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TEMPERATURE SENSITIVITY OF THE ASSEMBLY PROCESS OF THE ENVELOPED BACTERIOPHAGE φ6

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SUMMARY

1. Bacteriophage φ6 can produce plaques at temperatures up to about 30 °C. Above this temperature (31 or 32 °C), infected cells do not lyse. Temperature shift and pulse experiments (25 → 31 °C, 31 → 25 °C, and 31 → 25 → 31 °C) indicate that this temperature sensitivity for production of infectious virus particles occurs very late in infection and is reversible.

2. The use of spin-labeled hydrocarbons to probe the membranes of host cells and virus indicates the occurrence of a "phase transition" in both cellular and viral membranes at about 30 °C.

3. The phospholipid composition of infected cultures (at 25 or 31 °C) very late in infection is intermediate between that of uninfected cells and purified virus.

4. These results are discussed in relation to the assembly of φ6 membrane components.

INTRODUCTION

Bacteriophage φ6 is the only known bacterial virus that has a lipid-containing envelope similar to many of the mammalian viruses. The well-characterized bacteriophage PM2 also contains lipid, but the PM2 "membrane" is located at an internal region of the icosahedral virus particle. In bacteriophage φ6, however, the lipid is present in a membrane which envelopes the ribonucleoprotein core of the virion [1]. The φ6 envelope has the same fatty acid composition as the host cell, *Pseudomonas phaseolicola* HB10Y [1], but the phospholipid composition of φ6 differs from that of the host cell in that the virus contains a greater relative amount of phosphatidylglycerol [2].

We anticipate that φ6 may provide a useful system for studying various membrane-related aspects of the biology of the enveloped viruses. Of particular interest is the problem of assembly processes of enveloped viruses, specifically the envelope acquisition process.

As a start towards understanding the physical aspects of the φ6 envelope-acquisition process, we have begun investigating the effect of environmental conditions

on virus assembly. In this paper, we report the existence and explore the nature of a temperature sensitivity of the very late stages of the φ6 infectious cycle.

MATERIALS AND METHODS

Routine growth of cells and virus

The medium used for routine cell growth and virus production, NBY medium [1], contains 8 g nutrient broth, 2 g yeast extract, 0.5 g KH_2PO_4, 2 g K_2HPO_4, 5 g glucose, and 0.25 g $MgSO_4 \cdot 7H_2O$ per l of distilled water. TG9 medium, used for growth under radioisotope labelling conditions, contains 12.1 g Tris, 3 g KCl, 6 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g KH_2PO_4, 5 g glucose, and 20 mg each of arginine, proline, alanine, histidine, leucine, methionine, threonine, tryptophane, and cystine per l of distilled water. TG9 medium is adjusted to pH 7.1 by addition of HCl.

Ps. phaseolicola HB10Y was routinely grown in NBY medium at 25 °C with aeration. Stocks of bacteriophage φ6 were prepared by infecting a culture of 10^8 cells/ml with φ6 at a multiplicity of infection of 0.1. Following lysis, cellular debris was removed by low-speed centrifugation and the resulting supernatant was stored at 0 °C.

For plaque production on plates, bottom agar and top agar consist of NBY medium hardened with Bacto-agar at final concentrations of 1.5 and 0.5%, respectively. The infectivity of a suspension of virus and/or virus-infected cells was determined by mixing 0.1 ml of a dilution of the suspension with $3 \cdot 10^7$ HB10Y cells in 5 ml of melted top agar at 40 °C, then pouring into a plate containing 25 ml of hardened bottom agar. Following incubation of plates at 25 °C for 16 h, plaques are easily countable.

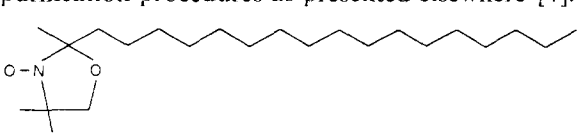
To assay for the ability of φ6 to cause cellular lysis at various temperatures, 10 ml cultures of HB10Y at 10^8 cells/ml growing at the appropriate temperature were infected with φ6 at a multiplicity of infection of 10. At various later times, the absorbance at 600 nm of an infected culture was monitored by use of a Bausch and Lomb Spectronic 20 colorimeter.

