THE NITRITE TEST AS APPLIED TO BACTERIAL CULTURES

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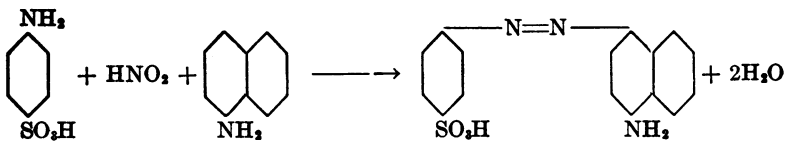
It has been observed by one of us (G. I. W.) when making the nitrate reduction test on bacterial cultures that there is sometimes a fading or disappearance of the red color of the positive test. The test used was the sulfanilic acid-alpha-naphthylamine method, according to the Manual of Methods of the Society of American Bacteriologists. In extreme cases the color appeared only at the point of contact of the incoming naphthylamine solution and the test broth. The question arose as to whether or not the test could properly be called positive and the following investigation was made to find the cause of, and if possible a remedy for, this fading.

The fading was always noticed at a time when such organisms as *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris* and the typhoid-paratyphoid group were being studied in the laboratory. Knowing that these organisms all produced more or less hydrogen sulfide, this compound was suspected as the bleaching agent. Accordingly a series of sixteen organisms were inoculated into lead acetate agar and nitrate broth tubes. Observations, after five days incubation, showed the following complete correlation between hydrogen sulfide production and the instability of the nitrite color (table 1). Where there was a very slight hydrogen sulfide production, the nitrite test color was permanent and where there was a large amount of hydrogen sulfide formed the nitrite test color faded immediately.

It was also found by using a series of sodium sulfide dilutions to which equal amounts of nitrite had been added that there was

a marked weakening of the color (table 2). Hydrogen sulfide, however, simply prevents maximum color development, probably by destroying a part of the nitrous acid, but does not directly cause the fading under discussion. Since the nitrite color is due to the production of p-sulfo-benzene-azo-alpha-naphthylamine according to the equation:



It would be expected that a reducing agent would destroy the color by producing a corresponding hydrazo compound. This would be operative in alkaline solution, but not under the conditions of the test.

Nevertheless, the correlation in table 1 shows hydrogen sulfide to be a factor, even though it is not the direct cause. In order to eliminate its effect, two samples of peptone were secured[1] which were devoid of cystine as shown by chemical analysis. Nitrate broth and lead acetate tubes were prepared with these peptones and the same sixteen organisms were inoculated into them. After five days incubation, the nitrate broths were tested with the standard reagents and none showed color fading, although some of the nitrite tests were so strong that a brown precipitate of the coloring matter soon developed. The lead acetate tubes were all negative. It was noted that with the strongly positive nitrite tests a single drop of naphthylamine solution gave a color which faded, but several additional drops produced a persistent color. This observation indicated that when a very small amount of the reagent was used the color faded; or, in other words, that in presence of a high concentration of nitrite the color fades. This view is borne out by the fact that where an ordinary nitrate broth tube shows fading, if a second tube of the same organism is diluted about one to five, thus diluting the nitrite formed, the second tube will not fade. These results all point to

[1] We are indebted to the Illinois State Water Survey for these peptones.

the conclusion that, in the presence of hydrogen sulfide, such high concentrations of nitrite are produced that the normal color suffers complete destruction.

TABLE 1

Correlation between fading of nitrite color and hydrogen sulfide production

	NITRITE TEST TIME OF FADING	H_2S PRODUCTION
1. *Escherichia coli*	1 minute	+
2. *Eberthella dysenteriae*	Permanent color	+ v. sl.
3. *Eberthella typhi*	½ minute	++
4. *Salmonella enteritidis*	½ minute	+++
5. *Salmonella pestis caviae*	1 minute	+++
6. *Salmonella suipestifer*	½ minute	+++
7. *Salmonella paratyphi*	1½ minutes	+ sl.
8. *Salmonella schottmülleri*	½ minute	+++
9. *Salmonella anatum*	½ minute	+++
10. *Salmonella pullorum*	1 minute	+
11. *Aerobacter aerogenes*	1½ minutes	+
12. *Proteus vulgaris*	½ minute	+++
13. *Alcaligines abortus*	Permanent color	+ v. sl.
14. *Proteus* X19	½ minute	+++
15. *Bacillus mesentericus*	Permanent color	+ v. sl.
16. Unknown spore former	2 minutes	+ sl.

