Growth of an Enveloped Mycoplasmavirus and Establishment of a Carrier State

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The growth of an enveloped DNA-containing mycoplasmavirus (MVL2 obtained from R. N. Gourlay) has been studied, by using the indicator host *Acholeplasma laidlawii* strain JA1. From virus one-step growth curves, artificial lysis experiments, and infected cell growth curves, it was found that virus infection is nonlytic. Newly infected cells grow slower and are osmotically more stable than uninfected cells. However, 4 to 6 h after infection, the cells reach a carrier state in which cell growth rate and osmotic fragility are indistinguishable from uninfected cells. Carrier cultures contain free virus. Every carrier culture cell gives rise to either a clone of carrier cells or a clone of MVL2-resistant cells.

Three enveloped mycoplasmavirus isolates have been reported (5, 6, 9). These are DNA viruses (7, 8) and have been designated group 2 mycoplasmaviruses (13). The other two groups of mycoplasmaviruses do not contain lipids: group 1 viruses are bullet-shaped particles, and group 3 viruses are polyhedral particles with short tails (reviewed by Maniloff and Liss, 13). Electron micrographs of negatively stained group 2 viruses show particles that are roughly spherical with an average diameter of 80 nm (7, 10; J. Maniloff, J. Das, and R. M. Putzrath, *In* K. Maramorosch, A. J. Dalton, and F. Haguenau, ed., *Insect and Plant Viruses: an Atlas*, in press).

Like the other two mycoplasmavirus groups, group 2 viruses form plaques on some strains of the mycoplasma *Acholeplasma laidlawii*. It has been shown that each plaque results from an infection by one group 2 virus (13); hence, viruses can be assayed as PFU.

In this paper, we present studies on the nature of the growth of MVL2, a group 2 mycoplasmavirus. These show that viral infection is not lytic, and the resulting cells can be propagated as a carrier culture.

MATERIALS AND METHODS

Cells and viruses. *A. laidlawii* strain JA1 was used as the indicator host and for virus propagation. This mycoplasma strain has been described by Liss and Maniloff (12).

MVL2 is the original group 2 mycoplasmavirus isolate (5) and was obtained from R. N. Gourlay.

Cells were assayed as colony-forming units (CFU) on tryptose agar plates, and viruses were assayed as PFU on lawns of *A. laidlawii* JA1. A lawn was made by spreading 1 ml of an overnight (late-logarithmic-growth phase) JA1 culture, which had been diluted 1:1 with tryptose broth, on a 10-cm petri dish containing tryptose agar. The petri dish was then dried for 15 to 30 min at 42°C. A 0.5-ml virus sample was spread on the lawn, and the plate was incubated overnight at 37°C. Plaques could then be easily seen; however, to facilitate counting, lawns were stained as described by Quinlan et al. (15).

To obtain a high-titer virus stock, 0.5 ml of a virus suspension (about 10^7 PFU/ml) was plated on a JA1 lawn and incubated overnight at 37°C. The plate was then overlaid with 9 ml of tryptose broth and kept at room temperature. After 24 to 48 h, the liquid was decanted and filtered through a 0.22-μm membrane filter (Millipore) to remove cells. The filtrate was used as the virus stock and contained about 10^{10} PFU/ml.

Media and buffers. Tryptose broth and agar plates (containing 1% glucose and 1% serum fraction) were used for cultivating cells, as described previously (11). Tris-NaCl buffer contained 0.05 M Tris and 0.15 M NaCl, adjusted to pH 7.4. Tris-EDTA buffer was 0.01 M Tris and 0.001 M EDTA, adjusted to pH 7.5.

