FACTORS INFLUENCING THE REDUCTION OF NITRATES AND NITRITES BY BACTERIA IN SEMISOLID MEDIA

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Although the ability to reduce nitrates has been used for the identification of bacteria since the extensive investigations of Maassen (1902), the literature is still replete with inconsistencies and contradictory findings. This may be illustrated by observations on members of the Brucella group which have been reported. Eyre (1912), Duncan and Whitby (1930) and Bergey (1930) have described Brucella as being unable to reduce nitrates. On the other hand Evans (1918), Lustig and Vernoni (1928) and Topley and Wilson (1929) have asserted that nitrates are occasionally reduced. In the recent experiences of ZoBell and Meyer (1931) every strain of a collection of over 400 typical representatives of the genus Brucella vigorously reduced nitrates. Studies on this biochemical character in the "Salmonella" group are no less perplexing. Tittsler (1930) has summarized similar diametrically opposite descriptions in the literature concerning the ability of *Bact. pullorum* and *Bact. gallinarum* to reduce nitrates. Lack of agreement on the nitrate-reducing properties of many other common microörganisms has been reported.

In view of these incompatibilities a series of studies was undertaken to ascertain the cause of the incongruity. Either the testing procedures are at fault, thus leading to discordant results; or the ability of bacteria to reduce nitrates is inconstant; or certain strains of a given variety reduce nitrates while other strains lack such a mechanism. Regardless of which is true, little reliance can be placed upon the test for the differentiation of

bacteria until these problems are solved. The present communication is primarily concerned with certain factors which influence the reduction of nitrates by microörganisms which have not been stressed by others.

EXPERIMENTAL

Approximately 600 cultures collected from various parts of the world were examined. Brucella and Salmonella predominated and two or more of 22 other common microörganisms were represented. A medium of the following composition was employed:

Bacto-peptone	2.0 grams
Beef-extract	1.0 gram
NaCl	3.0 grams
Agar	3.0 grams
KNO_3	1.0 gram
H_2O	1,000.0 cc.

The reaction was adjusted to pH 6.8. Preliminary experimentation indicated that the composition of the medium made very little difference except in nutrient properties as indicated by the multiplication rate. Practically any medium which supported a prolific growth was satisfactory. In exception to this, substances which alter the oxidation-reduction potential of the medium were important, as will be discussed later. The tubed media were uniformly inoculated with a 2 mm. loopful of a standardized suspension of the test organisms. Thus, each tube received approximately the same number of cells, objectionable clumps were avoided and the subsequent results were comparable. Nitrites were detected by the a-naphtholamine-sulfanilic acid method as described by Conn, *et al.* (1918) and the results were checked by the dimethyl-a-naphtholamine reagent recommended by Wallace and Neave (1927). In the absence of nitrites, nitrates were tested for by the zinc-reduction method: To the acidulated substrata containing the nitrite reagents about 20 mgm. of nitrate-nitrite-free zinc dust were added. The development of a pink color indicated the presence of nitrates. The importance of testing for nitrates when the nitrite test is negative merits emphasis. The procedure is advo-

cated as an auxiliary test by the Standard Method (1930) when other tests have failed.

It was found that many of the Brucella and Salmonella types responded negatively when tested for the presence of nitrite after their growth in nitrate media. In routine practice this would usually be considered as evidence of their inability to reduce nitrates. However, an application of the zinc-reduction test showed that the nitrates were likewise absent from the substrata. In fact many of these cultures affected the disappearance of 0.1

TABLE 1

The presence of gas, nitrites and nitrates in media following the growth of bacteria for four days

BACTERIUM	DESIGNATION	GAS	NITRITES	NITRATES
Bact. sanguinarium	3139	−	−	−
	4187	−	−	+
Bact. pullorum	1618	−	+	++
	Utah	−	−	−
Br. abortus	7122	−	+++	++
	Th. 10	−	+++	++
Br. suis	Da 87	+	−	−
	H. F. 4	+	−	−
Ps. pyocyanea	U. C.	+++	−	−
Ps. denitrificans	Iowa	+++	−	−
B. subtilis	No. 2	−	−	++++
Blank	Control	−	−	++++

per cent KNO_3 after two days of incubation. The only indication of the destruction of the nitrate molecule was the formation of gas by certain species. Table I illustrates typical findings.

