Studies with a Hydrophobic, Spin-Labeled Virucidal Agent

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A spin-labeled virucidal agent was synthesized, purified, and tested for its activity against the enveloped bacterial virus $\phi 6$ and herpes simplex virus. This compound, designated BPN, inactivated greater than 99% of $\phi 6$ and herpes simplex virus in vitro at concentrations as low as 0.1 mM. Detailed studies were carried out on the mechanism of inactivation of $\phi 6$ by BPN. These studies revealed that treatment of $\phi 6$ by BPN specifically removes a single envelope protein that is considered to be responsible for adsorption of the virus to the host cell. Related experiments with the $\phi 6$ host, *Pseudomonas phaseolicola* strain HB10Y, showed that this organism is insensitive to the effects of BPN. The basis for the differential sensitivity of $\phi 6$ to BPN, in comparison to the host cell, was investigated by electron spin resonance techniques. It was found that, for $\phi 6$, HB10Y, and their extracted phospholipids, BPN is localized in the hydrocarbon zones of the membrane bilayer. However, in the case of $\phi 6$, the rotational mobility of BPN is much reduced in comparison to that in HB10Y and the phospholipid preparations. Furthermore, an Arrhenius plot of rotational correlation time (τ_c) showed a marked discontinuity in slope at 31°C in the case of $\phi 6$, but not for the other samples studied. This suggests a strong interaction between the $\phi 6$ envelope proteins and the lipid domains in which BPN is localized. Calculations based on the known lipid and protein composition of $\phi 6$ indicate that there is an absence of "free-lipid" pools in the viral envelope. It is suggested that BPN localizes in free-lipid pools of cell membranes, where its presence is of little or no consequence, but that in $\phi 6$ the BPN perturbs the hydrophobic interactions between phospholipids and proteins in the envelope.

Many antiviral agents currently being developed are designed to interfere with viral nucleic acid metabolism (2, 15, 18, 20, 24). These compounds frequently have limited discrimination between viral and host cell functions, giving rise to potentially toxic, teratogenic, and immunosuppressive effects. In addition, such agents do not exclude the viral genome from the cell interior, so that the infected cell may be damaged, killed, or possibly transformed even though no new infectious virus is produced. For certain types of virus infections, virucidal agents may be more desirable in that they may inactivate the virus before its entry into an uninfected cell. In particular, these virucidal agents may be useful in the topical treatment of cutaneous infections such as the recurrent lesions of herpes simplex virus (HSV).

Presently in our laboratory we are investigating the mode of action of a certain class of molecules that have potential use as virucidal agents. These compounds have low water solubility, partition strongly into membrane structures, and appear to selectively perturb virus membrane functions in comparison to their effects on cell membranes. They are extremely potent inactivators of enveloped viruses at con-

centrations as low as 10^{-4} to 10^{-5} M in vitro, with little or no effects on host cells. One such compound is the common food additive butylated hydroxytoluene (BHT). Experiments performed in our laboratory have shown that BHT readily inactivates HSV types 1 (HSV-1) and 2 (HSV-2) and the lipid-containing bacteriophages $\phi 6$ and PM2 (3, 27). Other workers have shown that BHT is effective in vitro against Newcastle disease virus (1), cytomegalovirus (12), and Semliki Forest virus (12). In vivo, BHT is effective against Newcastle disease virus infections in chickens (1) and HSV-1 infections in hairless mice (23).

The elucidation of the mechanism of action of hydrophobic virucidal agents will require an understanding of the interaction between the molecule and the viral envelope as well as the ways in which this differs from the interaction between the molecule and the host cell membrane. To this end, we have designed and synthesized a hydrophobic, spin-labeled molecule that has virucidal properties. This compound, 1,3-diphenyl-2[5',5'-dimethyloxazolidinyl-N-oxyl]propane, designated BPN (see Fig. 1), inactivates $\phi 6$ and HSV in vitro and is effective against HSV cutaneous lesions in hairless mice. Furthermore,

FIG. 1. Structure of the antiviral spin label BPN.

the mechanism whereby it inactivates $\phi 6$ appears to be very similar to that of BHT. Since BPN is a spin label, it can be used in electron spin resonance (ESR) experiments to obtain information regarding the polarity of the environment in which the molecule is localized, the motional behavior of the molecule, and the effects of lipid-protein interactions on the spin label spectral parameters.

