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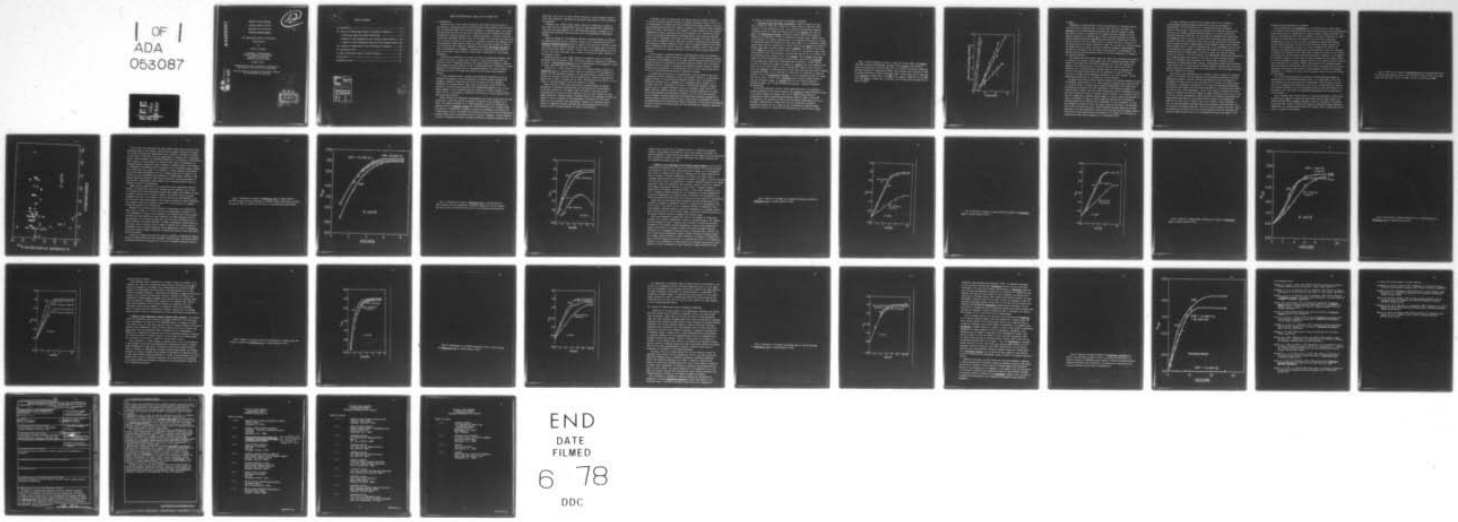

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OFFICE OF NAVAL RESEARCH

Contract N00014-75-C-0634

Work Unit No. NR 204-015

TECHNICAL REPORT NUMBER 6

The Physiological Bases for Microbial Barotolerance

by

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31 March 1978

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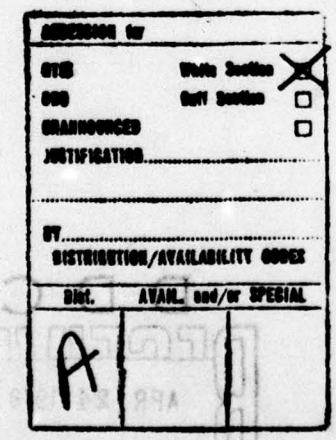


REPORT FOR PERIOD FROM 1 APRIL 1977 TO 31 MARCH 1978

I. Introduction

The major focus of our efforts during the past support period has been on the actions of compressed gases on microbial growth and metabolism, and this report will be concerned mainly with microbial responses to anesthetic gases. The impetus for this work came from our work of the previous year which indicated that growth inhibition by anesthetic gases is not a general anesthetic or narcotic effect but is instead a definably different action of the agents. Our past work was carried out with a variety of bacteria. The results of experiments reported here indicate that the distinction between growth inhibitory effects and general anesthetic effects can be made also with eukaryotic organisms, specifically Tetrahymena pyriformis. Thus, the distinction is probably a universal one, although we shall certainly have to work with a wider variety of cells before coming firmly to this general conclusion.

The practical importance of our work is related to deep-diving programs, such as those of the Navy, in which man is exposed to high-pressure gases for long periods of time in underwater habitats. The steady advance of hyperbaric medicine also presages long-term exposure of man to high-pressure gases. Certainly, it seems important in planning these sorts of prolonged exposures to be aware of growth inhibitory actions of the gases that differ from the better known anesthetic actions.

It seems reasonable here to present some background information to put our findings in perspective. Past 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. Miller {1972} has presented a narcotic potency hierarchy with Xe>N_2O>Kr>Ar>N_2>H_2>Ne or He. In fact, helium appears to have a negative narcotic potential of -0.045, compared to a value of +1.00 for nitrogen, as indicated by the studies of Brauer and Way {1970} with binary gas mixtures.

Growth inhibition of eukaryotic microorganisms and tissue culture cells by these gases shows a similar hierarchy of potency, but generally with nitrogen out of place. For example, Buchheit et al. {1966} presented a series for inhibition of mycelial growth of Neurospora crassa with Xe>Kr>Ar>>Ne>>N_2 or He. Although helium was found to be inhibitory, a pressure of nearly 300 atmospheres 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. Strangely, a helium pressure of only 40 atmospheres produced a definite inhibition of mycelial extension rate of

about 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 et al. {1967} found a similar series for inhibition of growth of HeLa cells with Xe or N_2O>Kr>Ar>>Ne, N_2 or He. High pressures {69 atmospheres} of Ne, H_2 or He were required for a 30% inhibitory response, and again, it is possible that inhibition could have been due to 69 atmospheres of hydrostatic pressure.

There are studies of the inhibitory effects of anesthetic gases for protozoa, but generally growth was not considered. However, if one assumes that cells of Paramecium multimicronucleatum would be unable to grow after the function of their contractile vacuole had been stopped by the action of the gases, then the data of Sears and Gittleson {1964} indicate a potency series with N_2O>Xe>Kr>>Ar, N_2 or He. In fact, the latter three gases had no effect on contractile vacuole function at the pressures tested.

Anesthetic gases can also inhibit growth of prokaryotic cells, as we found previously {Fenn and Marquis, 1968}. The potency series we obtained for inhibition of growth of Streptococcus faecalis was Xe or N_2O>Ar>N_2>He; helium appeared to have no effect apart from that due to hydrostatic pressure.

Schlamm et al. {1974} assessed the effects of normoxic helium on growth of Escherichia coli in a defined medium. Growth was stimulated by 68 atmospheres of helium, and the stimulation appeared to be due specifically to helium and not to hydrostatic pressure. The major action of helium was to reduce the lag phase of growth with no effect on growth rate or yield. The reduced lag was found to be the result of enhanced uptake of iron in the presence of helium.

Enfors and Molin {1975} have reported that compressed gases can inhibit germination of bacterial endospores and that this process of differentiation is extremely sensitive to the gases. Their data indicate that N_2O was more inhibitory than Ar, which was more inhibitory than N_2. Carbon dioxide also was highly inhibitory, possibly because of acidification of the suspensions. Helium was impotent, even at a pressure of 100 atmospheres. As indicated in last year's report, we have confirmed these observations. However, our experiments with a variety of vegetative bacteria indicated that spore germination is not more sensitive than is bacterial growth, at least of some species.

In general, then, it appears that the potency series for growth inhibition is nearly the same as that for narcotic or general anesthetic effects, and it was not unreasonable to consider that the two effects were related. However, there are some disturbing differences. For example, nitrogen is a moderately potent anesthetic gas but it seems to have little or no capacity to inhibit growth. If one considers combinations of gases, variances become more prominent. As we reported last year, helium is moderately stimulating for bacterial growth. This effect was expected on the basis of knowledge of the negative narcotic potential of helium. We did not expect that, in combination with a gas such as nitrous oxide, helium was strikingly potentiating for growth inhibition. This is just the opposite behavior that helium has in combination with nitrous oxide for general anesthetic action. Thus, helium antagonizes narcotic or general anesthetic action but enhances growth inhibitory actions.

In this report, we describe progress in defining more clearly the inhibitory actions of anesthetic gases for bacteria and an extension of our previous findings to eukaryotic microorganisms. One of the major advances during the past year on the project has been the realization that growth inhibitory effects are a distinctly different class of actions in comparison with general anesthetic effects. Results obtained last year pointed to the distinction, but we had not become fully aware of it or of its widespread importance in hyperbaric biology.

In considering the actions of compressed gases, one has always to deal with a hydrostatic pressure component. In fact, our primary interest is in hydrostatic pressure effects. In the particular experiments reported here, the effects of hydrostatic pressure can be considered to be negligibly small. However, one of our major aims is to investigate the effects of hydrostatic pressure on oxygen toxicity and on the growth inhibitory actions of anesthetic gases. The work of ZoBell and Hittle (1967) indicated a drastically increased sensitivity to oxygen for bacteria growing under hydrostatic pressure, and one of the most exciting recent findings in diving physiology is that hydrostatic pressure antagonizes or reverses general anesthetic effects. Both of these actions of hydrostatic pressure are physiologically and biochemically complex. Hopefully, our work with microorganisms can help to define the molecular bases for the interactions of pressure, oxygen and anesthetic gases.

II. Actions of High-pressure Gases on Prokaryotic Organisms.

A. Individual gases and general methodology. Anesthetic gases can cause both a slowing of growth and a reduction of growth yield for bacterial batch cultures. The data presented in Fig. 1 show relative sensitivities to growth inhibitory actions of oxygen and nitrous oxide for S. faecalis ATCC 9790, E. coli B and Staphylococcus aureus H growing in complex media. Here degrees of inhibition are expressed in terms of reductions in growth yields. Similar plots can be prepared for changes in growth rates. It is apparent that there is a range of sensitivity among the bacteria and that there seems to be a correlation between oxygen sensitivity and sensitivity to nitrous oxide with S. aureus more sensitive than E. coli, which is more sensitive than S. faecalis. The slopes of the lines for S. aureus and E. coli are rather steep, and so it is difficult to make a very strong case for much of a difference in sensitivity between the two. However, both are considerably more sensitive than is S. faecalis. For S. aureus and E. coli, oxygen is far more inhibitory than is nitrous oxide. However, for S. faecalis the two gases have essentially indistinguishable capacities to inhibit growth. In fact, it appears that oxygen acts against S. faecalis much as an anesthetic gas does, and as one would expect, it has a potency about equal to that of nitrous oxide. S. faecalis is a homofermentative, lactic-acid bacterium with little capacity to metabolize oxygen. It is reasonable to think that its resistance to oxygen is similar to that of Lactobacillus plantarum. It is the metabolic products of oxygen that are so toxic to cells, and L. plantarum avoids toxicity simply because it does not metabolize oxygen. S. faecalis does have some limited capacity to metabolize oxygen, but it also has protective enzymes - superoxide dismutase and peroxidase.