The fallacy of interpreting these data without information as to the presence of nitrates is self-evident. Even the minute amount of gas liberated was not perceptible except in semisolid media. Active nitrate-reducing organisms would have been considered as non-reducers. The Standard Method (1930) was designed to avoid this source of error by providing for the determination of nitrites on the first, second and fourth days. However,

the careful scrutinization of a large number of cultures under different conditions has shown that many bacteria and particularly *suis* type Brucella and certain forms of Pseudomonas concurrently destroy the nitrites as they are formed. Only at irregular intervals does the concentration of nitrites become sufficiently high to give a positive test even with sensitive reagents. Some of the microörganisms cause the quantitative disappearance of the nitrite ions of 0.01 per cent KNO_2 more rapidly than they reduce an equivalent amount of nitrate. Bronfenbrenner and Schlesinger (1920) recognized this factor and suggested the use of a control containing 0.002 per cent KNO_2 or $NaNO_2$. The standard Method (1930) now recommends a medium with 2 p.p.m. KNO_2 as an alternative when other methods have yielded inconclusive findings in order to ascertain if the bacteria destroy nitrites. Apparently a large number of common bacteria are endowed with this ability, ZoBell and Meyer (1932) recently pointed out that this property which obtains in the Brucella organism to a marked extent has probably misled some investigators who have described members of the genus as non-reducers. Present experiences reveal that nearly all strains of *Bact. pullorum, Bact. gallinarum, Bact. sanguinarium, Bact. aerogenes* and *Bact. saccharolyte* can cause the destruction of as much as 0.01 per cent $NaNO_2$ in five days. It is not unlikely that this factor is largely responsible for contradictory reports concerning the first two Salmonellas referred to above. Some bacterial species were encountered which destroy nitrites without exhibiting any aptitude for reducing nitrates. Thus *B. fecalis-alcaligenes* and *Vibro metchinokovi* were found to be in this category. Therefore it appears advisable to include in the regularly prescribed tests on the "Descriptive Chart" the test for the ability of bacteria to break down nitrites. Aside from serving as an indispensable adjunct to the nitrate reduction test, it would also furnish valuable supplementary biochemical criteria for the characterization of microörganisms.

That the physical consistency of the media is a factor of prime importance in bacterial growth and metabolism was demonstrated by the preparation of media of three different viscosities. The

first series consisted of firm gels containing 2.0 per cent agar, the second of gels of semisolid consistency with 0.3 per cent agar, while in the third no agar was used. Following the inoculation of these media with a uniform seeding of bacteria, evidence of multiplication first became perceptible in the semisolid series and continued to appear better in this medium. Some of the microaerophilic pathogens which were included in the experiment grew in the semisolid media but failed to proliferate in the liquid or solid media although the latter were identical in composition except for the gel structure. That the physical consistency of the media likewise influences the reducing activities of the bacteria

TABLE 2

Nitrate reduction after two days' incubation of bacteria in media of different viscosities and depths

BACTERIUM	DESIGNATION	LIQUID		SEMISOLID		SOLID	
		5 mm.	50 mm.	5 mm.	50 mm.	5 mm.	50 mm.
Bact. pullorum	No. 9	−	++	+	+++	+	++
Bact. aertrycke	N. Y.	−	++	++	++++	++	+++
Bact. aerogenes	No. 2	++	++	++	+++	++	+++
Bact. paratyphosum	Pigeon	+	+	+	+++	++	+++
Strept. fecalis	Calif.	−	−	−	−	−	−
Br. suis	No. 80	++	++	+	++++	++	+++
Br. abortus	F 16	−	−	−	++	−	+
Br. melitensis	Africa 1	−	−	−	+	−	−

was shown by the addition of redox indicators such as methylene blue and nitroanthraquinone in the proper dilution. The latter indicator was found to be preferable, since upon reduction the color passes through definite tinctorial stages from a colorless to a brownish-red compound. Invariably the dyes were reduced more rapidly in the semisolid substrata than in the solid or liquid media, probably due to the differential diffusion of atmospheric oxygen. The effect of the physical structure of the media on the reduction of nitrates by bacteria is illustrated by the data given in table 2. To demonstrate further the influence of the diffusion of atmospheric oxygen, the media were tubed so that the columns

were 5 and 50 mm. deep respectively. Although the differences were not always clear-cut, conclusive tendencies were shown. Nitrate reduction was better when the columns were 50 mm. deep than when there were only 5 mm. of media in the tube; reduction in degree and in percentage of positive tubes was highest in semisolid media and lowest in liquid media. The advantages of the deeper columns and of the semisolid consistency are directly attributable to their influence on the oxidation-reduction potential of the media as will be discussed more fully in a later communication. The available evidence stresses the important influence of the oxidation-reduction potential on the multiplication of bacteria (Hewitt (1930)). It is to be expected that the oxidation-reduction potential has even a greater effect upon nitrate reduction, a reaction which depends upon the electron-escaping tendency or fugacity of the system. This is indicated by the studies of Korsakova (1929) which deal with the mechanism of the reduction of nitrates. The reports of Tiulpanova-Mosevich (1930) indicate that *Thiobacillus denitrificans* loses much of its nitrate-reducing ability when cultivated under aerobic conditions, probably due to the unfavorable oxidation-reduction potential activated by the oxygen.

