

THE USE OF THE NITRATE-REDUCTION TEST IN CHARACTERIZING BACTERIA

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INTRODUCTION

For some time bacteriologists have used the ability to reduce nitrates as a criterion for distinguishing certain kinds of bacteria. The test has been regarded as of so much diagnostic value that it has been included in nearly all schemes of bacterial characterization, among the most important of which is the descriptive chart adopted by the Society of American Bacteriologists. An investigation of this test was begun as a result of some student work done under the direction of one of us (B) upon certain cultures of the colon group. Such very irregular results were obtained in regard to the power of reducing nitrates that the method of making the test was decided to be unsatisfactory. Further work was therefore undertaken, at first by students and volunteer assistants, and then by one of us (C) as a contribution to the work of the Committee on the Descriptive Chart appointed by the Society of American Bacteriologists. For the earlier part of the work much credit is due to Emma Edson Breed, H. M. Weeter, and H. V. Grant.

TECHNIC

1. *Formula of medium.* An attempt was made at first to follow the "standard" technic adopted by the American Public Health Association (1905, 1912). It was soon found, however, that a standard technic was sadly lacking. In the 1905 Report of the Committee on Standard Methods of Water Analysis the following directions are given:

Dissolve 1 gram peptone in 1 liter of tap water, and add 2 grams of nitrite-free potassium nitrate. It is convenient to prepare a stock solution of potassium nitrate by dissolving 4 grams of solid nitrate in 100 cc. of distilled water and use 5 cc. of this solution in the above formula.

A little arithmetic will show that to follow the direction given in the first sentence would give a 0.2 per cent solution, while that in the second sentence would give a 0.02 per cent solution of the nitrate. In the 1912 report an attempt was evidently made to correct this disagreement, because the two grams in the first sentence was changed to 0.2 gram; but by some slip the 4 grams in the second sentence was also changed to 0.4 gram, thus giving a 0.002 per cent solution. With this inconsistency there has naturally been a great variation in the "standard" nitrate broths used by different bacteriologists. This is shown by a survey of the literature. Thus Gorham (1901) recommends 1 gram of peptone and 0.2 gram of potassium nitrate (i.e., 0.02 per cent); while Chester (1901) recommends a formula different from any of the above—10 grams of peptone and 0.02 gram (i.e., 0.002 per cent) of *sodium* nitrate. Apparently in preparing a formula for nitrate broth the figures 1 for the peptone and 2 for the nitrate have been considered more important than the position of the decimal point in either case. This peculiar irregularity has already been mentioned by one of us (Breed, 1915).

In the early part of this work two different nitrate broths were used independently by two of the workers, each thinking he was following the standard technic; both contained 0.1 per cent peptone but one contained 0.2 per cent KNO_3 and the other 0.02 per cent KNO_3 . When the discrepancy was noticed, the two media were compared, as were other different nitrate media, all of them within the limits of variation of the formulae given above. Later, other arbitrary variations were made for special purposes, as will be explained later.

2. *The nitrite test.* Two different variations of the official nitrite test were used in the first part of this work, differing only in the quantity of the reagents used. Each variation was sup-

posed to be "standard" by the particular investigator employing it, owing to two different interpretations of the standard methods. On page 120 of the report for 1912 the following technique is given: "Remove 3 cc. of the culture to a clean test tube and add 2 cc. of each of the naphthylamine solution and the sulphanilic acid solutions described under the determination of nitrites. (see p. 22)." Three of the workers followed these directions exactly. Another, working independently, looked up page 22 and found that at this place the directions were to add 2 cc. to 100 cc. of the water to be tested, and concluded that the proportions of reagents given on page 120 must have been a mistake; so he used the reagents in the proportion of about 2 parts to 100 parts of the medium, i.e., a few drops to the culture tube. Later these two methods of using the reagents were compared, and the latter method was found to be the better. Using larger quantities of the reagent was found to dilute the nitrite present to such an extent as to obscure the reaction in cases where only small amounts of nitrite had been formed, especially when the reagents were old and somewhat discolored. As a result, in the latter part of the work, only a few drops of each reagent were added to the culture.

A still different test was used in some of the early part of the work: the potassium-iodide-starch test, as described by Erwin Smith (1905). This test compared very favorably with the official test, but proved a little less delicate and the reagents were found to deteriorate more rapidly; so its use was discontinued. This test is preferred by Harding (1910) just because it is less delicate; but as the present work progressed, the need of a delicate nitrite test was emphasized more and more clearly.

