

# Extreme Sensitivity of Enveloped Viruses, Including Herpes Simplex, to Long-Chain Unsaturated Monoglycerides and Alcohols

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Unsaturated monoglycerides and alcohols of chain lengths of 16 or 18 carbons were found to be extremely potent inactivators of two enveloped viruses, herpes simplex virus type 2 and bacteriophage  $\phi 6$ . The lipid-containing bacteriophage PM2 was also inactivated by some of these amphiphilic molecules. Treatment of herpes simplex virus type 2 with these compounds at concentrations as low as 0.2  $\mu\text{M}$  reduced virus survival to 50% in 30 min, making these agents the most potent inactivators of herpes simplex viruses discovered that are not cytotoxic to mammalian cells. Detailed characterizations of the effects of unsaturated monoglycerides and alcohols on bacteriophages  $\phi 6$  and PM2 showed that the inactivated  $\phi 6$  virion remained nearly intact but that PM2 was almost completely disrupted by the inactivating treatment. Some of the compounds inactivate the viruses even at low temperature (0°C). Excess amounts of diglycerides and phospholipids interfere with the inactivating abilities of some of the unsaturated monoglycerides and alcohols against  $\phi 6$  and PM2. Our findings suggest that the unsaturated monoglycerides and some of the unsaturated alcohols should be further studied as potential antiviral agents, particularly for application to herpesvirus-infected areas of the skin and accessible epithelium.

It was reported 30 years ago that an unsaturated fatty acid (oleic acid, 18:1,  $\Delta 9$  *cis*) inactivates influenza type A virions (16). Very little work on the potential antiviral activity of low concentrations of fatty acids and other hydrocarbon derivatives has been done until recently. We recently reported that oleic acid and palmitoleic acid (16:1,  $\Delta 9$  *cis*) are potent inactivators of the lipid-containing bacteriophage  $\phi 6$  (10) and inhibitors of the entry process of another lipid-containing bacteriophage, PR4 (8). The common food additive BHT (butylated hydroxytoluene) has been shown to inactivate a variety of lipid-containing viruses, including herpes simplex virus (HSV), Newcastle disease virus, bacteriophage  $\phi 6$ , and bacteriophage PM2 (2, 3, 14, 19). The methyl ester derivative of the polyene antibiotic amphotericin B inactivates vesicular stomatitis virus (6), and some saturated long-chain alcohols inactivate HSV,  $\phi 6$ , and PM2 (15). None of these agents inactivates non-lipid-containing viruses at the low concentrations that cause inactivation of the tested lipid-containing viruses.

We are conducting a comprehensive survey of the antiviral effects of fatty acid derivatives. We

report here that some unsaturated long-chain alcohols and monoglycerides exhibit extremely potent virucidal effects against HSV and bacteriophages  $\phi 6$  and PM2. We also report the results of studies on the mechanism(s) of these effects on the two lipid-containing bacterial viruses.

## MATERIALS AND METHODS

**Viruses and cells.** Bacteriophage  $\phi 6$  (18) contains a loose envelope structure composed of phospholipid and protein (1, 9, 12), making it structurally similar to the enveloped animal viruses. Bacteriophage PM2 (4) contains an internal lipid bilayer whose structure and composition have been studied in detail (5). The host bacteria for  $\phi 6$  and PM2 are, respectively, *Pseudomonas phaseolicola* HB10Y and the marine bacterium *Pseudomonas* BAL-31. We routinely used a thymidine, tryptophan auxotroph of BAL-31, designated TT167. We have previously reported our procedures for  $\phi 6$  (11) and PM2 (13). The non-lipid-containing bacteriophages T4 (host *Escherichia coli*) and bacteriophage  $\phi 23$  (host *P. phaseolicola* HB10Y) were routinely used in comparative studies. The growth rates of the host bacteria were not effected by the compounds used in these studies (7).

HSV type 2 (HSV-2) was obtained from John Docherty (Department of Microbiology and Cell Biology,

The Pennsylvania State University). Stocks were prepared by growth on monolayers of human embryonic lung (HEL) fibroblasts. The virus stocks were pelleted and resuspended in a small volume of buffer to remove serum that was present in the tissue culture medium. We have found in other experiments that serum can modify the quantitative aspects of virus inactivation by hydrophobic agents.

Routine plaque assays were carried out on monolayers of HEL cells in petri dishes with 0.5% methyl cellulose in the overlay medium.