We did find during the course of these experiments that it is possible to increase markedly the toxicity of oxygen for S. faecalis by adding phosphate buffer to the growth medium. For example, 20.4 atm of oxygen reduced growth in unsupplemented medium {tryptone-glucose-Marmite medium or TGM} by about 38%. In comparison, the reduction was 66% in the same medium supplemented with 0.1 M phosphate. Sensitivity to nitrous oxide also was increased in phosphate supplemented medium, but the change was not a striking one. Enhanced oxygen sensitivity could not be induced with nonbuffering salts such as NaCl, Na_2SO_4

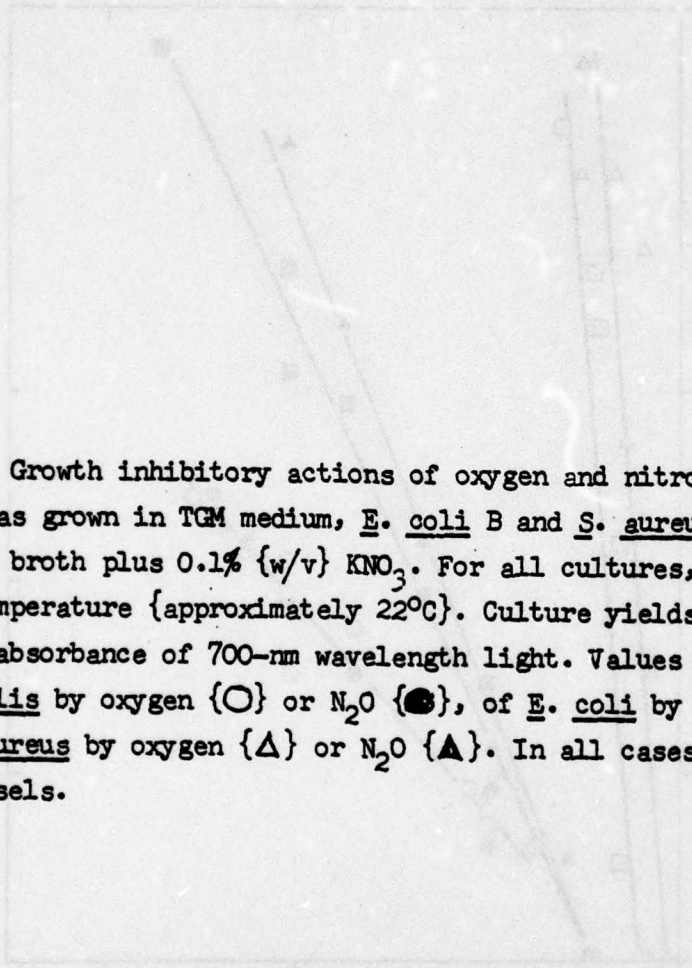
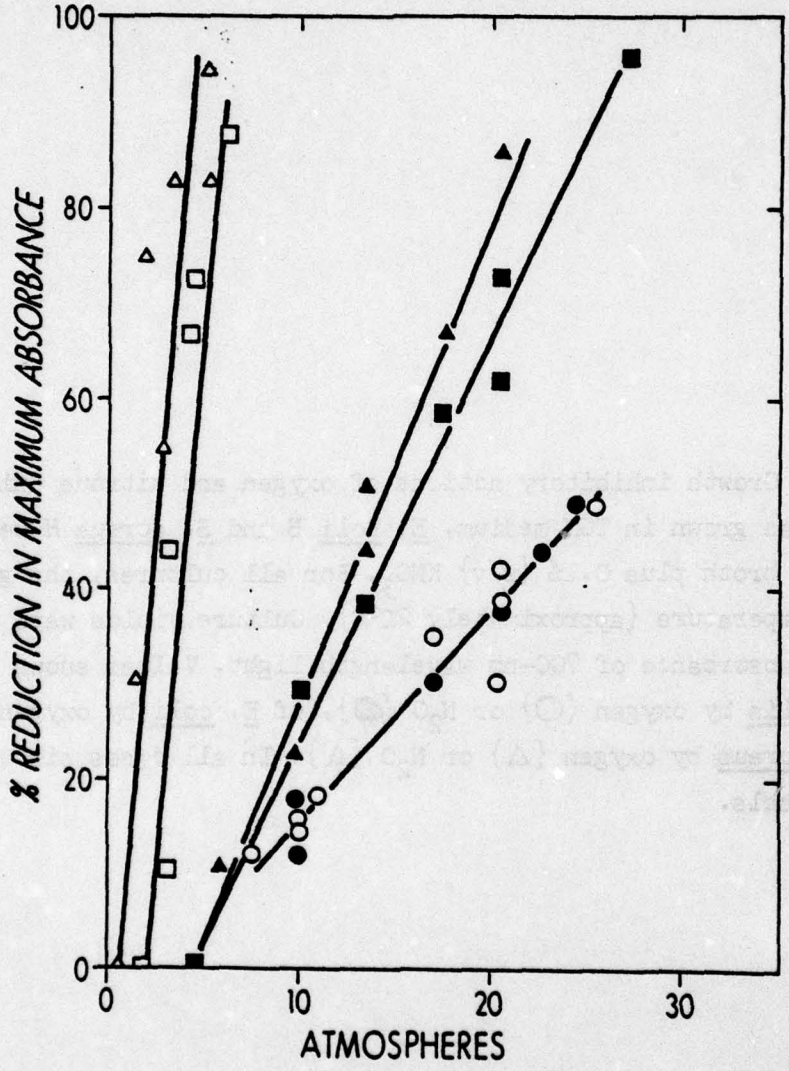


Fig. 1. Growth inhibitory actions of oxygen and nitrous oxide. S. faecalis ATCC 9790 was grown in TGM medium, E. coli B and S. aureus H were grown in tryptic soy broth plus 0.1% {w/v} KNO_3. For all cultures, the growth temperature was room temperature {approximately 22°C}. Culture yields were assessed in terms of maximal absorbance of 700-nm wavelength light. Values shown are for inhibition of S. faecalis by oxygen {○} or N_2O {●}, of E. coli by oxygen {□} or N_2O {■}, and of S. aureus by oxygen {△} or N_2O {▲}. In all cases air was present in the culture vessels.


% REDUCTION IN MAXIMUM ABSORBANCE
100
80
60
40
20
0
0
10
20
30
ATMOSPHERES

or $MgCl_2$.

Experiments in which metabolic acids were neutralized at intervals during the culture cycle indicated that the major effect here was a pH effect. S. faecalis cells in tryptone-Marmite solution used sufficient oxygen to yield Q_{O_2} values of about 3, measured with an oxygen electrode. Addition of 50 mM glucose resulted in an increased Q_{O_2} of about 15 µl/mg dry weight of cells/h. Oxygen utilization by these suspensions decreased with acidification to 77% of the pH-7 value at pH 6.0, to 66% at pH 5.5, to 13% at pH 5.0, to 5% at pH 4.5. The initial pH in our routine cultures is about 7.2, and there is a decline during the growth cycle to a value of about 4.8. However, phosphate supplementation buffered the cultures so that the final pH was about 6.2, and presumably, the growing bacteria produced greater amounts of the 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 4.9×10^9 cells/ml and exposed to 20 atm O_2 plus air or 20 atm N_2O plus air, there was no loss of viability over a four-hour period. However, after 24 hours of exposure, the count in the oxygen treated suspension had declined to 1.5×10^8 cells/ml and that in the N_2O-treated suspension declined to 1.1×10^8 cells/ml. Control suspensions exposed only to air showed no loss in viability.

E. coli B was chosen for more intensive studies of the growth inhibitory actions of anesthetic gases because of its high sensitivity indicated by the data of Fig. 1 and because there is a great deal of available information on its physiology, biochemistry and genetics. For most experiments, the bacterium was grown in a complex medium - tryptic soy broth {Difco Laboratories, Detroit, Michigan} to which was added 0.1% KNO_3 to serve as terminal electron acceptor. It is possible also to grow the organism in a defined, minimal medium. In general, 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. In each experiment, at least one assessment was made of total cell count, usually at the end of the experiment. Total counts were made by use of a Petroff-Hausser counting chamber and a phase microscope. For some experiments, viable counts were made also 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.}.

For many experiments, cultures were prepared with 50 ml of medium in cotton-plugged, 250-ml Erlenmeyer flasks with an initial population of approximately 5×10^7 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 {Aminco, Silver Springs, 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 minutes for sampling, a portion was removed, and the remainder was recompressed immediately. Measured temperature changes in cultures following decompression from, for example, 40 atmospheres of helium were at most only about 5°C. Of course, this cooling would be reversed on subsequent recompression of the culture. Following 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 O_2 or N_2O for 12 to 24 hours. 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

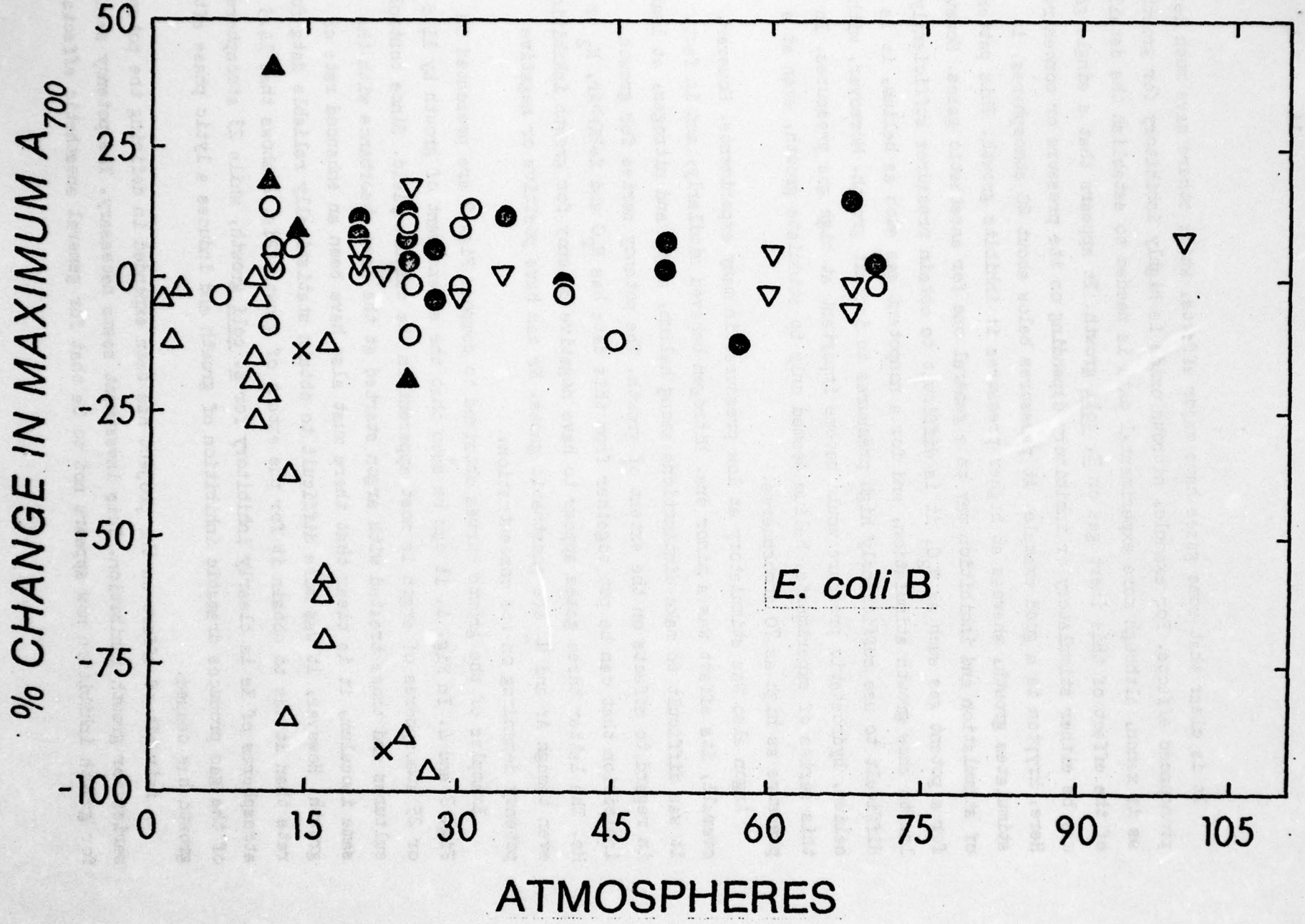
pressurization-depressurization procedure.

During experiments with Tetrahymena, to be described later in this report, it was observed that intracellular gas bubbles formed during and after decompression. These bubbles make the interpretation of optical density readings difficult, and there is some indication that they are harmful to the cells. Therefore, it seemed desirable to follow growth of the eukaryotic cells without decompression-recompression cycles. To carry out these measurements, Stephen Thom modified the two pressure cylinders we have that are fitted with optical windows. The culture to be pressurized is placed in a cotton stoppered test tube in the cylinder. Our Coleman spectrophotometer has been modified so that it is possible to position the cylinder so that light passes through the windows of the cylinder and through the test tube before hitting the photocell. Therefore, it is possible to determine changes in optical density without decompression and sample taking. It has been necessary to place a magnetic stirrer in the culture so that it can be mixed thoroughly for the optical density determinations. The windowed cylinder has proved useful for experiments with bacteria also, especially since we are now using xenon and krypton. These gases are very expensive, and with the windowed cylinders, it is possible to obtain an entire growth curve with only one filling of the chamber.

