

NITRITE REDUCTION BY *BACTERIUM DENITRIFICANS* IN RELATION TO OXIDATION-REDUCTION POTENTIAL AND OXYGEN TENSION

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Gaseous losses of nitrogen (N_2 or N_2O) from bacterial culture media and soils maintained under conditions commonly considered aerobic have been observed frequently (Meiklejohn, 1940; Corbet and Wooldridge, 1940; Korsakova, 1941; Broadbent, 1951; Broadbent and Stojanovic, 1952; Marshall *et al.*, 1953; and Collins, 1955). Not all workers, however, have considered such observations as proof that denitrification can occur under aerobic conditions, since the dissolved oxygen contents of the media were not determined. Furthermore, a low partial pressure of oxygen will often markedly inhibit denitrification by young cultures of bacteria, as has been shown by Sacks and Barker (1949), Wijler and Delwiche (1954), and repeatedly confirmed in this laboratory. Skerman *et al.* (1951) are probably the only workers who have measured the oxygen content of cultures. They observed no nitrate reduction in cultures of *Pseudomonas* if dissolved oxygen was present. Nitrite reduction was not studied. Arnold (1954) observed no evolution of nitrous oxide from well aerated soils but did obtain it where moisture was present in excess. Undoubtedly the varying results can be explained in part by variations among bacterial species and strains, and by the conditions under which the organisms were grown prior to study.

When oxygen is present not only does it compete with nitrate or nitrite, and thus reduces denitrification, but also it maintains the medium at a higher oxidation-reduction potential than would otherwise occur. This potential is also affected by the metabolic products excreted by the bacterial cells. The question arises, therefore, as to whether the inhibiting effect of oxygen on denitrification may not be due in part to the raising of the potential to a level that may be too high to permit nitrite reduction to occur. Very few studies of this type have been reported. Elema *et al.* (1934) found that *Micrococcus denitrificans*, when grown anaerobically, excreted metabolic products into the medium that estab-

lished a reversible oxidation-reduction system, but reported no aerobic studies. In work with *Bacterium denitrofluorescens*, Korochkina (1936) found that a high potential did not prevent denitrification.

Data are presented below on (a) the effect of various partial pressures of oxygen on nitrite reduction by a typical denitrifying bacterium, (b) the extent to which growth at different oxygen levels affects the reducing conditions in the medium, and (c) the importance of oxidation-reduction potential on the process.

MATERIALS AND METHODS

The organism used in these studies was *Bacterium denitrificans* Chester (NCTC strain 1656), obtained from N. R. Smith, formerly of this laboratory. It is listed in Bergey's *Manual of Determinative Bacteriology*, 6th ed., under the Appendix to Family IX, *Achromobacteriaceae*.

Since this organism grows poorly on all synthetic media tested, it was necessary to use a complex medium that had some poisoning effect. In all studies reported below a medium of the following composition was employed: sodium succinate, 0.85 per cent; peptone (Difco), 0.5 per cent; sodium nitrite, 0.1 per cent; and meat extract (Difco), 0.3 per cent.

The procedure used for obtaining active growing cells was to inoculate 100 ml of sterile medium contained in a 250-ml Erlenmeyer flask with 1 ml of a 24-hr culture. Air was replaced with purified nitrogen gas, the flask sealed, and incubated anaerobically for 24 hr at 28 C. Before placing the culture in the apparatus described below, it was adjusted to a turbidity of 85 per cent transmission (515 $m\mu$) by adding sterile medium.

