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Inactivation of the Lipid-Containing Bacteriophage PM2 by Butylated Hydroxytoluene

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Several factors have been investigated which are of significance in the inactivation of PM2, a lipid-containing bacterial virus, by butylated hydroxytoluene (BHT). Studies of the time dependence of inactivation during exposure to BHT showed that virus killing occurs rapidly, with the majority of the effect taking place in the first 5 min. The degree of inactivation is dependent upon the initial virus titer, the solvent from which BHT is added, and the presence of a variety of protective agents, including surfactants, bovine serum albumin, and bacterial cells. Sucrose gradient analysis of ^{32}P-labeled, BHT-treated virus was used to determine the degree to which the virion is disrupted by BHT. These experiments show that the ^{32}P-labeled molecules are converted into very slowly sedimentable material by BHT treatment, indicating complete destruction of the virus particle.

Butylated hydroxytoluene (BHT) is a small, hydrophobic molecule (Fig. 1) which is frequently used as an antioxidant. It is generally recognized as safe by the Food and Drug Administration (11) and is commonly added to foods to maintain freshness and prevent spoilage. We recently reported that BHT is a potent inactivator of the lipid-containing viruses PM2, $\phi 6$, and herpes simplex virus (13). It is effective, in vitro, at concentrations as low as 10^{-5} to 10^{-4} M, depending on the particular virus under study. These concentrations are in the range of those accumulated in the body fat of U.S. residents from dietary consumption of BHT as a food additive (2).

The potential use of BHT as an antiviral agent has prompted us to investigate further its mode of action. In the present study we have characterized several factors that influence its inactivation of PM2, a lipid-containing bacterial virus. In addition, experiments were carried out to determine the degree to which the PM2 virion is disrupted upon treatment with BHT.

MATERIALS AND METHODS

Virus and cells. Bacteriophage PM2 and its host, the marine bacterium *Pseudomonas* BAL-31, were discovered by Espejo and Canelo (5). Our original stocks were provided by Eugene Cota-Robles (University of California, Santa Cruz). A thymine-requiring derivative of BAL-31, designated PS1001, was obtained by trimethoprim selection (14). Its isolation and characteristics have been described (12). From PS1001 we isolated a PM2-resistant mutant, designated RA1, for some of the present work. RA1 was selected and characterized as a mutant that allows no detectable attachment of PM2 virus.

Media and culture methods. All experiments reported here were carried out in Medium 25, a chemically defined but somewhat enriched growth medium described previously (12). Medium 25 is buffered to pH 7.6 with tris(hydroxymethyl)aminomethane. For growth of RA1, thymidine was added at 50 μg/ml.

Samples were assayed for infectious virus by the agar overlay method. Bottom agar contained Q medium (12) hardened with 1.5% (wt/vol) agar (Difco). Top agar was composed of Q medium lacking yeast extract, hardened with 0.5% (wt/vol) agar.

Sucrose gradient analysis. Two types of sucrose gradients were used. For experiments with bacterial cells, samples were layered onto 5 to 35% sucrose gradients made in Medium 25 and centrifuged for 20 min in an International clinical centrifuge at a setting of 7. The tube was punctured from the bottom, and fractions were collected for assay. For experiments with PM2 virus, samples were layered onto 20 to 30% sucrose gradients made in 50% Medium 25 and centrifuged for 200 min at 25,000 rpm in a Beckman SW27 rotor.

Source of materials. All chemicals were obtained from commercial sources. $^{32}PO_4$ was supplied by New England Nuclear Corp. (Boston, Mass.). [^{3}H]BHT was prepared by Amersham/Searle (Arlington Heights, Ill.) using a catalytic tritonation procedure. Exchangeable tritium sites were removed by Amersham/Searle. The [^{3}H]BHT was purified by column chromatography using 200- to 325-mesh silica gel at a pH of 4. Diethylether-hexane (9:1, vol/vol) was the moving phase.

