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Effect of High Oxygen Tensions on the Growth of Selected, Aerobic, Gram-negative, Pathogenic Bacteria

SHELDON F. GOTTLIEB AND LEONARD M. PAKMAN

Departments of Physiology, Anesthesiology, and Microbiology, Jefferson Medical College, Philadelphia, Pennsylvania 19107

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The in vitro effects of high O_2 tensions (P_{O_2}) on aerobic, enteric pathogens were examined at pressures of up to 3 atm absolute. Organisms from the genera *Salmonella*, *Shigella*, and *Vibrio* were usually subjected to 24-hr exposures. Tensions of 0.87, 1.87, and 2.87 atm absolute of O_2 (plus traces of CO_2 and N_2) became progressively inhibitory for *Salmonella* and *Shigella* growth, but were bactericidal only for *V. comma* strains at tensions greater than 0.87 atm absolute of O_2. Growth inhibition of enteric organisms resulted from increased P_{O_2}, rather than pressure per se, and could be mitigated nutritionally; an appropriate carbohydrate source is at least partially involved. Further studies with vibrios indicated that such mitigation was independent of medium *p*H. In addition, a synergistic relationship existed between O_2 and sulfisoxazole when tensions from 0.87 to 2.87 atm absolute of O_2 were maintained for 3 to 24 hr. Synergism occurred even under nutritional conditions which negated growth inhibition by O_2 alone. Bactericidal concentrations of sulfisoxazole, in the presence of increased P_{O_2}, were reducible up to 4,000-fold. The combined procedure employed in this investigation, by use of an antimicrobial drug of known action, which also synergizes with O_2, plus nutritional studies, suggests a means for establishing a site of O_2 toxicity. These data support the concept that O_2 inhibition of growth represents a metabolic disturbance and that metabolic pathways involving *p*-aminobenzoic acid may be O_2-labile. Such an approach could also guide development of antimicrobial agents as O_2 substitutes for promoting synergism.

Recently, intensive efforts have been directed toward the in vitro and in vivo study of the effects of increased oxygen tensions, alone and in combination with antibiotics, on aerobic pathogenic bacteria (3), particularly those found in wound and burn infections (6, 8, 11, 12). Scant attention has been given to the effects of oxygen on the growth of the aerobic, facultatively anaerobic, gram-negative, intestinal pathogens. Moore and Williams (9, 10) reported that increased oxygen tensions to 0.92 atm had no growth-inhibitory effects on *Vibrio comma*, *Bacillus typhosus* (*Salmonella typhosa*), and the Flexner, Shiga, and Kruse strains of *Bacillus dysenteriae* (*Shigella dysenteriae*). However, for other forms of life, an inverse relationship exists between the oxygen tension and duration of exposure before the toxic effects of oxygen become manifest (4). Previous data also have shown that an organism's response to oxygen may be altered by its nutritional state (5) and by the presence of antibiotics (3).

The objectives of this investigation were threefold: (i) to explore the effects of increased oxygen tension (P_{O_2}) on the growth of aerobic, facultatively anaerobic, gram-negative enteric pathogens; (ii) to study the effects of nutrition on the growth of the enteric pathogens exposed to high P_{O_2}; and (iii) to investigate the interaction between P_{O_2} and sulfisoxazole. Sulfisoxazole (Sodium Gantrisin, Hoffman-La Roche, Inc., Nutley, N.J.) was selected as the antimicrobial agent, since *p*-aminosalicylic acid (PAS) synergizes with O_2, resulting in growth inhibition of drug-susceptible and -resistant strains of mycobacteria greater than that produced by either agent alone (3). Assuming that PAS may be acting on metabolic pathways involving *p*-aminobenzoic acid (PABA) utilization (2), we reasoned that other drugs that interfere with PABA utilization may also synergize with oxygen. Sulfisoxazole, like the other sulfa drugs, presumably exerts biological effects by also interfering with PABA metabolism (2).