**Media and buffers.** Experiments with  $\phi 6$ ,  $\phi 23$ , and T4 were carried out in NBY medium (18) which contains 8 g of nutrient broth, 2 g of yeast extract, 0.5 g of  $\text{KH}_2\text{PO}_4$ , 2 g of  $\text{K}_2\text{HPO}_4$ , 5 g of glucose, and 0.25 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per liter of distilled water. LS medium, which was used for  $\phi 6$  isotope-labeling experiments, contains 12.1 g of tris(hydroxymethyl)aminomethane, 1.5 g of KCl, 15 g of NaCl, 25 mg of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g of  $\text{NH}_4\text{Cl}$ , 5 g of glucose, 25 mg of  $\text{KH}_2\text{PO}_4$ , and 75 mg of  $\text{K}_2\text{HPO}_4$  per liter of distilled water, adjusted to pH 7.6.

Experiments with PM2 were carried out in Q medium (13), which contains 10 g of tryptone (Bacto, Difco), 5 g of yeast extract, 0.7 g of KCl, 26 g of NaCl, 1.5 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 12 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per liter of distilled water. M25S, used for producing radioisotope-labeled PM2 virions, is a standard supplemented minimal medium that has been described previously (13).

HSV-2 (strain 316-D) was exposed to compounds in a tricine-buffered salts solution (TBS) which contains 8 g of NaCl, 0.38 g of KCl, 0.1 g of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.1 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 4.5 g of *N*-tris(hydroxymethyl)methylglycine (tricine) per liter of distilled water. The pH was adjusted to 7.4 with NaOH.

**Virus treatment procedures.** The compounds used in this study are insoluble in water. Therefore, they were dissolved in ethanol at 100 times the desired final concentration and added to the aqueous virus suspension to give a final ethanol concentration of 1%, which itself results in no virus inactivation for any of the viruses used in this study. Virus concentrations for these experiments varied from  $10^5$  to  $10^7$  plaque-forming units (PFU) per ml. Over this range, no dependence of inactivation on virus concentration was detectable.

**Sucrose gradient analyses.** Stocks of bacteriophage  $\phi 6$  or PM2 labeled with  $^{32}\text{P}$  were purified and analyzed after treatment by sucrose gradient velocity sedimentation. Treated or untreated  $\phi 6$  samples were layered onto 15 to 30% sucrose gradients (in LS medium) and centrifuged at 27,000 rpm for 120 min in an SW27 rotor. PM2 samples were layered onto 20 to 30% sucrose gradients (in medium M25S) and centrifuged at 35,000 rpm for 120 min in an SW41 rotor.

**Sources of materials.** All of the alcohols and monoglycerides used in this study were obtained as the best grade supplied by Sigma Chemical Co., St. Louis, Mo., and were used without further purification. [ $^{32}\text{P}$ ]orthophosphoric acid (10 mCi/ml) was obtained from New England Nuclear Corp., Boston, Mass.

## RESULTS

**Virus inactivation by monoglycerides.** The lipid-containing bacteriophages  $\phi 6$  and

PM2, along with the non-lipid-containing bacteriophages T4 and  $\phi 23$ , have been shown to be valuable for preliminary screening of agents that might be expected to exhibit effects against lipid-containing viruses (14, 15). By this preliminary screening, we have found that a variety of unsaturated monoglycerides and alcohols are potent inactivators of lipid-containing viruses.

Figure 1 shows the concentration dependency of the inactivation of HSV-2 by monopalmitolein. The virus was exposed in a buffered salt solution to monopalmitolein at 25°C for 30 min and then assayed for surviving PFU. It is clear that monopalmitolein is a potent inactivator of HSV-2, with a concentration of approximately 1.8  $\mu\text{M}$  reducing virus survival to 50% under the treatment conditions. Graphs like that shown in Fig. 1 were used to determine the concentration of each monoglyceride tested that reduces HSV-2,  $\phi 6$ , or PM2 survival to 50% under the treatment conditions. These results, which are presented in Table 1, indicate that the unsaturated monoglycerides monopalmitolein, monoolein, and monolinolein are all extremely potent inactivators of HSV-2, but the saturated monoglycerides are not. Bacteriophage  $\phi 6$  is also very sensitive to these same agents, whereas PM2 is very sensitive only to monopalmitolein. T4 and  $\phi 23$  are insensitive to all the monoglycerides tested (data not shown).