RESULTS

Time dependence for BHT inactivation. In our earlier experiments (13), all samples were exposed to BHT for 30 min and then assayed, after dilution, for infectious virus. Figure 2

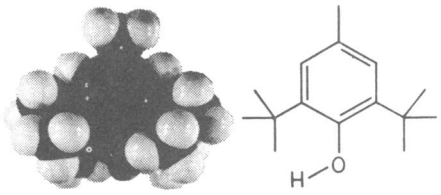

FIG. 1. *Structure of BHT.*

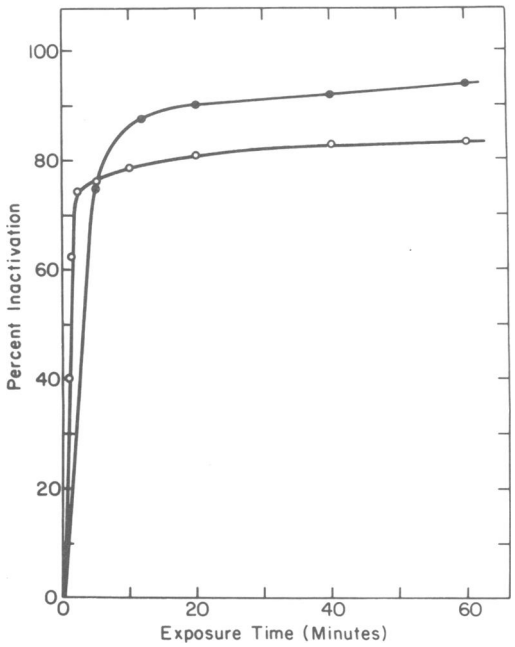

FIG. 2. *Inactivation of PM2 virus as a function of time of exposure to 0.1 mM BHT. From a solution of 10 mM BHT in 95% ethanol, 0.1 ml was added to 5 ml of Medium 25 and vortexed. Thirty seconds later (t = 0), 5 ml of a PM2 sample at 2 × 10⁵ PFU/ml was added, and the mixture was again vortexed. At various times after mixing the virus and BHT suspensions, samples were diluted and plated for plaques on Q plates with BAL-31 as an indicator host. Open circles and closed circles are for two independent experiments.*

shows data for samples diluted and plated at different times of exposure to 0.1 mM BHT. The inactivation occurs rapidly, with the majority of the effect taking place in the first 5 min. Some variation is seen, both in the time course and in the maximum level of inactivation, when 0.1 mM BHT is used. Two experiments illustrate this point in Fig. 2. When the concentration of BHT is increased to 0.2 mM, inactivation levels greater than 99% are consistently observed. The data in Fig. 2 indicate that, for PM2, little is gained by exposing the virus longer than about 10 min.

Effect of initial virus concentration. We found that the fraction of virus inactivated by a given BHT treatment is dependent upon the initial virus concentration present during the exposure. Such data for a BHT concentration of 0.2 mM are shown in Fig. 3. Up to a virus concentration of approximately 10^7 plaque-forming units (PFU) per ml, 0.2 mM BHT inactivates greater than 99% of PM2. Above this titer, the percentage of inactivation drops. We considered the possibility that cell debris present in crude viral lysates might be protecting the virus against BHT by some competition mechanism, but this apparently is not the case. Virus purified by velocity sedimentation on a sucrose gradient, and therefore free of any large amounts of contaminating cell debris, gave results that are identical, within experimental error, to those for crude lysates (Fig. 3). It should be pointed out that the actual number of

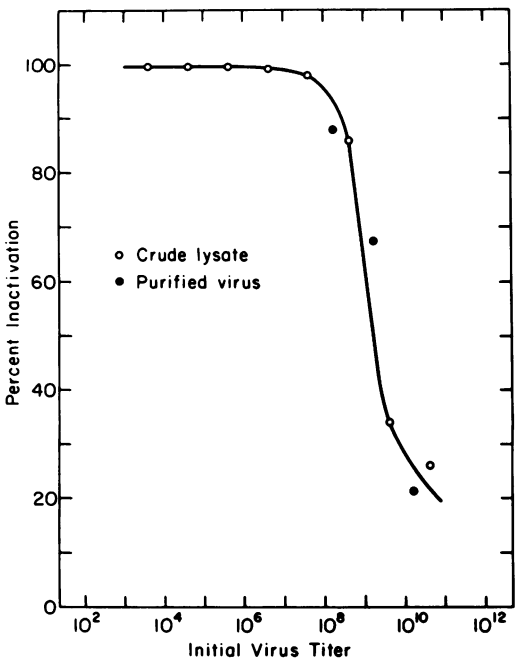

FIG. 3. *Inactivation of PM2 virus as a function of the initial virus titer. In one experiment (open circles), a crude virus lysate prepared in Medium 25 was diluted and used without purification of the virus. Samples (5 ml) of each dilution were treated with a final concentration of 0.2 mM BHT in a manner similar to that of Fig. 2. After a 30-min exposure, appropriate dilutions were plated for plaques. In a second experiment (closed circles), PM2 virus was pelleted, resuspended in a small volume, and purified by velocity sedimentation through a sucrose gradient as described in Materials and Methods. The peak fraction, containing about 2.5 × 10¹¹ PFU/ml, was diluted in Medium 25 and used as for the previous experiment.*

viruses inactivated is much greater at the higher initial titers, even though the percentage of inactivation is less.