MATERIALS AND METHODS

The organisms we used in our studies were randomly selected *V. comma* J 79 (Ogawa), J 38 (Ogawa), J 4124 (Inaba), J 5001 (Ogawa), J 4001 (Ogawa), J 75 (Ogawa), and J 76 (Inaba) furnished by K. Goodner (Department of Microbiology, Jefferson Medical College, Philadelphia, Pa.) plus the following laboratory strains: *Salmonella typhosa* J 15, *S. typhosa* J 1120-R, *S. paratyphi* A, two strains of *S. schoettmuelleri*, *S. oranienburg*, *S. senftenberg*, *Shigella dysenteriae*, and *S. flexneri*. Stock cultures of all vibrios were stored under mineral oil on solidified T_1N_1 medium, which is 1.0% Trypticase (BBL), 1.0% NaCl, and 2.0% agar, at *p*H 6.7. They were stored at room temperature (24 to 26 C). *Salmonella* and *Shigella* species stocks were maintained on Nutrient Agar (Difco), and were also stored under mineral oil at room temperature. The stock cultures and subsequent transfers were not monitored for smooth-rough variations although these stocks and their subcultures persistently gave reproducible results in the replicated experiments.

Growth experiments. Experimental media were distributed in 2.0-ml portions (total final volume) to cotton-plugged, optically matched test tubes (125 × 15 mm), and were sterilized by autoclaving at 121 C for 15 min. To decrease the diffusion limitation, the tubes in the high-pressure chamber were placed at an approximate 130° angle (5, 7) from the horizontal. Experiments were performed in duplicate or triplicate; data from typical experiments are presented in all the tables.

Sodium sulfisoxazole was dissolved in 0.033 M potassium phosphate buffer (*p*H 7.0) containing 1.0% Trypticase, 1.0% NaCl, and 0.2% yeast extract (Difco); hereafter, this solution, less the sulfisoxazole, is referred to as T_1N_1-YP medium (*p*H 6.8). This solution was sterilized by filtration and added aseptically to the medium-containing test tubes. Serial dilutions of the drug were made in T_1N_1-YP. Unless otherwise stated, T_1N_1-YP broth was the medium used in all drug studies.

Inocula for *V. comma* growth experiments were prepared by inoculating 5 ml of T_1N_1 broth with the appropriate strain, followed by incubation for 24 hr at room temperature. The cultures of each strain employed then contained the following numbers of colony-forming units per milliliter, as determined by plate counts employing Nutrient Agar: J 79, $7.17 \pm 2.03 \times 10^7$; J 38, $1.05 \pm 0.10 \times 10^8$; J 4124, $5.55 \pm 0.65 \times 10^4$; J 5001, $2.81 \pm 0.27 \times 10^8$. One drop (0.05 ml) of a 1:1,000 dilution (T_1N_1-YP broth as diluent) of these cultures was subsequently used to inoculate each tube of the experimental liquid media. The diluted inocula, together with the sulfisoxazole concentration range employed, were as recommended by Roche Laboratories, Nutley, N.J. (*personal communication*).

Inoculated liquid media were incubated at 37 C for 24 hr (unless otherwise noted) in a candle jar (CO_2 environment), an air incubator, or a high-pressure chamber (no. 614 Table-Top Hyperbaric Chamber, The Bethlehem Corp., Bethlehem, Pa.). This chamber was evacuated to a pressure of 76 mm of Hg, leaving a residual N_2 content of 60 mm of Hg (0.08 atm) in the chamber, and was filled to 1 atm absolute (760 mm of Hg) with a gas mixture consisting of 95% O_2 + 5.0% CO_2 (O_2-CO_2); for oxygen atmospheres greater than 1 atm absolute, 100% oxygen was superimposed on the original O_2 + CO_2 mixture in the chamber. For pressure control experiments, the desired pressure was attained by adding 100% N_2 to the air in the chamber, thus maintaining the partial pressure of O_2 (P_{O_2}) equivalent to air at 1 atm absolute. Temperature was maintained in the pressure chamber at 36 ± 1.5 C by a heating tape wrapped around the chamber and controlled by a nonindicating, adjustable temperature-sensor-controller (Fenwall Inc., Ashland, Mass.). Temperature was read on a thermometer suspended inside and viewed through the sight port of the chamber.

