ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1976, p. 96-101

Vol. 10, No. 1
Printed in U.S.A.

Inactivation of the Enveloped Bacteriophage ϕ6 by Butylated Hydroxytoluene and Butylated Hydroxyanisole

PAUL WANDA, JAMES CUPP, WALLACE SNIPES,* ALEC KEITH, TOM RUCINSKY,[1] LOUIS POLISH, AND JEFFREY SANDS

Biophysics Laboratory, Department of Biochemistry and Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802, and Physics Department and Molecular Biology Program, Lehigh University, Bethlehem, Pennsylvania 18015*

Received for publication 5 April 1976

Butylated hydroxytoluene (BHT) is a potent inactivator of the enveloped bacterial virus ϕ6 at concentrations as low as 3×10^{-5} M. The viral envelope is not removed by BHT treatment, in contrast to the effects of exposure to the detergent Triton X-100. BHT-treated viruses are morphologically indistinguishable from controls but are defective in their ability to attach to the host cell. Temperature at the time of exposure was found to be a crucial factor in the effectiveness of BHT against ϕ6. A precipitous drop in the degree of inactivation by 3×10^{-5} M BHT occurred when the temperature was lowered from 20 to 15 C. Calcium ions were found to potentiate the effect of BHT, particularly at lower temperatures where BHT alone was relatively ineffective. Barium and strontium, but not magnesium, were also effective in enhancing the activity of BHT. A structurally related molecule, butylated hydroxyanisole (BHA), was also found to inactivate ϕ6 virus, but higher concentrations were required than with BHT. Both BHT and BHA are commonly used as food additives, have apparent low toxicity to humans and other animals, and are potentially useful as antiviral agents.

It was recently reported from our laboratory that butylated hydroxytoluene (BHT) is a potent inactivator of lipid-containing viruses (11). This compound is generally recognized as safe by the Food and Drug Administration (5) and is widely used as an antioxidant in food and cosmetic preparations. The combination of effectiveness against viruses and apparent low toxicity to humans has generated considerable interest in the potential use of BHT as an antiviral agent, particularly for topical application to virus-infected areas of the skin. Consequently, we are characterizing the effects of BHT on lipid-containing viruses in vitro, hoping to improve our understanding of the mechanisms whereby BHT destroys viral infectivity.

In a detailed study of the effects of BHT on the bacterial virus PM2, it was found that virus particles are completely disrupted by BHT, with the viral deoxyribonucleic acid being released to the medium as a very slowly sedimentable material (1). Several factors which influence the inactivation of PM2 by BHT, including the initial virus titer, the time of exposure, and the presence of certain "protective" agents including surfactants, bovine serum albumin, and bacterial cells, were studied. Potentially important with regard to the use of BHT as an antiviral substance was the observation that its effectiveness is strongly dependent upon the solvent from which it is added to the culture medium.

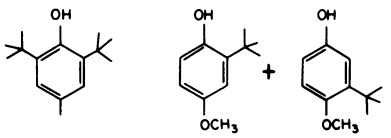
Different viruses have different susceptibilities to BHT, and the most susceptible one studied thus far is the enveloped bacterial virus ϕ6. This virus is bounded by a rather loose membrane structure (3, 13) and has many similarities to several animal viruses. We have now studied in more detail the inactivation of ϕ6 by BHT, and the results of this research are described here. In addition, we report that the related food additive butylated hydroxyanisole (BHA) also inactivates ϕ6, although higher concentrations are required than for BHT. The chemical structures of BHT and BHA are given in Fig. 1. BHA, as shown, is a mixture of two isomers.

MATERIALS AND METHODS

Viruses and cells. Bacteriophage ϕ6 was discovered by Vidaver et al. (13). Our initial stocks of ϕ6 and its host, *Pseudomonas phaseolicola* strain HB10Y, were kindly provided by Anne Vidaver.

Media and culture methods. Two types of media were used for the culture of HB10Y and ϕ6. V me-

[1] Present address: Washington University Medical School, Cancer Center, St. Louis, Mo. 63130.