WORK WITH THE COLON GROUP

In 1912, one of the students taking part in the work (E. E. B.) observed considerable irregularity in the nitrate-reduction test in the case of fifty different cultures of the colon group isolated from polluted water. These cultures included all four of the commonly recognized types, as distinguished by their ferment-

tative reactions with sucrose and dulcitol. They were tested in triplicate in 0.02 per cent nitrate broth (with 0.1 per cent peptone), some giving negative results in all three tubes, others giving inconsistent results, while the majority of the cultures gave consistent positive results as was expected. In 1913 the cultures giving no positive reactions the first time were tested again by the same student, using the same medium, and all but three of the cultures were found to produce nitrite in at least one out of three tubes. These three cultures and one other (which had shown nitrite in only one tube each time) were tested a third time with consistently positive results. These findings are listed in table 1 under the heading "Series of 1912-13." In all, about 64 per cent of the tests were positive, counting each tube in a set of triplicates as a single test. The fact that every culture gave a positive reaction in the end suggested that all might be nitrate-reducers, but that the methods of testing were such that they did not develop this ability in more than about two-thirds of the cases.

Later in 1913 (see table 1, Series of 1913) the same cultures were tested in triplicate again by another student (H. M. W.) using the same medium and the same methods generally, recording the results similarly. By this student 37 cultures were tested twice, 15 of them three times. Until the formula of the medium was varied, only about 37 per cent of the tests were positive. In regard to the individual cultures, there was practically no agreement with the results of the first student: only three cultures (nos. 11, 14 and 35) gave consistently positive reactions on both occasions.

In the course of this work it was noticed that no strain grew well in the medium used; so some preliminary work was done in varying the composition of the medium (last column under "Series of 1913," table 1). Twelve cultures that had given negative reactions in the majority of cases were tested again in a medium containing 0.2 per cent (instead of 0.1 per cent) peptone. The growth in this medium was noticeably better, and a distinct nitrite reaction was obtained in all 36 tubes.

TABLE 1

Nitrate reduction by organisms of the colon group in 0.02 per cent nitrate broth. Tests made in triplicate; + and - indicate presence and absence respectively of a distinct nitrite reaction in all three tubes; ± indicates a distinct nitrite reaction in two out of the three tubes, ∓ in only one of the three tubes. T indicates mere trace of nitrite.

CULTURE NUMBER	SERIES OF 1912-13			SERIES OF 1913				SERIES OF 1914		
	Peptone 0.1%			Peptone 0.1%			Peptone 0.2%*	Peptone 0.1%	Peptone 0.2%	Peptone 0.5%
	First test	Second test	Third test	First test	Second test	Third test				
1	-	+		-	-	+		∓	+	+
2	-	∓		-	∓			∓	+	+
3	+			-	+			∓	+	+
4	±	+		-	±			-	+	+
5	-	+		-	+†			±	+	+
6	±	±		-	T	+		-	+	+
7	+			-	-	+	+	+	+	+
8	-	+		±				-	+	+
9	+			-	T‡			-	+	+
10	-			-	∓			±	+	+
11	+			+	+			+	+	+
12	±			-	-	+		∓	+	+
13	+			-	±			+	+	+
14	+			T	+					
15	+			±				+	+	+
16	T?	+		-	T	-	+	+	+	+
17	-	+		-	+					
18	±			±				-	+	+
19	+			-	±†			-	+	+
20	-	+		-	∓	-	+	-	+	+
21	±	+		+				-	+	+
22	-	+		-	∓			+	+	+
23	+			-	T	+		±	+	+
24	+			-	±			-	+	+
25	-	+		-	+†			+	+	+
26	-	+		-	-	+	+			
27	∓	+		-	+			-	+	+
28	+			±				∓	+	+
29	+			-	-†	+	+	∓	+	+
30	+			-	∓			±	±	+
31	-	+		-	-	±	+	-†	+	+
32	+			∓	+			-	+	+
33	+			-				-	±†	+
34	-	+		-	+	+	+	±	+	+
35	+			+				+	+	+

TABLE 1—*Continued*

CULTURE NUMBER	SERIES OF 1912-13			SERIES OF 1913				SERIES OF 1914		
	Peptone 0.1%			Peptone 0.1%			Peptone 0.2%*	Peptone 0.1%	Peptone 0.2%	Peptone 0.5%
	First test	Second test	Third test	First test	Second test	Third test				
36	+			-	+			+	+	+
37	+			-	-	+	+	±	+	+
38	-	+		-	-	-	+	±	+	+
39	+			-	-	+	+	-	+	+
40	±			-	-			-	+	+
41	-	-	+	+	±†			+	+	+
42				-	T	-	+	-	+	+
43	-	-	+	-	+			-	+	+
44	+			-	+	+	+	+	+	+
45	-	-	+	+	-	?	+	±	+	+
46	±	±	+	+				-	+	+
47	+			±	T			-	+	+
48	+			+	±			±	+	+

* This medium contained only 0.01 per cent nitrate.

† One of the tubes showed nitrite present in mere traces only.

‡ One of the tubes showed a distinct positive reaction.