$\phi 6$ is a very useful model system for such studies because its structure has been well characterized, its morphological characteristics are similar to those of several animal viruses, and it can easily be purified in quantities sufficient for ESR analysis. This communication deals with the molecular interactions of BPN with the membranes of $\phi 6$ and its host, *Pseudomonas phaseolicola* strain HB10Y. Data pertaining to the physical and compositional changes induced in the virion by BPN are also presented. The results of these combined approaches suggest that the differential sensitivity of $\phi 6$ to BPN, in comparison with the host cell, may be due to the absence of pools of "free lipid" in the membrane of the virus.

MATERIALS AND METHODS

Virus, cells, and culture conditions. The enveloped bacteriophage $\phi 6$ and its host, *P. phaseolicola* strain HB10Y, were originally obtained from Anne Vidaver, Plant Pathology Department, University of Nebraska, Lincoln. Cells were cultured, and virus stocks were prepared in two types of media. V medium is an enriched medium containing 10 g of tryptone, 5 g of yeast extract, 7 g of NaCl, 1.5 g of KCl, and 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter of distilled water. V plates were composed of V medium hardened with 1.5% (wt/vol) agar (Difco Laboratories, Detroit, Mich.).

For preparing radioactively labeled virus, a defined but somewhat enriched medium was used. This medium, designated TBM21, contained 3 g of KCl, 6 g of NaCl, 2 g of NH_4Cl , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 HCl. For labeling with $^{32}\text{PO}_4$ and $[^3\text{H}]\text{glycerol}$, TBM21

was supplemented with all the naturally occurring amino acids except tryptophan (each at 20 mg/liter), adenosine and guanosine (each at 50 mg/liter), and uridine (100 mg/liter). For labeling with ^{14}C -labeled amino acids, the amino acid concentrations added to TBM21 were reduced to 1 mg/liter. $^{32}\text{PO}_4$ (carrier-free), 2- $[^3\text{H}]\text{glycerol}$ (200 mCi/mmol), and ^{14}C -labeled amino acid mixture (263 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Sucrose gradient analysis. Two types of sucrose gradients were used for the purification and analysis of $\phi 6$. For sedimentation velocity, samples were layered onto a preformed 15 to 35% sucrose gradient and centrifuged in a Beckman SW41-Ti rotor for 70 min at 32,000 rpm. For equilibrium density measurements, samples were layered onto a preformed 30 to 60% sucrose gradient and centrifuged for 8 h at 30,000 rpm in the SW41-Ti rotor. All gradients were prepared in 50% TBM21. After centrifugation, the bottom of the tube was punctured and 0.5-ml fractions were collected. The fractions were assayed for infectious virus by plaquing on HB10Y and for radioactivity by liquid scintillation spectrometry.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on the proteins of $\phi 6$ by the method of Laemmli (13). The sample solution contained 0.61 g of Tris, 2 g of sodium dodecyl sulfate, 5 ml of β -mercaptoethanol, and 5 mg of bromophenol blue, with the final volume brought to 100 ml with distilled water. The pH was adjusted to 7.0 with HCl. The samples were added to sample solution, placed in boiling water for 5 min, and then layered onto a 4% polyacrylamide stacking gel, under which was a 15% separating gel. Samples were electrophoresed for 4 h at 100 V. After electrophoresis the gels were fixed and autoradiograms were prepared. In some cases, the fluorography technique of Laskey and Mills (14) was utilized.

