JOURNAL OF BACTERIOLOGY, June 1975, p. 1301-1309
Vol. 122, No. 3

Printed in U.S.A.

Gas Vesicle Assembly in *Microcyclus aquaticus*

A. E. KONOPKA,* J. T. STALEY, AND J. C. LARA
Department of Microbiology, University of Washington, Seattle, Washington 98195

Received for publication 6 March 1975

When observed in the electron microscope intact gas vesicles appeared as transparent areas in whole cells of *Microcyclus aquaticus*, whereas vesicles collapsed by centrifugation were not discernible. Within 5 min of suspending cells containing collapsed vesicles in growth medium, small transparent vesicles were detected. By 15 min the average number of vesicles per cell was 15. This number remained relatively constant while the size of the vesicles increased until they attained their maximum diameter of 100 nm. At this time the vesicles, interpreted as biconical structures, began to elongate presumably due to the synthesis of the cylindrical midsection. Closely correlated with the time at which vesicles began to elongate was the initiation of smaller vesicles which resulted in a doubling of the number of vesicles per cell by 90 min. This evidence coupled with the isolation of a mutant which assembles only the conical portions of the vesicle suggests that assembly occurs in two distinct stages subject to genetic mutation. Protein and ribonucleic acid synthesis, and presumably adenosine triphosphate formation, were required for gas vesicle assembly. In addition, inhibition of protein or ribonucleic acid synthesis resulted in a loss of extant gas vesicles. Over the time course of our study, deoxyribonucleic acid synthesis was not required for gas vesicle assembly or stability.

Gas vacuoles are highly refractile, gas-containing cytoplasmic inclusions found in certain prokaryotic organisms. Representatives from a wide variety of prokaryotic groups including blue-green, purple, and green photosynthetic bacteria as well as heterotrophic bacteria contain this structure. Almost all gas vacuolate organisms are found in aquatic habitats and are nonmotile. Because the gas vacuole provides buoyancy to cells, its primary function may be to enable organisms to regulate their vertical position in a water column (16), although other functions have also been attributed to it (3, 13).

The vacuoles observed by light microscopy can be resolved by electron microscopy into a number of smaller structures called gas vesicles. These organelles have the shape of cylinders with conical ends and are composed of a single-layered membrane 2 nm wide. Chemical analyses indicate that the major, and perhaps sole, component of the vesicle membrane is protein (1, 4, 5). The protein occurs in subunits that are arranged in a linear pattern to form ribs running at right angles to the long axis of the vesicle. The membrane excludes cytoplasm from the interior but is freely permeable to gases so that the composition and pressure of the gas within the vesicle is the same as the ambient atmosphere (14). Thus, the structure is not supported by a positive gas pressure from within, but is rigid; it can, however, be collapsed when sufficient hydrostatic pressure is applied (15).

The aim of this investigation was to study the kinetics of gas vesicle formation and the effect of various inhibitors of protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) synthesis on this process. We chose to use the heterotrophic bacterium *Microcyclus aquaticus* for these studies because the gas vesicles of this organism can be readily observed in intact, whole cells by using the electron microscope. Therefore, in contrast to the blue-green bacteria, one can enumerate and measure the vesicles in individual cells.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. All strains were derived from *Microcyclus aquaticus* strain M, ATCC 27068. Strain S1 contains gas vacuoles under all cultural conditions (11). Cultures were routinely grown in CAGV medium, which contains 1 g of glucose, 1 g of vitamin-free Casamino Acids (Difco), 20 ml of modified Hutner's salt solution, and 10 ml of a vitamin solution per liter of medium (10). All cultures were incubated at 30 C on a shaker water bath, where S1 had a doubling time of 270 min. Medium DM contained 20 ml of modified Hutner's salt solution, 10 ml of vitamin solution, 1 g of glucose, 0.025 g of $(NH_4)_2SO_4$, and 0.07 g of Na_2HPO_4 per liter of culture.

Electron microscopy of cell-free vesicles. Cells

growing on CAGV agar plates were treated with 1 mg of ampicillin per plate (60 ml of medium per plate) for 2.5 h at 30 C. The cells were scraped from the plates into 0.01 M tris(hydroxymethyl)aminomethane, 0.02 M ethylenediaminetetraacetic acid, pH 8.1, and 1 mg of lysozyme per ml was added. After incubation at 37 C for 30 min, Triton X-100 was added to a final concentration of 1%, and the suspension was incubated for 50 min at 37 C. Samples of this lysate were stained with 1% uranyl acetate, and a drop was dried onto a Formvar-coated copper gird (200 mesh) and examined in a JEM-100B electron microscope at 60 kV.

