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CONTROL OF PHOSPHOLIPID SYNTHESIS AND VIRAL ASSEMBLY BY BACTERIOPHAGE PM2

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SUMMARY

The control of phospholipid synthesis exerted by the lipid-containing bacteriophage PM2 was studied by employing a glycerol-requiring auxotroph of the host cell *Pseudomonas* BAL-31. Double-isotope, pulse-label experiments suggest that cellular phospholipid polar groups are cleaved and replaced by glycerol during infection. A temperature sensitive virus mutant was isolated which cannot effect this polar group conversion at its restrictive temperature, 31.5 °C. This mutant lyses infected cells at 31.5 °C, but no stable virus particles are formed. This suggests that the phospholipid polar group conversion may be an essential feature for viral assembly in this system.

INTRODUCTION

The lipid-containing bacterial virus PM2 and its host, *Pseudomonas* BAL-31, both contain phosphatidylglycerol and phosphatidylethanolamine as their major phospholipids [1]. The relative amounts of these two phospholipids, however, are quite different in the virus and in its host. Bacteriophage PM2 contains about 67 % phosphatidylglycerol and 28 % phosphatidylethanolamine, whereas uninfected strain BAL-31 cells contain 23 % phosphatidylglycerol and 75 % phosphatidylethanolamine. During infection, cellular levels of phosphatidylglycerol rise and there is an appearance of significant quantities of phosphatidic acid [1]. It appears, therefore, that bacteriophage PM2 modifies cellular phospholipid synthesis to generate its own membrane structure.

Two aspects of phospholipid synthesis in bacteriophage PM2-infected cells are dealt with in this report. The first concerns the mechanism by which phosphatidylglycerol levels are elevated during infection. Double isotope, pulse-label experiments with a glycerol requiring derivative of strain BAL-31 show that phospholipids are synthesized or modified in infected cells by pathways not utilized in uninfected cells.

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The second aspect concerns the genetic control of phospholipid modification in infected cells and its relation to viral assembly. A temperature sensitive virus mutant which cannot effect phospholipid modification also cannot assemble stable virus particles at its restrictive temperature.

MATERIALS AND METHODS

Growth media

The two basic media used for these experiments have been described previously [2]. 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 contains various inorganic salts and is buffered to pH 7.6 with Tris–HCl. Medium 25-S contains, in addition, tryptophan at 20 μg/ml and thymidine at 50 μg/ml.

Isolation of bacterial strain GT12

Pseudomonas BAL-31 was mutagenized by exposure to 20 μg/ml nitrosoguanidine for 30 min in Q medium at 25 °C, a treatment that results in approximately 5 % survival. The cells were centrifuged, washed twice with Q medium, diluted 5-fold and grown 4 h in Q medium, containing 500 μg/ml glycerol. An aliquot was then centrifuged, washed 3 times in Medium 25, diluted 3-fold, and grown 1 h to further deplete the cells of glycerol. Penicillin G was then added at 60 000 units/ml for 4 h, resulting in 0.25 % survival. The cells were then diluted and plated. Colonies grown on Medium 25 plates supplemented with glycerol were replicated onto unsupplemented plates, and suspect colonies were tested by streaking. A glycerol auxotroph, designated GA1, was obtained.

A thymine requiring derivative of strain GA1 was isolated by treatment of a liquid culture with Trimethoprim [3]. Colonies grown on Medium 25 supplemented with glycerol and thymidine were tested for growth requirements by streaking, and the glycerol-thymidine double auxotroph, designated strain GT12, was obtained.

Isolation of virus mutant MF7

The bacterial virus PM2 was mutagenized by the following procedure. A 5-ml culture of *Pseudomonas* BAL-31 at approximately $1.5 \cdot 10^8$ cells/ml in Q medium was infected with bacteriophage PM2 at a multiplicity of infection of about 1. Five minutes later, nitrosoguanidine was added to a final concentration of 10 μg/ml. The culture was aerated for 50 min to allow one round of virus growth, which resulted in production of viable virus at about 20 % of the normal amount. The culture was then added to 40 ml of *Pseudomonas* BAL-31 at about 10^8 cells/ml and grown until total lysis occurred. The lysate was centrifuged to remove cell debris and dilutions were plated for plaques at 25 °C.

