Interactions of helium, oxygen, and nitrous oxide affecting bacterial growth

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Marquis, R. E., S. R. Thom, and C. A. Crookshank. 1978. Interactions of helium, oxygen, and nitrous oxide affecting bacterial growth. Undersea Biomed. Res. 5(2):189–198.—Helium at pressures of 20 to 70 atm in the presence of air was found to stimulate growth of *Streptococcus faecalis*, *Escherichia coli*, and *Staphylococcus aureus*, mainly by increasing the rate of exponential growth. However, at these same pressures, helium potentiated the growth-inhibitory actions of oxygen and nitrous oxide (N₂O). Oxygen was found to act essentially as an anesthetic gas in inhibiting growth of *S. faecalis*; its potency was approximately the same as that of N₂O, and it acted additively in combination with N₂O to inhibit the streptococcus. Oxygen proved to be more potent than N₂O in inhibiting the growth of *E. coli* and *S. aureus*, and each gas potentiated the action of the other. Oxygen sensitivity was correlated with N₂O sensitivity. Overall, our findings indicate that bacterial growth inhibition by anesthetic gases does not accurately reflect narcotic action.

Investigations of microbial growth inhibition by inorganic, anesthetic gases have led to the conclusion that the inhibitory potentials of the gases reflect their narcotic potentials. The narcotic potency series presented by Miller (1972) has Xe > N₂O > Kr > Ar > N₂ > H₂ > Ne or He. Brauer and Way (1970) used binary gas mixtures to show that helium actually has a negative narcotic potential of -0.045 compared with a value of +1.00 for nitrogen. Fenn and Marquis (1968) found a potency series for inhibition of growth of *Streptococcus faecalis* with Xe or N₂O > Ar > N₂; helium appeared to have little or no specific effect other than that due to hydrostatic pressure. Buchheit, Schreiner, and Doebbler (1966) presented a series for inhibition of mycelial growth of *Neurospora crassa* with Xe > Kr > Ar >> Ne >> N₂ or He. Although helium was found to be inhibitory, a pressure of nearly 300 atm was required for a 50% response, and it is possible that inhibition could have been due to hydrostatic pressure rather than to any specific effect of helium. However, a helium pressure of only 40 atm, approximately the pressure tested by Fenn and Marquis, produced a definite inhibition of mycelial extension rate of some 33%: from 4.8 to 3.2 mm/h. Further increases in helium pressure produced little more inhibition. Nitrogen was less potent than helium at these relatively low pressures. Breummer, Brunetti, and Schreiner (1967) found a similar series for inhibition of growth of HeLa cells with Xe or N₂O > Kr > Ar >> Ne, N₂, or He. High
pressures (69 atm) of Ne, N₂, or He were required for a 30% inhibitory response. Again, it is possible that inhibition at 69 atm could have been due simply to hydrostatic pressure.

Schlamm, Perry, and Wild (1974) have reported contrary effects of normoxic helium on growth of *Escherichia coli* in defined medium. Growth was stimulated by 68 atm of helium, and the stimulation appeared to be due specifically to helium and not to hydrostatic pressure. The major effect of helium was to reduce the lag phase of culture growth with no effect on growth rate or yield. The reduced lag was found to be due to enhanced uptake of iron.

Macdonald (1975) found that 100 atm of helium or hydrogen had no specific effect on cell division of *Tetrahymena pyriformis*. Some inhibition did occur but it was due to hydrostatic pressure. At higher gas pressures, the gases ameliorated inhibition due to hydrostatic pressure so that growth of cells compressed with the gases was faster than that of cells compressed in a gas-free system. Helium proved to be less effective in counteracting pressure than was hydrogen.

Enfors and Molin (1975) have reported that compressed gases can inhibit germination of bacterial endospores and that this process of differentiation was extremely sensitive. Their data indicate that N₂O was more inhibitory than Ar, which was more inhibitory than N₂. Carbon dioxide also was highly inhibitory, possibly because of acidification of the suspensions. Helium was impotent, even at a pressure of 100 atm.