It is worth mentioning again the problem of intracellular gas bubbles in experiments with decompressed eukaryotic cells. It is impossible to stop them from forming, even with extremely slow decompression. They introduce a serious error into experiments with eukaryotic cells, and it is important to be aware of this problem. Bacterial cells are sufficiently small so that gas bubbles do not form within them, and cycles of compression-decompression are not upsetting to culture growth.

Fig. 2 presents data on the effects of a series of anesthetic or noble gases on growth of E. coli B in tryptic soy broth with 0.1% {w/v} KNO_3. Again, the growth parameter used is maximal absorbance, which is a measure of the maximum biomass per unit volume of culture. The figure is incomplete because additional experiments are currently being carried out. However, there is sufficient data to come to a number of conclusions.

Fig. 2. Inhibition of growth of Escherichia coli B by high-pressure gases. All cultures were grown in tryptic soy broth with 0.1% {w/v} KNO_3. The gases tested were N_2O {△}, Xe {X}, Kr {▲}, Ar {○}, N_2 {▽} and He {●}.


50
25
0
-25
-50
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15
30
45
60
75
90
105
% CHANGE IN MAXIMUM A_{700}
ATMOSPHERES
E. coli B

It is clear that some gases have major effects, while others have much less pronounced effects. For example, nitrous oxide is highly inhibitory for growth, as is xenon, although more experimental data is needed to establish the details of the effect of this inert gas on E. coli growth. It appears that a single gas may be either stimulatory or inhibitory depending on its pressure or concentration. Here, krypton is a good example. At pressures below about 20 atmospheres, it stimulates growth, whereas at higher pressures it inhibits growth. This pattern of stimulation and inhibition may be a general one for anesthetic gases. However, for a potent gas such as N_2O, it is difficult to obtain pressures sufficiently low to show growth stimulation, and for a nonpotent gas such as helium, it is difficult to use sufficiently high pressures to inhibit growth. Moreover, with helium, hydrostatic pressure would become important at high gas pressures. In this series of experiments, helium seemed only to stimulate growth, even at a pressure as high as 70 atmospheres.

Argon also was stimulatory at low pressures in many experiments. However, overall, its effect was a minor one. Nitrogen behaved similarly, and in fact, it was difficult to make distinctions among helium, argon and nitrogen, at least in regard to effects on the extent of growth. The potency series for growth inhibition that can be put together from this data has N_2O and Xe>Kr>Ar, N_2 or He. The latter three gases appear to have negative potency for growth inhibition, even though Ar and N_2 are anesthetic gases. Kr can have positive or negative potency depending on its concentration.

Examples of the growth curves obtained to compose Fig. 2 are presented in Fig. 3 and 4. In Fig. 4, it can be seen that the enhancement of growth by 11.5 or 25 atmospheres of argon is most apparent in the culture yield. Since control cultures and those treated with argon started at the same absorbance with the same inoculum, it is clear that there must also have been an enhanced rate of growth. However, it was more difficult to obtain statistically reliable data for rate than it was to obtain it for the extent of growth. Fig. 4 shows that 14.5 atmospheres of Xe is clearly inhibitory for E. coli growth, while 23 atmospheres of the gas produces dramatic inhibition of growth and induces a lytic phase after growth has ceased.

A fair bit of effort on the project has been expended in defining the potency series for growth inhibition. This investment seems necessary. The potency series for growth inhibition now appears not to be that for general anesthetic effects.

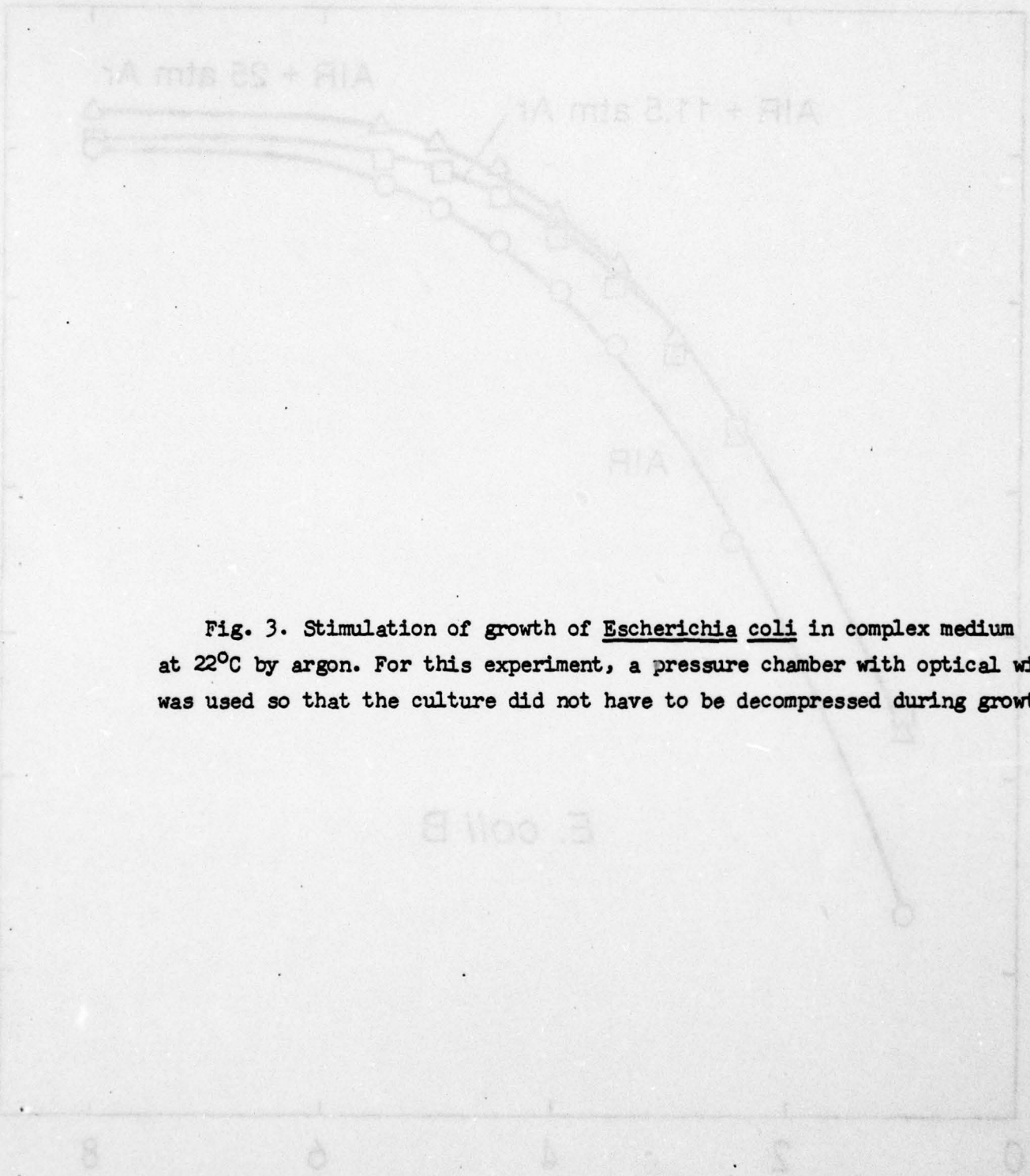
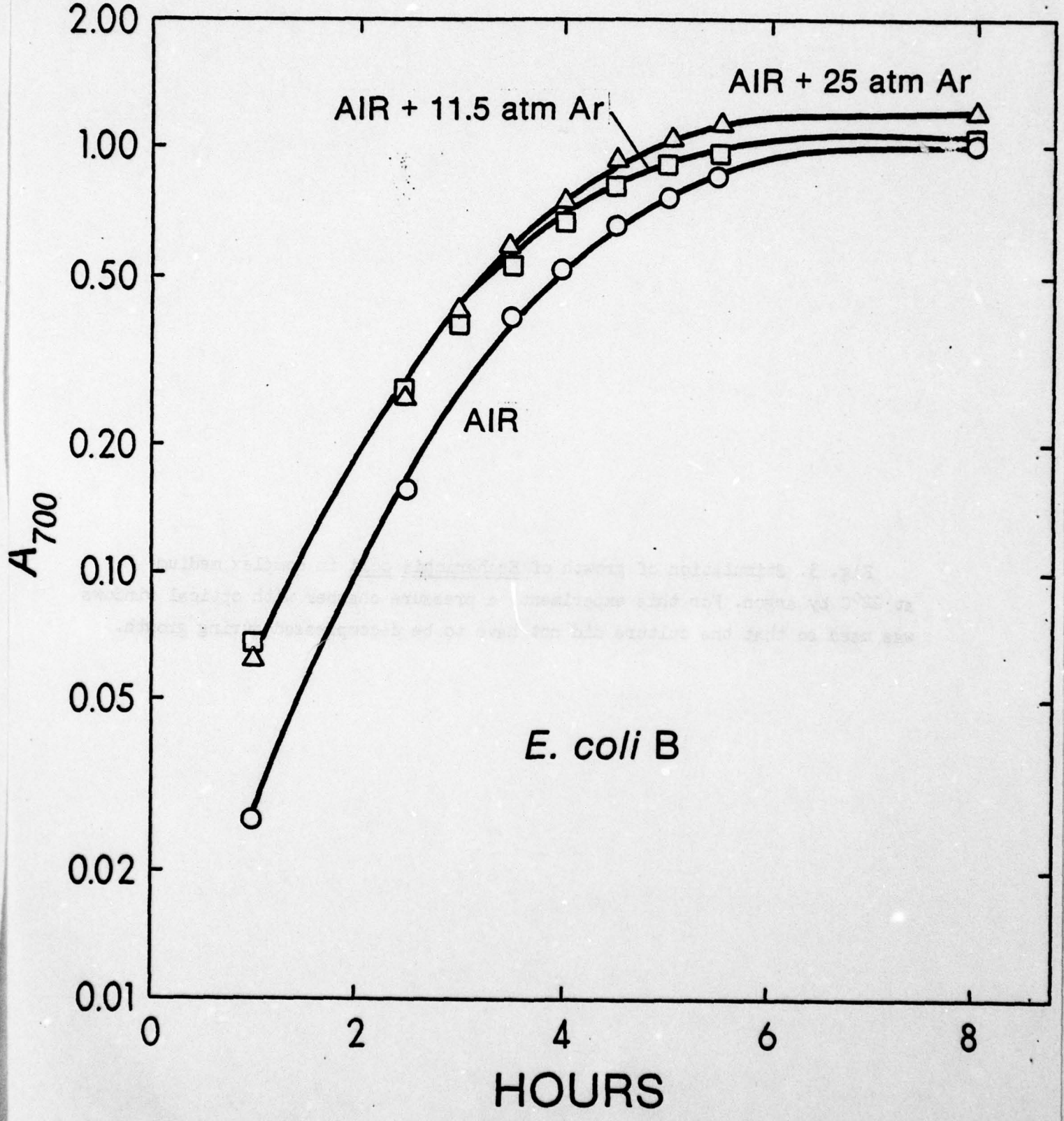


Fig. 3. Stimulation of growth of Escherichia coli in complex medium at 22°C by argon. For this experiment, a pressure chamber with optical windows was used so that the culture did not have to be decompressed during growth.