The temperature sensitive mutant MF7 was isolated as follows. About $3 \cdot 10^7$ cells were added (in 5 ml top agar) to Q plates and incubated at 31.5 °C for 2 h. At 32–35 °C, plaques of the mutagenized virus were picked with a toothpick and streaked onto two plates of cells in top agar. The plates were then incubated at 25 °C and 31.5 °C for about 6 h and then scored for streaks which showed no viral

growth at 31.5 °C. Stocks were grown from the corresponding 25 °C streaks and assayed for ability to produce plaques at 25 °C and 31.5 °C. Mutant MF7, obtained by this procedure, had the property of lysing infected cultures at 31.5 °C (multiplicity of infection > 1), but producing no infectious virus at that temperature.

Preparation of labeled virus

Virus were radioactively labeled by growth in the presence of [^{3}H]glycerol and ^{32}P. The lysates were centrifuged at low speed to remove cell debris and the virus were pelleted by centrifugation at 20 000 rev./min for 2 h in a SW40 rotor. The pellet was resuspended in 0.3 ml Medium 25 and, in some cases, passed through a P-100 column (made with Medium 25) to remove unincorporated label that remained after the initial pelleting step. The virus were purified by sedimenting through a 20–30 % sucrose gradient as described previously [2]. The virus obtained from the peak fractions, when analyzed for phospholipid content, contained phosphatidylglycerol and phosphatidylethanolamine in proportions within 15 % of these reported [1], and were therefore considered to be free of any significant amount of contaminating cellular phospholipid. Protein purity was of no interest in these experiments, and no examination of protein content was made.

Thin-layer chromatography

The chromatographic procedure of Braunstein and Franklin [1] employing silica gel GF thin layer plates with chloroform : methanol : water (65 : 25 : 4, v/v) as solvent was used for analysis of phospholipids. ^{32}P labeled components were detected and located on the plates by exposure of film to the developed plates. Spots were scraped from the plates, eluted repeatedly with methanol, and dried in scintillation counting vials. After additions of scintillation fluid, radioactivity measurements were made on a Beckman Model 240 liquid scintillation spectrometer. Corrections were made, when necessary, for ^{32}P counts occurring in the ^{3}H channel of the counter.

Sources of materials

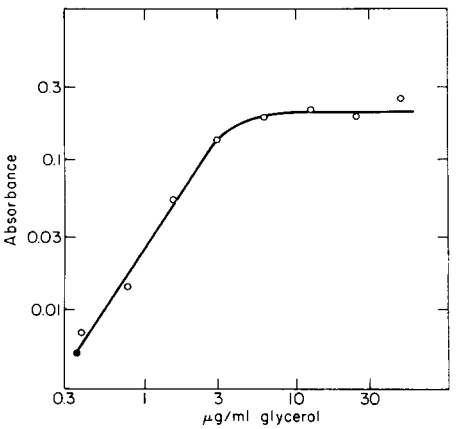
The column packing material P-100 was obtained from Bio-Rad Associates (Richmond, Calif.). [^{3}H]Glycerol (1 mCi/ml, 0.46 mg/ml) and $H_3{}^{32}PO_4$ (carrier free) were obtained from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Characterization of strain GT12

When supplied with glucose as a carbon source, strain GT12 requires low levels of glycerol for growth. Fig. 1 shows the absorbance of cultures after 20 h growth in varying amounts of glycerol. Maximal growth is achieved with about 6 μm/ml glycerol in the growth medium. Similar results were obtained with thymidine, with about 10 μg/ml producing maximal growth. We routinely culture strain GT12 in medium supplemented with 20 μg/ml glycerol and 50 μg/ml thymidine. When labeling with radioactive glycerol, the unlabeled glycerol level is reduced to 10 μg/ml.

