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Growth of a Bacterium Under a High-Pressure Oxy-Helium Atmosphere†

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Growth of a barotolerant marine organism, EP-4, in a glutamate medium equilibrated with an oxy-helium atmosphere at 500 atmospheres (atm; total pressure) (20°C) was compared with control cultures incubated at hydrostatic pressures of 1 and 500 atm. Relative to the 1-atm control culture, incubation of EP-4 at 500 atm in the absence of an atmosphere resulted in an approximately fivefold reduction in the growth rate and a significant but time variant reduction in the rate constants for the incorporation of substrate into cell material and respiration. Distinct from the pressurized control and separate from potential effects of dissolution of helium upon decompression of subsamples, exposure of the organism to high-pressure oxy-helium resulted in either a loss of viability of a large fraction of the cells or the arrest of growth for one-third of the experimental period. After these initial effects, however, the culture grew exponentially at a rate which was three times greater than the 500-atm control culture. The rate constant for the incorporation of substrate into cell material was also enhanced twofold in the presence of high-pressure oxy-helium. Dissolved oxygen was well controlled in all of the cultures, minimizing any potential toxic effects of this gas.

Most experimental investigations dealing with the activity of marine microorganisms at elevated hydrostatic pressure have used mixed bacterial populations, pure culture isolates collected from the sea surface, or isolates from the deep sea after maintenance for some time at normal atmospheric pressure (23, 25, 30, 31). To estimate accurately the metabolic activities of microorganisms at in situ pressures, however, it is important to consider the effects of decompression and recompression. To eliminate these variables, pressure-retaining microbiological samplers have been used for the retrieval of deep-sea microbial populations without decompression and with minimum temperature change (16). The samplers are subsequently used as laboratory incubation chambers in which the growth and metabolism of undecompressed mixed deep-sea populations are measured in a time course fashion under in situ conditions of temperature and pressure.

Results of such studies (15, 16) have confirmed earlier observations (13, 28) that microbial activities in the deep ocean are lower than those measured in decompressed parallel samples incubated at 1 atmosphere (atm). Further studies on the possible existence of barophilic bacteria and on the nature of barotolerance in general requires a technique of obtaining undecompressed pure cultures.

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The first step in this direction was made by the development of a pressure-retaining filtration sampler (14). The technical capability of transferring concentrated and undecompressed deep-sea water samples into prepressurized culture vessels (15, 16) now makes it possible to conduct a similar inoculation procedure for purification and isolation experiments.

In terms of logistics, the most effective way to obtain pure cultures under pressure is to streak undecompressed and concentrated samples onto the surface of a solid nutrient medium which is in equilibrium with an oxy-helium atmosphere.

Two phenomena potentially detrimental to bacteria in a hyperbaric isolation chamber are chemical toxicity of the more reactive gases (i.e., oxygen) and narcosis elicited by inert gases. Toxicity of oxygen at elevated partial pressure is well established in the literature (8, 11, 20, 32), and the avoidance of this potential difficulty in hyperbaric systems operating in the vicinity of 500 atm (total pressure) requires an accurate knowledge of the solubility properties of oxygen under these conditions. Helium has not been found to be narcotic at pressures of up to approximately 245 atm (3, 7, 9, 19-24). Little, however, is known of the effects of helium at pressures approaching and exceeding 500 atm.

Presented in this communication are the results of growth measurements on a marine organism incubated under an oxy-helium atmosphere at a 500-atm pressure. An investigation

dealing with the physical chemistry of oxygen in high-pressure systems is discussed elsewhere (25a).

MATERIALS AND METHODS

Hyperbaric growth chamber. The hyperbaric growth chamber (Fig. 1) is composed of a carbon steel reservoir pressure cylinder (RPC) partially filled with glycerol (gl), a stainless steel experimental pressure cylinder (EPC), and high-pressure valves (va through vf). The EPC was maintained at 20°C by using a refrigerated water bath. The experimental liquid medium (100 ml) was contained in a polycarbonate chamber housed within the EPC. The medium was stirred with a magnetic stirring bar. The gas and liquid phases were brought in contact with the outside through penetrations in the polycarbonate end cap (pec) and high-pressure ports in the EPC end cap (EC).