BHT BHA

FIG. 1. *Structures of BHT and BHA.*

dium contained 10 g of tryptone, 5 g of yeast extract, 7 g of NaCl, 1.5 g of KCl, and 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water. V plates were composed of V medium hardened with 1.5% (wt/vol) agar (Difco Laboratories, Detroit, Mich.).

A defined but somewhat enriched medium, designated TBM21, contained 3 g of KCl, 6 g of NaCl, 2 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of KH_2PO_4, 5 g of glucose, and 12.1 g of tris(hydroxymethyl)-aminomethane (Tris) base per liter of distilled water. The pH was adjusted to 7.6 with concentrated HCl. Medium TBM21 also contains all the naturally occurring amino acids except tryptophan (20 mg of each per liter) and the purine nucleosides adenosine and guanosine (50 mg of each per liter).

A Tris-buffered dilution solution, designated TB, contained the same salts as TBM21 but lacked glucose and the additional amino acid and nucleoside supplements.

Sucrose gradient analysis. Sucrose gradients (15 to 35%) were prepared in 50% medium TBM21. Virus samples were layered on top of the gradients and centrifuged for 180 min at 25,000 rpm in a Beckman SW27 rotor. The tube was punctured, and fractions were collected for assay.

Electron microscopy. A parlodian-carbon-coated grid was floated on the virus sample for approximately 5 min and then transferred, with a drop of sample adhering, to a drop of 0.2 M sodium cacodylate buffer, pH 7.0, containing 1% glutaraldehyde. After 5 min the grid was washed twice with distilled water and stained by transferring to a drop of 1% phosphotungstic acid for 1 to 2 min. Excess fluid was removed with filter paper, and the sample was dried in air. Electron micrographs were obtained with a Philips EM300 operating at 60 kV.

Source of materials. Carrier free $H_3{}^{32}PO_4$ was obtained from New England Nuclear Corp., Boston, Mass. BHT was purchased from Aldrich Chemical Co., Milwaukee, Wis., under the name 2,6-di-tert-butyl-4-methylphenol. BHA was obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Sedimentation properties of BHT-treated virus. Samples of BHT-treated virus were analyzed on sucrose gradients to determine the degree to which the virus particles are disrupted by BHT treatment. In particular, we were interested to know whether or not the viral envelope is removed during BHT treatment. It is known that the detergent Triton X-100 removes the membrane envelope from ϕ6, leaving only the inner icosahedral core (9). Therefore, Triton X-100-treated samples were also analyzed for comparative purposes.

A radioactively labeled virus stock was prepared in medium TBM21 supplemented with $^{32}PO_4$. After lysis cell debris was removed by low-speed centrifugation, and the virus was pelleted, resuspended in a small volume of medium TBM21, and purified on a sucrose gradient. The peak fractions of radioactivity (and plaque-forming units) were used in further experiments.

One sample of labeled virus was treated with 0.1 mM BHT for 30 min. This resulted in greater than 99% inactivation of the virus. Another sample was treated with 1% Triton X-100, again resulting in greater than 99% inactivation. The treated samples and an untreated control were layered onto sucrose gradients and analyzed as described in Materials and Methods. The results are shown in Fig. 2. For the control and the BHT-treated samples, the ^{32}P radioactivity forms a band centered about fraction 13 with very little radioactivity at the top of the gradient. In several experiments of this type, control and BHT-treated samples gave indistinguishable results, with never more than one fraction separating the peaks of radioactivity. Plating results showed that, for the control, the peaks of virus infectivity and ^{32}P radioactivity coincide, whereas the virus titer