In Q medium strain GT12 grows more slowly than strain BAL-31. At 25 °C the doubling times of strain GT12 and strain BAL-31 are about 84 and 36 min,



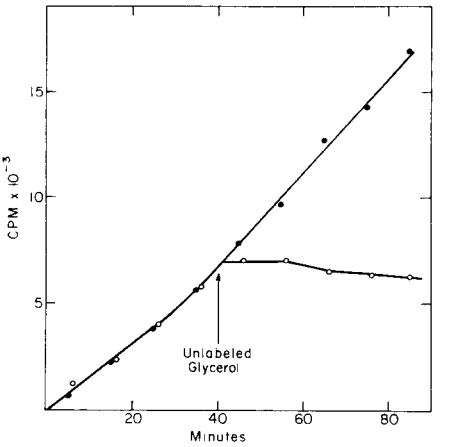


Fig. 1. Requirement of glycerol for growth of cell mutant strain GT12. A culture growing in Medium 25-S plus 20 μg/ml glycerol, at about $5 \cdot 10^7$ cells/ml, was centrifuged, resuspended in an equal volume of Medium 25-S, and diluted 10-fold into Medium 25-S supplemented with varying amounts of glycerol. After 20 h growth with aeration at 25 °C, the cultures were diluted 2-fold with Medium 25-S and the absorbance at 660 nm was measured with a Bausch and Lomb Spectronic 20 colorimeter.

Fig. 2. Termination of [^{3}H]glycerol incorporation by dilution with unlabeled glycerol. A culture of strain GT12 was grown in Medium 25-S plus 10 μg/ml glycerol to about 10^8 cells/ml. At time zero, [^{3}H]glycerol was added to a final activity of 1 μCi/ml and the culture was divided two ways. At $t = 40$ min unlabeled glycerol was added to one portion (○–○) at a final concentration 1.25 mg/ml. [^{3}H]Glycerol incorporation was determined by collecting 0.5 ml cells on a 0.22 μ Millipore filter, washing three times with 1 ml Medium 25, drying the filter, and counting by liquid scintillation spectrometry.

respectively. In Medium 25 supplemented with glycerol and thymidine, strain GT12 and strain BAL-31 have doubling times of about 120–150 and 60–80 min, respectively, at 25 °C.

Cultures of strain GT12 grown in Medium 25 supplemented with thymidine (50 μg/ml) and glycerol (10 μg/ml) incorporate glycerol almost exclusively into the lipid fraction, as shown by the following experiments. Cells were grown in the presence of 10 μCi/ml [^{3}H]glycerol, centrifuged, washed twice to remove unincorporated label, and the final pellet was extracted overnight with chloroform : methanol (2 : 1). More than 95 % of the ^{3}H counts were located in the extract. Purified PM2 virus grown on strain GT12 in the presence of [^{3}H]glycerol also contained ^{3}H counts largely in extractable form. When samples were dried by evacuation and extracted in chloroform : methanol (2 : 1, by vol.), 85–92 % of the ^{3}H counts were extractable. By the Folch wash procedure [4] using chloroform : methanol : water (8 : 4 : 3, by vol.), 92–97 % of the ^{3}H counts were located in the lower phase.

The addition of excess unlabeled glycerol to cultures growing in [^{3}H]-glycerol stopped further incorporation of label within a few minutes. This observation, shown in Fig. 2, allows the termination of labeling in pulse label experiments by simply flooding the culture with unlabeled glycerol.

Characterization of mutant MF7

The temperature-sensitive virus mutant MF7 will form plaques at 25 °C but

not at 31.5 °C, both these temperatures being permissive for wild type PM2 virus. Subsequent observations showed that host cells are killed when infected with mutant MF7 at both 25 °C and 31.5 °C. Furthermore, cultures infected with mutant MF7 at a high multiplicity of infection undergo lysis at both 25 °C and 31.5 °C. Fig. 3 shows the absorbance vs. time for cultures infected in Q medium at the two temperatures. Although lysis is as complete at 31.5 °C as at 25 °C, plating experiments show that the lysate made at 31.5 °C contains no infectious virus (above the input level).