In this paper, we present the results of experiments designed to assess the effects of helium on bacterial growth and its interactions with N₂O and O₂. These experiments are part of a general study of the relationship of growth inhibitory effects of anesthetics to their narcotic effects.

**MATERIALS AND METHODS**

*Streptococcus faecalis* ATCC 9790 was grown in a complex medium (TGM), which we have described previously (Marquis, Porterfield, and Matsumura 1973), containing tryptone, glucose, and Marmite, a commercial yeast extract from Bovril, Ltd., Montreal, Canada. *Escherichia coli* B and *Staphylococcus aureus* H were grown in tryptic soy broth (Difco Laboratories, Detroit, Michigan) with 0.1% (w/v) KNO₃. Growth was assessed in terms of absorbance of light of 700-nm wavelength measured with a Beckman DU spectrophotometer with cuvettes of 1-cm lightpath. Direct bacterial counts were performed with a Petroff-Hauser counter and a phase-contrast microscope. Viable counts were obtained by diluting samples with 1% (w/v) Difco peptone broth and plating 0.1-ml samples on the surfaces of trypticase-soy agar plates (Bioquest Co., Cockeysville, Md.).

Cultures were prepared with 50 ml of medium in cotton-plugged, 250-ml Erlenmeyer flasks and an initial population of approximately 5 × 10⁷ bacteria per ml, determined by direct counting. A sterile, Teflon-coated stirring bar was placed in each culture, and the flask was placed in a large, standard pressure cylinder of the type we have used previously (Marquis 1976) with an O-ring seal. The cylinders were constructed of nonmagnetic steel so that the cultures could be stirred to facilitate gas transfer. They hold a relatively large volume of gas, some 3200 ml. The cylinders were connected by means of high-pressure tubing through a two-way valve (Amino, Silver Spring, Md.) to tanks of compressed gas. In all experiments, the air that was initially in the pressure cylinders was not flushed out. The cylinders were pressurized with the desired gas, or mixture of gases, and then the two-way valves were closed to seal the cylinders. The high-pressure tubing was disconnected, and a pressure gauge was attached to each valve so that the actual pressure within the cylinders could be checked. All cultures were incubated at room temperature of approximately 22°C. Control, unpressurized
cultures were incubated on the bench next to the pressurized cylinders. Magnetic stirrers were used only to achieve initial gas transfer and not for long periods, so that the stirred cultures were not heated by the stirrer.

The cultures were decompressed over a period of about 5 min for sampling, a portion was removed, and the remainder was recompressed immediately. Measured temperature changes in cultures after decompression from, for example, 40 atm of helium were at most only about 5°C. Obviously, this cooling would be reversed on subsequent recompression of the culture. After decompression, the bacteria were observed with the phase microscope. No intracellular gas bubbles were seen in any of the experiments, nor was there evidence of gross cell damage except for bacteria that had been exposed to highly inhibitory levels of O₂ or N₂O for 12 to 24 h. Experiments in which cultures were repeatedly compressed and decompressed indicated that the procedure did not result in any significant reduction of total count or viable count or any change in culture absorbance. Also, in some experiments duplicate cultures were prepared and pressurized. One of them was then sampled repeatedly, while the other was sampled only once. Growth was essentially the same in the two cultures, indicating that any experimentally induced changes in growth were not to any significant degree the result of the pressurization-depressurization procedure.

Compressed gases were obtained from Air Products and Chemicals of Allentown, Pa. Helium was 99.995% pure, oxygen was medical grade and 99.6% pure. Nitrous oxide was 98% pure, with air as the major impurity.

Experiments on the effects of hydrostatic pressure alone were carried out in two ways. For some, a rubber plug was placed in a filled tube of culture, which was then compressed by use of compressed gases. More often, pressurization was performed with a hydraulic pump in a liquid system with the culture within a plastic syringe sealed at one end.