Resting cells were prepared by inoculating 250 ml of culture medium contained in 500-ml Erlenmeyer flasks, plugged with cotton, with 2 ml per flask of a 24-hr culture. After incubation for 24 hr at 28 C, the cultures were centrifuged, washed, and suspended in M/15 molar phosphate buffer of

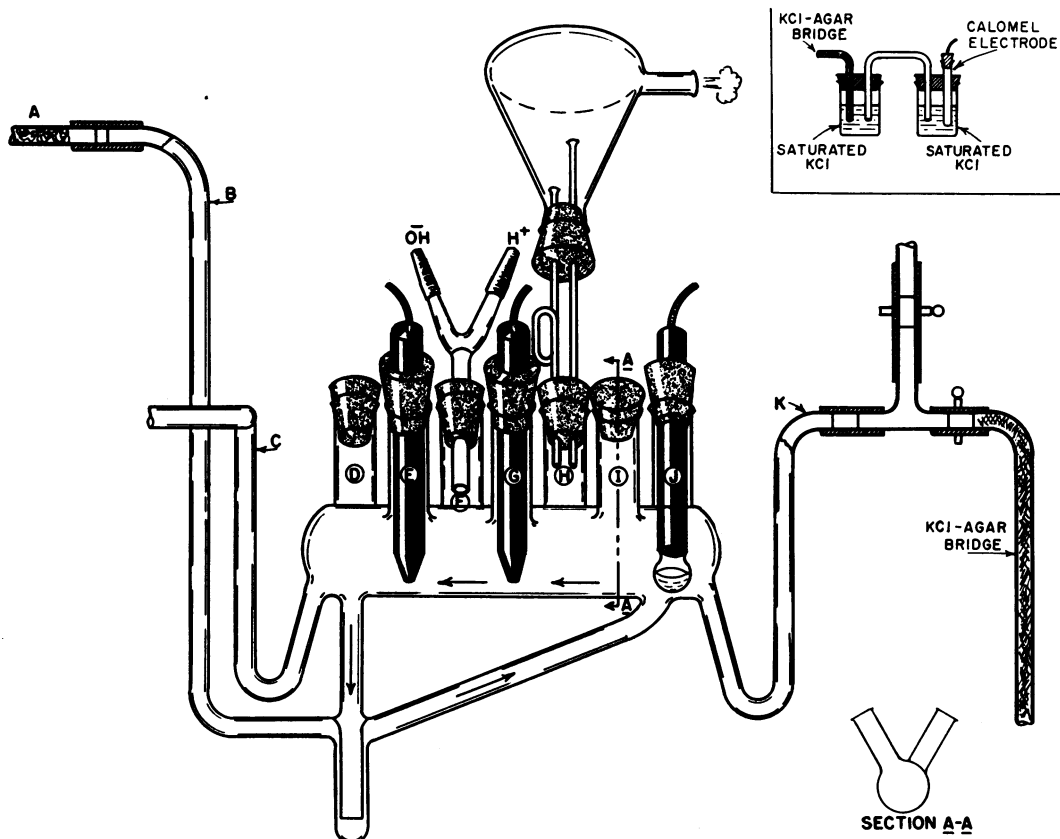


Figure 1. Culture vessel with additional apparatus used for the study of nitrite reduction, growth, and oxidation-reduction potentials in bacterial cultures.

pH 8, and the cell suspension adjusted with buffer to the desired per cent transmission. To these cell suspensions were added 0.1 per cent sodium nitrite and 0.85 per cent sodium succinate.

The culture vessel, shown in figure 1, is a specially constructed apparatus designed to provide complete aeration and stirring of the medium. It is a modification of the vessel employed by Hanke and Katz (1943). When filled to the desired level, which is a little more than half capacity, it contains 85 ml of medium. The different parts of the apparatus, designated by letters, are as follows: *A*, cotton-plugged tube for sterilization of the incoming gas used in aeration; *B*, gas inlet tube; *C* and *K*, medium bridges for contact with KCl-agar bridge (*S*); *D*, extra outlet; *E* and *G*, Beckman platinum wire electrodes; *F*, a Y-tube which is connected to acid and base containing burettes; *H*, a gas outlet and defoaming attachment (which, of course, is not needed if frothing is at a minimum); *I*, sampling outlet; and *J*, a