Virus adsorption. MVL2 virus was added to an overnight JA1 culture (late-logarithmic-growth phase), and, after mixing, a 0.5-ml sample was taken and plated for CFU and PFU to determine the multiplicity of infection (MOI). The culture was then incubated in a 37°C water bath. Two methods were used to measure unabsorbed viruses. (i) At 15-min intervals, a 4-ml sample was removed and centrifuged for 10 min at 12,000 × g at 4 to 6°C to pellet cells. The supernatant was assayed for PFU. (ii) At 15-min intervals, a 0.5-ml sample was removed and diluted 1,000-fold in Tris-EDTA buffer to lyse the cells. After 30 min at room temperature, the lysate was assayed for PFU. Similar adsorption rate constants were measured using both methods.

First-order rate constants were calculated from a least-squares analysis of the data by the formula $d \ln N/dt = -kC$, where N is the number of unadsorbed viruses at time t, C is the cell concentration, and k is the rate constant.

"**One-step growth.**" The one-step growth experiments were based on the protocol of Delbrück (2). Viruses and cells were mixed, and 0.5 ml was removed and assayed for PFU and CFU to determine the MOI. The culture was then incubated at 37°C for 30 min to allow virus adsorption. To reduce the amount of unadsorbed virus, the culture was centrifuged for 10 min at 12,000 × g at 4 to 6°C, and the cell pellet was suspended in fresh tryptose broth. The culture was diluted 10^5-fold in tryptose broth, to eliminate possible reinfection by progeny virus, and incubated at 37°C. At 15-min intervals, samples were removed and assayed for PFU to measure progeny virus production.

Artificial lysis. The experiments were based on those of Doermann (3). Viruses and cells were mixed, and 0.5 ml was removed and assayed for PFU and CFU to determine the MOI. The culture was incubated for 30 min to allow virus adsorption. To reduce the amount of unadsorbed virus, the culture was centrifuged at 7,700 × g for 10 min at 4°C. The cell pellet was suspended in fresh medium, and the culture was incubated at 37°C. At 15-min intervals, two samples were removed. One sample was diluted in medium and assayed for PFU and CFU. The other sample was treated to lyse the cells; this sample was diluted 1,000-fold in Tris-NaCl buffer, frozen (in liquid nitrogen) and thawed three times, and then assayed for PFU and CFU.

RESULTS

Preliminary considerations. For some of these studies, it was necessary to be able to lyse the cells of an infected mycoplasma culture without inactivating mature MVL2 viruses. The *A. laidlawii* cells are bounded only by a cell membrane (1), and thin-section electron micrographs show that MVL2 viruses are surrounded by a triple-layered envelope (7). Both have similar sensitivities to detergents and organics solvents; hence, these reagents cannot be used to inactivate cells without also inactivating viruses. Therefore, the sensitivity of cells and viruses to osmotic shock and freeze-thaw treatment was investigated (Table 1). Viruses were stable in Tris-EDTA and Tris-NaCl buffers, but a loss in titer was observed after freeze-thaw treatment. Cells were osmotically fragile in Tris-EDTA, but not in Tris-NaCl buffer. Freeze-thaw treatment in either buffer lysed the cells. It was found that cells are not lysed by freeze-thaw treatment in tryptose medium.

From these data (Table 1), Tris-EDTA treatment appeared to be the method of choice for cell lysis, because it lysed cells while causing no inactivation of viruses. However, as described below, cells become less osmotically fragile during the virus-growth-curve rise period. Therefore, in artificial lysis experiments, freeze-thaw treatment in Tris-NaCl buffer was used because (i) cell lysis by this procedure was found to be unaffected by viral growth, and (ii) the treatment inactivated substantially fewer viruses than cells.

Virus adsorption. After mixing viruses and cells at several cell densities and MOI values, adsorption was followed by assaying unadsorbed virus as a function of time. The first-order adsorption rate constants from three experiments are listed in Table 2. In subsequent virus growth experiments, a 30-min adsorption period was used, since this time allows adequate virus adsorption, but is significantly less than the virus latent period observed in the one-step growth experiments described below.

