JOURNAL OF VIROLOGY, Feb. 1978, p. 479-485
0022-538X/78/0025-0479$02.00/0

Vol. 25, No. 2
Printed in U.S.A.

Inhibitory Effect of Fatty Acids on the Entry of the Lipid-Containing Bacteriophage PR4 into *Escherichia coli*

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Received for publication 19 August 1977

Various unsaturated fatty acids (notably palmitoleic acid and oleic acid) interfered with plaque production by the lipid-containing bacteriophage PR4 on lawns of *Escherichia coli*. Addition of fatty acid to give 50 μg/ml (~0.2 mM) at the time of infection prevented phage replication. If, however, the fatty acid was added after infection, normal amounts of phage were produced. If the fatty acid was added (to 50 μg/ml) to the host cell culture a long enough time before infection such that the fatty acid concentration in the growth medium at the time of infection was reduced to ≤5 μg/ml (due to fatty acid incorporation by the host cells), normal phage replication occurred also. Neither palmitoleic acid nor oleic acid prevented PR4 attachment to *E. coli*. Several types of experiments indicated that it is the entry process of the virus that is inhibited by these fatty acids. Specifically, if the fatty acid was added at the time of infection, the host cells were not killed by the virus and no detectable amounts of viral protein were synthesized. In addition, experiments using purified radioisotope-labeled virions showed directly that entry is inhibited. Mutants of PR4 that did replicate in the presence of oleic acid arose spontaneously at a frequency of 10^{-6}. Three of these mutants that have been further characterized have protein and phospholipid compositions indistinguishable from those of wild-type PR4.

Lipid-containing bacterial viruses have been used in recent years as model systems for studying various aspects of enveloped viruses in general, including virion structures (1, 14), assembly processes (7, 12, 15), mechanisms of inactivation (8, 16, 17), and the inhibition of virus replication (7). We are especially interested in bacteriophage PR4, which contains lipid and can replicate in gram-negative bacteria, including *Escherichia coli*, that carry an appropriate drug resistance plasmid (3-5). We are studying the structure of phage PR4 and its mode of replication in *E. coli*. We have characterized the lipids of the virion (10) and have determined the molecular weights of the six major proteins detectable in the virus (6). In the midst of a recent study on PR4 replication in various "membrane" mutants of *E. coli* (11), we found that unsaturated fatty acid auxotrophs of *E. coli* are not susceptible hosts for PR4. Unsaturated fatty acids such as palmitoleic acid (16:1, Δ9, *cis*) or oleic acid (18:1, Δ9, *cis*) must be supplied in the medium for growth of these cells. As we show in this paper, the presence of significant concentrations of either of these fatty acids in the growth medium prevents the replication of phage PR4 in even "normal" (i.e., non-fatty acid auxotrophic) *E. coli* cells. The characterization of this inhibition of phage PR4 replication by these fatty acids is described.

MATERIALS AND METHODS

General. The *E. coli* K-12 derivative used in these experiments has been previously described (11). Strain PS2R requires tryptophan (amber), arginine (ochre), and purines (amber) for growth and contains the RP1 drug resistance plasmid. *E. coli* PS2R was grown in NBY, a rich nutrient broth-yeast extract medium previously described (10), or in supplemented low-sulfate medium (LS^+). LS medium contains, per liter of distilled water: Tris, 12.1 g; KCl, 1.5 g; NaCl, 15 g; $MgSO_4 7H_2O$, 25 mg; NH_4Cl, 1 g; glucose, 5 g; KH_2PO_4, 25 mg; and K_2HPO_4, 75 mg (adjusted to pH 7.6). Appropriate supplements were added to LS to produce LS^+ to allow PS2R growth.

Stocks of phages PR4 and T4 were grown in NBY or LS^+ medium in *E. coli* PS2R. Our procedures for growing and assaying both *E. coli* PS2R and phages have been previously described (10, 11). Assays for PFU in the presence of fatty acids were also performed, using only NBY top agar.

