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THE PHYSIOLOGICAL BASES FOR MICROBIAL BAROTOLERANCE.(U)
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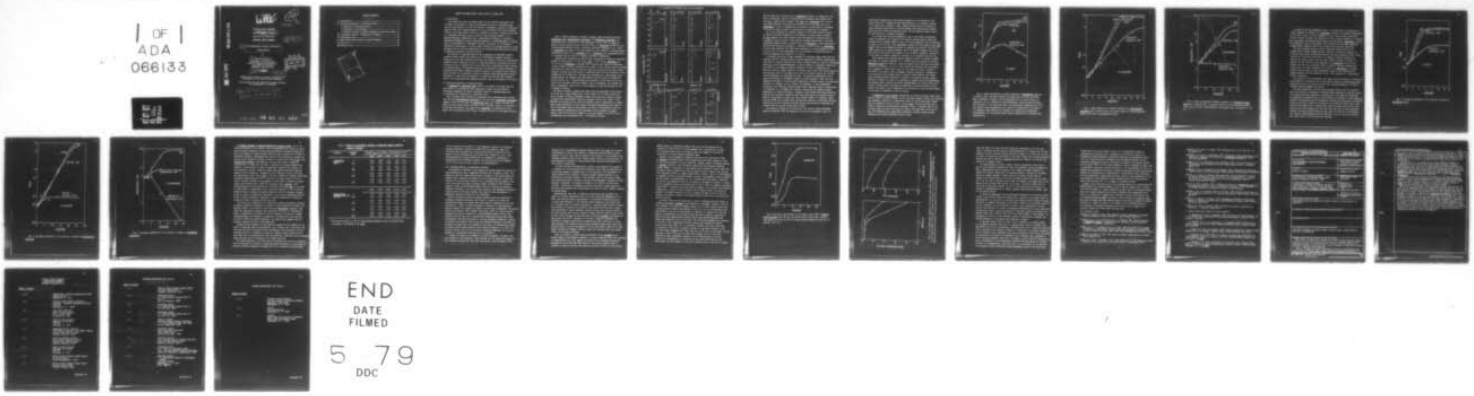

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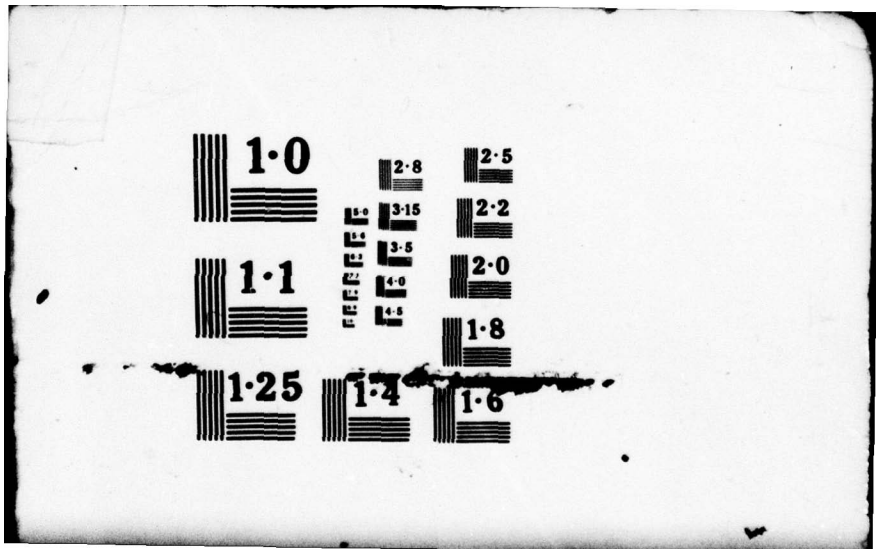
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(6) The Physiological Bases for Microbial Barotolerance.

by

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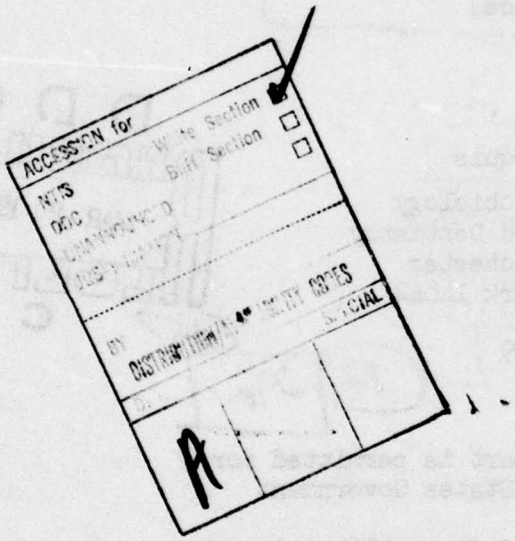


REPORT FOR PERIOD FROM 1 APRIL 1978 TO 31 MARCH 1979

I. Introduction

Once again this year, the annual report can be divided conveniently into two parts, one having to do with the biological actions of compressed gases and the other having to do with the effects of hydrostatic pressure on bacteria. Previous work with compressed gases led to the conclusion that their modifying effects on microbial growth cannot be considered as narcotic effects but are due to a definably different class of actions. This conclusion seems to be of general biological significance but is particularly pertinent in relation to deep-diving programs for which long-term exposure to compressed gases is planned and to the development of hyperbaric medicine. Certainly, if cell growth is inhibited by these gases, one would expect problems in prolonged exposures. These problems could be very much aggrevated by the potentiating interactions of gases in mixtures described in previous reports.

The effects of compressed gases on microorganisms are primarily specific chemical effects rather than effects due to hydrostatic pressure. However, a large part of our work during the past year has focused on effects of hydrostatic pressure itself, especially effects of long-term exposure. The results are very exciting in that it appears that we have been able to specifically adapt bacteria for improved growth in compressed cultures. The adaptation involves selection of naturally barotolerant variants initially present in the cultures used. However, these variants can be selected by means of repeated subculturing under pressure. Certainly, it seems that this advance is a major one that may offer answers to many of the vexing questions regarding microbial life in the depths of the ocean.

II. Microbial Responses to Compressed Gases

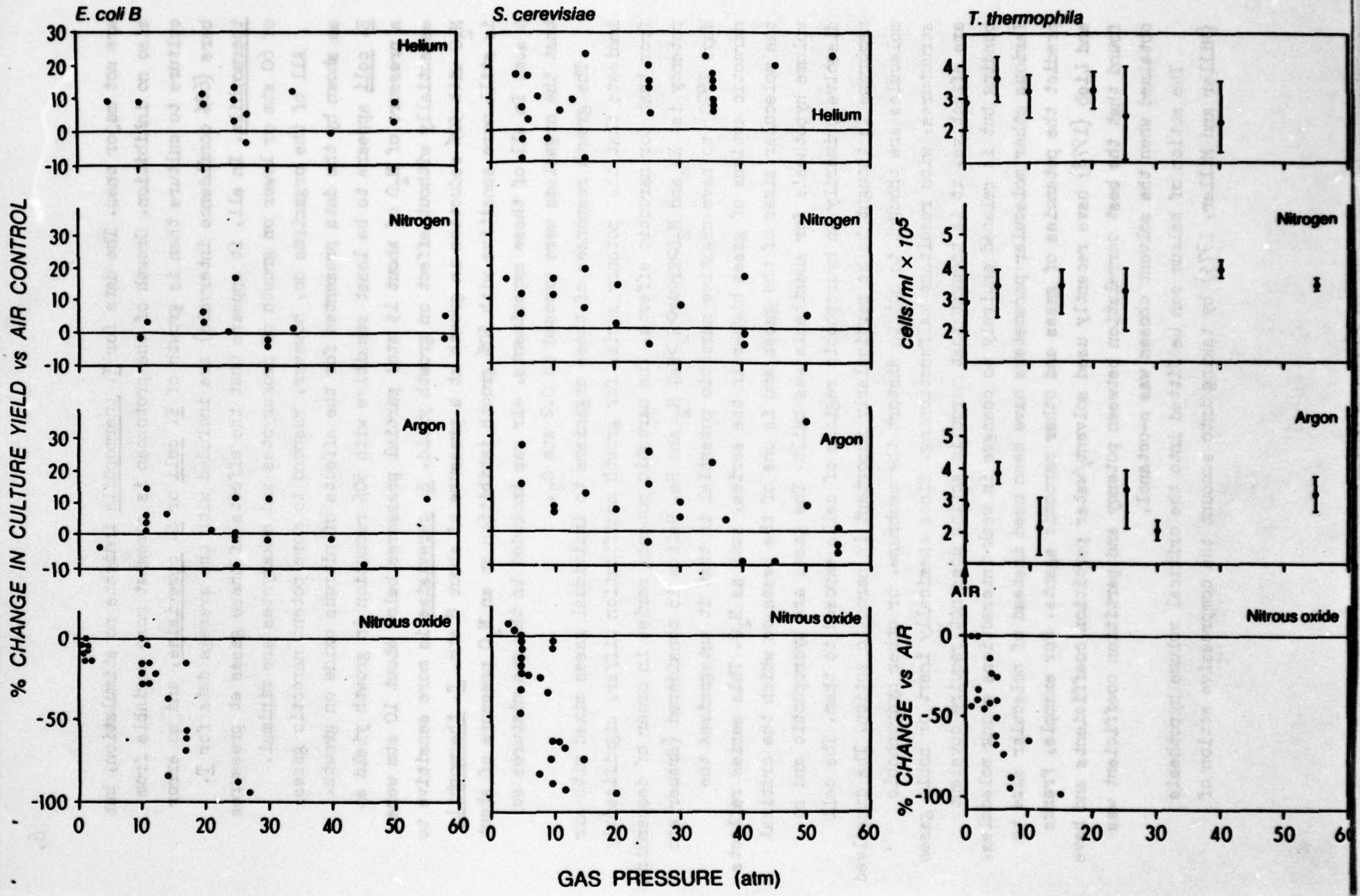
A. Responses to individual gases. Much of our effort in the past year has been directed to developing firmer experimental support for our previous conclusions regarding the growth modifying actions of compressed gases and to extending our observations to eukaryotic cells.

