ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1977, p. 523-528

Vol. 12, No. 4
Printed in U.S.A.

Inactivation and Inhibition of Replication of the Enveloped Bacteriophage ϕ6 by Fatty Acids

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Received for publication 10 May 1977

The enveloped bacteriophage ϕ6 has been shown to be an interesting model system for the study of chemical agents that might have specific antiviral effects against lipid-containing mammalian viruses. In this report, we describe two types of antiviral activity exhibited by several fatty acids against bacteriophage ϕ6. Oleic acid (18:1) and palmitoleic acid (16:1) were potent inactivators of the virus. Treatment with either fatty acid at 50 μg/ml at 25 or 0°C for 30 min reduced the virus titer to about 0.1% of the initial titer. Oleic acid at a concentration as low as 3 μg/ml ($\sim 10^{-2}$ mM) reduced the virus titer to <1% of the initial titer within 30 min. Ultracentrifugation analyses of ^{14}C-amino acid- and ^{32}P-labeled virus treated with oleic acid indicated that the virion is largely disassembled by the treatment. Myristic acid (14:0) and palmitic acid (16:0) did not inactivate ϕ6 at 50 μg/ml, but nevertheless did prevent ϕ6 plaque production. Single-step virus growth experiments in which fatty acid was added at various times before or after infection indicated that it was an early stage of the ϕ6 replication cycle that was inhibited by the presence of myristic acid and that the inhibition occurred only if the myristic acid concentration in the extracellular growth medium was $\geq$10 μg/ml. ϕ6 could attach to its host cell in the presence of myristic acid at 50 μg/ml. We conclude that the fatty acids that prevent ϕ6 replication probably do so by interfering with the entry of the viral genome into the host cell.

Lipid-containing bacterial viruses have been used in recent years as model systems for investigating the general properties of viruses that contain lipid as an integral structural component. Areas in which the use of lipid-containing bacteriophages have proven to be interesting include the mechanisms of inactivation and/or inhibition of replication of these viruses by some chemical agents that physically interact with membranes. Examples include the inactivation of bacteriophages ϕ6 and PM2 by the common food additive butylated hydroxytoluene and long-chain alcohols (3, 7, 8, 10) and the inhibition of the assembly process of PM2 by the small, spherical, hydrophobic molecule adamantanone (2). In all of these examples, the studies have been expanded to mammalian viruses with positive results (7, 8).

In this paper, we report that several free fatty acids are potent inactivators of the enveloped phage ϕ6 and that other fatty acids that do not inactivate the virus nevertheless prevent its replication. In addition, we describe experiments aimed at characterizing the mechanisms of inactivation and inhibition of replication. These effects of fatty acids on phage ϕ6 are interesting since ϕ6 is structurally similar to many enveloped mammalian viruses (1, 9).

MATERIALS AND METHODS

General. The medium used for routine cell growth and virus production, designated NBY medium (9), contains 8 g of nutrient broth, 2 g of yeast extract, 0.5 g of KH_2PO_4, 2 g of K_2HPO_4, 5 g of glucose, and 0.25 g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water. For isotope-labeling experiments, a glucose-minimal salts medium, designated TGN, was used. TGN contains 12.1 g of tris(hydroxymethyl)aminomethane, 1.5 g of KCl, 15 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 g of NH_4Cl, 5 g of glucose, 25 mg of KH_2PO_4, and 75 mg of K_2HPO_4 per liter of distilled water, adjusted to pH 7.6.

The basic techniques for the growth of *Pseudomonas phaseolicola* (the host for ϕ6) and bacteriophage ϕ6 in our laboratory have been described before (5, 6).

Fatty acid solutions at 1 mg/ml were made in distilled water in the presence of 4 mg of Tween 80 per ml.

Sucrose gradient analyses. Stocks of bacteriophage ϕ6 labeled with ^{14}C-amino acids (tryptophan, arginine, leucine, and threonine) or ^{32}P were purified by sucrose gradient velocity sedimentation. Vi-

rus samples were layered onto 15 to 30% sucrose gradients (in TGN medium) and centrifuged at 27,000 rpm for 90 min in a Beckman SW27 rotor. Tubes were punctured, and fractions were collected for radioactivity counting.

