The effect of high-pressure oxygen on chromogenic bacteria

LINDA L. REYNOLDS, M.T. (A.S.C.P.),
JOHN W. KING, M.D., PH.D.
Department of Clinical Pathology

MOST of the studies on the effect of oxygen under high pressure on bacterial infections have focused on the effect the oxygen might have on the experimental animal or on the human patient rather than on the infectious agent. Early studies on the effect of oxygen on bacteria were primarily aeration experiments using graded concentrations of oxygen at atmospheric pressures. Moore and Williams[1, 2] and Adams[3] studied the effect of oxygen on acid-fast bacteria and on *Pasteurella pestis* and concluded that oxygen had an inhibitory effect on the metabolism of these organisms. Karsner, Brittingham, and Richardson[4] investigated the effect of high partial pressures of oxygen on other bacteria; their results were inconclusive.

Wilson[5] aerated cultures of *Salmonella typhimurium* with increasing concentrations of oxygen, and found that cultures aerated with 100 percent oxygen produced approximately five times as much growth as did the control cultures that were not aerated. Levine[6] studied the effect of oxygen on *Bacillus subtilis* and concluded that increasing the concentration of oxygen in the atmosphere in which these cultures were grown to 100 percent had no significant effect on the rate of growth or sporulation of this organism. Smith and Johnson[7] grew *Serratia marcescens* in a glucose-citrate medium and found that cell concentration and live cell count reached a maximum when aerated at the rate of 6mM of oxygen per liter per minute. Both pigmented and nonpigmented strains behaved similarly, but no note was made as to the effect, if any, the concentration of oxygen had on the ability of the pigmented organisms to produce pigment. Recently, McDaniel, Bailey, and Zimmerli[8, 9] reported on the effect of oxygen supply on the growth of *Escherichia coli*. The amount of growth of that organism in broth cultures is dependent upon the oxygen made available by several methods of aeration and agitation and the use of various types of flasks. These authors present an excellent review of the published papers on the effect of oxygen supply in fermentation.

Ollodart and Blair[10] found that common gram-negative bacteria respond to increasing oxygen first by increased growth up to 1.1 to 1.3 atm of oxygen, and above this level by decreased growth. When exposed to 3 atm

of air, instead of pure oxygen, both *Escherichia coli* and *Pseudomonas aeruginosa* demonstrated enhanced growth in contrast to the inhibition produced by pure oxygen at these pressures.

While the studies on aerobic and facultatively anaerobic bacteria have shown that oxygen either stimulates growth or produces relatively slight effects on the organisms, it would be expected that studies with anaerobes would prove more fruitful. Several investigators[11-14] reported on the effect of high-pressure oxygen (HPO) on species of *Clostridium* and have reached the somewhat surprising conclusion that while HPO inhibits growth and toxin production in these organisms, the inhibition is not complete and the spores always recover activity when removed to more favorable conditions. McAllister and associates,[15] and Hopkinson and Towers[16] have demonstrated inhibition of growth of a whole series of common pathogenic bacteria that were subjected to HPO. Hess[17] investigated the effect of the amount of oxygen in the gas mixture used to produce bacterial aerosols of *Serratia marcescens,* and concluded that completely hydrated organisms were insensitive to pure oxygen at pressures up to 400 psi for 4 hours. No change in motility was observed as compared with cultures held under nitrogen. No comment was made in this paper about variation in pigment production as the result of HPO.

We wished to investigate the effect of HPO on bacteria and, because of our previous interest in the chromogenic gram-negative bacteria,[18] we decided to study that group of organisms.

The influence of the depth of the culture medium on the effect of oxygen on the bacteria was investigated after reading a recent report of Irvin and associates[19] which described the possible deleterious effects of the presence of wound exudates on the effectiveness of HPO therapy.

MATERIAL AND METHOD

Suitable cultures for study were available from isolations in our laboratory, from the American Type Culture Collection, and from the teaching collection of the Pennsylvania State University. Three cultures of *Chromobacterium violaceum,* four of *Serratia marcescens,* and three each of *Pseudomonas aeruginosa, Chromobacterium orangium, Serratia fuchsina, Serratia kilensis,* and *Flavobacterium aborescens* were subjected to HPO. Growth was measured by means of turbidity, and pigment production was compared with that of appropriate control cultures.

