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CALCIUM REQUIREMENT FOR ASSEMBLY OF THE LIPID-CONTAINING BACTERIOPHAGE PM2

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SUMMARY

The bacteriophage PM2 requires extracellular Ca^{2+} at concentrations greater than $3 \cdot 10^{-4}$ M for the production of viable virus, whereas the host cell *Pseudomonas* BAL-31 grows normally in medium containing $3 \cdot 10^{-5}$ M Ca^{2+} (low calcium). Virus attachment occurs normally in low calcium, the infected cultures partially lyse, but no infectious virus particles are released. Sucrose gradient analysis shows that lysates made in low calcium contain no PM2-like particles. The addition of calcium very late in the infectious cycle completely restores virus production to cultures infected in low calcium, whereas removal of calcium after infection prevents virus production. Our experiments indicate that Ca^{2+} is essential for some process late in the lytic cycle, such as the final assembly of stable, infectious PM2 particles.

INTRODUCTION

We are interested in the use of PM2, a bacterial virus that contains a lipid bilayer as part of its structure [1], as a potential model system for studying various aspects of membrane interactions. The PM2 infectious process involves virus attachment and entry (processes which are closely related to but may or may not involve membrane fusion), macromolecular biosynthesis, the genesis of infective virus particles, and cell lysis. The final steps in the genesis, or assembly, of the virus membrane present interesting problems in membrane dynamics. Apparently PM2 does not obtain its membrane by budding through the bacterial membrane, since mature virions can be seen inside the host cell prior to lysis [2]. Furthermore, the widely different phospholipid compositions of the virus and host cells [3] make it unlikely that sizable pieces of bacterial membrane are scavenged for use by the virus. It seems reasonable, therefore, that the PM2 membrane must be assembled around the inner portion of the virus particle, and the molecular forces and environmental factors that influence this assembly are largely unknown.

It was reported by Okada and Murayama [4] that Ca^{2+} is required for the successful fusion of mammalian cells induced by Sendai virus (strain Z, hemagglutinating virus of Japan). In the absence of calcium, viral infection takes place, but cell lysis

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rather than cell fusion occurs. These authors concluded from their experiments that calcium did not influence the "disconnection" of cell membranes at the virus absorbing site, but that calcium was essential as a stabilizing factor for the "reconnection" of cell membranes to produce cell fusion.

The requirement of calcium and ATP in maintaining the structural rigidity of biological membranes is well known. On this basis, Woodin and Wieneke [5] proposed that local areas of reduced structural rigidity resulting from the removal of calcium and ATP would promote membrane fusion. Poste and Allison [6] have incorporated these ideas in their more general theory for membrane fusion, in which particular emphasis is placed on the role of membrane calcium levels in regulating the process. Here again, one function of calcium is to stabilize the newly formed membrane after fusion has taken place.

Because of the known stabilizing feature of calcium and its apparent requirement for membrane fusion, we have investigated its role in the infectious process of PM2. The results of these experiments and some discussion of the dynamic aspects of membrane assembly are presented in this report.

MATERIALS AND METHODS

Virus and cells

Bacteriophage PM2 and its host, the marine bacterium *Pseudomonas* BAL-31, were gifts of Dr Eugene Cota-Robles. For the experiments described in this report, we used a thymine-requiring derivative of BAL-31, designated PS1001, which was isolated as follows. Approximately 10^7 BAL-31 cells were added to a bottom agar plate containing 36 μg/ml Trimethoprim [7]. Colonies appearing after incubation overnight at 25 °C, when tested, were found to require thymine. Single colony isolates of ten thymine requirers were tested for their rate of reversion to prototrophy. PS1001 was selected as a low-frequency reverting mutant which grew as rapidly as BAL-31 when supplied with thymidine but showed no growth in its absence.

Media

Cells and virus were routinely cultured in Q medium, which contained 0.7 g KCl, 26 g NaCl, 10 g Bacto-Tryptone, 5 g yeast extract, 1.5 g $CaCl_2 \cdot 2H_2O$, and 12 g $MgSO_4 \cdot 7H_2O$ per l of distilled water. Q plates contained Q medium hardened with 1.5% (w/v) Bacto agar. Top agar was composed of Q medium lacking yeast extract, hardened with 0.5% (w/v) Bacto agar.

