Differential Effects of Oxygen and Oxidation-Reduction Potential on the Multiplication of Three Species of Anaerobic Intestinal Bacteria

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The sensitivity of three strains of anaerobic intestinal bacteria, *Clostridium perfringens*, *Bacteroides fragilis*, and *Peptococcus magnus*, to the differential effects of oxygen and adverse oxidation-reduction potential was measured. The multiplication of the three organisms was inhibited in the presence of oxygen whether the medium was at a negative oxidation-reduction potential (Eh of -50 mV), poised by the intermittent addition of dithiothreitol, or at a positive oxidation-reduction potential (Eh of near $+500$ mV). However, when these organisms were cultured in the absence of oxygen, no inhibition was observed, even when the oxidation-reduction potential was maintained at an average Eh of $+325$ mV by the addition of potassium ferricyanide. When the cultures were aerated, the growth patterns of the three organisms demonstrated different sensitivities to oxygen. *P. magnus* was found to be the most sensitive. After 2 h of aerobic incubation, no viable organisms could be detected. *B. fragilis* was intermediately sensitive to oxygen with no viable organisms detected after 5 h of aerobic incubation. *C. perfringens* was the least sensitive. Under conditions of aerobic incubation, viable organisms survived for 10 h. During the experiments with *Clostridium*, no spores were observed by spore staining.

The methodology for the isolation and identification of anaerobic bacteria has experienced a tremendous growth in the past few years (1, 6, 8, 9, 18). In the development of these techniques there have been two major concerns: the elimination of atmospheric oxygen from the culture environment, and the utilization of reducing agents to maintain a lowered oxidation-reduction potential during the cultivation of the bacteria. In light of the emphasis placed on controlling these two factors, the question arises, which of the factors influences the growth of anaerobic bacteria? Is the presence of atmospheric oxygen inhibitory or, does an adverse oxidation-reduction potential in the medium interfere with the multiplication of anaerobic bacteria?

Several investigators have suggested a role for oxygen in toxicity for anaerobic bacteria (1–3, 6, 8, 10, 12, 13, 15). Other investigators have attributed toxicity to changes in the oxidation-reduction potential of the culture medium (7, 11, 17, 20). In many of these studies atmospheric oxygen was used to regulate the oxidation-reduction potential, precluding differentiation of the effects of these two factors in inhibiting the growth of anaerobes. In experiments where the effects of oxygen were distinguished from the effects of oxidation-reduction potential, oxygen, and not oxidation-reduction potential, was the growth limiting factor (4, 16).

It is the purpose of this investigation to determine whether atmospheric oxygen or adverse oxidation-reduction potential is inhibitory for several strains of anaerobic intestinal bacteria. Data are presented that indicate where emphasis should be placed in the development of new techniques in anaerobic methodology.

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MATERIALS AND METHODS

Organisms. Human fecal specimens were collected in paper collection cups and placed into a GasPak jar (BBL) which had been activated 2 h before. After reactivation with a new GasPak envelope, specimens were transported immediately to the anaerobe laboratory and were entered into an anaerobic glove box isolator similar to that described by Aranki et al. (1). The organisms were identified as *Clostridium perfringens*, *Bacteroides fragilis*, and *Peptococcus magnus* on the basis of morphology and by the cultural and biochemical

tests described by Holdeman and Moore (9) and Sutter et al. (18).