glass electrode. The apparatus is autoclaved with all fittings except the electrodes, which are sterilized separately. Clamps at *C* and *K* are closed and the agar portion of the bridge is placed in a small flask containing a saturated KCl-agar solution. Air is displaced during autoclaving, and upon cooling the fluid rises in the bridge tube. After solidification of the agar, the flask is removed and the tube is immersed in the saturated KCl solution that is connected by a U-bridge to a second KCl vessel containing the calomel half cell. A layer of mineral oil covers the KCl to prevent creeping of the salt. The platinum electrodes are cleaned with benzene, carbon tetrachloride, and sulfuric acid-dichromate solution, and are thoroughly rinsed after each operation. The electrodes are then immersed in mercuric chloride (1:1000) for 10 min, rinsed with sterile distilled water, and placed in the apparatus.

Sterile medium is inoculated with the standard

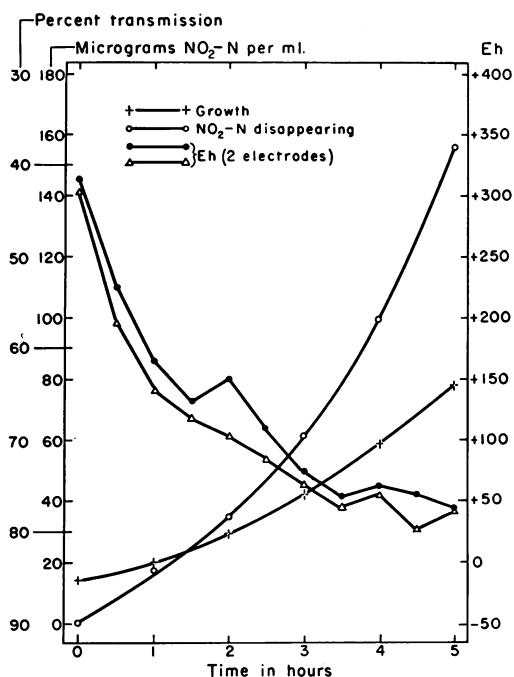


Figure 2. Growth, decrease in nitrite, and changes in oxidation reduction potential in a culture aerated with pure nitrogen gas.

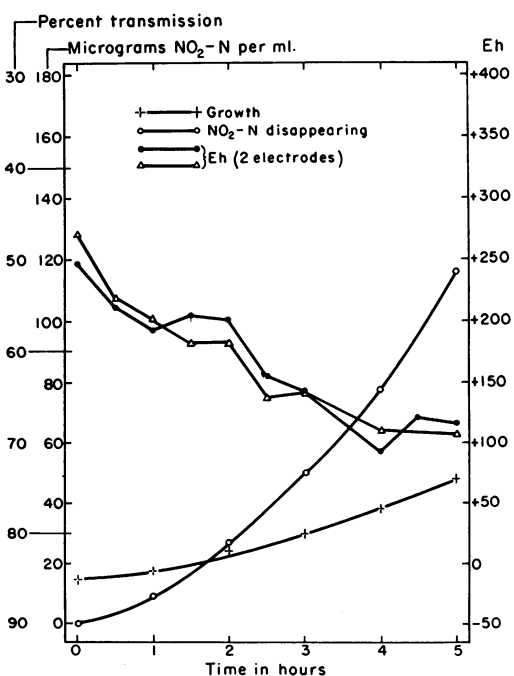


Figure 3. Growth, decrease in nitrite, and changes in oxidation reduction potential in a culture aerated with 0.5 per cent oxygen in nitrogen gas.

24-hr culture and adjusted to a turbidity of 85 per cent transmission using an Evelyn "photoelectric micro-colorimeter" and a 515-m μ filter. An 85-ml portion of this bacterial suspension is then placed in the apparatus, the clamps at *C* and *K* are removed, and the medium drawn into the bridge by means of the T-joint. Air is readily displaced through this joint which is then clamped, and the contact between the medium and the KCl-agar bridges is thereby maintained.