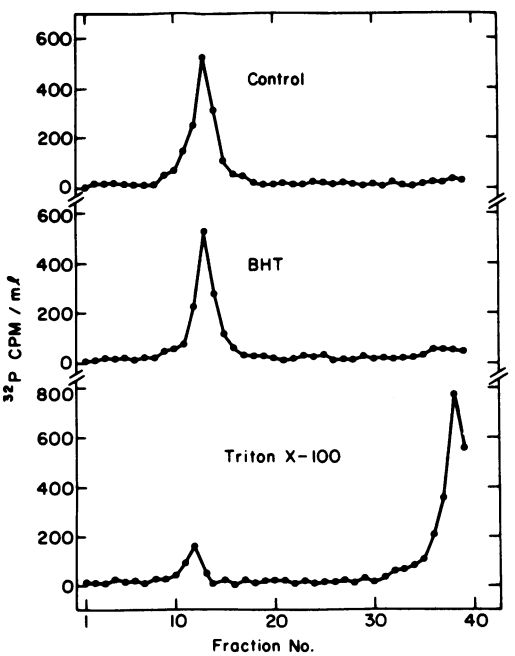


FIG. 2. *Sucrose gradient analysis of control, BHT-treated, and Triton X-100-treated samples of ϕ6 virus. The direction of sedimentation in this figure is from right to left.*

in the peak fractions of the BHT-treated sample was less than 1% of that for the control. Thus, sucrose gradient analysis shows no observable difference in the sedimentation properties of inactive, BHT-treated virus and the untreated, infectious virus.

The sample treated with Triton X-100 gave a gradient profile with ^{32}P radioactivity in two positions. A band centered about fraction 12, much reduced in total radioactivity compared to bands of control and BHT-treated samples, contained the ϕ6 cores (see next section). The material near the top of the gradient was not characterized further but was most likely the phospholipid material released from the virion by Triton X-100 treatment.

These data indicate quite strongly that the envelope of the ϕ6 is not removed by BHT treatment under conditions where viral infectivity is reduced to less than 1%. This conclusion is further substantiated by data described in the following section.

Electron microscopy studies. Samples from the peak fractions of the gradients of Fig. 2 were prepared and examined by electron microscopy. These experiments were carried out to further establish that the ϕ6 envelope was intact in BHT-treated virus and to detect any noticeable morphological differences that might exist between treated and control samples. Electron micrographs showed that BHT-treated samples were indistinguishable from controls, whereas Triton X-100-treated samples were lacking the viral envelope. We have quantified these observations by measuring the diameter of a large number of particles from each sample and displaying the data as a size distribution (Fig. 3). The size of ϕ6 particles has been reported to vary from about 60 to 100 nm, with the average being about 80 nm (3). A least-squares analysis of the data of Fig. 3 gives diameters of 81.8 ± 6.3 nm for control and 80.1 ± 6.6 nm for the BHT-treated samples. Within experimental error, these size distributions are identical, further establishing that the ϕ6 envelope is intact after BHT treatment. The particles observed after Triton X-100 treatment are smaller and more uniform in geometry, with a size distribution of 65.5 ± 2.7 nm and the morphological characteristics of virus cores.

Attachment studies. Experiments were carried out to determine whether inactive, BHT-treated virus particles can attach to the host bacterial cells. Radioactively labeled samples from the peak fractions of sucrose gradients such as those of Fig. 2 were used. The control or BHT-inactivated virus were mixed with HB10Y cells and incubated at 25 C. At different times, samples were centrifuged to pellet host cells and attached virus, and the supernatants were assayed for radioactivity associated with unattached virus particles. These measurements were used to calculate the percentage of virus attachment.

Date from two separate experiments are shown in Table 1. Although there is some scatter in the data, it is clear that the process of attachment is defective in BHT-inactivated virus. These experiments, therefore, establish that the portion of the ϕ6 virus life cycle where BHT is effective is in the attachment of virus particles to the host cell. Little is known about the attachment process for ϕ6, and an under-