Fig. 1 presents data obtained in experiments with individual gases showing that the yeast Saccharomyces cerevisiae and the protozoan Tetrahymena thermophila respond in much the same way that Escherichia coli does. Helium, nitrogen and argon all have little clearly definable effect on growth. There is an indication in the data that growth of E. coli and S. cerevisiae is stimulated by these gases, but the responses, even at gas pressures as high at 50 atmospheres (atm),

Fig. 1. Growth responses to helium, nitrogen, argon and nitrous oxide of Escherichia coli, Saccharomyces cerevisiae and Tetrahymena thermophila. All experiments were carried out at room temperature (about 22°C). E. coli was grown in tryptic-soy broth with 0.1% (w/v) KNO_3. S. cerevisiae was grown in the tryptone-glucose-Marmite broth we described previously (Marquis et al., 1973). T. thermophila was grown in the same medium but with 0.03% (w/v) ethylenediamine tetraacetate. Media for S. cerevisiae and T. thermophila were supplemented with 0.3 mg ampicillin per 100 ml to suppress bacterial contaminants.

Cultures were inoculated to yield initial populations of about the following numbers of cells per ml: 2×10^7 for E. coli, 1×10^5 for S. cerevisiae and 1×10^4 for T. thermophila. Overnight broth cultures were used for inoculation. A teflon coated, sterile, stirring bar was added to each culture, and the cultures were placed in steel pressure chambers. Gases were introduced into each chamber through a two-way valve from tanks of compressed gases. The air initially in the chambers was not flushed out. Cultures were stoppered with cotton plugs. After pressurization, the chambers were disconnected from the tanks by closing the two-way valves, and a pressure gauge was connected to each valve so that the actual final pressure within the chamber could be determined. The chambers were made of nonmagnetic steel, and so it was possible to stir the cultures by use of magnetic stirrers.

Two types of pressure chambers were used, both with gas-tight, O-ring seals. The first was a large chamber with a total volume of 3.2 liters, which held 50-ml cultures in 250-ml, Erlenmeyer flasks. The second was a smaller chamber of about 70-ml capacity which held 3-ml cultures in 10-ml test tubes. It was equipped with windows and fit into the modified cuvette compartment of a Perkin-Elmer spectrophotometer so that optical absorbance measurements with light of 700 nm wavelength could be made without the need for decompression. With the large chambers decompression and recompression were necessary for sample removal. This process did not appear to cause any damage to the cells if the decompression was accomplished over a five-minute interval.


E. coli B
S. cerevisiae
T. thermophila
Helium
Nitrogen
Argon
Nitrous oxide
% CHANGE IN CULTURE YIELD vs AIR CONTROL
cells/ml × 10⁵
AIR
% CHANGE vs AIR
GAS PRESSURE (atm)

are not major ones. The data for T. thermophila indicate no stimulation, but also no inhibition. Growth of the protozoan is somewhat more variable from culture to culture than is growth of E. coli or S. cerevisiae, and so error bars {68% confidence intervals} are included with the average data for T. thermophila. In all, it appears that the effects of these gases at pressures of 60 atm or lower on growth of prokaryotes and eukaryotes are minimal.

All of the organisms do, however, respond to more potent narcotic gases, as shown by the data presented for the effects of nitrous oxide on growth. E. coli appears to be least sensitive with 50% reduction in growth yield at a pressure of N_2O of about 15 atm; partial pressures below about 10 atm were essentially without effect on growth yield. S. cerevisiae is more sensitive to N_2O with 50% reduction in growth at a pressure of about 8 atm. T. thermophila is still more sensitive with 50% growth inhibition at an N_2O pressure of about 4 atm. In all of these experiments, air was included in the gas mixtures so that the organisms were exposed to 0.2 atm O_2.

The growth responses of these organisms to individual gases mixed with air indicate that the potency series for growth modification differs significantly from that for narcotic effects. The narcotic potency series in order of descending potency is: Xe and N_2O>Kr>Ar>N_2>Ne, H_2 and He. Helium is considered {Brauer and Way, 1970} to have negative narcotic potential in that it antagonizes the narcotic actions of gases higher in the series, such as N_2O. This series reflects the molecular sizes of the gases and is one of the bases on which the critical volume hypothesis for anesthesia was built. The gases are hydrophobic and so dissolve primarily in hydrophobic regions of cells exposed to them. The cell membrane is thought to be particularly important in narcotic action. The dissolved molecules are thought to act to distort the membrane, or other hydrophobic structures, with resultant malfunctioning. Since chemically inert or noble gases are effective, it is thought that narcotic action does not require covalent bonding but is related entirely to changes in weak interactions among molecules. Various other molecular parameters have been used instead of molecular size to predict the potencies of gases and other narcotic agents. For example, Franks and Lieb {1978} have recently used solvent/water partition coefficients and have found that the best correlation between potency and partition coefficient was obtained when the solvent chosen was n-octanol.

The action of helium can be fitted into the critical volume hypothesis {Miller and Miller, 1975} by taking into account the compressive action of

hydrostatic pressure which could oppose expansion of the hydophobic site. Helium would act to expand the site when dissolved in it. However, because of the small size of helium, a high partial pressure would be required to cause sufficient distortion for malfunctioning. This high partial pressure would more than offset the expansive effect of the dissolved helium, and so the gas would have a negative potential.

It is clear from the data presented in Fig. 1 that growth modification cannot be predicted on the basis of the narcotic potency series. Argon and nitrogen did not inhibit growth of any of the test organisms, even at pressures as high as 50 to 60 atm. A few experiments carried out with krypton and E. coli suggest that the gas stimulates growth at pressures below about 15 atm but is highly inhibitory at higher pressures. Thus, for growth inhibition the cross-over point in the series from negative or nil potency to positive potency is at the level of krypton, whereas for narcosis it is between He and Ne or H_2.

Other workers have reported growth inhibitory effects of helium, nitrogen and argon. For example, Buchheit et al. {1966} found that growth of the mold Neurospora crassa was inhibited. Argon was highly potent and could stop growth entirely at pressures of 10 to 15 atm. Helium and nitrogen were less potent, and a pressure of either gas of about 30 atm reduced the rate of growth to some 75% of the control value. Strangely, further increases in pressure up to 120 atm had little further effect on growth. These workers, and others, have interpreted growth inhibition in terms of the narcotic potency series. However, it seems to us that it is difficult to justify such as interpretation. The potency series obtained for growth inhibition does not strictly follow the narcotic potency series. Moreover, as described in the next section of this report, the effects of gas combinations are certainly not what one would expect on the basis of knowledge of narcotic action.

