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EFFECT OF LIPID ALKYL CHAIN PERTURBATIONS ON THE ASSEMBLY OF BACTERIOPHAGE PM2

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SUMMARY

The lipid-containing bacteriophage PM2 can produce infectious virus in cultures infected at temperatures up to 31.5 °C, but not at 34 °C. Its host, *Pseudomonas* BAL-31, grows at 34 °C and cultures infected at that temperature undergo lysis. Sucrose-gradient analysis shows that 34 °C lysates contain no PM2-like particles. Temperature-shift experiments establish that the thermally sensitive process is late in infection when virus assembly is taking place.

Adamantanone, a small hydrophobic molecule that perturbs membrane hydrocarbon zones, prevents the production of infective virus. Concentrations which prevent virus production have no effect on host-cell growth or stability of mature virions. Adamantanone exerts its effects late in the infectious cycle, and lysates made in its presence contain no PM2-like particles. These experiments, carried out at 25 °C, indicate that adamantanone prevents the assembly of stable PM2 virus.

Spin-label studies suggest that the lipid alkyl chains of the host-cell membrane are in an "ordered" state at temperatures below about 33 °C and undergo a transition to a "disordered" state above that temperature. Furthermore, the addition of adamantanone perturbs the hydrocarbon zones, producing a greater degree of disorder even below 25 °C. Our findings suggest that the cell membrane can function and grow with the lipid alkyl chains in either the "ordered" or "disordered" state, but that the "ordered" state must be maintained for PM2 assembly to occur.

INTRODUCTION

The lipid-containing bacteriophage PM2 presents an interesting and somewhat unique problem in studies of membrane assembly. The mature virion, which contains a phospholipid bilayer as part of its structure, is assembled within the host cell prior to lysis [1]. The unique features associated with the production of the PM2 bilayer

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may best be seen by contrasting this process with that of cellular membrane growth. In the case of the cell, during growth, a bilayer structure is ever present and newly synthesized phospholipids may be inserted into this preexisting membrane. Although the details of cell-membrane growth are not known, it is not difficult to describe plausible models for expansion of an established bilayer structure. With PM2, however, the problem would appear more complex. By some means, the initiation and subsequent growth of a lipid bilayer must be effected in a region in space where none previously existed. Furthermore, the material for this bilayer must be extracted to some extent from the host-cell membrane, since PM2 is known to derive its phospholipids at least in part from preexisting cellular membrane components [2–4]. An understanding of the energetics and physical forces involved in this transfer of lipid material from one bilayer structure to another is of general interest to the study of membrane-membrane interactions.

We are interested in the dynamic aspects of assembly of the PM2 virus particle, particularly the genetic and physical factors controlling the assembly process. It might be anticipated that more stringent conditions would be required for assembly of the PM2 particle than those required for maintaining the integrity of mature virions or, for that matter, the structure and function of the host-cell membrane. In accord with this, we found previously that high levels of Ca^{2+} ($> 3 \cdot 10^{-4}$ M) are required for PM2 assembly, while much lower levels ($< 3 \cdot 10^{-5}$ M) are sufficient for host-cell growth and for stability of the mature virus particle [5]. For virus production, the high levels of Ca^{2+} are essential only during the final stages of infection, when viral assembly is taking place. Ca^{2+} is known to stabilize membrane structures (see ref. 6 for review) and a greater degree of stabilization is apparently required during viral assembly than at other times.

In the present work we have investigated further the question of stability requirements for PM2 assembly, in contrast to the requirements for cell growth and the stability of mature virus particles. Of interest here was the degree of order in the host-cell membrane hydrocarbon zones that is necessary for successful virus assembly. The degree of order was modified by (a) varying temperature and (b) introducing adamantanone, a nearly spherical, lipid-soluble, perturbing molecule into the membrane hydrocarbon zones. Spin-label analysis was used to detect lipid alkyl chain perturbations produced by high temperatures and by the perturber molecule. Our results suggest that cell growth can proceed with the lipid hydrocarbon zones in either the "ordered" or "disordered" state, but that the "ordered" state must be maintained for PM2 assembly to occur.

MATERIALS AND METHODS

Organisms, growth media, and culture methods

Wild-type *Pseudomonas* BAL-31 and bacteriophage PM2 were used for most experiments. A thymine-requiring mutant of BAL-31, designated PS1001, was used for labeling cultures with [^{3}H]thymidine. The isolation and characterization of PS1001 have been described previously [5].

