JOURNAL OF BACTERIOLOGY, Aug. 1980, p. 841-846
0021-9193/80/08-0841/06$02.00/0

Vol. 143, No. 2

Rupture of the Cell Envelope by Induced Intracellular Gas Phase Expansion in Gas Vacuolate Bacteria

BARBARA B. HEMMINGSEN[1]* AND EDVARD A. HEMMINGSEN[2]

Department of Microbiology, San Diego State University, San Diego, California 92182[1]; and The Physiological Research Laboratory, Scripps Institution of Oceanography, La Jolla, California 92093[2]

Using a new approach, we estimated the physical strength of the cell envelopes of three species of gram-negative, gas vacuolate bacteria (*Microcyclus aquaticus*, *Prosthecomicrobium pneumaticum*, and *Meniscus glaucopis*). Populations of cells were slowly (0.5 to 2.9 h) saturated with argon, nitrogen, or helium to final pressures up to 100 atm (10, 132 kPa). The gas phases of the vesicles remained intact and, upon rapid (1 to 2 s) decompression to atmospheric pressure, expanded and ruptured the cells; loss of colony-forming units was used as an index of rupture. Because the cell envelope is the cellular component most likely to resist the expanding intracellular gas phase, its strength can be estimated from the minimum gas pressures that produce rupture. The viable counts indicated that these minimum pressures were between 25 and 50 atm; the majority of the cell envelopes were ruptured at pressures between 50 and 100 atm. Cells in which the gas vesicles were collapsed and the gas phases were effectively dissolved by rapid compression tolerated decompression from much higher gas saturations. Cells that do not normally possess gas vesicles (*Escherichia coli*) or that had been prevented from forming them by addition of L-lysine to the medium (*M. aquaticus*) were not harmed by decompression from gas saturation pressures up to 300 atm.

In contrast to the extensive amount of information available on the chemistry, biosynthesis, structure, and function of the cell walls and envelopes of bacteria, relatively little is known about the physical characteristics of these important cellular constituents. The elasticity of the envelope was demonstrated by Wámoscher (22) during micro-dissection of bacteria and by Isaac and Ware (11), who stretched gram-negative bacteria two to three times their original length without rupture; upon release of the tension, the cells returned to their original size and shape. The elastic cell envelope allows some swelling and contraction when cells are exposed to media of differing osmotic pressures (14), but it is sufficiently rigid to be able to maintain the shape of the cell against surface tension forces that tend to produce a spherical shape in nonrigid objects the size of bacteria (15); when the cell envelope is wholly or partially removed, very often cells that are normally rod-shaped become spherical (1, 24).

The cell envelope is strong enough to resist rupture or even deformation by turgor pressures of several atmospheres in gram-negative bacteria (14, 19, 20) and up to about 30 atm (3,040 kPa) in gram-positive bacteria (14, 16). Most species can withstand hypotonic conditions without lysis, but should the cell envelope be removed or weakened, the cells usually swell and burst if the osmotic stress is applied rapidly (1, 2).

Although some minimum cell envelope strengths can be inferred from such observations, no attempt appears to have been made to directly determine the strength of the cell envelope in the absence of osmotic stress. It has been suggested that rupture somehow can be produced in bacteria and other microorganisms by exposing the cells to gas supersaturations (e.g., references 4 and 5), but it has been demonstrated (6, 9) that this is not feasible due to the difficulty or impossibility of nucleating the required gas phase within the cells.

However, an intracellular gas phase is present in those species of gram-negative bacteria capable of forming highly refractile, hollow, cytoplasmic inclusions bounded by rigid nonunit membranes that are freely permeable to gases (21). These gas vesicles might make it feasible to introduce large pressures within the cells. In the present study we explored this possibility by subjecting three species of gas vacuolate bacteria to various gas saturation and decompression conditions for the purpose of inducing rupture of the cells. Rupture was assessed by determining the loss of viable cells in the experimental population after decompression. The limited amount of fine structural and biochemical data available (10, 13, 18) suggests that the three

species of interest have a fairly typical gram-negative cell envelope. Such envelopes usually consist, from the inside out, of the cytoplasmic membrane; a thin layer of peptidoglycan, the bag-shaped macromolecule that encloses the cell and is generally thought to confer rigidity and strength to the cell envelope (15, 17, 23); and an outer membrane composed of protein, phospholipid, and lipopolysaccharide in varying amounts (3). Inasmuch as the cell envelope most likely provides the major resistance to intracellular gas phase expansion during decompression, its approximate strength can be related to the minimum gas pressure required for rupture.