2.00
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0.02
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A_{700}
AIR + 25 atm Ar
AIR + 11.5 atm Ar
AIR
E. coli B
0
2
4
6
8
HOURS

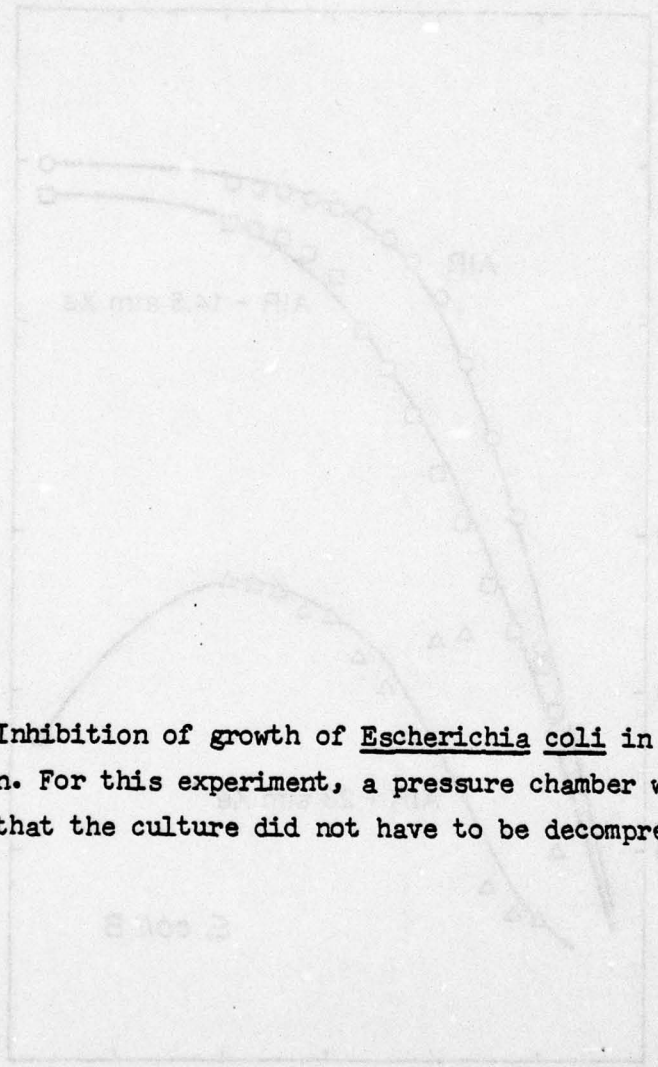
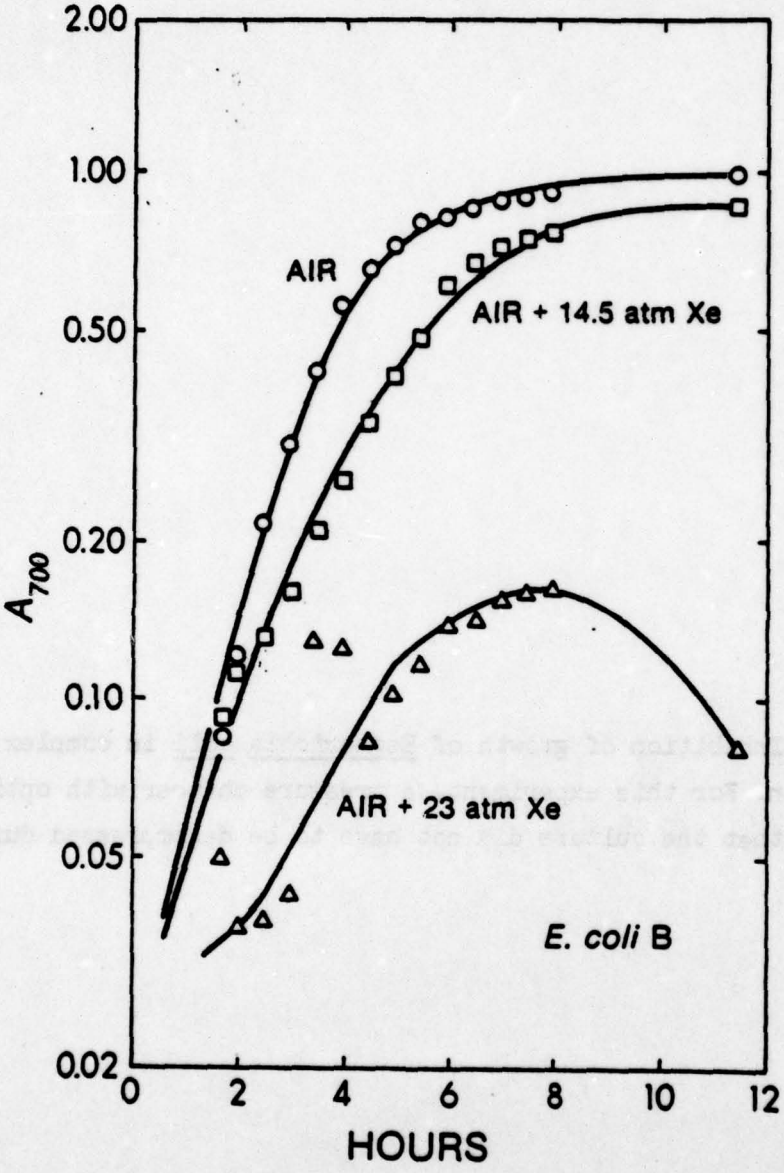


Fig. 4. Inhibition of growth of Escherichia coli in complex medium at 22°C by xenon. For this experiment, a pressure chamber with optical windows was used so that the culture did not have to be decompressed during growth.


2.00
1.00
0.50
0.20
0.10
0.05
0.02
A_{700}
AIR
AIR + 14.5 atm Xe
AIR + 23 atm Xe
E. coli B
0
2
4
6
8
10
12
HOURS

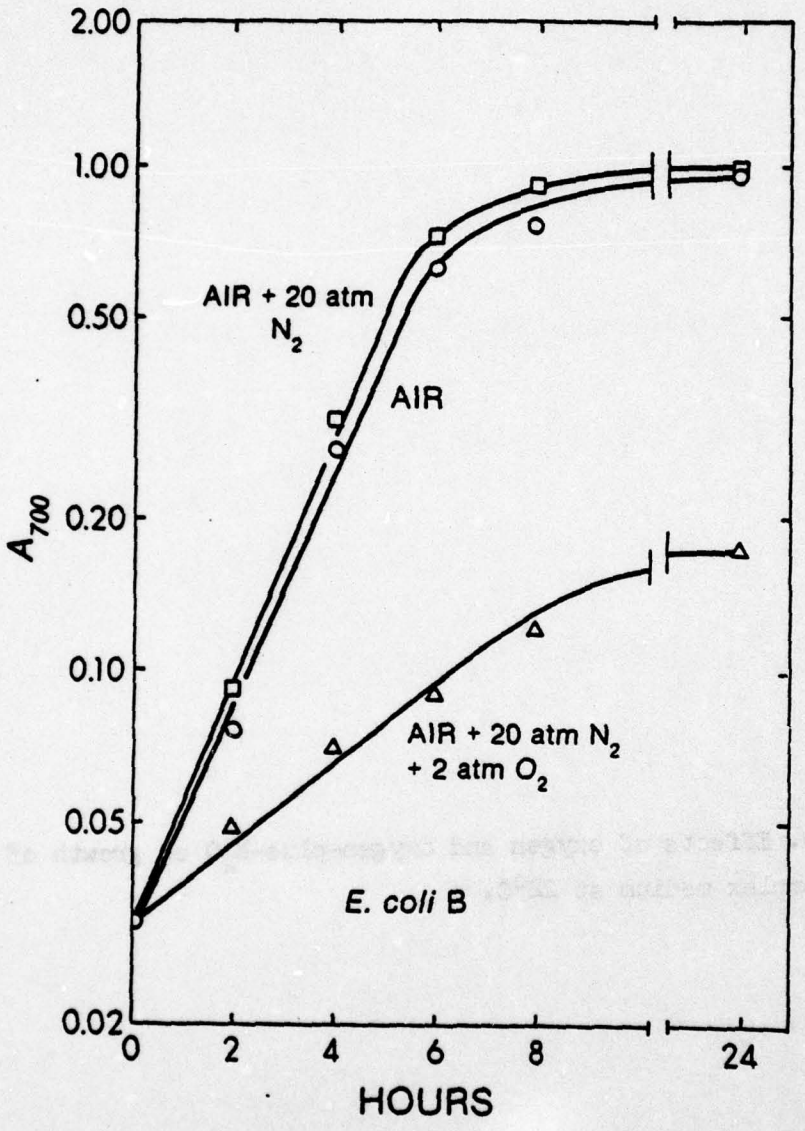
Helium is the only gas that has negative narcotic or anesthetic potential. However, helium, argon and nitrogen all seem to have negative potentials for growth inhibition. These observations suggest that the target for growth inhibition may be different from that for general anesthesia, and results obtained with gas combinations support this view.

B. Effects of gas combinations with elevated oxygen pressure. For all of the experiments described above, the air that was initially in the pressure chambers was retained so that the final partial pressure of oxygen was 0.2 atmospheres. Also, when the chambers were opened for sampling, air was again introduced and was not flushed out during pressurization. As shown by the data of Fig. 1, increased oxygen pressure was inhibitory for growth of E. coli B, and the inhibition rose from nil at about two atmospheres to complete stoppage of growth at an oxygen pressure of about six atmospheres. When nitrogen, argon or helium were combined with oxygen, they greatly enhanced its toxic action. A dramatic example of potentiation by nitrogen is presented in Fig. 5. Here twenty atmospheres of N_2 acts to slightly stimulate growth, but the combination of twenty atmospheres of N_2 and two atmospheres of O_2 produces severe inhibition. Two atmospheres of O_2 alone is without inhibitory effect, and so N_2 can be said truly to be potentiating.

Gases that are more potent growth inhibitors also are potentiating for O_2 toxicity. An example is presented in Fig. 6. As expected, two atmospheres of O_2 had no effect on the extent or rate of growth. However, in combination with a very slightly inhibitory pressure of N_2O {6.8 atmospheres}, it markedly reduced both the extent and the rate of growth. Again, there was a marked potentiation of the growth inhibitory action of oxygen by an anesthetic gas.

When lower pressures of oxygen were used, it was possible to obtain some increase in the extent of growth due to action of anesthetic gases. For example, the data presented in Fig. 7 indicate that the extent of growth was actually increased in the presence of three atmospheres of argon and one atmosphere of oxygen, with little or no change in growth rate. When the oxygen level was increased to two atmospheres, there was still an increased yield. However, the rate of growth was significantly reduced. Further increases in oxygen pressure or in argon pressure resulted in growth inhibition. An example is presented in Fig. 8. When the oxygen pressure was at a noninhibitory level of two atmospheres, addition of ten atmospheres of argon resulted in noticeably slower growth, while addition of thirty atmospheres of argon resulted in marked reduction of both

Fig. 5. Effects of nitrogen and nitrogen-plus-oxygen on growth of Escherichia coli in complex medium at 22°C.


2.00
1.00
0.50
0.20
0.10
0.05
0.02
A_{700}
AIR + 20 atm N_2
AIR
AIR + 20 atm N_2 + 2 atm O_2
E. coli B
0
2
4
6
8
24
HOURS