Where attempts were made to control the oxidation reduction potential of the culture medium electrolytically, as done by Hanke and Katz (1943), the apparatus was altered to contain a platinum foil electrode at *D*, and the customary KCl-agar bridge at *C*. An additional KCl vessel from the bridge at *K* contained another platinum foil electrode, and current was supplied from a 45-volt dry cell battery. Control of the current was realized through use of a fine adjustment rheostat. The current was cut off during pH and oxidation reduction potential readings. Since this method of controlling the potential of the medium was not satisfactory for the problem under investigation because of dissociation of the nitrite, only brief reference is made to it below.

All experiments were conducted at 28 C, and at pH 8.0, which was controlled by additions of 0.5 N HCl or NaOH as required. Before adopting a pH of 8.0, several experiments were conducted in which growth and denitrifying capacity were measured at various oxygen tensions and pH levels. It was found that, independent of the oxygen tension, the organism showed no variation in activity between a pH of 7.0 and 8.5; growth was negligible at pH 6.5 or below, and activity was diminished at pH 9.0. The gas mixture, which was introduced at the maximum practical rate, produced excellent stirring and mixing. At hourly intervals a sample of the medium was removed for nitrite-nitrogen analysis and for turbidity determination.

All oxidation reduction potential measurements were made on a Beckman "model H-2 pH meter" adapted for this determination. Readings were made usually at intervals of 15 or 30 min. All Eh readings (referred to the normal hydrogen electrode as zero) are expressed in millivolts.

Quantitative nitrite-nitrogen analyses were made by the usual Griess-Ilosvay method using an Evelyn photoelectric macro-colorimeter and the 515-m μ filter.

RESULTS

In figures 2 to 6 are shown typical 5-hr growth and nitrite reduction curves for *B. denitrificans* grown at oxygen partial pressures between 0 and 20 per cent. Included also on the figures are curves showing the oxidation-reduction potentials recorded on duplicate electrodes. Growth increased as the partial pressure of oxygen increased except that at the 0.5 per cent level it was slightly poorer than where none was present. Oxygen markedly inhibited denitrification; at 0.0, 0.5, 6, 10 and 20 per cent oxygen the μg of nitrite-nitrogen per ml reduced in 5 hr were 156, 117, 29, 5 and 0, respectively. The oxidation reduction potential curves show a consistent tendency for the Eh to drop with time at all constant oxygen partial pressures. Nitrite reduction occurred at potentials of approximately +200 mv and lower, but there was no indication that the potential is a determining factor. Duplicate electrodes usually gave closely agreeing results indicating that substances were being given off that tended to poison the potential of the medium.

Attempts were made to maintain the potential electrolytically, as done by Hanke and Katz

(1943), but the quantity of current required to hold a low Eh value was sufficient to destroy much of the nitrite. Instead, varying oxygen-nitrogen mixtures (Knight, 1930) were used. Growth and nitrite utilization curves at controlled potentials of approximately Eh +140, +200, and +340 mv are given in figures 7 to 9. The quantities of nitrite reduced in 3 hr in the order of increasing Eh values were 44, 20, and 13 μg per ml, respectively. These results would seem to eliminate potential as a limiting factor in nitrite reduction by *B. denitrificans*. Obviously this organism uses oxygen preferentially over nitrite just as Skerman *et al.* (1951) observed with *Pseudomonas* grown on nitrate.

Denitrification studies were also conducted with "resting," or nonproliferating cells, which under anaerobic conditions showed immediate rapid denitrification. In two such experiments, 50 and 64 μg of nitrite-nitrogen were denitrified in 3 hr, and 84 and 101 μg in 5 hr. In these experiments the Eh readings were erratic since washing had removed most materials that act as poisoning agents. Where the potential was poised by the Knight method at near +45 mv, 20 μg of nitrite-