"**One-step growth.**" The one-step virus growth protocol allows the general features of the virus infection to be examined for both lytic and nonlytic viruses. Typical MVL2 one-step growth curves are shown in Fig. 1 and 2. In 17 experiments, with MOI values from 0.4 to 90, there was a latent period of about 120 min

TABLE 1. *Survival of cells and viruses in buffers*

Buffer treatment	Fraction survival		
	A. laidlawii cells	MVL2 virus	Carrier culture cells
None	1.00	1.00	1.00
Tris-EDTA[a]	5.3×10^{-5}	0.97	3.1×10^{-5}
Tris-EDTA + freeze-thaw[b]	$<10^{-4}$	0.09	$<10^{-4}$
Tris-NaCl[a]	0.95	1.03	0.72
Tris-NaCl + freeze-thaw[b]	8.0×10^{-3}	0.26	3.4×10^{-3}

[a] Samples were diluted 1,000-fold into buffer and held 10 min at room temperature before assaying for CFU or PFU. Tris-EDTA buffer was 0.01 M Tris, 0.001 M EDTA (pH 7.5), and Tris-NaCl buffer was 0.05 M Tris, 0.15 M NaCl (pH 7.4).
[b] Samples in buffer were frozen and thawed three times before assaying for CFU or PFU.

TABLE 2. *Adsorption rate constants for MVL2*

Experiment	Cells (CFU/ml)	MOI	k (cm³/min)
1[a]	2.0×10^8	2.4	1.6×10^{-10}
2[a]	6.1×10^7	1.1	4.4×10^{-10}
3[b]	3.4×10^8	0.002	1.1×10^{-10}
Theoretical[c]			2.0×10^{-9}

[a] Unadsorbed viruses were assayed after cells were removed by centrifugation.
[b] Unadsorbed viruses were assayed after cells were osmotically lysed.
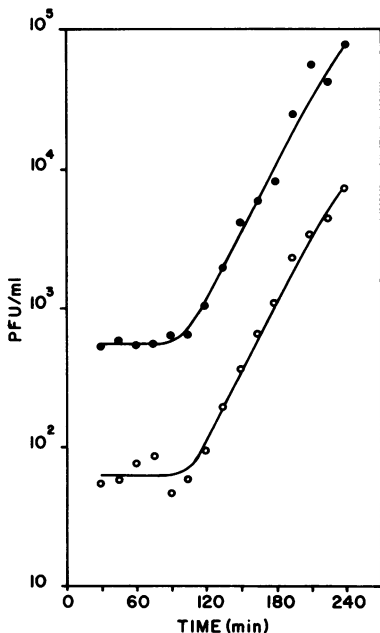[c] Calculation of theoretical rate constant described in Discussion.

FIG. 1. One-step growth of MVL2. Cells were infected at an MOI of either 6 (●) or 0.4 (○), and virus growth was measured as described in the text.

(mean ± standard deviation of 118 ± 29 min). This was followed by an exponential release of progeny virus, lasting 4 to 6 h. During this rise period, about 1,000 PFU were released per infected cell (Fig. 2). It should be noted that the approximate 6-h time between virus adsorption and the end of the rise period corresponds to less than two cell-generation times (as will be discussed below). About 6 h after the start of infection, the rate of progeny virus appearance decreases (Fig. 2). This state of slow virus release persists, and the further study of these carrier cell cultures will be considered below.

Artificial lysis. The gradual release of virus observed in one-step growth experiments suggested that virus infection might be a nonlytic process. Such a nonlytic infection cycle would imply the lack of a pool of intracellular mature viruses. The possible existence of intracellular progeny viruses during the viral latent period was examined by artificially lysing infected cells. For these experiments, during the virus growth curve, each time a sample was removed for PFU assay a parallel sample was removed, diluted in Tris-NaCl buffer, frozen and thawed, and assayed for PFU. Hence, the unlysed sample measured the number of infected cells plus the number of extracellular viruses, and the lysed sample measured the number of intracellular viruses plus the number of extracellular

viruses. To verify cell lysis throughout the experiments, all samples were also assayed for CFU.