All liquid and solid culture media were inoculated, and cultures were exposed to pure oxygen at 45 psi in suitable pressure jars. The periods of exposure ranged between 24 and 72 hr. All inocula were from 24-hr broth cultures either of nutrient broth or of trypticase soy broth. Control cultures were prepared at the same time and treated identically as the cultures

exposed to HPO, except that the control cultures were incubated under normal atmospheric conditions. Daily readings of the turbidity of the exposed cultures were compared with the control cultures, and any variation in pigment production was noted. All incubation was carried out at room temperature (25 C), as many of the bacterial strains studied produced pigment better at this temperature than at 37 C, and all cultures grew well at the lower temperature.

RESULTS

In almost all experiments, the control cultures reached maximum growth and pigment production within three days of inoculation, while most cultures exposed to oxygen at increased pressures were delayed in growth and showed little pigment at that time (*Fig. 1*). *Chromobacterium violaceum* produced almost no pigment in three days' incubation after exposure for 48 hr to HPO. The control cultures at that time were turbid and deeply pigmented. *Chromobacterium orangium* was somewhat less sensitive to the effect of HPO. The differences in its pigmentation do not show so well in the black-and-white photograph (*Fig. 1*) as in the fresh culture; there was variation, and this culture also showed the effect of HPO in the inhibition of pigment production.

Table 1 presents some representative results of these experiments. The original test culture of *Pseudomonas aeruginosa* showed no inhibition by HPO either in growth or in pigment production. However, one of three other test cultures of this organism subsequently did show inhibition both of growth and of pigment production, results that agreed with those of previous workers[8, 16] who have reported delayed growth of this organism under high oxygen tension. All the rest of the cultures showed various degrees of sensitivity to HPO as evidenced by delay both in growth and in pigment production. In no case were the bacteria killed by HPO, as all subcultures grew normally. Similar results were obtained with nutrient

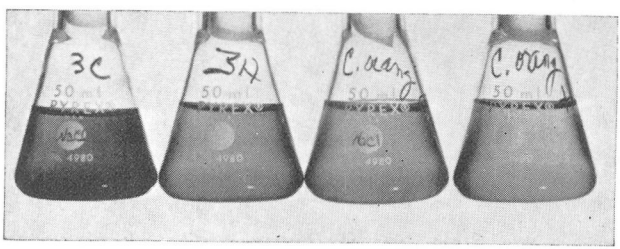

Fig. 1. Comparison of pigment produced in *Chromobacterium violaceum* and in *Chromobacterium organium* after 5-day incubation. Cultures 1 and 3 were exposed to 48 hr of HPO; cultures 2 and 4 were controls. The cultures were grown in 50 ml of trypticase soy broth.

Table 1.—*Representative reactions of chromogenic bacteria exposed to HPO as compared to control cultures (50 ml of trypticase soy broth dispensed in 150-ml Erlenmeyer flasks)*

Organism	Exposure to high-presure oxygen					
	36 hr		72 hr		Control	
	Growth	Pigment	Growth	Pigment	Growth	Pigment
Pseudomonas aeruginosa	+	+	+	+	+	+
Chromobacterium violaceum	0	0	0	0	+	+
Chromobacterium orangium	0	0	+	+	+	+
Serratia marcescens	+	Trace	+	+	+	+
Serratia fuchsina	0	0	+	0	+	+
Serratia kilensis	0	0	+	0	+	+
Flavobacterium arborescens	0	0	+	+	+	+

Table 2.—*The effect on cultures of exposure of media to HPO before and after inoculation.* (The instrument used was a Coleman Jr. spectrophotometer set at 505 λ.)

Organism	Medium* exposed to HPO for 24 hr, transmittance of light, %				
	Before and after inoculation		After inoculation only		No exposure to HPO
	Capped†	Uncapped	Capped†	Uncapped	Capped†
Chromobacterium violaceum	98	98	97	97	79
Serratia kilensis	96	99	67	70	72
Escherichia freundii	96	97	71	68	68
Proteus vulgaris	71	83	67	65	68

* The medium was trypticase soy broth in 50-ml quantities in 150-ml Erlenmeyer flasks.
† Capped cultures were plugged with foam plastic stoppers.

broth and with nutrient broth supplemented with yeast extract, phosphate salts, and serum. Cultures on trypticase soy broth or on agar reacted similarly, although solid media were not so desirable as liquid because, during decompression, bubbles appeared in the medium and the plates were then difficult to interpret.