The medium was equilibrated with air at 20°C, placed in the EPC, and allowed to stir for approximately 60 min within the cylinder under an air atmosphere. Oxy-helium (partial pressure of oxygen, 0.043 ± 0.002 atm) from mixing chamber 1 (mc1) was introduced into the system via a hypodermic needle inserted through a rubber septum in the inlet tee (in). The gas was directed through inlet valves vb, vc, vd, and the exit valve ve at a flow rate of approximately 1 liter per min. Valve vd was closed after approximately 10 to 20 s of flushing. The partial pressure of oxygen in the exit gas was monitored in chamber mc2 with a Teflon membrane-covered oxygen polarographic electrode (e) which was calibrated against the atmosphere (2, 17). Flushing was continued until the partial pressure of oxygen of the exit gas remained constant for at least 5 min (total flushing time, approximately 10 min). With the gas still flowing, the connection between valve ve and the EPC was removed, and radiolabeled substrate (see below) was introduced into the culture through the channel in end caps EC and pec. Valve ve was reattached, the gassing needle was removed from the inlet (in), and, within 5 s, valves ve, vb, and vc were sequentially closed. Stirring was terminated during these gassing procedures to minimize reduction of the dissolved oxygen in the growth medium in response to the low partial pressure of oxygen of the flushing gas. The septum of the inlet was replaced by a high-pressure plug (precautions were taken to prevent entrance of air), and the RPC and EPC were independently precharged to 100 atm from a commercial tank of helium (He) through valves vb and vd, respectively. The gas phases of the RPC and EPC were reunited by opening valve vc and further compressed by opening valve va and hydraulically introducing glycerol into the RPC via pump Hyd. Stirring was reinitiated to facilitate equilibration of the medium and the gas phase. The approach to equilibrium, determined from separate oxygen mea-