B. Responses to gas mixtures. Although He, N_2 and Ar have only rather small effects alone on microbial growth in air, they have dramatic effects when combined with minimally effective or noneffective levels of more potent gases. Sample data are presented in Fig. 2,3 and 4. For E. coli, 10 atm of N_2O had little effect on growth in the experiment described in Fig. 2. However, when 30 atm of argon, which by itself has almost no effect, was mixed with the 10 atm of N_2O, the combination was dramatically inhibitory. Clearly, argon strongly potentiates the growth inhibitory action of N_2O.

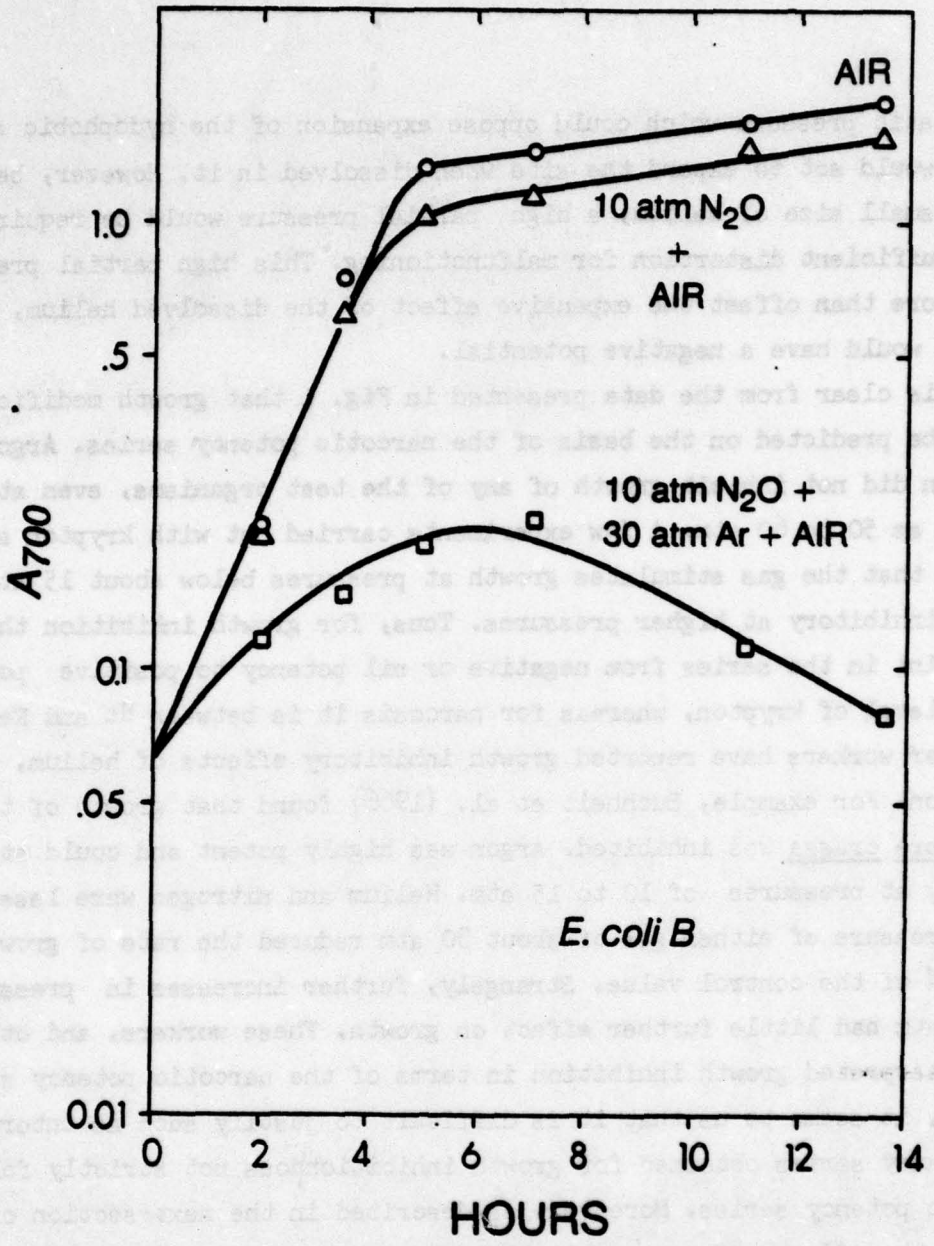


Fig. 2. Argon potentiation of growth inhibition of Escherichia coli due to nitrous oxide. The growth medium was tryptic-soy broth with 0.1% {w/v} KNO_3. Absorbance of light of 700 nm wavelength by the culture {A_{700}} was assessed by use of a Beckman DU spectrophotometer. Large pressure cylinders with capacity of 3.2 liters were used. The experimental temperature was 22°C. The chambers were decompressed over a period of approximately five minutes for sampling. After samples were removed, the chambers were immediately repressurized, and the cultures were stirred magnetically to accelerate gas equilibration.

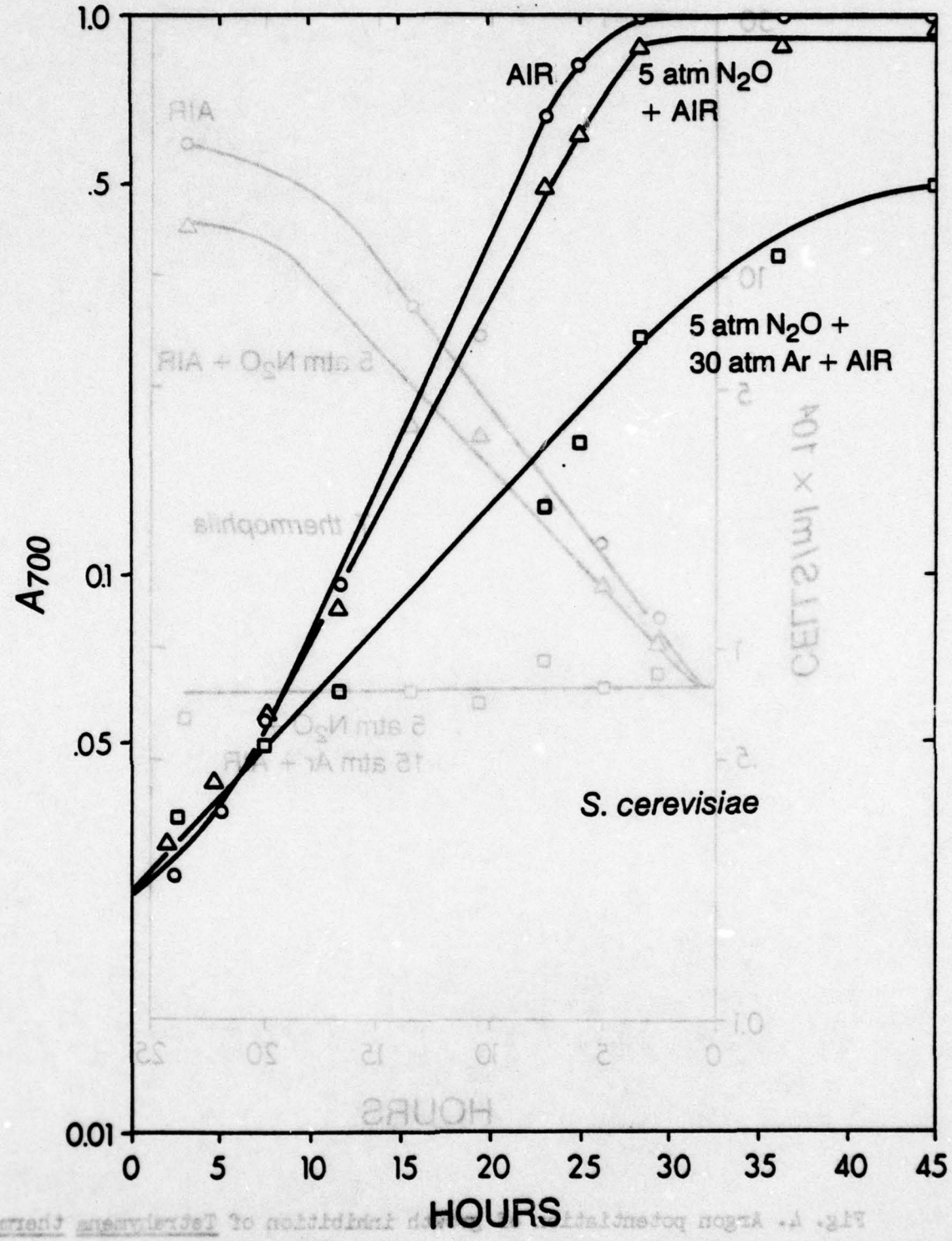


Fig. 3. Argon potentiation of growth inhibition of Saccharomyces cerevisiae due to nitrous oxide. The growth medium was tryptone-glucose-Marmite broth with 3 μg ampicillin per ml.