Three chemically defined media were routinely used. The basic minimal medium, STG medium, contained 0.7 g KCl, 1.1 g NH_4Cl, 1.5 g $CaCl_2 \cdot 2H_2O$, 9.9 g $MgCl_2 \cdot 6H_2O$, 26 g NaCl, 23 mg Na_2SO_4, 12.2 g Tris, 2 g glucose, and 25 mg KH_2PO_4 per l of distilled water. The pH of STG medium was adjusted to 7.6 by addition of HCl. Medium 25, a chemically defined but somewhat enriched medium, consisted of STG medium supplemented with 20 μg/ml of the amino acids alanine, arginine, asparagine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine (all L-isomers); 50 μg/ml of adenosine and guanosine; and 100 μg/ml of uridine. A medium containing no added divalent ions, designated ND medium, is identical to medium 25 except that $CaCl_2$ and $MgCl_2$ are omitted. Medium

25-S and medium ND-S refer to these respective media supplemented with 20 μg/ml tryptophan and 50 μg/ml thymidine. For all experiments reported here, ND medium and ND-S medium were supplemented with $MgCl_2$ at 10^{-2} M and $CaCl_2$ at a concentration appropriate for the experiment. Conditions indicated "(+)Ca" are for a calcium concentration of 10^{-2} M.

The residual Ca^{2+} concentration in ND medium, as measured with a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer, was $3 \cdot 10^{-5}$ M. This residual calcium level was present for all experiments designated "(−)Ca" in this report.

Virus attachment

10-ml cultures of PS1001 were grown in ND-S medium with and without added calcium to about 10^8 cells/ml. The cultures also contained 1% Tergitol (Nonidet P40, Union Carbide Co.), which prevents PM2 from sticking to Millipore filters. Tergitol at a concentration of 1% contributed no significant amount of calcium to our medium. At various times after adding PM2 at a low multiplicity of infection (m.o.i.), 2 ml of the infected culture was filtered through a 0.22 μm Millipore filter to collect cells and attached virus, and the filtrate was assayed for plaque-forming units (p.f.u.).

In other experiments (data not shown) for which Tergitol was omitted from the cultures, the cells and attached virus were removed by centrifugation. This process was slower than filtering, but gave approximately the same results.

DNA synthesis

Stocks of PM2 prepared in Medium 25-S were pelleted by centrifuging for 2 h at 20 000 rev./min in a SW41 rotor and were resuspended overnight in ND-S medium lacking added calcium. 10-ml cultures of PS1001 at 10^8 cells/ml, grown in ND medium supplemented with 10 μg/ml of tryptophan and thymidine, with and without calcium, were infected with PM2 at a m.o.i. of 10. An uninfected culture was maintained as a control. 15 min later, 100 μCi of [^{3}H]thymidine was added to each culture. 45 min after infection (prior to lysis) 10 ml of 10% trichloroacetic acid was added and 30 min later the precipitate was collected on a 0.22-μm Millipore filter. The filter pad with the precipitate was washed twice with cold 5% trichloroacetic acid and twice with ethanol, dried, and then counted by liquid scintillation spectrometry. In some cases the cells were lysed by sonication prior to the addition of trichloroacetic acid. Except for this last step, our procedure is similar to that used by Franklin et al. [8].

Sucrose gradient analysis

Cultures of PS1001 were grown in ND medium supplemented with 10 μg/ml tryptophan and thymidine, with and without added calcium. A stock of PM2 in ND medium was used to infect the cultures at a m.o.i. of 10 in the presence of 100 μCi [^{3}H]thymidine. After lysis, cell debris was removed by low speed centrifugation and the clarified lysate was then centrifuged at 20 000 rev./min for 2 h in a SW41 rotor to pellet virus particles. The resuspended pellet (in 0.3 ml ND medium) was layered onto the top of a 20–30% sucrose gradient in 50% ND medium and centrifuged for 160 min at 35 000 rev./min in a SW41 rotor. Half-ml fractions were collected and assayed for radioactivity.