Effect of cells on virus inactivation. We further investigated the possibility that cells can protect PM2 against BHT, making use of mutant RA1, a derivative of BAL-31 to which PM2 does not attach. Virus at an initial titer of 10^6 PFU/ml were exposed to 0.2 mM BHT with various concentrations of RA1 present. The data, shown in Fig. 4, indicate that 2×10^8 cells/ml reduce the inactivation by about 50%, whereas at cell concentrations less than 2×10^7/ml no protective effect is observed.

Protective agents. Several agents were found to protect PM2 from BHT, including a number of surfactants and the protein bovine serum albumin. These data are summarized in Table 1. The surfactants appear to solubilize the BHT and may therefore reduce the amount of BHT interacting with the virus particles. Our usual procedure for adding BHT to virus samples involves mixing 0.1 ml of a 20 mM solution of BHT in ethanol with 5 ml of medium, followed by mixing 1:1 with a virus solution. This always results in a somewhat turbid suspension of BHT. When Tergitol or the Tweens are present, no such turbidity is observed. It may be, as experiments described later will suggest, that the turbid, microcrystalline BHT particles are essential for inactivation of PM2.

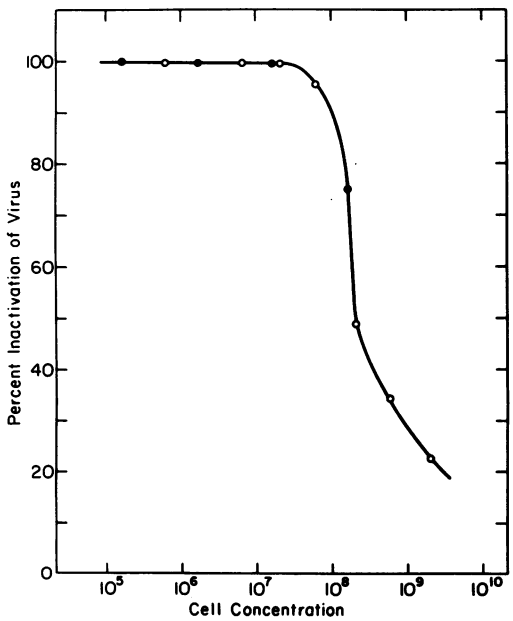

FIG. 4. *Virus inactivation by BHT in the presence of different concentrations of cells. The mutant RA1 was grown to a cell concentration of approximately 2×10^8 CFU/ml, centrifuged, and resuspended in a smaller volume of Medium 25. Various dilutions were mixed with aliquots of a virus suspension, and the mixtures were treated with BHT at a final concentration of 0.2 mM, according to the procedure in Fig. 2. After 30-min of exposure, dilutions were plated for plaques with BAL-31 as an indicator host. The initial virus concentration for all samples was 10^6 PFU/ml. Open and closed circles are for the two independent experiments.*

TABLE 1. *Effects of various agents on virus inactivation by BHT*[a]

Protective agent	Concn (%)	Inactivation (%)
None		>99
Tergitol	1	23
Triton X-100	1	<5
Tween 40	1	<5
Tween 80	1	<5
Tween 20	1	<5
	0.1	14
	0.001	33
	0.00001	67
BSA	1	<5
	0.1	<5
	0.01	8
	0.001	17

[a] BHT was added from ethanol to give a final concentration of 0.2 mM BHT, 1% ethanol. BSA, Bovine serum albumin.