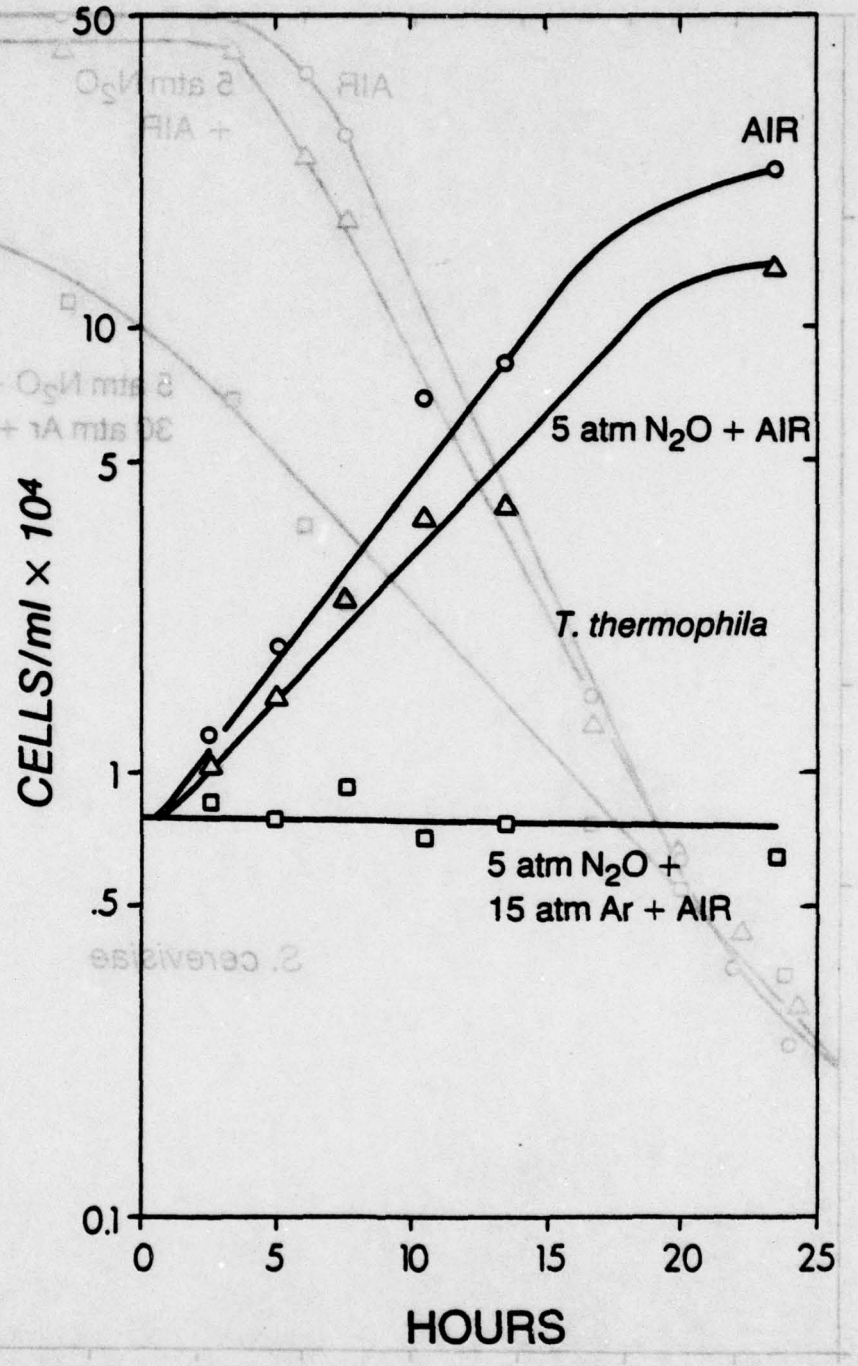


Fig. 4. Argon potentiation of growth inhibition of Tetrahymena thermophila due to nitrous oxide. The growth medium was tryptone-glucose-Marmite broth with 0.03% (w/v) ethylenediamine tetraacetate and 3 µg ampicillin per ml.

As shown by the data presented in Fig. 3, the same sort of interaction is clearly apparent for growth of S. cerevisiae. Because of the greater sensitivity of the yeast to N_2O, a partial pressure of only 5 atm was used, with little effect on growth. However, the addition of 30 atm of argon resulted in a mixture that slowed growth and reduced growth yield. The protozoan T. thermophila is still more sensitive, and the combination of 5 atm N_2O and 15 atm argon completely stopped growth, whereas 5 atm of N_2O alone was only somewhat inhibitory.

The data presented in Fig. 5,6 and 7 show that these gases also act dramatically to enhance oxygen toxicity. Here N_2 is used as an example, but He and Ar show similar potentiating actions. The data presented in Fig. 5 show that 28 atm of N_2, which has little or no effect on growth, enhances the toxicity of 2 atm O_2, which also has little effect on growth by itself. As shown by the data in Fig. 6, the responses of S. cerevisiae are even more extreme. One atm of O_2 has little effect on growth, but when combined with 15 atm of N_2, it almost completely stops growth. T. thermophila is somewhat difficult to work with in these experiments because of its sensitivity to oxygen. However, the data presented in Fig. 7 show clearly the potentiating action of nitrogen.

The results of other experiments have shown that the efficacy of potentiation can be related to molecular size in that argon is better at potentiating growth inhibition by N_2O or O_2 than is N_2, which is a better potentiator in terms of required partial pressures than is He.

The major importance of the new data presented here is that it indicates that the responses of bacteria to compressed gases described in previous reports are probably universal responses of growing cells. For speed and convenience in acquiring the required data, we have confined our attention to microorganisms. However, the responses shown by prokaryotic bacteria are shown also be eukaryotic yeasts and protozoa. Yeasts are considered to be related to plant cells, while protozoa are related more to animal cells. Work is planned with tissue culture cells and with whole animals. We feel that they will show the same responses. However, our major efforts during the next year or so will be to characterize the responses physiologically and biochemically, and for this work, microbes will again prove to be the best test organisms.