Oxygen utilization was monitored with a Model-53, oxygen-electrode assembly from Yellow Springs Instrument Co. (Yellow Springs, Ohio).

RESULTS

Effects of helium on bacterial growth

Figure 1 presents data from a typical experiment showing stimulation of bacterial growth by helium. Here it is apparent that 23.8 or 40.8 atm of helium in the presence of air significantly increased the rate of exponential growth of S. faecalis and slightly increased the yield. In eight experiments, there was an average increase in final absorbance of the cultures from 0.865 ± 0.027 to 0.887 ± 0.126 and an average decrease in doubling time from 1.65 ± 0.15 to 1.23 ± 0.24 h. Figures after the ± sign indicate 95% confidence limits, which primarily reflect variations from experiment to experiment. In no single experiment was growth in the pressurized culture less than or equal to that in the control culture. Doubling times were estimated from the slopes of linear portions of plots of the logarithm of Aₗ₉₀ versus time. These stimulatory effects were essentially independent of pressure over the range from 20 to 70 atm, the highest pressure tested in this series of experiments. Over this same range, hydrostatic pressure had no effect, or a very slight inhibitory effect above 50 atm. In essence, the same results were obtained with S. aureus and E. coli, although stimulation was slightly less for the former organism and slightly more for the latter in relation to the results obtained with S. faecalis. Our findings, then, agree with those of Schlamm et al. (1974). However, it seems that the helium effect here is not related to enhanced uptake of iron, since we used complex media, and addition of iron did not enhance growth.
Fig. 1. Stimulation of growth of *S. faecalis* in TGM medium by helium; \(A_{700}\) values indicate absorbance of 700-nm-wavelength light by the cultures.

**Growth inhibition by oxygen and nitrous oxide**

Surprisingly, the effects of these two gases on growth and viability of *S. faecalis* were similar. Figure 2 shows the typical pattern of inhibition, with slowing of exponential growth and reduction in culture yield, in these experiments by 14.7 and 20.4 atm \(N_2O\). The inhibitory potential of oxygen for *S. faecalis* was essentially the same as that of \(N_2O\), as indicated by the yield data presented in Fig. 3. Rate data gave a similar picture. As indicated in Fig. 3, oxygen was much more toxic than \(N_2O\) for *E. coli* and *S. aureus*. It is apparent in Fig. 3 that sensitivity of \(N_2O\) is correlated with oxygen sensitivity.

When oxygen and nitrous oxide were combined, their effects on growth of *S. faecalis* were essentially additive. However, they appeared to be potentiating for each other in inhibiting growth of *E. coli* or *S. aureus*. The example presented in Fig. 4 shows that 2 atm of oxygen had only a relatively small effect on *E. coli* growth. However, there was a considerable reduction in growth rate and culture yield when 2 atm of \(O_2\) were combined with 6.8 atm \(N_2O\), which itself had little effect on growth. Very similar results were obtained for *S. aureus*, but of course the bacterium was more sensitive to the combined or separate gases.

During the course of these experiments, we found that it was possible to increase markedly the oxygen sensitivity of *S. faecalis* by adding phosphate buffer to the growth medium. For example, 20.4 atm of oxygen reduced growth in unsupplemented TGM medium by about 38%,
but in medium supplemented with 0.1 M phosphate, the reduction was 66%. The effect could not be induced with other salts such as NaCl, Na₂SO₄, or MgCl₂. Sensitivity to N₂O was increased also in the phosphate-supplemented medium, but the change was not as striking one.