Solvent effects. BHT has very low solubility in water but may be readily dissolved in ethanol, ether, acetone, dimethyl sulfoxide (Me_2SO), and other organic solvents. We found that the inactivation of PM2 by 0.2 mM BHT is dependent upon the solvent from which the BHT is introduced into the medium. Solutions (20 mM) of BHT in several solvents were prepared, and these were added to a final concentration of 1% solvent, 0.2 mM BHT to viral samples as described above. The results are shown in Table 2. Control experiments showed that PM2 is not inactivated by 1% solutions of

TABLE 2. *Virus inactivation by BHT added from different solvents*

Solvent	Absorbance[a]	Inactivation (%)[b]
Ethanol	0.20	>99
Me_2SO	0.22	>99
Acetone	0.15	78 ± 4
Ether	0.02	<5

[a] Measured at 620 nm with a Bausch and Lomb Spectronic 20 colorimeter.

[b] Samples were exposed at an initial virus concentration of 10^6 PFU/ml. Data shown are the average of three independent experiments.

ethanol, ether, Me_2SO, or acetone when added by the same procedure. It is interesting to note from the data of Table 2 that a correlation exists between the percentage of inactivation and the turbidity of the solution, again suggesting that BHT microcrystals may be important for virus inactivation.

Time of mixing. The turbidity that appears when a solution of BHT in ethanol is mixed with medium gradually disappears with time. This seems to be the result of an aggregation of the microscopic BHT crystals into larger particles, forming a precipitate which floats to the top. This loss of turbidity is hastened by mechanical agitation, such as bubbling the solution with air or nitrogen gas. We carried out an experiment to determine the effectiveness of a BHT suspension for inactivating PM2 as a function of time after the BHT was added to medium from an ethanol solution. The results are shown in Fig. 5. It can be seen that a BHT suspension prepared in this way is about half as effective at inactivating PM2 after standing without agitation for 30 min. Our interpretation of this experiment is that the small microcrystals of BHT that result from mixing the ethanol solution with medium are the most effective at virus inactivation, and that these aggregate into larger, less effective structures upon standing.

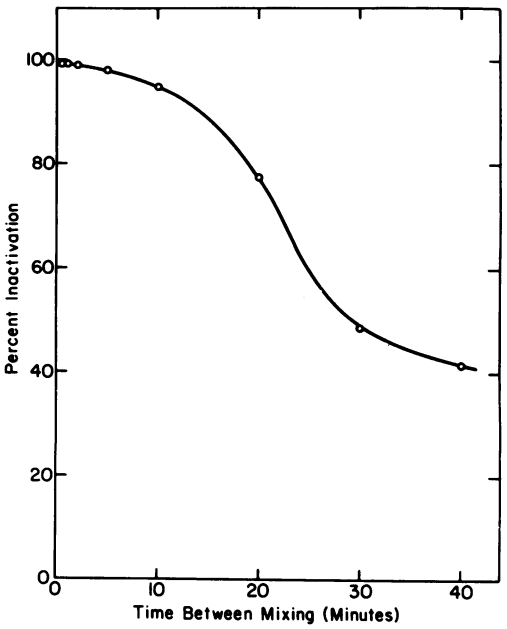

FIG. 5. *Inactivation of PM2 virus as a function of time between mixing. BHT (0.6 ml) at 20 mM in ethanol was added to 30 ml of Medium 25 at time 0. At various times thereafter, 3 ml of the BHT suspension was added to 3 ml of PM2 virus at 2 × 10⁶ PFU/ml. Each sample was diluted and plated for plaques 30 min after the BHT and virus suspensions were mixed. Time on the horizontal axis is the period between mixing the BHT with medium and mixing this suspension with virus.*

Effect of BHT-treated virus on cell survival. Even though BHT-inactivated virus cannot form plaques on BAL-31, we considered the possibility that the inactive virus is capable of attaching to and killing the host cell. Many virus mutants, for example, can kill a nonpermissive host cell on which they cannot form plaques. A sample of PM2 was treated for 30 min with 0.2 mM BHT at an initial virus titer of 10^8 PFU/ml, resulting in about 84% inactivation. Three samples of BAL-31 at 6×10^7 colony-forming units (CFU)/ml were mixed with an equal volume of (i) medium, (ii) PM2 at 10^8 PFU/ml, or (iii) BHT-treated PM2. After 20 min, dilutions were plated for surviving bacteria. The results are shown in Table 3. It is clear from these data that BHT-treated PM2 are not capable of killing the host cells. The 7% cell inactivation by BHT-treated PM2 is most likely due to the 16% virus that survived the BHT treatment.