The idea suggested by the last mentioned tests was followed up by a third student (H. V. G.) the next year. Parallel tests were made in nitrate broth containing 0.1, 0.2, and 0.5 per cent peptone respectively (Series of 1914, table 1). With 0.1 per cent peptone only 42 per cent of positive results were obtained, and the agreement of the individual cultures with their previous behavior was no greater than it had proved to be the preceding year. With 0.2 per cent peptone 98.5 per cent of the tests (129 out of 131 tubes) gave positive results, every culture showing a positive reaction in at least two of the tubes. With 0.5 per cent peptone all of the cultures gave positive reactions in all three of the triplicate tubes. Further tests not listed in the table were made with a medium containing 0.1 per cent peptone and 0.2 per cent (instead of 0.02) per cent nitrate. This use of ten times the original amount of nitrate was found to have no influence on the results, the irregularity proving as great as with 0.1 per cent peptone and 0.02 per cent nitrate.

The conclusion reached by this work was that inconsistent results of the nitrate-reduction test may be expected with organisms of the colon group unless enough peptone is present to furnish these bacteria with conditions favorable to their vigorous growth. With 0.1 per cent peptone the growth was generally poor; with 0.2 per cent fairly good; and with 0.5 per cent very good. The amount of nitrate present apparently had little influence on the results. The important matter was a vigorous growth of the organisms; and under conditions allowing vigorous growth all the cultures of the colon group tested proved to reduce nitrate to nitrite.

WORK WITH BACILLUS CEREUS FRANKLAND

As an organism to contrast with those of the colon group, *B. cereus* Frankland¹ was chosen. *B. cereus* grows well in the presence of considerable organic matter, but it does not seem to require the large amounts of nitrogenous material that the colon organisms do. The cultures of *B. cereus* used were isolated by one of us (C) from soil.

Ten out of 130 cultures, apparently all *B. cereus*, failed to produce nitrite when tested, promptly after isolation from soil, in broth containing 0.1 per cent peptone and 0.2 per cent KNO_3 . One of these ten cultures (which we will denote culture x) was tested again two years later (table 2, test 2) together with several typical *B. cereus* cultures (which we will call cultures A to G respectively) that had produced nitrite the first time. This work was done by a different investigator (H. V. G.) from the one who made the original tests. In order to see whether the explanation for the disagreement previously found might be due to the same cause as the disagreement in the case of the colon organisms, the four following nitrate broths were used:

0.1 per cent peptone, 0.2 per cent nitrate
0.1 per cent peptone, 0.02 per cent nitrate
0.2 per cent peptone, 0.02 per cent nitrate
0.5 per cent peptone, 0.02 per cent nitrate

¹ This organism was identified by means of the characteristics previously described (Conn 1917).

TABLE 2
Nitrite production by B. cereus in various nitrate media. Tests made in duplicate. Each + or - sign indicates presence or absence of nitrite in all three tubes of one set. The sign ± indicates disagreement among three parallel tubes

	ORIGINAL TESTS 1911-13		SECOND TEST, 1914		THIRD TEST, 1914		FOURTH TEST, 1914		FIFTH TEST, 1915			SIXTH TEST, 1918		SEVENTH TEST, 1918			
	0.2%	0.1%	0.2%	0.1%	0.2%	0.1%	0.2%	0.1%	0.2%	0.1%	0.02%	0.02%	0.2%	0.1%	0.1%	0.1%	
Medium containing { KNO ₃ ... { Peptone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture A.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture B.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture C.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture D.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture E.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture F.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture G.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture H.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture X.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture Y.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture a.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture b.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture c.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture d.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culture x.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* This medium contained 1 per cent glucose.

† Nitrate-peptone agar. Test made on agar slants.

‡ Five different nitrate agars used, containing no added organic matter except sugar. Tests made on agar slants. Each medium inoculated in triplicate and the three tubes tested on different days. Each + or - sign indicates presence or absence of nitrite in all fifteen tubes.

Each culture was inoculated in triplicate into each medium; and clear-cut positive nitrite reactions were obtained in all cases, even with culture x.

A few months later (test 3, table 2) cultures A and B, together with seven other typical nitrite-positive *B. cereus* cultures² were tested again in the first of the nitrate broths listed above, all giving such clear-cut positive reactions that no further investigation of these cultures were made. At the same time, six other cultures were retested in this same medium, and also in media of the following composition:

0.5 per cent peptone, 0.2 per cent nitrate;
0.5 per cent peptone, 1 per cent glucose; 0.2 per cent nitrate.

One of these six cultures (culture X, table 2) had given a positive reaction the first time, while the others (cultures a, b, c, d and x) had given negative reactions. The results showed no nitrite from cultures a, b, c, d, and none from culture X, while nitrite was found with culture x, as had been the case in test 2. In other words all the cultures except X and x showed agreement with the original test; but X had changed from nitrite-positive to nitrite-negative, x from nitrite-negative to nitrite-positive.