Source of materials. Myristic (14:0), myristoleic (14:1), palmitic (16:0), palmitoleic (16:1), oleic (18:1), and linoleic (18:2) acids were obtained from Sigma Chemical Co., St. Louis, Mo. [^{32}P]orthophosphoric acid (10 mCi/ml), L-[^{14}C]arginine (1.67 mCi/mg), L-[^{14}C]threonine (1.67 mCi/mg), DL-[3-^{14}C]tryptophan (0.167 mCi/mg), L-[^{14}C]leucine (2.27 mCi/mg), and [^{3}H]palmitic acid (1.33 mCi/mg) were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Effect of fatty acids on ϕ6 plaque production. We initially observed that the addition of fatty acids to give 50 μg/ml at the time of plating for ϕ6 plaque production on lawns of *P. phaseolicola* in NBY or TGN agar prevented the development of visible plaques. This inhibition of plaque production occurred for all the fatty acids we tested except linoleic acid (18:2) and was not due to Tween 80, which was present in all the fatty acid preparations (Table 1).

Effect of fatty acids on ϕ6 viability. To determine whether the inhibition of ϕ6 plaque production by any of myristic (14:0), myristoleic (14:1), palmitic (16:0), palmitoleic (16:1), and oleic (18:1) acids was due to virus inactivation, we incubated suspensions of ϕ6 in the presence of fatty acid at 50 μg/ml for 30 min at room temperature and then assayed for virus survival. Myristic, myristoleic, and linoleic acids had little, if any, virus-inactivating abilities at 50 μg/ml, but palmitoleic and oleic acids were very potent inactivators of ϕ6 at this concentration (Table 2). It thus appears that there are at least two modes of fatty acid inhibition of ϕ6 replication: (i) some fatty acids (16:1 and 18:1) are very efficient inactivators of the virus and thus probably inhibit phage replication in this way, and (ii) some other fatty acids (14:0 and 16:0) have no significant inactivating effect on the virus but nevertheless prevent phage replication. These two mechanisms of inhibition of ϕ6 growth are described in the remainder of this report.

Effect of concentrations of palmitoleic and oleic acids on ϕ6 inactivation. We performed virus inactivation experiments like those reported in Table 2 for different concentrations of palmitoleic (16:1) and oleic (18:1) acids. The results (Fig. 1) show that oleic acid is a much more potent inactivator of ϕ6 than is palmitoleic acid. Indeed, even at a concentration of 3 μg/ml (10.6 μM), ϕ6 survival was <1% after 30 min of exposure to oleic acid.

TABLE 1. *Effect of fatty acids on ϕ6 plaque production*

Plate[a]	ϕ6 plaquing efficiency (%)[b]
Control	100
Tween 80	100
14:0	<0.001
14:1	<0.001
16:0	<0.001
16:1	<0.001
18:1	<0.001
18:2	45

[a] Plates contained 10 ml of NBY agar (0.5%). Fatty acids were added to give 50 μg/ml (with 200 μg of Tween 80 per ml).

[b] Tabulated as percent (compared with control) appearance of visible plaques after 24 h of incubation at 25°C.

TABLE 2. *Effects of fatty acids on ϕ6 viability*

Fatty acid[a]	Survival (%)[b]
None	100
None, Tween 80 (200 μg/ml)	100
14:0	100
14:1	40
16:0	100
16:1	0.1
18:1	0.1
18:2	100

[a] Fatty acids were added to NBY medium to give 50 μg/ml. Tween 80 was present at 200 μg/ml for all fatty acid tests.

[b] Tabulated as relative number of plaque-forming units present after 30 min of incubation at room temperature.

Effect of temperature on ϕ6 inactivation by palmitoleic and oleic acids. The physical properties of membranes have been shown in many studies to be strongly dependent on temperature. For the phenomenon discussed here of the inactivation of an enveloped virus by fatty acids, it might be expected that the inactivation would be very dependent on temperature due to a "rigid" structure of the viral envelope at low temperature and a "fluid" structure at higher temperature. We investigated the inactivation of ϕ6 by palmitoleic acid and oleic acid at 0°C as compared with the inactivation at 25°C. The results (Table 3) show that there is apparently no significant temperature effect when fatty acids at 50 μg/ml (0.18 mM) are used to inactivate ϕ6.

Sedimentation properties of oleic acid-treated virus. Sucrose gradient analyses of the inactivation of ϕ6 by oleic acid were carried out to determine the extent to which the virus particle is disrupted by this fatty acid. Separate stocks of ϕ6 labeled with ^{32}P ([^{32}P]ortho-

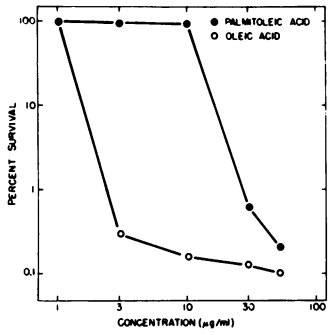

FIG. 1. *Effect of concentrations of palmitoleic and oleic acids on bacteriophage φ6 inactivation. Portions of fatty acid-Tween 80 solutions were added to NBY medium at 25°C to give various fatty acid concentrations. At time zero, φ6 was added to give approximately 10^7 plaque-forming units per ml. Thirty min after φ6 addition, samples were diluted and assayed for plaque-forming units.*