Growth in liquid media, as contained in the optically matched test tubes, was measured turbidimetrically with an Evelyn colorimeter (Ribicon Co., Philadelphia, Pa.) at 660 mμ. To facilitate turbidimetric measurements, it was necessary to adjust the volume of the original 2.0-ml cultures to 4.0 ml by the addition of identical, uninoculated, sterile broth. Thus, the final optical density (OD) values as given in this paper represent a 1:2 dilution of the actual growth.

Bactericidal or bacteriostatic effects of the gaseous environment, alone or in combination with sodium sulfisoxazole, were determined in duplicate by transferring 0.10 ml from those tubes devoid of visible growth (before volume adjustment) to tubes containing 10 ml of Brain Heart Infusion broth (Difco), supplemented with 0.2% yeast extract (Difco), and incubated at room temperature for a minimum of 72 hr. The appearance of growth after this period indicated a bacteriostatic effect of the experimental conditions (although some members of the population may have been killed); we interpreted the absence of growth to signify that the previous experimental conditions were bactericidal.

For studies involving inhibition of surface growth by O_2, agar plates were employed and inoculated by the streak dilution technique, with 24-hr-old Nutrient Broth (Difco) cultures as the source of the inoculum. After 24-hr gaseous exposures within the chamber (37 C), surface growth on these plates was compared to the control plates incubated in air (37 C, 24 hr); the latter cultures always demonstrated profuse growth over most of the surface. We assumed that plates which, upon removal from the chamber, showed questionable growth (less than 10 minute colonies or a suggestive haze within a small area of heaviest inoculum) were subjected to inhibitory or bacteriostatic conditions, if profuse growth subsequently developed within less than 24 hr of air incubation at 37 C. Conditions were considered to have been bactericidal if plates showed no growth upon removal from the chamber and did not grow upon subsequent air incubation (24 hr, 37 C).

RESULTS

Initial experiments designed to determine the susceptibility of some enteric pathogens to the

growth-inhibitory effects of oxygen revealed that with surface cultures on Nutrient Agar (NA) at 1 atm absolute, a gas mixture consisting of 0.87 atm absolute of O_2 + 0.05 atm absolute of CO_2 + 0.08 atm absolute of N_2 was inhibitory to the growth of five of the seven *V. comma* strains examined. *Salmonella* and *Shigella* species were generally more resistant to this O_2 tension (Table 1). All cultures were plated in duplicate and continuously exposed to the gaseous environment for 24 hr at 37 C. On NA, at 2 or 3 atm absolute, O_2 (1.87 and 2.87 atm absolute, respectively, CO_2 and N_2 as above) tended to become bacteriostatic for *Salmonella* and *Shigella* species and bactericidal only for the vibrios. Thus, on subsequent re-incubation of the cultures exposed to 2.87 atm absolute of O_2 in 1 atm absolute of air for 24 hr at 37 C, all previously inhibited enteric organisms grew profusely, except for *V. comma* strains—all of which failed to grow. Even at 1.87 atm absolute, O_2 was bactericidal for five of the seven vibrios used, but for none of the other enteric organisms.

Our studies with all enteric bacteria examined showed that O_2 inhibition of growth could be mitigated by use of a medium more nutritionally enriched than NA, i.e., Brain Heart Infusion Agar (BHIA). In controls (candle jar or air, at 1 atm absolute), all strains of *V. comma* grew well on NA and BHIA within 24 hr at 37 C. In the presence of 0.87 and 1.87 atm absolute of O_2 (24-hr exposures, 37 C), these organisms grew profusely, but only on BHIA. At 2.87 atm absolute of O_2, *Vibrio* did not grow on NA; but growth did appear on BHIA with all vibrios tested, except for J 4124.

Although the evaluations of growth recorded in Table 1 were somewhat subjective, it seemed that increased O_2 tensions up to 3 atm absolute became increasingly inhibitory to the growth of all enteric bacteria examined but more inhibitory to vibrios. The O_2 inhibitory effect is, however, less manifest with a nutritionally enriched medium and may thus be at least partially overcome. Because vibrios are more sensitive to O_2 than other enteric bacteria examined, vibrios were selected for more intensive investigation.