Four types of media were used in the work reported here. Q-medium is an enriched medium containing tryptone broth, yeast extract, and various inorganic salts. Medium 25, a defined but somewhat enriched medium, contains all the naturally

occurring amino acids except tryptophan and, in addition, adenosine, guanosine, and uridine. Medium 25 is buffered to pH 7.6 with Tris · HCl. M3-medium is similar to Medium 25 but lacks several amino acids, and is used when labeling cultures with radioactive amino acid mixtures. ND-medium is similar to Medium 25 but contains no added divalent ions. For experiments in which the Ca^{2+} concentration was varied, ND-medium was supplemented with $MgCl_2$ at 10^{-2} M and $CaCl_2$ at the concentration required for a particular experiment. Recipes for all these growth media have been reported [5].

Cultures were assayed for plaque-forming units (p.f.u.) by the agar overlay method. Bottom agar was composed of Q-medium hardened with 1.5 % (w/v) Bacto agar. Top agar contained Q-medium, lacking yeast extract, hardened with 0.5 % (w/v) Bacto agar.

Adamantanone was obtained from Aldrich chemical company. Analysis by thin-layer chromatography using silica-gel g with a chloroform moving phase revealed a single spot indicating that the adamantanone was of reasonably high purity.

Sucrose-gradient analysis

Lysates prepared in the presence of radioactive nutrients were analyzed by velocity sedimentation through sucrose gradients to detect the presence of PM2 virus and PM2-like particles. Our procedure, employing 20–30 % sucrose gradients in Medium 25, has been reported [5].

Electron spin resonance (ESR) measurements

ESR experiments were carried out at X-band microwave frequencies with a Japan Electrons Optics Laboratory spectrometer, Model JESME 1X. The sample temperature was controlled with a variable temperature apparatus constructed in our laboratory. Continuous monitoring of the sample temperature was accomplished with a thermistor probe. Our estimated accuracy in temperature measurement is ± 0.5 °C.

Rotational motion times (τ_c) for the spin label were calculated from measurements of the heights and widths of the ^{14}N hyperfine lines, as taken from first-derivative spectra. The equation used was

$$\tau_c = 6.5 \cdot 10^{-10} W_1 \left[\left(\frac{h_1}{h_{-1}} \right)^{\frac{1}{2}} - 1 \right]$$

where h_1 and h_{-1} are the first-derivative heights of the low-field and high-field hyperfine lines, respectively. W_1 is the first-derivative peak-to-peak width of the low-field line, in gauss. The expression above is similar to the one used in previous experiments from our laboratory [7], but makes use of the low- and high-field spectral parameters rather than the mid- and high-field lines. This alleviates problems that arise when the mid-field line has greater area than the low- and high-field lines. This condition frequently results when a portion of the spin-label molecules collect in pools, giving rise to spin-exchange effects.

RESULTS

Temperature dependence for virus production

BAL-31 can grow at temperatures up to about 35 °C, but productive virus

infections will not occur at temperatures this high. At 25 °C, cells infected at a low multiplicity of infection (m.o.i.) release about 100 to 200 p.f.u. per input p.f.u. during the first round of virus production. These virus are released between about 50 and 80 min post infection. At 31.5 °C, the average burst size of infectious virus is reduced to about 50, while at 34 °C no increase in the number of p.f.u. is observed in the infected culture. We have taken 25 and 34 °C as permissive and restrictive temperatures for virus production by wild-type PM2 and have investigated the nature of the temperature sensitivity involved.

Attachment studies showed that PM2 attaches about equally well at 25 and 34 °C. Furthermore, cultures infected at a high m.o.i. show lysis at both temperatures, as shown in Fig. 1. Lysis is not as complete at 34 °C as at 25 °C, but this cannot

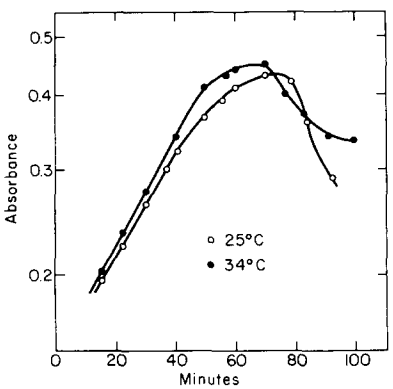


Fig. 1. Lysis of PM2-infected cultures of BAL-31 at 25 and 34 °C. For this experiment, cultures growing in Q-medium were infected at a m.o.i. of 10 when the absorbance was approx. 0.3. The absorbance was measured at 660 nm with a Bausch and Lomb Spectronic 20 colorimeter.

account for the complete loss of virus production at the higher temperature. We carried out temperature-shift experiments to determine the portion of the infectious cycle where the thermal sensitivity for virus production occurs. Single-step growth curves for a variety of temperature-shift conditions, including both shift-up and shift-down experiments, are shown in Fig. 2.