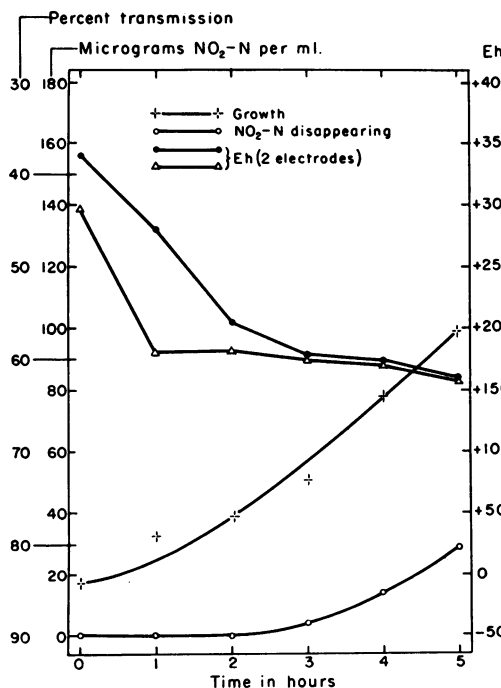


Figure 4. Growth, decrease in nitrite, and changes in oxidation reduction potential in a culture aerated with 6 per cent oxygen in nitrogen gas.

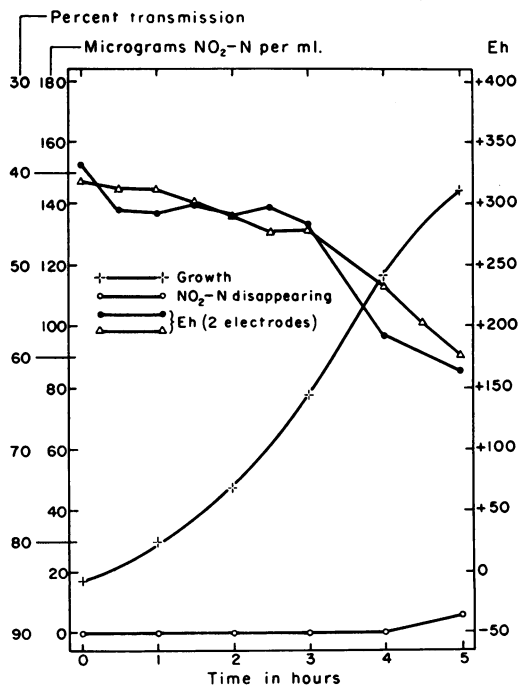


Figure 5. Growth, decrease in nitrite, and changes in oxidation reduction potential in a culture aerated with 10 per cent oxygen in nitrogen gas.

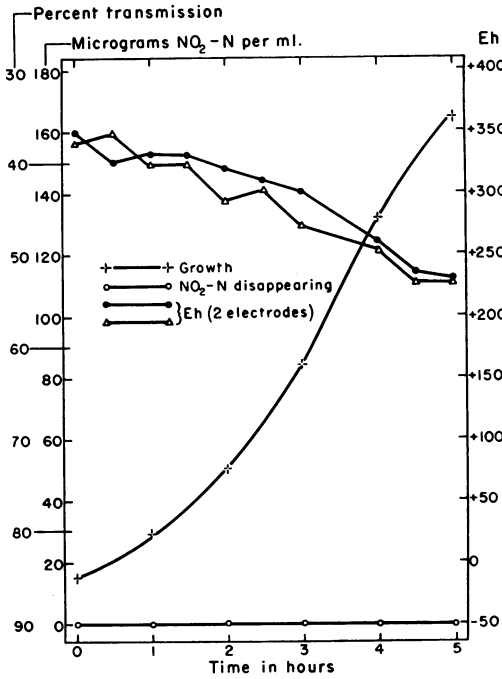


Figure 6. Growth, decrease in nitrite, and changes in oxidation reduction potential in a culture aerated with 20 per cent oxygen in nitrogen gas.