Population determinations of control cultures. Growth curves of each of the three bacteria were prepared during each experiment. The control cultures were grown in 250 ml of Trypticase soy broth enriched with hemin, 0.1 mg/100 ml; menadione, 0.05 mg/100 ml; sodium carbonate, 0.042 mg/100 ml; dithiothreitol (DTT), 0.1%. A magnetic stirring bar was placed in the culture flask to provide continuous stirring during incubation at 37 C in the anaerobic glove box isolator. One milliliter of a 10^{-3} dilution of an overnight culture of each species provided a final concentration of approximately 10^4 organisms/ml. Population determinations were made by preparing 10-fold serial dilutions in a reduced diluent described by Holdeman and Moore (9). A 0.1-ml quantity of each dilution was spread with a bent glass rod on the surface of a Trypticase soy agar plate enriched with hemin, 0.1 mg/100 ml; menadione, 0.05 mg/100 ml; sodium carbonate, 0.042%; DTT, 0.1%; sheep blood, 5%. Determinations were made at 0, 2, 5, and 10 h after inoculation. After overnight incubation at 37 C in the anaerobic glove box isolator, the colonies that developed on the surface of the plates were counted with an electric colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Population determinations in aerated cultures with negative oxidation-reduction potential. An apparatus was designed to permit aeration, addition of reducing agent, and measurement of the bacterial population, pH, and oxidation-reduction potential of the culture without resultant contamination (Fig. 1). The major part of the apparatus is a reaction kettle (Kimax) consisting of a 4,000 ml capacity bottom and a cover with one opening in the center and three equally spaced openings near the outside margin. The cover and bottom have smooth ground
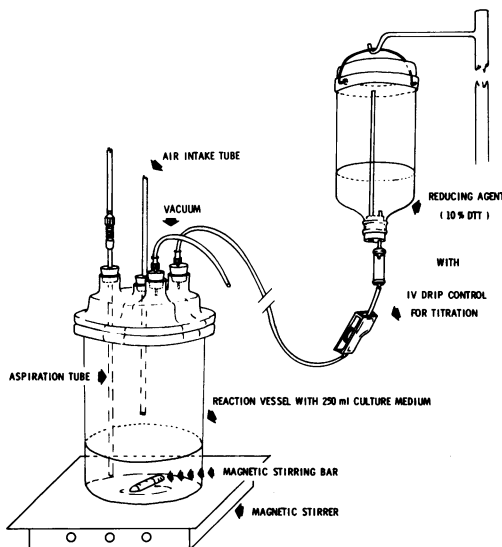

FIG. 1. *Apparatus for maintaining negative oxidation-reduction potentials under aerated conditions.*

flanges for a tight seal. Each cover opening is fitted with a rubber stopper in which a hole is drilled. Through two of the stoppers glass tubing is inserted; one is for aspiration of culture samples for population determinations and pH and oxidation-reduction potential measurements, and the other serves to impinge air onto the culture medium after the air has been filtered through a cotton plug in the upper end of the tube. Air flow is created by a vacuum drawn through the third opening. The final opening supports a titration unit through which reducing agent is intermittently added. Continuous stirring of the culture medium within the apparatus is accomplished with a magnetic stirring bar. Before inoculation, 250 ml of enriched Trypticase soy broth was added to this system and was prereduced by placing the apparatus in the anaerobic glove box isolator for 48 h. After inoculation of the broth to a final concentration of about 10^4 organisms/ml, the apparatus was removed to an air incubator and placed on a magnetic stirrer situated within the incubator. Aeration and intermittent addition of 10% DTT at a rate of 1 drop/min through a 21-gauge needle was started after a portion was removed for determination of inoculum size. Subsequent portions were removed at 2, 5, and 10 h after inoculation for population determinations as described earlier. At the same time 5 ml of culture were transferred to a disposable test tube for measuring both pH and oxidation-reduction potential with a Beckman expandomatic pH meter. Determination of pH was made with a combination glass electrode (Beckman model 39142). A polished combination platinum electrode (Beckman model 39186), standardized with a solution of potassium hydrogen phthalate saturated with quinhydrone as described by Tabatabai and Walker (19), was used to measure oxidation-reduction potential. Oxidation-reduction potential measurements were made after the combination platinum electrode was allowed to equilibrate for 5 min. The values obtained were expressed in terms of Eh (standard hydrogen electrode) by use of a correction factor, 214 mV. The addition of DTT to the medium was regulated during incubation to maintain an average Eh of -50 mV. After collection of the final 10-h sample, two additional 5-ml portions were removed to separate sterile culture tubes. One tube was incubated anaerobically for 48 h to determine if viable cells remained, while the other tube was inoculated with approximately 10^4 organisms using a 0.001-ml graduated inoculation loop and was incubated anaerobically to check for an inhibitory activity by the culture medium.