Production and purification of labeled virus. ^{32}P- and ^{35}S-labeled phages were produced as follows. *E. coli* PS2R cultures were grown to 10^8 cells per ml in 100 ml of LS^+ medium and infected with phage at a multiplicity of infection (MOI) >1. At the time of infection, either 10 μCi of [^{32}P]phosphate or 10 μCi of $Na^{35}SO_4$ per ml was added to each culture. After lysis,

cellular debris was removed by low-speed centrifugation, and the virions were pelleted by centrifuging at 20,000 rpm for 1 h in an SW27 rotor. The pellets were resuspended in 2 ml of LS medium overnight, layered onto 15 to 30% linear sucrose gradients (in LS medium), and centrifuged at 25,000 rpm for 90 min in an SW27 rotor at 4°C. Two-milliliter fractions were collected and assayed for radioactivity. The peak fractions were layered onto 30 to 60% linear sucrose gradients (in LS medium) and centrifuged at 25,000 rpm for 6 h in an SW27 rotor at 4°C. Peak fractions from these gradients were diluted and stored at 4°C or centrifuged into a pellet for further analysis.

Lipid and protein analyses. Thin-layer chromatographic analysis of ^{32}P-labeled phage lipids (10, 15) and sodium dodecyl sulfate-polyacrylamide slab gel electrophoretic analysis of ^{35}S-labeled phage proteins (6) have been previously described.

Sources of materials. [^{32}P]phosphate (carrier free, 10 mCi/ml), $Na^{35}SO_4$ (10 mCi/mmol of sulfur in 1 ml of water), and [^{3}H]oleic acid (7.35 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). All fatty acids and Tween 80 were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Effects of fatty acids on phage plaque production. PR4 produces clear plaques on lawns of *E. coli* PS2R. To determine the plaque production ability of PR4 in the presence of various fatty acids, phages were plated in NBY top agar with 50 μg of fatty acid per ml added at the time of plating. Fatty acid concentrations of 50 μg/ml have no measurable inhibitory effect on the growth rate of *E. coli* PS2R cells. In addition, none of the fatty acids tested inhibits bacteriophage T4 plaque production. The information presented in Table 1 shows that various unsaturated fatty acids interfere with PR4 plaque production to varying extents. Palmitoleic acid and oleic acid are the most effective, totally preventing the appearance of visible plaques. Saturated fatty acids do not inhibit PR4 replication, and the number, location, and configuration of the double bond(s) in the unsaturated fatty acids are important in determining the degree of inhibition.

Effect of fatty acid concentration on PR4 replication. We carried out single-step phage growth experiments to determine the effect of palmitoleic and oleic acid concentrations on PR4 replication when the fatty acid is added at the time of infection. Figure 1 shows the effect of various concentrations of these fatty acids on virus yield. Palmitoleic or oleic acid at a concentration of 50 μg/ml reduces the PR4 yield to essentially zero. A fatty acid concentration of 10 μg/ml results in a yield of roughly 15% in oleic acid and 40% in palmitoleic acid (compared to a 100% yield in the presence of no fatty acid or a saturated fatty acid). The quantitative drop in PR4 yield with an increase in fatty acid concentration could be a result of either a reduced burst size in the presence of these fatty acids or a reduced probability of successful infection.