The data of Fig. 2 show clearly that the time of thermal sensitivity is very late in the infectious cycle. In Fig. 2a, a culture shifted from 25 to 34 °C at 25 min post-infection shows no increase in virus titer. In Fig. 2b, a culture shifted from 34 to 25 °C as late as 30 min post infection produces virus at nearly the same time and in the same quantity as for 25 °C throughout. Cultures shifted to 25 °C at 40 and 50 min, when many cells are lysing, begin producing infectious virus within minutes. It appears that the lower, permissive temperature is required only at the very late stages of infection, when PM2 particles are being assembled.

Lysates made at 25 and 34 °C were analyzed on sucrose gradients to determine whether inactive, PM2-like particles are present after lysis of a 34 °C culture. The results (Fig. 3) show that no such particles are formed at the higher temperature. We interpret the results of the temperature-shift and sucrose-gradient experiments to

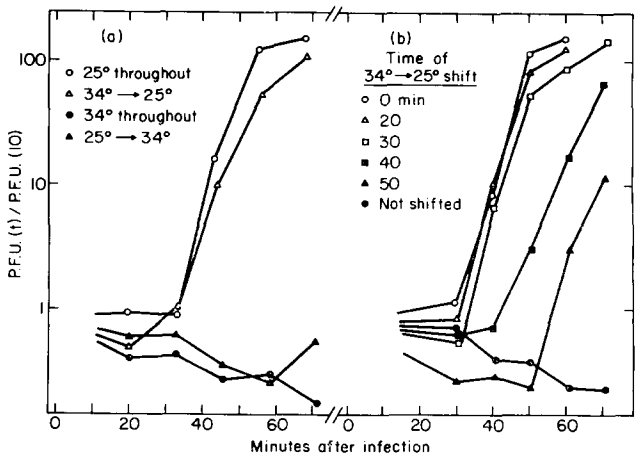


Fig. 2. Effect of temperature shifts (25 °C ↔ 34 °C) on the production of infectious PM2 virus. Cultures of BAL-31 at 10^8 cells/ml at either 25 or 34 °C were infected with PM2 at a m.o.i. of 10^{-3}. At times indicated on the figure, aliquots were shifted in temperature. Samples were diluted through iced Q-medium at times specified on the ordinate and assayed for p.f.u. at 25 °C. Data are expressed as the p.f.u. at time (t) relative to the input p.f.u., as measured 10 min after infection. (a) 25 °C ↔ 34 °C shifts 20 min after infection; (b) 34 °C ↔ 25 °C shifts at various times after infection.

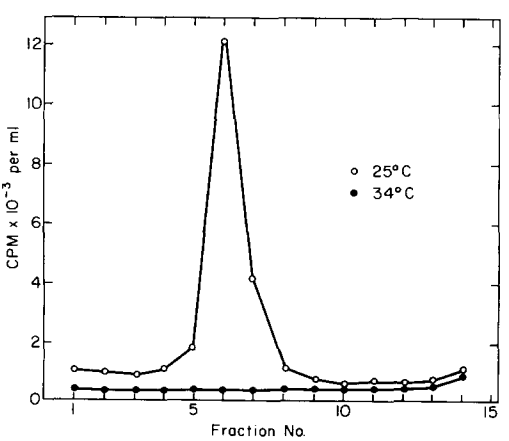


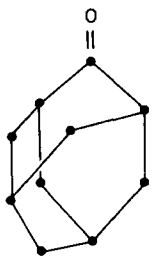
Fig. 3. Sucrose-gradient analysis of 25 and 34 °C lysates of PM2-infected cultures of strain PS1001. 6-ml cultures at 10^8 cells/ml in M3-medium were infected with PM2 at a m.o.i. of 10 in the presence of 50 μCi ^{3}H-labeled protein hydrolysate. Following lysis and removal of cell debris by low-speed centrifugation, the lysates were pelleted, resuspended, and analyzed on sucrose gradients as described previously [5].

mean that the thermally sensitive step for production of PM2 is in the process of assembling stable virus particles.