In order to detect any PM2-like particles lacking DNA, lysates were also made with a ^{14}C-labeled protein hydrolysate in M3 medium, which is similar to Medium 25 but lacks asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine. lysine, phenylalanine, serine, tyrosine, and valine.

Calcium accumulation by PS1001

10-ml samples of ND-S medium containing various amounts of calcium were inoculated with 10^7 cells/ml in the presence of 5 μCi ^{45}Ca and 200 μCi ^3H_2O. Following growth to 10^8 cells/ml, the cells were pelleted by low speed centrifugation, the supernatant was saved for radioactivity measurements, and the pellet was resuspended in 1 ml ND medium. The resuspended pellet and an aliquot of the supernatant were assayed for ^{45}Ca and ^3H radioactivity. Corrections were made for ^{45}Ca counts in the ^3H channel of the liquid scintillation spectrometer.

Source of radioactivity

[^3H]Thymidine (9.82 Ci/mmole), ^3H_2O (1 Ci/ml), and $^{45}CaCl_2$ (6.36 Ci/g) were obtained from New England Nuclear Corp. (Boston, Mass.). ^{14}C-labeled protein hydrolysate was obtained from Schwartz-Mann (Orangeburg, N.Y.).

RESULTS

Requirement of calcium for virus production

The doubling time of PS1001 in ND-S medium, as measured by absorbance at 660 nm, is about 2 h at 25 °C. Within experimental error, the growth rate is the same in media containing either 10^{-2} M Ca^{2+} or the residual level of $3 \cdot 10^{-5}$ M Ca^{2+}.

Fig. 1 shows viable virus production as a function of time after infection for cultures under (+)Ca and (−)Ca conditions. For this and other figures, the data are presented as the p.f.u. titer at a given time relative to the input p.f.u. titer. When calcium is present, an average burst of approximately 200 p.f.u. occurs between 40 and 60 min after infection. Without calcium, there is an initial rise in p.f.u. such that, by 60 min, the culture contains about 60 p.f.u. per input virus. Virus production does not continue beyond this point, and the viable virus titer drops sharply to the extent that, by 100 min, there are only 5 p.f.u. per input virus. We have no explanation for the rise and fall in p.f.u. These data show that calcium concentrations quite sufficient for cell growth are insufficient for normal virus production.

The results of experiments that establish the amount of calcium necessary for virus production are shown in Fig. 2. A significant increase in p.f.u. measured at 90 min, relative to input p.f.u., is seen only when the calcium concentration in the medium is greater than $3 \cdot 10^{-4}$ M. This concentration is at least 10 times as great as that necessary for cell growth. Maximal virus yield for the conditions of Fig. 2 occurs for calcium concentrations of $1.5 \cdot 10^{-3}$ M and higher.

Virus attachment, DNA synthesis and cell lysis

Direct measurement of the attachment of virus to cells under (+)Ca and (−)Ca conditions shows that calcium is not necessary in the medium for attachment to occur. The results of these experiments are shown in Fig. 3 for the case where