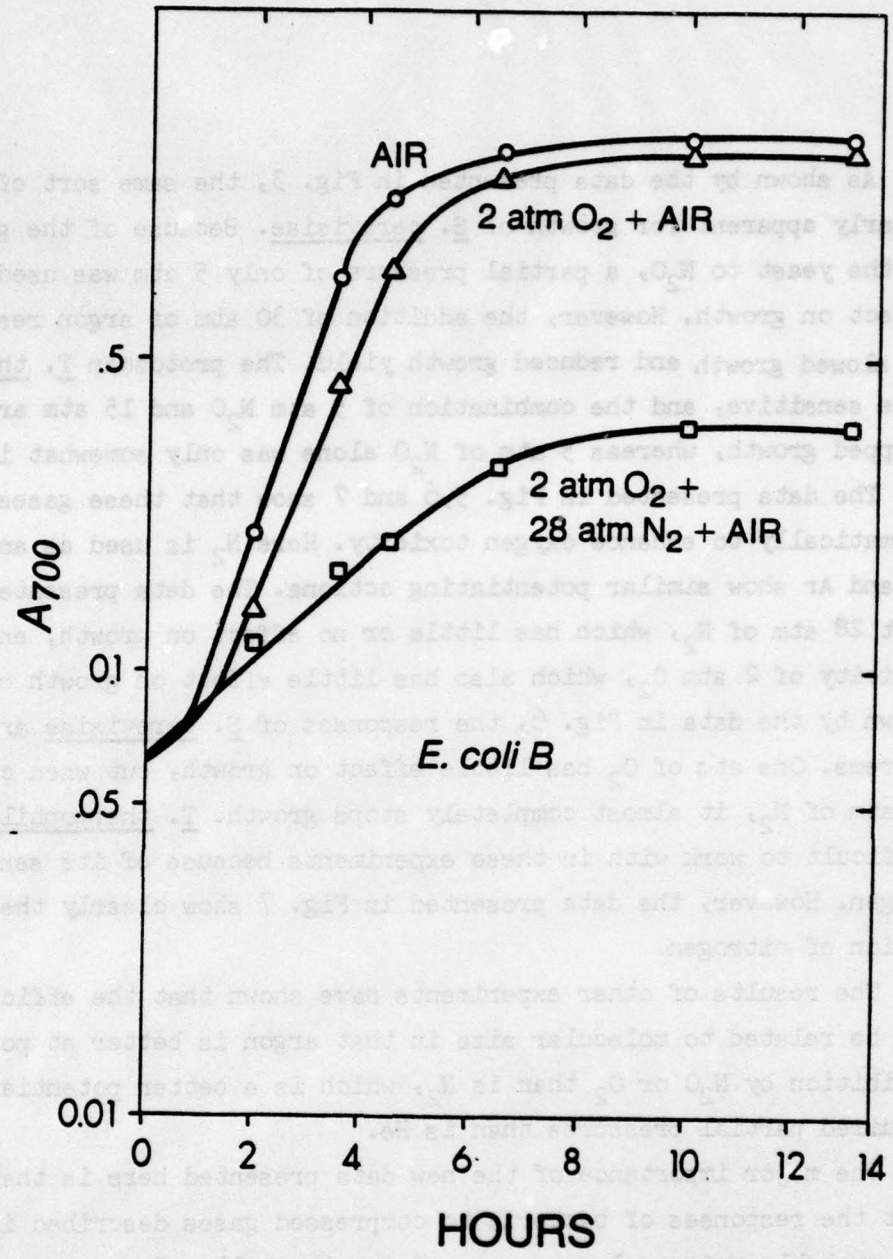


Fig. 5. Nitrogen potentiation of the toxicity of oxygen for Escherichia coli B.

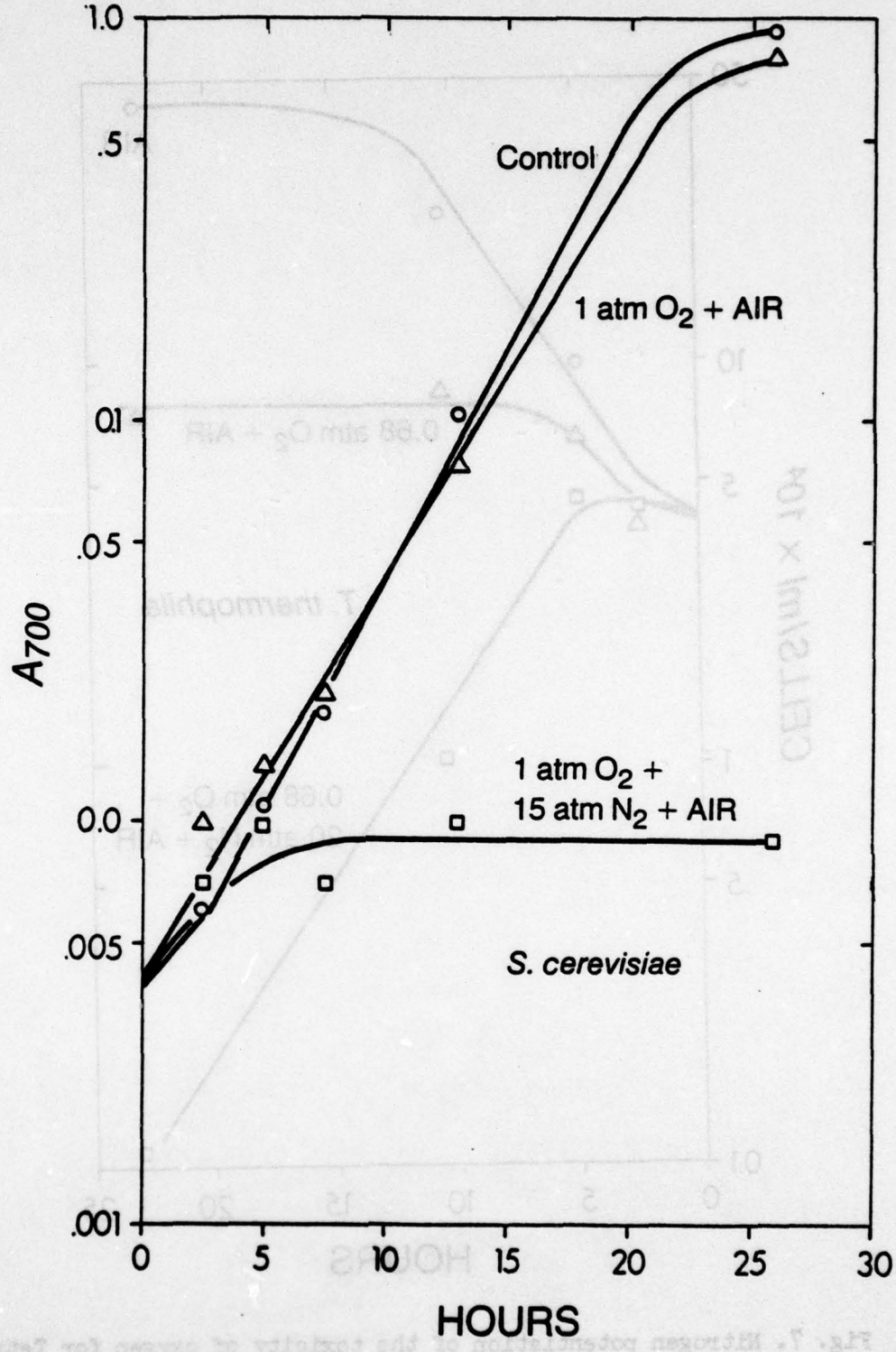


Fig. 6. Nitrogen potentiation of the toxicity of oxygen for Saccharomyces cerevisiae.