Quantitation of gas vacuolation. Samples (1 ml) were fixed with iodine solution (5 g of I_2 and 10 g of KI in 100 ml of water) at a final concentration of 0.5% (vol/vol) and stored at 4 C until they were examined. For electron microscopic examination, a drop of the sample was dried onto a Formvar-coated copper grid and examined as above. Electron micrographs were taken of representative fields and examined by using a dissecting microscope. Individual gas vesicles were measured by using an ocular micrometer. Between 30 and 100 cells were examined to determine the average number of gas vesicles per cell, and 50 to 100 individual gas vesicles were measured to compute the mean length and width of the gas vesicles in a sample.

The surface area of a gas vesicle was determined by using the following formulae, assuming that the conical portions of the gas vesicles were right circular cones. The surface area (SA) of the conical portions of the vesicle is given by $SA = 2\pi(W/2)S$, where $S = [(W/2)^2 + (L/2)^2]^{1/2}$; W is the width of the vesicle and L is the length. The SA of the cylindrical midpiece was calculated by using the following formula, $SA = 2\pi(W/2)(L\text{-}100)$. ($L$-100) is the length of the cylindrical midpiece, since the length of the conical portion in these vesicles was constant and equal to 100 nm. Values are given in square nanometers.

RNA and DNA synthesis. Nucleic acid synthesis was followed by labeling with [^{3}H]adenine (New England Nuclear, Boston, Mass.), 27.4 Ci/mmol. Strain S2, an adenine auxotroph, was grown in CAGV plus 10 μg of adenine per ml to a density of 10^8 cells/ml and [^{3}H]adenine was added to a final specific activity of 0.25 Ci/mmol. To determine total nucleic acid incorporation, 0.1-ml samples were added to 2 ml of cold 10% trichloroacetic acid and put on ice for 1 h. A drop of carrier DNA (herring sperm) was added, and the samples were filtered onto a 0.22-μm (pore size) membrane filter (25 mm in diameter; Millipore) presoaked in 5% trichloroacetic acid plus 50 μg of adenine per ml. The filters were washed two times with 5% trichloroacetic acid plus adenine, once with 1 N KOH, once with 95% ethanol, and finally with 5% trichloroacetic acid plus adenine, dried, and counted in Liquifluor (New England Nuclear, Boston, Mass.). Samples were counted in a Beckman LS-100 scintillation counter.

Incorporation into DNA was determined by taking a 0.2-ml sample, adding 0.1 ml of 0.2 M ethylenediaminetetraacetic acid and 0.1 ml of lysozyme (10 mg/ml). This was placed on ice for 15 min; 0.6 ml of 1.2% Triton X-100 and 0.06 ml of Pronase (1 mg/ml) were added, and the sample was incubated at 37 C for 60 min. One milliliter of 1 N KOH was added, and the sample was incubated overnight at 37 C, after which it was neutralized with 1 N HCl, precipitated with an equal volume of cold 20% trichloroacetic acid, and placed on ice for 60 min. The sample was filtered, washed, and counted as described for total incorporation samples. Incorporation into RNA was calculated as the difference between total incorporation and DNA incorporation.

Mutant isolation. Cells were grown in DM broth to a density of 10^8 cells/ml, 10 ml was added to a glass petri dish, and the sample was irradiated for 90 s with a Hanovia 8341 25-watt ultraviolet light source at a distance of 58 mm while rotating the dish 120 rpm. This period of irradiation caused 99% killing, and the survivors were plated and incubated on CAGV medium in the dark.

RESULTS

Structure of gas vesicles. Negatively stained preparations of gas vesicles from *M. aquaticus* revealed that they have the same morphological features typical of gas vesicles of other prokaryotes (Fig. 1), appearing as cylinders with conical endpieces. Vesicles are occasionally somewhat distorted when observed in whole cell preparations (Fig. 2), but we have resorted to the examination of vesicles in whole cells because it is the most direct method of estimating (i) the number of vesicles per individual cell and (ii) the size (i.e., surface area) of vesicles in a cell.

Kinetics of gas vesicle assembly. When cells of S1 are growing exponentially in CAGV medium, each cell contains approximately 75 vesicles measuring 250 by 100 nm. To study the assembly process, vesicles in such cells were collapsed by centrifugation at 12,000 $\times$ g for 10 min at 4 C, and gas vesicle formation was followed upon resuspension in CAGV at 30 C (the rate of formation of vesicles was the same whether cells were centrifuged at 4 or 25 C, and cell growth resumed at the same rate, i.e., 0.22 doublings per h, after suspension). Immediately upon resuspension (time zero) whole cells examined in the electron microscope were devoid of vesicles indicating that collapsed vesicles are not detectable by this procedure. Vesicles were seen in about half the cells after 5 min and by 10 min nearly all cells contained them. At 15 min, all cells contained vesicles but the number of vesicles was less than the 75 seen per cell before collapse, and ranged from 14 to 34 in 12 different experiments.