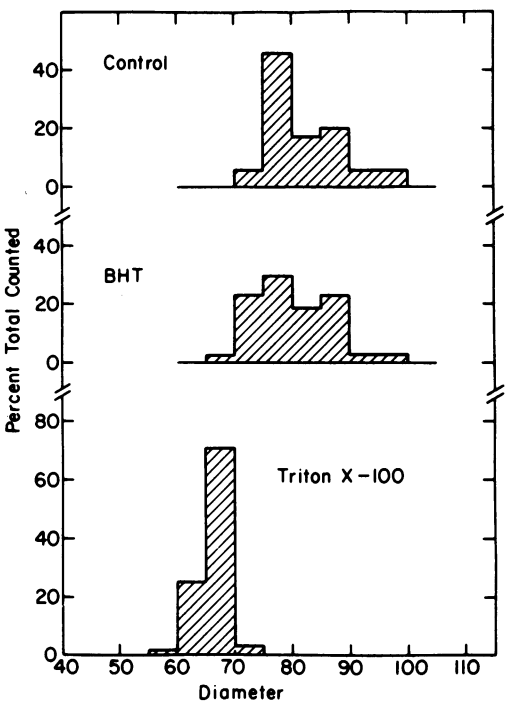


FIG. 3. *Size distribution of ϕ6 virus particles as determined from electron micrographs. The numbers of particles measured were 35, 44, and 65 for control, BHT-treated, and Triton X-100-treated samples, respectively. The horizontal axis is given in nanometers.*

TABLE 1. *Attachment of control and BHT-treated virus to host cells*

Time (min)	Expt	Attachment (%)	
		Control	BHT-treated
30	1	19	4
60	1	40	0
45	2	72	12
75	2	77	8

standing of the mechanism whereby BHT inhibits the specific virus-host cell interaction will require further investigations.

Temperature effects. The physical properties of membrane structures are strongly dependent on temperature. Hydrophobic molecules can partition more favorably into the hydrocarbon zones of membrane lipids at higher temperatures than at lower temperatures (12). We carried out experiments to determine the effects of temperature on the inactivation of ϕ6 by BHT. In one experiment virus survival was measured after different times of exposure to 0.05 mM BHT at 0 and 25 C. These results show a striking difference in survival at the two temperatures (Fig. 4). After a 5-min exposure at 25 C the survival is less than 0.1%, whereas a 2-h exposure at 0 C results in no more than 50% inactivation. We next determined the effects of a 30-min exposure to 0.03 mM BHT at various temperatures in the range 0 to 33 C; the results are shown in Fig. 5. Up to about 15 C there is very little inactivation under these conditions. A precipitous drop in survival occurs between 15 and 20 C, with greater than 99% inactivation at all temperatures above 20 C. These results make it clear that temperature is a crucial parameter in the inactivation of ϕ6 by BHT.

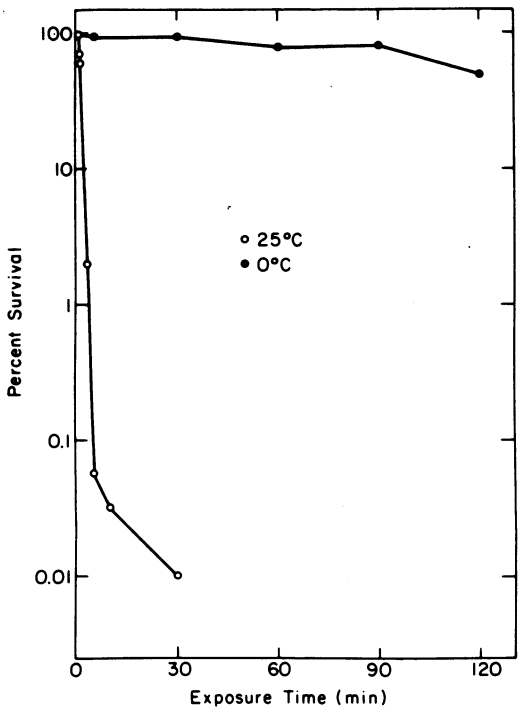


FIG. 4. *Survival of ϕ6 virus as a function of time of exposure to 0.03 mM BHT at 0 and 25 C. From a solution of 3 mM BHT in 95% ethanol, 0.1 ml was added to 5 ml of V medium and vortexed gently. Thirty seconds later (t = 0) 5 ml of a ϕ6 sample at about 2 × 10^{10} plaque-forming units per ml (in V medium) was added, and the mixture was again gently vortexed. At various times after mixing the virus and BHT suspensions, samples were diluted appropriately in TB buffer at the same temperature used for exposure and plated for plaque-forming units on V plates with HB10Y cells as an indicator host. Data are presented as percentage of survival compared to control samples in the absence of BHT.*