TABLE 3. *Inactivation of φ6 by fatty acids at 0 and 25°C*

Fatty acid[a]	Survival (%)[b]	
	0°C	20°C
None	100	100
16:1	0.10	0.14
18:1	0.15	0.10

[a] Tween 80 was present in control tubes at 200 μg/ml, and fatty acids were present at 50 μg/ml.

[b] Percent survival after 30 min of incubation at indicated temperature.

phosphoric acid) and ^{14}C-amino acids were prepared and purified by velocity sedimentation in a sucrose gradient. Aliquots of purified virus were incubated for 30 min at 25°C in the presence and absence of oleic acid at 50 μg/ml. The aliquots were then analyzed on sucrose gradients. The results for ^{32}P-labeled φ6 are shown in Fig. 2, and the results for ^{14}C-amino acid-labeled φ6 are shown in Fig. 3. The ^{32}P gradients are an indication of the sedimentation properties of the viral ribonucleic acid and phospholipid. For untreated virus, the ^{32}P was found associated with the peak of infectious virus (fraction 8 in Fig. 2). For the oleic acid-treated virus, however, almost all the ^{32}P was part of more slowly sedimenting material, presumably viral fragments containing the viral ribonucleic acid and/or phospholipid. The situation was similar for Fig. 3, which shows that the viral proteins in the oleic acid-treated sample were also parts of slowly sedimenting material. The presence of a large amount of ^{14}C at the very top of the gradient containing the oleic acid-treated virus suggests that some of the φ6 proteins may have been present in uncom-

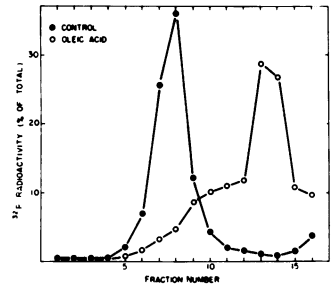

FIG. 2. *Sucrose gradient velocity sedimentation analysis of oleic acid-inactivated ^{32}P-labeled φ6. ^{32}P-labeled φ6 virions were purified in a 15 to 30% sucrose gradient (in TGN medium) and then split into two samples. One sample was incubated in the presence of oleic acid at 50 μg/ml for 30 min at 25°C. The control sample was incubated for 30 min at 25°C in the absence of added oleic acid. The two samples were then layered onto 15 to 30% sucrose gradients and centrifuged at 27,000 rpm for 90 min in an SW27 rotor. Two-milliliter fractions were collected and assayed for radioactivity. Sedimentation is from right to left.*

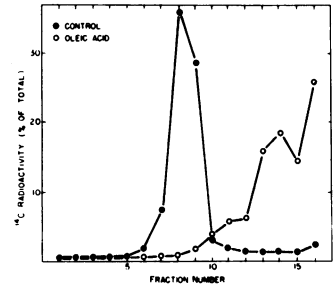

FIG. 3. *Sucrose gradient velocity sedimentation analysis of oleic acid-inactivated ^{14}C-amino acid-labeled φ6. The procedure was the same as that for Fig. 2.*

plexed form. The net amount of radioactivity recovered from each of the two sets of gradients was approximately (within 5%) the same for the control and fatty acid-treated samples, implying that no significant amounts of viral material in the fatty acid-treated sample escaped detection due to such possible phenomena as binding to the walls of the centrifuge tube. Taken together, Fig. 2 and 3 imply that the physical integrity of bacteriophage φ6 is largely destroyed by treatment with oleic acid at a concentration that has no measurable ill effect on the host cell.

Inhibition of φ6 replication by myristic and palmitic acids. Among the fatty acids tested (Table 1), myristic (14:0) and palmitic (16:0) acids did not, at 50 μg/ml, inactivate φ6, but nevertheless did prevent plaque production. Thus, these fatty acids interfere with some stage of the φ6 replication cycle. Figure 4 shows the concentration dependencies of the