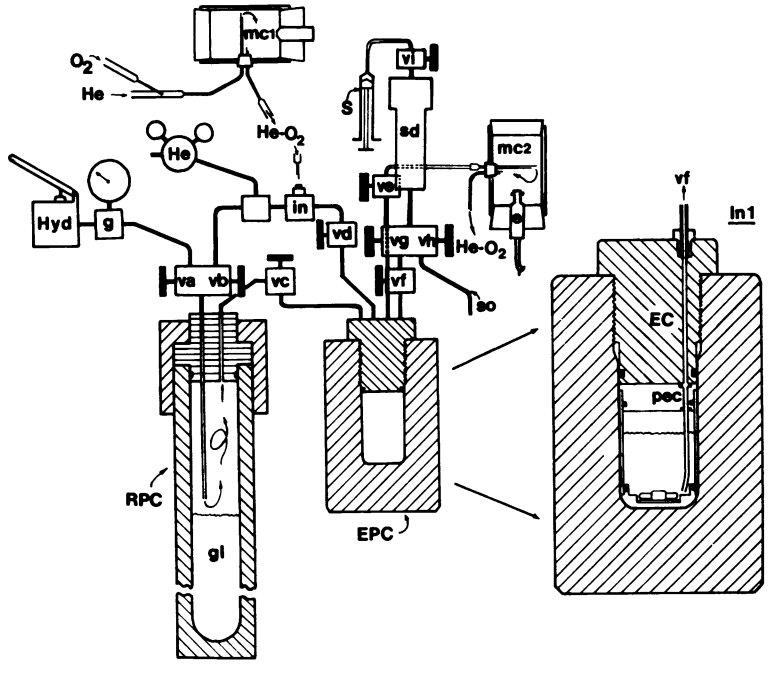

FIG. 1. *Hyperbaric growth chamber. RPC, Reservoir pressure cylinder; EPC, experimental pressure cylinder; Hyd, gl, and g, hydraulic pump, hydraulic fluid glycerol, and pressure gauge, respectively; va through vi, high-pressure valves; He, 100-atm source of helium; mc1 and mc2, gas mixing chambers; 3, membrane polarographic oxygen electrode; in, oxy-helium inlet tee; sd, subsampling device (see reference 26); S, syringe for measuring subsample volume; so, subsample outlet. Components detailed in insert 1 (In1): EC, end cap; pec, end cap of the polycarbonate growth chamber. A description of the operation of the hyperbaric system is presented in the text.*

surements, followed an asymptotic exponential function, with a rate constant of 3.5×10^{-2} min^{-1}. Equilibrium was 95% complete within 1.4 h.

Device sd, valves vg, vh, and vi, and syringe S were used to withdraw 6-ml subsamples from the compressed culture. Except for the method of decompression, the procedure of Taylor and Jannasch (26) was used for this operation. During subsampling the pressure was maintained constant in the EPC by activation of the hydraulic pump. The subsample was decompressed by allowing the floating piston contained in device sd (see reference 26 for details) to be displaced to its uppermost position near valve vi over a period of 15 min (total volume of the sample chamber increased to 12 ml). This effected a slow decompression of the subsample in which the dissolution of helium proceeded in a controlled fashion and provided the driving force for the movement of the piston. At the end of the decompression period, the subsampling device was detached from the EPC between valves vg and vf and inverted, and the helium (pressure, less than 4 atm) was slowly vented through valve vg. The subsampling device was reinverted to the upright position, and the fully decompressed subsample was expelled through valve vg by depressing syringe S as previously described (26).

The two pressure vessels in the hyperbaric system are designed to withstand 3,200 atm of pressure and may be operated at 800 atm of pressure with a fourfold margin of safety.

Measurement of growth. Experiments were conducted at 20°C in the basal medium of Wirsen and Jannasch (29) modified as described by Taylor and Jannasch (26). Yeast extract was omitted from the medium in this series of experiments. An exponentially growing culture of the marine organism EP-4 (26) was used to inoculate 500 ml of medium (containing 500 μm glutamate) to a population density of 10^5 viable cells per ml. After aeration at 20°C for approximately 10 min, the inoculated medium was dispensed into preautoclaved polycarbonate growth chambers to be incubated (i) under hydrostatic pressure (26; the stirring motor-containing plunger was modified to be autoclavable), (ii) under hyperbaric conditions (Fig. 1), and (iii) at 1 atm in the absence of a gas phase. Just before the initiation of the experiment, [*U*-^{14}C]glutamate (260 μCi/μmol; 385 nmol/ml) was introduced into the inoculated 500 μM glutamate growth medium contained in the growth chambers (final activity, approximately 0.05 μCi/ml). The helium and hydrostatic pressure cultures were concomitantly compressed to 500 atm over a period of 20 min. Zero-time subsamples were taken from these cultures at 20 min after the completion of compression. Subsamples for zero-time analysis were taken from the 1-atm control cultures at approximately 10 min after the introduction of radiolabeled substrate.

Viable count, incorporation of radiolabel into trichloroacetic acid-precipitable cell material, and production of radiolabeled carbon dioxide were measured as previously described (26). Direct counts were determined by the acridine orange technique by using the procedure described by Watson et al. (27). Apparent colony-forming units were determined by counting single cells, cell pairs, and clumps of cells as one. The total number of cells was also determined from the sum of all of the cells present as individuals, in pairs, and in clumps.