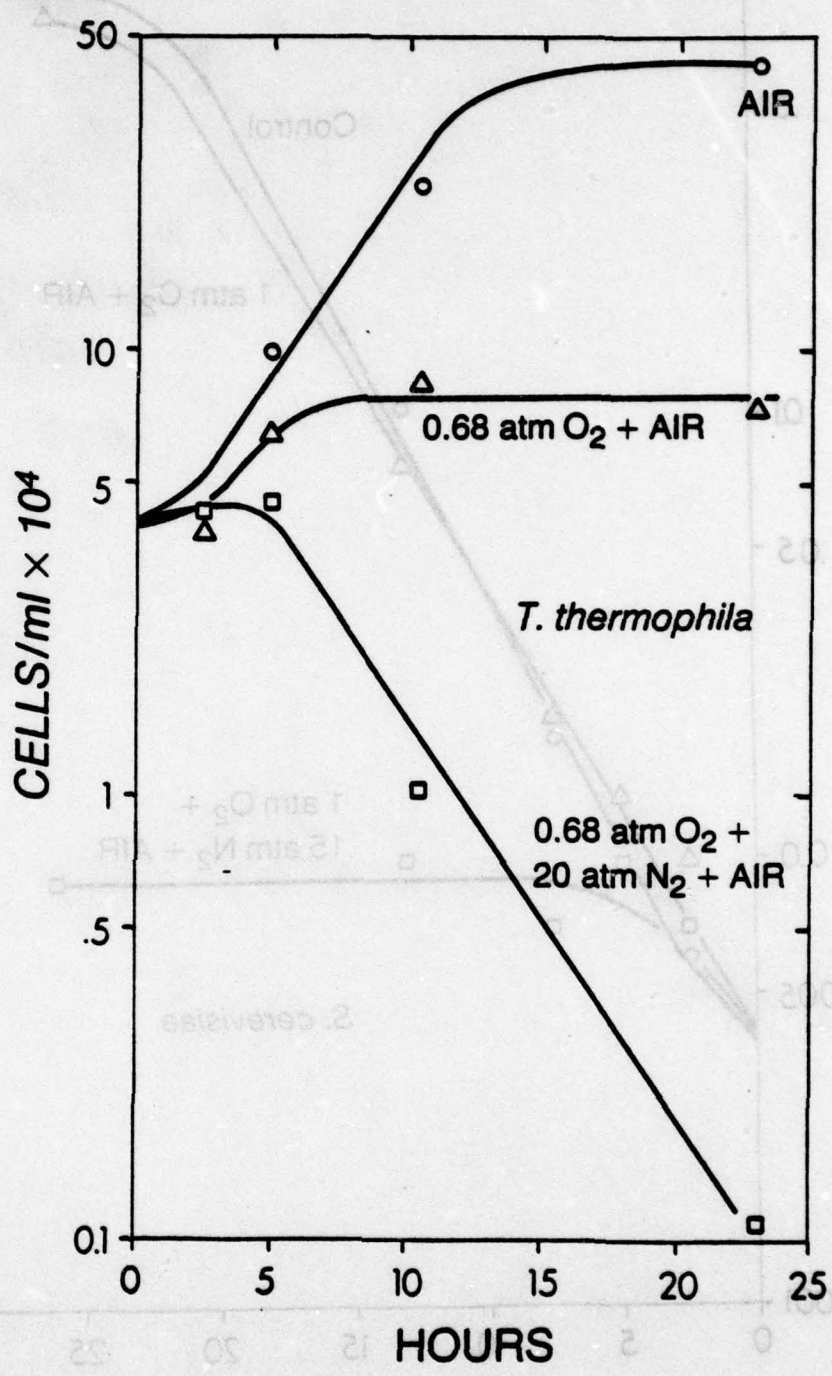


Fig. 7. Nitrogen potentiation of the toxicity of oxygen for Tetrahymena thermophila.

C. Pressure reversal of growth inhibition by narcotic agents. One of the defining hallmarks of the narcotic action of anesthetics is reversal by hydrostatic pressure. Therefore, we have been interested in determining whether or not it is possible to reverse growth inhibition due to anesthetics by means of hydrostatic pressure. Preliminary data indicate that hydrostatic pressure can reverse growth inhibitory actions of aliphatic alcohols for both E. coli B and Streptococcus faecalis. Unfortunately, the data also reveal a number of complications. It seems that one must be selective in chosing pressures and inhibitor concentrations in order to demonstrate reversal. For example, the data presented in Table 1 indicate clearly that growth inhibition caused by heptanol can be reversed by pressures of 100 or 200 atm. In fact, the data are impressively clear. For E. coli at one atm, 0.055% {v/v} heptanol essentially completely suppressed growth, but at 100 atm, full growth equal to that of control cultures occurred in the presence of this level of inhibitor. Similar results were obtained with 0.150% {v/v} heptanol for S. faecalis, which constitutionally is less sensitive to the alcohol than is E. coli. In contrast to these effects of pressures of 100 or 200 atm, pressures of 300 or 400 atm seemed actually to enhance the growth inhibitory action of heptanol. However, we want to carry out additional experiments to more fully document the effects of 300 or 400 atm pressures, especially at higher temperatures at which the inhibitory effects of pressure itself are less.

The view we are obtaining of the interactions between hydrostatic pressure and growth inhibitory anesthetics is similar to that developed by Kirkness and Macdonald {1972} on the basis of their work with Tetrahymena organisms. They found that hydrostatic pressure could potentiate the action of subinhibitory doses of anesthetics or nitrogen but that it reversed the effects of higher concentrations on cell division. It seems that the system must have more than one site of action for growth inhibition. Our initial data suggest that the bacterial systems also must be multisite ones.

During the year progress has been made on a number of other aspects of the study of compressed gases and anesthetics, but it seems best to wait until next year when more data is available before presenting a full discussion. We have continued efforts to determine the bases for enhanced oxygen toxicity due to inert gases but the experimentation has been difficult, especially since compressed gases interfere with the workings of the oxygen electrode.

The major importance of the data that is presented here is that it indicates that the responses of bacteria to compressed gases described by us previously are probably universal responses of growing cells. For convenience and efficiency,

Table 1. Effects of hydrostatic pressure on bacterial growth inhibition caused by heptanol.

Organism	Pressure (atm)	Maximum A_{700} at heptanol concentrations (v/v) *				
		0.000	0.030	0.040	0.050	0.055
Escherichia coli B	1	0.54	0.48	0.40	0.30	0.14
	400	0.19	0.05	0.03	0.03	0.03
	1	0.54	0.51	0.47	0.34	0.21
	300	0.25	0.13	0.08	0.06	0.04
	1	0.54	0.48	0.42	0.25	0.06
	200	0.54	0.54	0.54	0.54	0.54
	1	0.55	0.45	0.39	0.25	0.08
	100	0.52	0.52	0.54	0.54	0.54

		0.000	0.080	0.100	0.125	0.150
Streptococcus faecalis ATCC 9790	1	0.69	0.62	0.34	0.05	0.02
	400	0.45	0.32	0.29	0.21	0.05
	1	0.64	0.62	0.40	0.18	0.05
	300	0.57	0.42	0.23	0.14	0.05
	1	0.70	0.70	0.64	0.12	0.04
	200	0.64	0.59	0.55	0.52	0.46
	1	0.62	0.62	0.34	0.09	0.01
	100	0.65	0.68	0.70	0.70	0.68

* Cultures were grown at 22°C in tryptone-glucose-Marmite broth. 0.1% (w/v) KNO_3 was added to the medium for *E. coli*.

we have confined our attention to microorganisms. However, the responses shown by prokaryotic bacteria are shown also by eukaryotic yeasts and protozoa. Work is planned with tissue culture cells at some time in the future. For the upcoming year, our efforts will continue to be on basic characterization of growth modification by anesthetics, especially on physiological and biochemical aspects, and for this work, microorganisms are by far and away the best subjects for experimentation.

III. Biological Effects of Hydrostatic Pressure

The question of whether or not bacteria can become adapted for growth at high pressures has been a remarkably difficult one to answer. In fact, the answer is still not clear despite nearly a century of work since Certes and Regnard first considered the problem in their pioneering work on deep-sea bacteria. It is known that bacteria can be recovered from all depths of the ocean, even from the deepest trenches. ZoBell and Morita {1957} obtained evidence, based on most-probable-number counts of bacteria from deep-sea sediments incubated at one atm and under pressure, that obligately barophilic bacteria exist in the Deep. However, they were not able successfully to subculture barophilic bacteria in the laboratory.

Work in the past few years has raised questions about the existence of obligately barophilic bacteria, which would, of course, be clearly and irreversibly adapted for growth under high pressure. Apparatus has now been developed by a number of research groups for retrieval of undecompressed deep-sea samples. For example, the group at Woods Hole Oceanographic Institution {Taylor and Jannasch, 1976; Jannasch and Wirsen, 1977} has built retrieval apparatus that can be connected to a high-pressure-transfer set-up. Thus, bacteria from the Deep can be retrieved and transferred without ever being decompressed. They have been unable to recover obligately barophilic bacteria or bacteria that differ in major ways from ordinary laboratory or terrestrial bacteria. In other words, the bacteria do not seem to be specifically adapted to function better under pressure. Other workers {Schwarz et al., 1976} have isolated bacteria from the gut contents of deep-sea amphipods. The bacteria appeared to be moderately baroduric but not barophilic.

A number of people have attempted to isolate barophilic or highly baroduric mutants in the laboratory. These attempts are not generally reported in the literature because they have been unsuccessful. Pope and Ogrinc {1975}

reported that a streptomycin-resistant mutant strain of E. coli has increased barotolerance in terms of its ability to synthesize protein under pressure. However, it is not clear that the growth of this strain is also enhanced under pressure relative to that of the parent strain. Early on in this project, we used mutagens in attempts to obtain mutants with enhanced barotolerance but were unable to obtain any.

For most microbial barophysiologic studies, microorganisms are exposed to to pressure for only relatively short periods of time. They are maintained at one atm and exposed to pressure only for the interval of the experiment. It seemed important for attempts to isolate pressure adapted bacteria in the laboratory to determine whether or not bacteria could be cultivated through many subcultures under pressure. Long-term culturing under pressure has been achieved previously, for example by Chumak et al. {1968}, but with mixed cultures. Therefore, we attempted long-term culturing of single species of bacteria under pressure with decompression for only the brief periods required for subculturing.