The combination of preinoculation and postinoculation exposures of the medium to HPO produced even greater inhibition than postinoculation exposure alone (*Table 2*). *Chromobacterium violaceum* was greatly inhibited by preinoculation exposures to HPO. While *Serratia kilensis* was inhibited by preinoculation exposure, it revived much more rapidly, so that its growth as measured by turbidity equalled that of the control at 24-hr incubation in HPO. *Escherichia freundii,* which was included in the

experiment as a representative of the enteric bacteria, and is relatively resistant to HPO, was similarly inhibited, while *Proteus vulgaris* was not affected by the preinoculation exposure of the medium to HPO.

Cultures of *Serratia marcescens* were prepared in Petri dishes to a depth of 0.1, 0.2, 0.4 or 0.8 cm. When such cultures were exposed to HPO, there was more inhibition of growth in the organisms grown in the 0.1-cm deep plates than in the cultures prepared in deeper layers. *Table 3* summarizes the data in a representative experiment with a freshly isolated, highly pigmented strain of *Serratia marcescens*. Pigment production was uniformly inhibited in all cultures studied even after 48 hr of incubation under atmospheric conditions. Presumably pigment production depends upon the activity of a more highly oxygen-sensitive mechanism than do cell growth and division. The apparently slightly more complete recovery of the 0.1-cm deep cultures as compared to the cultures prepared in deeper layers may be an artifact produced by greater relative evaporation of the medium in the thin-layer cultures as compared with the relative evaporation of the deeper cultures.

Inhibition of *Serratia marcescens* at 20 psi was negligible, whereas the results at 32 psi were more striking, but still did not reach the degree seen in cultures held at 45 psi. Pigment production was not inhibited at 20 psi, but was inhibited almost as effectively at 32 psi as at higher pressures. *Table 4* shows a representative experiment illustrating these reactions.

In an attempt to determine whether the inability to produce pigment in HPO exposure cultures was or was not due to mutation and subsequent selection, cultures of pigment-inhibited cultures of *Chromobacterium violaceum* and *Serratia marcescens* were streaked on trypticase soy agar plates

Table 3.—*The effect of depth of culture medium on oxygen inhibition of* Serratia marcescens

Depth of culture, cm	Culture growth,* %				
	Experimental	Control		Experimental	Control
0.1	100	18	Cultures removed to atmospheric conditions for 48 hr	7	3
0.2	96	2		18	8
0.4	95	4		15	3
0.8	92	2		22	6

* Growth expressed as percentage of transmitted light.

The experimental cultures were held under 45 psi oxygen for three days, after which they were removed to atmospheric conditions and held an additional 48 hr. Readings were taken at the end of the HPO incubation and at 48 hr after exposure to HPO.

The medium used was nutrient broth dispensed in plastic Petri dishes having an area of 25 sq cm. Thus 2.5 ml of medium dispensed in a Petri dish gave a culture depth of 0.1 cm.

Table 4.—*Growth of* Serratia marcescens *at different pressures of oxygen*

Depth of culture, cm	Growth, percentage of transmittance of light*		
	45 psi	32 psi	Control
0.1	98	84	28
0.2	98	84	36
0.4	96	75	47
0.8	93	60	61

* Of 1/25 dilution of a 24-hr culture at 550 λ.

so that pigmentation of individual colonies could be observed. All colonies appeared to develop the same degree of pigmentation and it was not possible to obtain stable nonpigmented variants of these organisms.

Experiments using pigmented *Staphylococcus aureus* showed no alteration of pigment or coagulase production in any of the three strains tested. The cultures subjected to HPO were delayed slightly in growth but no changes in mannitol fermentation or antibiotic sensitivity could be detected in the exposed cultures. McAllister and associates[15] had previously noted pigment inhibition in *Staphylococcus aureus.*

CONCLUSIONS

Exposure of chromogenic gram-negative bacteria to high pressures of oxygen (HPO) resulted in inhibition of growth, which was most complete when the culture medium was held under HPO for a period before inoculation, or when the medium was dispensed in shallow layers. In shallow cultures, the organisms did not recover completely even after 48 hr under normal atmospheric conditions. At the same time, pigment production was inhibited. The recovery of the ability to produce pigment was delayed even longer than the ability to grow. When cultures that were nonpigmented due to HPO exposure were inoculated into other media, they regained their pigment-producing ability and the subcultures grew quite normally. When such cultures were streaked on solid media, so that isolated colonies were produced, the colonies were all pigmented uniformly, which suggests that the production of nonpigmented cultures by HPO is not the result of the development of an oxygen-resistant nonpigmented mutant.

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