We analyzed lysates of mutant MF7 made at 25 °C and 31.5 °C to determine whether any stable, virus-like particles are produced at the higher temperature. In separate experiments, viral protein or viral DNA was labeled with either ^{14}C-labeled amino acids or ^{3}H-labeled thymidine. When ^{3}H-labeled thymidine was used, the virus were grown on strain PS1001, a thymine-requiring derivative of strain BAL-31 described previously [2]. When ^{14}C-labeled amino acids were used, cultures were grown in M3 medium [2], which is similar to Medium 25 but lacks several amino acids, Lysates made at 25 °C and 31.5 °C were centrifuged first at low speed to remove cell debris and then at high speed to pellet any virus-like particles. The pellets were analyzed on sucrose gradients, as seen in Fig. 4 for the case where radioactive amino acids were used. Similar results were obtained with [^{3}H]thymidine. It is clear from the data of Fig. 4 that no stable particles with sedimentation properties similar to those of bacteriophage PM2 are produced in 31.5 °C lysates of mutant MF7. This suggests that the genetic defect in mutant MF7 is one that prevents the assembly of normal virus at 31.5 °C.

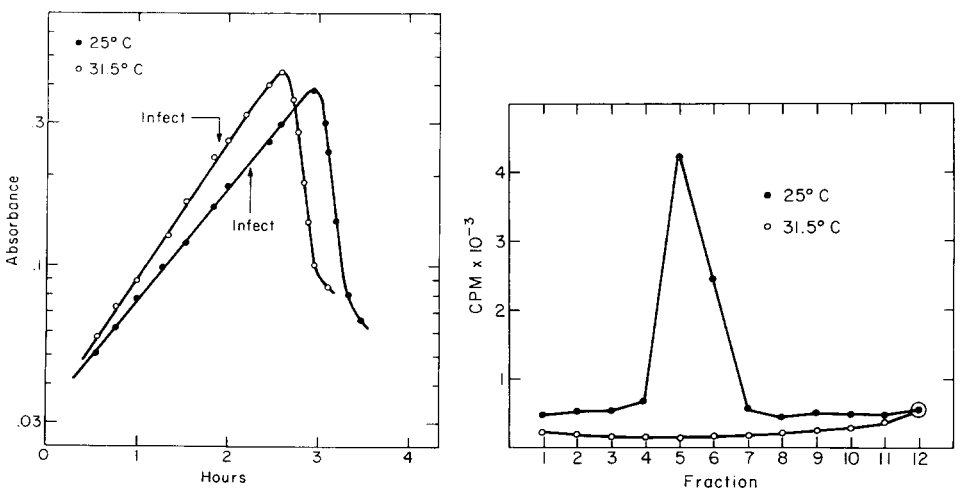


Fig. 3. Lysis of host cells by temperature sensitive virus mutant MF7 at its permissive (25 °C) and restrictive (31.5 °C) temperatures. Cultures growing in Q medium were infected at a multiplicity of infection of approx. 10 at the times indicated.

Fig. 4. Sucrose gradient analysis of mutant MF7 lysates made at two temperatures. Five-ml cultures of PS1001 in M3 medium were infected with mutant MF7 at a multiplicity of infection of 10. 10 μCi of ^{14}C-labeled amino acid mixture was added to each culture at the time of infection. After lysis, the cultures were centrifuged at low speed to remove cell debris and the virus were pelleted by centrifugation at 20 000 rev./min for 2 h in an SW40 rotor. The resuspended pellet in 0.3 ml Medium 25 was layered onto a 20–30 % sucrose gradient in 50 % STG medium [2] and centrifuged for 160 min at 35 000 rev./min in a SW40 rotor. 0.5-ml fractions were collected and assayed for radioactivity.