The differences in ability of BHIA and NA to supplement growth of *V. comma* J 38 and J 79 in the presence of increased O_2 tensions (in separate experiments) were not caused by the differences in *p*H of the two media. Adjusting the *p*H of NA with Na_2HPO_4 from its usual value of 6.7 to 7.4 (the *p*H of BHIA) still did not confer on this medium the ability to support growth of the organisms in the presence of a high P_{O_2}. In

TABLE 1. *Growth of enteric bacteria on the surface of Nutrient Agar (NA) and Brain Heart Infusion Agar (BHIA) plates upon exposure to increased oxygen tensions*[a]

Organism	Oxygen tension (atm absolute)						Controls	
	0.87		1.87		2.87		NA	BHIA
	NA	BHIA	NA	BHIA	NA	BHIA		
Vibrio comma J38	±[b]	+	−[c]	+	−[c]	+	+	+
V. comma J 79	−[b]	+	−[c]	+	−[c]	+	+	+
V comma J 5001	+	+	−[b]	+	−[c]	+	+	+
V. comma J 4124	−[b]	+	−[c]	+	−[c]	−[c]	+	+
V. comma J 4001	+	+	−[b]	+	−[c]	+	+	+
V. comma J 75	−[c]	+	−[c]	+	−[c]	+	+	+
V. comma J 76	±[b]	+	±[c]	+	−[c]	+	+	+
Salmonella typhosa J 15	+	+	±[b]	+	±[b]	+	+	+
S. typhosa J 1129-R	+	+	±[b]	+	±[b]	+	+	+
S. paratyphi A	−[b]	+	−[b]	−[b]	−[b]	−[b]	+	+
S. schottmuelleri J 1158	+	+	+	+	±[b]	+	+	+
S. schottmuelleri J 129	+	+	+	+	−[b]	−[b]	+	+
S. oranienburg	+	+	+	+	±[b]	+	+	+
S. senftenberg	+	+	±[b]	+	−[b]	+	+	+
Shigella dysenteriae	+	+	±[b]	±[b]	−[b]	−[b]	+	+
S. flexneri	−[b]	±[b]	−[b]	−[b]	−[b]	−[b]	+	+

[a] All O_2 exposures were at 37 C for 24 hr with 0.05 atm absolute of CO_2 and 0.08 atm absolute of N_2 present; controls were done in a candle jar at 1 atm absolute. Degrees of growth: +, profuse growth; −, no growth; ±, questionable growth. Growth occurring on BHIA was always more luxuriant than on NA.

[b] Markedly inhibitory or bacteriostatic conditions.

[c] Bactericidal conditions.

contrast, on BHIA at *p*H 6.7 (adjusted with NaH_2PO_4), these two vibrio strains were able to grow even under 2.87 atm absolute of O_2.

Subsequent incubation of O_2-exposed vibrio cultures on NA to 1 atm absolute of air did not result in growth. Apparently, O_2 exerts bactericidal effects on vibrio when cultures are grown on a medium nutritionally less complex than BHIA.

Candle jar or hyperbaric chamber atmospheres had no significant effect on the *p*H of NA, BHIA, or other media used in these tests; occasional random fluctuation of ± 0.2 *p*H units was observed after a 24-hr incubation period.

The inhibition of growth noted with all organisms in the three genera examined was caused by the increased P_{O_2} and not by elevated pressures per se. This was demonstrated by the appearance of similar levels of growth on suitable agar or in broth media when the organisms were incubated for 24-hr periods either at 1 atm absolute in a candle jar or at 3 atm absolute in a gaseous environment consisting of 0.2 atm absolute of O_2 + 0.05 atm absolute of CO_2 + 2.75 atm absolute of N_2 (P_{O_2} as in 1 atm absolute of air).

Exposure of uninoculated NA to 2.87 atm absolute of O_2 for 24 hr did not cause any alteration of this medium that adversely affected growth, following subsequent vibrio inoculation and incubation in air or candle jar. Other control experiments indicated that a vacuum drawn to 76 mm of Hg had no measurable effect on the growth of any organism. Oxygen inhibition of growth was not due to other possible differences in incubation conditions within the high-pressure chamber, as compared to candle jar incubation; similar growth responses were noted for organisms incubated for 24 hr at 37 C either in the chamber at 1 atm absolute of air or in the candle jar.