Effects of calcium ions. Several divalent ions, notably Ca^{2+}, interact strongly with membrane components and may therefore modify the response of membranes to other molecules. We investigated the effects of Ca^{2+} on the inactivation of ϕ6 by BHT and found a considerable modification of the antiviral activity of BHT in the presence of Ca^{2+}. At low temperatures, where 0.03 mM BHT is relatively inactive in the absence of added Ca^{2+}, the addition of Ca^{2+} in concentrations from about 5 to 50 mM greatly enhanced the inactivation by BHT. Ca^{2+} alone at these concentrations was without effect. The data of Fig. 5 illustrate the effect of 50 mM Ca^{2+} on BHT inactivation versus temperature. At 15 C, the percentage of survival is reduced from 88 to 2.5% by the addition of 50

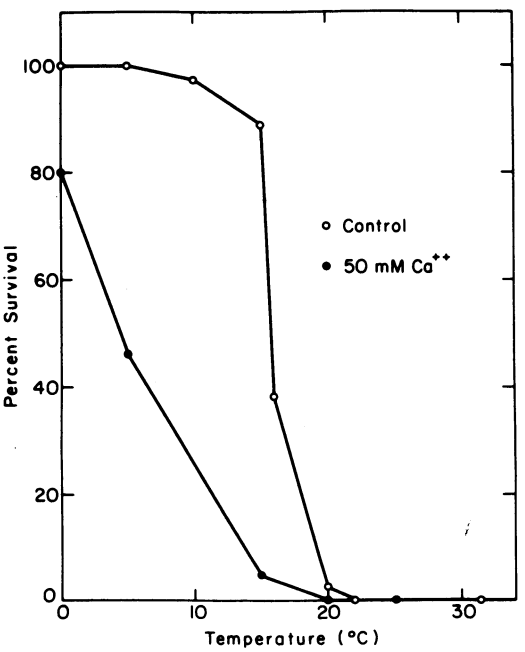


FIG. 5. *Survival of ϕ6 virus after exposure to 0.03 mM BHT for 30 min at different temperatures. Exposures were carried out in V medium as described in Fig. 4, with and without 50 mM added calcium chloride. The residual level of Ca^{2+} in V medium, as measured by flame photometry, is about 0.3 mM.*

mM Ca^{2+}. These observations may be of considerable importance in the design of antiviral preparations using BHT as the active agent.

In many systems barium and strontium, but not magnesium, can substitute for calcium in certain aspects of membrane structure and function (6, 7, 10). We have found similar results in the present work. Virus was exposed at 15 C to 0.03 mM BHT in the presence of ions at different concentrations. Data for ion concentrations of 5 and 50 mM, presented in Table 2, show similar enhancement of BHT activity by Ca^{2+}, Ba^{2+}, and Sr^{2+}, but much less enhancement by Mg^{2+}. At higher concentrations Mg^{2+} shows a mild potentiating effect, but this is smaller even at 50 mM than the effect of Ca^{2+} at 5 mM.

Inactivation of ϕ6 by BHA. BHA has some structural similarities to BHT and is about equally widespread as a food additive. Experiments were carried out to determine whether BHA is effective at inactivating ϕ6 virus. Figure 6, which presents data for a 30-min exposure to different concentrations of BHA and BHT, shows that BHA does inactivate ϕ6 but that somewhat higher concentrations are required as compared to BHT.

Samples of BHA-treated virus were analyzed on sucrose gradients as described in the BHT studies. No differences were observed in the sedimentation properties of control and BHA-treated samples under conditions where exposure to BHA reduced the plaque-forming units to less than 1%. This suggests similar mechanisms for BHT and BHA activity. No further analysis of BHA-treated virus was carried out.

DISCUSSION

The envelope of ϕ6 has been described as a "membranous, compressible, saclike structure" surrounding the virus core (13). Its fatty acid composition is nearly identical to that of the host cell, with 16- and 18-carbon, saturated and monounsaturated fatty acids accounting for about 95% of the total fatty acids (13). Both the ϕ6 and the host cell have phosphatidylethanolamine and phosphatidylglycerol as their major phospholipids, but the proportions are quite different in the two cases. ϕ6 has an unusually high proportion of phosphatidylglycerol. This negatively charged phospholipid makes up about 57% of the total lipids (8).