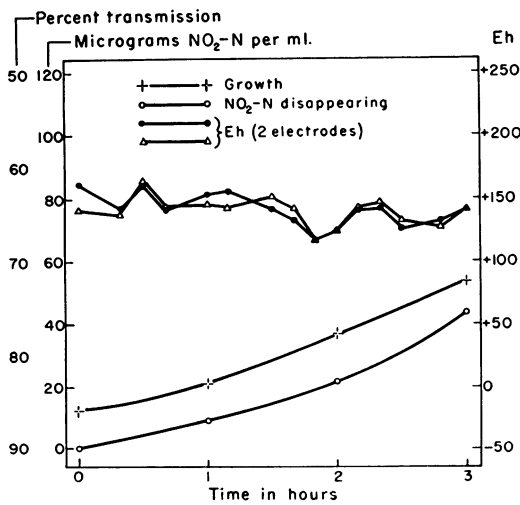


Figure 7. Growth and decrease in nitrite in a culture having the potential controlled at approximately Eh + 140 mv.

nitrogen per ml was reduced in 3 hr by the resting cells. Where the Eh was held at near +350 mv only slightly less denitrification occurred even though more oxygen was needed to maintain this potential than was required at the +45 mv level.

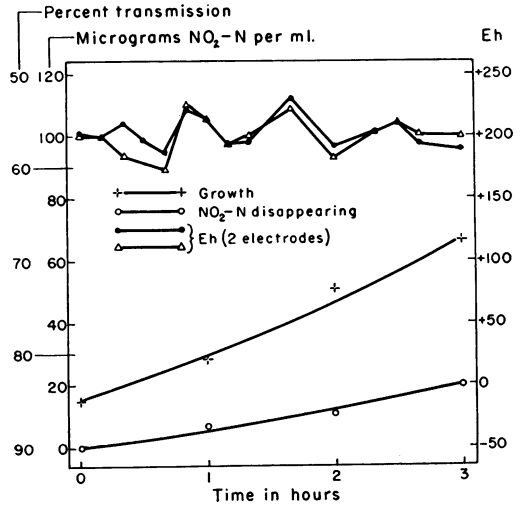


Figure 8. Growth and decrease in nitrite in a culture having the potential controlled at approximately Eh + 200 mv.

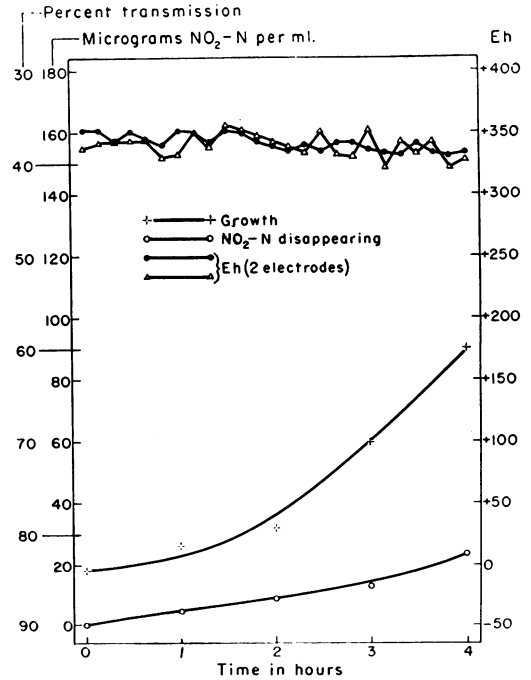


Figure 9. Growth and decrease in nitrite in a culture having the potential controlled at approximately Eh + 340 mv.

DISCUSSION

The only fact brought out above that seems to need further discussion is the matter of denitrification in the presence of oxygen.

In the present experiments excellent aeration

is believed to have been provided, since the specially designed apparatus permitted a large volume of gas to flow through all parts of the medium with vigorous agitation. The effectiveness of the aeration is shown in figure 6, where, in spite of the heavy cell concentration, the dissolved oxygen content of the culture medium was high enough to prevent nitrite reduction. Furthermore, the growth rate was rather constant for 4 hr until the cell concentration became so heavy that a logarithmic growth rate could no longer be expected.