Population determinations under anaerobic conditions with a positive oxidation-reduction potential. In these experiments, growth curves of each organism were determined as described for control experiments except potassium ferricyanide was added to the culture medium instead of DTT. Potassium ferricyanide was added in a concentration of 0.1% just before inoculation of the culture medium to a final concentration of approximately 10^4 organisms/ml. During incubation, sufficient potassium ferricyanide was added to the culture medium to maintain an average Eh of $+325$ mV throughout

the experiment. Population determinations were made at 0, 2, 5, and 10 h after inoculation. At the same time, oxidation-reduction potential and pH measurements were made within the anaerobic glove box isolator with electrodes connected by wire leads to a pH meter located outside the glove box isolator.

Population determinations in aerated cultures with a positive oxidation-reduction potential. These experiments were performed as described for aerated cultures with negative oxidation-reduction potentials except DTT was not added to the medium.

RESULTS

The differential effects of oxygen and a positive oxidation-reduction potential on the multiplication of C. perfringens, B. fragilis, and P. magnus were determined by culturing these organisms in various environments where oxygen concentrations and oxidation-reduction potential levels were individually regulated. Control cultures represented optimum conditions for multiplication, i.e., anaerobic incubation of cultures in enriched Trypticase soy broth.

Figure 2 represents a summary of the experiments with Clostridium. Under control conditions C. perfringens populations reached levels of 10^9 organisms/ml after 10 h of incubation at 37 C. The first phase involved growing this organism in an aerated culture medium poised at a negative oxidation-reduction potential (average Eh of -50 mV) over a 10-h period. The initial population of 10^3 organisms/ml decreased steadily until after 5 h of incubation only half the number of viable cells remained. After 10 h of incubation no viable cells could be detected. At this time a broth check was performed. The portion of the medium incubated anaerobically remained clear after 48 h, whereas the portion inoculated with the organism produced turbidity overnight at 37 C in the anaerobic glove box isolator. A spore stain of the 10-h sample was made, and no spores were observed. To examine the effects of a positive oxidation-reduction potential, C. perfringens was inoculated into an anaerobic medium poised at an average Eh of $+325$ mV by the periodic addition of 10% potassium ferricyanide over a 10-h period. The growth curve was almost identical with that of the control culture. After 10-h of incubation, the colony count was approximately 10^9 organisms/ml. The final phase of the experiment was conducted by inoculating C. perfringens into an aerated culture medium without DTT, resulting in a culture Eh of near $+500$ mV. The population decreased at the same rate as the aerated culture with a negative oxidation-reduction potential. After 10 h of incubation, no viable organisms could be detected.

The results with B. fragilis are summarized in Fig. 3. After 10 h of growth, the anaerobic control culture reached 10^6 to 10^7 organisms/ml. When grown in an aerated culture medium, maintained at an average Eh of -50 mV, the Bacteroides population decreased in number so that 5 h after inoculation no viable organisms could be detected. An identical growth pattern was observed when the organisms were inoculated into an aerated medium without DTT at a positive oxidation-reduction potential. However, when inoculated into an anaerobically incubated medium adjusted to an average Eh of $+325$ mV with potassium ferricyanide, growth was similar to that observed in control cultures, reaching 10^6 to 10^7 organisms/ml in 10 h. Broth checks after aerobic incubation were made and no inhibition was observed.