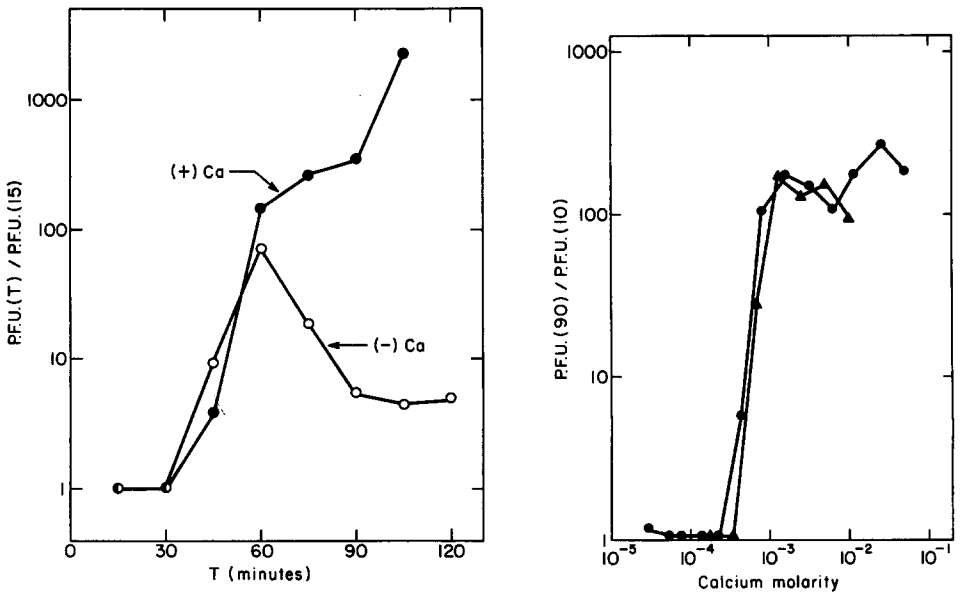

Fig. 1. Total p.f.u. relative to input p.f.u. as a function of time (T) for cultures of PS1001 infected with PM2. Cells were grown in ND-S medium with and without added calcium to a concentration of about 10^8 cells/ml at 25 °C. PM2 stocks were diluted at least 10^4 in ND medium and added (time zero) at a final concentration of about 10^4 p.f.u./ml. At various times, an aliquot of the infected culture was diluted into Q medium and assayed for p.f.u. on PS1001. The number of total p.f.u. in the infected culture at 15 min is taken as the input titer.

Fig. 2. Effect of calcium concentration on virus production. Cells grown in ND-S medium to a concentration of about $5 \cdot 10^7$ cells/ml were added to an equal volume of ND-S medium supplemented with a known concentration of calcium and allowed to grow for 1 h. PM2 stocks were diluted in ND medium and added at time zero to a final concentration of about $5 \cdot 10^4$ p.f.u./ml. At times 10 and 90 min the cultures were assayed for total p.f.u. as described in Fig. 1. The results of two separate experiments are shown as circles and triangles.

cells and attached virus were removed by filtration. (Stability of PM2 is similar under (+)Ca and (−)Ca conditions.)

The effect of calcium on DNA synthesis was determined by measuring the uptake of [^{3}H]thymidine into trichloroacetic acid precipitable material under (+)Ca and (−)Ca conditions. Franklin et al. [8] showed that total DNA synthesis in infected cultures of BAL-31 is markedly increased over the level in uninfected cultures, and that the newly synthesized DNA is mostly viral DNA. By our procedure, trichloroacetic acid precipitates from (a) an infected culture under (+)Ca conditions, (b) an infected culture under (−)Ca conditions, and (c) an uninfected culture, containing, respectively, (a) 117 300 cpm, (b) 97 500 cpm, and (c) 7500 cpm of incorporated [^{3}H]thymidine. This suggests that calcium is not essential for the synthesis of viral DNA.

Cultures of PS1001 infected with PM2 at an m.o.i. of 10 under (+)Ca conditions lyse about 50 min after infection at 25 °C, as monitored by absorbance at 660 nm (Fig. 4). Under (−)Ca conditions, lysis still occurs, although it is much less synchronous and appears to be incomplete. The absorbance in a (+)Ca infected culture