MATERIALS AND METHODS

Bacteria. The two species of obligately aerobic, heterotrophic gas vacuolate bacteria, *Prosthecomicrobium pneumaticum* (ATCC 23633) and *Microcyclus aquaticus* (ATCC 27068), were obtained from the American Type Culture Collection, Rockville, Md. *M. aquaticus* was maintained at 30°C in the Casamino Acids medium recommended by the American Type Culture Collection; *P. pneumaticum* was maintained at 25°C in the defined medium recommended by the same source. Both were transferred about every 4 weeks. Cultures 3 to 5 or 5 to 7 days old, respectively, incubated in stationary tubes containing 8 ml of the recommended medium, were used in each experiment; the cells floating near the surface in a dense layer were removed by gentle suction and diluted approximately 1:3 in sterile distilled water. From 95 to 99% of the cells in the population had gas vesicles, as judged by their bright appearance in a phase-contrast microscope. Viable counts were performed by spreading small amounts of serial dilutions in sterile distilled water on the surface of the appropriate liquid medium solidified with 1.2% (wt/vol) Noble agar. Plates spread with *P. pneumaticum* were incubated at 25°C for 10 to 20 days before colonies were counted; colonies of *M. aquaticus* were counted after 3 to 4 days of incubation at 30°C.

The rod-shaped strain of *Meniscus glaucopis*, an aerotolerant, anaerobic, heterotrophic, gas vacuolate bacterium, was supplied by R. L. Irgens and was maintained on basal growth medium that had been autoclaved with 1% $CaCO_3$ and 1.2% Noble agar (both wt/vol) (10); after cooling, and before the plates were poured, filter-sterilized vitamin B_{12} was added to a final concentration of 1 μg/liter (Irgens, personal communication), and an autoclaved stock solution of maltose was added to a final concentration of 0.3%. Stock cultures, streaked on plates of this medium, were kept in a GasPak 100 Anaerobic System (BBL Microbiology Systems, Cockeysville, Md.) at 30°C and transferred every 4 to 6 weeks. Experimental suspensions were prepared by suspending several colonies from 5- to 8-day-old streak plates in liquid basal growth medium; usually about two-thirds of the cells in such suspensions contained gas vesicles. Dilutions for viable count determinations were carried out in liquid basal growth medium. The spread plates were incubated at 30°C in an anaerobic incubator filled with N_2 and CO_2 (approximately 89 and 11%, respectively) for 7 to 9 days.

For experiments lasting more than 0.5 h, the experimental suspensions of all three species were prepared in 4.5-ml amounts; 0.5 ml was removed for viable count determinations, and then 2.0 ml was pressurized and 2.0 ml was stirred at the same speed at atmospheric pressure. Viable counts were performed on each at the end of the experiment. All microscopic observations were made with a phase-contrast microscope at × 1,000.

The species without gas vacuoles, *Escherichia coli*, was obtained and handled as described previously (6). For certain experiments, cells of *M. aquaticus* without gas vesicles were obtained by growing the cells in 100 ml of a defined medium which contained 50 μg of L-lysine per ml (12); the culture was incubated in a 250-ml Erlenmeyer flask on a reciprocal shaker (about 100 strokes per min) at 30°C for 2 days. No gas vacuoles could be seen in the cells. Konopka (12) has shown that under these growth conditions no gas vesicle protein can be detected by immunological methods. The culture was diluted with distilled water and otherwise treated as described above.

Experimental procedure. The experimental apparatus and procedures have been described elsewhere (6, 9). Briefly, 2 ml of the bacterial suspension was placed in a 10-ml glass dish contained within a 12-ml stainless steel chamber. The chamber was closed and flushed with argon, nitrogen, or helium of 99.99% or better purity. After flushing for 1 to 2 min, the gas was admitted to the equilibration pressure either rapidly, within 40 s (fast compression), or slowly, in a stepwise fashion with an 8-min pause between each step for equilibration (slow compression). This latter procedure was designed to preserve the gas phase within the cytoplasm of gas vacuolate bacteria, or any potential nucleation site within the cytoplasm of non-gas vacuolate bacteria, that otherwise might be dissolved by the rapid application of hydrostatic pressure without accompanying high dissolved-gas tensions. The slow compression was carried out in steps as follows: for equilibration pressures up to 50 atm, the pressure steps ranged from 1.3 to 8 atm; for pressures from 50 to 100 atm and 100 to 200 atm, the steps ranged from 9 to 15 and 15 to 30 atm, respectively. Commercial tank pressures of about 150 atm were boosted to the required levels by compressing the gas in an accumulator by means of a hydraulic water pump. All experiments were carried out at room temperature.

