EFFECT OF EXCESS NITRITE ON TESTS FOR INDOLE AND THE CHOLERA RED REACTION[1]

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The classical cholera red reaction was first described by Bujwid (1887) who noted the development of a rose-violet color upon the addition of hydrochloric, nitric, or sulfuric acids to a broth culture of the common bacillus. Diagnostic significance was immediately placed on the test since the reaction did not occur with cultures of *Bacillus anthracis*, *B. subtilis*, air-borne micrococci, or with fecal bacteria. Dunham (1887) reviewed these results, established the use of sulfuric acid as the test reagent, and further recommended the use of the test for the identification of *Vibrio comma*. The test depends upon the simultaneous production of indole from tryptophan and nitrites from nitrates by the organism. The reaction between nitrous acid, produced by acidification of nitrites, and indole yields nitroso-indole; hence, the development of a pink to violet color.

We have observed that cultures of *V. comma* strains Inaba and El Tor produce neither the classical cholera red reaction nor positive tests for indole (using Kovacs reagent) when grown in the commercial indole-nitrite medium (BBL). However, positive tests for indole were obtained when these strains were grown in peptone water. Since the only major difference between the two media appeared to be the presence of 0.1 per cent potassium nitrate in the former medium, the influence of nitrate and nitrite concentration on the indole and cholera red tests was investigated. In a similar study of the cholera red reaction, Paladino-Blandini (1906) observed the reaction to cease when indole and nitrite concentrations became disproportionate. Gallut (1948) later observed that the nature of the color of the cholera red reaction depended upon the ratio of indole to nitrite concentrations, the color changing from orange to red to violet as the ratio was changed from less than 1 to greater than 2 in the presence of 0.004 per cent indole. However, we know of no references which pertain to the action of nitrites on tests for indole in bacterial cultures.

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EXPERIMENTAL METHODS

Indole reactions were determined by the methods of Kovacs (1928); Böhme (1905), the Ehrlich-Böhme test; and Holman and Gonzales (1923), the Gnezda test. The cholera red test was performed by the addition of 2 to 3 drops of concentrated sulfuric acid to the preparations. The presence of nitrites was determined by the method of Griess-Ilosvay, cited by Wilson and Miles (1955). All tests, with the exception of the Gnezda test, were performed on 1-ml aliquots from the cultures or indole-nitrite preparations. The latter were prepared from pure crystalline indole (Eastman White Label) and reagent grade sodium nitrite. All cultures were grown in peptone water, pH 7.2, consisting of 1 per cent peptone (Difco) and 0.5 per cent sodium chloride, unless otherwise indicated.

RESULTS

The Inaba and El Tor strains of *V. comma* were grown in peptone water containing varying concentrations of sodium nitrate at both pH 7.2 and 8.0 for 22 hr. The indole, nitrite, and cholera red reactions for both strains were comparable at each pH level (table 1). Indole and cholera red tests were negative on cultures yielding strong nitrite reactions. The cholera red reaction was strong only in the medium originally containing 0.001 per cent sodium nitrate below which the reaction was limited by excessively low concentrations of nitrite. All Gnezda tests for indole were positive.

TABLE 1

Indole, nitrite, and cholera red tests on cultures of Vibrio comma inaba and El Tor containing varied amounts of sodium nitrate

Per cent $NaNO_3$	Kovacs Indole Test*		Gnezda Indole Test		Nitrite Test		Cholera Red Test	
	pH 7.2	pH 8.0	pH 7.2	pH 8.0	pH 7.2	pH 8.0	pH 7.2	pH 8.0
0.1	−	−	+	+	++++	++++	−	−
0.01	−	−	+	+	++++	++++	−	−
0.001	++	+++	+	+	+++	+++	++	++
0.001	++++	++++	+	+	+	+	±	+
0.00001	++++	++++	+	+	±	±	−	−
None added	++++	++++	+	+	±	±	−	−

* The Ehrlich-Böhme test yielded comparable results.