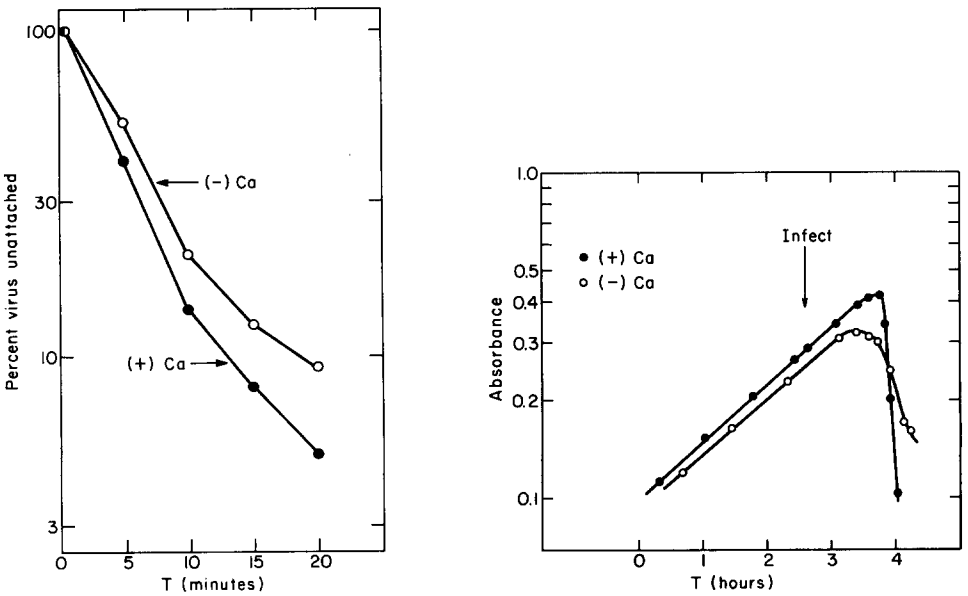

Fig. 3. Attachment of PM2 to PS1001 in media with and without added calcium. The number of unattached virus at various times has been normalized to the number at 0.5 min.

Fig. 4. Effect of calcium on the lysis of cultures of PS1001 infected with PM2. Absorbance was measured at a wavelength of 660 nm with a Bausch and Lomb Spectronic 20 colorimeter.

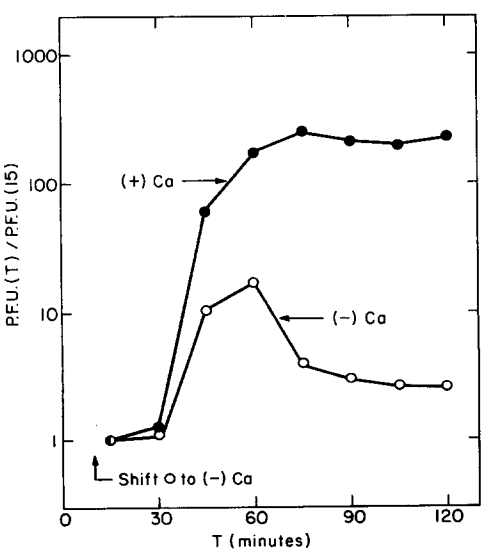

Fig. 5. Effect of shifting an infected culture out of calcium. Cells were grown in ND-S medium supplemented with 10^{-2} M calcium to a concentration of about 10^{8} cells/ml. PM2 was added (time zero) at a final concentration of about 10^{8} p.f.u./ml and 10 min later aliquots of the infected culture were diluted 10^{5} into ND-S medium with and without added calcium (10^{-2} M). At various times the cultures were assayed for total p.f.u. as described in Fig. 1.

drops about a factor of four during a period of 20 min; in a (−)Ca infected culture, the absorbance drops about a factor of two during a period of 40 min. Although lysis is somewhat impaired without calcium, the effects of calcium on lysis cannot account for the requirement of calcium for virus production.

Calcium shift experiments

Infected cultures were shifted in or out of calcium at different times after infection in order to determine when in the infectious cycle calcium is essential. Fig. 5 shows the results of an experiment in which part of a culture was shifted to (−)Ca conditions 10 min after infection. This shift was accomplished by diluting a (+)Ca culture a factor of 10^5 into ND-S medium. At the same time, the control culture was diluted by the same amount into ND-S medium containing 10^{-2} M calcium. The results are very similar to those shown in Fig. 1, and show that the presence of calcium early in the infectious cycle is not sufficient to restore virus production.

Fig. 6 shows the results of adding calcium at different times to cultures infected under (−)Ca conditions. The addition of calcium as late as 60 min after infection completely restores the production of viable virus particles. The experiments of Fig. 6 clearly show that calcium is essential only for the very final stages of infection.