Initially the gas vesicles appeared as small units 33 nm long (Fig. 2A). Between 15 and 65 min the numbers per cell remained relatively constant, but the size increased to a maximum

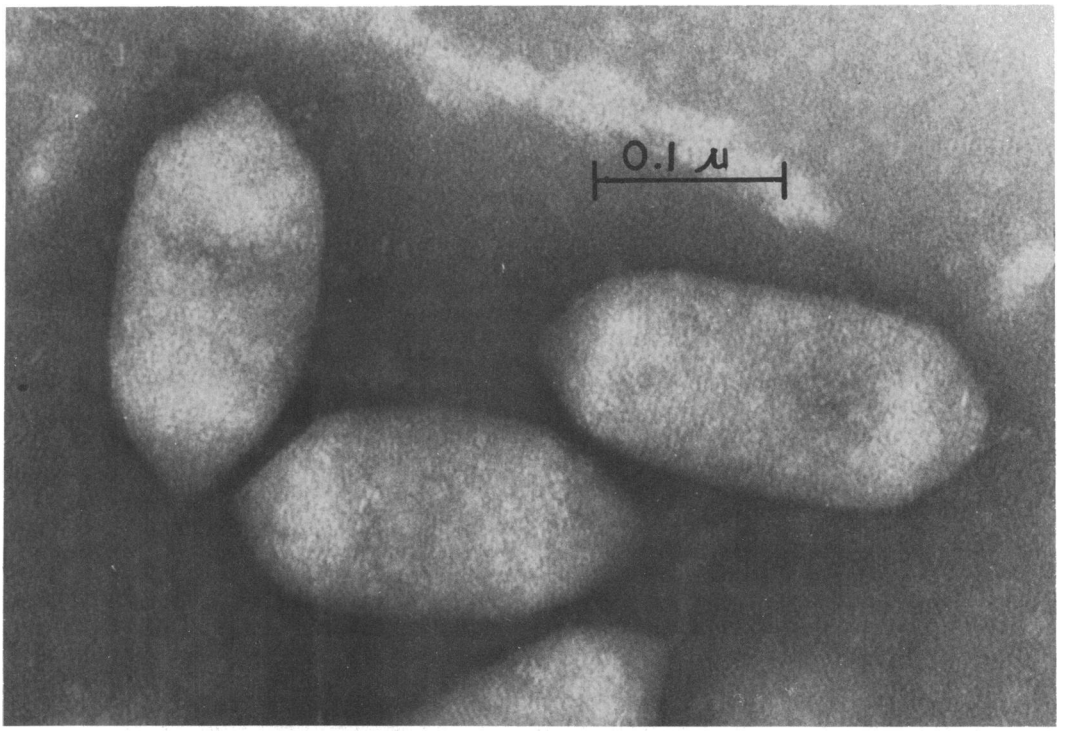

FIG. 1. *Cell-free gas vesicles of strain S1, stained with uranyl acetate (×305,000).*

diameter of 100 nm. At subsequent time intervals, (Fig. 2C and D) elongated vesicles (i.e., with cylindrical midsections) were found. The maximum length was normally 250 nm, although occasionally vesicles as long as 325 nm were observed.

To quantitate the rate of assembly of gas vesicles, estimates were made of the surface area of vesicle membrane at various time intervals. To accomplish this the vesicles were assumed to be biconical structures when their length was less than 100 nm and cylinders with conical endpieces when their length was greater (see formulae in Materials and Methods). This definition was based upon measurements of the conical endpieces of gas vesicles that also contained cylindrical midpieces and also from the gas vesicles of strain S8, in which the gas vesicles lack cylindrical midpieces. The surface area should be directly proportional to the number of protein subunits in a vesicle. When the average surface area of an individual vesicle was plotted versus time after collapse, a linear relationship was obtained after a 10-min lag (Fig. 3).

Though the number of gas vesicles per cell was relatively constant at 15 from 15 to 50 min after collapse, at 90 min (Fig. 2C) there were a number of small vesicles in addition to the expected complement that had begun to elongate. These small vesicles are most probably the result of new initiations rather than the degradation of existing vesicles since the average number of vesicles per cell doubled between 50 and 90 min (Fig. 4). Because a gas vesicle cannot be seen until at least 5 min after its initiation, the exact time that assembly of a new vesicle began was estimated from its dimensions assuming that new vesicles were assembled at the same rate as those initiated at time zero. From these data the rate of initiation of gas vesicles was calculated (Table 1). The rate of initiation was low and relatively constant from 15 to 65 min and then increased sixfold after 65 min, the time at which the vesicles reached their maximum width and had begun to elongate.