Sucrose gradient analysis of BHT-treated virus. The experiments described here were undertaken to determine the degree to which the PM2 virus particle is disrupted by BHT treatment. This required the use of radioactively labeled, purified virus. We also used [^{3}H]BHT in some of this work to obtain an estimate of the degree of binding of BHT to active virus particles when a BHT treatment resulting in only partial inactivation is administered.

PM2 lysates were prepared in the presence of $^{32}PO_4$ and centrifuged at low speed to remove cell debris. The virus was pelleted, resuspended in a small volume of medium, and layered onto a sucrose gradient. Fractions were collected and assayed for radioactivity and PFU. Both ^{32}P radioactivity and PFU appeared as a single band in the region of fraction 12. The peak fraction was diluted and used for further experiments.

In one experiment a 10-fold dilution of fraction 12 was treated for 30 min with 0.2 mM BHT containing 0.5 μCi of [^{3}H]BHT per ml at final concentration. An untreated sample was maintained as a control. The BHT treatment

TABLE 3. *Survival of BAL-31 to BHT-treated virus*

Sample	CFU/ml	Survival (%)
Cells plus medium	3.0×10^7	100
Cells plus PM2	8.3×10^6	28
Cells plus BHT-treated PM2	2.8×10^7	93

resulted in less than 50% inactivation, as expected from the data in Fig. 3, because the initial virus titer was about 2.5×10^9 PFU/ml. The BHT-treated and control samples were analyzed on sucrose gradients; the results are shown in Fig. 6. For the control, the ^{32}P radioactivity forms a band centered about fraction 12, with very little radioactivity at the top of the gradient. Plating results showed that the peak of virus infectivity coincided with the peak of radioactivity. For the BHT-treated sample the peak at fraction 12 is diminished and a new band very near the top of the gradient appears. Plating results showed that the particles in the peak at fraction 12 are equally infectious as those for the control. These data indicate that BHT inactivates PM2 by completely disrupting the virion, releasing the ^{32}P-labeled nucleic acid and phospholipid material into a form that will sediment no more than about 10% of the way down this gradient.

BHT has a density less than that of the gradient solution, and the ^3H radioactivity is located near the top of the gradient. It is interesting to note the absence of any detectable incorporation of [^3H]BHT into the PM2 virus particles that survive the BHT treatment. We have quantified this using the data of Fig. 6 and the plating results. Our estimate is that the PM2 particles contain less than 10^5 BHT molecules/PFU. It is not surprising, therefore, that we do not detect PM2-associated BHT in this way, in that 10^5 BHT molecules correspond to an amount equivalent in weight to about 50% that of the virus.

Additional and more convincing evidence that the PM2 particles are disrupted by BHT was obtained in a second experiment, in which the