Effect of adamantanone on virus production

Adamantanone (structure shown below) is a small, nearly spherical molecule

that is readily solubilized in membrane hydrocarbon zones. A similar molecule, adamantane, was previously shown to perturb lipid alkyl chains in membranes [8], and our spin-label experiments described later show that adamantanone causes a similar disordering of the BAL-31 membrane hydrocarbon zones. We therefore studied the effect of adamantanone on PM2 production.



At concentrations up to $6 \cdot 10^{-3}$ M adamantanone is not toxic to PM2 nor to BAL-31. The growth rate of BAL-31 is slightly reduced at that concentration. At $3 \cdot 10^{-3}$ M adamantanone the cells grow, within experimental error, at their normal rate. Data for the effects of adamantanone on PM2 stability and BAL-31 growth are shown in Fig. 4.

When $6 \cdot 10^{-3}$ M adamantanone is added to a culture prior to infection, no infectious virus are produced. If the m.o.i. is high, the cultures partially lyse, although

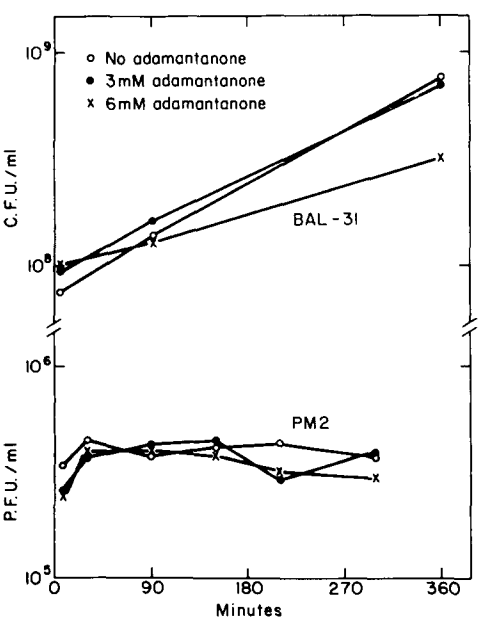


Fig. 4. Effect of adamantanone on BAL-31 growth and PM2 stability. Cells and virus were exposed to adamantanone in Medium 25, and at various times aliquots were diluted and plated for colonies and plaques, respectively.

lysis does not appear as complete as in the absence of adamantanone. We measured the concentration dependence for the inhibition of virus production by adamantanone, and the results are shown in Fig. 5. Plotted in Fig. 5 is the average burst size, measured as p.f.u. at 90 min relative to input p.f.u., for different adamantanone concentrations. $3 \cdot 10^{-3}$ M adamantanone reduces virus production to about 20 % of the normal level, and $6 \cdot 10^{-3}$ M completely inhibits virus production.

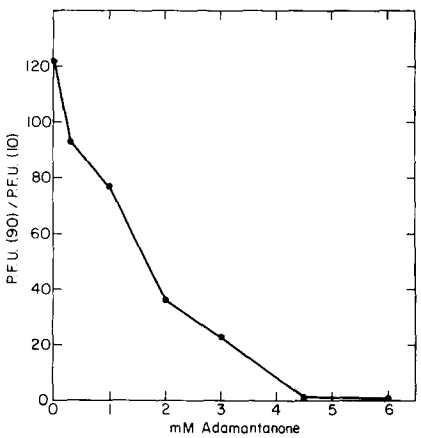


Fig. 5. Effect of different adamantanone concentrations on the production of infectious PM2 virus. The average burst size is measured as p.f.u. at 90 min divided by the input p.f.u., as measured at 10 min after infection.