Phage purification

A 4-l lysate of φ6, produced at 25 °C, was concentrated by polyethyleneglycol–NaCl treatment [1]. It was then purified by velocity sedimentation in a 15–35% sucrose gradient [2], dialyzed overnight against Buffer A [1] and then re-purified by centrifugation in a 30–60% sucrose gradient (in TG9 medium) by spinning at 27 000 rev./min for 9 h in a SW27 rotor. The peak fraction of infectivity collected from this second gradient contained 10^{13} p.f.u./ml. These purified virus particles were then diluted in TG9 medium and pelleted by centrifugation at 20 000 rev./min for 2 h in a Type 30 rotor.

Spin label experiments

A hydrocarbon spin label oxazolidine, 2N19, was used as a membrane probe. This compound was synthesized using the procedure of Keana et al. [3] and general purification procedures as presented elsewhere [4].



For probing cellular membranes, 25 ml of cells were grown at 25 °C to about 10^8 cells/ml, then centrifuged and resuspended in 0.1 ml of TG9 medium. Spin label was added to a final concentration of about 10^{-4} M. Likewise, the phage membrane was probed by resuspending a purified phage pellet in 0.05 ml TG9 medium, and adding spin label to a final concentration of about 10^{-4} M.

A Japan Electron Optics electron paramagnetic resonance x-band spectrometer JES-ME-1X equipped with a laboratory-constructed variable temperature controller accurate to better than ± 0.5 °C was employed.

Approximations of rotational correlation time (τ_c) were carried out using the expression

$$\tau_c = KW_1 \left[\left(\frac{h_1}{h_{-1}} \right)^{\frac{1}{2}} - 1 \right]$$

which is similar to the form used before [5] except that h_1 and W_1, the first derivative low field line height and line width, replace h_0 and W_0, the first derivative midfield line height and width, in order to avoid errors introduced by concentration dependent line broadening mechanisms which affect only the midline. K is a constant that depends on the microwave frequency, the anisotropic hyperfine tensor terms, and the anisotropic g terms for the particular spin label.

Determination of phospholipid composition of lysate

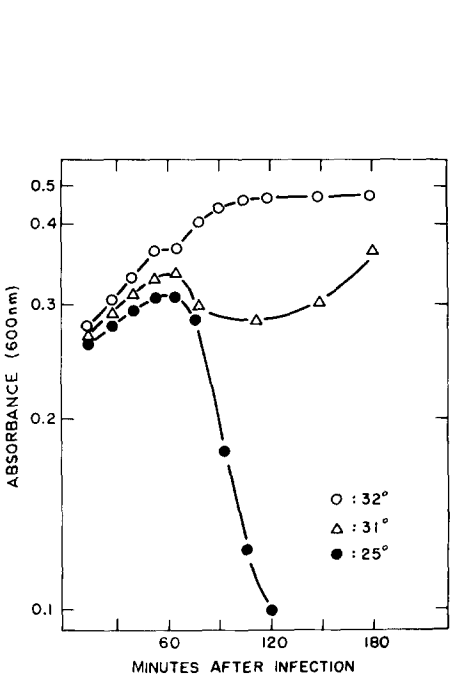
50 μCi of $^{32}PO_4^{3-}$ was added to 50 ml cultures of HB10Y cells in TG9 medium at $5 \cdot 10^7$ cells/ml at 25 or 31 °C. 30 min later, φ6 (in TG9 medium) was added to give a multiplicity of infection of 20. One uninfected culture was maintained as a control. The absorbance of the culture infected at 25 °C was followed, and all the cultures were transferred to 0 °C when the absorbance of the 25 °C-infected culture fell below 0.05 (from a high of about 0.3 immediately prior to the commencement of lysis). Following overnight storage at 0 °C, the cultures were dried and phospholipids were extracted and analyzed as described previously [2].

