

EFFECT OF GASEOUS ENVIRONMENT ON GROWTH AND CATALASE CONTENT OF *VIBRIO FETUS* CULTURES OF BOVINE ORIGIN¹

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Isolation of *Vibrio fetus* is desirable in making a positive diagnosis of vibriosis; however this is often made difficult by (1) the presence of other microorganisms, especially when liquid media are used, and (2) the failure of solid media to support growth of some strains on primary culture under the generally accepted method of incubation in jars in which 5 to 10 per cent of the air is replaced with carbon dioxide. Recent reports of Bryner and Frank (1955a) and Reich *et al.* (1956) show that growth of *V. fetus* of bovine origin is increased in an atmosphere in which the oxygen content is reduced below that of air. The relation of different concentrations of carbon dioxide and oxygen in the gaseous environment to growth remains to be determined. For this reason the effect of atmospheres containing various levels of carbon dioxide, hydrogen, nitrogen and oxygen on growth of *V. fetus* on blood agar was observed. The results are reported here.

MATERIAL AND METHODS

Cultures. Of the 34 cultures used, 32 were isolated from aborted bovine fetuses and two from bull semen. All of the cultures reduced nitrates, were catalase positive and failed to produce hydrogen sulphide.

Culture medium. Blood agar was prepared by adding 10 per cent citrated ox blood to sterile melted nutrient agar (pH 7.0) which was cooled to 45 C. The medium was dispensed in 15 ml amounts into 25 by 150 mm test tubes and slanted to give a standard surface. The slants were incubated at 37 C for 24 hr before use and inoculated by streaking a 4 mm loopful of a 48-hr-old culture in 5 ml of thiol medium (shaken before

use) over the entire surface except the lower one cm.

Gaseous environment. The inoculated blood agar slants were placed in vacuum jars and, with the aid of a mercury manometer, the atmospheres within the jars were adjusted to the desired concentrations of the several gases at 96 per cent of atmospheric pressure. For anaerobic conditions the vacuum jars were evacuated and washed twice with nitrogen, palladium asbestos was placed in the vacuum jars and the nitrogen replaced with 10 per cent carbon dioxide and 86 per cent hydrogen. Carbon dioxide-free atmospheres of mixtures of oxygen, hydrogen and nitrogen were obtained by bubbling the gases through a 30 per cent solution of potassium hydroxide and placing a container of the solution in the vacuum jars.

Determination of growth. After 72 hr incubation at 37 C, growth was washed from each slant with 5 ml of Sorensen's phosphate buffer solution (pH 7.0) containing 0.3 per cent formalin. Turbidity was determined with a Lumetron (Model 400-A) using the red filter (wave length 650 m μ). All tests were made in duplicate and the figures given represent the average of two tests.

Catalase test. Inoculated blood agar slants were incubated in atmospheres containing 10 per cent carbon dioxide and 15, 10, 5, or 1 per cent oxygen, respectively. Nitrogen was added as needed to establish 96 per cent of atmospheric pressure. After 72 hr incubation at 37 C, growth was suspended in normal saline and diluted to 50 per cent of light transmittance using the red filter. Five ml of this suspension was transferred to a Smith fermentation tube and 5 ml of a 3 per cent solution of hydrogen peroxide were added. The amount of liquid displaced by the production of oxygen by the action of catalase was observed after 30 min at room temperature.

Hydrogenase test. Growth from blood agar slants that were incubated at 37 C for 72 hr in an at-

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mosphere of 5 per cent oxygen, 10 per cent carbon dioxide and 81 per cent hydrogen was suspended in normal saline and diluted to a density of 2.0 on the MacFarland Nephelometer scale. Two ml of $m/15$ phosphate buffer (pH = 8.0) and 1 ml of a 10^{-4} molar methylene blue chloride solution were placed in a Thunberg tube and 1 ml of the bacterial suspension was placed in the side arm cap. The tubes were evacuated and refilled with hydrogen. This procedure was repeated three times and then the tubes were inverted to mix the contents. The tubes were incubated in a 37 C water bath and observed after 1 hour. If reduction of methylene blue occurred, the procedure was repeated using helium instead of hydrogen. A positive test for hydrogenase was based on reduction of methylene blue within 1 hour under hydrogen with no reduction in 2 hr under helium.

RESULTS

Growth in air. Under ordinary atmospheric conditions, none of the 34 cultures grew on blood agar slants. Moderate growth occurred in liquid thiol and thioglycolate media.

Anaerobic conditions. In the absence of oxygen, but in the presence of 1 to 60 per cent carbon dioxide, no growth occurred either on blood agar or in thiol and thioglycolate media.

Carbon dioxide required for growth. In carbon dioxide-free atmospheres containing 1 to 60 per cent oxygen no growth occurred either on blood agar or in the liquid media.

Optimum oxygen tension. Inoculated blood agar slants were incubated in atmospheres in which the percentage of oxygen was reduced to from 1 to 15 per cent by replacing air in the jars with the required amounts of either carbon dioxide, hydrogen or nitrogen. The results are shown in figure 1.