Many organic solvents and surfactant substances will inactivate lipid-containing viruses, frequently by extracting or removing the membrane components. Our experiments show that BHT does not remove the ϕ6 envelope but inactivates the virus by a different mechanism. After BHT treatment, virus particles are unable to attach to host cells. Electron micrographs of crude lysates from untreated preparations reveal virus particles attached to pili of host bacterial cells (13). It is not known whether this association is due to specific attachment sites on the virus and cell or whether other polar or hydrophobic interactions of a less specific nature are involved. With regard to the effect of BHT on ϕ6 attachment the question remains as to whether BHT is interacting with the membrane lipids, proteins, or both. BHT has been shown to exert disordering effects on lipid alkyl chains in micellar structures (2) and in the hydrocarbon interior of membrane bilayers (4). Interactions of this type in the ϕ6 mem-

TABLE 2. *Effect of divalent ions on the inactivation of ϕ6 by BHT at 15 C*

Ion	Concn added (mM)[a]	Survival (%)
None		88
Calcium	5	32
	50	4.5
Barium	5	47
	50	8.5
Strontium	5	30
	50	22.5
Magnesium	5	100
	50	42.5

[a] The residual levels of calcium and magnesium in V medium are about 0.3 and 3 mM, respectively.

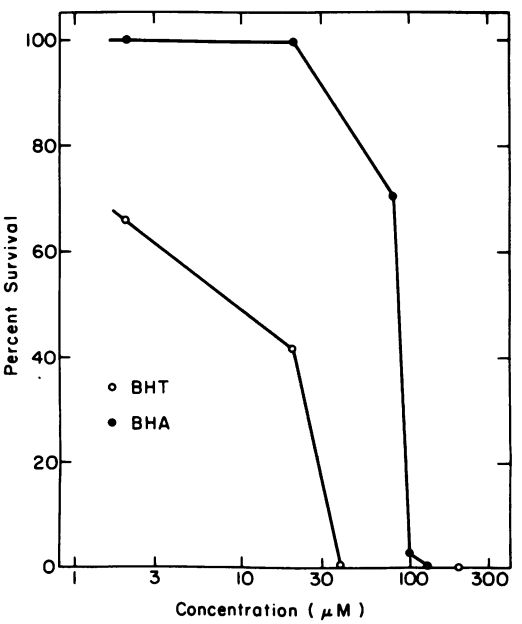


FIG. 6. *Survival of ϕ6 virus to various concentrations of BHT and BHA at 25 C. Exposures were carried out as described for Fig. 5. The exposure time was 30 min.*

brane could, in an indirect manner, alter the conformation or orientation of virus-attachment proteins through modifications of the lipid-protein interactions. Other possibilities are that the attachment sites are masked by BHT or that an attachment protein or glycoprotein is completely removed from the virion by BHT treatment. The proteins of ϕ6 have been characterized to some extent, including three proteins that are removed by Triton X-100 treatment and which, therefore, may be part of the envelope (9). Similar studies of BHT-treated virus would help elucidate the mechanism of BHT activity.

The reduced potency of BHT at lower temperatures may be due to a decrease in the ability of the BHT molecules to partition into the viral membrane. At low temperatures the alkyl chains of membrane phospholipids are more ordered, the interchain van der Waals forces are greater, and hydrophobic molecules with less favorable packing geometry are expected and have been found to have reduced partitioning into the hydrocarbon zones (12). BHT has very low solubility in aqueous media and was delivered to the virus in our experiments by adding the compound from a concentrated solution in ethanol. Under these conditions the kinetics of exchange of the BHT molecules into the viral membrane are no doubt quite complex.