Test 4 was made a few weeks later. This test included cultures b, c, x and X, together with a fifth, which from its similarity to culture X will be denoted as culture Y. Cultures b, c, x and X gave the same results as in test 3. Culture Y, like culture X, had given a positive nitrite reaction at the time of isolation two years earlier, but now showed no production of nitrite.

No further tests of culture Y were made, but cultures X, a, b, c, d and x were tested again about a month later (test 5). This test was made in six different nitrate broths, the four used in test 2 together with two additional broths as follows:

0.2 per cent peptone, 0.2 per cent nitrate
0.5 per cent peptone, 0.2 per cent nitrate

² Not listed in Table 2, because no further work was done with these seven cultures.

All the cultures except culture c gave the same results as in the two preceding tests, no difference being observed between their behavior in the different media. Culture c gave distinctly negative results with the four media used in test 2; but with the two new media (that is, the media with 0.2 per cent nitrate combined with more than 0.1 per cent peptone) a discrepancy was observed on the tenth day, one of the two duplicate tubes of each medium tested giving a positive nitrite test.

Four years later culture a was tested again, still with negative results. In the meanwhile the advantage of agar for making the test in doubtful cases (see p. 277) had been learned, and so this culture at this time was tested also on beef-extract-peptone agar to which 0.1 per cent KNO_3 had been added. The results were still negative. Good growth, however, was obtained in all cases.

Test 7 was made a few months later. Three organisms were tested this time: H, a typical nitrite-positive *B. cereus* culture that had not been tested before since immediately after isolation five years before; culture X and culture b. These cultures were tested at this time in six different agar media, all but one of them containing no added organic matter except some sugar. Culture H gave positive results in all the media used; cultures X and b gave negative results throughout although one tube out of nine in the case of culture b gave a positive nitrite reaction on the seventh day (due probably to an impurity). One of the media used contained no possible source of ammonia except the nitrate, so a Nessler test was made to see if the nitrate had been converted into ammonia without accumulation of nitrite. This test also was negative.

As a result of this work it was concluded that with *B. cereus* the explanation of the irregularities is not as simple as in the case of the colon organisms. *B. cereus* grows well in almost any medium and ordinarily reduces nitrate to nitrite. Certain cultures, however, seem to lack this reducing power, either temporarily or permanently, although they grow well in the media used. In general, constantly negative or constantly positive results have been found with any particular culture. This sug-

gests that there may be two different species so closely related that they can be distinguished only by means of the nitrate test. Three cultures, however (X, Y, and x) and possibly a fourth (c) gave inconsistent results. Although this disagreement may possibly have been due to a contamination that ran out the original organism, the agreement in all other respects with the original descriptions makes this explanation doubtful. No other explanation of the irregularity has been found, however, unless it be assumed that cultures of *B. cereus* may lose or gain the power of reducing nitrates when cultivated in the laboratory.

WORK WITH FLUORESCENT PSEUDOMONADS

During the course of a study of soil bacteria, numerous cultures of fluorescent pseudomonads were obtained that appeared to be closely related although differing in certain particulars. One of the most noticeable points of difference was that some produced nitrite in nitrate broth containing 0.1 per cent peptone while others produced no nitrite under these conditions. With these organisms the growth was always fairly good; nevertheless it was felt that the difference might be due to causes similar to those affecting nitrate-reduction by the colon organisms. Upon testing the cultures in broth containing larger amounts of peptone (as much as 1 per cent) the same distinction between nitrate-producers and non-nitrite-producers held true; but in this broth a further difference appeared, some of the organisms producing gas (presumably free N), others producing no gas. In other words three groups of fluorescent pseudomonads were found upon inoculation into 1 per cent peptone solution containing nitrate: (1) producing nitrite and gas; (2) producing nitrite but no gas; (3) producing neither nitrite nor gas (in appreciable quantities).

As the fluorescent organisms grow rather better on the surface of agar than in a liquid medium, further work was carried on in a nitrate agar containing beef-extract 0.3 per cent, peptone 1 per cent, KNO_3 0.1 per cent. The nitrite test was made by pouring the reagents upon the surface of the agar slant after incubation;

gas was detected by means of bubbles and cracks in the medium. One day of incubation was enough to bring out the nitrite reaction with vigorous nitrate-reducers; but cultures were generally kept until the seventh day. This agar slant test was found to give the same results obtained with the 1 per cent peptone broth cultures; the same three groups were found as before.

TABLE 3

Composition of nitrate media used in tests listed in tables 4-7. Figures indicate grams per liter.