**Virus inactivation by unsaturated long-chain alcohols.** Snipes et al. (15) previously reported that low concentrations of saturated alcohols of chain lengths of 10 to 14 carbons inactivated HSV and  $\phi 6$ , whereas PM2 was inactivated by the 10- and 12-, but not 14-carbon saturated alcohols. We have extended this study to include unsaturated long-chain alcohols (Table 2).

HSV-2 is very sensitive to almost all of the unsaturated alcohols tested from chain lengths of 14 to 20 carbon atoms and containing one (*cis* or *trans*) to four double bonds at various positions along the alkyl chain.  $\phi 6$  is also extremely sensitive to all of these compounds, but PM2 is inactivated only by palmitoleyl alcohol. These unsaturated alcohols are more potent inactivators of HSV-2 and  $\phi 6$  than are the saturated alcohols reported previously (15) and tested again during these experiments (Table 2).  $\gamma$ -Linolenyl alcohol is the most potent compound against HSV-2 of any of the compounds tested during this and previous (14, 15) work.

**Effects on cells.** Monolayers of HEL cells were treated for 2 h with the monoglycerides and alcohols at 150  $\mu\text{M}$  and then observed for the appearance of any noticeable cytopathic effect. No cytopathic effect was observed for any of the compounds at this concentration, which is approximately 100-fold higher than the con-

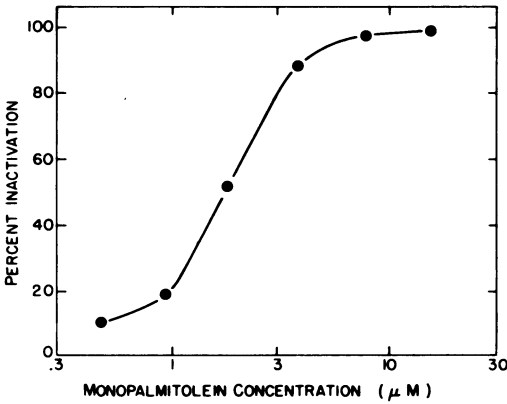


FIG. 1. Inactivation of HSV-2 by different concentrations of monopalmitolein.

TABLE 1. Concentrations required for 50% inactivation of viruses by monoglycerides (30-min exposure at 25°C)

Monoglyceride	Concn (μM)		
	HSV-2	φ6	PM2
14:0	44	36	36
16:0	>75	30	>75
16:1 (9)	1.8	1.5	3.0
18:0	>75	>75	>75
18:1 (9)	1.0	1.4	14
18:2 (9, 12)	1.1	0.7	14

TABLE 2. Concentrations required for 50% inactivation of viruses by unsaturated alcohols (30-min exposure at 25°C)

Alcohol	Concn (μM)		
	HSV-2	φ6	PM2
14:0	4.7	2.0	>75
14:1 (9)		0.9	>75
16:1 (9)	2.1	0.8	40
18:1 (9)	4.1	0.8	>75
18:1 (9 trans)	1.9	0.4	>75
18:2 (9, 12)	1.0	1.9	>75
18:3 (9, 12, 15)	1.0	1.9	>75
18:3 (6, 9, 12)	0.2	0.4	>75
20:4 (5, 8, 11, 14)	3.5	0.7	>75

centrations which cause significant inactivation of HSV-2. It is thus clear that the virus is much more susceptible than the host cell to these compounds. In addition, no detectable decrease in the number of viable cells was observed when the cells were exposed for 24 h to the compounds at the low concentrations that cause virus inactivation.

**Effects of temperature.** Because the physical properties of membranous structures are highly temperature dependent, we investigated