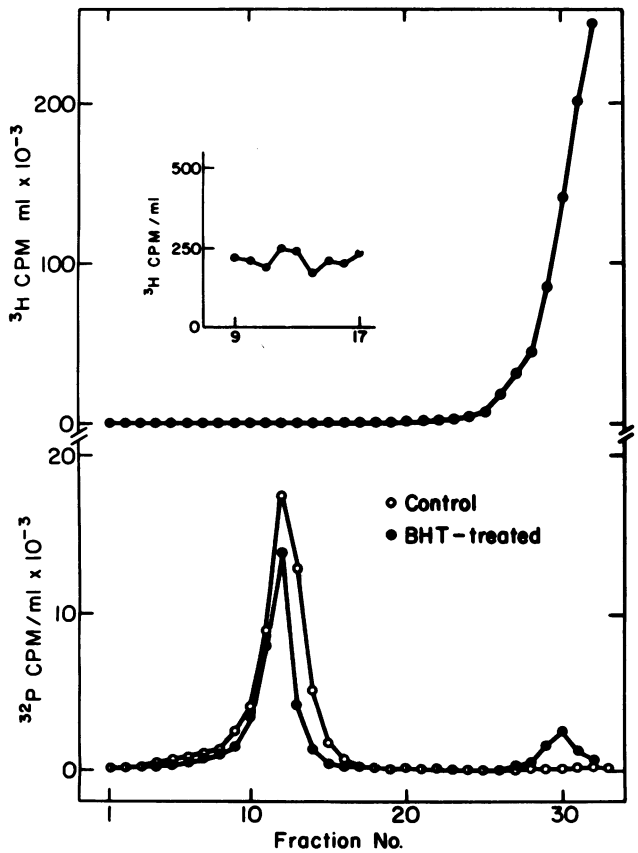

FIG. 6. *Sucrose gradient analysis of control and BHT-treated PM2 virus. The peak fraction from a purification gradient was diluted fivefold in Medium 25 and divided into two portions. One was treated for 30 min with [^3H]BHT at a final concentration of 0.2 mM and 0.5 μCi/ml by the procedure described in Fig. 2; the other was diluted twofold with medium and maintained as a control. One-milliliter samples were analyzed on sucrose gradients. Correction was made for ^{32}P counts that are registered in the ^3H channel of the counter. The peak fractions 10 through 14 had combined PFU of 1.34×10^9 and 1.08×10^9 for the control and BHT-treated samples, respectively. (Inset) ^3H radioactivity for fractions 9 through 17 on an expanded scale.*

virus sample was diluted 100-fold for BHT treatment. Under these conditions, the inactivation was greater than 99%. Figure 7 shows the sucrose gradient analysis of the BHT-treated sample and reveals that there are no detectable particles in the region of fraction 12. The ^{32}P radioactivity is all located about three fractions from the top of the gradient in a very slowly sedimenting form.

Cell growth in the presence of BHT. Some experiments were carried out to determine the effect of BHT on growth of the host cell BAL-31. The concentration of BHT used was 0.2 mM, a level that inactivates greater than 99% of PM2 virus. A culture growing exponentially was divided into two parts, and one was treated with BHT by the same procedure used to treat virus. Initially, as is seen in Fig. 8, the absorbance of the BHT-treated culture is quite high but drops rapidly to that of the control. This initial high absorbance is due to the turbidity of the BHT microcrystals that form when the ethanol-BHT solution is added to the medium. Within 30 min, most of the turbidity due to the BHT has disappeared as the microcrystals aggregate into larger particles that float to the top. Afterwards, the absorbance measurements show that the BHT-treated cells grow at a rate that is identical, within experimental error, to the control.

At several times during the experiment, samples were diluted and plated for colonies. These data, shown in the upper portion of Fig. 8, confirm the lack of any significant effect of BHT on the survival or growth of the host cell. Additional plating experiments on more dilute cell cultures (data not shown) established that 0.2 mM BHT is not toxic to BAL-31 at cell concentrations as low as 10^3 CFU/ml.

Uptake of BHT by host cells. Low-speed sucrose gradient analysis was used to provide some estimate of the degree to which BHT is taken up by or becomes associated with the host cell. These experiments were prompted by the observation (Fig. 4) that cells can protect the virus against inactivation by BHT. To facilitate rapid location of the cells on the gradient, the culture was briefly labeled with ^{32}P.

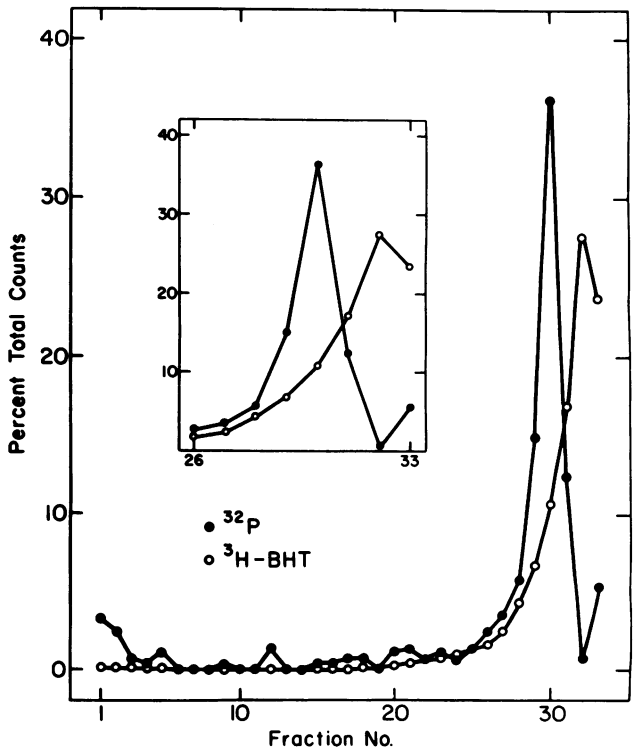

FIG. 7. *Sucrose gradient analysis of BHT-treated PM2 virus. Experimental details are the same as Fig. 6, except that the sample was diluted 50-fold rather than fivefold before BHT treatment. In this figure, data are expressed as percentage of the total radioactivity recovered from the gradient. The total counts/min recovered were* 2.8×10^3 *and* 3.4×10^6 *for* ^{32}P *and* ^3H, *respectively. Fractions 26 through 33 are shown with an expanded horizontal axis in the inset.*