Materials. All chemicals were of reagent or analytical grade. [*U*-^{14}C]glutamic acid was purchased from New England Nuclear Corp., Boston, Mass. Helium (purity, 99.995%) and oxygen (purity, 99.5%) were obtained from Union Carbide Corp., Linde Div., Hyannis, Mass. The following crucial items were obtained from the companies indicated: no. OC-14 carbon steel pressure reactor from High Pressure Equipment, Erie, Pa.; hydraulic pump 46-12180SP and pressure gauge 47-18320 from American Instrument Co., Silver Spring, Md.; valves vc, vd, ve, vf (20SV-4071), and va–vb, vg–vh (20SV-4074), vee type, 316 stainless steel, from Autoclave Engineers, Erie, Pa. The EPC was machined by Abco Tool and Die Co., Inc., Hyannis, Mass., from Nitronic-50 stainless steel (Armco Steel Corp., Baltimore, Md.).

RESULTS AND DISCUSSION

Growth of a barotolerant marine microorganism, EP-4, under an oxy-helium atmosphere at 500 atm (20°C) was compared with growth of control cultures incubated at 1 and 500 atm of hydrostatic pressure. Four independent methods of measurement were employed: (i) viable count, (ii) direct count, (iii) incorporation of radiolabel from [^{14}C]glutamate into trichloroacetic acid-precipitable cell material, and (iv) respiration of [^{14}C]glutamate into [^{14}C]carbon dioxide (except for the culture incubated under hyperbaric conditions).

The data in Fig. 2 represent the results of two replicate experiments which were conducted 1 week apart. In all instances the responses of the cultures were the same within experimental error, except perhaps for the incorporation data from the culture incubated under 500 atm of hydrostatic pressure. These observed differences, however, do not detract from the basic conclusions of the paper.

The rate constants for growth were determined directly from the slopes of the (logarithmically transformed) cell count data in Fig. 2 and are presented in Table 1. Although the rate constants calculated from the direct and viable counts were not significantly different from one another, the trend of the data suggests that the viability (initially ≥90% and equal to viable count/direct count in colony-forming units × 100) of all of the cultures decreased slightly with time. The constant ratio between the colony-forming units and the total cells which were determined (see above) from microscopically enumerated cells revealed that neither hydrostatic pressure nor a high-pressure atmosphere interfered with the separation of dividing cells. Neither filament formation nor pleomorphism was observed in any of the cultures.