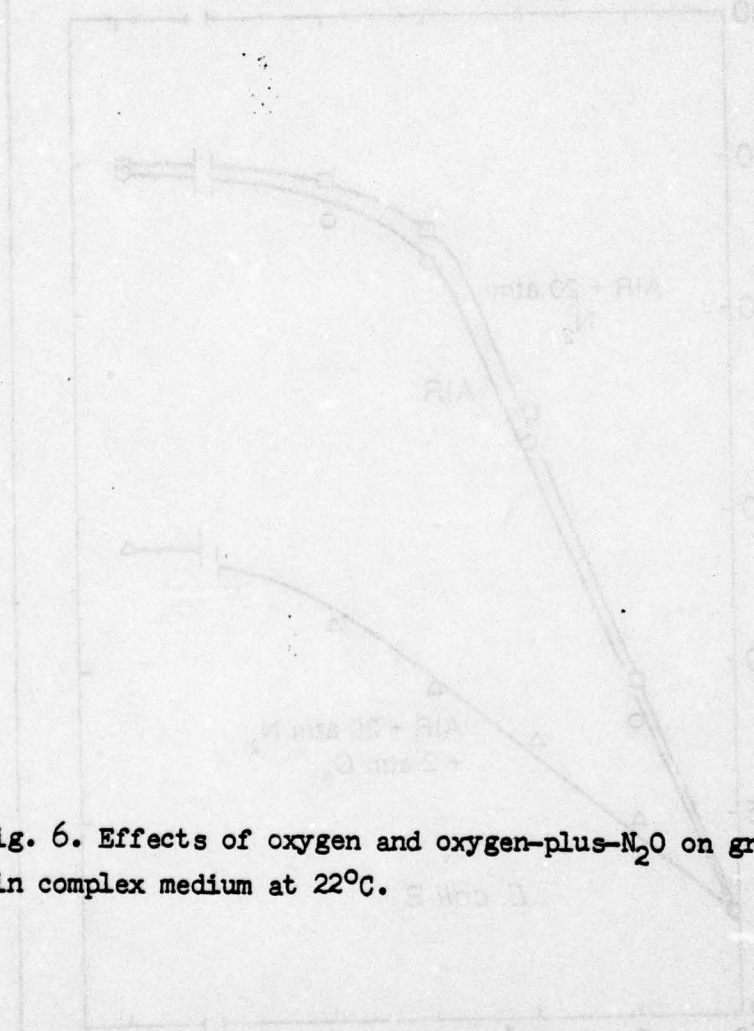
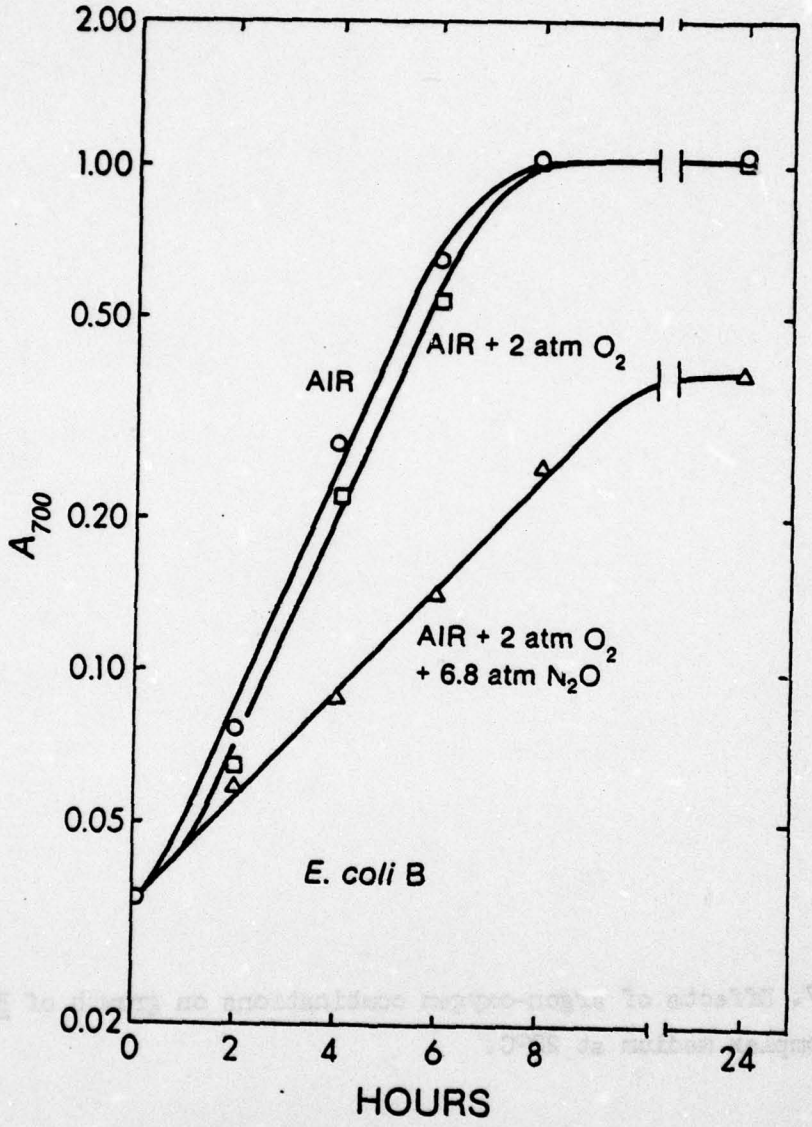


Fig. 6. Effects of oxygen and oxygen-plus-N_2O on growth of Escherichia coli in complex medium at 22°C.


2.00
1.00
0.50
0.20
0.10
0.05
0.02
A_{700}
AIR
AIR + 2 atm O_2
AIR + 2 atm O_2
+ 6.8 atm N_2O
E. coli B
0
2
4
6
8
24
HOURS

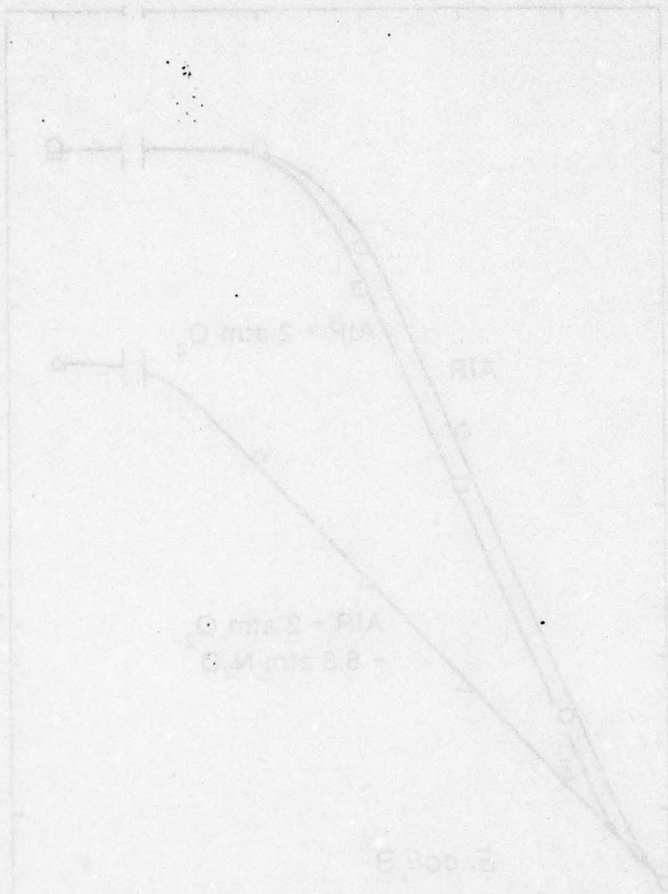
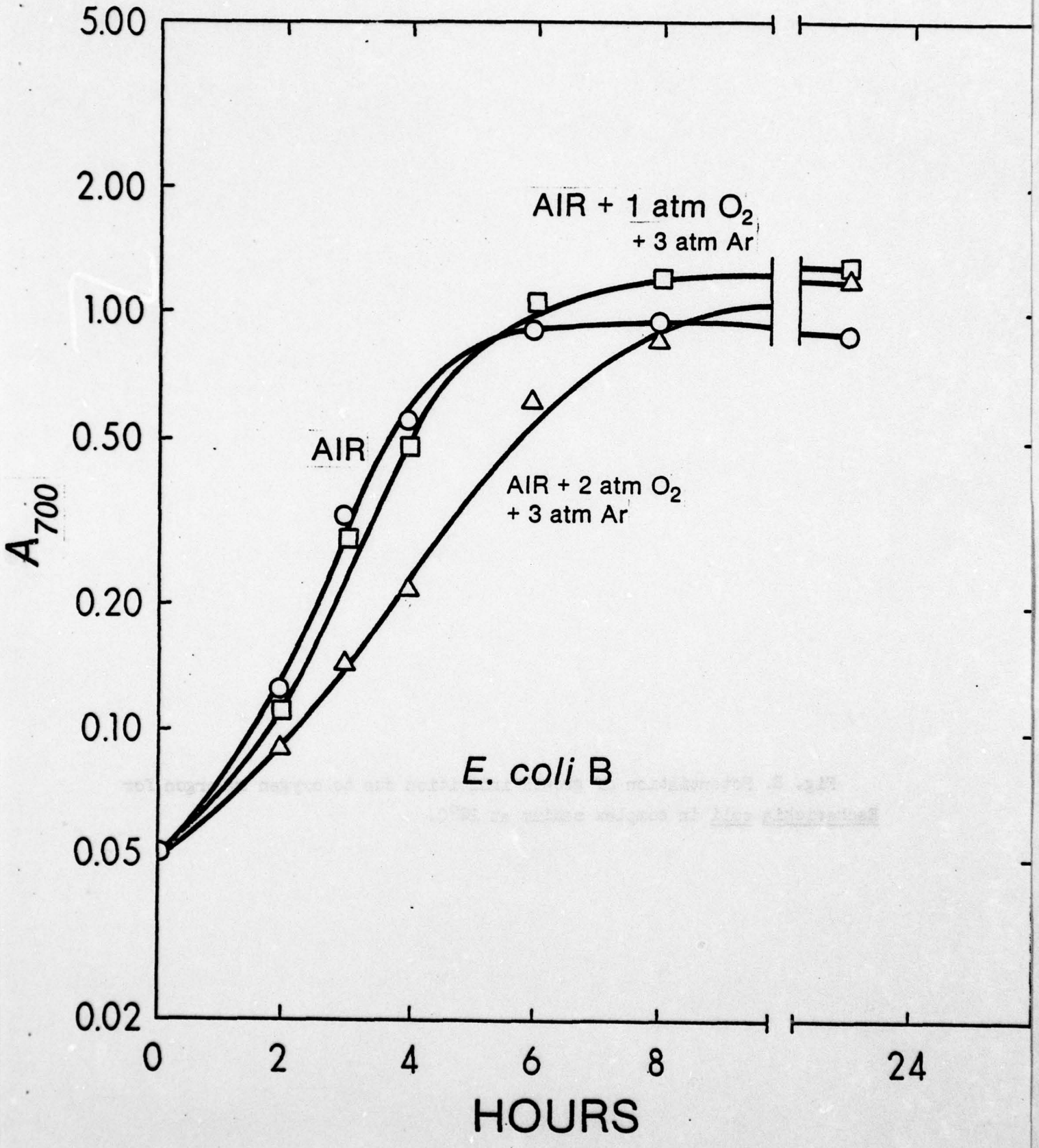


Fig. 7. Effects of argon-oxygen combinations on growth of Escherichia coli in complex medium at 22°C.


5.00
2.00
1.00
0.50
0.20
0.10
0.05
0.02
A_{700}
AIR + 1 atm O_2
+ 3 atm Ar
AIR
AIR + 2 atm O_2
+ 3 atm Ar
E. coli B
0
2
4
6
8
24
HOURS