Figure 3 shows a typical artificial lysis experiment. During the latent period, the number of PFU in the lysed sample was due to the background of free, unadsorbed virus. In the rise period, the number of PFU in the lysed sample increased to equal the number of PFU in the unlysed sample, reflecting the release of progeny viruses. These experiments are consistent with the interpretations that there is no intracellular pool of completed virus and that virus maturation and release are coincident. Moreover, virus growth was not accompanied by a loss in cell titer.

Osmotic fragility of infected cells. One of the methods of cell lysis investigated for artificial lysis experiments was osmotic shock. Although uninfected cells are osmotically fragile and lyse when diluted 1,000-fold into Tris-EDTA buffer (see Table 1), it was found that infected cells were several orders of magnitude less fragile; hence, osmotic shock could not be used in artificial lysis experiments.

To examine cell osmotic fragility as a function of virus growth, the artificial lysis experiment was repeated at high MOI (to assure that every cell was infected), with the sample taken for cell lysis at each time being osmotically shocked by diluting 1,000-fold in Tris-EDTA buffer (Fig. 4). During the first 3 h of virus growth, there is at most a doubling in the cell titer. However, the cells become progressively
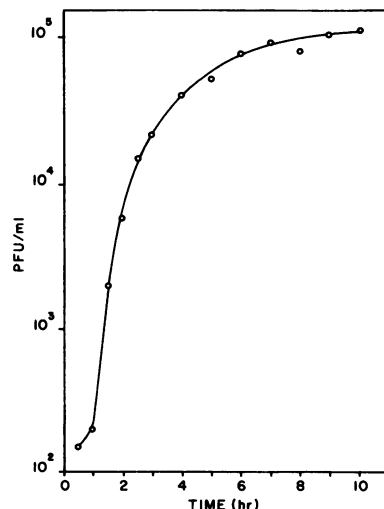

FIG. 2. One-step growth of MVL2 leading to establishment of a carrier culture. Cells were infected at an MOI of 8, and virus growth was measured.
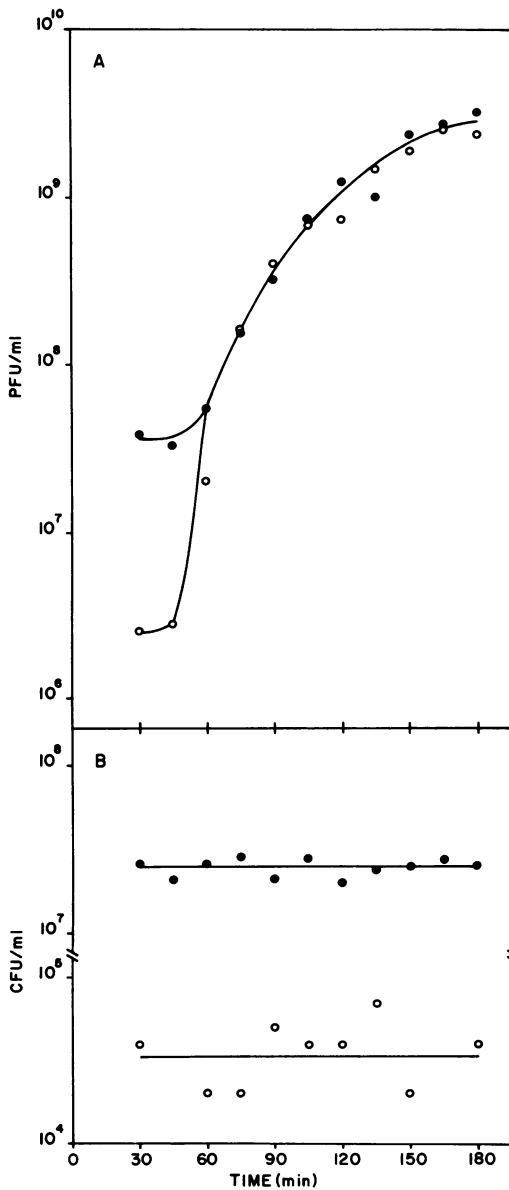
FIG. 3. *Artificial lysis of MVL2-infected cells. At each time during the virus growth curve (MOI = 4), two samples were removed. One sample was assayed directly (●), and the other was put through three cycles of freezing and thawing to lyse the cells (O) before assay. All samples were assayed for infectious viruses (A) and viable cells (B). The titers of the virus assays on the lysed samples were corrected for virus inactivation due to the freeze-thaw procedure (shown in Table 1).*