The data of Skerman *et al.* (1951), using a polarograph to determine oxygen, may also be cited as possible evidence of excellent aeration in our experiments. In their studies, which were in some respects similar to those reported here, they found that approximately 18 sec were required for a heavy 8-hr culture of *Pseudomonas* to reduce the oxygen concentration of the medium from 8 ppm (saturated with air) to zero, and the decrease was uniform per unit of time; the corresponding period for a 12-hr culture was 6 sec. The initial concentration (8 ppm) could be restored by vigorous aeration, sometimes with air and sometimes with oxygen, within 2 to 3 min. It seems, therefore, that in our vigorously aerated cultures nitrite reduction occurred in culture media that were nearly in equilibrium with the oxygen in the gas phase. Furthermore, reduction was brought about by aerated resting cells where the rate of removal of oxygen from the cell suspension was much less. It is well to point out that these conclusions do not conflict with the findings of Skerman *et al.* (1951) that denitrification occurred only when the oxygen concentration was "zero." They studied nitrate reduction only and used a different organism than that employed in the present studies. Furthermore, regardless of their emphasis on "zero" oxygen, these Australian workers point out that "an organism may use two alternative respiratory mechanisms at the same time, provided conditions are satisfactory for both." In connection with their own experiments they state that "it is obvious that oxygen must still be entering the solution and be utilized by the cells even though the oxygen concentration is at a 'zero' level, which permits simultaneous utilization of nitrate." In other experiments they observed that nitrate reduction decreased as oxygen supply increased, as Sacks and Barker (1949) and others have reported.

Although it was obvious from the data presented above (figures 3 to 5 and 7 to 9) that

simultaneous use of the two hydrogen acceptors, oxygen and nitrite, was occurring, this fact was positively established by manometric and analytical studies conducted according to the usual Warburg technique. In one 2-hr experiment, with a heavy suspension of resting cells, 180 μg of nitrite-nitrogen were used by the organisms, and the manometer showed a decrease of 389 μL in gas volume. If it be assumed that all of the 180 μg of nitrite-nitrogen was converted into nitrogen gas, then the oxygen consumption during the 2-hr period was 533 μL . In anaerobic control vessels gas production from nitrite actually corresponded to 85 per cent of the theoretical maximum. These manometric experiments indicate that oxygen acts as a noncompetitive inhibitor of nitrite reduction, as Stickland (1931) found was true for *Escherichia coli* grown on nitrate, but further research would be needed for definite proof of this statement.

From the practical standpoint, perhaps the most important fact established by the work with *B. denitrificans* is that the organism can use both oxygen and nitrite simultaneously, although the former is utilized preferentially. Whatever the dissolved oxygen level may have been in these studies, nitrite was reduced in cultures that were simultaneously using large amounts of oxygen. In field soils, where denitrification is an important economic problem, presumably anaerobic conditions would not be required for gaseous forms of nitrogen to be released from nitrates or nitrites. It would not be well, however, to assume from laboratory experiments with masses of adapted cells that denitrification under aerobic soil conditions is of major importance.

SUMMARY

Experiments are reported with *Bacterium denitrificans* showing the simultaneous changes occurring in cell numbers, oxidation reduction potential, and nitrite concentration of the medium as a result of growth during 3- to 5-hr periods. In a few experiments "resting cells" were also used. A special growth chamber, which was very effective in the aeration of cultures, is described.

This organism was found to be capable of reducing nitrite to gaseous forms of nitrogen very rapidly. This reduction occurred with both growing and resting cells at potentials held fairly constant at levels varying between Eh + 340 and + 340 mv. Oxidation reduction potential was, therefore, not a limiting factor in controlling the

process, although the bacterial cells excreted materials into the medium that lowered the potential. Attempts to poise the potential electrolytically were unsuccessful, and hence it was necessary to employ aeration with oxygen-nitrogen mixtures.

The organism is capable of using simultaneously both molecular oxygen and nitrite but the former is utilized preferentially. Marked denitrification occurred when cultures were vigorously aerated with a mixture of 6 per cent oxygen in nitrogen gas. Obviously, anaerobic conditions are not required in order for nitrite reduction to occur.

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