The optimum oxygen tension was found to be 5 per cent when this level was obtained by replacing 75 per cent of the air in the jars with either carbon dioxide, hydrogen or nitrogen. Atmospheres consisting of 25 per cent air and either 75 per cent hydrogen or nitrogen contained enough carbon dioxide to support growth.

Of the three gases used to reduce the oxygen content of the atmosphere within the jars, carbon dioxide gave slightly better growth and nitrogen slightly poorer growth than hydrogen. In the atmospheres of hydrogen and air, growth of 4 of the 34 cultures was greater than in atmospheres

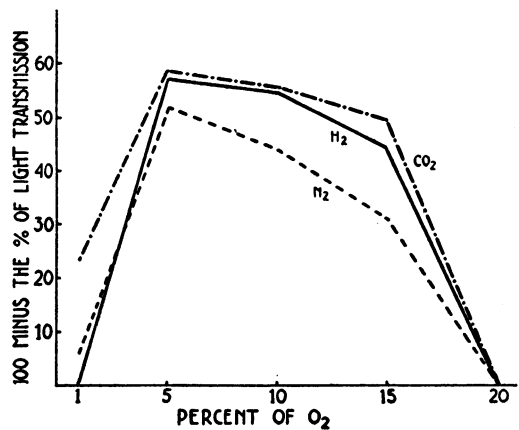


Figure 1. Growth of *Vibrio fetus* in atmospheres in which the oxygen content was reduced by partially replacing air with nitrogen, hydrogen and carbon dioxide.

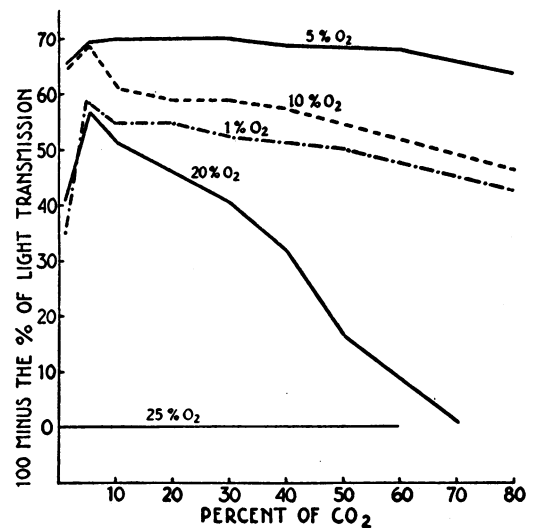


Figure 2. Growth of *Vibrio fetus* in atmospheres containing 1 to 25 per cent oxygen and 1 to 80 per cent carbon dioxide.

in which the oxygen tension was reduced with either carbon dioxide or nitrogen.

Optimum carbon dioxide tension. Cultures on blood agar were grown in atmospheres in which the carbon dioxide content was adjusted to from 1 to 80 per cent and the oxygen content to 1, 5, 10, 20 and 25 per cent. When necessary, nitrogen was used to adjust the pressure within the jars to 96 per cent of atmospheric pressure. The results are given in figure 2.

The optimum carbon dioxide tension was 10 to 30 per cent in the presence of 5 per cent oxygen, and 5 per cent in the presence of 1, 10 and 20 per cent oxygen.

Increasing the carbon dioxide content of the atmosphere beyond 30 per cent decreased the amount of growth slightly in the presence of 5 per cent oxygen, moderately in the presence of 1 and 10 per cent oxygen and markedly in the presence of 20 per cent oxygen.

No growth occurred in atmospheres containing 25 per cent oxygen regardless of the carbon dioxide content.

Effect of hydrogen on growth. Cultures on blood agar were grown in atmospheres containing from 1 to 40 per cent hydrogen with 10 per cent carbon dioxide and 5, 10 and 20 per cent oxygen, respectively. The pressure within the jars was adjusted to 96 per cent of atmospheric pressure by adding nitrogen as needed. Of 34 strains, only four showed a slightly greater growth response in the presence of hydrogen. Increasing the hydrogen concentration from 1 to 40 per cent did not increase growth.

Hydrogenase was demonstrated in suspensions of cells of only the four strains that showed increased growth when cultured in atmospheres containing hydrogen. Subculturing of the other 30 strains every three days, for five transfers, in atmospheres containing 5 per cent oxygen, 10 per cent carbon dioxide and 81 per cent hydrogen

did not result in either the production of hydrogenase or increased growth in the presence of hydrogen. Hydrogen was not essential for growth of any of the strains.

Effect of partial vacuum on growth. Cultures were incubated in atmospheres containing 5 to 15 per cent oxygen and 10 per cent carbon dioxide with the nitrogen content adjusted to give partial vacuums of 0, 25, 50 or 75 per cent of atmospheric pressure. The levels of vacuum used had no significant effect on growth.