TABLE 1. *Effects of fatty acids on PR4 plaque production*

Fatty acid[a]	PR4 plaquing efficiency (%)[b]	PR4 plaque morphology
None	100	Large, clear
None, Tween 80[c]	100	Large, clear
Saturated fatty acid[d]	100	Large, clear
Myristoleic acid (14:1, Δ9, *cis*)	100	Large, clear
Palmitoleic acid (16:1, Δ9, *cis*)	<0.001	No visible plaques
Oleic acid (18:1, Δ9, *cis*)	<0.001	No visible plaques
Elaidic acid (18:1, Δ9, *trans*)	60–80	Medium size, slightly cloudy
Vaccenic acid (18:1, Δ11, *cis*)	20–40	Very small, very cloudy
Linoleic acid (18:2, Δ9, *cis*, Δ11, *cis*)	20–40	Small, cloudy
Linolenic acid (18:3, Δ9, 12, 15)[e]	10–20	Very small, very cloudy
Arachidonic acid (20:4, Δ5, 8, 11, 14)	20–40	Small, cloudy
Erucic acid (22:1, Δ13, *cis*)	100	Large, clear

[a] Fatty acids added to give 50 μg/ml at time of plating.

[b] All plaquing efficiencies are tabulated as percentage of plaquing efficiency in the absence of fatty acid.

[c] Tween 80 (final concentration, 0.2 mg/ml) present in all cases to enhance fatty acid solubility.

[d] All even-numbered carbon atom saturated fatty acids from caproic acid (6:0) to lignoceric acid (24:0).

[e] Linolenic acid approximately 6 to 8% *trans* isomers.

Temporal dependency of fatty acid effect on PR4 replication. To determine the stage of the PR4 infectious process with which these fatty acids interfere, we performed in vivo phage production experiments with palmitoleic and oleic acids present only at specific times. Figure 2 shows the effect of adding (to give 50 μg/ml) oleic acid at various times, pre- and postinfection, to a culture of approximately 10^8 cells per ml infected with PR4 at an MOI <1. PR4 production is totally inhibited if oleic acid is added at the time of infection or 50 min before infection, but not if oleic acid is added at 30 or 60 min after infection. Oleic acid added at 110 min before infection does not inhibit PR4 production, indicating that either (i) oleic acid is taken up by the cells, thus lowering the oleic acid concentration in the medium at the time of infection, or (ii) the cell in some way adjusts to the presence of oleic acid and this adjustment

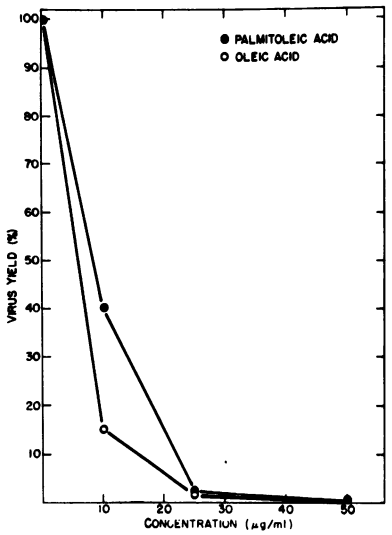

FIG. 1. *Effect of palmitoleic and oleic acid concentration on PR4 replication. Ten-milliliter cultures of E. coli PS2R were grown in NBY medium at 37°C with aeration to 10^8 cells per ml. At t = 0, 10^8 PFU of PR4 were added to each cell culture along with fatty acid at the appropriate concentration. At t = 20 min, all cultures were diluted by adding 0.1 ml to 10 ml of NBY medium plus fatty acid at the appropriate concentration. At various times, samples were diluted and immediately plated for plaque production using E. coli PS2R as host cell. Virus yield is defined as the PFU (120)/PFU(30) in the presence of the appropriate fatty acid concentration divided by the PFU (120)/PFU(30) in the absence of fatty acid, expressed as a percentage.*