Preparation of phospholipids. Phospholipids were extracted from HB10Y by the method of Kates (8). The phospholipids of this bacterium are composed of approximately 56% phosphatidylethanolamine, 29% phosphatidylglycerol, and 15% cardiolipin (19). After extraction, the lipids were dried and taken up as multilayers as previously described (11).

Since the quantity of $\phi 6$ necessary to yield sufficient phospholipids for ESR analyses is excessive, a phospholipid preparation designed to mimic that of $\phi 6$ was prepared by the following procedure. HB10Y phospholipids were extracted from cells grown in the presence of $^{32}\text{PO}_4$ and $[^3\text{H}]\text{glycerol}$. The phospholipids were purified by thin-layer chromatography on Silica Gel GF plates and identified by their R_f values and the $^3\text{H}/^{32}\text{P}$ ratio. The phosphatidylethanolamine and phosphatidylglycerol were eluted from the plates, counted for radioactivity, and recombined in the proportion of 38% phosphatidylethanolamine and 62% phosphatidylglycerol. Except for the small contribution of cardiolipin (8%), this is characteristic of the phospholipid composition of $\phi 6$ (19). Since the fatty acid compositions of $\phi 6$ and HB10Y are identical (26), we consider these mimicked $\phi 6$ phospholipids as an adequate representation of actual $\phi 6$ phospholipids. Multilayers of this preparation were also made for spin label studies. It should be noted, however, that

such multilayers may not exhibit the phospholipid asymmetry that is often found in viral membranes.

Synthesis of BPN. The spin-labeled compound BPN was synthesized by the method of Keana et al. (9). The precursor compound, 1,3-diphenylacetone, was purchased from Aldrich Chemical Company, Milwaukee, Wis. In a typical synthesis, 3 g of 1,3-diphenylacetone, 30 mg of benzoylsulfonylchloride, 20 g of anhydrous CaCl_2 , and 30 ml of 2-amino-2,2-dimethylpropanol were refluxed in 200 ml of toluene for 3 days in a Soxhlet apparatus. The mixture was cooled to room temperature and extracted with aqueous sodium bicarbonate, and the organic solvent phase was concentrated on a rotary evaporator. The product was then treated with 2.5 g of *p*-chloroperbenzoic acid with simultaneous addition of 50 ml of ether in a pressure-compensating funnel. After a 2-day oxidation period at room temperature, the mixture was extracted with sodium bicarbonate and washed with water. The organic phase was again concentrated in a rotary evaporator, and the product was purified on a column of SilicAR CC-7 (Mallinkrodt Chemical Works, St. Louis, Mo.), using chloroform as the moving phase. The fractions were tested for ESR signal and checked for homogeneity by thin-layer chromatography. The appropriate fractions were dried, dissolved in ethanol, and stored at 4°C until used.

Spin label measurements. ESR measurements were carried out on a Japan Electron Optics Laboratory spectrometer, model JES-ME-1X, operating at X-band microwave frequencies. The sample temperature was controlled to $\pm 0.5^\circ\text{C}$ with a variable temperature device constructed in our laboratory.

Rotational correlation times (τ_c) were determined by two methods. In the fast tumbling range ($\tau_c < 2.4 \times 10^{-9}$ s), the equation

$$\tau_c = 6.5 \times 10^{-10} W_0[(h_0/h_{-1})^{1/2} - 1]$$

was used (10). In the intermediate tumbling range (2.4×10^{-9} s $< \tau_c < 1.8 \times 10^{-8}$ s), values of τ_c were determined by comparison with a calibration curve for BPN in glycerol-water mixtures, using handbook values for the viscosity of these mixtures. In the regions where the two methods overlap, determinations made by the two methods were in good agreement.