In a liquid medium, oxygen inhibition of growth was also obtained, thereby permitting easier quantitation. T_1N_1 broth was capable of supporting similar growth of all vibrios at 1 atm absolute in the candle jar (or air), or 3 atm absolute consisting of 1 atm absolute of air + 2 atm absolute of N_2. When strain J 79 was used in more extensive nutritional studies, we found that this broth would not support growth, as measured after 24 hr at 37 C, in a 3 atm absolute milieu consisting of 2.87 atm absolute of O_2 + 0.05 atm absolute of CO_2 + 0.08 atm absolute of N_2 unless this medium was first enriched with 0.2% (w/v) yeast extract or 0.37% (w/v) Brain Heart Infusion broth (BHIB; commercial powder).

Alone, 1.0% yeast extract did not support growth of J 79 under 2.87 atm absolute of O_2, whereas 2.0% yeast extract did support growth. Both concentrations of yeast extract supported growth at 0.2 atm absolute of O_2. Moreover, a 1.48% BHIB, in the absence of all other constituents, supported growth of J 79, under 2.87 atm absolute of O_2, equal to or greater than that obtained in 1 atm absolute candle jar controls. These data suggest that inhibitory O_2 effects on growth could be reversed nutritionally.

T_1N_1 broth, prepared in 0.033 M potassium phosphate buffer at *p*H 7.0, enriched by 0.2% succinic acid, 0.2% citric acid, 0.2% DL-glutamic acid, 0.4% lactic acid, or 0.6% sodium acetate (stock solutions neutralized to *p*H 7.0 with KOH prior to use) still failed to support the growth of J 79 at 2.87 atm absolute of O_2 to an extent equal to or greater than control candle jar cultures at 1 atm absolute. In contrast, supplementing T_1N_1 with 0.2% glucose, 0.2% fructose, or 0.2% sucrose resulted in growth at 2.87 atm absolute of O_2 to an extent equal to or greater than control candle jar cultures at 1 atm absolute. We found that an appropriate carbon source could negate the requirement for complex and uncharacterized substances as contained in yeast extract; this indicates that a completely synthetic medium might be used to promote growth at elevated O_2 tensions and to establish the nutritional requirements necessary to overcome O_2 inhibition. A synthetic medium has been suggested for the routine growth of vibrios (Finkelstein and Lankford, Bacteriol. Proc., p. 49, 1955). Also, these data suggest that the efficacy of BHIB in supporting growth of vibrios under 2.87 atm absolute of O_2 may be caused by a triggering action of the glucose present in this medium.

The question of oxygen enhancement of or interference with drug action was investigated, employing strains of *V. comma* in broth (T_1N_1-YP). We found that this broth supported growth of *V. comma* J 79 at increased P_{O_2}.

The data in Table 2 reveal that at 1 atm absolute (candle jar), 10 μg of sulfisoxazole per ml inhibited growth of *V. comma* J 79, thus decreasing growth by approximately 50%. The bacteriostatic concentration range was $>625 \leqq 1{,}250$ μg/ml, and the bactericidal range was $>5{,}000 \leqq 10{,}000$ μg/ml. Alone, 2.87 atm absolute of O_2 also diminished growth by approximately 50%. A combination of 2.87 atm absolute of O_2 and sulfisoxazole showed a marked synergistic effect between these two agents; the bacteriostatic concentration range for sulfisoxazole in the presence of 2.87 atm absolute of O_2 was decreased to $>20 \leqq 39$ μg/ml, and the bactericidal range was also diminished to $>39 \leqq 78$ μg/ml. At 0.87 and 1.87 atm absolute of O_2, the bacteriostatic concentra-

tion of sulfisoxazole decreased to >156 ≦ 313 μg/ml; the bactericidal ranges were similarly diminished to >1,250 ≦ 2,500 μg/ml and >313 ≦ 625 μg/ml, respectively. We noted that, at O_2 tensions of 0.87 and 1.87 atm absolute, O_2 alone was not inhibitory; in contrast, O_2 at these tensions markedly enhanced growth.