TABLE 2

Effect of sodium sulfide on the nitrite color

MILLIGRAMS OF H_2S PER LITER OF SOLUTION	COLOR IN 10 MINUTES AS A PERCENTAGE OF THE CONTROL TUBE
0.0	100
5.0	95
9.6	90
14.4	85
19.2	70
24.0	50
28.9	40
33.6	20
38.5	10

Chemical tests have confirmed this and shown that an excess of of nitrous acid attacks the p-amino group of the coupled naphthylamine, the resulting diazonium salt breaking down with the for-

mation of the corresponding hydroxy-azo derivative; some further coupling may also occur. This view is supported by the fact that alpha-naphthol coupled with sulfanilic acid gives a brownish-yellow color strongly resembling the faded nitrite test. Spectrophotometrically the absorption maxima of the two solutions are coincident, though the faded nitrite test shows a slight secondary absorption attributable to the small additional coupling. Furthermore, fading in high nitrite concentrations was avoided by using naphthylamine in which the amino group had been protected by forming the mono-acetyl derivative. Coupling is slow, however, so that this remedy is not practical.

These facts suggest the use of some other compound for the coupling reaction. Of the common azo colors, the yellows and browns are unsuitable because they are difficult to detect in a normal yellow or brown nitrate broth. Dimethylaniline, however, gives a red color (free acid of methyl orange) very similar to the standard test. It possesses the advantages of stability and of giving a color which cannot fade because the amino group is protected. However, it will not detect less than one part of nitrite nitrogen in fifty million and probably would not be acceptable for this reason.

As the object of this investigation was to improve the test now in use, nitrate tubes inoculated with the sixteen organisms were tested by other methods found in the literature. The Bismarck brown test as suggested by Griess (1878) and adapted by Preusse and Liemann (1878) does not contrast well with the normal color of the broth when only small amounts of nitrite are present. The Letts and Rea (1914) modification of the old Fresenius' iodide-starch method would be satisfactory if the reagent had greater permanency. The neutral red test suggested by Rochaix (1909) produces a very distinctive color, but it fades too rapidly. The benzidine method of Armani and Barboni (1911) was not tried as its yellow color with small nitrite concentrations would be a distinct disadvantage. The old Riegler reagent (1897) is probably not sensitive enough for general use, and the same criticism would apply to the indole test of Dane (1911) as recommended by Rosenthaler and John (1915).

None of the known tests mentioned above seem to offer a suitable substitute and the dimethylaniline lacks the desired sensitiveness. The sensitivity of any nitrite test used in bacteriological characterization affords a point for argument. Some investigators doubt the significance of exceedingly minute amounts of nitrite in cultures. For example, Harding (1910) favored the starch-iodide test because it was less delicate than the standard test. Conn and Breed (1919) on the other hand have emphasized the need for a very delicate test and the Committee on Bacteriological Technic of the Society of American Bacteriologists apparently are in agreement. As a result, dimethyl-alpha-naphthylamine was tried. It is a compound like dimethylaniline, but with a higher molecular weight which would

TABLE 3

Sensitivity of the standard and the proposed test

NITRITE DILUTION	STANDARD	PROPOSED
1:40 million	Distinct	Distinct
1:80 million	Distinct	Distinct
1:100 million	Faint	Faint
1:200 million	Faint	Very faint

All readings made after ten minutes.

give it a more intense color and consequently greater sensitivity. The results were so favorable that the recommendation that it be used in place of alpha-naphthylamine is offered without hesitation.