Temperature shift experiments showed that the temperature-sensitive function in mutant MF7 is required only during the later stages of development. The data of Fig. 5 illustrate this point with cultures shifted either up (25 °C to 31.5 °C) or down (31.5 °C to 25 °C) about halfway through the infectious cycle. It is clear that a temperature of 31.5 °C for times up to 25 min has no effect on production of viable virus if the temperature is lowered at that time. Furthermore, a temperature of 31.5 °C starting at 25 min completely prevents the production of viable virus. Additional experiments in which cultures infected at 31.5 °C were shifted to 25 °C at times throughout the infection cycle showed that a permissive temperature for the last few minutes prior to lysis was sufficient for production of viable virus. The temporal location of the genetic defect in mutant MF7 is consistent with the defect being one that prevents normal virus assembly.

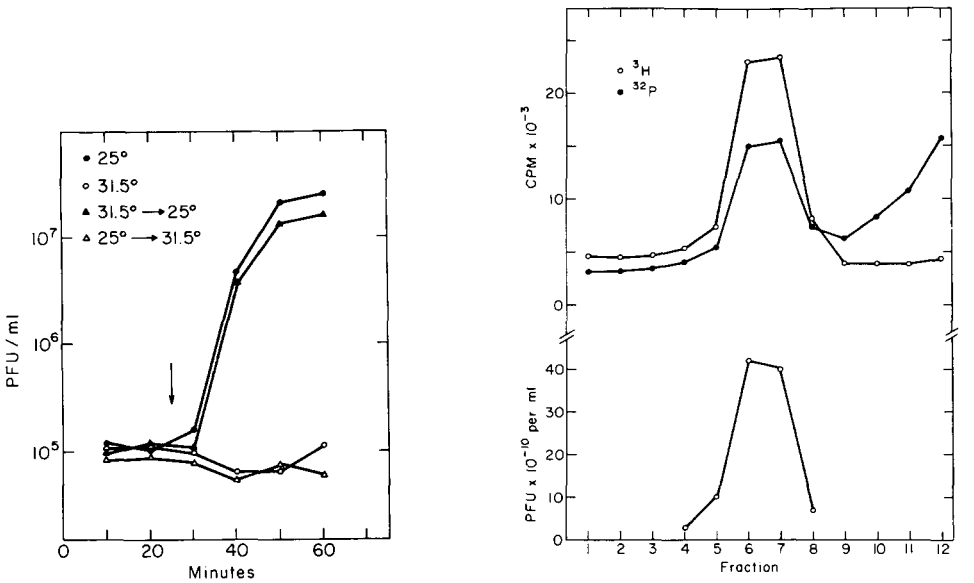


Fig. 5. Temperature shift experiments with virus mutant MF7. At time zero cultures of PS1001 in Medium 25 were infected with mutant MF7 at a multiplicity of infection of about 10^{-3}. Cultures at 25 °C and 31.5 °C were either maintained at the same temperature throughout the experiment (○–○ and ●–●) or shifted to the other temperature 25 min after infection (△–△ and ▲–▲). The cultures were assayed for plaque forming units (PFU) by plating with PS1001 and incubating at 25 °C.

Fig. 6. Sucrose gradient of double labeled bacteriophage PM2. In this experiment both [^{3}H]glycerol (10 μCi/ml) and ^{32}P (3 μCi/ml) were added to a culture of strain GT12 prior to infection and labeling conditions were maintained for both isotopes throughout the experiment at a multiplicity of infection of 10. Labeling conditions were maintained for both isotopes throughout the infectious cycle in this experiment. PFU, plaque forming units.

Origin of viral phospholipids

Pulse label experiments with ^{32}P and [^{3}H]glycerol, in cultures of strain GT12 infected with bacteriophage PM2, were carried out to investigate the temporal origin of viral phospholipids. The cultures were labeled continuously with ^{32}P and [^{3}H]-glycerol was added at various times before or during infection. In some cases, [^{3}H]-

TABLE I

TEMPORAL ORIGIN OF VIRAL PHOSPHOLIPID GLYCEROL

^{32}P was present throughout in all experiments.