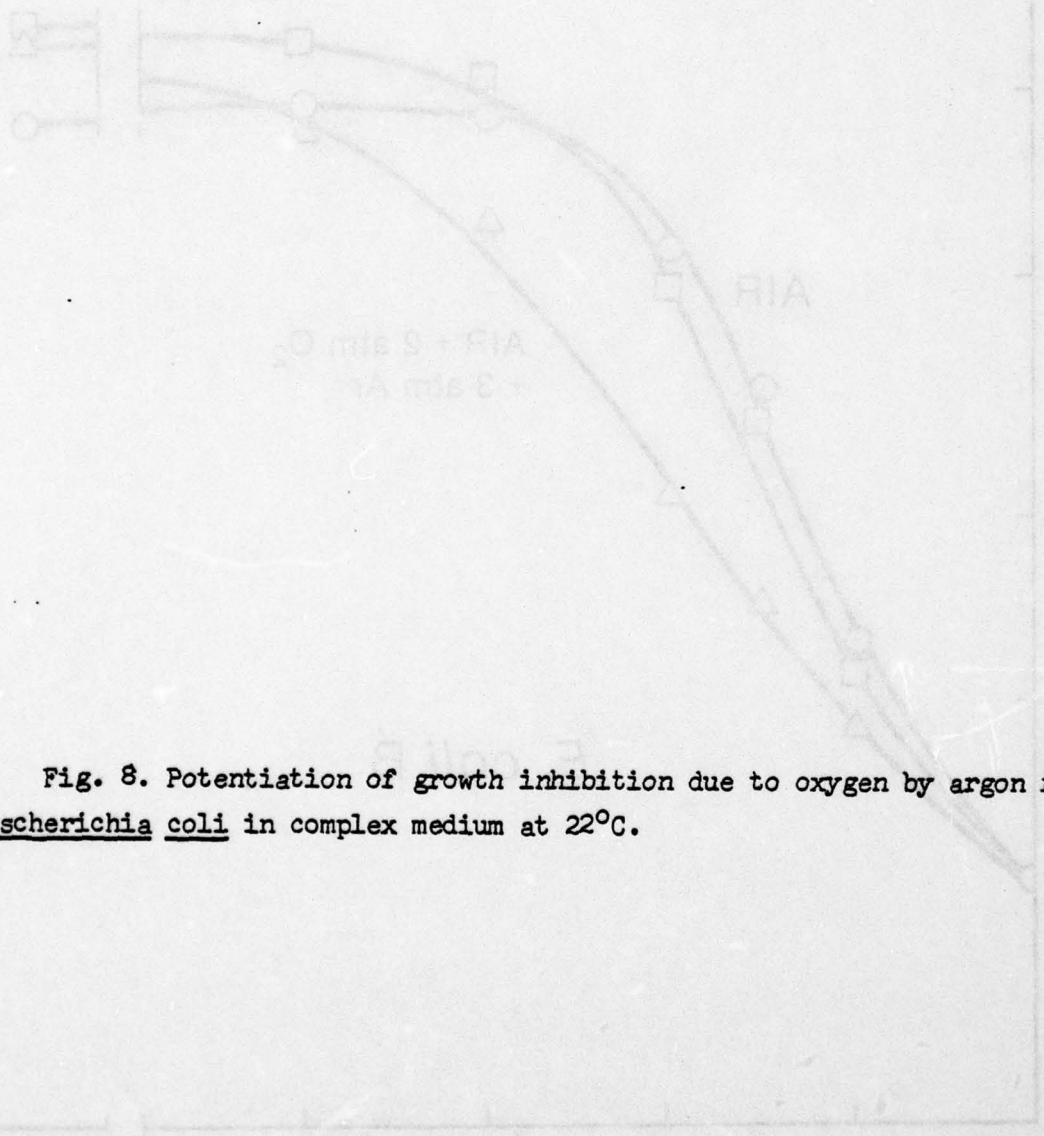
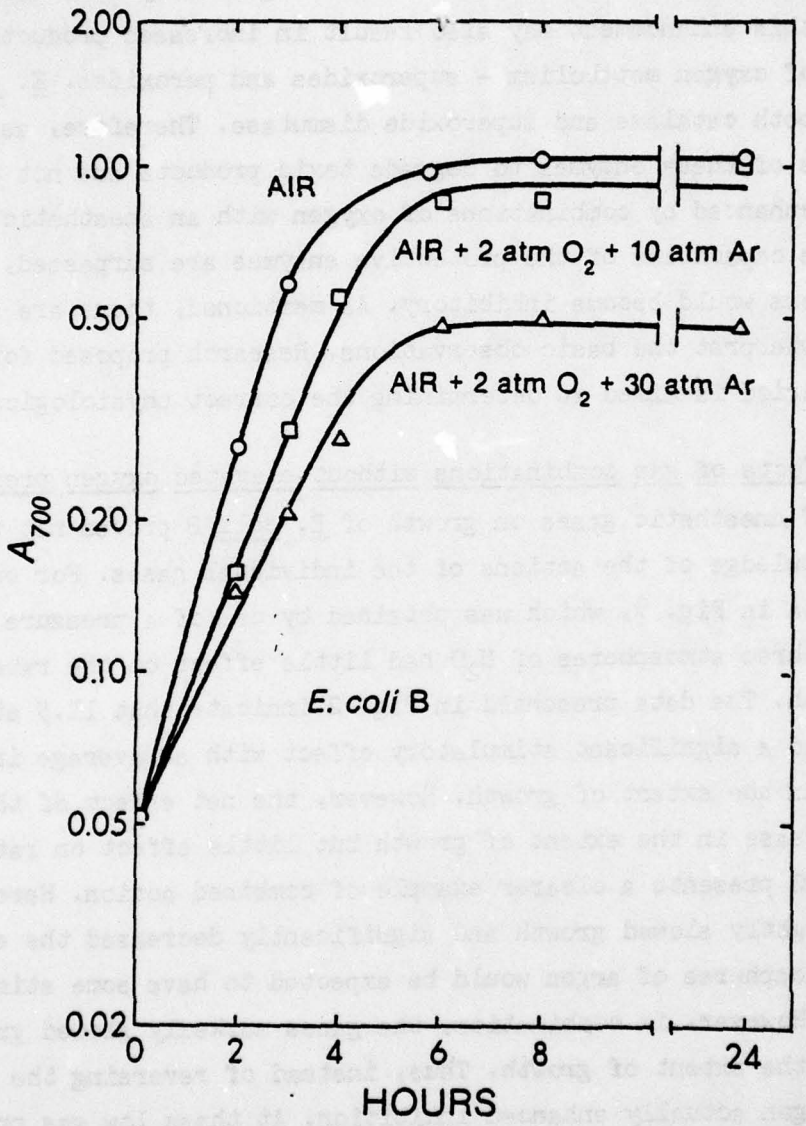


Fig. 8. Potentiation of growth inhibition due to oxygen by argon for Escherichia coli in complex medium at 22°C.


2.00
1.00
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0.20
0.10
0.05
0.02
A_{700}
AIR
AIR + 2 atm O_2 + 10 atm Ar
AIR + 2 atm O_2 + 30 atm Ar
E. coli B
0
2
4
6
8
24
HOURS

rate and extent of growth.

These findings can be interpreted in a number of ways. For example, it is possible that a gas such as argon may enhance oxygen utilization by E. coli or induce the organism to use oxygen in an energetically more effective manner. However, this enhancement may also result in increased production of toxic products of oxygen metabolism - superoxides and peroxides. E. coli has protective enzymes, both catalase and superoxide dismutase. Therefore, as long as the capacities of these enzymes to degrade toxic products are not exceeded, growth would be enhanced by combinations of oxygen with an anesthetic gas. However, when these capacities of the protective enzymes are surpassed, then such combinations would become inhibitory. As mentioned, there are alternative ways to interpret the basic observations. Research proposed for the upcoming support period is aimed at determining the correct physiological interpretation.

C. Effects of gas combinations without elevated oxygen pressure. The combined actions of anesthetic gases on growth of E. coli B proved not to be predictable from a knowledge of the actions of the individual gases. For example, as shown by the data in Fig. 9, which was obtained by use of a pressure chamber with optical windows, three atmospheres of N_2O had little effect on the rate or extent of E. coli growth. The data presented in Fig. 2 indicate that 11.5 atmospheres of krypton had a significant stimulatory effect with an average increase of somewhat over 25% in the extent of growth. However, the net effect of the combined gases was a decrease in the extent of growth but little effect on rate.

Fig. 10 presents a clearer example of combined action. Here, ten atmospheres of N_2O slightly slowed growth and significantly decreased the extent of growth. Twenty atmospheres of argon would be expected to have some stimulatory effect {Fig. 2}. However, in combination, the gases markedly slowed growth and markedly decreased the extent of growth. Thus, instead of reversing the inhibitory action of N_2O, argon actually enhanced inhibition. At these low gas pressures, one would expect little effect of hydrostatic pressure, and it seems that the enhancement of the growth inhibitory potential of N_2O must be due specifically to argon and not to hydrostatic pressure.

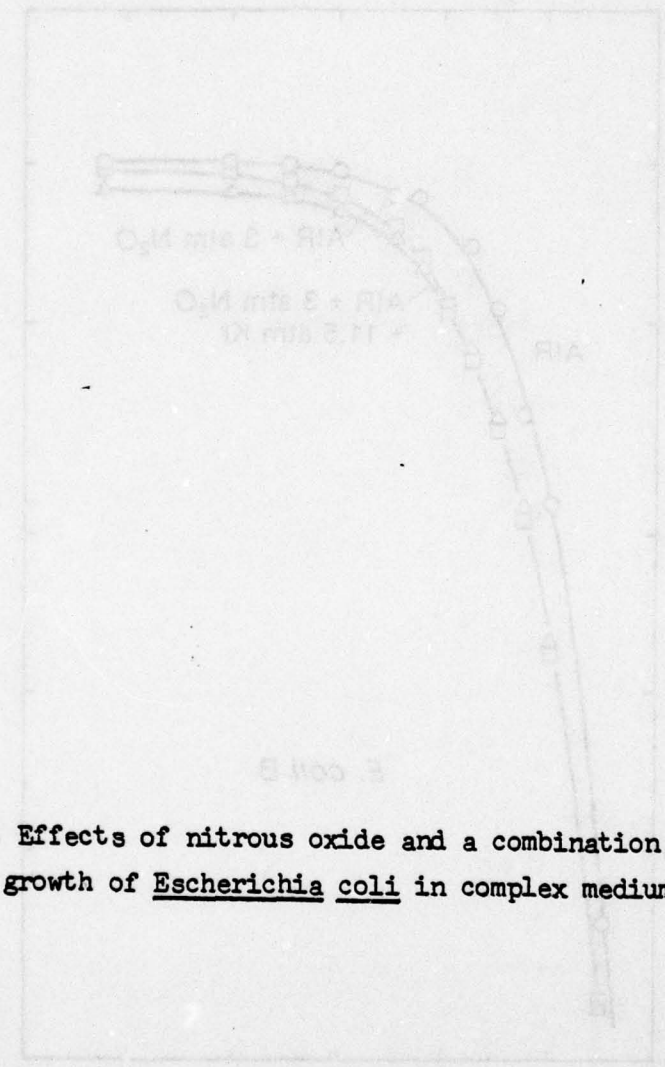
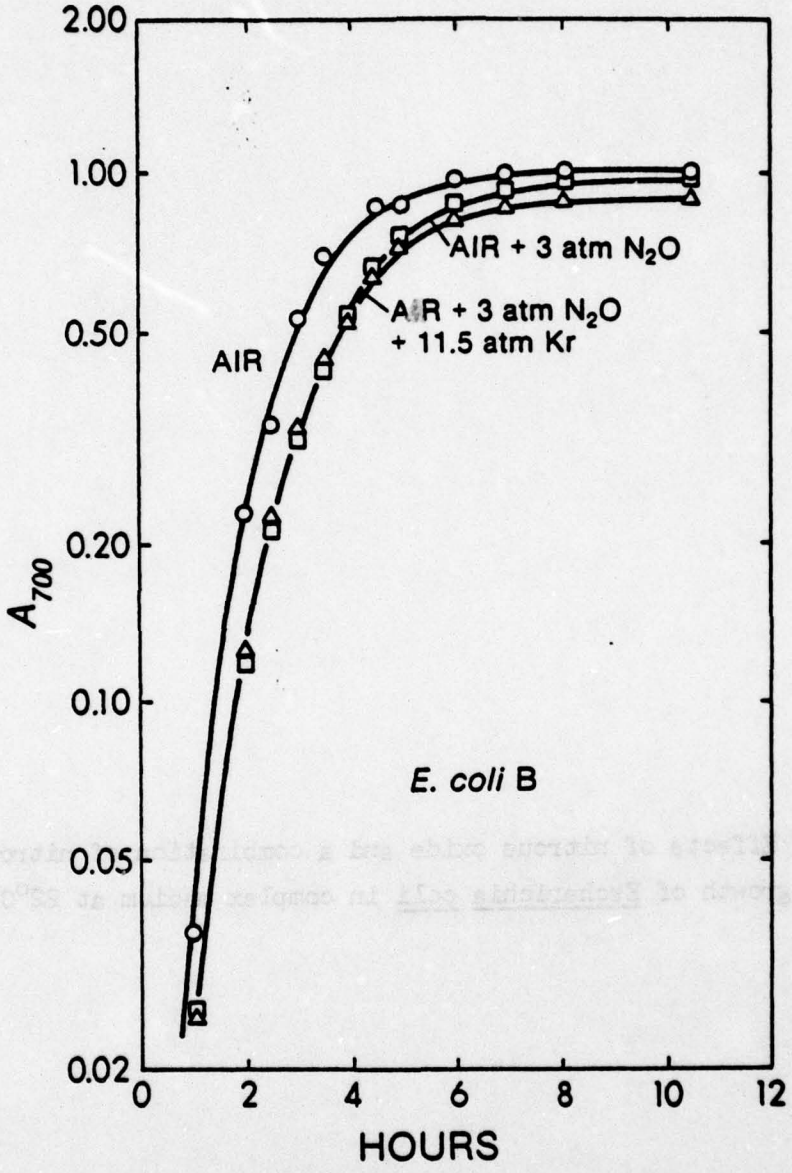