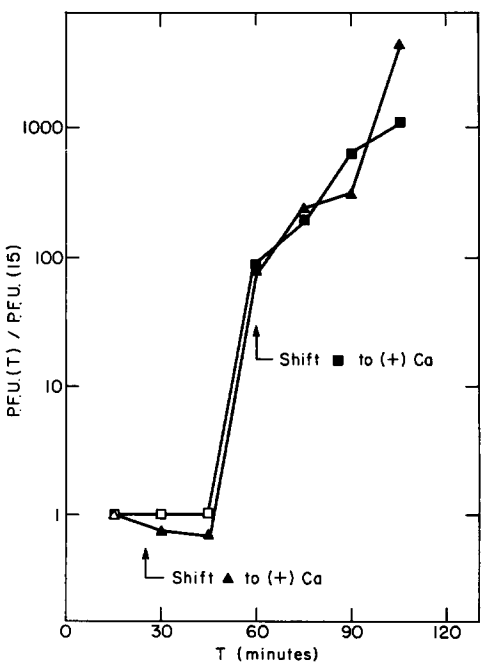

Fig. 6. Effect of adding calcium at two different times to cultures infected under (−)Ca conditions. Cells grown in ND-S medium to about 10^8 cells/ml were infected (time zero) with PM2 at a final concentration of about 10^4 p.f.u./ml. The cultures were assayed at various times for total p.f.u. as described in Fig. 1. Calcium was added at a final concentration of 10^{-2} M to one culture at 25 min after infection (triangles) and to another culture at 60 min after infection (squares). Data shown are an average of two experiments. Controls for these experiments, in which calcium was either absent or present throughout, were also performed and comprise part of the data for Fig. 1.

Requirement of calcium for production of PM2-like particles

Lysates made under (+)Ca and (−)Ca conditions were analyzed by velocity sedimentation through sucrose gradients to determine whether inactive, PM2-like particles are present after lysis of a (−)Ca infected culture. In separate experiments [^{3}H]thymidine and ^{14}C-labeled amino acids were used for radioactive labels. Fig. 7 shows the results for lysates made in the presence of [^{3}H]thymidine, and clearly indicates that no DNA-containing PM2-like particles are present. Experiments with labeled amino acids likewise showed the absence of any PM2-like particles. These results by themselves indicate either that no particles are assembled under (−)Ca conditions or, as supported by the results shown in Figs 1 and 6, that any particles which are assembled, disintegrate under conditions where viable PM2 particles, produced in (+)Ca, are stable.

Calcium content of PM2 particles

Lysates were made in the presence of ^{45}Ca and analyzed on sucrose gradients to detect the presence of any large amounts of calcium in the PM2 particles. The peak gradient fraction from a 10-ml lysate made in the presence of 100 μCi/ml of ^{45}Ca and $3 \cdot 10^{-3}$ M unlabeled calcium contained 240 cpm/ml above background and $3.5 \cdot 10^{10}$ p.f.u./ml. From this, we calculate that less than 100 Ca^{2+} ions per infectious unit are bound stably to the virus particles. Calcium bound in a rapidly exchangeable manner would not have been detected by these experiments. Any readily exchangeable calcium associated with the virion, however, cannot be essential for viral stability, since virus produced under (+)Ca conditions remain viable when placed in medium without added calcium.

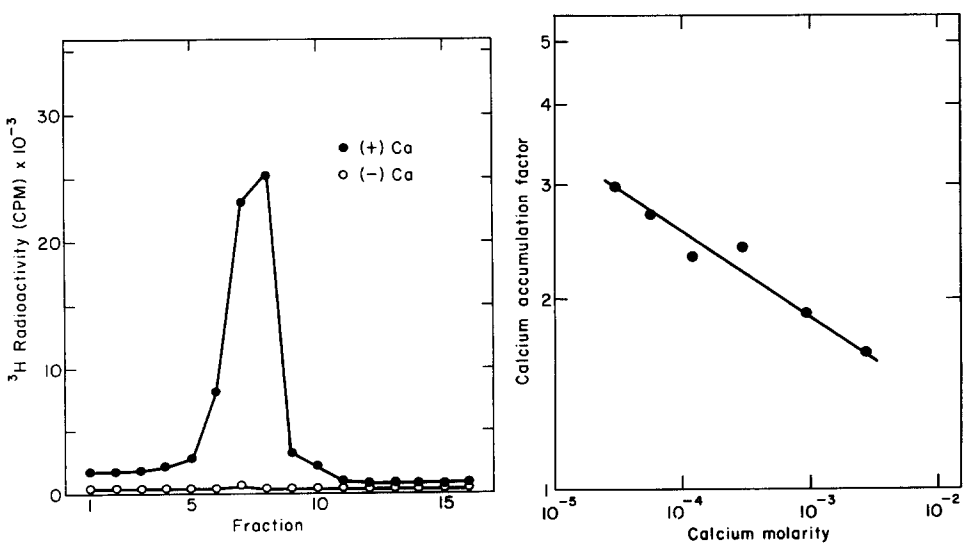

Fig. 7. Sucrose gradient analysis of viral lysates made with and without added calcium to the medium. Viral DNA was labeled by infecting cells in the presence of [^{3}H]thymidine.