the temperature dependencies of the inactivation of bacteriophages φ6 and PM2 by unsaturated monoglycerides and alcohols. The unsaturated alcohols, in ethanol solution, were added to nutrient medium containing φ6 at ~10<sup>7</sup> PFU/ml at zero time. At later times, samples were diluted and assayed for number of PFU of φ6. Figure 2 shows the kinetics of the inactivation of φ6 at 0 and 25°C by elaidyl alcohol and γ-linolenyl alcohol, which are the two most potent inactivators of φ6 (at 25°C) described in this report. Although the kinetics of φ6 inactivation are very similar for these two agents at 25°C, there is a significant difference at 0°C: specifically, elaidyl alcohol (at 3.8 μM) does not inactivate φ6 at 0°C but γ-linolenyl alcohol does, albeit at a reduced rate compared to 25°C. In a more detailed analysis of the temperature dependency of the inactivation of φ6 by these two alcohols, unsaturated alcohols were added to NBY medium containing φ6 at ~10<sup>7</sup> PFU/ml at various temperatures. Fifteen minutes later, samples were diluted and assayed for PFU. Figure 3 shows that the antiviral activity of elaidyl alcohol is strongly temperature dependent in the range from 5 to 15°C. The activity of γ-linolenyl alcohol is much less sensitive to temperature in the range from 0 to 30°C. Potentially relevant to these findings are the melt temperatures of these pure compounds: elaidyl alcohol is a solid

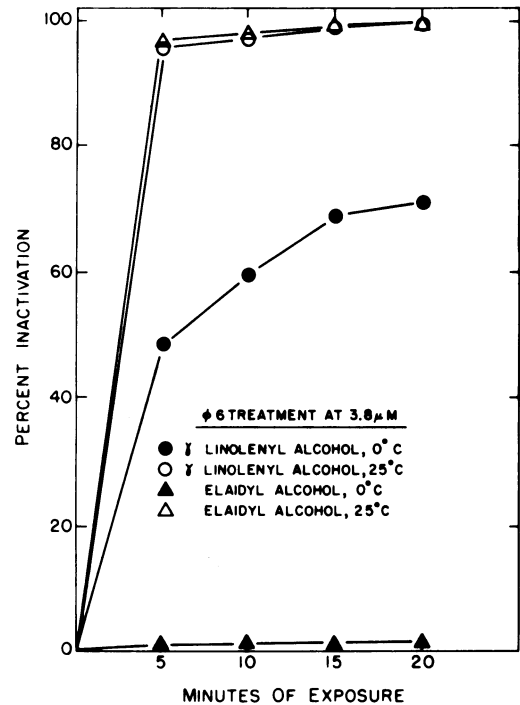


FIG. 2. Kinetics of inactivation of phage φ6 by γ-linolenyl alcohol and elaidyl alcohol at 25 and 0°C.

over this temperature range (melting temperature  $\approx 37^\circ\text{C}$ ), whereas  $\gamma$ -linolenyl alcohol is a liquid (melting temperature  $< -5^\circ\text{C}$ ).

Using a procedure similar to that described above, we investigated the temperature dependency of the inactivation of  $\phi 6$  and PM2 by monopalmitolein, which is the most potent inactivator of PM2 found to date. The results (Fig. 4) for the inactivation of PM2 by two concentrations of monopalmitolein show that there is a plateau in the temperature dependency in the range 10 to 15°C (the data shown are an average of four experiments, each of which gave the same qualitative results). No plateau occurs in the temperature dependency of the inactivation of  $\phi 6$  by this compound.

**Sedimentation properties of inactivated virus.** To determine the degrees of physical disruption of the virions caused by exposure to unsaturated alcohols and monoglycerides, sucrose gradient velocity sedimentation analyses on  $^{32}\text{P}$ -labeled, purified virus preparations were performed. Analysis of  $\phi 6$  virions inactivated by an unsaturated fatty acid (oleic acid [10]) were performed for comparison with the unsaturated monoglycerides and alcohols. The results of the

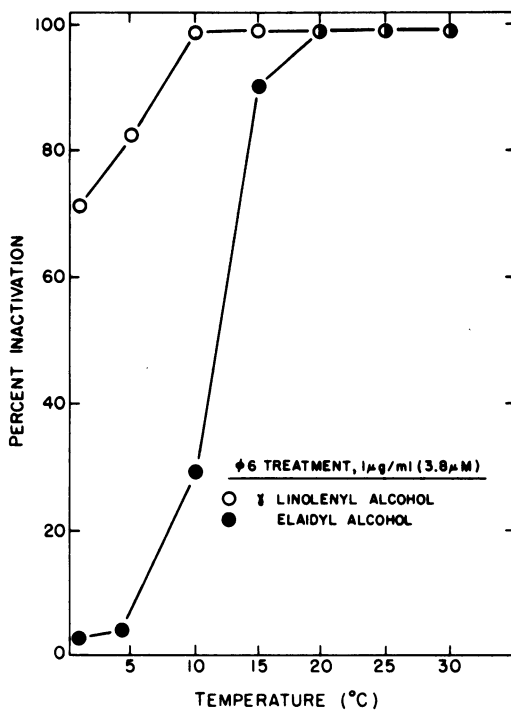


FIG. 3. Temperature dependency of the inactivation of phage  $\phi 6$  by  $\gamma$ -linolenyl alcohol and elaidyl alcohol. The data shown are an average of three experiments.