The suspensions were equilibrated at full pressure for 20 to 30 min by using a magnetic stirrer at 250 rpm. Separate experiments have shown that this is about twice as long as is needed for complete equilibration of water samples of equivalent volumes. After equilibration, the gas in the chamber was discharged rapidly, and complete decompression was obtained within 1 to 2 s (fast decompression); the exhaust gas was trapped by a bed of cotton to prevent contamination of the laboratory. In some experiments, the gas was discharged slowly in steps (slow decompression) by reversing the slow compression sequence; these latter experiments required about 5 h. After decompression was complete, samples of the suspension in the chamber and of the control suspension were taken for microscopic observation and for viable count determinations.

RESULTS

Populations of gas vacuolate cells that were slowly saturated with argon and rapidly decompressed experienced little loss of viable cells when the saturation pressures were 25 atm or less (Fig. 1, open bars); with *M. aquaticus* and *M. glaucopis*, even 50 atm did not yield a clear effect. With higher argon saturations, many cells of all three species were killed, and with 100 atm the survival was less than 40%. Concurrent with this decrease in colony-forming units was the appearance of phase-light, cell-shaped objects among the phase-dark cells that had lost their gas vacuoles. The characteristics of these two different cell types were not further examined, nor were their relative numbers estimated.

When the cell populations were compressed rapidly rather than slowly, the decompression generally caused considerably less killing (Fig. 1, solid bars). Even after decompression from 100-atm saturation, the majority of the cells survived; these cells lacked phase-bright gas vacuoles but otherwise appeared normal, and only a few phase-light, cell-shaped objects were present. Somewhat more killing was observed with *P. pneumaticum* possibly because the prosthecae characteristic of this species (18) were damaged by external bubbles that formed in the suspending fluid during fast decompression from the higher gas saturations.

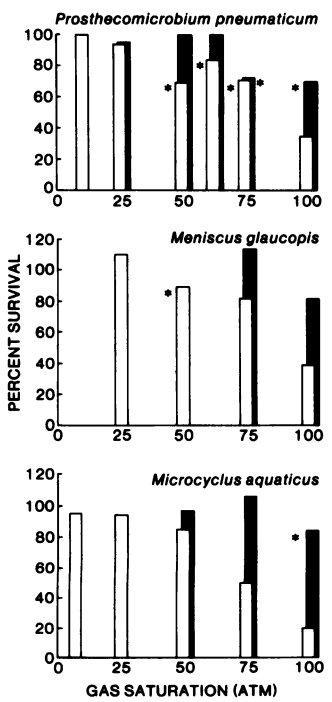

FIG. 1. *Effects on the survival of three species of gas vacuolate bacteria after fast decompression of gas-saturated cell suspensions. The suspensions were either compressed slowly (open bars) or rapidly (solid bars) to the full gas saturation pressure. (*) represents the mean of two separate experiments.*

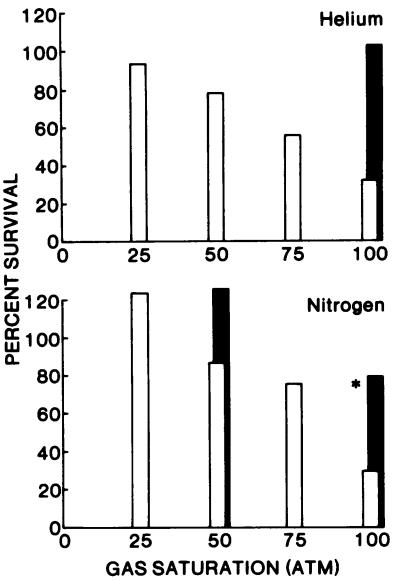

FIG. 2. *Effects on the survival of the gas vacuolate bacterium, M. aquaticus, after fast decompression of cell suspensions saturated with either nitrogen or helium. The suspensions were either compressed slowly (open bars) or rapidly (solid bars) to the full gas saturation pressure. (*) represents the mean of three separate experiments.*

The effects of gases other than argon were tested on *M. aquaticus* (Fig. 2). Although nitrogen and in particular helium vary substantially from argon in their physical properties, the patterns of decrease in viable cells were generally the same as for argon. However, fast compression and saturation with helium to 100 atm had no detectable effect.