Fig. 8. Accumulation of calcium by PS 1001 as a function of total calcium concentration in the growth medium. The "calcium accumulation factor" equals the ratio of ^{45}Ca to ^3H_2O in the cell pellet divided by the ratio of ^{45}Ca to ^3H_2O in the growth medium.

Accumulation of calcium by PS1001

Experiments were carried out with ^{45}Ca to determine whether the calcium concentration inside the host cell is different from the concentration in the medium. $^{3}H_2O$ was used as a secondary label, and it was assumed that $^{3}H_2O$ is neither accumulated nor excluded by cells in preference to water. Therefore, if calcium is neither accumulated nor excluded, the ratio of ^{45}Ca to ^{3}H inside the cell will be identical to that same ratio outside the cell. If the ratio inside exceeds the ratio outside, calcium is accumulated; if the ratio inside is less than the ratio outside, calcium is excluded. These ratios of radioactivity were measured for cultures containing varying amounts of unlabeled calcium, and the results are shown in Fig. 7. Cells grown in medium containing $3 \cdot 10^{-5}$ M total calcium contain about three times as much calcium per unit volume as the medium does. For higher external calcium concentrations the accumulation factor is less. We expect that accumulation factors from Fig. 7 are minimum values, since any residual medium left with the pellet in our procedure would tend to reduce the value obtained for the ratio of ^{45}Ca to ^{3}H inside the cells relative to that ratio outside.

Replacement of calcium by other divalent ions

All the experiments described above were carried out in the presence of 10^{-2} M $MgCl_2$, so it is clear that Mg^{2+} cannot replace the Ca^{2+} requirement for the production of PM2. Indeed, Mg^{2+} is a necessary requirement for growth of the host cell. We investigated the possibility that other divalent ions might replace Ca^{2+} by measuring the yield of p.f.u. at 90 min after infection relative to the input p.f.u. Cultures contained the chlorides of various ions at a concentration of 10^{-2} M, and control cultures contained, in addition, 10^{-2} M calcium. The results are shown in Table I. Strontium and barium are both capable of replacing calcium, but negative results were obtained with all other divalent ions tested. It appears that ions which cannot

TABLE I

VIRUS PRODUCTION IN THE PRESENCE OF DIVALENT IONS

Ions were added as the chloride at a concentration of 10^{-2} M.

Divalent ion	Virus yield*	
	(−)Ca**	(+)Ca***
None	3.5	165
Ca^{2+}	158	225
Mn^{2+}	0.7	0.9
Cu^{2+}	0	0
Sr^{2+}	285	260
Cd^{2+}	0.4	0.6
Zn^{2+}	0	0.3
Co^{2+}	0.2	0.2
Ba^{2+}	177	133
Ni^{2+}	0	0.1

* Average of two experiments; measured as the ratio of p.f.u. at 90 min to p.f.u. at 10 min.
** No additional calcium added.
*** 10^{-2} M Ca^{2+} was added in addition to the ion shown in the first column.

replace calcium are in fact inhibitory, since no increase in p.f.u. occurs in their presence even when calcium is present.

DISCUSSION

The experiments presented here suggest very strongly that high levels of calcium are essential for the virus assembly process during PM2 infection. The presence of calcium during the final moments of the infectious cycle is sufficient to yield stable, infective virus particles that cannot be formed in its absence. This discussion,therefore, will concentrate on the role that calcium might have in alleviating some of the problems that can be anticipated in the assembly of a lipid-containing structure like PM2.