less osmotically fragile during the virus latent and rise periods. Although cell osmotic fragility decreases by about four orders of magnitude during virus growth, most of the cells in the infected culture remain sensitive to osmotic shock.

Growth of infected cells. Previous experiments (Fig. 3B and 4) have shown that infected cells remain viable during virus growth. To examine the growth of infected cells, viruses and cells were mixed at an MOI of 30. After 1 h for virus adsorption, the culture was diluted 100-fold, incubated at 37°C, and assayed for CFU as a function of time. A parallel uninfected culture was also assayed for CFU. As seen in Fig. 5, infected cells grow slower than uninfected cells and reach a lower final stationary-phase titer. The infected cells doubling time is 4 to 5 h, as compared to about 1.5 h for uninfected cells.

Virus carrier state. In carrying out one-step growth experiments, it was noted that, after the rise period, virus release continued at a slow rate (see Fig. 2). Infected cultures could be maintained by daily transfer (each transfer involving a 100-fold dilution into fresh medium) and are referred to as carrier cell cultures. The culture used for the experiments described in this section has been kept through over 100 transfers and reaches a daily titer of about 10^8 CFU/ml and 10^7 to 10^9 PFU/ml.
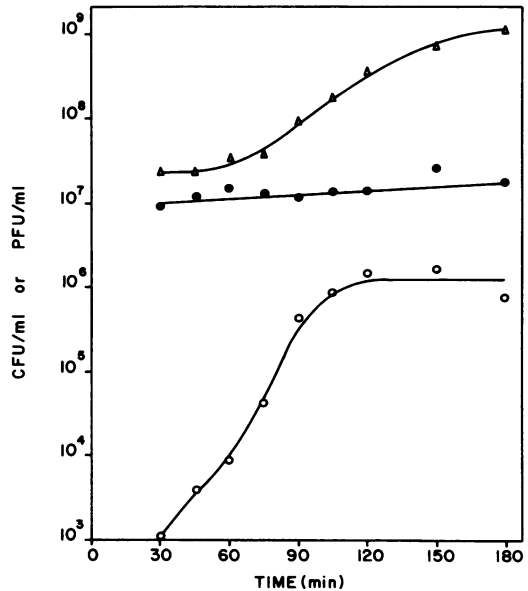