RESULTS

Virucidal activity of BPN. The inactivation of $\phi 6$ upon exposure to various concentrations of BPN was studied by the following procedure. A solution of BPN was prepared in ethanol at 100 times the desired final concentration. This solution was diluted into V medium or a Tris-buffered salts solution (27), and approximately 30 s later virus was added to give a titer of about 10^7 plaque-forming units/ml. Control samples were treated with 1% ethanol by the same procedure and showed no loss in infectivity. The samples were exposed to BPN for various lengths of time at 25°C, after which appropriate dilutions were plated for infectious virus on lawns of HB10Y. We have found the inactivation

of $\phi 6$ by BPN to be very rapid, with more than 90% of the maximum inactivation by a given concentration of BPN occurring during the first 10 min of exposure. For that reason, samples were routinely exposed for 30 min and assayed for plaque-forming units.

Figure 2 shows the percent survival of $\phi 6$ as a function of exposure to various concentrations of BPN by this procedure. At concentrations of 100 μM and greater, more than 99% of the virus is inactivated. Thus, BPN is a potent virucidal agent *in vitro*. We have also found that BPN inactivates HSV types 1 and 2 at concentrations similar to those required for $\phi 6$. Furthermore, experiments with HSV-1 cutaneous infections in hairless mice showed that BPN is effective in shortening the duration and reducing the intensity of the lesions. These results with HSV (data not shown) establish that the virucidal activity of BPN is not limited to $\phi 6$.

Although $\phi 6$ is sensitive to BPN, the growth of its host is unaffected by the presence of virucidal concentrations of the compound. The growth rate of HB10Y in V medium is the same in the presence and absence of 200 μM BPN, a concentration twice that required to inactivate greater than 99% of $\phi 6$ (Fig. 3). We have also observed that this concentration of BPN causes no apparent cytopathic effect to cultured human embryonic lung cells that are used for plaquing HSV in our laboratory.

Sucrose gradient analysis. Samples of $\phi 6$ labeled with ^{32}P were analyzed on sucrose density gradients to determine whether treatment with BPN removes the viral envelope. The three samples analyzed were (i) untreated virus, (ii) virus treated with 200 μM BPN, and (iii) virus

FIG. 2. Inactivation of $\phi 6$ upon exposure to various concentrations of BPN. The virus was exposed for 30 min in V medium at 25°C, at an initial titer of approximately 10^7 plaque-forming units/ml. Appropriate dilutions were plated for plaque-forming units on lawns of HB10Y cells.

FIG. 3. Growth of HB10Y in the presence (○) and absence (●) of 0.2 mM BPN. At various times, samples from cultures growing at 25°C in V medium with aeration were diluted and plated for colony-forming units (CFU).

treated with 1% Triton X-100. The exposure of $\phi 6$ to 1% Triton X-100 is known to remove the viral envelope (25, 27) and served as a positive control in these experiments. To obtain a high level of inactivation of $\phi 6$ by BPN at the high virus titers required for these experiments, the samples were dialyzed against a larger volume of V medium containing 200 μ M BPN. Control and Triton X-100 samples were treated similarly. By this procedure, we were able to obtain greater than 99% inactivation of $\phi 6$ by both BPN and Triton X-100 at $\phi 6$ titers up to 10^{10} plaque-forming units/ml.

Sucrose density gradients for the three samples are shown in Fig. 4. For the untreated control, the band of 32 P radioactivity centered on fraction 8 corresponds to the peak of infectivity. For the Triton X-100 treated sample, the band is located at a greater density, as expected for nucleocapsids, and the total amount of 32 P radioactivity is reduced compared with that of the control. For the BPN-treated sample, the particles band at a density even lower than that of the control, and the 32 P radioactivity in the peak is approximately the same as that of the controls. These results, which were repeated several times, suggest that the $\phi 6$ envelope is not removed by BPN treatment, but that the physical properties of the virion are modified in some manner.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples of $\phi 6$ were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine whether treatment with BPN caused any detectable changes in the viral protein composition. For these experiments, the virus was labeled with 14 C-labeled amino acids and purified on sucrose gradients. Treated and untreated samples were analyzed on gels of various concentrations to obtain optimum separation of all the $\phi 6$ proteins. In some cases the protein bands were visualized by conventional autoradiography, and in others the fluorography technique for amplifying film exposure was employed. Figure 5 shows a fluorogram which clearly illustrates the only change we have observed in $\phi 6$ brought about by BPN treatment. It is evident from these results that protein P3 is completely removed from $\phi 6$ upon exposure to BPN.