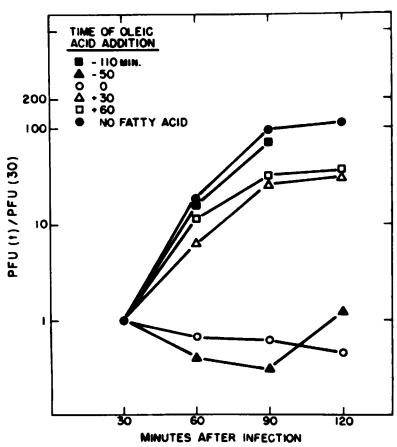

FIG. 2. *Temporal dependency of the effect of oleic acid on PR4 replication. Cultures of E. coli PS2R were grown in NBY medium at 37°C with aeration. Oleic acid (to give 50 μg/ml) was added at the indicated times pre- and postinfection. At appropriate times after infection, samples were diluted and plated for plaque production using E. coli PS2R as host cell.*

allows PR4 production. Similar results were obtained from experiments using 50 μg of palmitoleic acid per ml added at various times pre- and postinfection.

We also did experiments to show the effect of shifting out of oleic or palmitoleic acid at 45 min after infection for the case of the fatty acids added at the time of infection. The results of these experiments (data not shown) indicated that the inhibition of PR4 production by oleic or palmitoleic acid is reversible, as evidenced by PR4 production after the fatty acids are removed from the culture. There is a time delay in the recovery of PR4 production similar to the period of time in the presence of the fatty acid, suggesting that the infectious process cannot begin until the fatty acid is removed from the medium.

Uptake of [^{3}H]oleic acid by *E. coli* PS2R. The ability of PR4 to replicate in *E. coli* PS2R when oleic or palmitoleic acid is added at 110 min before infection suggests that most of the fatty acid may be removed from the medium by the cells during this interval, leaving a low concentration of fatty acid in the medium at the time of infection. To determine the rate of uptake of oleic acid by *E. coli* PS2R, a mixture of oleic acid and [^{3}H]oleic acid (to give a net oleic acid concentration of 50 μg/ml) was added to cells growing in NBY or LS medium at 10^8 cells per ml. At various times, 5-ml samples were centrifuged, and the supernatants were assayed for [^{3}H]oleic acid. The results (data not shown) indicated that approximately 90% of the [^{3}H] oleic acid is incorporated by (or bound to) the cells within 30 min of the time of addition. This fairly rapid uptake of oleic acid by PS2R could explain the loss of inhibition of PR4 production when the fatty acid is added 110 min before infection.

Attachment kinetics of PR4 in the presence of fatty acid. The data on the temporal dependency of the fatty acid inhibition of PR4 production suggest that an early stage of the infectious process is inhibited by the presence of the fatty acids. We therefore tested the ability of PR4 to attach to *E. coli* PS2R in the presence of oleic or palmitoleic acid (at 50 μg/ml, added at the time of mixing phage with cells, as in the experiments on the inhibition of phage replication described earlier). The results (Fig. 3) clearly indicate that neither oleic acid nor palmitoleic acid inhibits the attachment of phage PR4 to *E. coli* PS2R. Thus, some postattachment but nevertheless early stage of the infectious process is inhibited by the presence of these fatty acids in the growth medium.

Effect of fatty acids on loss of cell colony-forming ability due to phage infection. The

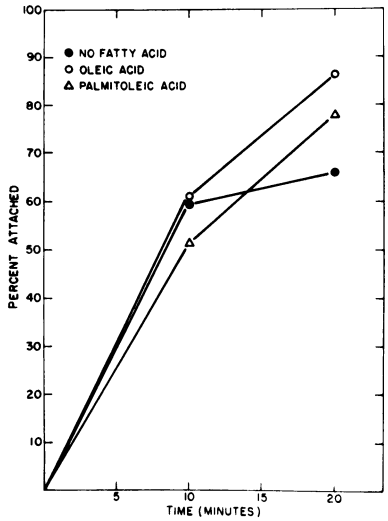

FIG. 3. *Attachment of PR4 to E. coli PS2R in the presence of palmitoleic or oleic acid. Ten-milliliter cultures of E. coli PS2R were grown in NBY medium at 37°C with aeration to 2 × 10^8 cells per ml. At t = 0, 10^7 PFU of PR4 were added to each culture with or without fatty acid (50 μg/ml). At various times, samples were centrifuged, the supernatants were assayed for (unattached) phage, and the results were converted to percent PR4 attached.*