TABLE 2

Effect of nitrite concentration on indole and cholera red tests on cultures of Escherichia coli

Per cent $NaNO_2$ Added to Culture Before Testing	Kovacs Indole Test	Nitrite Test	Cholera Red Reaction	Gnezda Indole Test
0.1	−	++++	−	+
0.01	−	++++	−	+
0.005	−	++++	−	+
0.001	±	+++	−	+
0.0005	++	+++	+	+
0.0001	++++	++	+	+
0.00005	++++	+	−	+
None	++++	−	−	+
0.1% $NaNO_3$	++++	−	−	+

TABLE 3

Results of Kovacs test for indole on varying mixtures of indole and sodium nitrite

Molarity of Sodium Nitrite	Molarity of Indole				
	0.0005	0.00025	0.0001	0.00005	0.000025
0.00001	++++	++++	++++	++	−
0.000025	++++	++++	++++	+	−
0.00005	++++	++++	++++	±*	−
0.0001	++++	++++	±*	−	−
0.00025	++++	±*	−	−	−
0.0005	++*	−	−	−	−

* Orange color reaction.

To ascertain whether or not the inhibiting action of nitrites on the Kovacs indole test was peculiar to the vibrio, 48-hr cultures of *Escherichia coli* were prepared and varying amounts of sodium nitrite added just prior to testing. Results (table 2) again showed the inhibiting effect of the nitrite ion in concentrations greater than 0.001 per cent on the Kovacs test. The slight indole reaction at 0.001 per cent nitrite concentration was probably the result of the loss of volatile indole during the long incubation period. The nitrate ion had no effect on the indole test. Again, all Gnezda tests for indole were positive.

The reactions were then studied with chemicals alone using varying amounts of indole and sodium nitrite (tables 3 and 4). The data indicate that positive tests for indole are inhibited by the presence of nitrite in excess of the molar concentration of indole present when Kovacs reagent was employed. It was noted (table 3) that the color of the Kovacs test was orange when molar equivalent amounts of indole and nitrite were present, red when indole was in excess, and negative when nitrite was in excess. The strongest cholera red reaction (table 4) occurred when the molar ratio of indole to nitrite was approximately 2:1. The intensity of the reaction diminished

TABLE 4

Results of the cholera red reaction on varying mixtures of indole and sodium nitrite

Molarity of Sodium Nitrite	Molarity of Indole					
	0.0005	0.00025	0.0001	0.00005	0.000025	0.00001
0.000005	−	−	−	−	−	−
0.00001	±	±	±	±	+	−
0.000025	+	+	+	+	±	−
0.00005	++	++	++	±	−	−
0.0001	++++	++++	+	±*	−	−
0.00025	++++	+	±*	−	−	−
0.0005	++†	+*†	±*	−	−	−

* Orange color reaction.

† Red precipitate on standing.

when the ratio was changed in either direction, but more sharply when excess nitrite was present. As noted by Gallut (1948), an orange reaction occurred in the region of slight nitrite excess.

DISCUSSION

The results have shown that the Kovacs test for indole is negative when the molar concentration of nitrite is greater than that of indole. The cholera red reaction is similarly inhibited by excess nitrite concentration. Further, it was noted that the presence of excess nitrite had no effect on the production of indole as indicated by the independence of the Gnezda test to nitrite concentration. The latter test depends upon the action of volatile indole upon an oxalic acid-saturated paper strip suspended above the culture which becomes pink in the presence of indole.

These data imply that the concentration of nitrates in media used for the Kovacs indole test becomes important whenever the molar concentration of nitrites may become greater than that of indole; for example, (a) when the test organisms reduce nitrate only to nitrite as in the case of the vibrios, (b) when further reduction of accumulated nitrites is delayed, and (c) when tests for indole are desired within a short period of incubation. In such cases, false negative tests for indole might occur if media such as the BBL Indole-Nitrite Medium are employed. It is recommended that tests for indole should not be performed with media containing more than 0.001 per cent inorganic nitrate unless the Gnezda test is employed; this amount in conjunction with a satisfactory peptone has also been found optimum for the performance of the cholera-red test, and is in agreement with the following statement found in *Methods for Medical Laboratory Technicians* (1951): "Moreover, the peptone-water medium used for the cholera red tests must contain an adequate amount of nitrate, but not *an excess*. . . To insure a medium that will be satisfactory for the test any peptone that gives good indole tests may be used with the addition of 0.001 per cent (not more or less) sodium nitrate."

SUMMARY

It was found that the Kovacs test for indole was inhibited in the presence of molar concentrations of sodium nitrite in excess of the quantity of indole present. A similar effect was observed in the cholera red test. The presence of nitrites in concentrations to 0.1 per cent had no notable effect on inhibiting indole production since positive Gnezda tests for indole were consistently obtained. It is recommended that the concentration of nitrates in indole media should not be greater than 0.001 per cent if reagents containing paradimethylaminobenzaldehyde are to be used for the indole test.

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