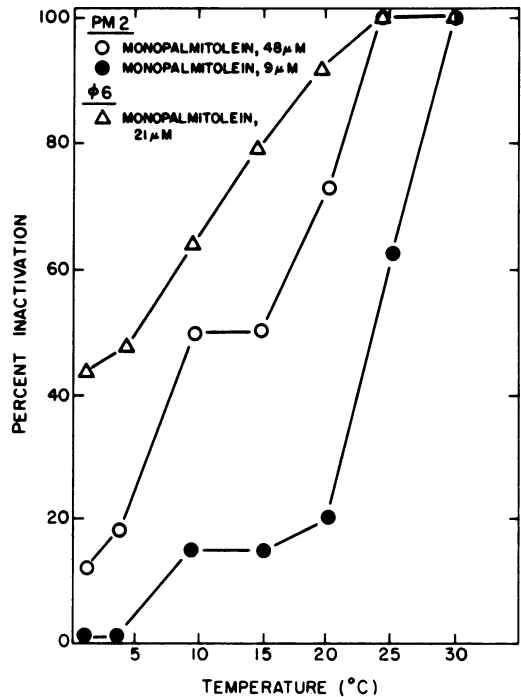


FIG. 4. Temperature dependencies of the inactivation of PM2 and  $\phi 6$  by monopalmitolein. The data shown are an average of four experiments.

analyses on  $\phi 6$  (Fig. 5) show that whereas oleic acid significantly disrupts the virion (probably by completely removing the envelope),  $\phi 6$  virions inactivated by monoolein or  $\gamma$ -linolenyl alcohol sediment nearly like infectious virions except for a small amount of material that remains at the top of the gradient. This suggests that exposure of  $\phi 6$  to either of these compounds results in the removal of some of the envelope lipids (and perhaps proteins), but that the virion remains nearly intact. These nearly intact virions are, however, unable to attach to host cells (data not shown).

Figure 6 shows the gradient analysis of PM2 virions that have been inactivated by monopalmitolein. Because nearly all of the virus material remains at the top of the gradient, we conclude that treatment of PM2 with this low concentration of monopalmitolein results in a nearly complete disassembly of the PM2 particle.

**Interference effects.** Relevant to any possible future use of these types of hydrocarbon derivatives as virucidal agents is the extent to which other molecules might interfere with the ability of the agent to inactivate a given virus. We have investigated this aspect of the effects of unsaturated alcohols and monoglycerides

against  $\phi 6$  and PM2. We screened for interfering effects of a carbohydrate (sucrose), a protein (bovine serum albumin), and lipid. Distearin or diolein was added to nutrient medium containing  $\phi 6$  at  $\sim 10^7$  PFU/ml. Approximately 1 min later, monolinolein was added. Samples were incubated for 30 min at 25°C and then diluted and assayed for PFU. Similar results were obtained if the monoacylglyceride and diacylglyceride were first mixed together (in ethanol solution) and then added to the virus suspension. Neither sucrose nor bovine serum albumin (at 100  $\mu\text{g/ml}$ ) had any interfering effect, but an interfering effect of lipid was found. Figure 7 shows that diglycerides (which do not inactivate  $\phi 6$  or PM2) can interfere with the inactivation of  $\phi 6$  by monolinolein. The unsaturated diglyceride diolein is a better interferer than is the saturated diglyceride of the same chain length, distearin. The inactivating and potentially interfering compounds were mixed together in the aqueous medium and incubated at the indicated