Effect of inhibitors on RNA and DNA synthesis. The ability of rifampin, mitomycin C, and nalidixic acid to inhibit DNA or RNA synthesis in *M. aquaticus* was investigated by following incorporation of [^{3}H]adenine into these macromolecules in strain S2, an adenine auxotroph. Rifampin (25 μg/ml) inhibited RNA

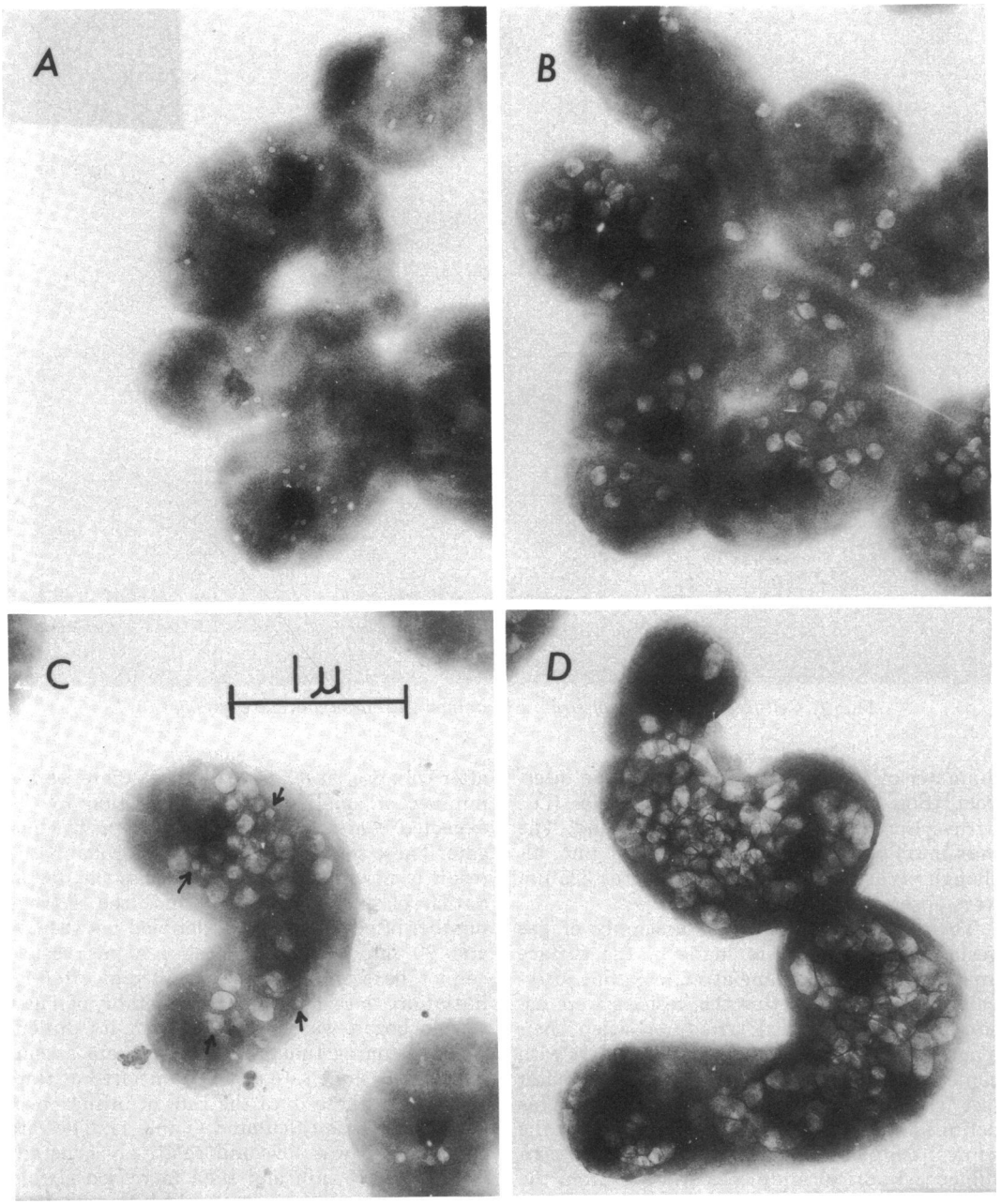

FIG. 2. *Assembly of gas vesicles in strain S1. Cells were centrifuged to collapse existing gas vesicles and the reappearance of vesicles was monitored by using electron microscopy of whole-cell preparations. Typical cells are shown at (A) 15, (B) 60, (C) 90, and (D) 200 min after resuspension. Note that when cells have numerous vesicles the vesicles become overlapped (D). The arrows in (C) designate some of the newly initiated vesicles.*

synthesis 95% 20 min after addition. Of the inhibitors tested, only mitomycin C inhibited DNA synthesis specifically. At a concentration of 25 μg/ml, it reduced the rate of DNA synthesis by 87% after 15 min, although the rate of RNA synthesis was not affected for 90 min.