Data were taken to determine the time during the infectious cycle that adamantanone was inhibitory. It can be seen from Fig. 6 that the addition of adamantanone at any time prior to lysis prevents virus production. This is consistent with adamanta-

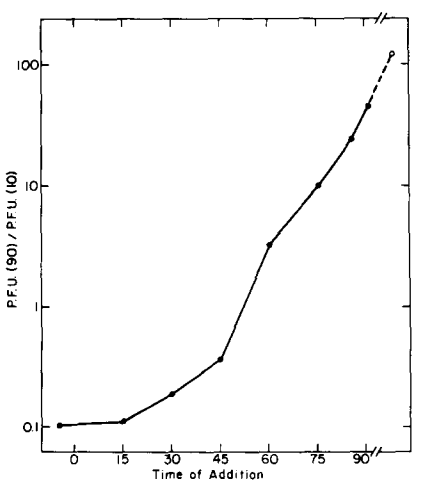


Fig. 6. Average burst size as a function of time of adamantanone addition. A culture of BAL-31 cells in Medium 25 was infected at time zero with PM2 at a m.o.i. of 10^{-3}. Adamantanone was added to aliquots at various times after infection to a final concentration of $6 \cdot 10^{-3}$ M.

none exerting its effect near the end of the infectious cycle. We studied the effect of adamantanone on virus attachment (data not shown), and found that concentrations which prevent or reduce virus production have no effect on attachment. We also removed adamantanone at various times after infection by large dilutions into adamantanone-free medium, and found that virus production is restored, to some extent, if adamantanone is removed prior to about 50 min. These experiments all indicate that adamantanone acts late in the infectious cycle, when PM2 particles are being assembled.

To determine whether non-infectious, PM2-like particles are produced when adamantanone is present, lysates were prepared and analyzed on sucrose gradients. Fig. 7 shows that, in the presence of adamantanone, the lysate contains no particles of a size comparable to PM2. We interpret these results to mean that adamantanone inhibits PM2 production by preventing the assembly of stable virus particles.

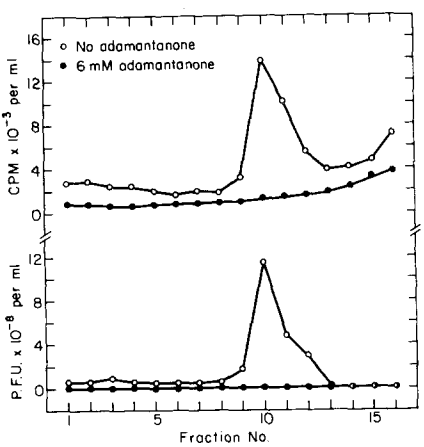
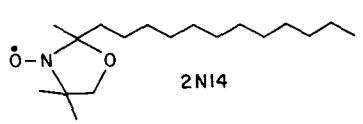


Fig. 7. Sucrose-gradient analysis of viral lysates made in the presence and absence of $6 \cdot 10^{-3}$ M adamantanone. Fractions taken from the gradients were analyzed for radioactivity and p.f.u. ^{3}H-labeled amino acids were used as a label.

Spin-label studies

The effect of temperature and adamantanone on the hydrocarbon zones of the host-cell membrane was investigated with the nitroxide spin label 2N14 shown below. This spin label has no detectable water solubility, but is readily solubilized in membranous structures. Its mobility, as measured by its rotational correlation time, τ_c, can



2N14

be taken as a relative indication of the fluidity or degree of disorder in the region of the lipid alkyl chains. Small values of τ_c correspond to greater freedom of motion for the spin label.

Values of τ_c were measured for 2N14 at various temperatures and the data, displayed on an Arrhenius plot, are shown in Fig. 8. Without adamantanone present we consistently observe a discontinuity in slope on the Arrhenius plot at about 33 °C. Such discontinuities are generally associated with a transition from an ordered structure within the membrane hydrocarbon region, existing below the transition temperature, to a more disordered state above the transition temperature [9]. The temperature

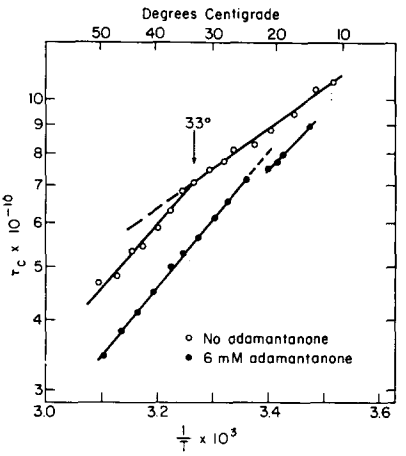


Fig. 8. Arrhenius plot of the rotational correlation time (τ_c) for BAL-31 cells spin-labeled in the presence and absence of $6 \cdot 10^{-3}$ M adamantanone. A 50-ml culture of BAL-31 in Medium 25 was divided and adamantanone was added to one portion. The cells were centrifuged, resuspended in a small volume of the supernatant, and spin-labeled with 2N14. Potassium ferricyanide was added to prevent spin reduction.

of this transition for the BAL-31 membrane is, within experimental error, identical to the temperature above which PM2 assembly cannot occur. A reasonable interpretation of our results is that the host-cell lipid alkyl chains must be in an ordered state, as exists below the "phase transition" observable by ESR, in order for PM2 assembly to take place.