Bacteria that are incapable of forming gas vesicles (*E. coli*) or that can be prevented from producing them (*M. aquaticus* grown in defined medium with lysine) were not affected after either slow or fast compression to an argon saturation pressure of 100 atm followed by fast decompression. Cells of *M. aquaticus* that were grown in the same defined medium without lysine produced gas vesicles as expected, and, after either slow or fast compression, they were nearly as sensitive to decompression from 100 atm of argon as cells grown in the Casamino Acids medium. Cells that lacked gas vesicles before the experiments were unaffected even by rapid decompression from 300 atm of argon (Table 1); in contrast, only 3 to 5% of gas vacuolate cells of *M. aquaticus* and *P. pneumaticum* survived, and large numbers of phase-light, cell-shaped

TABLE 1. *Fast compression of bacteria with and without gas vacuoles to a gas saturation pressure of 300 atm of argon, followed by fast decompression*

Species	Gas vacuoles	Colony-forming units per ml		% Survival
		Before compression	After decompression	
E. coli	Absent	2.3×10^7	3.0×10^7	130
M. aquaticus	Absent[a]	3.8×10^8	3.6×10^8	95
	Present[b]	1.5×10^8	0.4×10^7	3
	Present[c]	4.3×10^8	2.0×10^7	5
P. pneumaticum	Present	4.6×10^7	1.6×10^6	3.5
M. glaucopis	Present	9.5×10^7	2.9×10^7	30.5

[a] Cells were grown in defined medium with 50 μg of L-lysine per ml (12).

[b] Cells were grown in the same defined medium without added lysine; more than 99% had gas vacuoles.

[c] Cells were grown in the Casamino Acids medium; more than 98% had gas vacuoles.

objects were observed. The effect was less pronounced with *M. glaucopis*, probably because such a large percentage of cells in the initial suspensions did not have gas vacuoles as judged by their appearance in the phase-contrast microscope.

Slow stepwise compression of *M. aquaticus* and *M. glaucopis* to 200 atm of argon, followed by slow decompression to atmospheric pressure, resulted in little or no killing, indicating that hydrostatic pressure and gas exposure per se had no effect. However, in a suspension of *P. pneumaticum*, 16% of the cells were killed and the survivors required approximately twice the usual time to form colonies; thus, the long-term exposure of this species to experimental factors other than gas phase expansion may have had some adverse effects.

Experiments were carried out to estimate the resistance of the gas vesicle membrane to explosion by an expanding gas phase. Suspensions of cells of *M. aquaticus* and of *P. pneumaticum* that had been subjected to slow compression with argon using stepwise increases well below the critical threshold for gas vesicle collapse were rapidly decompressed. Loss of phase brightness was taken as evidence of gas vesicle explosion. Our preliminary results indicated that the critical threshold for explosion of the vesicles in vivo was no more than about 12 and 25 atm, respectively, whereas the pressures needed to collapse the vesicles are roughly 10 and 20 atm lower (13, 20).

DISCUSSION

Bacteria that do not form gas vesicles or that are prevented from forming them were remarkably resistant to gas supersaturations induced by fast decompression from gas saturation pressures of up to 300 atm (Table 1; 6). Even though such gas supersaturations lead to profuse, spontaneous nucleation of bubbles in aqueous solutions (7, 8), the cells were not seriously affected, as shown by their subsequent ability to form colonies. The absence of killing suggests that cytoplasm is stable and resists gas phase nucleation even at such extreme gas supersaturations.

Bacteria that do form gas vesicles were affected markedly by fast decompression from considerably lower gas saturation pressures (Fig. 1). This strongly suggests that the stepwise gas saturation did maintain, as intended, the gas phases of the vesicles. Although the pressure increases for each step in the beginning stages were kept below the value for inward collapse of the gas vesicle membrane, estimated as 5 to 6 atm for *P. pneumaticum* (20) and as 2 atm for *M. aquaticus* (13), collapse probably occurred later on when larger pressure steps were utilized to avoid excessive equilibration times. But the gas contents of the vesicles then were sufficiently high to prevent their dissolution. Under such circumstances, their stabilization may be aided by a decreased surface tension from gradual aggregation of polar molecules around the gas phase or, alternatively, by molecular "shells" or "skins" (25).

Once an intracellular gas phase has been stabilized and has gained a substantial gas pressure, the sudden removal of the hydrostatic pressure by rapid decompression will create a large pressure difference between the inside and outside of the cell as the pressure of the internal gas phase is transmitted hydraulically throughout the cell. These pressure differences had few serious consequences for any of the gas vacuolate species examined when decompressions were carried out from saturation pressures up to about 50 atm. However, when the pressures were higher, many of the cells in each population were killed by the outward force of the expanding internal gas phase, which presumably ruptured the cells.