RESULTS

Virus production at various temperatures

The host cell for bacteriophage φ6, *Ps. phaseolicola* HB10Y, can grow well at temperatures up to 34 °C. On agar plates, φ6 can produce large clear plaques on a lawn of HB10Y cells at 25 °C, cloudy plaques at 29 °C, and no visible plaques at 31 °C. Thus, temperatures at or slightly above 31 °C prevent normal virus production but not cellular growth and division.

Preliminary experiments indicated that this temperature sensitivity of φ6 production was not due to lack of virus attachment to the host cell at 31 °C, as will become clear as the results are described. To determine whether φ6 could still cause lysis of HB10Y cells at 31 °C and above, we infected cultures of cells with φ6 at a multiplicity of infection of ten, incubated the liquid cultures with aeration at various temperatures, and followed the absorbance of the cultures as a function of time after infection. The results of these experiments are shown in Fig. 1. At 25 °C, lysis begins at about 70 min after infection and is complete by about 120 min. At 31 °C, the



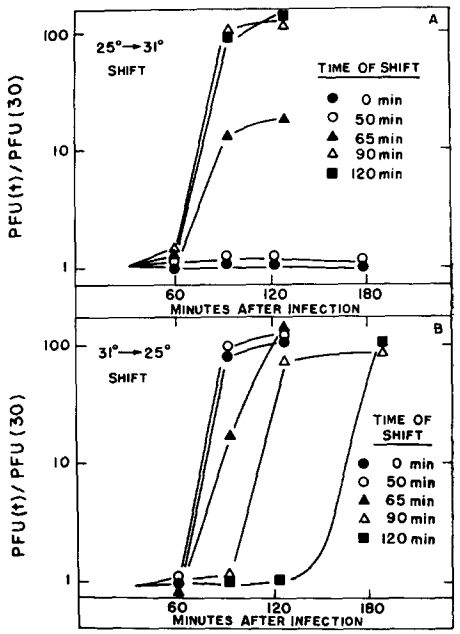


Fig. 1. Ability of bacteriophage $\varphi 6$ to cause cellular lysis at various temperatures. The absorbance of infected cultures at 600 nm was recorded, for which an absorbance of 0.3 corresponds to about $1 \cdot 10^8$ cells/ml.

Fig. 2. Effect of temperature shifts at various times after infection on the production of infectious virus particles from $\varphi 6$-infected cultures of HB10Y cells. The ordinate is plotted as the ratio of p.f.u. in the culture at the time of assay to the p.f.u. present at 30 min after infection.

absorbance drops slightly beginning at about 70 min, but significant lysis does not occur. At 32 °C, there is no drop in absorbance, merely a slight lag at about 60–70 min after infection. Thus, the defect in a 31 °C infection is such that it prevents significant cellular lysis from occurring at this temperature.

Analysis of single step virus growth

To determine the stage of the infectious cycle of $\varphi 6$ which is sensitive to temperatures at or above 31 °C, single step virus growth experiments were conducted under conditions of temperature shifts at various times during infection. The effect of shifting an infected culture from 25 to 31 °C at various times after infection is shown in Fig. 2a. A shift to 31 °C as late as 50 min completely prevents release of infectious virus particles, while a shift to 31 °C at 65 min, just as lysis is about to commence, reduces the yield of virus by 80 %. Fig. 2a thus indicates that a temperature of 31 °C very late in the infectious cycle prevents release of infectious virus particles.

The results of shift down experiments, where cultures infected at 31 °C were shifted to 25 °C at various times after infection, are shown in Fig. 2b. As shown in the figure, a shift from 31 to 25 °C as late as 50 min allows for production of a normal amount of infectious virus particles at the normal time (70–90 min). If a culture is

held at 31 °C beyond this time, lysis is delayed, and virus production does not occur until after a subsequent shift to 25 °C. These data further support the indication of Fig. 2a that the temperature sensitivity of the φ6 infectious cycle occurs late in infection.