From the work of Mindich et al. (16) it is known that P3 is an envelope protein and that it is probably involved in the adsorption of virus to the host cell. We carried out experiments to determine the extent to which BPN-treated $\phi 6$ is capable of adsorption. Purified, radioactively labeled control and treated samples were incubated with HB10Y for 40 min at 25°C, after which the cells and adsorbed virus were removed by low-speed centrifugation. The supernatants

FIG. 4. Sucrose density gradients of control, BPN-treated, and Triton X-100 treated $\phi 6$. HB10Y cells prelabeled by growth in TBM21 containing 5 μ Ci of 32 P_O, per ml were infected with $\phi 6$ in TBM21 top agar containing 10 μ Ci of 32 P_O, per ml. After lysis, agar and cell debris were removed by low-speed centrifugation, and the virus was pelleted in the ultracentrifuge. The pellet was suspended in V medium, and portions were treated by dialysis as described in the text. The samples were pelleted, layered onto 30 to 60% sucrose gradients, and centrifuged as described in the text. Highest density is to the left.

FIG. 5. Fluorogram of a polyacrylamide gel after electrophoresis of control and BPN-treated $\phi 6$. A viral lysate was prepared in TBM21 top agar containing ^{14}C -labeled amino acids at $0.5 \mu\text{Ci/ml}$. After low-speed centrifugation, the virus was pelleted and purified by successive velocity and density gradients as described in the text. After treatment with BPN, the sample and an untreated control were pelleted, suspended in sample solution, and electrophoresed as described in the text. Although protein P2 is not visible on this fluorogram, other gels run under different conditions established that P2 is unaffected by BPN treatment.

were assayed for radioactivity of unadsorbed virus and compared to the radioactivity in the virus suspension before exposure to the cells. It was found that about 85% of the radioactivity remained in the supernatant for BPN-treated $\phi 6$, whereas only 35% was recovered for the untreated control. These data indicate that the biological inactivation of $\phi 6$ by BPN is most likely a result of the inability of treated virus to adsorb to the host cell.

Spin label studies. The ESR spectrum of BPN exhibits a three-line hyperfine pattern characteristic of that for nitroxide spin labels. Figure 6 shows the first-derivative spectra for BPN in aqueous solution and in a purified preparation of $\phi 6$. The hyperfine coupling constant A_N can be used as an indication of the polarity of the environment in which the spin label is localized. Table 1 gives values of A_N for BPN in several solvents of varying polarity (as indicated

by their dielectric constants), in the virus and cell samples, and in the corresponding lipid preparations. These data indicate that the spin label is localized in the hydrocarbon zones of the membrane bilayer of $\phi 6$ and HB10Y preparations.

The motional characteristics of BPN were determined at various temperatures by measuring the rotational correlation time, τ_c . This parameter gives an indication of the microviscosity of the environment surrounding the spin label and the interaction of the spin label with the matrix. Figures 7 and 8 show Arrhenius plots of τ_c for $\phi 6$, HB10Y, and the corresponding phospholipid preparations. For HB10Y (Fig. 7), the slopes of the lines for the intact cells and the extracted phospholipids are essentially identical, although the spin label rotational mobility is somewhat greater in the lipids than in the cells. Over the temperature range of 15 to 40°C , no discontinuities in the τ_c values or in the slope of

FIG. 6. First-derivative ESR spectra of BPN in aqueous solution and in $\phi 6$. The virus was prepared in V medium, precipitated with polyethylene glycol 6000, collected on a 75% sucrose shelf, and purified by successive velocity and density gradients. The $\phi 6$ sample was diluted in a Tris-buffered salts solution (27), treated with 0.05 mM BPN, and pelleted for ESR analysis. At the high virus titer used here, the treatment with BPN caused less than 10% inactivation. Measurements of the hyperfine coupling constant (A_N) were taken between the lowfield and midfield lines of spectra such as these.