Figure 4 illustrates the results of the experiments with P. magnus. Procedures were the same as those used to test the Clostridium and Bacteroides species. The figure shows that identical growth patterns were obtained in anaerobically incubated medium at an Eh of -200 mV or at an Eh of $+325$ mV. Populations reach 10^8 organisms/ml after 10 h of incubation. In aerated cultures, with or without DTT, there were no viable organisms detected after 2 h of incubation. Broth checks of the culture medium showed no inhibiton caused by the medium.
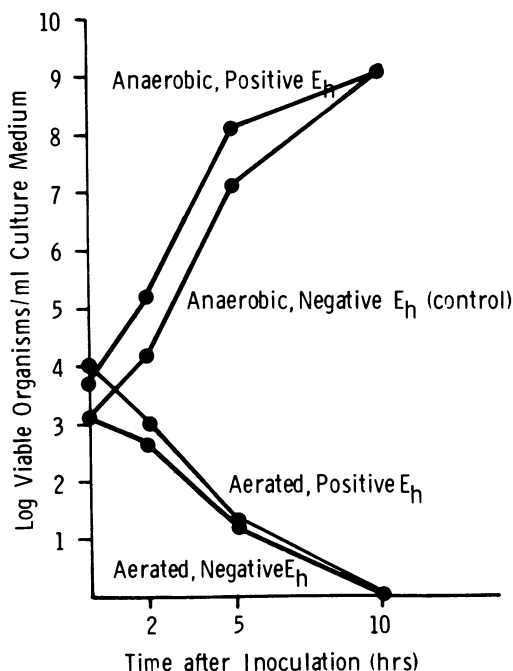

FIG. 2. The differential effects of oxygen and positive oxidation-reduction potential on the multiplication of C. perfringens.
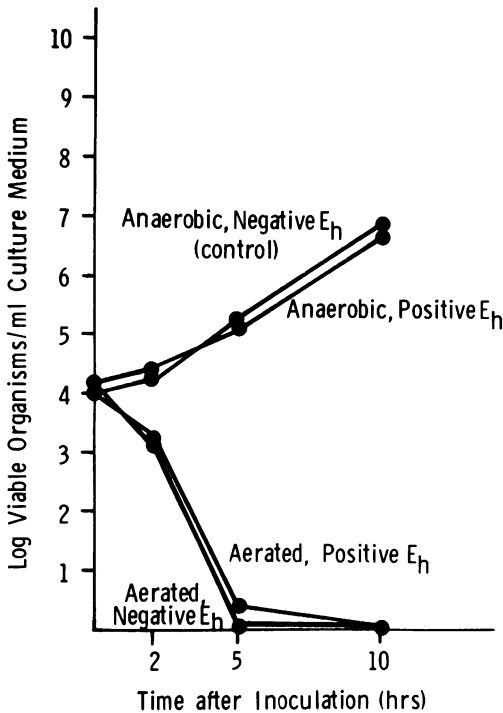
FIG. 3. *The differential effects of oxygen and positive oxidation-reduction potential on the multiplication of B. fragilis.*

In summary, all three organisms reacted similarly to the effects of oxygen and oxidation-reduction potential. Multiplication was inhibited in the presence of oxygen in both the negative and positive ranges of oxidation-reduction potential. However, in the absence of oxygen at either negative or positive oxidation-reduction potentials, multiplication occurred.

DISCUSSION

In this study three representative species of anaerobic bacteria were utilized. In addition to *Clostridium,* the genus *Bacteroides* was selected as a representative of the anaerobic gram-negative bacilli. The third organism, *P. magnus,* a gram-positive coccus, represents a group of anaerobic organisms considered to be more sensitive to oxygen than either *Clostridium* or *B. fragilis.* All three species were freshly isolated from normal human feces with minimal exposure to atmospheric oxygen. *C. perfringens* was selected for these experiments since it rarely sporulates, even under adverse conditions.

Our studies demonstrate that a significant inhibition of multiplication of all three test organisms occurred in the presence of air whether

the oxidation-reduction potential was negative (Eh of −50 mV) or positive (Eh of near +500 mV). At 10 h, no viable cells could be detected in any of the aerated cultures. In the absence of air, on the other hand, the organisms multiplied despite the oxidation-reduction potential of the culture medium. The procedures used in this study clearly separate the effects of oxygen and oxidation-reduction potential on the organisms, and demonstrate the inhibitory effect of oxygen.