INGREDIENTS	MEDIA SYMBOLS*					
	P	DM	D	DL	S	DA
Agar†.....	15.0	15.0	15.0	15.0	15.0	15.0
Peptone.....	10.0					
Glucose.....		10.0	10.0	10.0		10.0
Lactose.....				5.0		
Sucrose.....					10.0	
KNO ₃	1.0	1.0	1.0	1.0	1.0	1.0
CaCl ₂		0.5	0.5	0.5	0.5	0.5
MgSO ₄		5.0				
K ₂ HPO ₄		5.0	0.5	0.5	0.5	0.5
NH ₄ Cl.....						2.0
Beef extract.....	3.0					

* In these media symbols, the letters represent the significant ingredients, as follows: P, peptone; D, glucose; M, magnesium sulphate; L, lactose; S, sucrose; A, ammonium chloride.

† "Bacto-agar" (a purified agar sold by the Digestive Ferments Co.) was used in all except Medium P.

For further testing six cultures were selected, two of each group. These six cultures will be denoted in this paper as follows:

- AA } Producing nitrite and gas in nitrate-peptone media.
- BB }
- A } Producing nitrite without gas in nitrate-peptone media.
- B }
- a } Producing neither nitrite nor gas in nitrate-peptone media.
- b }

These six cultures were retested not only on the nitrate-peptone agar previously used (test 1, table 4) but also on agar containing no nitrogen (disregarding impurities) except 0.1 per cent KNO_3 and with no added organic matter except sugar (tests 2-5, table 4). The object was to learn whether the nitrate was converted to ammonia. The reports of the Committee on Water Analysis (A. P. H. A. 1905, p. 150; 1912, p. 120) have called attention to the need of making the ammonia test, but have added that ammonia may also come from the peptone. Kligler (1913) emphasized the importance of this source of error. As the fluorescent organisms are all ammonifiers, it is impossible to tell whether the ammonia present in a nitrate-peptone medium comes from the nitrate or the peptone. Hence it was decided to grow them under conditions where there could be no source of ammonia except the nitrate, so that its presence would show reduction of the nitrate even though there was no accumulation of nitrite. The first media tried contained glucose (media DM, and DL, table 3) and were found to be unsatisfactory for the ammonia test because no glucose could be obtained free from ammonia. Lactose was found to have the same disadvantage, so finally sucrose (medium S, table 3) was employed as a source of energy.

Even before a satisfactory ammonia-free medium was obtained, some very interesting results were procured (see table 4). On these media all six cultures, even including strains a and b, were found to give the nitrite test, sometimes in mere traces, but often in appreciable quantities, as early as twenty-four hours after inoculation. The test on the ammonia-free medium S (test 4, table 4) gave the following results: Cultures AA and BB showed the presence of ammonia, as well as the nitrite and gas demonstrated on the peptone media; cultures A and B showed a very strong nitrite reaction, but a weak ammonia reaction after the fourth day; cultures a and b showed a moderate nitrite reaction and an ammonia reaction slightly stronger than with cultures A and B. The ammonia reactions were in no case strong enough to prove that the organisms were converting the nitrate rapidly into ammonia.

TABLE 4
 Nitrate reduction by *Ps. fluorescens* in 0.1 per cent nitrate agar. First test.* Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, - absent, ? doubtful.

	MEDIUM P.			MEDIUM DM.			MEDIUM DL.			MEDIUM S.				MEDIUM DA.			
	Nitrite present in			Nitrite present in			Nitrite present in			First day	Second day		Fourth day		Nitrite present in		
	1 day	4 days	4 days	4 days	7 days	1 day	2 days	3 days	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	1 day	2 days	3 days
Culture A.....	+++	T	-	-	+	+++	+++	+++	+++	?	+++	-	+++	T	+++	+++	+++
Culture B.....	+++	+++	-	-	+	+++	+++	+++	+++	?	+++	-	+++	T	+++	+++	+++
Culture a.....	-	-	T	T		+	T	+	+	?	+	+	+	+	-	-	-
Culture b.....	-	-	T	T		+	T	+	+	?	+	+	+	+	-	-	-
Culture AA.....	+	T†	+	+	+	+	+	+	+	?	+	+	+	+	+	+	+
Culture BB.....	+++	T†	-	-		+	+++	+++	+++	+	+	-	+	T	+	+	+

* In this test the five media were inoculated separately, on different days.

† Gas production shown by presence of cracks in the agar.

A further test (test 5, table 4) was then made on a synthetic agar (medium DA, table 3) containing nitrate, glucose, and ammonium chloride. Cultures A, B, a and b gave the same reactions (i.e., either nitrite-positive or nitrite-negative) as on peptone media; of the gas-producers, BB behaved as on the other media, but AA failed to produce gas. In other words, it was shown that cultures a and b were prevented from producing nitrite on the synthetic medium by the addition of ammonium chloride. This test was considered to show that some strains of fluorescent organisms, although capable of reducing nitrate, do not attack it if there is a more easily available source of nitrogen present.³ In this connection it is interesting to note that the nitrite reaction of these two organisms was stronger on the sucrose medium than on the media containing those sugars which had ammoniacal impurities.