The synergistic interaction of O_2 and sulfisoxazole was not strain-specific. However, the data in Table 2 reveal that *V. comma* strain J 38, like J 79, was affected by O_2, alone and in combination with sulfisoxazole. Similar results were obtained with strains J 4124 and J 5001. Depending upon the strain of *V. comma*, the bactericidal concentration of sulfisoxazole, acting in the presence of 2.87 atm absolute of O_2, was usually decreased 1/100 to 1/4,000 of that required at 1 atm absolute (candle jar). Strains J 38 and J 4124 were more sensitive than J 79 to the growth-inhibitory effects of O_2 alone; at 2.87 atm absolute of O_2 (although the organisms remained viable), J 38 and J 4124 did not grow, but J 79 did. Also, strain J 5001 was more sensitive to O_2 than strain J 79, but it apparently was not as sensitive as J 38 and J 4124. These differences in sensitivity to O_2 were unrelated to inocula size. Sensitivity to O_2 was independent of the organism's serotype (Ogawa and Inaba). Furthermore, it is clear, that at 0.87 and 1.87 atm absolute, O_2 enhanced the growth of strain J 38 as it did that of J 79.

The synergistic effects of O_2 and sulfisoxazole could be observed at 2.87 atm absolute O_2 even under conditions by which the growth-inhibitory effect of O_2 (alone) was obviated by replacing the T_1N_1-YP medium used in the earlier sulfisoxazole-O_2 studies with BHIB (Table 3). This latter medium nutritionally mitigated growth inhibition by O_2; at 2.87 atm absolute of O_2, the growth in BHIB of *V. comma* J 79 and J 38 was approximately twice as great as that which occurred at 1 atm absolute (candle jar control), measured after 24 hr at 37 C. Nevertheless, sulfisoxazole was potentiated in its action so that between 30- and 100-fold less was required for bacteriostatic activity and 10-fold less for bactericidal activity in the presence of O_2, as compared to candle jar controls.

We next undertook a study to ascertain the effects on vibrios of short, intermittent exposures to oxygen in the presence of sulfisoxazole, as opposed to a single 24-hr period (3). *V. comma* J 79 and J 38 were subjected to 3 atm absolute of the O_2-CO_2-N_2 mixture for two 3-hr periods, with the exposures separated from one another by incubation for 3 hr in air at 1 atm absolute. After the second O_2 exposure, the cultures were placed in a candle jar at 1 atm absolute and examined for growth 24 hr after the initial inoculation. Throughout, incubation temperatures were 37 C. This experiment was repeated later, limiting O_2 exposures to a single 3-hr period at

TABLE 2. *Effect of increased oxygen tensions and sulfisoxazole concentration on the growth of Vibrio comma J 79 and J 38*[a]

Sulfisoxazole (μg/ml)	Oxygen tension (atm absolute)						Controls	
	0.87		1.87		2.87		J 79	J 38
	J 79	J 38	J 79	J 38	J 79	J 38		
0	0.28	0.52	0.35	0.42	0.05	0[b]	0.13	0.33
2.5	0.27	0.44	0.27	0.41	0.03	0[c]	0.13	0.19
5	0.27	0.29	0.27	0.34	0.02	0[c]	0.11	0.14
10	0.23	0.18	0.24	0.20	0.01	0[c]	0.06	0.10
20	0.11	0.07	0.16	0.03	0.01	0[c]	0.03	0.07
39	0.06	0.06	0.05	0[b]	0[b]	0[c]	0.04	0.06
78	0.03	0.05	0.03	0[b]	0[c]	0[c]	0.03	0.04
156	0.01	0.03	0.01	0[c]	0[c]	0[c]	0.03	0.03
313	0[b]	0[b]	0[b]	0[c]	0[c]	0[c]	0.02	0.02
625	0[b]	0[b]	0[c]	0[c]	0[c]	0[c]	0.01	0.01
1,250	0[b]	0[b]	0[c]	0[c]	0[c]	0[c]	0[b]	0[b]
2,500–5,000	0[c]	0[c]	0[c]	0[c]	0[c]	0[c]	0[b]	0[b]
10,000	0[c]	0[c]	0[c]	0[c]	0[c]	0[c]	0[c]	0[c]

[a] Growth expressed as optical density units; T_1N_1-YP broth used with incubation at 37 C for 24 hr. All O_2 exposures were with 0.05 atm absolute of CO_2 and 0.08 atm absolute of N_2 present; Controls were done in a candle jar at 1 atm absolute.