FIG. 4. *Osmotic fragility of MVL2-infected cells. Cells were infected at an MOI of 90, and the artificial lysis protocol was followed, except that lysis was by 1,000-fold dilution in Tris-EDTA buffer. The unlysed samples were assayed for PFU (△) and CFU (●), and the lysed samples were assayed for CFU (O). PFU assay of the lysed samples gave results similar to those of Fig. 3.*
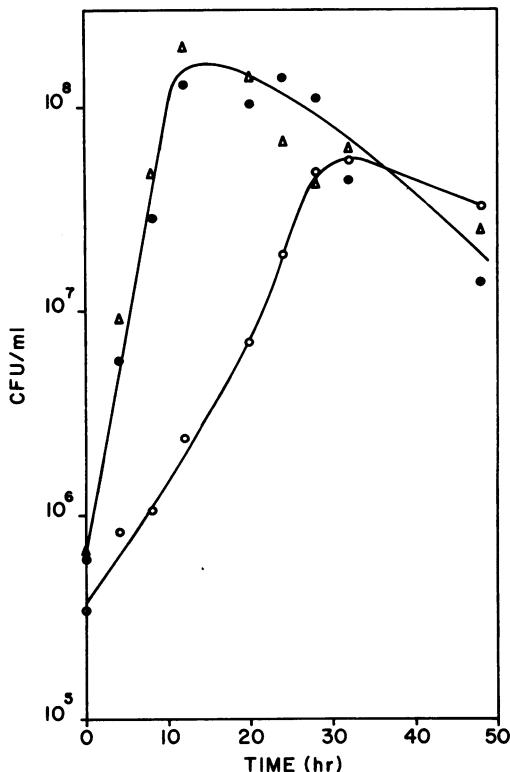
FIG. 5. *Growth of uninfected, infected, and carrier culture cells. Symbols: (●) uninfected cells, (○) cells infected at an MOI of 30, and (△) carrier cells. A single curve is drawn for the uninfected and carrier culture sample points.*

Cell growth and osmotic fragility were examined to see whether carrier culture cells have properties like uninfected or infected ones. The growth curve of carrier culture cells (Fig. 5) was indistinguishable from uninfected cells; the growth rate of carrier cells was that of an uninfected culture, rather than of a newly infected culture. Similarly, the osmotic fragility of carrier cells (measured as survival in Tris-EDTA buffer) was the same as that of uninfected cells (see Table 1), rather than of newly infected cells (see Fig. 4).

To examine the nature of the virus carrier state, experiments were carried out to ask: (i) what is the level of free virus in carrier cultures; (ii) does high dilution of carrier cells into fresh medium change the virus carrier state so as to give rise to the rapid release of viruses seen in newly infected cells; and (iii) what fraction of cells in a carrier culture is capable of producing viruses?

The amount of free virus in a carrier culture was determined by removing cells by filtration

(through a 0.22-μm membrane filter [Millipore]) and assaying the filtrate for PFU. Such filtration of cell cultures was found to remove all CFU, and filtration of virus suspensions passed about 90% of the PFU. About 60% of the PFU of carrier cultures passed through these filters; hence, carrier cultures contain free viruses. That each carrier culture cell carries virus or is resistant to MVL2 infection was shown directly in the experiments described below.

The effect of dilution on the virus carrier state was examined by diluting a carrier culture 10^7-fold into fresh medium. No rapid increase in virus titer, similar to that found in a virus growth experiment (see Fig. 1), was observed after dilution. Instead, both virus and cell titers increased at approximately the same rate.

The fraction of carrier culture cells able to produce virus was determined by washing the cells to remove free virus (this removes more than 99% of the filterable PFU) and then diluting and dispensing the cells into 214 tubes of fresh medium such that each tube received less than one cell. Each tube was scored for the presence of both cells and virus. From these data (Table 3), it can be seen that tubes containing no cells had no virus, confirming that essentially all free viruses were removed by the washing procedure. Of the 40 tubes that contained cells, 35 also contained virus. Since the probability of a tube receiving no cell was 174/214 = 0.813, from the Poisson distribution, the average number of cells put into each tube was 0.21; of the 40 tubes receiving cells, statistically 36 tubes received one cell, 4 tubes received two cells, and no tube received more than two cells. Parenthetically, in this experiment the number of free viruses (filterable PFU) remaining after washing was 10^4-fold less than the number of

TABLE 3. *Summary of experiment to determine fraction of carrier culture cells capable of producing virus*[a]

Contents of tubes	No. of tubes
No cells, no virus	174
No cells, virus	0
Cells, no virus	5
Cells, virus	35

[a] Cells were washed five times with fresh medium (to remove free virus), diluted, and dispensed into tubes of fresh medium (such that each tube would receive only a small number of cells). The tubes were incubated at 37°C. The presence of cells in a tube was determined by the appearance of turbidity after 3 to 4 days. At this time, each tube was tested for virus by plating a drop on an indicator lawn.