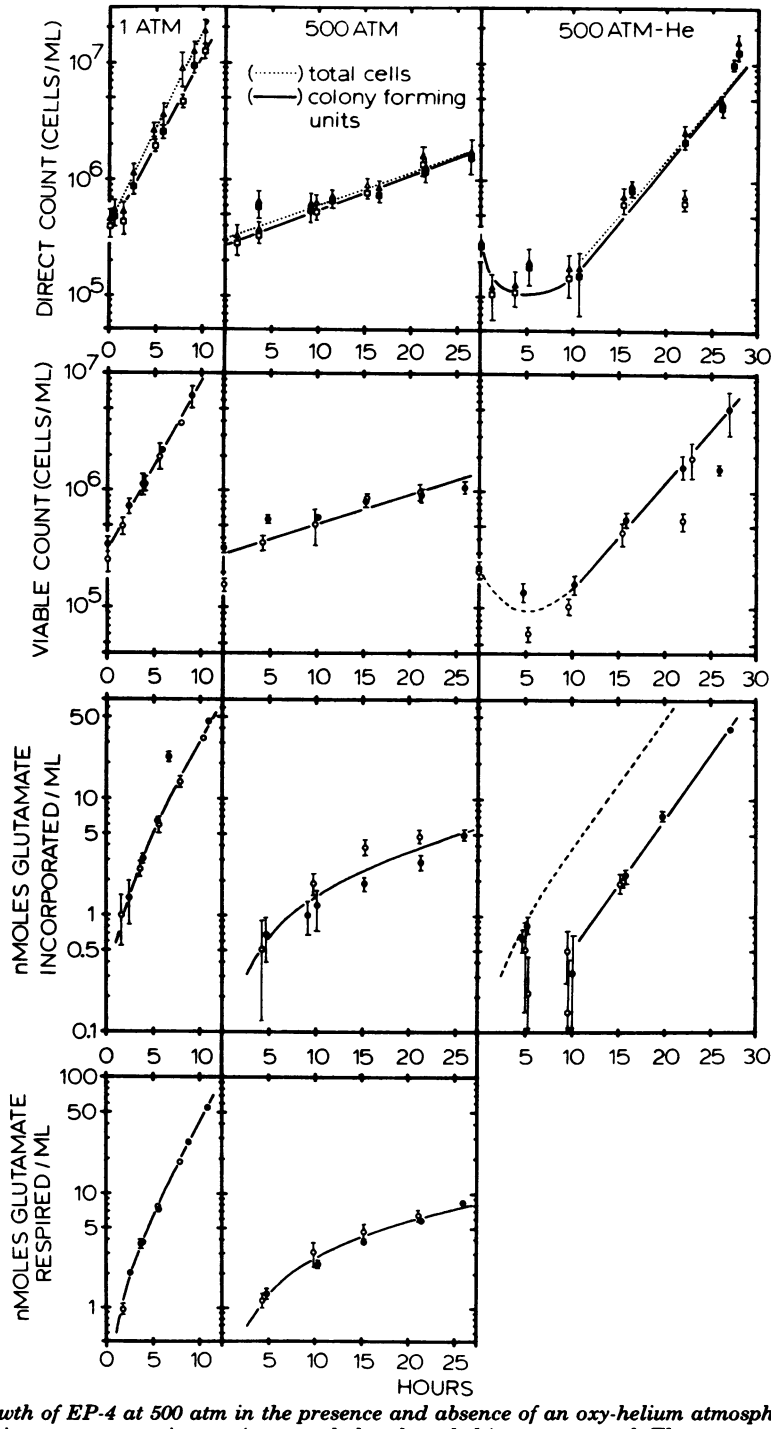

FIG. 2. *Growth of EP-4 at 500 atm in the presence and absence of an oxy-helium atmosphere. Data from two separate time course experiments (open and closed symbols) are presented. The curves were calculated from equation 1 employing best-fit values of k and R. The growth medium contained 500 μM glutamate [U-^{14}C]glutamate to yield the following specific activities: (i) open symbols, 266 ± 6, 162 ± 18, and 182 ± 9 dpm/nmol in the cultures incubated at 1 and 500 atm of hydrostatic pressure and at 500 atm in the presence of an oxy-helium atmosphere (500 atm-He), respectively; (ii) closed symbols, 220 ± 4, 198 ± 4, and 213 ± 8 dpm/nmol at 1 and 500 atm and 500 atm-He, respecitvely. The partial pressure of oxygen in the pressurized atmosphere was 0.22 ± 0.01 atm, which corresponds to a dissolved oxygen concentration of 227 ± 10 μM. The data are expressed as the average of duplicate subsamples. The error bars in the viable cell count represent the discrepancy between duplicates. The error bars in the metabolic data represent the error propagated from the uncertainties in the independent variables used for calculation. In some instances the accounted error lies within the confines of the symbol. Pressure was measured by gauge and was accurate to better than 5%. On the basis of previous observations (26) it was concluded that the carbon dioxide measured was a product of respiration and not simple decarboxylation.*

TABLE 1. *Growth and metabolism constants of EP-4 growing at a pressure of 500 atm*[a]