TABLE 2. *Effect of palmitoleic and oleic acids on loss of E. coli PS2R colony-forming ability due to PR4 infection*

Virus added	Fatty acid added	% Cell survival[a]
None	None	100
None	Palmitoleic	100
None	Oleic	100
PR4 (MOI = 5)	None	28
PR4 (MOI = 5)	Palmitoleic	100
PR4 (MOI = 5)	Oleic	100

[a] Percent cell survival is defined as the ratio of CFU per milliliter that form as a result of the indicated treatment to the CFU per milliliter that form for the case of no virus and no fatty acid added. Phage and fatty acid (to give 50 μg/ml) were added simultaneously to cell cultures at 10^8 CFU/ml. The mixtures were incubated for 40 min at 37°C, then diluted by a factor of 10^5 to 10^6, and plated for colony formation on NBY agar.

finding that palmitoleic and oleic acids do not inhibit PR4 attachment to *E. coli* PS2R coupled with the earlier data indicating that it is an early stage of the infectious process that is inhibited by these fatty acids suggested to us that the inhibited stage might be the entry process. One indication of successful entry (and subsequent viral genome expression) is the death (loss of colony-forming ability) of the host cell. We assayed for the loss of cellular colony-forming ability due to PR4 infection in the presence and absence of fatty acids. A fairly high MOI (4) was used in these experiments to achieve a significant degree of cell death. The results (Table 2) show that the presence of either palmitoleic or oleic acid totally prevents the killing of the host cells by PR4, supporting the hypothesis that it is an early stage of the PR4 infectious process that is inhibited by the presence of these fatty acids.

Assay for PR4 entry in the presence of oleic acid. To directly determine whether the inhibition of PR4 replication by these fatty acids is at the stage of viral entry into the host cell, we followed the fate of radioisotope labels when ^{32}P- or ^{35}S-labeled purified PR4 virions are mixed with *E. coli* PS2R in the presence or absence of oleic acid at 50 μg/ml. The ^{32}P- and ^{35}S-labeled virus was purified through successive linear sucrose gradients as described in Materials and Methods. Samples of these labeled phage preparations were mixed with cells to give an MOI ≈1, incubated for 20 min at 37°C, and then cooled and treated by one of two (sonic treatment + EDTA [9]; lysozyme + EDTA [2]) *E. coli* spheroplasting procedures. All supernatants from each procedure with each isotope were assayed for radioactivity. The summarized results of these experiments are presented in Table 3. The significant results are as follows: (i) in the presence of oleic acid, essentially no ^{32}P (nearly equal amounts of viral DNA and lipid) is found in the resuspended final cell pellet (S4, spheroplasts) for either spheroplasting technique, whereas significant amounts (10 to 26%) are found in these final pellets in the absence of oleic acid; (ii) significantly higher amounts of both ^{32}P and ^{35}S are released in the initial wash in saline or buffer (S2) in the presence of oleic acid. The first result confirms that the entry of viral DNA into *E. coli* PS2R does not occur in the presence of oleic acid at 50 μg/ml. The second result indicates that both viral protein and viral DNA and/or lipid are fairly easily released from the cell surface in the presence of oleic acid.

Taken together, these data show that oleic acid inhibits the entry process of bacteriophage PR4. An additional result which is consistent with this conclusion is that palmitoleic and oleic acids prevent the appearance of any detectable amount of viral protein synthesis if the fatty acid is added at the time of infection (data not shown).