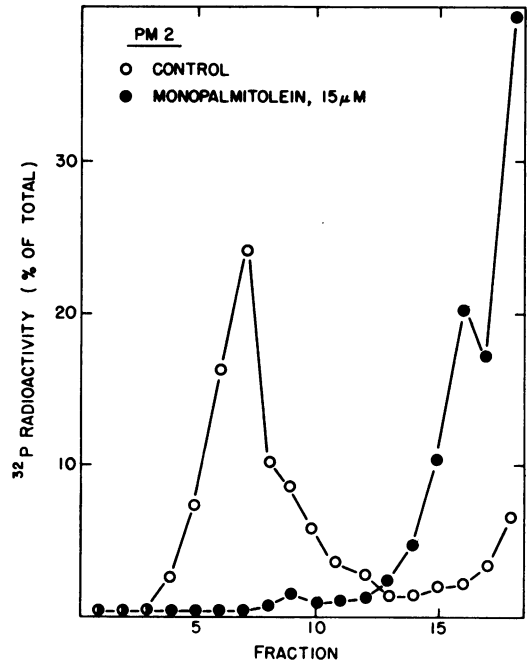


FIG. 6. Sucrose gradient velocity sedimentation analysis of inactivated  $^{32}\text{P}$ -labeled PM2 virions. Sedimentation is from right to left.

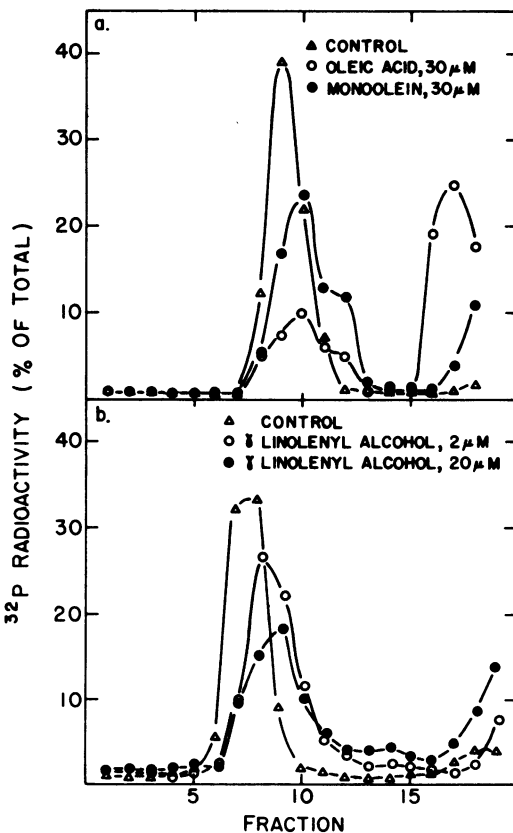


FIG. 5. Sucrose gradient velocity sedimentation analysis of inactivated  $^{32}\text{P}$ -labeled  $\phi 6$  virions. Sedimentation is from right to left.

temperature for 2 min. Virus was then added and exposed to the compounds for 15 min. In a more comprehensive survey of such interference effects, Table 3 shows that diolein (if present in excess compared with the inactivating agent) interfered with the inactivation of  $\phi 6$  and PM2 by the most potent inactivating agents at 25°C much better than it did at 37°C. Several other hydrocarbon derivatives similar to  $\gamma$ -linolenyl alcohol and elaidyl alcohol did not interfere with the inactivation of  $\phi 6$  by these agents. A phospholipid, dipalmitoylphosphatidylcholine, interfered with the inactivation of  $\phi 6$  by all three agents. Unlike the result with diolein, dipalmitoylphosphatidylcholine interfered better at 37 than at 25°C.

DISCUSSION

Recurrent HSV infections, both type 1 and type 2, remain without effective treatment. Several agents that inhibit synthesis of viral DNA offer some promise, but in general this class of drugs has potential side effects that limit their desirability for general use. Consequently, the development of new approaches to antiviral chemotherapy, including the topical use of hydrophobic, membrane-active compounds such as those described here, seems worthwhile. To this end, our experiments have been directed toward

the structural parameters that optimize virucidal activity as well as other factors that influence the effectiveness of any given compound.