To further investigate the nature of this temperature sensitivity late in infection, temperature pulse experiments were conducted. These experiments were similar to those described in Fig. 2b, i.e. infection at 31 °C followed by a shift to 25 °C at some later time, except that the culture was shifted back up to 31 °C 15 min after the 31 → 25 °C shift. That is, these experiments investigated the effect of a short pulse of permissive (25 °C) temperature at various times in an infection at the non-permissive (31 °C) temperature. The results of these experiments are shown in Fig. 3. Pulses of permissive temperature at any time during or after which normal virus production would be occurring at 25 °C result in rapid release of infectious virus particles. For example, if the 25 °C pulse occurs from 105 to 120 min after infection, significant virus release (approx. 35 p.f.u./infected cell) occurs by the end of the pulse. If the 25 °C pulse occurs at even later times, the release of infectious virus particles occurs at a slower rate and results in quantitatively less production of infectious virus particles. The results shown in Figs 2b and 3 indicate that the defect in a 31 °C infection is reversible, in that virus particles can be released within 15–30 min of a shift from 31 to 25 °C even later than the normal lysis time.

We investigated the possibility that the temperature sensitive defect is in lysozyme activity by producing lysis artifically. Cells were infected at 31 °C and 90 min later, when a shift to 25 °C would have resulted in rapid release of infectious virus, the culture was lysed by sonication under conditions that do not reduce the viability

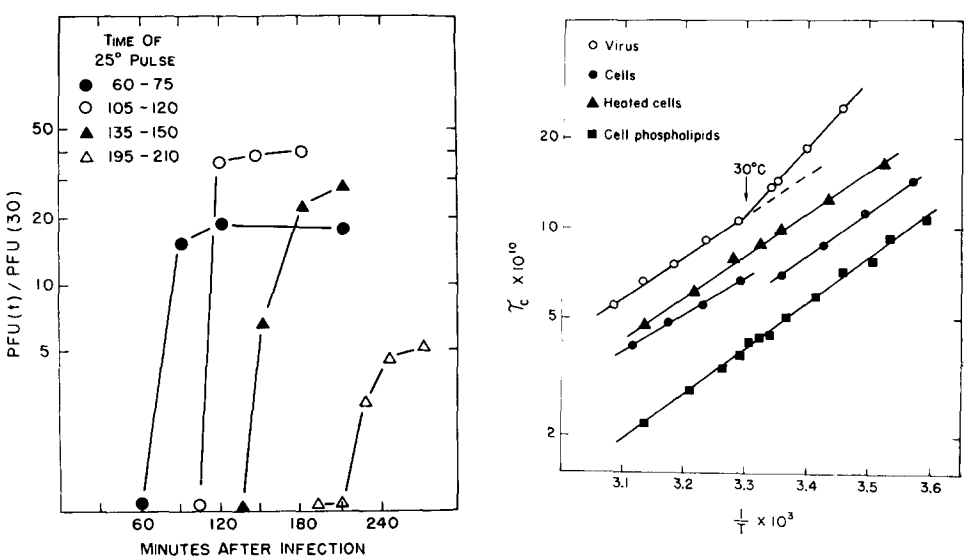


Fig. 3. Effect of a short interval at 25 °C during a 31 °C infection. In all cases, cultures infected at 31 °C were given a 15-min pulse at 25 °C at various times after infection.

Fig. 4. Arrhenius plots of the motion parameter τ_c of the spin-labelled hydrocarbon 2N19 in *Ps. phaseolicola* HB10Y cells grown at 25 °C, HB10Y cells grown at 25 °C and then heated at 65 °C for 10 min, bacteriophage φ6 produced at 25 °C, and phospholipids extracted from cells grown at 25 °C.

of virus particles. No infectious virus were released. This indicates that cells late in infection, at 31.5 °C, do not contain mature virions ready for release upon lowering of the temperature to 25 °C.