Experiments in which metabolic acids were neutralized at intervals during the culture cycle indicated that the main effect was a pH effect. *S. faecalis* has relatively limited capacity to reduce oxygen, and this capacity is reduced at low pH. For example, *S. faecalis* cells in tryptone-Marmite solution used sufficient oxygen to yield Qₒₒ values of about 3, measured with an oxygen electrode. Addition of 50 mM glucose resulted in an increased Qₒₒ of about 15 µliter/mg dry weight of cells/h. Oxygen utilization decreased with acidification to 77% of the pH 7 value at pH 6.0, to 66% at pH 5.5, to 33% at pH 5.0, to 5% at pH 4.5. Initial culture pH values were about 7.2, and the values declined during the growth cycle to about 4.8. However, phosphate supplementation buffered the cultures so that the final pH was about 6.2 and presumably, the cells produced greater amounts of toxic metabolites of oxygen in the buffered cultures.

Both oxygen and nitrous oxide caused cell death over relatively long periods of exposure. For example, when *E. coli* cells were suspended in minimal medium without a fuel source at an initial concentration of 4.9 × 10⁹ cells/ml and exposed to 20 atm O₂ plus air or 20 atm N₂O plus air, there was no loss of viability over a 4-h period. However, after 24 h of exposure, the count in the oxygen-treated suspension had declined to 1.5 × 10⁹ cells/ml and that in the N₂O-treated
Fig. 3. Growth inhibitory actions of O₂ and N₂O. Culture yields were assessed in terms of maximal absorbance of 700-nm-wavelength light. Values shown are for inhibition of *S. faecalis* by oxygen (○) or N₂O (●), of *E. coli* by oxygen (□) or N₂O (■), and of *S. aureus* by oxygen (△) or N₂O (▲). In all cases air was present in the culture vessels.

Suspension declined to 1.1 × 10⁸ cells/ml. Control suspensions exposed only to air showed no loss in viability.

**Potentiating action of helium**

Although helium alone stimulated growth, it exacerbated the inhibitory effects of N₂O or O₂ when combined with either of these gases. A typical example is presented in Fig. 5, which shows that 10.2 atm of N₂O had some inhibitory effect on *E. coli* growth, principally by extending the lag time by about 0.3 h and reducing culture yield by about 20%. However, a combination of 10.2 atm N₂O and 20.4 atm helium extended the lag time by some 2.4 h compared with the 1-atm culture, and caused an increase in mass doubling time from 1.2 to 2.0 h with a yield decrease of some 53%. In a comparison experiment, 20.4 atm of helium alone had little effect on lag time but decreased the mass doubling time from 1.2 to 1.1 h and increased the yield by 13%.

Helium also potentiated the inhibitory action of O₂. Typical data for *E. coli* are presented in Fig. 5. Again, potentiation was apparent in the increase in mass doubling time and the decrease in yield.

Helium also enhanced the inhibitory potential of oxygen or nitrous oxide for *S. aureus* and *S. faecalis*. Of course, the latter bacterium was relatively resistant to O₂ or N₂O, but still, the
Fig. 4. Inhibition of growth of *E. coli* in trypticase-soy broth plus 0.1% (w/v) KNO₃ by oxygen and a combination of oxygen and nitrous oxide.

addition of helium to these gases produced mixtures that were more rather than less inhibitory compared to the helium-free gases.

**DISCUSSION**

The data presented here show that the previously reported negative narcotic potential of helium for animals is reflected in a stimulatory action of the gas for microbial growth. The action appears to be due specifically to helium and not to hydrostatic pressure, but is peculiar in being pressure-independent over the range from 20 to 70 atm. Buchheit et al. (1966) described inhibitory actions of helium that were pressure-independent over a similar range.