The sixth test (table 5) was run a few months later. This test was a repetition of tests nos. 2 to 5, using the same four media together with one other glucose agar only slightly different (medium D, table 3). The same six cultures were tested, together with four others (laboratory cultures isolated from soil nearly six years previously): two gas-formers CC and DD, and two that were nitrite-negative in peptone media, c and d. Of these four cultures, DD was especially interesting. It had been kept under cultivation in the laboratory for the longest time of all, and when first tested on medium P (nitrate-peptone agar) six years after isolation, was found to be a weak gas-producer. During the next few months its power of gas-production apparently diminished until when the work recorded in table 5 was done it failed to produce gas on any medium. The possibility of a contamination being present and causing this discrepancy is not entirely excluded.

The four cultures AA, BB, A, B, a, and b, gave results agreeing fairly well with the earlier tests, except in regard to the gas-

³ Another possible explanation is that growth is so rapid in the presence of ammonium chloride that these organisms are able to use up the nitrite as fast as produced. This theory is not a satisfactory explanation, however, because the growth of Cultures a and b was better in the absence than in the presence of ammonium chloride in this medium.

TABLE 5
Nitrate reduction by Ps. fluorescens in 0.1 per cent nitrate agar. Second test. Cultures inoculated in triplicate, but only one tube tested each day.*
Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, - absent, ? doubtful

CULTURE	MEDIUM DM.				MEDIUM D.				MEDIUM DL.				MEDIUM S.				MEDIUM DA.				
	Nitrite present in				Nitrite present in				Nitrite present in				Nitrite present in				Nitrite present in				
	2 days	4 days	5 days	7 days	2 days	4 days	5 days	7 days	2 days	4 days	5 days	7 days	2 days	4 days	5 days	7 days	2 days	4 days	5 days	7 days	
A.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
a.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
b.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
c.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
d.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
AA.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
BB.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CC.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
DD.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

* In this test the five media were inoculated simultaneously.

† Gas production shown by presence of cracks in the agar.

production by AA and BB on certain media, a point which seems open to considerable variation. The two old cultures, c and d were found to be even less vigorous nitrate-reducers than a and b. Culture c produced no nitrite on any medium and only a trace of ammonia on medium S; culture d showed nitrite and ammonia on medium S, but no nitrite on any other medium. Culture c grew very poorly on medium S, a fact which undoubtedly explains its failure to reduce the nitrate on this synthetic medium.

It was therefore concluded that vigorous growth of the fluorescent organisms is as necessary as it is with the colon organisms, in order to get a satisfactory test for nitrate-reduction; but that a more common cause of error is that cultures do not always reduce the nitrate if a more readily available source of nitrogen is present. In the present work the difference between those cultures that could reduce nitrate under all conditions and those that could do so only in the absence of other sources of nitrogen remained constant; but, as the two sets of cultures resembled each other in all other respects and as various gradations were found between those that showed no nitrite in the presence of mere traces of ammonia and those that produced it even in the presence of peptone, it does not seem to be a question of two distinct species.

WORK WITH AN ORANGE CHROMOGENIC PSEUDOMONAS

Another organism chosen for study because of its irregularity in respect to the nitrite test was an orange chromogenic pseudomonas, a form very common in soil and water. It has recently been studied in this laboratory and a description of it is now in course of publication (Conn and Bright, 1919). It is concluded to be identical with *Bacillus caudatus* Wright (1895), although it has a polar flagellum. This organism grows so poorly in liquid media of all sorts that nitrate broth was realized from the beginning to be an unfair medium to use in the nitrate-reduction test, and the early irregular results were considered to be due to the poor growth in this broth. For that reason beef-

extract-peptone agar containing 0.1 per cent nitrate (medium P, table 3) was used in subsequent work.

A special study was first made of eight different strains of this organism, four of which strains had been separated into two or three substrains immediately after isolation from the soil, all of these substrains having been kept distinct during the laboratory cultivation. Including these separate substrains, 15 different cultures were studied. It was found (see test no. 1, table 6) that they could be divided into two groups, those producing abundant nitrite on nitrate-peptone agar and those showing no nitrite on this medium. The cultures may be listed as follows:

Group 1.—Producing nitrite

- A. A typical chromogenic strain.
- B. A typical chromogenic strain.
- C₁ } Two substrains of a typical chromogenic strain.
- C₂ }
- D₁ } Three substrains of a typical chromogenic strain.
- D₂ }
- D₃ }
- X₁ } Three substrains of a non-chromogenic strain.
- X₂ }
- X₃ }

Group 2.—Not producing nitrite

- a₁ } Three substrains of a chromogenic strain.
- a₂ }
- a₃ }
- b A chromogenic strain.
- c A chromogenic strain

On nitrate-peptone agar the agreement between the different substrains of any one original strain was complete. Some of the strains grew more vigorously than others. In general there was no correlation between vigor of growth and production of nitrite, although it was striking that after a few months cultivation in the laboratory all the nitrite-negative cultures died.