Assay for binding of oleic acid to bacteriophage PR4. Oleic acid might be expected to be inhibiting PR4 entry by associating with (i) the host cell surface, (ii) the surface of the

TABLE 3. *Effect of oleic acid on association of phage PR4 with E. coli PS2R*

Phage label[a]	Spheroplasting procedure[b]	Oleic acid concn (μg/ml)	Distribution (%) of radioactivity in supernatants[c]			
			S1	S2	S3	S4
^{35}S	Sonic treatment-EDTA	0	49.5	18	25.5	7
		50	45.5	31	19	4.5
	Lysozyme-EDTA	0	42.5	14	15	28.5
		50	52	28.5	14.5	5
^{32}P	Sonic treatment-EDTA	0	36.5	7.5	45.5	10.5
		50	64	22.5	11.5	2
	Lysozyme-EDTA	0	53	11.5	9.5	26
		50	67	22	9	2

[a] PR4 labeled with ^{35}S or ^{32}P were produced and purified as described in the text.

[b] The two spheroplasting procedures used are those described by Lugtenberg et al. (9) and Birdsell and Cota-Robles (2).

[c] Purified ^{35}S- or ^{32}P-labeled PR4 virions were mixed with *E. coli* PS2R cells (log phase) to give an MOI of 1. Oleic acid to give 50 μg/ml was simultaneously added to half of the cultures. Cultures were incubated with aeration for 20 min at 37°C and then subjected to one of two procedures that produce spheroplasts of *E. coli*. The first procedure (9) consisted of the following. A culture was centrifuged to pellet the cells, the supernatant of this initial centrifugation being labeled S1. The pellet was resuspended in 0.9% NaCl and centrifuged. The resulting supernatant is S2. The pellet was resuspended in 50 μM Tris-hydrochloride (pH 8.5) containing 2 mM EDTA. Under cooling, this suspension was subjected to sonic treatment for four 15-s intervals. The mixture was then centrifuged at 1,500 × *g* for 10 min, the supernatant (some cell envelope material) being labeled S3. The pellet (spheroplasts) was resuspended in water and labeled S4. The second procedure (2) consisted of the following. A culture was centrifuged to pellet the cells, the supernatant of this initial centrifugation being labeled S1. The pellet was resuspended in 10 mM Tris-hydrochloride (pH 8.0) and centrifuged. The resulting supernatant is S2. The pellet was resuspended in 10 mM Tris-hydrochloride (pH 8.0) containing 0.5 M sucrose. Lysozyme was added to give 20 μg/ml, and the mixture was incubated at room temperature for 7 min, a 10 mM concentration of Tris-hydrochloride (pH 8.0) containing 2 mM EDTA was added, and the mixture was incubated for 12 more min at room temperature. The culture was mixed well by Vortex action and centrifuged at 2,000 × *g* for 5 min, the supernatant (some cell envelope material) being labeled S3. The pellet was resuspended in water and labeled S4. These two procedures are not necessarily equally effective in producing spheroplasts, and the quantitative variation in our data between the two techniques shows this. The data shown here are the summarized results of two experiments, each of which gave essentially the same results.

phage, or (iii) both the cell and phage surfaces. We have previously described the rapid association of oleic acid with *E. coli* PS2R. To determine if significant and strong binding of oleic acid to page PR4 also occurs, we mixed various amounts of [^{3}H]oleic acid with samples of 10^{10} PFU of PR4, incubated these mixtures for 30 min at 37°C, and then analyzed the mixture by sedimentation through a 15 to 30% linear sucrose gradient. In all cases (data not shown), no peak of radioactivity was observed to cosediment with the virus. Calculations showed that we would not detect such a peak if up to five oleic acid molecules were bound per virion. Therefore, at most, only a few oleic acid molecules are binding to each virion in the experiments reported in this paper. In addition, the fact that PR4 replication is not inhibited by preinfection treatment of the virus with fatty acid suggests that the inhibition of entry of PR4 by these fatty acids is due to (i) the interaction of the fatty acid with the cell and/or (ii) a very weak interaction of the fatty acid with the virus that is readily reversed when the free oleic acid concentration is significantly reduced. Further experiments will be necessary to distinguish between these two possibilities.