Pressure	Measurement	Growth parameter[b]			
		k (h^{-1})	Generation time (h)	R_{resp} (fmol of glutamate respired viable $cell^{-1}$ h^{-1})	R_{incorp} (fmol of glutamate incorporated viable $cell^{-1}$ h^{-1})
1 atm	Viable count	0.48 ± 0.08	2.09 ± 0.35		
	Direct count	0.52 ± 0.08	1.91 ± 0.30		
	Respiration	0.48 ± 0.03	2.07 ± 0.13	1.60 ± 0.12	
	Incorporation	0.47 ± 0.03	2.12 ± 0.14		1.31 ± 0.09
500 atm	Viable count	0.085 ± 0.009	12 ± 1		
	Direct count	0.100 ± 0.018	10 ± 2		
	Respiration	0.140 ± 0.006	71 ± 31	0.91 ± 0.09	
	Incorporation	0.054 ± 0.014	19 ± 5		0.42 ± 0.04
500 atm-He	Viable count	0.31 ± 0.05	3.3 ± 0.5		
	Direct count	0.32 ± 0.05	3.1 ± 0.5		
	Incorporation	0.35 ± 0.02	2.8 ± 0.2		0.94 ± 0.08

[a] Calculated from the data in Fig. 2.

[b] Values are mean ± standard deviation. The standard deviations of the parameters were determined from the error propagated from the uncertainty in the independent variables used for calculation.

Incorporation and respiration were measured in terms of the total radiolabel accumulated into cell material and carbon dioxide, respectively. Under conditions of balanced growth, metabolism rate and growth rate information may be obtained by using an integrated growth equation (26): $TC = (RNo/k\ln 2)\ (e^{kt\ln 2} - 1)$ (equation 1) or $\ln(TC) = \ln(e^{kt\ln 2} - 1) + \ln(RNo/k\ln 2)$ (equation 2), where TC equals the total metabolite converted by time t in nanomoles per milliliter, R equals the metabolism rate constant in nanomoles of metabolite converted per viable cell per hour, No equals the initial viable cell density in cells per milliliter, k equals the growth rate constant h^{-1}, and ln2 equals the natural logarithm of 2. As can be seen from equation 2, a plot of the data in the form $\ln(TC)$ versus t will result in a curve whose slope asymptotically approaches $k\ln 2$ as $e^{kt\ln 2}$ becomes significantly larger than one. The growth rate constant may be accurately obtained from the slope only after $kt\ln 2 \geq 3$ generations have occurred. Thus, the data which may be used for analysis are limited. The metabolism data of the 500-atm hydrostatically pressurized cultures (Fig. 2) cannot, for example, be used for graphically determining k because only two generations of growth have transpired. To avoid this restriction, k and R were estimated by using nonlinear regression methods (5) based upon equations 1 and 2. Computations were conducted by using program BMD07R (1970 version for a Xerox Sigma 7 computer) from Dixon (reference 4, p. 387–396).

Growth rate and metabolism rate constants obtained from the metabolism data in Fig. 2 are compiled in Table 1. The growth rate constants for the 1-atm control culture which were determined from the independent cell population and metabolism measurements agreed well with one another (average value of k, 0.49 ± 0.10 h^{-1}; average generation time, 2.05 ± 0.44 h), and within the described limits of error the values for R were time invariant. Thus, within the limits of the detectability of the methods, the criteria for obtaining valid estimates of k and R from metabolism data were adequately upheld. A similar comparison made with the culture incubated under 500 atm of hydrostatic pressure, however, revealed widely divergent values of k and suggests that the culture was in a state of highly imbalanced growth. Emphasis was placed upon the growth rate constants which were determined directly from the cell population measurements (average k, 0.092 ± 0.010 h^{-1}; average generation time, 11 ± 2 h). With this chosen growth rate constant, the metabolism rate constants for respiration and incorporation into trichloroacetic acid-precipitable cell material (R_{resp} and R_{incorp}, respectively) were calculated from equation 1. As would be expected in cases of imbalanced growth, R_{resp} and R_{incorp} were not constant in time, but rather decreased from the values in Table 1 at $t \approx 0$ to an R_{resp} of 0.40 ± 0.10 fmol $cell^{-1}$ h^{-1} and an R_{incorp} of 0.28 ± 0.05 fmol $cell^{-1}$ h^{-1} at 25 h.