Unfortunately, this sort of long-term subculturing under pressure has a number of problems associated with it. We found previously {Matsumura et al., 1974} that bacteria become hypersensitive to the growth inhibitory actions of acids under pressure. At near maximal growth pressures, this sensitivity is so extreme that it is nearly impossible to maintain liquid cultures because the bacteria are inhibited by metabolic acids before they have had a reasonable opportunity to grow. Therefore, during the past year, we have started to use stab cultures with inocula stabbed in single lines in tubes of solid media. By use of stab cultures, we have been able to grow S. faecalis at pressures as high as 950 atm at 37°C. Moreover, it has been possible to maintain the bacterium in repeated subcultures at pressures as high as 850 atm. At these high pressures, the cultures contain many abnormally shaped cells. Since cell division seems to be more barosensitive than is cell growth, many of the cells appear enlarged and elongate. However, there also are many cells that appear normal in the phase microscope.

These new results provide further indication that S. faecalis has an amazing range of barotolerance dependent on growth conditions. When the bacterium is using pyruvate as a fuel supply, its growth can be completely stopped by pressures as low as 200 atm. With ribose as fuel source, growth occurs at pressures as high as 450 atm. With glucose, lactose or maltose as fuel source,

growth occurs at pressures as high as 550 atm, and this maximum can be increased to about 750 atm if 50 mM magnesium ion or calcium ion is added to the growth medium. Now we are able to grow the organism at 950 atm in stab cultures in tryptone-glucose-Marmite agar.

However, a more remarkable finding of the work of the past year is that S. faecalis can become adapted during long-term growth under pressure so that it is more barotolerant. We have called the primary strain of pressure adapted S. faecalis studied to date the APR-11 strain {acid and pressure resistant variant number 1, subvariant 1}. The growth curves presented in Fig. 8 show that the APR-11 strain is better able than the parent strain to grow at 450 atm and room temperature {22°C} in complex medium with an initial pH of 6. The difference is apparent in both the rate and extent of growth. The generation time for APR-11 and the parent is approximately 0.6 h at one atm under these growth conditions. At 450 atm, the generation time for the parent is increased to nearly 4 h, while that of the APR-11 strain is increased only to 1.8 h. Thus the apparent activation volume $\{\Delta V^{\ddagger}\}$ calculated from these data for growth of the parent is 103 ml/mole, compared with a value of only 60 ml/mole for the APR-11 mutant. The extent of growth of the APR-11 mutant at 450 atm is nearly the same as that at one atm. However, the extent of growth of the parent strain at 450 atm is only about 10% that at one atm.

In effect, then, we have been able to select a pressure-resistant mutant or variant by growing S. faecalis under pressure for prolonged periods of time in stab cultures. The resistant organism appears to be a stable mutant rather than a physiologically adapted form because it does not lose its barotolerance when subcultured at one atm for many months. In addition, it appears that very few cells in a population of the parent culture are able to grow at a pressure as high as 750 atm. Samples from a parent culture were diluted and mixed with molten agar medium at 43°C in test tubes. The tubes were stoppered and incubated at 750 atm at room temperature. Visible colonies were apparent in one-atm control tubes after about 48 h of incubation. However, even after seven days of incubation, there were no apparent colonies in the tubes incubated at 750 atm. This result suggested that fewer than one in 10^8 cells in the parent culture were able to grow at 750 atm, at least from a single cell or a few cells deposited in agar. Subsequent incubation of the 750-atm tubes at one atm resulted in the appearance of colonies, but only about 10% as many as one would expect from the numbers in the tubes incubated only at one atm. It seemed

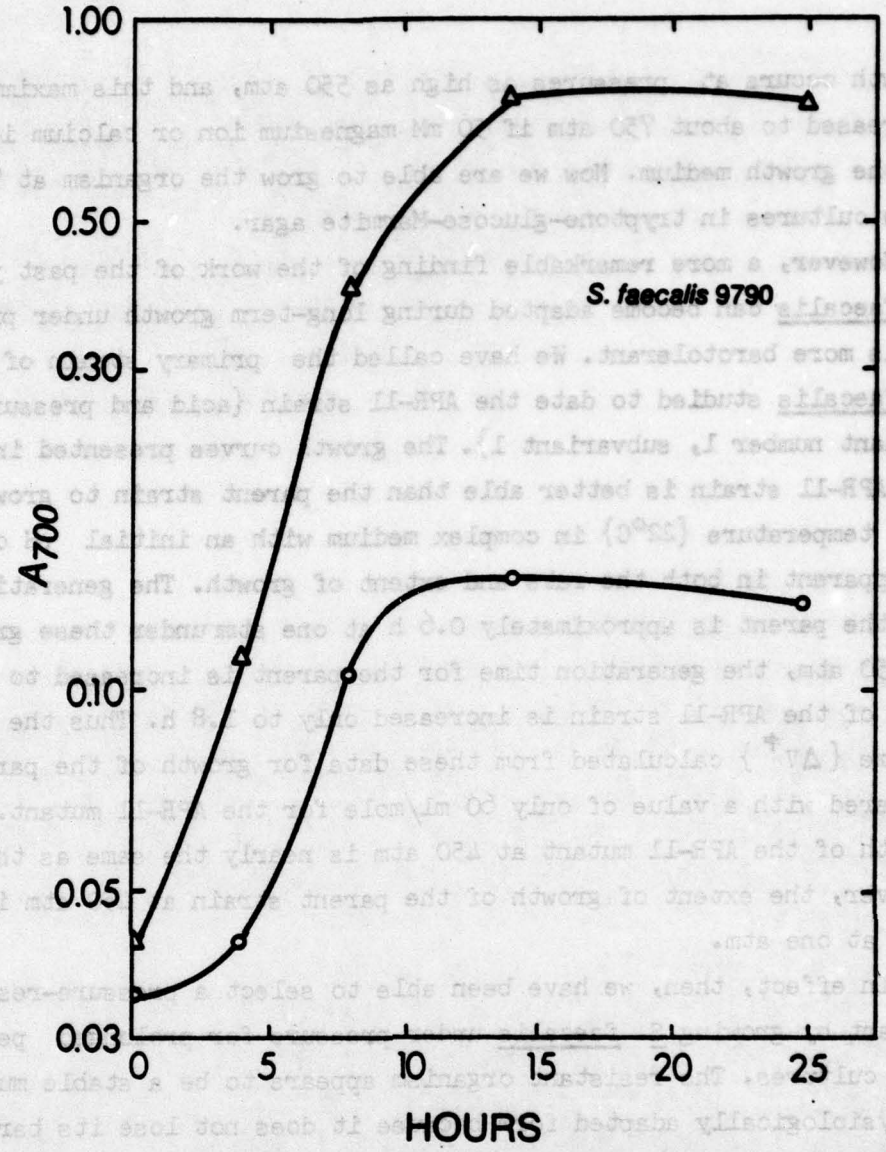


Fig. 8. Relative barotolerance of the parent strain {○} of Streptococcus faecalis ATCC 9790 and the derived APR-11 strain {△}. Cultures were grown at 450 atm and 22°C in tryptone-glucose-Marmite broth with an initial pH of 6.0.