A similar pattern of results was obtained when various helium or nitrogen supersaturations were induced in gas vacuolate cells of the representative species, *M. aquaticus* (Fig. 2, open bars). Argon, helium, and nitrogen represent a wide range of solubilities and diffusivities. Thus, the decompression time of 1 to 2 s employed in the present study appears to be fast enough so that even the small helium atoms must take part in the initial expansion of the intracellular gas phase rather than immediately rushing out of the cells.

Whereas slow stepwise compression maintained the intracellular gas phase, rapid expo-

sure of gas vacuolate bacteria to the hydrostatic pressures accompanying the gas saturation pressures used in the present study would collapse the vesicles at once (13, 19, 20) and would dissolve the internal gas phases. Yet some killing occurred upon fast decompression from an argon saturation pressure of 100 atm (Fig. 1, solid bars) and considerable killing from 300 atm (Table 1). These observations suggest that under conditions in which gas vesicles are collapsed immediately during compression, intracellular gas phases are also formed, provided the gas supersaturations are sufficiently high. These gas phases may originate from traces of gas trapped within the remnants of the vesicle membrane, or the remnants alone may serve as nucleation sites. Whatever the mechanism, gas phase initiation after fast compression differs fundamentally from the situation that exists during slow compression, in which an actual gas phase is maintained as a physical entity throughout the entire compression-decompression procedure.

A value may be placed on the strength of the cell envelope, the cellular component which most likely withstands the outwardly directed pressure of the expanding gas phase, by assuming that the percentage of decrease in colony-forming units in the cell suspensions corresponds to the percentage of cells that are ruptured. Neglecting the minute (in the present context) interfacial tensions acting on the cell envelope, the external pressure upon complete decompression will be zero (gauge pressure), and the internal pressure will be equal to the pressure in the intracellular gas phase minus the inward pressure of the cytoplasmic surface tension that would tend to oppose the expansion. The cytoplasmic surface tension appears to be relatively unimportant in withstanding the gas phase expansion, as was indicated by the ease with which dissolution of the gas phase was avoided during the slow compression process. The presence of a gas vesicle membrane around the gas phase would not matter provided its strength in resisting explosion is less than that of the cell envelope, as our observations indicated was the case. The net force acting per unit area of the cell envelope will be approximatly equal to the pressure of the intracellular gas phase, which in turn is equal to the gas tension in the cytoplasm and the extracellular medium as defined by the equilibration (gauge) pressure. Thus, by taking the lowest predecompression gas saturation pressure that produces killing upon decompression as the threshold for rupture, the cell envelopes of the three gram-negative bacteria examined are strong enough, as a minimum, to withstand internal pressures between 25 to 50 atm, but most rupture between 50 and 100 atm. The fact that a considerable fraction of the cells survived after 100-atm saturation could be due to their possession of very strong cell envelopes; more likely such cells lost their gas phases during the slow compression or, as in the case of a certain percentage of the population of *M. glaucopis*, they lacked gas vesicles at the beginning of the experiments.

We believe that it will be feasible to refine the present approach for determining the strength of the cell envelope and to relate the values obtained, for example, to the age of the cells. However, before attempting to do so, more information should be gained on the state of the vesicles as the cells undergo compression to establish optimum schedules for maintenance of the internal gas phase. Although the contribution of turgor pressure would not interfere in a major way with the estimates of cell envelope strength, corrections of several atmospheres may be necessary since turgor pressures of gram-negative bacteria have been placed in the range of 1 to 6 atm (14, 19, 20). Future investigations utilizing the present experimental approach should also address the intriguing possibility that, under some conditions, part or most of the cytoplasm is expelled from the ruptured cells, thereby allowing the mostly intact envelopes to be separated from cytoplasmic constituents.

ACKNOWLEDGMENTS

We thank R. L. Irgens, Southwest Missouri State University, for kindly providing the culture of *M. glaucopis* and for suggestions that were helpful in its cultivation.

This work was supported in part by a Faculty-Grant-in-Aid of Research to B.B.H. and by Public Health Service grant HL-16855 from the National Institutes of Health to E.A.H.