CFU, and, since each tube received only 0.21 CFU, statistically the possibility that any tube received both a cell and a virus can be ignored.

The 40 tubes that contained cells, as determined by the appearance of turbidity after 3 to 4 days of incubation at 37°C, represent 40 clones from the carrier culture. These 40 tubes, each containing about 10^8 CFU/ml, were tested for the presence of virus by plating a drop from each tube on an indicator lawn. The virus test showed that 5 of the 40 clones did not produce PFU. This was confirmed by finding no plaques after repeated platings of 0.5 ml of these cultures on indicator lawns. Considering that these five clones came from a carrier culture, which had been transferred over 100 times and which had high titers of free virus, they probably represent virus-resistant cells in the carrier culture.

Since 35 of the 40 clones from the original carrier culture were able to produce plaques on indicator lawns, it is concluded that most carrier culture cells are able to give rise to clones of carrier cells: those that cannot may be virus resistant. It was noticed that the plaque data on the 35 clones of carrier cells fell into two classes. In 14 cases, a confluent plaque was observed, as would be expected for a drop of culture carrying a high titer of virus. However, in 21 cases, a small number of discrete plaques were seen where the drop had been placed. Therefore, some of the carrier culture cells produced clones in which the number of PFU was several orders of magnitude less than the number of CFU. Further experiments are in progress to examine the stability and molecular basis of this carrier state and will be reported in a separate communication.

DISCUSSION

The studies described here show that infection of A. laidlawii cells by the enveloped mycoplasmavirus MVL2 is not lytic. In contrast, the other reported nonlytic viruses of procaryotes do not contain lipids: e.g., filamentous coliphages (reviewed by Marvin and Hohn, 14) and the bullet-shaped group 1 mycoplasmaviruses (reviewed by Maniloff and Liss, 13). Furthermore, the other viruses of procaryotes that are enveloped (e.g., φ6; see 18) or lipid containing (e.g., PM2; see 4) are lytic. Hence, MVL2 is the only reported virus of procaryotes similar to the enveloped, nonlytic animal viruses. This may be a consequence of the fact that the mycoplasmas are the only procaryotes without cell walls and, therefore, have cell surfaces resembling animal cells.

From the three virus adsorption experiments (Table 2), the average first-order adsorption rate constant is 2×10^{-10} cm^3/min. To relate this to the number of virus-cell collisions, a theoretical rate constant has been calculated based on collision kinetics (as described by Stent, 16). This calculation assumes that every collision between virus and cell leads to adsorption, and, hence, the computation is based on the number of collisions between the approximately spherical MVL2 virus (0.08-μm diameter) and a sphere (1.0-μm diameter) whose surface is equal to that of the mycoplasma cell. The calculated theoretical adsorption rate constant is 2×10^{-9} cm^3/min. This is an order of magnitude greater than the experimental result; therefore, under these experimental conditions only about 10% of the potential MVL2 virus-cell collisions lead to adsorption. This is in contrast with the group 1 mycoplasmaviruses (nonlytic, bullet-shaped virions) for which theoretical and experimental rate constants are essentially the same and for which every virus-cell collision must result in adsorption.

In these experiments, the A. laidlawii indicator cells have been observed in three different states: uninfected, newly infected, and carrier cells. The newly infected cells, which are producing progeny viruses during the rise period, are characterized by a longer doubling time and an increased osmotic stability relative to uninfected cells. The basis for these cellular changes is not known. However, it should be noted that the cell membrane area is only equivalent to that of about 150 MVL2 particles. Therefore, during this period of rapid virus production, a large fraction of newly synthesized membrane must be for viral envelopes.