Fig. 9. Effects of nitrous oxide and a combination of nitrous oxide and krypton on growth of Escherichia coli in complex medium at 22°C.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A_{700}
0
2
4
6
8
10
12
HOURS
AIR
AIR + 3 atm N_2O
AIR + 3 atm N_2O
+ 11.5 atm Kr
E. coli B

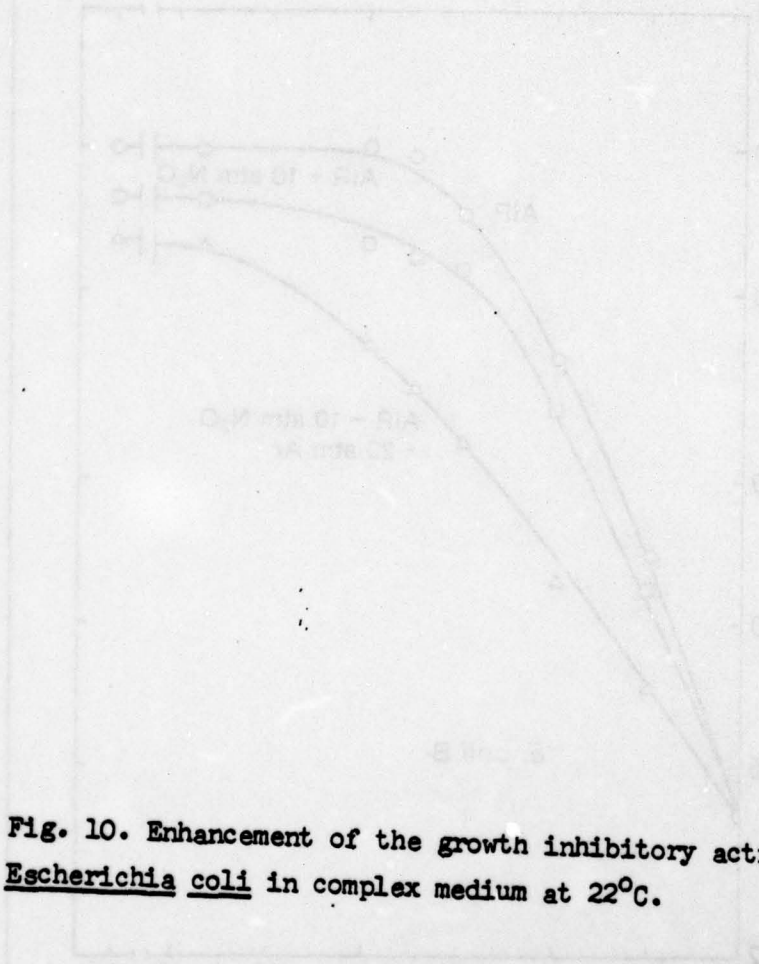
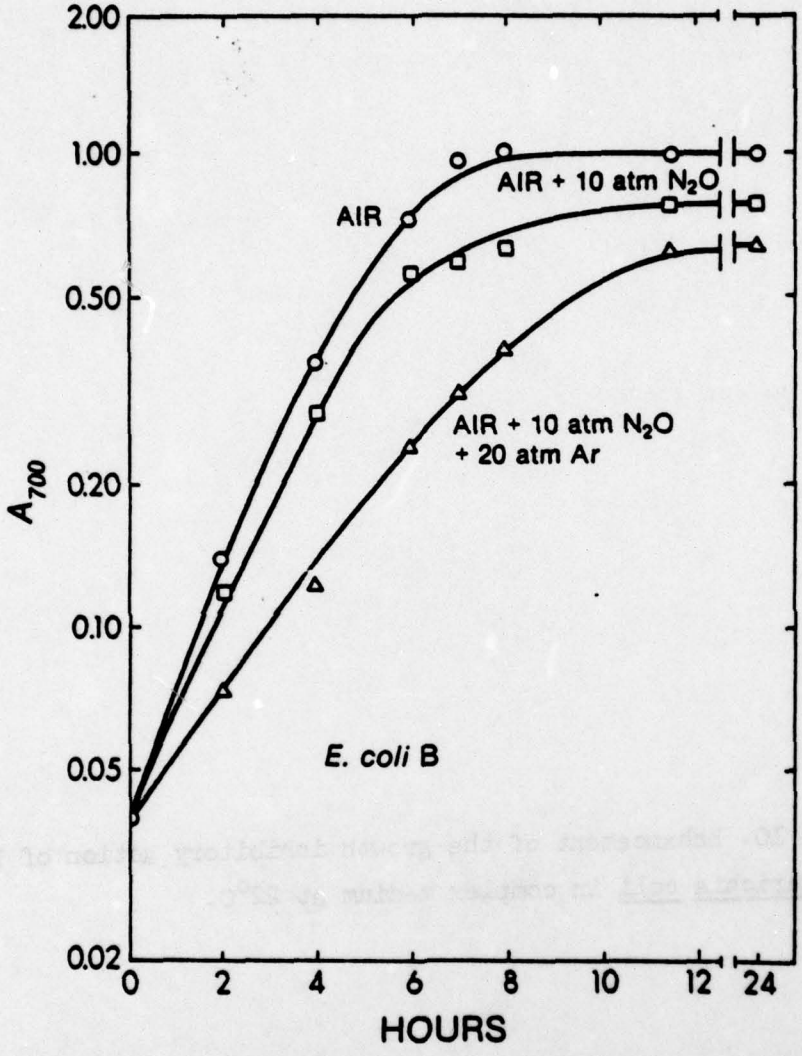


Fig. 10. Enhancement of the growth inhibitory action of N_2O by argon for Escherichia coli in complex medium at 22°C.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A_{700}
AIR
AIR + 10 atm N_2O
AIR + 10 atm N_2O
+ 20 atm Ar
E. coli B
0
2
4
6
8
10
12
24
HOURS

In combinations of anesthetic gases, nitrogen proved to be peculiar in that it had a mild antagonistic effect. The data presented in Fig. 11 show, again, that ten atmospheres of N_2O decreases the extent of growth of E. coli. Addition of twenty atmospheres of nitrogen to the N_2O reversed to a large extent the inhibition of growth. Again, it seems that the action cannot be one of hydrostatic pressure but must be a specific gas effect. Even helium enhances the inhibitory action of N_2O, and it is only N_2 that reverses it in what appears to be a nitrogen-specific action.

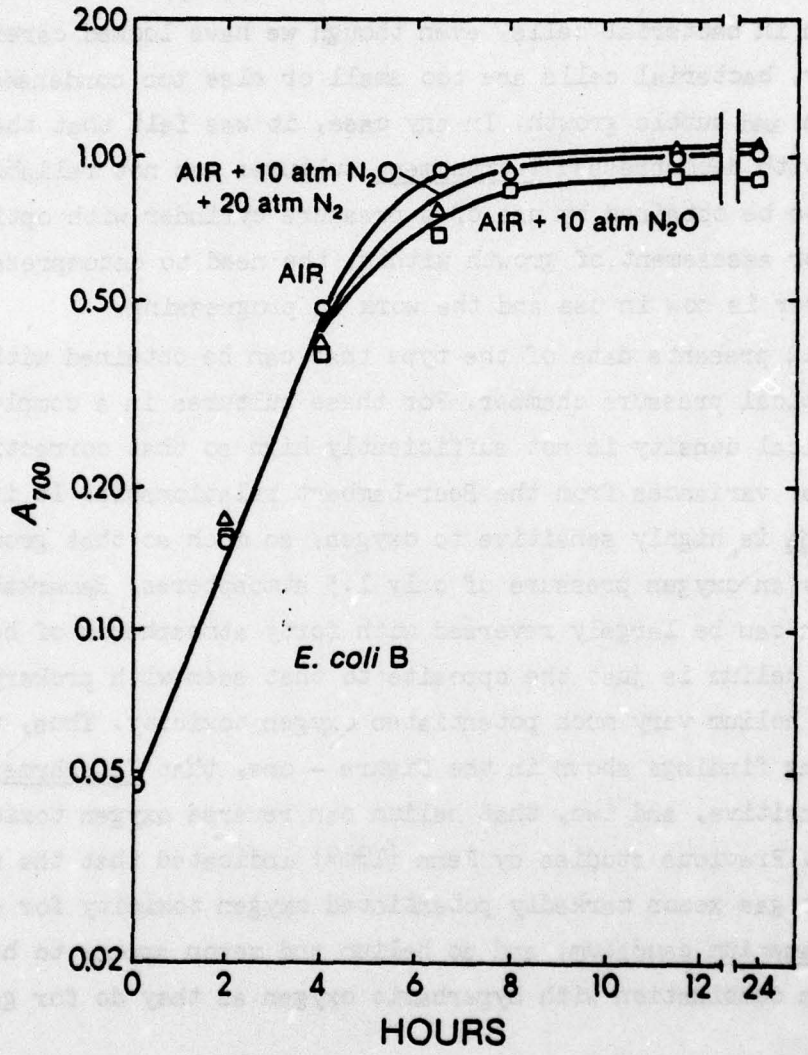
III. Actions of High-pressure Gases on Eukaryotic Organisms.

Nearly all of the past studies of the physiology of anesthetic gas effects have been carried out with eukaryotic organisms. In fact, our earlier work {Fenn and Marquis, 1968} seems to have been the first in which prokaryotic cells were used. Offhand, one might expect that these two varieties of organisms, prokaryotes and eukaryotes, might differ in their responses to anesthetic gases. Certainly, the two have radically different cellular structures. Moreover, prokaryotes lack structures such as microtubules, and it is felt that microtubules are involved in the eukaryotic responses to hydrostatic pressure and to anesthetic gases. Pressure acts to cause depolymerization of microtubules to component tubulins, and this depolymerization, which is accompanied by an unusually large volume change of some 90 to 400 ml per mole of tubulin, is thought to be the primary lesion resulting in inhibition of motility, mitosis and cell division. Anesthetic gases also affect microtubules {Miller and Miller, 1975} and can cause depolymerization. However, relatively high levels of anesthetics are required to cause depolymerization.

Currently, there is a strong feeling that the most important actions of anesthetic gases involve the cell membrane and that it is at this locus that hydrostatic pressure acts to antagonize anesthesia. Prokaryotic membranes differ from eukaryotic membranes in a number of respects, notably by the absence of sterols and polyunsaturated fatty acids in most species. Therefore, one might expect differences in responses to anesthetic gases between the two types of cells. However, the gross compositions of the membranes of the two types are similar, and one could alternatively expect similar responses. Only through comparative studies can the needed information be gained.

For the purpose of extending our previous studies with prokaryotes to eukaryotes, we choose Tetrahymena pyriformis as prime test species. It is relatively easy to cultivate, and there is past work on its responses to

Fig. 11. Reversal of the growth inhibitory effect of N_2O by nitrogen for Escherichia coli in complex medium at 22°C.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A_{700}
AIR + 10 atm N_2O
+ 20 atm N_2
AIR
AIR + 10 atm N_2O
E. coli B
0
2
4
6
8
10
12
24
HOURS

anesthetic gases {Kirkness and Macdonald, 1972}. As indicated previously, we ran into major problems with Tetrahymena which we had not encountered previously in our work with bacteria. Decompression of Tetrahymena cultures, even very slow decompression, resulted in the formation of intracellular gas bubbles, and there was some evidence of cell disruption. Gas bubbles were never seen in bacterial cells, even though we have looked carefully for them. Presumably, bacterial cells are too small or else too condensed to allow for nucleation and bubble growth. In any case, it was felt that the initial data obtained with decompressed Tetrahymena cultures was not reliable, and new data had to be obtained by use of a pressure cylinder with optical windows that allowed for assessment of growth without the need to decompress the culture. This chamber is now in use and the work is progressing.