TABLE 6

Nitrate reduction by the orange chromogenic *Pseudomonas* in 0.1 per cent nitrate agar. First test.* Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T, trace, - absent, ? doubtful.

	MEDIUM F.			MEDIUM DM.			MEDIUM DL.			MEDIUM S.						MEDIUM DA.				
	Nitrite present in			Nitrite present in			Nitrate present in			First day		Second day		Fourth day		Seventh day		Nitrite present in		
	1 day	4 days	7 days	1 day	2 days	3 days	7 days	Ni- trite	Am- monia	Ni- trite	Am- monia	Ni- trite	Am- monia	Ni- trite	Am- monia	Ni- trite	Am- monia	4 days	7 days	
Culture A.....	++	+++	++					+	+	+	+	+	+	+	+	+	+	+	+	+
Culture B.....	T	++	+	++	++	++		+	+	+	+	+	+	+	+	+	+	+	+	+
Culture C ₁	++	+++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture C ₂	+	+++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture D ₁	+	+++	++	T	T	T		+	+	+	+	+	+	+	+	+	+	+	+	+
Culture D ₂	+	+++	++	T	T	T		+	+	+	+	+	+	+	+	+	+	+	+	+
Culture D ₃	+	+++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture X ₁	-	+++	++				-													
Culture X ₂	-	+++	++				-													
Culture X ₃	-	+++	++				-													
Culture a ₁	-	-	T																	
Culture a ₂	-	-	T																	
Culture a ₃	-	-	T																	
Culture b.....	-	-	T																	
Culture c.....	-	-	T																	

* In this test the five media were inoculated separately on different days.

These fifteen cultures were tested upon synthetic nitrate media (tests 2-5, table 6), the same media used for the fluorescent organisms. Medium DM (test 2) gave rather surprising results, as nitrite was absent in all but two cases, and in one of these two cases was present in mere traces. This was not due to poor growth in all cases, for very good growth was obtained with cultures A and B and with all substrains of C and D; whereas culture X₂ grew poorly but gave the nitrite test. A later repetition of this test (see table 7) with A, B and all substrains of X showed nitrite to be produced by cultures A and B, but that the nitrite reaction with culture B disappeared after the first day. This suggests that the meaning of the negative reaction in test 2 (table 6) may have been in many cases that the cultures were examined after the nitrite had disappeared.

The poor growth of strains X, a, b, and c, noticed on this medium, was observed on all the other synthetic agars used.

This was undoubtedly due to the fact that these media contained no added organic matter except the sugars; for these four strains were found to be unable to attack any sugar.

The cultures were then tested on medium DL (test 3, table 6). A distinct nitrite reaction was obtained promptly with cultures A, B, C₁, C₂, D₁, D₂, and D₃ and on the seventh day with the non-chromogenic strain X₂. No trace of nitrite was observed with any of the cultures which had not produced nitrite in the earlier tests. The correlation between vigor of growth and strength of the nitrite reaction was quite marked. Test 4 was made upon medium S, containing sucrose instead of glucose and lactose, sucrose being used because of its freedom from ammoniacal impurities. Tests for both nitrite and ammonia were made. The results were very striking. Nitrite (at least in traces) was observed with every culture except one of the substrains (X₁) of the non-chromogenic strain. Appreciable amounts of ammonia were observed with every culture, showing nitrate-reduction to have occurred in all cases. As in the previous test, vigor of growth was distinctly correlated with the strength of the nitrite reaction, growth in this case tending to be rather better than on the lactose-glucose medium.

TABLE 7

Nitrate reduction by the orange chromogenic *Pseudomonas* in 0.1 per cent nitrate agar. Second test.* Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, - absent; ? doubtful.

	MEDIUM P.				MEDIUM DM.				MEDIUM D.				MEDIUM DL.				MEDIUM S.				MEDIUM DA.				
	Nitrite present in				Nitrite present in				Nitrite present in				Nitrite present in				Nitrite present in				Nitrite present in				
	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	
Culture A...	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture B...	T																								
Culture E...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture F...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture G...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Culture X ₁ ...				+																					
Culture X ₂ ...																									
Culture X ₃ ...																									
Culture X ₄ ...																									

*In this test the six media were inoculated simultaneously.

Later (test 5, table 6) a few of the cultures were tested upon agar (medium DA) containing ammonium chloride as well as nitrate and glucose. Nitrite was found with all seven of the strains that showed nitrite on nitrate-peptone agar (medium P), also with culture b, the one strain tested that had not shown nitrite on medium P, but not with the one substrain (X_2) of the non-chromogenic organism that was tested.

A few months later those cultures that were still alive were tested again (table 7), together with a few other strains of the same organism. This is a very difficult organism to keep living under laboratory conditions, and only cultures A, B, X_1 , X_2 , and X_3 were still alive. The strains which had failed to produce nitrite on the medium P had all died. The new cultures studied were:

E }
F } Three recent isolations from soil, all giving a strong nitrite
G } reaction on Medium P when first isolated.