Infected cells reach what appears to be a stable virus carrier state. These carrier cells have a growth rate and osmotic fragility indistinguishable from uninfected cells. However, the carrier culture contains free viruses and infectious centers, and every cell in the carrier culture is able to give rise to either a clone of carrier cells or of virus-resistant cells. Since newly infected cells release virus at a much faster rate than carrier culture cells, the rate of viral replication must be regulated within each carrier culture cell. Therefore, the mycoplasmavirus carrier state resembles those systems in which every cell carries a virus, rather than those in which the culture is maintained by an equilibrium between virus-infected cells and virus-sensitive uninfected cells (reviewed by Walker, 17).

LITERATURE CITED

1. Carstensen, E. L., J. Maniloff, and C. W. Einolf. 1971. Electrical properties and ultrastructure of mycoplasma membranes. Biophys. J. 11:572–581.
2. Delbrück, M. 1940. The growth of bacteriophage and lysis of the host. J. Gen. Physiol. 26:643–660.
3. Doermann, A. H. 1952. The intracellular growth of bacteriophages. I. Liberation of intracellular bacteriophage T4 by premature lysis with another phage or with cyanide. J. Gen. Physiol. 22:365–384.
4. Franklin, R. M., M. Salditt, and J. A. Silbert. 1969. Structure and synthesis of a lipid-containing bacteriophage. I. Growth of bacteriophage PM2 and alterations in nucleic acid metabolism in the infected cell. Virology 38:627–640.
5. Gourlay, R. N. 1971. Mycoplasmatales virus-laidlawii 2, a new virus isolated from Acholeplasma laidlawii. J. Gen. Virol. 12:65–67.
6. Gourlay, R. N. 1972. Isolation and characterization of mycoplasma viruses, p. 145–156. In Ciba Foundation Symposium, Pathogenic mycoplasmas. Elsevier, Amsterdam.
7. Gourlay, R. N., D. J. Garwes, J. Bruce, and S. G. Wyld. 1973. Further studies on the morphology and composition of mycoplasmatales virus-laidlawii 2. J. Gen. Virol. 18:127–133.
8. Hutkova, J., V. Drasil, A. Melichar, and J. Smarda. 1973. Determination of some biophysical parameters of the newly isolated mycoplasmatales virus MV-Lg-pS2-L 172. Stud. Biophys. 39:217–222.
9. Liska, B. 1973. Isolation of a new mycoplasmatales virus. Stud. Biophys. 34:151–155.
10. Liska, B., and L. Tkadlecek. 1975. Electron microscopic study of a mycoplasmatales virus, strain MV-Lg-pS2-L 172. Folia Microbiol. (Prague) 20:1–7.
11. Liss, A., and J. Maniloff. 1971. Isolation of mycoplasmatales viruses and characterization of MVL1, MVL52, MVG51. Science 173:725–727.
12. Liss, A., and J. Maniloff. 1973. Infection of A. laidlawii by MVL51 virus. Virology 55:118–126.
13. Maniloff, J., and A. Liss. 1974. Comparative structure, chemistry and evolution of mycoplasmaviruses, p. 583–603. In E. Kurstak and K. Maramorosch (ed.), Viruses, evolution and cancer. Academic Press Inc., New York.
14. Marvin, D. A., and B. Hohn. 1969. Filamentous bacterial viruses. Bacteriol. Rev. 33:172–209.
15. Quinlan, D. C., A. Liss, and J. Maniloff. 1972. Eagle's basal medium as a defined medium for mycoplasma studies. Microbios 6:179–185.
16. Stent, G. S. 1963. Molecular biology of bacterial viruses. W. H. Freeman and Company, San Francisco.
17. Walker, D. L. 1968. Persistent viral infection in cell cultures, p. 99–110. In M. Sanders and E. H. Lennette (ed.), Medical and applied virology. Warren H. Green, Inc., St. Louis.
18. Vidaver, A. K., R. K. Koski, and J. L. Van Etten. 1973. Bacteriophage ϕ6: a lipid-containing virus of Pseudomonas phaseolicola. J. Virol. 11:799–805.