Motion of spin-labeled hydrocarbons in φ6 and host cell membranes

The results of the temperature shift and pulse experiments described above indicate that the temperature sensitivity of the φ6 infectious cycle occurs very late in infection, suggesting that the sensitivity involves some aspect of viral assembly. We probed the fluidity of the φ6 membrane and the membranes of the host cell by use of a spin labeled hydrocarbon, 2N19. As Fig. 4 shows, the hydrocarbon environment probed by 2N19 is more fluid in the host cell than in the virion. Moreover, for both φ6 and HB10Y cells, a discontinuity in the slope of the Arrhenius plot is observed at about 28–32 °C and appears to coincide with the temperature above which φ6 particles are not assembled.

Phospholipids extracted from HB10Y cells allow greater spin label motion than cell membranes. Furthermore, no phase transition is observed for the extracted phospholipids. This suggests an involvement of membrane proteins in determining the degree of fluidity of the hydrocarbon zones of native membrane of the host cell. Further evidence for this comes from the observation that heat-treated cells also do not exhibit the phase transition in the region near 30 °C (Fig. 4).

Phospholipid composition of lysates

A necessary prerequisite to a physical understanding of the envelope aquisition process of φ6 and effects of temperatures on this process is a knowledge of the biochemical composition of (i) host cell membranes, (ii) the φ6 membrane, and (iii) the membranes present very late in infection. We have previously reported the phospholipid composition of the membranes of HB10Y cells and φ6 [2]. We found that HB10Y and φ6 each contain three major classes of phospholipid: phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol (cardiolipin). However, in HB10Y cells, phosphatidylethanolamine is the most prevalent (56–70 % of total phospholipid), while in φ6, phosphatidylglycerol is the most prevalent (55–60 %). In addition, the phospholipid composition in HB10Y cells appears to be approximately the same in both the cytoplasmic membrane and the outer membrane (unpublished data). While this indicates that φ6 does not acquire its envelope from a random, unaltered region of cellular membrane material, it suggests several possibilities for the biochemical steps involved in the envelope acquisition process. To shorten the list of these possibilities, we have now investigated the phospholipid composition of a culture very late (i.e. after lysis at 25 °C) in the infectious cycle. Cells were grown in medium containing ^{32}P for one generation at 25 and 31 °C. Then φ6 was added at a multiplicity of infection of 20 and aeration was continued until lysis of the 25 °C culture was complete (4 h in TG9, drop in absorbance at 600 nm from 0.3 to 0.04). The phospholipid composition of each culture (a 25 °C lysate, a 31 °C infected cell culture, and a culture of uninfected cells) was then determined as previously described [2]. The results of this experiment are shown in Table I.

The results of these experiments indicate that there is some net synthesis of phosphatidylglycerol during φ6 infection at both 25 and 31 °C. If this were not so, the phospholipid composition of a 25 °C lysate (virus + cellular debris + any unlysed

TABLE I

PHOSPHOLIPID COMPOSITION OF φ6-INFECTED HB10Y CELLS

Source of phospholipids	Number of independent determinations	% of total phospholipid (average of two experiments)		
		DPG*	PE**	PG***
HB10Y Cells	4	10	68	22
φ6	4	8	34	58
Lysate produced at 25°	2	8	55	37
Cells infected for 4 hours at 31°	2	6	56	38

* DPG, diphosphatidylglycerol.
** PE, phosphatidylethanolamine.
*** PG, phosphatidylglycerol.

cells) should be the same as that of uninfected cells. However, phosphatidylglycerol is not synthesized to such an extent that the entire cellular membrane becomes as rich in this phospholipid as the φ6 membrane, for if this were so, the phospholipid composition of a 25 °C lysate should be nearly the same as that of purified φ6 particles. Our observation that phosphatidylglycerol levels are elevated during infection at both 25 and 31 °C shows that the temperature sensitivity for virus production is not due to lack of phosphatidylglycerol synthesis.

DISCUSSION

The phospholipid composition of *Ps. phaesiolicola* HB10Y is modified during infection by the membrane-bounded phage φ6, resulting in an increased proportion of phosphatidylglycerol (increases from 22 to 37 %). The virus particles resulting from successful infection have a still higher concentration of phosphatidylglycerol (58 %). The events required to produce mature virions include a mechanism to concentrate this phospholipid in a suitable way expedient to the final steps of assembly. We believe an understanding of the logistics of phosphatidylglycerol during phage assembly may well by central to understanding the dynamic mechanisms of such membrane assembly processes.