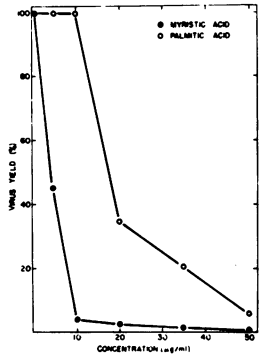

FIG. 4. *Effects of various concentrations of myristic and palmitic acids on φ6 replication. Cultures of P. phaseolicola growing in NBY medium at about 10^8 cells per ml were infected at time zero with φ6 at a multiplicity of infection of ~0.001. Also at time zero, myristic acid or palmitic acid was added to give various concentrations. At various times, dilutions from the culture were assayed for plaque-forming units (PFU). "Virus yield" is here defined as the ratio of PFU in the culture at 120 min to the PFU in the culture at 30 min, normalized to 100% for the case of no myristic acid present.*

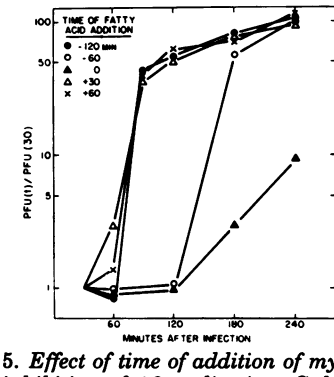

FIG. 5. *Effect of time of addition of myristic acid on the inhibition of φ6 replication. Cultures of P. phaseolicola in NBY medium at 25°C were infected with φ6 at time zero (cell concentration, approximately 1.5×10^8 cells per ml; cellular generation time, approximately 90 min). At various times before or after infection, myristic acid was added to give 50 μg/ml. At the indicated times, samples from the cultures were diluted into NBY medium and immediately plated for plaque production. The data are presented as the ratio of plaque-forming units (PFU) in a culture at time t to the plaque-forming units in that culture at 30 min.*

inhibition of virus growth when fatty acid is added to a culture at the time of infection. Myristic acid prevented virus replication at concentrations as low as 10 μg/ml, whereas palmitic acid was inhibitory only at concentrations greater than about 20 μg/ml.

Effect of time of addition of myristic acid on inhibition of φ6 replication. To determine what stage of the φ6 infectious cycle was inhibited by these fatty acids, we added myristic acid (to give 50 μg/ml) to cultures at various times before and after infection with φ6. The results of these experiments (Fig. 5) indicate that the time of addition of fatty acid was important in preventing φ6 replication. If fatty acid was added after infection, at 30 or 60 min (normal lysis time is at 70 to 90 min), there was no measurable inhibition of virus replication. There was also no inhibition if fatty acid was added 120 min before infection. If fatty acid was added 60 min before infection, virus replication was inhibited for an interval of 60 to 90 min, but, eventually, normal amounts of new virus were produced. Significant "long term" inhibition was observed only if fatty acid was added very near to the time of infection.

The data indicating full virus replication when fatty acid was added after infection suggest that one of the very first steps of the infectious cycle, either attachment or entry, is the stage inhibited by fatty acids. The data indicating full virus replication when fatty acid is added a "long time" before infection suggest that the disappearance of fatty acid from the growth medium (via cellular incorporation) resulted in loss of the inhibitory effect. We conducted fatty acid uptake experiments to test this idea. Using the same cellular concentrations as those in Fig. 5, we measured the rate of incorporation of ^{3}H-fatty acids (total fatty acid concentration of 50 μg/ml) into *P. phaseolicola*. The results indicated that, under the conditions of Fig. 5, ~40% of the fatty acid was incorporated by 30 min, ~75% was incorporated by 60 min, and ~90% was incorporated by 120 min. These data correlate fairly well with the loss of inhibitory effect for fatty acid added before infection, suggesting that if the fatty acid concentration in the growth medium at the time of infection was less than about 5 to 10 μg/ml (for myristic acid), virus attachment, entry, and subsequent replication proceeded normally; if the fatty acid concentration in the growth medium at the time of infection was greater than about 5 to 10 μg/ml (for myristic acid), one of the early steps (perhaps attachment or entry) was inhibited.

φ6 attachment in presence of myristic acid. To attempt to distinguish between the possibilities that myristic acid inhibited φ6 replication by preventing either attachment or entry, we measured the efficiency of attachment of φ6 to its host cell in the presence and absence of myristic acid. The results indicated that the presence of myristic acid in the growth medium at 50 μg/ml inhibited φ6 attachment some-

what. (The attachment rate constant of ϕ6 in the presence of myristic acid was approximately 55 to 75% that of ϕ6 in the absence of the fatty acid.) This relatively small effect is not enough, however, to explain the inhibition of replication of the virus (attempts to do these experiments using a higher myristic acid concentration [100 μg/ml] were unsuccessful due to ϕ6 inactivation by high concentrations of this fatty acid). It thus appears that entry might be the main stage of the ϕ6 replication cycle inhibited by the presence of myristic acid (and, perhaps, palmitic acid).