[b] Bacteriostatic conditions.

[c] Bactericidal conditions.

2.87 atm absolute. In both experiments and with both strains, we observed synergism between O_2 and sulfisoxazole (Table 4). The bacteriostatic and bactericidal concentrations of sulfisoxazole were decreased, as compared to controls incubated at 1 atm absolute. Oxygen inhibition of vibrio growth was not noted under these shorter exposure conditions. However, a comparison of the data in Table 4 reveals that, with longer exposures to O_2, lesser amounts of sulfisoxazole are required for bactericidal and bacteriostatic activity.

TABLE 3. *Effect of increased oxygen tension and sulfisoxazole concentration on the growth of Vibrio comma strains in the absence of oxygen inhibition of growth*[a]

Sulfisoxazole (μg/ml)	Strain J 79		Strain J 38	
	Test atmosphere	Control	Test atmosphere	Control
0	0.11	0.06	0.19	0.11
2.5	0.12	0.07	0.18	0.10
5	0.08	0.05	0.07	0.09
10	0.06	0.06	0[b]	0.09
20	0[b]	0.06	0[b]	0.06
39	0[b]	0.03	0[b]	0.01
78	0[b]	0.03	0[b]	0.01
156	0[c]	0.02	0[c]	0.01
313	0[c]	0.02	0[c]	0[b]
625–5,000	0[c]	0[b]	0[c]	0[b]
10,000	0[c]	0[c]	0[c]	0[c]

[a] The test atmosphere was 0.05 atm absolute of CO_2, 0.08 atm absolute of N_2, and 2.87 atm absolute of O_2; controls were done in a candle jar at 1 atm absolute. Growth expressed as optical density units; BHI broth used with incubation at 37 C for 24 hr.

[b] Bacteriostatic conditions.

[c] Bactericidal conditions.

DISCUSSION

The data that we have presented illustrate that increased oxygen tensions can inhibit the growth of species of *Salmonella*, *Shigella*, and *Vibrio*. Individual species within a given genus vary in their responsiveness to high oxygen tensions (4); thus, this responsiveness to high oxygen tensions might be an additional characteristic for differentiating bacteria (3, 4). Also, the data presented in this paper indicate that individual strains of a given species will differ in their response to increased oxygen tensions. This was observed with several strains of *V. comma*, although more detailed studies are required to determine whether this is also true for strains of species within the genera *Salmonella* and *Shigella*.

The inhibition of growth of *V. comma* at 2.87 atm absolute of O_2 in T_1N_1-YP broth contrasts sharply with the enhancement of growth noted at 0.87 and 1.87 atm absolute of O_2 (24-hr exposures). These data, plus the absence of O_2 inhibition of growth observed with limited (3 hr)

TABLE 4. *Inhibitory sulfisoxazole concentrations (μg/ml) for Vibrio comma strains as a function of exposure intervals to increased oxygen tension*

Conditions of incubation[a]	Strain J 79		Strain J 38	
	Bacteriostatic	Bactericidal	Bacteriostatic	Bactericidal
Candle jar (1 atm absolute) for 24 hr (control)	>625 ≦ 1,250	>5,000 ≦ 10,000	>625 ≦ 1,250	>5,000 ≦ 10,000
CO_2 (0.05 atm absolute) + N_2 (0.08 atm absolute) + O_2 (2.87 atm absolute)				
For 3 hr, followed by candle jar for 21 hr[b]	>156 ≦ 313	>1,250 ≦ 2,500	>156 ≦ 313	>1,250 ≦ 2,500
For two 3-hr periods, interrupted by 3 hr in air, and followed by 15 hr in candle jar[b]	>156 ≦ 313	>1,250 ≦ 2,500	>39 ≦ 78	>625 ≦ 1,250
For 24 hr continuous exposure[c]	>20 ≦ 39	>39 ≦ 78	0	>0 ≦ 2.5

[a] All cultures incubated in T_1N_1-YP broth at 37 C in the presence or absence of sulfisoxazole and examined turbidimetrically 24 hr after inoculation.