The enhancement of BHT activity by calcium ions is not readily understandable. Calcium is generally thought to stabilize membrane structures, so it might be expected that the presence of calcium would dimiminish BHT activity. Although the nature of the potentiating effect of calcium remains open to question, some considerations in light of recent molecular studies of Ca^{2+}-phospholipid interactions seem appropriate. Ohnishi and Ito (6), using the spin label technique, have investigated the interaction of Ca^{2+} ions with negatively charged phospholipids in artificial membranes containing phosphatidylserine and phosphatidylcholine. These investigators found that Ca^{2+} induces a phase separation in the bilayer structure, such that regions of phosphatidylserine aggregates bridged by Ca^{2+} chelates coexist with a more fluid phase of phosphatidylcholine molecules. The strong binding of Ca^{2+} to the negative phospholipids provides very efficient charge neutralization, and the surface of the aggregated phase is described as being hydrophobic. Of particular interest is the fact that Ba^{2+} and Sr^{2+}, but not Mg^{2+}, ions induced similar phase separations. The ϕ6 membrane contains a large proportion of negatively charged phosphatidylglycerol, and similar phase separations could occur in the presence of Ca^{2+} ions. The resulting hydrophobic surface of the Ca^{2+}-phosphatidylglycerol aggregates might then promote the exchange of BHT into the viral membrane, thereby increasing virus inactivation. Modifications of BHT activity by Ca^{2+} and other ions is an area that requires further investigation.

The data presented here for the inactivation of ϕ6 by BHA is the first report that this substance has antiviral properties. For ϕ6, BHA is less active than BHT at comparable concentrations. It may be that other viruses, with different membrane structures, will be equally or more susceptible to BHA than BHT. In light of the apparent low toxicity of BHA, this substance may deserve further study as a potential antiviral agent.

ACKNOWLEDGMENTS

This research was supported by ERDA contract no. E(11-1)2311 and by grant BMS 74-05587 from the National Science Foundation.

LITERATURE CITED

1. Cupp, J., P. Wanda, A. Keith, and W. Snipes. 1975. Inactivation of the lipid-containing bacteriophage PM2 by butylated hydroxytoluene. Antimicrob. Agents Chemother. 8:698-706.
2. Eletr, S., M. A. Williams, T. Watkins, and A. D. Keith. 1974. Perturbation of the dynamics of lipid alkyl chains in membrane systems: effect on the activity of membrane-bound enzymes. Biochim. Biophys. Acta 339:190-201.
3. Ellis, L. F., and R. A. Schlegel. 1974. Electron microscopy of *Pseudomonas* ϕ6 bacteriophage. J. Virol. 14:1547-1551.
4. Hammerstedt, R., R. Amman, T. Rucinsky, W. Snipes, P. Morse II, J. Lepock, and A. Keith. 1976. Characterization of bovine sperm membranes using spin labels and electron spin resonance spectroscopy: effect of butylated hydroxytoluene and cold shock on membrane structure. Biol. Reprod. 14:381-397.
5. Kermode, G. O. 1972. Food additives. Sci. Am. 226:15-21.
6. Ohnishi, S., and T. Ito. 1974. Calcium induced phase separations in phosphatidylserine-phosphatidylcholine membranes. Biochemistry 13:881-887.
7. Okada, Y., and F. Murayama. 1966. Requirement of calcium ions for the cell fusion reaction of animal cells by HVJ. Exp. Cell Res. 44:527-551.
8. Sands, J. A. 1973. The phospholipid composition of bacteriophage ϕ6. Biochem. Biophys. Res. Commun. 55:111-116.
9. Sinclair, J. F., A. Tzagoloff, D. Levine, and L. Mindich. 1975. Proteins of bacteriophage ϕ6. J. Virol. 16:685-695.
10. Snipes, W., J. Cupp, J. A. Sands, A. Keith, and A. Davis. 1974. Calcium requirement for assembly of the lipid-containing bacteriophage PM2. Biochim. Biophys. Acta 339:311-322.
11. Snipes, W., S. Person, A. Keith, and J. Cupp. 1975. Butylated hydroxytoluene inactivates lipid-containing viruses. Science 187:64-66.
12. Tinberg, H. M., L. Packer, and A. Keith. 1972. Role of lipids in mitochondrial energy coupling: evidence from spin labeling and freeze-fracture electron microscopy. Biochim. Biophys. Acta 283:193-205.
13. Vidaver, A. K., R. K. Koski, and J. L. Van Etten. 1973. Bacteriophage ϕ6: a lipid-containing virus of *Pseudomonas phaseolicola*. J. Virol. 11:799-805.