TABLE 1. Nitrogen hyperfine coupling values (A_N) for the spin label BPN in solvents of various polarity and in $\phi 6$, HB10Y, and the corresponding lipid preparations

Solvent	Dielectric constant	A_N (gauss)
Water	78.5	15.5
Ethylene glycol	37.7	15.2
Ethanol	24.3	14.6
Ether	4.3	13.8
Heptane	1.9	13.7
$\phi 6$		13.7
$\phi 6$ lipids		13.7
HB10Y		13.7
HB10Y lipids		13.7

FIG. 7. Arrhenius plots of τ_c for BPN in HB10Y and its extracted phospholipids. The preparations were treated with 0.05 mM BPN under conditions that correspond to approximately one spin label per 200 phospholipid molecules. Potassium ferricyanide (10 mM) was added to the cell preparation to prevent biochemical reduction of the spin label.

FIG. 8. Arrhenius plots of τ_c for BPN in ϕ_6 and a preparation of its mimicked phospholipids. The sample of ϕ_6 was prepared and treated as described in the legend for Fig. 6.

the line are observed. This is expected for membranes having a heterogeneous composition of fatty acyl chains. For ϕ_6 (Fig. 8) the lower line for the mimicked phospholipids is also straight over the temperature range studied. The slope of the line is somewhat less than that for HB10Y cells and phospholipids. This may be due to a reduced degree of long-range interactions in the ϕ_6 lipids because of charge repulsion from the

greater percentage of phosphatidylglycerol in these bilayers. The most striking effect is observed for the intact spin-labeled virus, as seen in the upper portion of Fig. 8. The rotational motion of BPN is much reduced in comparison to that of the mimicked lipids, and a sharp discontinuity in the slope occurs at approximately 31°C. These data suggest that the phospholipids in ϕ_6 interact strongly with membrane proteins and that this interaction is reflected as reduced rotational mobility of the spin label BPN. Since the ϕ_6 lipids show no break in the Arrhenius plot at 31°C, the effect observed for the intact virus could be due to an abrupt change in lipid-protein interactions, or perhaps to a reversible conformational change of some membrane protein(s) that modifies the rigidity of surrounding phospholipid molecules. Calculations to be presented in the discussion indicate that essentially all the ϕ_6 phospholipids are expected to be in close proximity to membrane proteins.

DISCUSSION

For any antimicrobial agent to be of clinical value, it must be effective against the infectious organism at concentrations and under conditions that are comparatively harmless to the host. We have observed somewhat empirically that a number of hydrophobic, quasi-spherical molecules strongly perturb virus membrane structures without significantly affecting host membrane functions. These molecules, such as BHT and the spin label BPN, are not surface-active compounds with detergent-like properties. They are uncharged, have very low water solubility, and partition strongly into biological membranes, yet they have geometrical characteristics that prevent their orderly stacking in the alkyl chain region of the lipid bilayer. Although BHT and BPN both have antioxidant properties, it seems unlikely that this is related to their antiviral activity, since other structurally similar molecules that are not antioxidants also inactivate enveloped viruses. The major question at hand is the basis for their selective inactivation of enveloped viruses in contrast to their inconsequential effects on cells.

The three-dimensional structure of BPN and its physical properties are not dissimilar to those of BHT. All our studies show that these two molecules inactivate ϕ_6 in a very similar and probably identical manner. For both BPN (data presented here) and BHT (27; P. Wanda, Ph.D. thesis, The Pennsylvania State University, University Park, 1978), the treatment of ϕ_6 does not remove the viral envelope, but results in the specific removal of the structural protein P3 from the envelope of the virus. As a result, the

virus is no longer capable of adsorbing to the host cell. Thus, information obtained about the mechanism of action of BPN may apply to similar studies of BHT and other structurally related virucidal agents.