Spin-labeled cells containing $6 \cdot 10^{-3}$ M adamantanone gave lower values of τ_c and did not show the distinct discontinuity in slope on the Arrhenius plot (Fig. 8). The slope with adamantanone present is nearly identical to that which exists at temperatures above 33 °C in the absence of adamantanone. A slight discontinuity in τ_c values, with little change in slope, is seen at a lower temperature for the preparation containing adamantanone. The data of Fig. 8 indicate that adamantanone is an effective perturber of lipid alkyl chains, introducing a state of disorder in the membrane at lower temperatures which is similar to the state normally existing only above 33 °C. We suggest that adamantanone prevents PM2 assembly by disordering the hydrocarbon zones in the host membrane.

Low temperature studies and Ca^{2+} effects

We considered the possibility that the disordering effects of adamantanone might be sufficiently overcome at low temperatures to allow virus production. BAL-31

can grow, at reduced rates, down to temperatures below 4 °C, and productive PM2 infections will occur with extended burst times down to that temperature. Fig. 9 shows the effect of $6 \cdot 10^{-3}$ M adamantanone on PM2 production in the low temperature range. Cultures were assayed for input p.f.u. at 10 min postinfection and for virus production 24 h later. The results show that adamantanone prevents virus production over the entire temperature range.

Since Ca^{2+}, a membrane stabilizer, is required for PM2 assembly whereas adamantanone, a membrane perturber, inhibits assembly, we investigated their antagonistic behavior with regard to virus production in a direct manner. A concentration

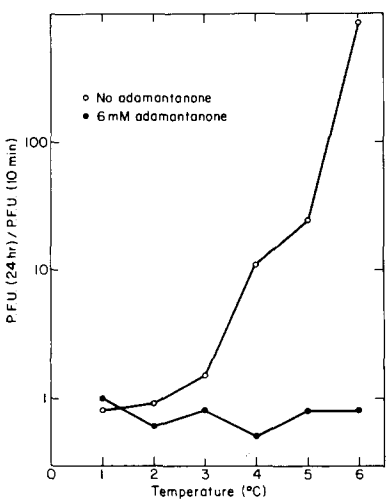


Fig. 9. Effect of adamantanone on production of infectious PM2 at low temperatures. The concentration of adamantanone used was $6 \cdot 10^{-3}$ M. Cultures of BAL-31 cells at 10^8 cells/ml were infected with PM2 at a m.o.i. of 10^{-3} and assayed for p.f.u. at 10 min and 24 h after infection.

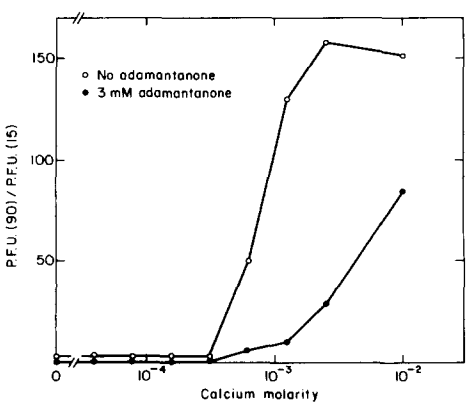


Fig. 10. Effect of adamantanone on production of infectious PM2 virus with different levels of added Ca^{2+}. A culture of BAL-31 in ND medium (plus Mg^{2+}) was divided and adamantanone was added to one portion to a final concentration of $3 \cdot 10^{-3}$ M. Various amounts of Ca^{2+} were added to aliquots of each, and 30 min later PM2 virus was added at a m.o.i. of 10^{-3}. The cultures were assayed for p.f.u. at 10 min and 90 min postinfection.

of $3 \cdot 10^{-3}$ M adamantanone, which allows virus production at about 20 % of the normal level in Medium 25, was used and the Ca^{2+} concentration was varied. The results are shown in Fig. 10. With adamantanone present, nearly 10 times as much Ca^{2+} is required for virus production at the same level as in its absence. The data of Fig. 10 provide convincing evidence that the requirement for Ca^{2+} and the inhibitory effect of adamantanone are both involved with the same aspect of the production of infectious PM2 virus.