[^{3}H]glycerol present	$^{3}H/^{32}P$	Percent
Throughout	3.46	100
Added 10 min after infection	0.91	26
Diluted out 10 min before infection	1.84	53

glycerol incorporation was terminated by the addition of excess unlabeled glycerol. The virus were then purified (see Materials and Methods) and the peak gradient fractions were analyzed for ^{32}P and ^{3}H radioactivity and for viable virus titer. Data of this type, as shown in Fig. 6, could then be used to determine the temporal origin of the viral phospholipid glycerol. Phospholipids were extracted from the peak fractions and the $^{3}H/^{32}P$ ratio was determined. By normalizing the $^{3}H/^{32}P$ ratio of pulse label experiments to that for continuous labeling with both isotopes, the percent of phospholipid glycerol produced at any time could be determined. These data, shown in Table I, show that more than half of the [^{3}H]glycerol appearing in viral phospholipids was pre-existing cellular phospholipid synthesized prior to infection. There is some incorporation, into viral phospholipid, of [^{3}H]glycerol added after infection. It should by noted that [^{3}H]glycerol can appear in two locations in viral phospholipid glycerol, and the possibility remains that the basic phospholipid structure, except for the polar group, could largely or totally come from cellular phospholipids synthesized prior to infection.

Stability of cellular phospholipids during infection

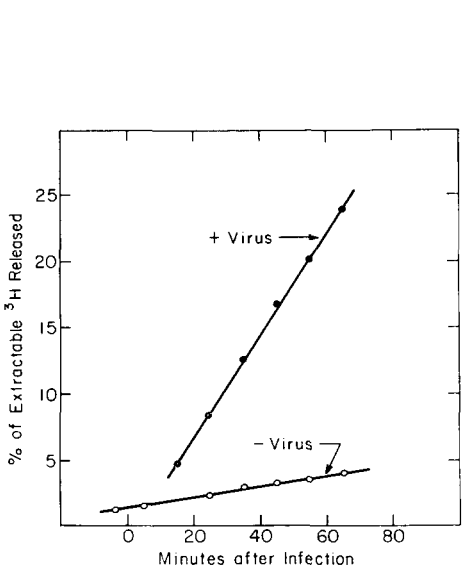
When cultures of cells prelabeled with [^{3}H]glycerol are infected with PM2 virus, there is a stimulated release of ^{3}H from the cell into the medium (Fig. 7). There was some variability in the degree to which infection increased ^{3}H release, but the addition of PM2 virus always increased the release by about a factor of two or more. We carried out experiments to determine whether mutant MF7, at 31.5 °C, can cause the same increase in ^{3}H release. The data for a time 35 min after infection are shown in Table II. This virus mutant is as capable of stimulating the release of ^{3}H from cellular phospholipids at its restrictive temperature as at its permissive temperature.

TABLE II

STIMULATED RELEASE OF ^{3}H FROM CULTURES INFECTED WITH MF7

Conditions of experiment as described for Fig. 7; samples taken 35 min after infection.

	^{3}H Release (cpm)	
	25 °C	31.5 °C
Infected with MF mutant	4382	4708
Uninfected	2175	2350
Stimulation factor	2.02	2.00



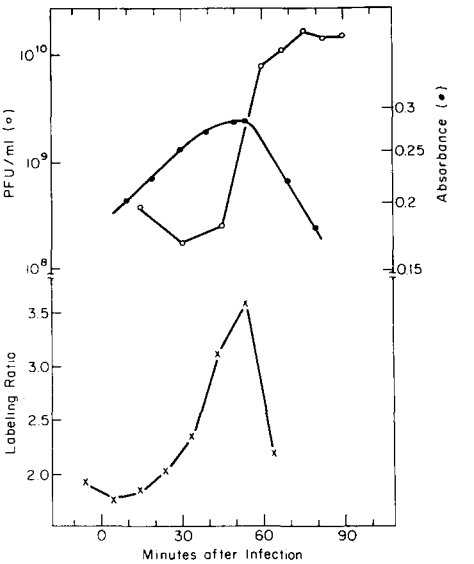


Fig. 7. Stimulated release of ^{3}H from a culture of strain GT12 infected with bacteriophage PM2. A culture of strain GT12 was prelabeled by growth in Medium 25-S plus 10 μg/ml glycerol and 2 μCi/ml [^{3}H]glycerol for 2 h. The cells were centrifuged, washed twice in Q medium, and resuspended in a volume of Q medium equal to the original culture volume. An aliquot was taken for immediate phospholipid extraction and the remainder divided two ways. One culture was infected with PM2 virus at a multiplicity of infection of about 10 ($t = 0$); the other was maintained as a control. At various times an aliquot of the culture was filtered through a 0.22 μ Millipore filter to collect cells and the filtrate was assayed for radioactivity. Data are presented as the percent of ^{3}H released to the medium relative to the amount extractable as phospholipid material.