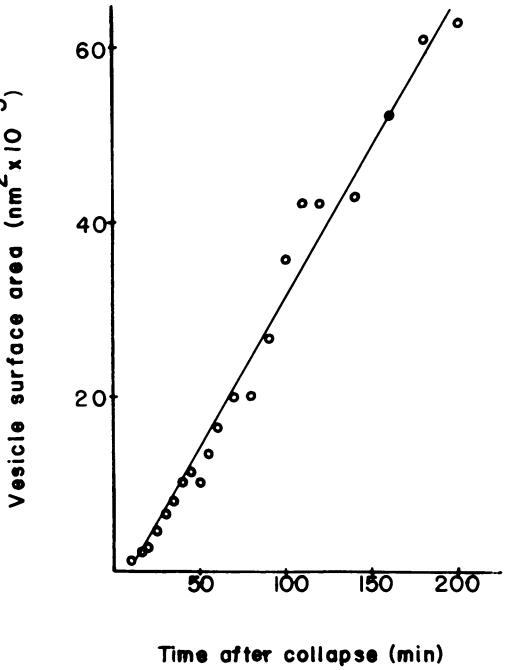

FIG. 3. *Increase in the average surface area of a gas vesicle initiated at time zero during their assembly. Cells of S1 were centrifuged to collapse existing gas vesicles, and the reappearance of vesicles was monitored by using electron microscopy. The length of each gas vesicle was determined from measurement in electron micrographs. Time zero is the moment when cells whose vesicles had been collapsed were resuspended in fresh CAGV. The surface area of the vesicles was calculated from formulae as described in text. Confidence limits (95%) were calculated for the 20-, 55-, 90-, 120-, and 160-min samples. The limits were ± 4 to 6% of the sample mean.*

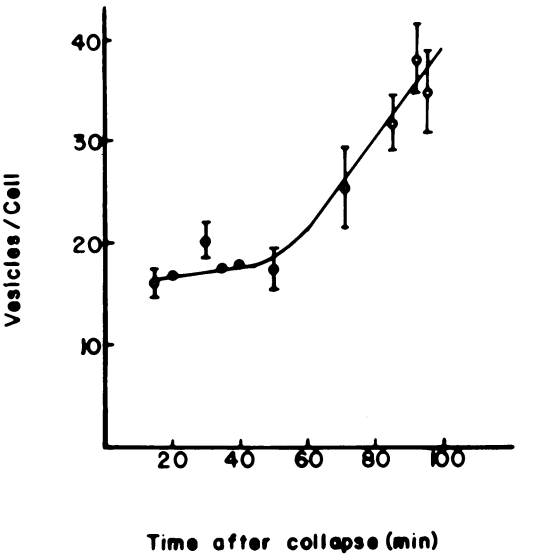

FIG. 4. *The average number of gas vesicles reassembled per cell after destruction of existing vesicles. Cells of S1 were centrifuged and suspended in CAGV (time zero), and the appearance of gas vesicles was monitored by electron microscopy. The bars indicate 95% confidence limits.*

TABLE 1. *Rate of initiation of gas vesicles in S1*[a]

Time interval (min)	No. initiated per min/cell[b]
15–65	0.14
65–80	0.81

[a] Cells were centrifuged to collapse existing vesicles and suspended in CAGV medium, and electron micrographs of samples taken during incubation were examined.

[b] This value was obtained by tabulating the number of vesicles initiated before (15 to 65 min) and after (65 to 80 min) the conical endpieces initiated at zero time were completed. This number was divided by the number of cells examined to obtain the rate. The values represent the average from eight samples from each of two experiments.

Mitomycin C was also bactericidal at this concentration (Table 2). Nalidixic acid (50 μg/ml) inhibited RNA synthesis by 94% after 20 min and also reduced the rate of DNA synthesis.

Inhibition of gas vesicle assembly. The kinetics of gas vesicle assembly were studied in the presence of several inhibitors. The experimental design was similar to that described above, except that after centrifugation the cells were resuspended in CAGV with one of the following additions: 100 μg of chloramphenicol, 25 μg of rifampin, or 25 μg of mitomycin C per ml, or 0.01 M KCN; the control had no additions. Figure 5 shows the results of this study. Inhibition of DNA synthesis by mitomycin C did not affect gas vesicle assembly over a 13-h period. In contrast, KCN totally inhibited gas vesicle assembly. The effects of chloramphenicol (or streptomycin) and rifampin (or nalidixic acid) on gas vesicle assembly were more complex. Inhibition of protein or RNA synthesis affected the rate of assembly of gas vesicles; the degree of inhibition was greater with chloramphenicol than rifampin (77 versus 38%). Chloramphenicol also caused a large reduction in the number of gas vesicles initiated in a cell, whereas in rifampin-treated cultures a normal complement of gas vesicles was initiated, but

TABLE 2. *Effect of mitomycin C on viability and cell number of M. aquaticus*

Time (min)[a]	Count	
	Viable[b]	Direct[c]
0	3.4×10^8	3.5×10^8
15	4.0×10^6	3.5×10^8
180	1.2×10^6	4.0×10^8
360	8.7×10^5	4.2×10^8

[a] Minutes after addition of 25 μg of mitomycin C per ml.