Effect of gaseous environment on catalase content of cultures. Since catalase production has been shown to be a characteristic of *V. fetus* by Reich (1954) and Bryner and Frank (1955b), cultures grown in atmospheres containing 10 per cent carbon dioxide and 1, 5, 10 and 15 per cent oxygen, respectively, were tested for catalase (figure 3).

Maximum catalase content of suspensions of *V. fetus* cells was obtained in the atmosphere that contained 1 per cent oxygen, although maximum growth did not occur in this gaseous environment. As the oxygen content of the atmosphere was increased, the amount of catalase in the cell suspensions decreased. An average of 25 mm of oxygen was released by the cultures grown in the presence of 15 per cent of oxygen as compared to 64 mm for cultures grown in an atmosphere containing 1 per cent oxygen.

Cultivation of four catalase negative vibrios (isolated from bull semen) in atmospheres containing 1 to 10 per cent oxygen did not result in catalase production.

DISCUSSION

For the isolation of *V. fetus* of bovine origin the common practice has been to increase the carbon dioxide tension to about 10 per cent and not to alter the oxygen concentration significantly. From the results reported here, the optimum oxygen-carbon dioxide environment was provided by reducing the oxygen tension to 5 per cent and increasing the carbon dioxide tension to 10 per cent.

Four of the 34 strains of *V. fetus* produced hydrogenase. Molecular hydrogen does not normally occur in appreciable amounts in the presence of molecular oxygen. Consequently hydrogen is usually found in areas of anaerobiosis or where it is being produced by microorganisms. *V. fetus* does not grow anaerobically; however, in the

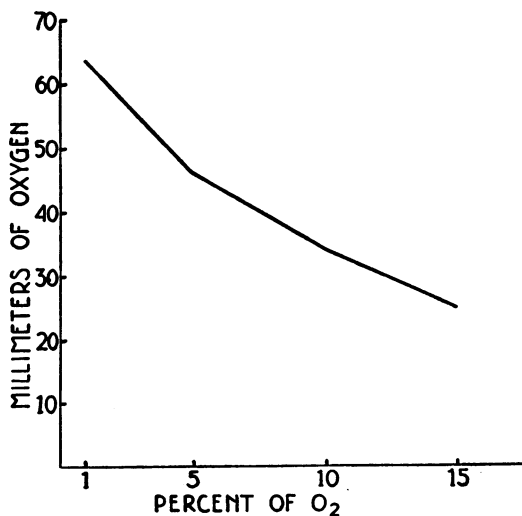


Figure 3. Catalase content of cultures of *Vibrio fetus* grown in atmospheres containing 1 to 15 per cent oxygen and 10 per cent carbon dioxide.

genital tract of bulls and cows it is associated with a variety of other organisms. It may be that in the genital tract of certain cows the bacterial flora may result in the release of hydrogen and, if *V. fetus* is also present, the strain may acquire the ability to use hydrogen. However, in the experiments reported herein cultivation in the presence of hydrogen did not result in cultures acquiring the ability to use hydrogen. Because hydrogen is not essential for the growth of *V. fetus*, its use increased the growth only slightly for a small number of strains, and on account of its danger, hydrogen is not recommended for incorporation in the gaseous atmosphere for *V. fetus*.

It appears that the optimum gaseous environment for the growth and isolation of *V. fetus* of bovine origin consists of 5 per cent oxygen, 10 per cent carbon dioxide, with or without adjusting the pressure within the jars to 96 per cent of atmospheric pressure by the addition of nitrogen. Use of nitrogen is preferred because, under conditions of partial vacuum, leakage of air into the jar may increase the oxygen tension above the optimum level. Preliminary studies show that culturing under this gaseous environment has greatly increased the chance of isolating *V. fetus* from semen and other specimens.

Based on rH, Vincent *et al.* (1952) found that nine cultures classed as *V. fetus* belonged to three respiratory types; three were strict aerobes, five were micro-aerophiles and one was a strict anaerobe. None of the 34 bovine strains used in this study grew in the absence of oxygen.

The production of catalase by *V. fetus* was first noticed by Reich (1954) and was shown by Bryner and Frank (1955b) to be a useful tool in the differentiation of *V. fetus* from saprophytic vibrios. The amount of catalase that can be demonstrated in a culture of *V. fetus* depends on

the oxygen tension in which it is cultured. However, the fact that the culture produces catalase and not the quantity produced is significant.

SUMMARY

Cultures of *Vibrio fetus* of bovine origin required both oxygen and carbon dioxide for growth.

The optimum gaseous environment for growth of *V. fetus* on blood agar was provided by an atmosphere consisting of 5 per cent oxygen and 10 per cent carbon dioxide, either under partial vacuum, or adjusted to 96 per cent of atmospheric pressure by adding hydrogen or nitrogen.

Four of 34 strains grew only slightly better in an atmosphere containing hydrogen. For this reason and because of the danger involved, use of this gas is not recommended.

Reduced oxygen content of the gaseous environment increased the amount of catalase released by *V. fetus* cells but did not cause catalase negative vibrios to become catalase positive.

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