As obtained from the Eastman Kodak Company, dimethyl-alpha-naphthylamine is a slightly viscid liquid, specific gravity 1.016, with a faint kerosene odor and colorless except for a slight bluish fluorescence. The reagent, prepared by dissolving 6 cc. in one liter of 5 N acetic acid, is stable and apparently unaffected by exposure to light and air. The color produced with traces of nitrite is similar to the standard test. In high nitrite concentrations, the color does not fade because the amino group is protected. The colored compound does, however, tend to precipitate in high concentrations, but instead of turning brown or being entirely

destroyed as is the case with the standard test, enough of the dimethyl color remains in solution to give a dark red mixture which may be diluted with water to a clear red solution. The color production with very small amounts of nitrite is a little slower than the standard test, but the sensitivity of the two is alike (table 3). The sensitivity of the standard test was placed by Warington (1881) at 1:100 million though higher dilutions developed color on standing. Determinations have shown 1:100

TABLE 4

Comparison of the standard and the proposed test

	STANDARD TEST	PROPOSED TEST
1. *Escherichia coli*.....	Faded to yellow	Deep red; slight precipitate
2. *Eberthella dysenteriae*....	Brown precipitate	Deep red solution
3. *Eberthella typhi*.....	Faded to yellow	Deep red; slight precipitate
4. *Salmonella enteritidis*....	Faded to yellow	Deep red; slight precipitate
5. *Salmonella pestis caviae*..	Faded to yellow	Deep red: slight precipitate
6. *Salmonella suipestifer*...	Faded to yellow	Deep red; slight precipitate
7. *Salmonella paratyphi*....	Faded to yellow	Deep red; slight precipitate
8. *Salmonella schottmülleri*.	Faded to yellow	Deep red; slight precipitate
9. *Salmonella anatum*.....	Faded to yellow	Deep red; slight precipitate
10. *Salmonella pullorum*.....	Faded to yellow	Deep red; slight precipitate
11. *Aerobacter aerogenes*.....	Nearly faded	Deep red; slight precipitate
12. *Proteus vulgaris*.....	Faded to yellow	Deep red; slight precipitate
13. *Alcaligines abortus*.....	Brown precipitate	Deep red solution
14. *Proteus* X19.....	Faded to yellow	Deep red; slight precipitate
15. *Bacillus mesentericus*....	Brown precipitate	Deep red solution
16. Unknown spore former..	Nearly faded	Deep red; slight precipitate
17. Control.....	Very faint color	No color in 10 minutes

million in two minutes, 1:300 million in ten minutes and 1:500 million in thirty minutes. The proposed dimethyl compound shows 1:80 million in two minutes, 1:200 million in ten minutes and 1:400 million in thirty minutes. It is assumed that most bacteriologists allow a ten minute reaction period before taking a final reading on the nitrite test. Making allowance for the natural color of the broth, the standard test and the proposed modification are both sensitive to one part of nitrite nitrogen in one hundred million of solution. Sterile control tubes of nitrate

broth, when incubated for five days, not infrequently give faint positive tests with such delicate reagents.

Duplicate tubes of nitrate broth, made with ordinary sulfur containing peptone, were inoculated with the same series of organisms and tested for nitrite after five days incubation by both the standard and the new reagents (table 4). In the standard test the usual fading was noticed but in the proposed test it was not. In both tests the color appeared within thirty seconds after the addition of the reagents showing that with these organisms the tests are not only sufficiently delicate but that there is a margin of sensitivity that is adequate.

DISCUSSION

It has been shown that the fading in the standard nitrite test is caused by an excess of nitrite. In a great many cases the color fades almost instantly and could very easily be overlooked by the busy laboratory worker making a number of tests. It has also been shown that in cultures of hydrogen sulfide producing bacteria this fading is more marked. This condition is not due to the organism itself but to the action of the hydrogen sulfide formed. The trouble is alleviated by the use of a sulfur free medium. A new test is recommended that does not have the disadvantages of the standard test.

CONCLUSIONS

1. In high nitrite concentrations, the color resulting in the standard nitrite test is destroyed on account of the destruction of the amino group of the naphthylamine.

2. The production of high nitrite concentrations is favored by the use of peptones containing sulfur and, conversely, small amounts of nitrous acid escape detection in the presence of hydrogen sulfide.

3. Dimethyl-alpha-naphthylamine gives a stable reagent of high sensitivity and produces a permanent nitrite color.

4. It is recommended, therefore, in place of alpha-naphthylamine.

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