For membrane-bound viruses that become enveloped by budding through either the nuclear or cytoplasmic membrane of the cells, the phospholipid molecules can remain basically in the stable bilayer configuration throughout the process. Only at the final stage, where the newly enclosed virion is freed from the cell membrane structure, is it required that the bilayer structure be momentarily broken. By contrast, the genesis of a new membrane must start with the aggregation or assembly of phospholipid molecules, either newly synthesized or scavenged from another membranous material. While the stability of the final product may be apparent, the ability to initiate its formation in light of the dynamic motion of membrane phospholipids is not easily comprehended. For PM2, the problem is compounded by the presence of 64% phosphatidylglycerol [3, 9], a negatively charged phospholipid, in the bilayer of the mature virion. The following calculations reveal the true magnitude of this problem and lead us to conclude that the protein layer external to the lipid bilayer must be positively charged. Subsequent to these considerations, a likely role for calcium during the process of assembly will become apparent.

The energy associated with an electric field in any region of space is given by

$$\text{Energy} = \int_V \tfrac{1}{2}\varepsilon E^2 \mathrm{d}V$$

where E is the electric field intensity, ε is the permittivity, and V is the volume it occupies. The energy of charged species in aqueous solution is vastly reduced by ordering of water dipoles about the charge, thereby reducing the volume of integration to a negligible quantity. Only in this way can free ions exist in solution. The lipid bilayer of PM2 contains about 7000 phospholipid molecules, about 4480 of which are phosphatidylglycerol*, located in a region between radii of 200 and 240 Å in the virus particle [1]. The particle radius is 300 Å, due to the protein layer outside the lipid bilayer [1]. The energy associated with the electric field from the phosphatidylglycerol molecules is 21 125 eV, or about 5 eV per phosphatidylglycerol molecule, in the region of space containing the bilayer and the external protein (see Appendix). This prodigious quantity strongly indicates that the external protein has considerable positive charge which reduces the energy. We calculate** that an average of 6.3 positive charges per protein molecule would be sufficient to neutralize the negative charge of the bilayer.

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* Based on the percentage of phosphatidylglycerol in PM2 from ref. 9.

** Based on 710 copies of protein species II suggested by Datta et al. [10] to lie outside the lipid bilayer.

During assembly, calcium could have a vital role in stabilizing the lipid bilayer prior to deposition of the protein coat. It seems highly unlikely that the complete bilayer is formed before any protein is added and more likely that assembly of the bilayer and deposition of the protein coat occur as coordinated events. Calcium may be necessary for charge cancellation only momentarily, and not as an integral part of the virus structure. Our observation that no significant amount of calcium is bound stably to the virion, coupled with the fact that virus formed under (+)Ca conditions are stable after calcium is removed from the medium, supports the point of view that calcium is not the charge-cancelling cation in the complete particle. It appears that calcium is needed only when the membrane components are in a state of change, as seems to be the case during membrane fusion [6]. In this regard, it is interesting to note that Okada and Murayama [4] report that barium and strontium can replace the calcium requirement for Sendai virus-induced cell fusion, and we find that these same ions can replace the requirement for calcium in the production of PM2.

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APPENDIX

Taking the phospholipid content of PM2 as 118 μg phospholipid per mg virus [9], the particle molecular weight as $4.5 \cdot 10^7$ [9], and an average phospholipid molecular weight of 750, we calculate that there are about 7000 phospholipid molecules per virion. The bilayer is assumed to extend from a radius (r) of 200 Å to a radius of 240 Å, with the external protein layer between radii 240 and 300 Å, as depicted by Harrison et al. [1]. The inner half of the bilayer accounts for 45% of the bilayer, or 3175 phospholipid molecules; the outer layer accounts for the remaining 3825 phospholipid molecules. Since the negative phosphatidylglycerol molecules account for 64% of the phospholipid molecules [9], we base the calculation on 2032 electron charges at $r = 200$ Å and 2448 electron charges at $r = 240$ Å. The volume of integration for the inner layer of charge is the volume in the space between $r = 200$ and 300 Å; for the outer layer of charge, the volume is in the space between $r = 240$ and 300 Å. The sum of these two contributions, taking a value of $\varepsilon = 4\varepsilon_0$ for the permittivity, is 21 250 eV per virion, or 4.74 eV per phosphotidylglycerol molecule. For comparison, the energies of a covalent bond and a hydrogen bond are about 4 eV and 0.2 eV, respectively.

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