Fig. 8. Modification of phospholipid synthesis during virus infection. Aliquots of a culture of strain GT12 were given 10-min exposure to both [^{3}H]glycerol (3 μCi/ml) and ^{32}P (1 μCi/ml) before and at various times during infection with PM2 at a multiplicity of infection of about 10. The "labeling ratio" is defined in the text.

Phospholipid synthesis in infected cells

Pulse label, double isotope experiments were carried out to investigate the nature of phospholipid synthesis in infected cells. In these experiments both ^{32}P and [^{3}H]glycerol were added simultaneously to an aliquot of the culture either before or at some time during infection. Ten minutes later this aliquot was iced to retard further incorporation. The cells were centrifuged, washed twice with medium, centrifuged again, and extracted overnight with chloroform : methanol (2 : 1, by vol.). The extract was lyophilized and the lipids were taken up in a small volume of chloroform and chromatographed.

A parameter for analysis was chosen which minimizes any errors involved with recovery of phospholipids from the chromatogram. For each sample the phosphatidylglycerol and the phosphatidylethanolamine were eluted and their ^{3}H/^{32}P ratios determined. For normal metabolic synthesis of these compounds [5], the ^{3}H/^{32}P ratio for phosphatidylglycerol should be twice that for phosphatidylethanolamine. We found this to be the case for uninfected cultures. The "labeling ratio", defined here as ^{3}H/^{32}P (phosphatidylglycerol) divided by ^{3}H/^{32}P (phosphatidyl-

ethanolamine), was always within 10 % of two for uninfected cells.

When 10-min pulses were given to infected cells, the labeling ratio rose sharply and reached levels as great as 3.5 in some experiments (Fig. 8). Virus production and cell lysis as measured by absorbance are also shown for the experiment of Fig. 8. The maximum labeling ratio is reached at the time that the most virus are being released to the medium. The subsequent decline in the labeling ratio was reproducible and suggests that cell debris remaining after lysis has phospholipids that were synthesized by the pathways of uninfected cells. This latter point, however, is not established directly by these experiments because the cultures may contain some uninfected cells.

Cells infected with the virus mutant MF7 did not show the elevated labeling ratio at the restrictive temperature, 31.5 °C, but did at 25 °C. This is shown in Fig. 9. In this experiment we also determined the relative amounts of phosphatidylglycerol and phosphatidylethanolamine being synthesized during each time interval, as shown in Fig. 9. At 31.5 °C about 22–24 % of the phospholipid being synthesized is phosphatidylglycerol. At 25 °C, the value rises to about 40 % during infection. We conclude that the elevated labeling ratio observed in these experiments is involved with and likely is an essential aspect of the mechanism whereby bacteriophage PM2 increases phosphatidylglycerol levels for generation of its membrane.

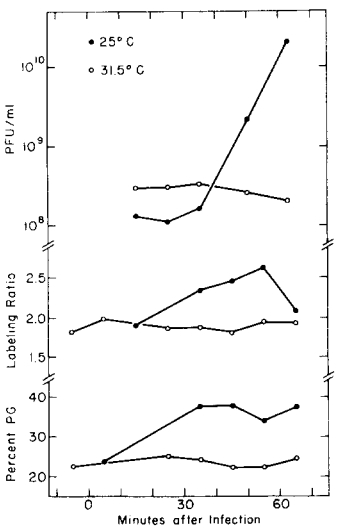


Fig. 9. Temperature sensitivity of the phospholipid modification process in virus mutant MF7. Samples were treated as for Fig. 8. The percent phosphatidylglycerol in infected cultures at 25 °C and 31.5 °C is also given. PFU, plaque forming units.