[b] Determined by duplicate plate counts on CAGV agar.

[c] Determined by microscopic count by using a Petroff-Hauser counting chamber.

the number of gas vesicles per cell decreased with time. Thus, when the data were analyzed by computing the total area of gas vesicle membrane/cell (*SA* × number/cell), rifampin inhibits gas vesicle assembly by 84% and chloramphenicol by 98%. When these inhibitors were added to cells containing gas vacuoles, the vacuoles remained intact in the presence of KCN or mitomycin C, but were completely lost in chloramphenicol-treated cultures, and partially lost in the presence of rifampin. Twelve hours after the addition of chloramphenicol, 85% of the cells had fewer than 15 gas vesicles per cell whereas before the drug was added, the average number per cell was 75. If 25 μg of mitomycin C per ml was added in addition to 100 μg of chloramphenicol per ml, gas-vacuolate cells lost vesicles more slowly than in cultures treated with chloramphenicol alone.

Gas vesicle mutant that lacks the cylindrical midpiece. Gas vacuolate cells produce opaque colonies on solid media because of light scattering by the gas vacuoles. After ultraviolet irradiation, *M. aquaticus* was grown on CAGV plates, and the colonies were examined for changes in opacity. In this manner, strain S8, which only assembles the conical endpieces, was isolated (Fig. 6). We have examined several hundred cells of S8 by electron microscopy and have never seen gas vesicles with cylindrical midpieces.

DISCUSSION

The formation of gas vesicles in *M. aquaticus* appears to occur in two distinct stages. Initially gas vesicles are biconical structures and retain this form until they measure 100 by 100 nm. The second stage of vesicle assembly involves the formation of the cylindrial midpiece, resulting ultimately in a structure measuring 250 by 100 nm.

Centrifugation of gas vacuolate cells results in the presence of a large number of collapsed vesicle membranes, whose fate during the reformation of gas vesicles must be resolved. Reinflation of collapsed gas vesicles in *Anabaena flos-aquae* has been ruled out by studies of the membrane's permeability to gas and the rigid nature of the vesicles (14). Reinflation would require that the membrane be gas-tight while the vesicle was being inflated, yet the vesicles of

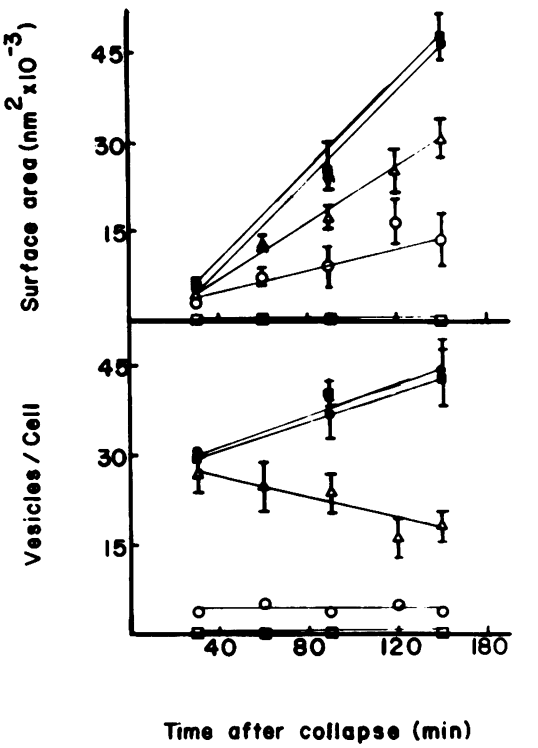

FIG. 5. *Effect of inhibitors on gas vesicle assembly in S1. Exponentially growing cells of S1 were centrifuged to collapse existing gas vesicles and suspended in CAGV with additions of* (○) *100 μg of chloramphenicol per ml,* (△) *25 μg of rifampin per ml,* (●) *25 μg of mitomycin C per ml, or* (□) *0.01 M KCN; a control culture was incubated in CAGV with no additions* (■). *The number of gas vesicles per cell and their dimensions were determined from electron micrographs of samples taken during subsequent incubation. The average surface area of an individual vesicle was computed as described in the text. The bars indicate 95% confidence limits. If the limits are not shown, they are smaller than the values encompassed by the plotted point.*