Brauer and Way (1970) found that the narcotic effects of inert gases in binary mixtures were linearly additive when the test response was the righting reflex of CD-1 mice. In fact, they used the additivity rule to calculate the negative narcotic potential of helium. Our findings indicate that the additivity rule is not applicable to bacterial growth studies because helium potentiates the action of nitrous oxide rather than reversing it. We had reported previously (Fenn and Marquis 1968), based largely on results obtained with xenon, that inert gases potentiate oxygen toxicity. Helium also is potentiating for oxygen toxicity. Nitrous oxide was potentiating for oxygen damage to *E. coli* and *S. aureus* but only additive with oxygen in damaging *S. faecalis*. 
Fig. 5. Potentiating effects of helium on inhibition of growth of E. coli by oxygen (left panel) and nitrous oxide (right panel).
In our previous study with *S. faecalis* 9790, the organism was much more oxygen-sensitive. It was possible formerly to obtain a 50% reduction in the extent of growth with only 3 to 4 atm of \( O_2 \), whereas now some 25 atm is required. In fact, *S. faecalis* now behaves superficially in the same way that *Lactobacillus plantarum* does. This latter bacterium has little or no protective superoxide dismutase or catalase (McCord, Keele, and Fridovich 1971) but it is still highly oxygen-tolerant simply because it does not metabolize oxygen and so does not produce toxic metabolic products. *S. faecalis* does use some oxygen, even though it is primarily a homofermentative, lactic-acid bacterium. However, it has protection against oxygen metabolites in the form of superoxide dismutase and peroxidase (Fridovich 1975). In all, it seems that oxygen acted against *S. faecalis* in our experiments with unsupplemented TGM medium primarily as an anesthetic gas with a potency about equal to that of nitrous oxide. Bennett and Ackles (1970) have previously assessed the narcotic effects of hyperbaric oxygen for man.

Bacteria appear from our data to be relatively insensitive to anesthetic gases. Anesthetic effects in man can be achieved with \( N_2O \) or Xe at pressures of 1 atm or less. Xenon pressures required for 50% inhibition of growth have been found to be only 0.8 atm for *N. crassa* (Buchheit et al. 1966) and about 2 atm for HeLa cells (Breummer et al. 1967). \( N_2O \) should be only slightly less potent than Xe, and in our past studies with *S. faecalis*, we found that 6 to 8 atm of Xe or \( N_2O \) were required for 50% inhibition of growth. However, in the present study, about 26 atm of \( N_2O \) were required for 50% inhibition. *E. coli* and *S. aureus* were more sensitive and required 16 and 14 atm, respectively, for 50% reduction in maximal optical density of cultures in complex media. The basis for this apparent difference between prokaryotic and eukaryotic cells is not known, although bacteria tend to be more condensed cells with lower water contents. Even single-celled protozoa are relatively sensitive. Sears and Gittleson (1964) found that only approximately 3.5 atm of Xe were required to reduce by 50% contractile vacuole activity in *Paramecium multimicronucleatum*. Presumably, growth would require a functional vacuole.

Fenn (1969) found that \( N_2O \) and Xe potentiated the toxic effects of oxygen for *Paramecium caudatum*, and so this protozoan behaved as did the bacteria used in our study. Fenn (1965) had mentioned being surprised to find a correlation between oxygen toxicity and nitrogen narcosis. We also have been made curious by the correlation between oxygen sensitivity and \( N_2O \) sensitivity for the admittedly limited sample of bacteria we tested. Perhaps the exploration of this correlation will reveal some common site of action.

Supplementary data and information pertaining to this project are presented in ONR Progress Report 5 (ADA037840) available from Defense Documentation Center, Cameron Station, Alexandria, Virginia 22314. Our work was supported by contract N00014-75-C-0634 from the Office of Naval Research. S. R. Thom is a Medical Scientist Trainee under U.S. Public Health Service grant GM-02263. —Manuscript received for publication September 1977. Revision received December 1977.

l’action de l’autre. Une corrélation entre la sensibilité à l’oxygène et la sensibilité au protoxyde d’azote s’observe aussi. En général, nos résultats montrent que l’inhibition de la croissance bactérienne par des gaz anesthésiques ne sert pas d’indice exacte de l’action narcotique.

**REFERENCES**