DISCUSSION

As a basis for discussing the nature of phospholipid synthesis in bacteriophage PM2-infected cultures, it is perhaps useful to list the following possibilities for the origin of viral phospholipids:

(1) Viral phospholipids are obtained by selective uptake of preexisting cellular phospholipids.

(2) Viral phospholipids are synthesized de novo after infection.

(3) Viral phospholipids are generated by modification of preexisting cellular phospholipids.

While these three possibilities are distinct, the generation of the bacteriophage PM2 membrane might involve more than one of these processes.

Espejo and Canelo, in a study of the origin of bacteriophage PM2 phospholipid, concluded that the viral phospholipid is not synthesized de novo but is derived from pre-existing cellular phospholipids [6]. This conclusion was based on the observation that the specific activity of phospholipid from virus produced from ^{32}P-prelabeled bacteria was essentially the same as that in the host bacteria labeled before infection. Our data are not in disagreement with those of Espejo and Canelo, but rather provide additional information on the origin of viral phospholipid glycerol. Slightly more than half of the glycerol component of viral phospholipids is synthesized prior to infection (Table I). This means that neither (1) nor (2) listed above can uniquely describe the origin of viral phospholipid, but does not eliminate the possibility that (1) and (2) in combination are sufficient.

The data of Figs 7 and 8 strongly suggest that pre-existing cellular phospholipids are modified during infection. The strongest evidence for phospholipid modification comes from the data of Fig. 8. The most reasonable interpretation of the sharp increase of the labeling ratio is that phospholipid polar groups are cleaved from pre-existing, unlabeled phospholipids, and that labeled glycerol is then added to the resultant phosphatidic acid. This would produce phosphatidylglycerol that is ^{3}H-labeled at the polar group position but unlabeled at the phosphorous positions, and would result in a labeling ratio greater than two. Whether or not phosphatidylethanolamine is produced in the same manner, i.e., by addition of ethanolamine to phosphatidic acid, would not affect this interpretation since no ^{3}H would be incorporated into phosphatidylethanolamine by this process. The mechanism of phospholipid modification proposed here is consistent with and supported by the observation of Braunstein and Franklin [1] that phosphatidic acid levels rise in infected cultures.

In view of the results presented for virus mutant MF7, some considerations of the role that phospholipid modification plays in assembly of the virus membrane seems appropriate. At 31.5 °C, the release of ^{3}H is stimulated in cultures infected with mutant MF7 (Table II), but apparently the phospholipid modification process is not completed (Fig. 9). No increase in phosphatidylglycerol levels is observed at this temperature, and no stable virus-like particles are assembled (Fig. 4). This would suggest that phospholipid modification is probably an essential feature for assembly of this virus.

The following considerations are consistent with the suggestion that phospholipid modification is an integral part of bacteriophage PM2 membrane assembly. Although bacteriophage PM2 contains a high proportion of phosphatidylglycerol in comparison to the host, there are more than sufficient amounts of this phospholipid in the cell membrane to produce a full burst of virus. We estimate that 100 virus particles requires only about 5.5 % of the phosphatidylglycerol from the membrane of a single cell*. Nevertheless, when increased phosphatidylglycerol production is

* Calculation based on bacterial plasma membrane of $1\,\mu \cdot 3\,\mu$ rod-shaped cell with 50 $Å^2$ per phospholipid molecule. Virus phospholipid content as calculated in ref. 2.

blocked as in mutant MF7 infections at 31.5 °C, no virus particles are assembled. It may be that polar groups are cleaved from cellular phospholipids, that the resulting phosphatidic acid is used as a basis for viral phospholipids, and that the addition of glycerol is an integral part of transferring the phosphatidic acid to the developing virus membrane. The primary problem appears to be in getting the phosphatidylglycerol and phosphatidylethanolamine localized in the virus membrane bilayer in the required proportions rather than in producing enough phosphatidylglycerol.

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