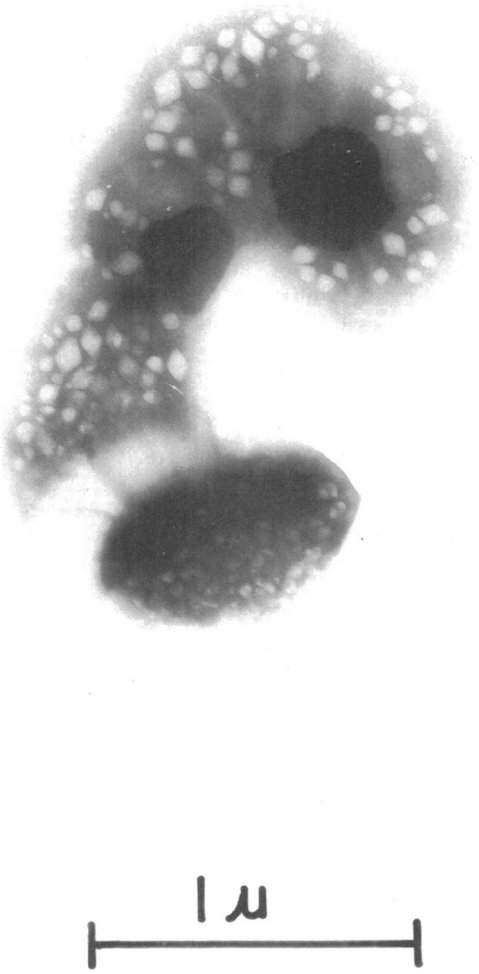

FIG. 6. *Gas vesicles of strain S8. Only the conical endpieces of the vesicles are assembled; compare these vesicles to the largest vesicles seen in Fig. 2D, which are completed vesicles containing a cylindrical midsection.*

A. flos-aquae were permeable to gas both entering and leaving the vesicle. We assume that the vesicles of *M. aquaticus* are similarly permeable to gas. The collapsed vesicles could be a source of protein subunits for assembly of new vesicles, although de novo protein synthesis is required for gas vesicle reformation and the simplest explanation for this requirement is that the subunit protein must be synthesized.

Though there were about 75 vesicles per cell in *M. aquaticus* S1 prior to collapse, the number initially formed soon after suspension in growth medium ranged from 14 to 34 in a series of experiments. This indicates that exponentially growing cells of this strain have fewer sites for initiation than the number of extant vesicles.

The average number of vesicles per cell remained relatively constant from 15 to 50 min after collapse. Then from 50 to 90 min the number of gas vesicles doubled. These new initiations occurred well before the extant vesicles reached their maximum length. Indeed, our evidence indicates that the new initiations began at the same time that the original vesicles attained their maximum diameter, i.e., at a time when they began to synthesize the cylindrical midsection. Therefore, these data indicate that the synthesis of the conical endpieces and the cylindrical midsection are more distinct than previously considered (7, 8, 12). That the two processes have different requirements for assembly is also supported by the isolation of S8 which only assembles the conical endpieces.

The two stages might differ in having (i) different protein subunits in the conical and cylindrical pieces (a hypothesis which is not supported by chemical studies of the vesicle protein from several organisms [1, 4, 5]), (ii) unique enzymes involved in the assembly of protein subunits, or (iii) distinct enzymes that modify the protein subunits to produce different conformations for the cones and cylinders. Although any one of the hypotheses is compatible with the isolation of a gas vesicle mutant that only assembles the conical endpieces, the correlation of new vesicle initiation with completion of stage 1 is most likely in the case of (ii). With regard to (iii) it is noteworthy that the vesicle protein of *Halobacterium halobium* has been reported to contain tightly bound phosphate and galactose (5), which might alter the conformation of the vesicle subunits just as glycogen phosphorylase of mammalian cells and glutamine synthetase of *Escherichia coli* can be modified by low-molecular-weight compounds (9).

The rate of assembly of a gas vesicle was constant after 10 min. This constant rate of assembly was not anticipated for two reasons. First, we have presented evidence that gas vesicle assembly consists of two distinct processes, and there was no a priori reason to believe that these stages would proceed at the same rate. Also, there was an increase in the number of gas vesicles per cell from 15 to 40 during the time of the experiments. One would expect the assembly rate of a gas vesicle to decrease when the number of assembly sites doubled if the

availability of subunits limited the rate of assembly. Since the rate was constant, either the availability of subunits did not limit assembly or the subunits were produced at an increased rate when the number of vesicles increased.