DISCUSSION

For the routine examination of bacteria for their nitrate-nitrite splitting propensities the following procedure has yielded excellent results: The organisms are cultured in semisolid media containing 0.1 per cent KNO_3 and the necessary nutrient constituents to insure their multiplication. Instead of testing for the presence of nitrites on any prescribed day, the test is performed only after good growth has occurred and the time required for this will vary tremendously with different microörganisms. Either a-naphtholamine or dimethyl-a-naphtholamine with sulfanilic and acetic acids have been consistently satisfactory. If no color appears after a few minutes to indicate the presence of nitrites, about 20 mgm. of zinc dust are added directly to the substrata which already contains the nitrite reagents. Nitrates, if present, are thereupon shown by the development of the pink color.

Another medium which contains 0.002 per cent KNO_2 is always inoculated and tested to see if nitrites are destroyed.

Applying the principles described above, it has been found that the nitrate-reducing ability of microörganisms is a fairly constant characteristic. Results from the examination of the 600 cultures have been duplicated and repeated six months later with an accuracy approaching 100 per cent. Strains of given varieties collected from various parts of the world were quite alike in their ability or lack of ability to reduce nitrates. Thus, every strain of 420 Brucella reduced nitrates while others, like representatives of the sarcinae and certain streptococci, were uniformly negative. Therefore it seems that if precautionary measures are taken, absolute reliance can be placed on the nitrate tests for the characterization of bacteria. The ability of some microörganisms to destroy nitrites concomitantly as they are produced from nitrates is probably responsible for many contradictory findings and provisions must be made to avoid this source of error.

The superiority of semisolid media for the cultivation of bacteria recommends this physical consistency for the nitrate reduction test. Semisolid media combine most of the advantages of solid and liquid media and are for most purposes easier to handle than either of the latter. In summarizing some of the advantages of media containing small percentages of agar Hitchens (1921) pointed out that since such a medium retards the diffusion of oxygen, any degree of aerobiosis is procurable at some level of the medium column. Semisolid media permit the movement of motile organisms and the ready diffusion of nutrients and waste products. Hitchens found that many fastidious parasites which failed to multiply in liquid or solid media grew luxuriantly in semisolid media. Media which contain 0.2 to 0.3 per cent agar are particularly useful for studying the reduction of nitrates because in addition to enhancing multiplication they also favor the reducing activities of microörganisms. Many bacteria have been encountered which, although they fail perceptibly to reduce nitrates in liquid or solid media, react in semisolid media. Furthermore semisolid media are good indicators of gas production. Due to the viscosity which retards convection currents gas

bubbles are visibly retained while in liquid or solid media minute amounts of the gas frequently escape unobserved. Also, the relationship of the organisms to atmospheric oxygen can be ascertained by noting the zone at which there is maximum proliferation. Aerobes will grow on or near the surface while facultatives and anaerobes will be found proportionately deeper. Thus it can be determined whether the organisms are able to derive their oxygen demands from the nitrate molecule as manifested by pseudo-anaerobic growth. All of these points furnish additional criteria for the identification of species. Finally, in testing for the presence of nitrates by the zinc method, the gel causes the zinc dust to be suspended throughout the medium and thus increases its sphere of action.

CONCLUSIONS

1. Approximately 600 strains of bacteria which have been examined were found to be constant in their ability to reduce nitrates.

2. Many species of microörganisms destroy nitrites as they are formed from nitrates so that nitrite tests are frequently negative although nitrates have been reduced. This property which obtains in the Brucella and in the Salmonella groups is in part responsible for the contradictory reports concerning their nitrate reducing ability.

3. It is recommended that a test for the presence of nitrates invariably be made in conjunction with the test for nitrites when the latter is negative.

4. The amplified test for the ability of bacteria to reduce nitrites is of sufficient import to warrant its inclusion in the Descriptive Chart.

5. The multifold advantages of semisolid media for the nitrate reduction test warrants its use as a Standard Method.

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