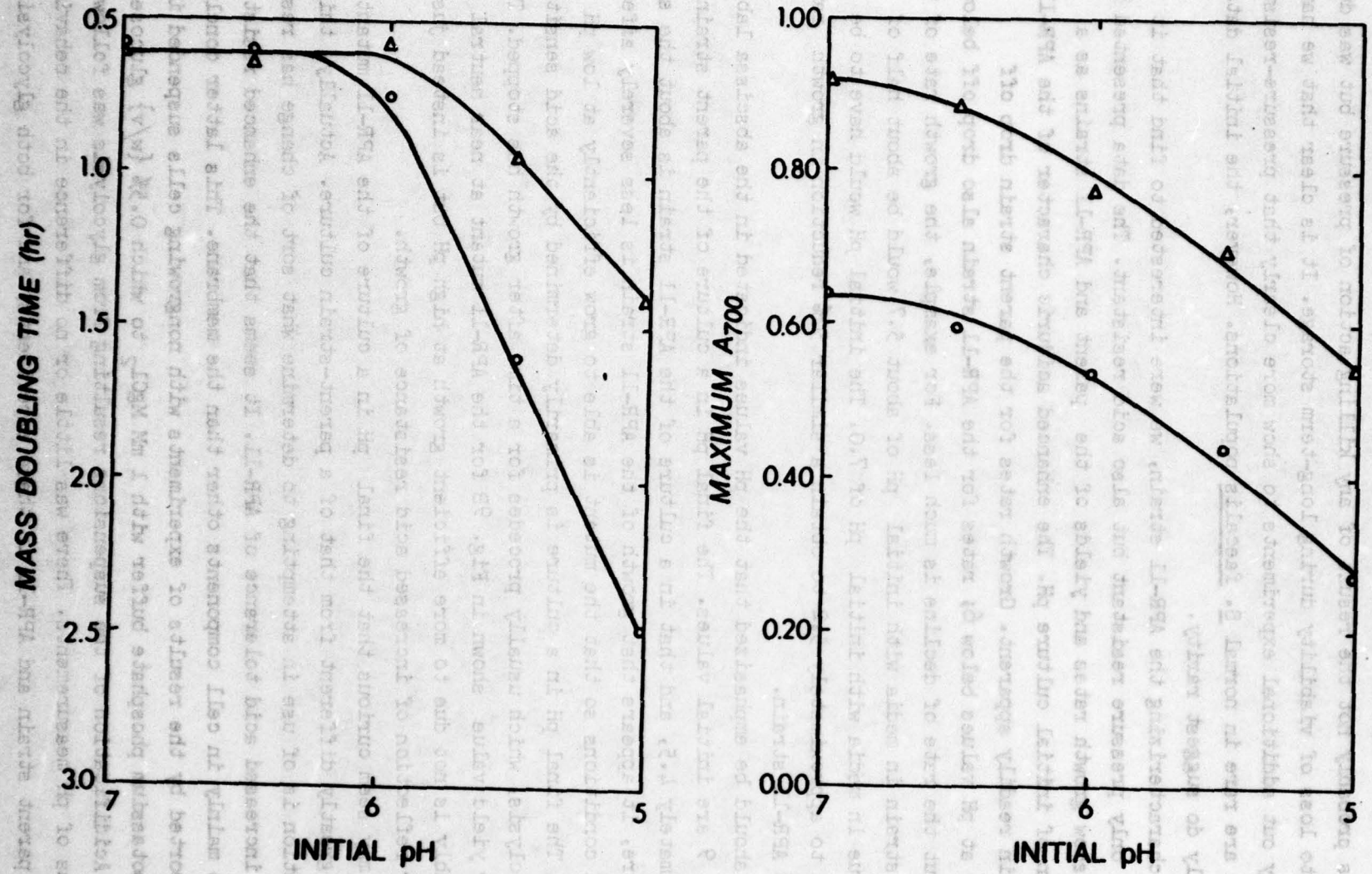


Fig. 9. Acid sensitivity of growth of the parent strain {○} of Streptococcus faecalis ATCC 9790 and the derived APR-11 strain {△}. Cultures were grown at one atm and 22°C in tryptone-glucose-Marmite broth with the initial pH values indicated by the abscissa scale.

then that 90% of the cells had died during the incubation at 750 atm. This death is probably not the result of any killing action of pressure but was due simply to loss of viability during long-term storage. It is clear that we have to carry out additional experiments to show more clearly that pressure-resistant mutants are rare in normal S. faecalis populations. However, the initial data certainly do suggest rarity.

In characterizing the APR-11 strain, we were interested to find that it was not only pressure resistant but also acid resistant. The data presented in Fig. 9 show growth rates and yields of the parent and APR-11 strains as a function of initial culture pH. The enhanced aciduric character of the APR-11 strain in readily apparent. Growth rates for the parent strain drop off sharply at pH values below 6; rates for the APR-11 strain also drop off below pH 6, but the rate of decline is much less. For example, the growth rate of the parent strain in media with initial pH of about 5.7 would be about half of the value in media with initial pH of 7.0. The initial pH would have to be reduced to approximately 5.2 to obtain a similar 50% reduction in growth rate for the APR-11 strain.

It should be emphasized that the pH values indicated in the abscissa labels in Fig. 9 are initial values. The final pH in a culture of the parent strain is approximately 4.5, and that in a culture of the APR-11 strain is about the same. Therefore, it appears that growth of the APR-11 strain is less severely affected by acid conditions so that the mutant is able to grow efficiently at low pH values. The final pH in a culture is primarily determined by the acid sensitivity of glycolysis, which usually procedes for a time after growth has stopped. The greater yield value shown in Fig. 9B for the APR-11 mutant at near neutral pH presumably is not due to more efficient growth at high pH but is instead just another reflection of increased acid resistance of growth.

It may seem curious that the final pH in a culture of the APR-11 mutant is not greatly different from that of a parent-strain culture. Actually, this information is of use in attempting to determine what sort of change has resulted in the increased acid tolerance of APR-11. It seems that the enhanced resistance must be mainly in cell components other than the membrane. This latter conclusion is supported by the results of experiments with nongrowing cells suspended in 20 mM potassium phosphate buffer with 1 mM $MgCl_2$ to which 0.5% {w/v} glucose was added. Acidification of the suspensions resulting from glycolysis was followed by means of pH measurements. There was little or no difference in the behavior of the parent strain and APR-11 in these experiments, and for both glycolysis

stopped when the pH had dropped to about 3.9. The glycolytic system is relatively acid sensitive with a minimum pH for activity of about 6 for streptococcal cells. Normally, protons are moved out of glycolyzing streptococci by a proton extrusion system involving the membrane ATPase, and the protoplast membrane then acts as a barrier to reentry of the protons {Harold and Van Brunt, 1978}. If the barrier function is short circuited by agents such as gramicidin, glycolysis becomes sensitized to the environmental pH. Thus, it appears that streptococci are able to carry out glycolysis in acid media mainly because the cytoplasmic pH is maintained at a near neutral value. Therefore, the acid sensitivity of glycolysis by intact cells is a good indicator of the functionality of the membrane in proton extrusion and as a proton barrier. These functions appear not to be changed in the APR-11 mutant.

Clearly, one of our major jobs in the upcoming period is to characterize the APR-11 mutant. Our current feeling is that it may be a mutant that is better able than the parent to produce ammonia from amino acids in the growth medium and so in a sense produces its own buffer. However, more experimental work has to be carried out to support this conclusion. We are in the process also of isolating pressure-resistant variants of other bacteria, including E. coli. This work is rather slow and tedious because of the need to increase the growth pressure in a number of steps. It does appear to be a feasible approach to the isolation of resistant variants, and we hope to have a range of such variants prepared for study within the next year.

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The results of experiments carried out during the past year gave further support to our view that the modifying actions of compressed gases on microbial growth cannot be considered narcotic actions but belong instead to a definably different class of actions. A major component of this work involved the use of eukaryotic microorganisms.

Helium, nitrogen and argon at pressures as high as 60 atmospheres were found to have relatively little effect {slightly stimulatory} on growth of Escherichia coli, Saccharomyces cerevisiae or Tetrahymena thermophilia. In

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the potency series for narcotic action, which has the order N_2O or Xe>Kr> Ar>N_2>Ne, H_2 or He, there is a cross-over from negative potency to positive potency between He and Ne or H_2. For growth effects, this cross-over from stimulatory or nil action to inhibitory action occurs at the level of Kr rather than H .

Although He, N_2 or Ar alone have only minor effects on growth, in combination with more potent gases such as N_2O they exert a dramatic potentiating action. The order of effectiveness for potentiation has the series Ar>N_2>He. These gases also dramatically potentiate the toxic action of oxygen on growth. The potentiation can be demonstrated with prokaryotes {Escherichia coli} and also with eukaryotes that are phylogenetically related to plants {Saccharomyces cerevisiae} or to animals {Tetrahymena thermophila}.

During the past year, it has been possible to show that hydrostatic pressures of 100 or 200 atmospheres act to reverse the growth inhibitory effects of liquid narcotic agents of the aliphatic alcohol series. Higher pressures seem to enhance inhibition, and the data suggest multisite targets for growth modifying effects of narcotic agents.

Substantial advances have been made during the past year also in our study of the biological effects of hydrostatic pressure. In this work, we are concerned with pressure effects rather than with specific gas effects, and efforts are made to exclude gases from the test systems under study. By means of long-term cultivation of Streptococcus faecalis in agar stab cultures at pressures of 800 to 900 atmospheres, we have been able to select a variant bacterium with enhanced barotolerance. This variant is a stable mutant that occurs in small numbers in the parent population. Interestingly, it is not only baroduric but also aciduric. This combined resistance can be related to our previous findings that bacteria become sensitized under hydrostatic pressure to the growth inhibitory action of metabolic acids. The technique of prolonged culture in agar stabs under pressure with periodic transfer to new medium at one atmosphere offers a means for isolation of barotolerant variants of many microorganisms.

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