Since BPN is a spin label, the nature of its interaction with virus and cell membranes can be explored. BPN is localized in zones of identical polarity in both the virus and cells as well as their extracted counterparts (Table 1). A hyperfine coupling of 13.7 gauss indicates low polarity characteristic of the hydrocarbon zone of the membranes. The motional characteristics (Fig. 7 and 8), although varying somewhat in the systems studied, are typical for that of spin labels in lipid regions and do not reflect the degree of immobilization characteristic of binding to protein molecules. Thus, the virucidal agent appears to be localized in similar regions in both virus and cells, and the difference in its motional properties is likely due to the effects of proteins on the lipid molecules surrounding the spin label.

Some insight into the basis for the greater immobilization of BPN in $\phi 6$ compared to that of the mimicked $\phi 6$ lipids and the cell systems may come from considerations of the lipid and protein composition of the viral envelope. Calculations based on the percent composition of protein and lipid in the envelope of $\phi 6$ (A. Vidaver, personal communication) reveal that there is only enough lipid to occupy about 30% of the surface of the envelope. By calculating the number of copies of each membrane protein from literature data (21), we estimate that there are approximately five phospholipid molecules for each membrane protein molecule. Although the specific arrangement of the proteins in the $\phi 6$ envelope is not known, it would nevertheless appear that essentially all of the phospholipid molecules are "boundary lipids" (7), being adjacent to and having strong associations with the surface of membrane proteins. Such a picture is consistent with our ESR data showing greatly reduced freedom of motion for BPN in $\phi 6$. Likewise, the discontinuous behavior of τ_c on the Arrhenius plot of Fig. 7 can be interpreted as reflecting the interactions between proteins and lipids in close proximity.

The HB10Y membrane appears to be more characteristic of the fluid mosaic structure, having some regions in which liquid-crystalline arrays of phospholipids predominate. One explanation for the insensitivity of HB10Y to BPN and BHT would be that these molecules are primarily localized in the free-lipid zones and have little or no effect on lipid-protein interactions and membrane protein function. Since such free-lipid zones appear not to be present in

$\phi 6$, the partitioning of the molecules into its envelope may be much more effective in disrupting lipid-protein interactions. One result could be the release of a membrane protein, as observed here for P3.

It is particularly interesting that, of the five envelope proteins of $\phi 6$ (25), only P3 is removed by BPN and BHT. Experiments were carried out using reported methods for removing membrane proteins to investigate the nature of the binding of P3 to the membrane lipids. It was found (data not shown) that (i) 2 M KCl, (ii) 50 mM EDTA, (iii) pH 3.5, and (iv) pH 11 were ineffective in removing P3. These are conditions that are known to release proteins that are associated with membranes through ionic interactions with the polar surface (17, 22). On the other hand, treatment with low concentrations (0.01%) of a nonionic detergent (Triton X-100), which is known to solubilize intrinsic membrane proteins (6), specifically removed P3. At higher concentrations, Triton X-100 removed the entire $\phi 6$ envelope. These data indicate that P3 is held in the membrane by relatively weak hydrophobic forces that are readily diminished by the interactions of BPN and BHT.

It remains to be seen whether other enveloped viruses are similarly devoid of free-lipid domains. Recently, data have been reported suggesting that Semliki Forest virus may not have a significant amount of lipid not engaged in lipid-protein interactions (4, 5). It is interesting to note in this regard that this virus is also inactivated by BHT (12). If the absence of free-lipid domains in viral membranes is widespread and is generally associated with sensitivity to membrane perturbers, then this class of agents may have a broad spectrum of activity against enveloped viruses. Nevertheless, it may be that each particular virus will be optimally inactivated by a hydrophobic molecule having certain geometrical features, and the proper design of such molecules will be enhanced by understanding the nature of their interactions with virus and cell membrane structures.

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