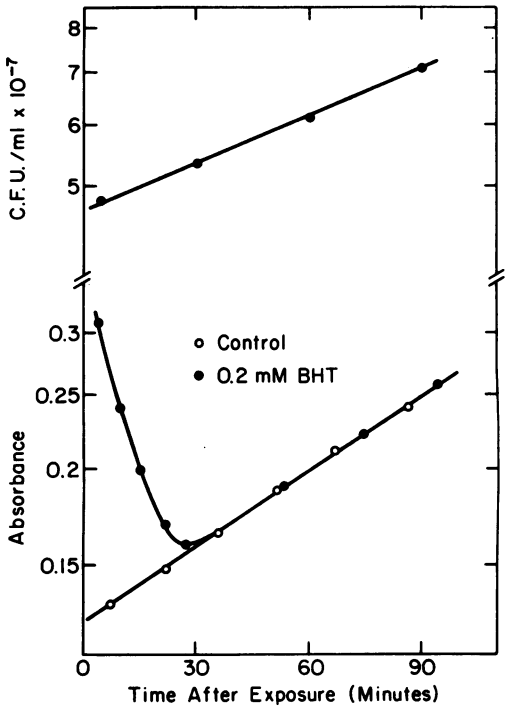

FIG. 8. *Growth of Pseudomonas BAL-31 in the presence and absence of 0.2 mM BHT. The absorbance was measured at 620 nm with a Bausch and Lomb Spectronic 20 colorimeter. Both the control and BHT-treated cultures were bubbled with air throughout the experiment.*

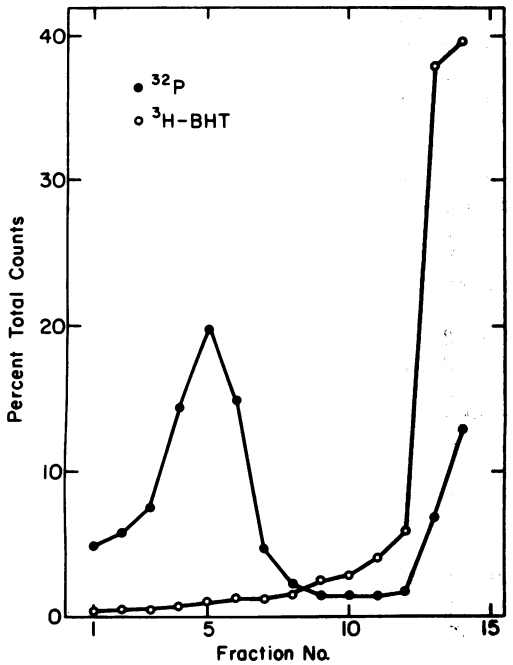

FIG. 9. *Sucrose gradient analysis of BHT-treated BAL-31 cells. A culture growing in Medium 25 at an absorbance of 0.3 was labeled for 15 min with $^{32}PO_4$. The cells were centrifuged, washed twice, treated for 30 min with [^{3}H]BHT at 0.2 mM (0.05 μCi/ml), and analyzed on a sucrose gradient. Data are expressed as percentage of total radioactivity recovered from the gradient. The total counts/min recovered were 8.6 × 10^4 and 3.3 × 10^6 for ^{32}P and ^3H, respectively.*

The labeled cells were centrifuged and washed twice with medium to remove unincorporated radioactivity. After treatment with 0.2 mM BHT containing [^{3}H]BHT for 30 min, the culture was analyzed on a sucrose gradient, as described in Materials and Methods.

Figure 9 shows the results of an experiment in which the cell concentration during exposure to BHT was 6 × 10^7 CFU/ml. A well-defined band of ^{32}P radioactivity shows the location of cells on the gradient, and plating results gave a coincident peak for CFU. It can be seen from the ^3H radioactivity that an insignificant amount of BHT has been taken up by or irreversibly associated with the cells. We estimate that no more than 7%, and most likely much less, of the total BHT is cell associated in this experiment.