[b] No inhibition of growth due to O_2 alone was observed with either stain when using these shorter O_2 exposure periods.

[c] Oxygen alone was slightly inhibitory to the growth of J 79 but was bacteriostatic for J 38 during such long O_2 exposures.

periods of exposure to 2.87 atm absolute of O_2, suggest the existence of a critical relationship between the partial pressure of oxygen and the exposure time before the deleterious effects of O_2 become manifest.

These data support the concept that, in the enteric organisms we examined, O_2 toxicity can represent a metabolic disturbance, because the resultant growth inhibition can be mitigated nutritionally (5). Hyperoxia alone (2.87 atm absolute of O_2, 24-hr exposure) was either bacteriostatic or it significantly reduced the growth rate with *Salmonella* and *Shigella* inoculated on NA; but when these oxygen-inhibited cultures were later incubated in air, growth occurred.

Therefore, it is unlikely that O_2 reacted with media components to produce significant concentrations of growth-inhibitory substances. If these conditions had been present, there should not have been growth on BHIA, nor would the rapid and profuse growth often seen on NA (usually within 24 hr) occur upon subsequent incubation in air.

In the case of *V. comma* (under similar conditions as those used for *Salmonella* and *Shigella*) hyperoxia was bactericidal for the seven strains examined. However, with all genera we investigated, the inhibitory effects of O_2 depended on the degree of enrichment of the growth media—the majority of even the *V. comma* strains grew to some degree on BHIA.

The mechanism whereby the nutritionally enriched media manifested protection against O_2 inhibition of growth must await identification of the active components. The data suggest that a carbohydrate may be one factor involved. Our findings that nutritional enrichment supports growth of aerobic vibrios in the presence of increased O_2 tensions are consistent with the results of Fletcher and Plastridge (1). Their investigations showed that, in contrast to a chemically defined medium, yeast extract agar doubled the tolerance of microaerophilic vibrios to 0.2 atm absolute of O_2.

We found that oxygen altered the responsiveness of *V. comma* to sulfisoxazole, because under conditions in which there was no O_2 inhibition of growth during the 24-hr exposure period, there was still a marked synergistic effect between these two agents. Also, we observed this O_2-induced synergism with intermittent O_2 exposures. Whether O_2 will synergize with PABA antagonists to inhibit the growth of bacteria other than *V. comma* and mycobacteria (3) has not yet been demonstrated.

The observations that two widely separated genera, such as *Mycobacterium* (3) and *Vibrio*, respond in a similar manner to O_2 in the presence of drugs known to interfere with PABA metabolism suggests that metabolic pathways involving PABA may be particularly sensitive to increased O_2 tensions. Schreiner (11), by use of antibiotics (not yet shown to be directly involved with PABA metabolism), was unable to demonstrate synergism with increased O_2 tensions utilizing *Staphylococcus aureus*. Nevertheless, our suggestion does not preclude the possibility of O_2 acting at other sites (4). Thus, *V. comma*, like *Achromobacter* species P 6 (5), may serve as a model system for studying the cellular and subcellular mechanisms of O_2 toxicity. Detailed studies of the mechanism of O_2 synergism with PAS or sulfisoxazole may provide a new approach for the development of drugs that are also able to synergize with PABA antagonists and are thus able to replace the need for cumbersome, mechanical equipment to supply the currently necessary increased O_2 tensions. The observation that oxygen exerts profound effects on gram-negative enteric pathogens, as demonstrated by the growth-inhibitory effects and increased sensitivity to sulfisoxazole, raises the question as to whether exposure to increased oxygen tensions (in the presence or absence of drugs) may also alter the pathogenicity of the organisms.

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