Fig. 12 presents data of the type that can be obtained with Tetrahymena in the optical pressure chamber. For these cultures in a complex medium the final optical density is not sufficiently high so that corrections have to be made for variances from the Beer-Lambert relationship. It is apparent that Tetrahymena is highly sensitive to oxygen, so much so that growth is completely stopped by an oxygen pressure of only 1.5 atmospheres. Remarkably, this inhibition can be largely reversed with forty atmospheres of helium. This action of helium is just the opposite to that seen with prokaryotic cells for which helium very much potentiates oxygen toxicity. Thus, there are two interesting findings shown in the figure - one, that Tetrahymena is extremely oxygen sensitive, and two, that helium can reverse oxygen toxicity for the protozoan. Previous studies by Fenn {1969} indicated that the more potent anesthetic gas xenon markedly potentiated oxygen toxicity for another protozoan, Paramecium caudatum, and so helium and xenon appear to have opposite actions in combination with hyperbaric oxygen as they do for general anesthetic effects.

During the next year, we shall extend our work with eukaryotic organisms to define interactions of pressure, oxygen and anesthetic gases as they affect growth and to attempt to uncover some of the physiological and biochemical details of the responses of the organisms. It is possible that yeast may prove to be the most convenient test organism for many of these studies, although we certainly intend to continue to use Tetrahymena. Clearly, also, we shall be concerned with differences in the responses of prokaryotic and eukaryotic organisms.

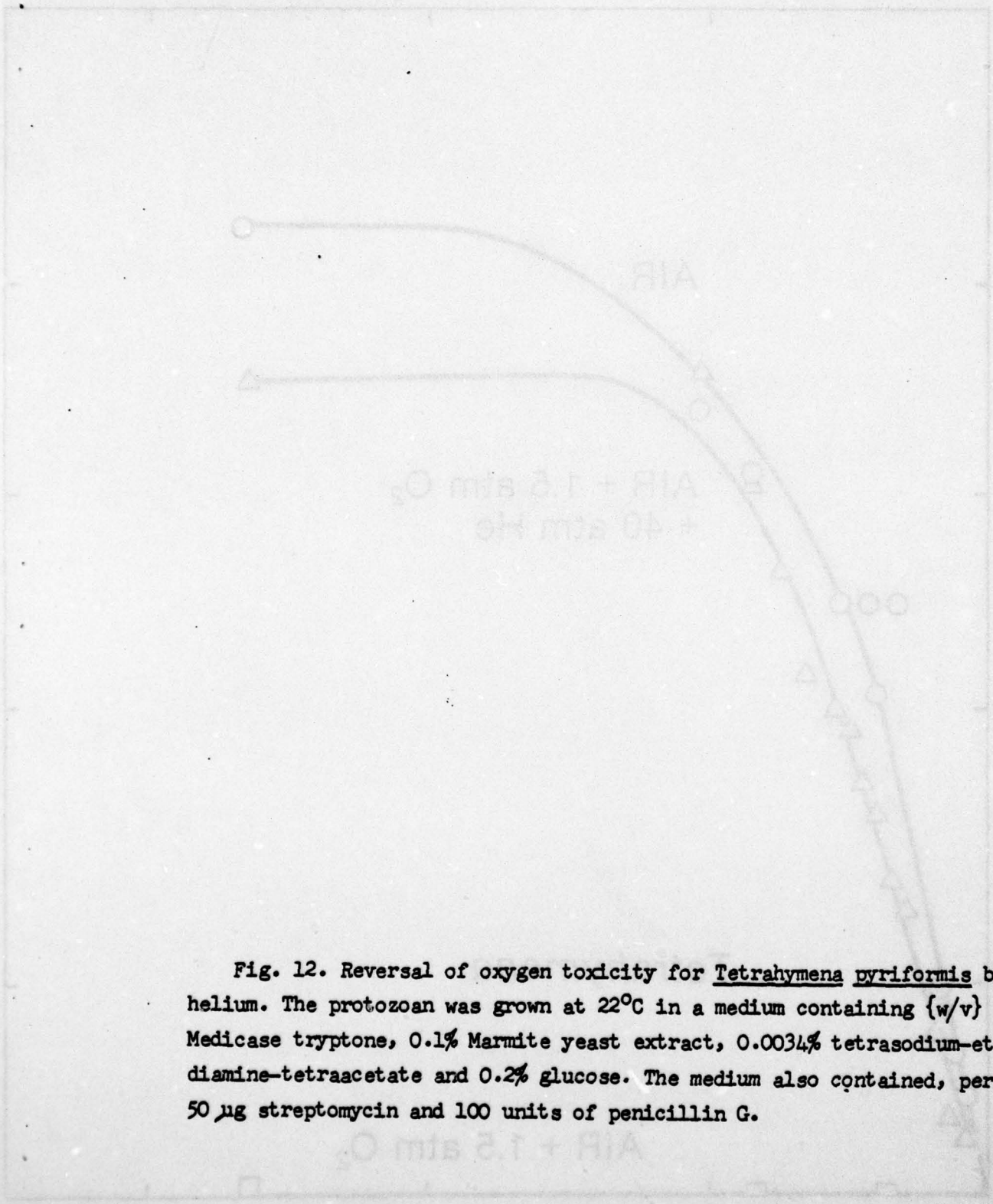
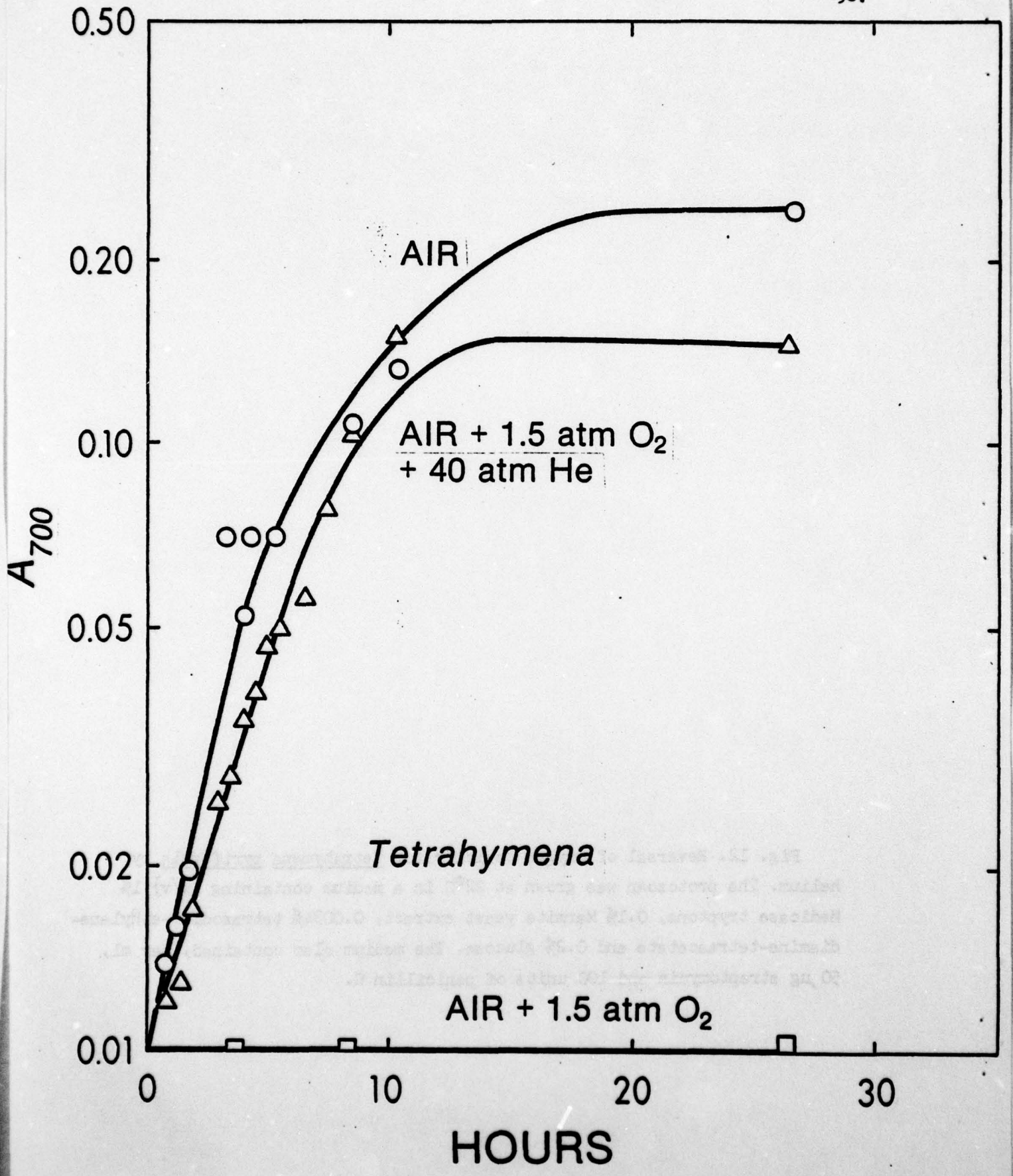


Fig. 12. Reversal of oxygen toxicity for Tetrahymena pyriformis by helium. The protozoan was grown at 22°C in a medium containing (w/v) 1% Medicase tryptone, 0.1% Marmite yeast extract, 0.0034% tetrasodium-ethylene-diamine-tetraacetate and 0.2% glucose. The medium also contained, per ml, 50 µg streptomycin and 100 units of penicillin G.


0.50
0.20
0.10
0.05
0.02
0.01
A_{700}
AIR
AIR + 1.5 atm O_2
+ 40 atm He
Tetrahymena
AIR + 1.5 atm O_2
0
10
20
30
HOURS

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Attempts to relate growth inhibitory actions of anesthetic gases to their narcotic potencies revealed that the two are not well correlated. Instead, it was apparent that bacterial growth inhibition by the gases is a unique class of actions that can be distinguished from general anesthetic or narcotic actions. The potency series determined for inhibition of growth of Escherichia coli B in complex medium at 22°C had N_2O and Xe>Kr>Ar, N_2 or He. The latter three gases had negative potencies in that they stimulated

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growth rather than inhibiting it. In the narcotic potency series only helium has a negative potency. Kr also could stimulate growth at pressures less than twenty atmospheres, but it was inhibitory at higher pressures. The net conclusions here are that the potency series for growth inhibition differs from that for anesthesia and that different intracellular sites of action are likely.

Experiments with a series of bacteria indicated that there is a range of sensitivity to nitrous oxide and that nitrous oxide sensitivity is correlated with oxygen sensitivity. Staphylococcus aureus was more sensitive to the gases than was E. coli, and both were considerably more sensitive than was Streptococcus faecalis. For the streptococcus, which has only minimal capacity to metabolize oxygen and has protective peroxidase and superoxide dismutase enzymes, oxygen seemed to act mainly only as an anesthetic gas with a potency about equal to that of nitrous oxide.

The uniqueness of the growth inhibitory action was evident also in the effects of gas combinations on E. coli growth. Helium and anesthetic gases, including argon and nitrogen, were found to potentiate oxygen toxicity. For example, two atmospheres of oxygen or twenty atmospheres of nitrogen each alone had no effect or a slightly stimulatory effect on growth. When combined, they were highly inhibitory and almost completely suppressed growth. Combinations of anesthetic gases acted in a more than additive manner in inhibiting growth. Nitrogen was an exception in that it antagonized the action of, for example, nitrous oxide. were conducted

Preliminary experiments with the protozoan Tetrahymena pyriformis indicated that decompression after exposure to hyperbaric gases resulted in the formation of intracellular gas bubbles, which were never seen in decompressed bacterial cells. Therefore, to study the effects of high-pressure gases on Tetrahymena, it was necessary to construct a pressure chamber with optical windows so that growth could be monitored without the need for decompression and sample taking. Initial experimental results indicated that helium can reverse oxygen toxicity for Tetrahymena, which proved to be highly oxygen sensitive. Thus, helium behaved oppositely for eukaryotic cells than it did for prokaryotes.

During the next year, we shall continue work on the interactions of hydrostatic pressure, oxygen and anesthetic gases that affect growth with an aim to defining in more detail the basic responses of microorganisms, identifying major biochemical and physiological targets and comparing the responses of eukaryotic microorganisms with those of prokaryotes.

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