We present a consideration of possible mechanisms by which the phospholipid composition of φ6 may become spatially ordered and relate these postulates to our findings:

I. Assembly of φ6 is a direct process where part or all of the phospholipid components go directly into the three dimensional structure without existing first as a cell membrane associated two dimensional structure.

II. The appropriate phospholipid composition is generated in the bacterial cytoplasmic membrane prior to final assembly into a mature virion. (1) Phosphatidylglycerol is accumulated in localities in the bacterial membrane and is maintained due to phospholipid physical properties for a time-duration adequate for assembly. (a) These phospholipid patches are produced by localized synthesis of phosphati-

dylglycerol. (b) The phospholipid patches are produced by a phase separation of phosphatidylglycerol which was synthesized and located throughout the bacterial membrane to form regions rich in phosphatidylglycerol. (2) A barrier to the diffusion of phospholipids composed of a protein network maintains a high concentration of locally synthesized phosphatidylglycerol. (3) Aggregated φ6 proteins located in the bacterial membrane preferentially bind phosphatidylglycerol thereby creating local zones rich in the content of this phospholipid. With regard to the temperature sensitivity for virus production, there are two possibilities. (a) The protein–protein association may be preferentially temperature sensitive. (b) The protein–phosphatidylglycerol interaction may be preferentially temperature sensitive.

I. Direct three dimensional assembly

Geometrical considerations would lead one to believe that a two dimensional organization is more simple and in the assembly process would precede the final three dimensional phage geometry. While this consideration is logical the possibility remains that some process or mechanism may exist by which the phospholipid molecules are synthesized or collected directly onto the three dimensionally structured phage particle (I above). Although this possibility remains, we will not treat if further here.

II. Two dimensional assembly occurs first

The temperature sensitivity of the final events of phage φ6 assembly, the coincidence of this temperature sensitivity with an ESR-detectable "phase transition" in cellular and viral membranes, and the loss of this transition after protein denaturation by heat suggest that this virus assembly process is tightly coupled to membrane structural ordering of the phage components in the bacterial membrane before the three dimensional viral structure is produced.

One way (1a) to achieve this is to have local patches of phosphatidylglycerol enzymatically produced at the site of assembly and for the physical properties of the local phospholipid domains to have an adequate time-dependent stability to allow for assembly of all the other required components before diffusion processes cause dilution of the phosphatidylglycerol-rich patch. Another and similar process (1b) is that the physical properties of the membranes of infected cells result in a phase separation into regions abundant in phosphatidylglycerol and other regions scarce in this phospholipid. Indeed, such phase separations might even occur in uninfected cells. Recent data on phospholipid bilayers and vesicles [7, 8] and *Escherichia coli* membranes [9] indicate that such phase separations may be commonplace in membranes that contain charged phospholipids such as phosphatidylglycerol.

(2) A protein aggregation pattern where a network of protein is constructed containing a population of trapped or semi-trapped phospholipid molecules constitutes a local zone ideal for containing high proportions of nascent phosphatidylglycerol. Such zones would facilitate the two dimensional assembly of phage membrane components and could function as a diffusion barrier for an adequate time to allow completion of the viral assembly process.

(3) Another possibility is that the primary information for concentrating phosphatidylglycerol is contained in binding sites specific for this phospholipid on a species of membrane protein. These proteins would probably also have protein–

protein binding sites to facilitate containment of a region corresponding to the size of a mature φ6 virion. Since this postulate requires two sets of binding sites, either or both could be preferentially sensitive to temperature and likewise either or both could be sensitive to the physical state of the membrane lipids.

All three possibilities discussed above are consistent with our observations and at this point in the development of knowledge about φ6 assembly all seem plausible. We realize that the results presented here could be explained several other ways than discussed above. Future experiments on the φ6 assembly process will be designed to distinguish among the various possibilities and to further elucidate the process of envelope formation in this system.

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