The unsaturated monoglycerides and some of

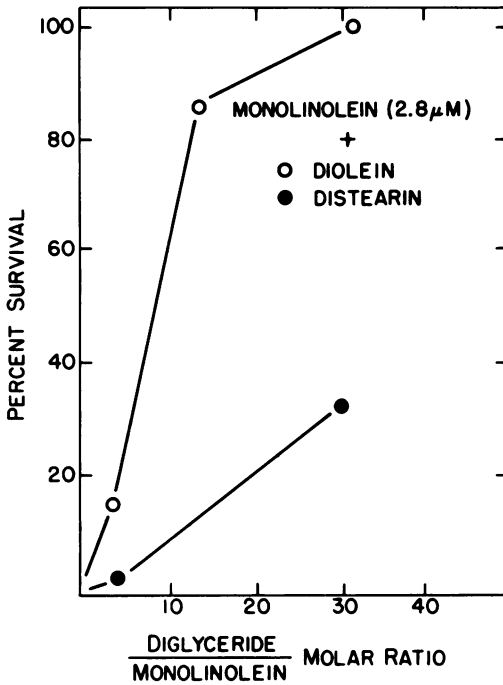


FIG. 7. Effect of the presence of diacylglycerides on the inactivation of  $\phi 6$  by a monoacylglyceride.

the unsaturated alcohols are the most potent inactivators of HSV yet discovered that are not toxic to host cells. The importance of the presence of one or more double bonds is especially evident for the monoglycerides. The 16- and 18-carbon saturated alkyl chain compounds monopalmitin and monostearin are inactive against HSV-2, whereas the three unsaturated compounds monopalmitolein, monoolein, and monolinolein are extremely potent inactivators. Most of the unsaturated alcohols are also more potent than the respective saturated alcohols (15), with  $\gamma$ -linolenyl alcohol being the most potent inactivator of herpesviruses ever reported.

The detailed studies carried out on the inactivation of the lipid-containing bacteriophages  $\phi 6$  and PM2 gave some interesting results. The sedimentation analyses performed on inactivated virions showed that  $\phi 6$  virions inactivated by a potent unsaturated alcohol ( $\gamma$ -linolenyl alcohol) or monoglyceride (monoolein) remain nearly intact but have lost the ability to attach to host cells, whereas phage PM2 is nearly totally disrupted by monopalmitolein. The measured temperature dependencies of inactivation of these phages may be related to the ability of the compound to partition from its micellar aggregates (16) into the viral lipid bilayer, thereby causing limited disruption of the external  $\phi 6$  envelope but much greater disruption, and hence virus disassembly, of the internal PM2 lipid bilayer. These sedimentation analyses results are similar to those we previously found for the inactivation of  $\phi 6$  (19) and PM2 (3) by BHT (at

TABLE 3. Effects of lipid on the inactivation of  $\phi 6$  and PM2 by unsaturated alcohols and monoglycerides

Virus	Inactivating compound (concn, $\mu\text{g}/\text{ml}$ )	Other compound present (50 $\mu\text{g}/\text{ml}$ )	% Survival at:	
			25°C	37°C
$\phi 6$	$\gamma$ -Linolenyl Alcohol (1)	None	2	1
		Stearyl alcohol	2	1
		Diolein	75	4
		Phosphatidylcholine	80	100
	Elaidyl Alcohol (1)	None	2	1
		Stearyl alcohol	2	1
		Elaidyl acetate	2	1
		Diolein	75	1
		Phosphatidylcholine	45	100
	Monoolein (1)	None	4	1
		Diolein	100	50
		Triolein	40	5
Phosphatidylcholine		50	100	
PM2	Monopalmitolein (15)	None	10	<1
		Diolein	70	4
		Triolein	15	<1
		Phosphatidylcholine	70	30

higher concentrations than those used in the experiments reported here).

The interference by some diglycerides and phospholipids with the inactivation of  $\phi 6$  by the unsaturated alcohols and monoglycerides might be due to the ability of the alcohol or monoglyceride to partition into the diglyceride or phospholipid aggregates, thus giving mixed aggregates that have nearly the same physicochemical properties as the aggregates of pure diglyceride or phospholipid. Because the alcohols and monoglycerides are probably forming micelle aggregates while the diglycerides and phospholipids are perhaps aggregated into vesicles of heterogeneous size (17), the fusion of the micelles into the relatively abundant vesicles could result in loss of virucidal activity. These results may be significant in relation to any future clinical use of these unsaturated alcohols and monoglycerides as virucidal agents.

Our *in vitro* results show that  $\gamma$ -linolenyl alcohol is extremely active against HSV and that several unsaturated monoglycerides are also effective. All of these compounds are nontoxic to cultured HEL cells. The monoglycerides are common natural products.

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#### ADDENDUM IN PROOF

Welsh, Skurrie, and May have recently reported (*Infect. immun.* 19:395-401) that at least a significant part of the virucidal activity of human milk resides in the monoglyceride and free fatty acid fractions.

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