Compared with the response at 1 atm, the mean generation time of EP-4 was increased 5.3-fold when incubated at 500 atm of hydrostatic pressure. In terms of R, both the respiratory and assimilatory processes were decreased by pressure. However, because both R_{incorp} and R_{resp} decreased upon continued incubation of the compressed culture, the apparent magnitude of the pressure effects upon metabolism was time dependent. For example, relative to the metabolism rate constants of the control culture, there

was an approximately 1.8-fold decreased R_{resp} and a 3.1-fold decreased R_{incorp} soon after the compression to 500 atm. Later in the growth there were decreases of approximately 4.0- and 4.7-fold in R_{resp} and R_{incorp}, respectively.

The responses of EP-4 to hydrostatic pressure described here agree in trend with earlier observations (26). Recalculation of the earlier data by using the procedures employed in this communication did not significantly affect the results except for increasing the generation time at 500 atm from 4.4 ± 0.4 to 6.0 ± 1.8 h. This change caused an insignificant increase in the calculated metabolism rate constants (6%).

The presence of an oxy-helium atmosphere at high pressure resulted in a response quite unlike that of the 500-atm control culture (Fig. 2). There was an average 2.5-fold loss in viable cells in the initial period of incubation, paralleled by a similar loss in the total cells, indicating that cell lysis had occurred. After 10 h, however, the culture grew exponentially (average k, 0.31 ± 0.06 h^{-1}; average generation time, 3.2 ± 0.6 h) at a rate threefold more rapid than that of the hydrostatic pressure control culture. The growth and metabolism rate constants for the helium culture (Table 1) were calculated by using the above-described nonlinear regression techniques. Because the culture was in an apparent state of imbalanced growth for the first 10 h, only data after this period were analyzed. The variables t_m, No_m, and TC_m were employed for computation, in which $t_m = t - 10$ h; No_m = the viable count at $t = 10$ h (1.5×10^5 cells per ml); and TC_m = (TC at $t > 10$ h) − (TC at $t = 10$ h). The growth rate constant agreed within 13% of that determined from population measurements. The value of R_{incorp} for the helium culture was intermediate between those found for the 1- and 500-atm control cultures. Potential increases in k and R_{incorp} (12, 18) during the 15-min decompression period would have affected the metabolism data by less than 0.5%.

It is interesting to note that at zero time the direct and viable cell counts in the helium culture were very close to those observed with the 500-atm control culture. The zero-time subsamples were taken 20 min after compression when the culture was approximately 50% equilibrated with the high-pressure atmosphere. It was not until approximately 1 h later, when equilibrium was essentially complete, that the full effects of the atmosphere became manifest. The observed loss of cells was most likely due to the helium carrier gas and not to oxygen. The solubility of oxygen in compressed aqueous systems is known (25a), and the concentration of oxygen in all of the cultures was well controlled. The initial oxygen concentrations were 222 ± 5, 222 ± 5, and 227 ± 10 μM for the 1-atm, 500-atm, and 500-atm-helium cultures, respectively. These concentrations of oxygen have not been shown to be toxic to biological systems (11), even at elevated hydrostatic pressure (32). Oxygen was not limiting at any time during the experiment.