Gas vesicle assembly can be prevented by inhibiting protein and RNA synthesis and presumably by interference with adenosine triphosphate formation (by KCN), but assembly is not affected by inhibition of DNA synthesis. Since protein is the major, if not sole, constituent of gas vesicle membranes, it is not surprising that protein synthesis is necessary for gas vesicle assembly. Apparently de novo protein synthesis is required to reform gas vesicles in cells after collapse of existing gas vesicles, since treating cultures with chloramphenicol resulted in a 98% decrease in gas vesicle formation. The newly synthesized proteins may be the vesicle subunits, or they might be other proteins required for assembly, since the collapsed vesicles could be a source of subunits. Inhibition of RNA synthesis affects gas vesicle assembly since protein synthesis ceases when no mRNA is made. In the presence of these inhibitors, some gas vesicle assembly does occur. This could be explained by (i) the utilization of the subunits of collapsed vesicles to a very limited extent, (ii) the existence of a pool of vesicle subunits in the cell, or (iii) incomplete inhibition of protein synthesis. Note that the effects of rifampin and chloramphenicol on the number of vesicles initiated after centrifugation were quite different. Rifampin-treated cultures contained nearly a normal complement of gas vesicles after 30 min, although incorporation of [^{3}H]adenine into RNA showed that RNA synthesis was inhibited by 95% after 20 min. Apparently the protein synthesis that did occur during this period was sufficient to allow normal vesicle initiation. The decrease in vesicle numbers in rifampin-treated cultures could result from vesicle degradation or dilution of the number per cell by cell division. In either case, there is no increase in vesicle numbers, although individual vesicles increased in size. Energy generation has also been shown to be necessary for gas vesicle assembly in *H. halobium*, in that 2,4-dinitrophenol and anaerobic conditions prevented reappearance of gas vacuoles in cells whose vacuoles had been destroyed (6).

Treatment of gas-vacuolate cells with chloramphenicol or rifampin caused degradation of intact gas vesicles. Thus, the gas vesicle is not a static structure but appears to exist in a state of dynamic equilibrium requiring continued protein synthesis to maintain its integrity. Our data suggest that degradation of gas vesicles involves the reverse reaction of assembly, since the average size of extant gas vesicles decreased after treatment with chloramphenicol. Mitomycin C should also promote degradation of gas vesicles since it is reported to cause breakdown of DNA (2), and thus RNA and protein synthesis must cease after a period of time in mitomycin-treated cultures. Gas vesicles should then be degraded in these cultures. This effect was not seen and, in fact, addition of mitomycin decreased the ability of chloramphenicol to cause gas vesicle degradation. Thus, in addition to preventing DNA synthesis, mitomycin may have a secondary effect in that it prevents degradation of existing vesicles.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM-00511 from the National Institute of General Medical Sciences and National Science Foundation grant GB-30313.

LITERATURE CITED

1. Buckland, B., and A. E. Walsby. 1971. A study of the strength and stability of gas vesicles isolated from a blue-green alga. Arch. Mikrobiol. **79**:327-337.
2. Gale, E. F., E. Cundcliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring. 1972. The molecular basis of antibiotic action, p. 246-252. John Wiley & Sons, Ltd., New York.
3. Houwink, A. L. 1956. Flagella, gas vacuoles, and cell wall structure in *Halobacterium halobium*; an electron microscopic study. J. Gen. Microbiol. **15**:146-150.
4. Jones, D. D., and M. Jost. 1970. Isolation and chemical characterization of gas vacuole membranes from *Microcystis aeruginosa* Kuetz. emend. Elenkin. Arch. Mikrobiol. **70**:43-64.
5. Krantz, M. J., and C. E. Ballou. 1973. Analysis of *Halobacterium halobium* gas vesicles. J. Bacteriol. **114**:1058-1067.
6. Larsen, H., S. Omang, and H. Steensland. 1967. On the gas vacuoles of the halobacteria. Arch. Mikrobiol. **59**:197-203.
7. Lehman, H. 1972. Kinetik des Aufbaus einer Proteinmembran. Untersuchungen an Gasvakuolen des blaugrünen Alge *Microcystis aeruginosa*. Ber. Detsch. Bot. Ges. **84**:615-627.
8. Lehmann, H., and M. Jost. 1971. Kinetics of the assembly of gas vacuoles in the blue-green alga *Microcystis aeruginosa* Kuetz. emend. Elenkin. Arch. Mikrobiol. **79**:59-68.
9. Stadtman, E. R. 1970. Mechanisms of enzyme regulation in metabolism, p. 409-415. *In* P. D. Boyer (ed.), The enzymes, vol. 1. Academic Press Inc., New York.
10. Staley, J. T. 1968. *Prosthecomicrobium* and *Ancalomicrobium:* new prosthecate freshwater bacteria. J. Bacteriol. **95**:1921-1942.
11. Van Ert, M., and J. T. Staley. 1971. Gas-vacuolated strains of *Microcyclus aquaticus*. J. Bacteriol. **108**:236-240.
12. Waaland, J. R., and D. Branton. 1969. Gas vacuole development in a blue-green alga. Science **163**:1339-1341.

13. Waaland, J. R., S. R. Waaland, and D. Branton. 1970. Gas vacuoles. Light shielding in blue-green algae. J. Cell Biol. **48**:212–215.
14. Walsby, A. E. 1969. The permeability of the blue-green algal gas vacuole membrane to gas. Proc. R. Soc. Ser. B **173**:235–255.
15. Walsby, A. E. 1971. The pressure relationships of gas vacuoles. Proc. R. Soc. Ser. B **178**:301–326.
16. Walsby, A. E. 1972. Structure and function of gas vacuoles. Bacteriol. Rev. **36**:1–32.