DISCUSSION

Several observations must be kept in mind in attempting to formulate ideas regarding the mechanism whereby the PM2 lipid bilayer is assembled. Some of those relevant to a discussion of the results presented here are: (1) The PM2 virion contains 67 % phosphatidylglycerol and 28 % phosphatidylethanolamine, whereas the host-cell membrane contains 23 % phosphatidylglycerol and 75 % phosphatidylethanolamine [10]. (2) 2 h after infection, when host-cell lysis has already begun, the concentration of phosphatidylglycerol in the infected cells rises to only 26 % and the concentration of phosphatidylethanolamine drops to 65 % [10]. (3) PM2 derives its phospholipid material to some extent from cellular phospholipids [2–4]. (4) The PM2 particle matures inside the host cell rather than obtaining membrane material by budding through the cell membrane [1].

A most intriguing aspect of the problem of PM2 assembly is how the virus can obtain a phospholipid composition so different from that of the host cell while at the same time using cell-membrane material. One possibility, which we will discuss in connection with the present work, is that local pools of high phosphatidylglycerol content are produced in the cell membrane during infection, and that these are the phospholipid regions that become a part of the virus. It is known, however, that phospholipids diffuse laterally at a high rate in membranes, and some consideration must be given to this point.

Several workers using various techniques [11–14] have estimated that the two-dimensional diffusion coefficient for phospholipids in a bilayer is in the range of 10^{-7} to 10^{-10} cm^2/s. The rapid diffusion of phospholipid molecules suggested by these values would tend to randomize the location of the different phospholipid species, placing a definite lifetime on pools of high phosphatidylglycerol concentration that might be produced during infection. The magnitude of this problem can be estimated from the following calculations. If, during assembly, a maturing PM2 particle utilizes a patch of cell-membrane material that is one-tenth of the total viral membrane, this patch has an area* of approx. $3.5 \cdot 10^{-12}$ cm^2. Assuming a diffusion coefficient of 10^{-8} cm^2/s, we estimate that the patch must be incorporated into the virion within approx. 0.35 ms after its formation, otherwise the phospholipid species would become randomized. It seems likely, therefore, that some means exists for stabilizing the pools against the randomizing effects of diffusion.

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* Based on 7000 phospholipid molecules per virion, as calculated in ref. 5, and a cross-sectional area of 50 $Å^2$ per phospholipid molecule.

Ohnishi and Ito, using the electron-exchange effect with spin-labeled phospholipids, have recently observed the Ca^{2+}-induced clustering of individual phospholipid species in artificial membranes [15]. The process is referred to by those authors as a lateral phase separation and is attributed to an interaction between Ca^{2+} and the negatively charged phosphatylserine molecules. The phase separation could also be affected by Ba^{2+} and Sr^{2+}, but not Mg^{2+}. In our studies of the Ca^{2+} requirement for PM2 assembly [5], we found that Ba^{2+} and Sr^{2+}, but not Mg^{2+}, can replace Ca^{2+} for the assembly of infectious PM2 particles. These similarities suggest that one role of Ca^{2+}, and perhaps the most important one, might be in aggregating the negatively charged phosphatidylglycerol molecules into pools within the host-cell membrane.

It is to be expected that the rate of lateral diffusion of phospholipids in the BAL-31 membrane would be much greater in the disordered state, above 33 °C, than in the ordered state below that temperature. Likewise, the perturbation produced by adamantanone might lead to more rapid lateral diffusion at temperatures below 33 °C. We suggest, as a possibility, that the disorder produced both thermally and by the perturbing effects of adamantanone may prevent the formation of local pools rich in phosphatidylglycerol for a length of time sufficient to allow their incorporation into the PM2 particle. This, in effect, could inhibit the assembly of stable PM2 particles under these conditions. Temperature-dependent phase separations have been described earlier in defined phospholipid systems [16, 17] and have been inferred in biological membranes.

Membrane proteins might also be involved, either directly or indirectly, in the formation of pools rich in phosphatidylglycerol, and some aspects of this potential involvement have been discussed in connection with the assembly of the membrane-bounded bacterial virus $\phi 6$ [18]. The data presented here do not rule out the possibility that adamantanone is associating with some specific membrane protein and exerts its effect on assembly in that way.

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