Previous experience with this hyperbaric system has revealed that dissolution of helium from the subsample upon rapid decompression (2 min) resulted in the destruction of cells. Although increasing the decompression time to 15 min significantly reduced cell loss, it is likely that these effects have not been eliminated completely. It is, in fact, not unreasonable to suggest that the observed response of the helium culture may in part be due to decompressive losses of a fraction of the cells. The metabolism data from the helium culture, however, do not support this as the major reason. Assume, for example, that (i) helium had no detrimental effects upon the incubating culture and growth proceeded exponentially with a generation time of 2.8 h (initial population, 2.2×10^5 viable cells per ml; Fig. 2), and (ii) subsampling resulted in a constant 2.5-fold loss in cells. The metabolism rate constant presented in Table 1 was calculated under the assumption that No_m was representative of the cell population within the incubating culture. If, however, the true value of No_m were 2.5-fold higher than that observed, the determined value of R_{incorp} would have to be reduced to 0.38 fmol per viable cell in the culture per h. Substitution of the above parameters into equation 1 should provide an estimate of the amount of trichloroacetic acid-precipitable cell material which would be measured if outgassing were the sole detrimental effect of helium. It should be noted that approximately 50% of the trichloroacetic acid-precipitable cell material released from destroyed cells is retained by the filter (determined from an independent series of experiments). This, however, has no influence upon the present argument because this systematic effect, irrespective of the degree to which it occurs, would be automatically included in the calculation of the metabolism constant. The resulting predicted curve is indicated by the dashed line at the bottom of Fig. 2. The expected amount of trichloroacetic acid-precipitable cell material was sevenfold greater than that observed, indicating that under the present experimental conditions the helium initially interfered with the functioning of the culture. The observed detrimental effects were apparently temporary because upon continued incubation there was a definite and significant enhancement of growth of EP-4 relative to the 500-atm control culture.

Although it is not possible at the present time to unequivocally explain the above results, one

of the two following mechanisms is most likely responsible.

It can be supposed, for instance, that once equilibrated with the oxy-helium atmosphere, approximately 85% of the cells were killed, a portion of which possibly lysed. If it were concluded that the closed-circle data point near 5 h (Fig. 2) was erroneously high, the trend of the viable count data of the helium culture would lend support to this contention. The loss in microscopically observable cells, as well as the increased experimental uncertainty in the viable count data, could result in part or in whole from lysis during decompression. The observed viable population would therefore be systematically low. Against a background of approximately 1×10^5 to 1.5×10^5 mostly nonviable cells per ml, the increase in cells resulting from growth of the remaining viable fraction would not be detectable before approximately 10 h. The response of the culture could alternatively be explained as follows. The initial presence of a high-pressure oxy-helium atmosphere temporarily suspended growth and, therefore, significant incorporation of glutamate into cell polymers. Viability, however, was for the most part retained. As incubation continued, the culture underwent an adaptive change and began to grow at the observed rate after approximately 10 h. Again cell loss from decompression would be invoked. A combination of the above effects is of course possible. To establish the mechanism which is operative, a line of investigation is currently being pursued in which the ability of EP-4 to form colonies under a high-pressure oxy-helium atmosphere will be compared with agar shake cultures incubated under hydrostatic pressure. The effects of outgassing upon decompression will be eliminated as a variable and thus make possible an assessment of whether helium at high pressure initially results in the nonviability of a large fraction of the cells.

The enhanced rate of growth of the helium cultures relative to that which was observed in the pressurized controls agrees in trend with the recent observations of Marguis et al. (20). When comparisons were made with air-grown and hydrostatic pressure control cultures, they observed small but significant increases in the growth rates and cell yields of *Streptococcus faecalis, Staphylococcus aureus,* and *Escherichia coli* when incubated in the presence of 20 to 40 atm of helium (partial pressure of oxygen, 0.2 atm). They did not experience any initial detrimental affects of helium. It is premature at this time to suggest a mechanism for the enhanced growth of cultures incubated under elevated partial pressures of helium.

In summary, it must be concluded that helium is not physiologically neutral as a means of compressing bacteria to pressures as high as 500 atm. Separate from potential effects of decompression, initial exposure of EP-4 to a high-pressure oxy-helium atmosphere resulted in either loss of viability or the arrest of growth for nearly 10 h. The effects of the gas, however, are not entirely detrimental, as the growth rate and the rate of incorporation of substrate into cell polymers is ultimately several-fold enhanced relative to hydrostatic pressure control cultures.

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