LITERATURE CITED

1. **Brenner, S., F. A. Dark, P. Gerhardt, M. H. Jeynes, O. Kandler, E. Kellenberger, E. Klieneberger-Nobel, K. McQuillen, M. Rubio-Huertos, M. R. J. Salton, R. E. Strange, J. Tomcsik, and C. Weibull.** 1958. Bacterial protoplasts. Nature (London) **181**:1713–1715.
2. **Corner, T. R., and R. E. Marquis.** 1969. Why do bacterial protoplasts burst in hypotonic solutions? Biochim. Biophys. Acta **183**:544–558.
3. **DiRienzo, J. M., K. Nakamura, and M. Inouye.** 1978. The outer membrane proteins of gram-negative bacteria: biosynthesis, assembly, and functions. Annu. Rev. Biochem. **47**:481–532.
4. **Foster, J. W., R. M. Cowan, and T. A. Maag.** 1962. Rupture of bacteria by explosive decompression. J. Bacteriol. **83**:330–334.
5. **Fraser, D.** 1951. Bursting bacteria by release of gas pressure. Nature (London) **167**:33–34.
6. **Hemmingsen, B. B., and E. A. Hemmingsen.** 1978. Tolerance of bacteria to extreme gas supersaturations. Biochem. Biophys. Res. Commun. **85**:1379–1384.
7. **Hemmingsen, E. A.** 1977. Spontaneous formation of bubbles in gas-supersaturated water. Nature (London) **267**:141–142.
8. **Hemmingsen, E. A.** 1978. Effects of surfactants and electrolytes on the nucleation of bubbles in gas-supersaturated solutions. Z. Naturforsch. **33a**:164–171.
9. **Hemmingsen, E. A., and B. B. Hemmingsen.** 1979.

Lack of intracellular bubble formation in microorganisms at very high gas supersaturations. J. Appl. Physiol. Respir. Environ. Exercise Physiol. **47**:1270–1277.

10. **Irgens, R. L.** 1977. *Meniscus*, a new genus of aerotolerant, gas-vacuolated bacteria. Int. J. Syst. Bacteriol. **27**:38–43.
11. **Isaac, L., and G. C. Ware.** 1974. The flexibility of bacterial cell walls. J. Appl. Bacteriol. **37**:335–339.
12. **Konopka, A. E.** 1977. Inhibition of gas vesicle production in *Microcyclus aquaticus* by L-lysine. Can. J. Microbiol. **23**:363–368.
13. **Konopka, A. E., J. C. Lara, and J. T. Staley.** 1977. Isolation and characterization of gas vesicles from *Microcyclus aquaticus*. Arch. Microbiol. **112**:133–140.
14. **Mitchell, P., and J. Moyle.** 1956. Osmotic function and structure in bacteria. Symp. Soc. Gen. Microbiol. **6**: 150–180.
15. **Rogers, H. J.** 1979. Biogenesis of the wall in bacterial morphogenesis. Adv. Microb. Physiol. **19**:1–62.
16. **Rose, A. H.** 1976. Osmotic stress and microbial survival. Symp. Soc. Gen. Microbiol. **26**:155–182.
17. **Salton, M. R. J.** 1964. The bacterial cell wall. Elsevier Publishing Co., Amsterdam.
18. **Staley, J. T.** 1968. *Prosthecomicrobium* and *Ancalomicrobium*: new prosthecate freshwater bacteria. J. Bacteriol. **95**:1921–1942.
19. **Walsby, A. E.** 1971. The pressure relationships of gas vacuoles. Proc. R. Soc. London Ser. B **178**:301–326.
20. **Walsby, A. E.** 1976. The buoyancy-providing role of gas vacuoles in an aerobic bacterium. Arch. Microbiol. **109**: 135–142.
21. **Walsby, A. E.** 1978. The gas vesicles of aquatic prokaryotes. Symp. Soc. Gen. Microbiol. **28**:327–357.
22. **Wámoscher, L.** 1930. Versuche über die Struktur der Bakterienzelle. Z. Hyg. Infektionskr. **111**:422–460.
23. **Weidel, W., and H. Pelzer.** 1964. Bagshaped macromolecules—a new outlook on bacterial cell walls. Adv. Enzymol. **26**:193–232.
24. **Weiss, R. L.** 1976. Protoplast formation in *Escherichia coli*. J. Bacteriol. **128**:668–670.
25. **Yount, D. E., T. D. Kunkle, J. S. D'Arrigo, F. W. Ingle, C. M. Yeung, and E. L. Beckman.** 1977. Stabilization of gas cavitation nuclei by surface-active compounds. Aviation Space Environ. Med. **48**:185–191.