The results also demonstrate a gradation of oxygen sensitivity among these bacteria. Loesche (12) observed two general oxygen sensitivity patterns among anaerobic bacteria which he termed strict and moderate anaerobiosis. The group designated as strict anaerobes did not grow at oxygen tensions greater than 0.5%, whereas moderate anaerobes were capable of growth at oxygen levels as high as 2 to 8%. A third group, classified as aerotolerant, included organisms capable of growth at oxygen levels above those of the moderate anaerobes. Fredette et al. (5) determined oxygen sensitivity by studying the effects of hyperbaric oxygen on various species of anaerobic bacteria multiplying in deep agar. Their results indicated that a
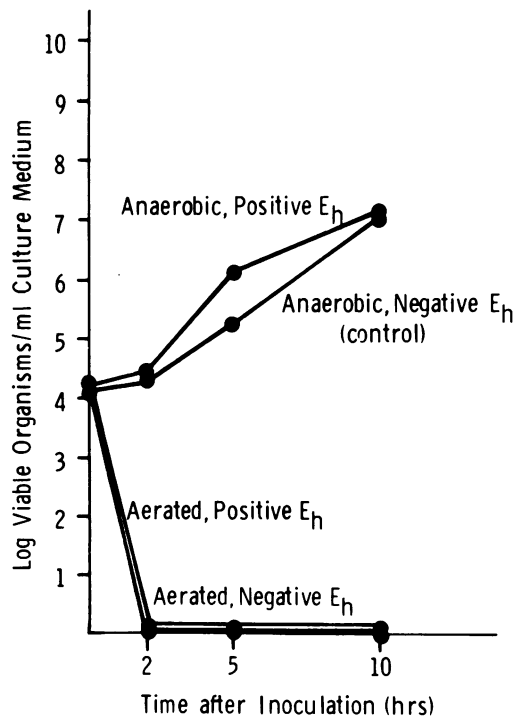

FIG. 4. *The differential effects of oxygen and positive oxidation-reduction potential on the multiplication of P. magnus.*

gradation in sensitivity to hyperbaric oxygen exists, with *C. perfringens* being least sensitive and *B. fragilis* most sensitive. Our results demonstrate a similar pattern. *P. magnus* was most sensitive to oxygen, *B. fragilis* was moderately sensitive, and *C. perfringens* was least sensitive to oxygen.

This study confirms that DTT and potassium ferricyanide are suitable reagents for the control and maintenance of negative and positive oxidation-reduction potentials, respectively, in culture media. The absence of toxicity for these organisms at concentrations below 2%, and its stability in air, make DTT a useful reducing agent. Potassium ferricyanide is also nontoxic for these organisms at concentrations below 2% and its constant and predictable rate of reduction under anaerobic conditions makes it very suitable for use in studies of this type.

With the availability of methods for the study of the differential effects of oxygen and oxidation-reduction potential on the multiplication of anaerobic bacteria, additional investigations on other examples of anaerobic bacteria are feasible. Studies utilizing various concentrations of oxygen will provide information concerning the degree of oxygen sensitivity.

A positive oxidation-reduction potential provides evidence that a culture medium may be in an oxidized state; it does not, per se, appear to be the limiting factor in the growth of anaerobic bacteria. In an era of anaerobic bacteriology where emphasis is often placed on the culture medium with a low oxidation-reduction potential by the addition of reducing agents, this result is significant. It indicates a need to realize that reducing agents might not adequately provide the conditions necessary for anaerobic growth, since oxygen and its resulting products may actually be the important factors that interfere with the growth of anaerobic bacteria. Culture procedures that involve the purging of oxygen from the environment need to be emphasized, not only during inoculation and incubation, but during collection as well.

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