In another experiment, where the cell concentration during exposure to BHT was 6 × 10^8 CFU/ml, less than 10% of the BHT was associated with the cells. This is a cell concentration that can protect PM2 virus from inactivation by BHT (Fig. 3). These data are seemingly contradictory, but may be explainable in the following way. If the inactivation of PM2 virus is due to its interaction with the very small microcrystals of BHT, these may represent a relatively small fraction of the total BHT present during treatment. Cells may also interact and associate with these smaller particles, in preference to the larger BHT aggregates, and effectively remove them from solution. In this way the cells could protect the virus from inactivation by BHT, at the same time removing only a small fraction of the total BHT from solution.

DISCUSSION

The bacterial virus PM2 contains a lipid bilayer as an integral part of its structure (9). The virion contains four major structural proteins (3) and a circular, double-stranded deoxyribonucleic acid molecule (6). An inner core, which can be isolated under certain conditions, is composed of two proteins and the viral deoxyribonucleic acid (10). Around this core there is a lipid bilayer structure consisting mainly of phosphatidylglycerol and phosphatidylethanolamine (1). External to the lipid bilayer, a pro-

tein coat, icosahedral in shape, is located (9). Protein spikes, thought to be the means by which PM2 attaches to its host cell, protrude from the virion. The particle diameter is about 60 nm (5).

The presence of a lipid bilayer in PM2 makes this virus susceptible to many organic solvents, as are a variety of lipid-containing animal viruses. BHT, being a hydrophobic, bulky molecule nearly spherical in shape, is expected and has been found (4) to have perturbing effects on membrane structures. Nevertheless, BHT has an apparent low level of toxicity to cells, a conclusion supported by some of the work presented here. Its effective inactivation of lipid-containing viruses may therefore be of some potential importance.

One aspect of the work reported here was a characterization of several factors that influence the antiviral activity of BHT. This was carried out, to some extent, to provide a rational basis for further experiments with other systems. From our experiments it is clear that a number of factors must be considered when studying the antiviral activity of BHT. For inactivation of PM2, the action takes place within a few minutes after virus is added to a BHT suspension. The degree of inactivation is dependent on the initial virus titer, the solvent from which BHT is added, and the presence of a variety of "protectors," including surfactants, bovine serum albumin, and bacterial cells. Suspensions of BHT are most active against PM2 immediately after they are prepared and lose their effectiveness on standing.

Many of these experiments support the point of view that only a small portion of the BHT suspension prepared by our procedure, most likely very small microcrystalline particles, are effective for inactivating PM2. First, the degree of turbidity produced when the BHT suspension is formed from different solvents (Table 2) seems related to inactivation efficiency. Second, surfactants which reduce the turbidity, presumably by solubilizing the BHT, protect PM2 against inactivation (Table 1). Third, the loss of antiviral activity of a BHT suspension upon standing can be explained by the aggregation of these active microcrystals into larger structures (Fig. 5). This is observed as a decrease in turbidity. And fourth, the observation that cells can protect PM2 under conditions where only a small fraction of the total BHT is taken up by or adsorbed to the cells strongly suggests that only a small fraction of the BHT is participating in virus inactivation. Further evidence along these lines was provided by experiments (data not presented) in which the BHT suspension was passed through a 0.22-μm filter before adding to the virus. The filtration removed the larger BHT crystals, including a good part of the light-scattering material. ^{3}H radioactivity counts showed that only 20% of the total BHT passed through the filter. However, the filtered solution was still active against PM2.

A second aspect of this work was a study, using sucrose gradient analysis, of the means by which BHT inactivates PM2. The results show that BHT-treated virus is so disrupted that the viral material sediments only about 10% of the way down the gradient. The sedimentation coefficient of the inner core of PM2 has been reported as 133S, as compared to 290S for the complete particle (8). From these values we can estimate, in rough approximation, that inner cores would sediment to the region of fraction 24 in our gradient. Viral deoxyribonucleic acid, on the other hand, has a sedimentation coefficient of about 28S (7) and should sediment to the region of fractions 30 and 31 on our gradient. We conclude, on this basis, that the inner core of PM2 is not intact after BHT treatment, that the particle structure is completely disrupted under these conditions, and that the band near the top of the gradient is largely the released viral deoxyribonucleic acid.

The mechanism of BHT inactivation may well be different with other viruses. For example, work in progress with ϕ6, an enveloped bacterial virus that is extremely susceptible to BHT, indicates that this virus is not totally disrupted, but that the BHT-treated particles cannot attach to the host cells.

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