X_4 A fourth substrain of the non-chromogenic strain, obtained by inoculating X_3 into sterile soil and reisolating after a few weeks.

These nine cultures were inoculated into the same media used in the earlier test and also into medium D.

The only essential difference noted from the results of the earlier tests was that the non-chromogenic cultures had now lost their power of producing nitrite upon any of the media tested, even including medium P. Cultures A, X_1 , X_2 , and X_4 still gave distinct ammonia reactions in medium S, and X_3 showed a trace of ammonia.

The conclusion drawn from these tests is that this pseudomonad is neither like the colon organisms nor like the fluorescent bacteria in respect to the nitrate-reduction test. Some available organic matter must be present in order to allow good growth; and yet it is possible for some strains to produce good growth without giving the nitrite test. There is evidence that in some cases, at least, this is because the nitrite is converted into ammonia as fast as produced, so that there is no accumulation of nitrite.

CONCLUSIONS

As a result of this work the conclusion was drawn that the nitrate-reduction test, as made by testing for nitrite in "standard" nitrate broth after a definite period of incubation, is not as simple as generally supposed. This test is open to several sources of error:

1. *Poor growth.* Any organism must be tested in some medium in which it makes good growth. If it grows poorly, the results are likely to be variable (as with the colon organisms in media containing less than 0.2 per cent peptone), and under such conditions absence of nitrite is of no significance. It is probably impossible to find any one medium in which all bacteria make satisfactory growth.

2. *Presence of more readily available nitrogen.* Some of the cultures studied (fluorescent pseudomonads) seem to be able to reduce nitrate only in the absence of ammoniacal (or amide) nitrogen. Their ability to reduce it does not show, therefore, on ordinary peptone media. Such behavior may sometimes be of diagnostic importance; but the fluorescent cultures studied which showed this characteristic differed in no other observed respect from typical *Ps. fluorescens* (vigorous nitrate-reducer) and are not thought to belong to a separate species.

3. *Reduction without accumulation of nitrite.* Some organisms (like certain strains of "*B. caudatus*") utilize the nitrite as fast or almost as fast as produced. It may thus be assimilated, converted into ammonia, or converted into free nitrogen. Free nitrogen can generally be detected by gas bubbles in the liquid or cracks in the agar. Ammonia can be detected only if the organism is growing in an ammonia-free medium containing no source of ammonia other than the nitrite; but many organisms are unable to grow under such conditions. Assimilation of the nitrite (either as nitrite or as ammonia) cannot be detected by any simple test.

In case of each of the four species (or groups of species) studied, a different explanation was found necessary to account for cultures showing no nitrite. Only in one case, that of *B. cereus*, did

investigation show the possibility of two species being concerned, one differing from the other in its ability to reduce nitrate. Inasmuch, therefore, as nitrate-reduction on any medium or under any condition whatsoever indicates an organism as a nitrate-reducer, the general conclusion of the present work is that no organism can be safely called a non-nitrate-reducer except as the result of exhaustive tests, too time-consuming to be made in routine bacteriological investigations.

REFERENCES

- AMERICAN PUBLIC HEALTH ASSOCIATION 1905 Report of committee on standard methods of water analysis. *J. Inf. Diseases*, Suppl. 1, 1-27. See p. 109.
- AMERICAN PUBLIC HEALTH ASSOCIATION 1912 Standard methods for the examination of water and sewage. Second Edition. New York. See p. 135.
- BREED, R. S. 1915 The standard method of determining nitrate reduction. Paper read at Philadelphia meeting of Soc. of Amer. Bacteriologists *Abstr. in Sci.*, N. S. **41**, 661.
- CHESTER, F. D. 1901 Manual of determinative bacteriology. New York. See p. 34.
- CONN, H. J. 1917 The spore-forming bacteria in soil. *N. Y. Agr. Exp. Sta.*, Tech. Bul. 58.
- CONN, H. J., AND BRIGHT, J. W. 1919 The ammonification of manure in soil. *N. Y. Agr. Exp. Sta.*, Tech. Bul. 67.
- GOHRAM, F. P. 1901. Laboratory course in bacteriology. Philadelphia. See p. 92.
- HARDING, H. A. 1910 The constancy of certain physiological characters in the classification of bacteria. *N. Y. Agr. Exp. Sta.*, Tech. Bul. 13. See p. 34.
- KLIGLER, I. J. 1913 The source of ammonia and nitrites in a nitrate-peptone solution. *Amer. J. Pub. Health*, **8**, 788-792.
- SMITH, ERWIN. 1905 Bacteria in relation to plant diseases. Washington. See p. 63.
- WRIGHT, J. H. 1895 Report on the results of an examination of the water supply of Philadelphia. *Nat. Acad. Sci.*, *Memoirs*, 7, 422-482. See p. 444.