DISCUSSION

In several earlier studies, the enveloped bacteriophage ϕ6 was shown to be an interesting model system for studying the effects of several potential antiviral agents (7, 8, 10). Butylated hydroxytoluene and various long-chain alcohols were shown to be potent inactivators of ϕ6. Upon extension to a study of mammalian viruses, both butylated hydroxytoluene and the long-chain alcohols were found to have inactivating abilities against herpes simplex virus type 1, which is, morphologically, somewhat similar to bacteriophage ϕ6, each having a lipid-protein envelope on its surface. In this paper, we have extended the list of the common chemical agents that have an inhibitory effect on ϕ6 to include various fatty acids. Specifically, we found that low concentrations (<100 μM) of oleic and palmitoleic acids cause rapid inactivation of ϕ6 and that similar concentrations of myristic acid or palmitic acid, while not inactivating the virus, nevertheless do inhibit ϕ6 replication. All of these effects occur at concentrations that do not inhibit the growth of the host cell.

Our data on the inactivation of ϕ6 by fatty acids indicate that oleic acid (18:1) is the most potent of the common fatty acids at inactivating ϕ6. Oleic acid at ~10 μM reduces the titer of a ϕ6 stock by >99% within 30 min. Our sucrose gradient sedimentation analyses of oleic acid-inactivated ϕ6 indicate that the mechanism of inactivation involves the physical disruption of the ϕ6 virion into subviral fragments. It is known that, in the type of gradients used in these experiments, ϕ6 core particles (virion lacking the lipid-protein envelope) sediment with about the same sedimentation coefficient value as intact virions (due to smaller size but greater density of core as compared with virion). Our gradients do not show the presence of virus cores, suggesting that the virus disruption caused by oleic acid treatment is severe. The inactivation of ϕ6 by oleic acid is thus quite different from the inactivation of ϕ6 by butylated hydroxytoluene, for which the virus stays nearly intact, even retaining most of its lipid-protein envelope (10). The degree of disruption of the ϕ6 virion by the long-chain alcohols has not been reported.

Myristic (14:0) and palmitic (16:0) acids have an inhibitory effect on ϕ6 replication of a type that has not been seen previously. The presence of these fatty acids at 50 μg/ml prevents ϕ6 plaque production but does not cause virus inactivation. Experiments aimed at determining the stage of the infectious cycle that is inhibited by these fatty acids showed that the inhibition of a round of virus replication occurs only if the fatty acid is added at about the time of infection, suggesting that it is an early event, such as attachment or entry, that is inhibited. Furthermore, if the fatty acid is added a long enough time before infection, such that the fatty acid concentration in the growth medium at the time of infection is very low ($\leq$10 μg/ml), no inhibition occurs. We showed that the ϕ6 attachment rate is only slightly lower in the presence of myristic acid, suggesting that entry is the main stage of the infectious cycle that is inhibited by this fatty acid. It is possible that some early event after entry is the stage that is inhibited, but entry seems to be the most logical choice, since the inhibition is dependent on the fatty acid concentration in the growth medium.

These inhibitory effects on the ϕ6 replication cycle (presumably at the level of entry) by myristic acid and palmitic acid are overcome once the fatty acid concentration in the growth medium drops to a subinhibitory level. The complete inhibition of plaque production by these fatty acids is explainable, since the fatty acid concentration in the agar falls to the subinhibitory level only when the cell concentration reaches about 2×10^8 cells per ml (as calculated from our fatty acid uptake experiments). This high cell concentration only occurs near the end of the incubation period, long after virus replication would have had to begin to result in production of visible plaques.

The physical mechanism whereby myristic acid may be preventing entry is not clear. It is interesting, however, that fatty acids also appear to prevent entry of the lipid-containing bacteriophage PR4 (unpublished data, this laboratory) into *Escherichia coli*, even though the lipid region in PR4 is not on the surface of the virus, as it is in ϕ6. If the general mechanism of genome entry of lipid-containing bacterial viruses involves fusion between the viral lipid bilayer region and a cellular membrane, it is

possible that this step is inhibited by the presence of the fatty acids.

This report provides the first indication that fatty acids can inactivate and inhibit the replication of a lipid-containing virus. Since fatty acids do not have toxic effects on some cells at the concentrations used here (but see reference 14), it appears that fatty acids should be tested further, using several lipid-containing mammalian viruses, to investigate the possible clinical use of fatty acids as inhibitory agents against certain viruses.

ACKNOWLEDGMENT

This work was supported by grant PCM 76-12546 from the National Science Foundation.

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