Oleic acid-resistant mutants of bacteriophage PR4. We tried to isolate mutants of PR4 that can successfully infect *E. coli* PS2R in the presence of palmitoleic or oleic acid. To detect such "fatty acid-resistant" mutants of the phage, we plated PR4 (5×10^7 PFU) onto lawns of *E. coli* PS2R in the presence of oleic or palmitoleic acid at 50 μg/ml. PR4 plaque production was totally inhibited by palmitoleic acid, but plaques were formed on oleic acid plates at the rate of 1 per 10^6 added PFU. Three oleic acid-resistant mutants of PR4 were selected and rechecked for infectivity in the presence of oleic acid. Each of the oleic acid-resistant mutants has a plating efficiency of approximately 20% and a "cloudy" plaque morphology in 50 μg of oleic acid per ml. None of these mutants forms visible plaques in the presence of 50 μg of palmitoleic acid per ml. These mutants provide an indication that there might be some subtle differences in the inhibition of PR4 entry by oleic and palmitoleic acids.

To determine whether any of these three oleic acid-resistant mutants of PR4 have a signifi-

cantly different protein or lipid composition as compared to wild-type PR4, we purified ^{32}P- and ^{35}S-labeled preparations of PR4 and each of the mutants and analyzed them for phospholipid (by thin-layer chromatography and subsequent autoradiography) and protein (by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and subsequent autoradiography) compositions, as described before for wild-type PR4 (6, 10). We were unable to detect any significant differences in quantitative phospholipid composition, structural protein molecular weights, and relative abundances of the six structural proteins (data not shown) between any of these mutants and the wild-type virus. These results coupled with the 20% plating efficiency and the "cloudy" plaque morphology of all of the oleic acid-resistant mutants detected (several hundred) suggest that a conformational alteration on or in the PR4 virion can partially, but not totally, overcome the inhibition of entry caused by the presence of oleic acid.

DISCUSSION

We have shown that various unsaturated fatty acids totally or partially inhibit the replication of the lipid-containing bacteriophage PR4 without altering the *E. coli* generation time or "inactivating" the virus. Palmitoleic and oleic acids are the most potent inhibitors of all the fatty acids tested. To inhibit phage replication, the fatty acids must be present at a sufficient concentration in the growth medium at the time of infection. These fatty acids do not prevent the attachment of phage PR4 to *E. coli* PS2R but do inhibit the phage entry process. This inhibition of entry was shown indirectly by the lack of phage-induced cell "death" (loss of colony-forming ability) in the presence of oleic or palmitoleic acid and directly by the fate of the ^{32}P and ^{35}S from labeled phage added to cells in the presence or absence of oleic acid. In addition, we showed that while oleic acid is rapidly incorporated into (and/or binds to) *E. coli*, no binding of oleic acid directly to phage PR4 was detected.

Recent electron microscopic studies by Bradley (3) and Bradley and Cohen (4) indicate that PR4 probably attaches to pili determined by the necessary cellular drug resistance plasmid. The first stages of the PR4 replication process thus might involve initial virus binding to the side or tip of the pilus and subsequent translational and re-orientational motion of the phage on the pilus to achieve proper positioning for entry. The presence of lipid in the PR4 virion suggests that fusion between the cell surface membrane and the phage "membrane" might occur during entry to allow the deposition of the phage DNA into the cell. Our finding that oleic acid inhibits entry and enhances the release of phage components from the cell surface upon washing could be the result of an inhibition either of "proper positioning" or some subsequent step such as pilus retraction or cell membrane-viral "membrane" fusion. An elucidation of the exact stage of entry that is inhibited by fatty acid might be possible by more detailed electron microscopic analyses.

ACKNOWLEDGMENTS

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

Loren Schragger provided excellent technical assistance in several experiments. The Journal's reviewers provided several excellent and helpful recommendations.

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