Modulation by Small Hydrophobic Molecules of Valinomycin-Mediated Potassium Transport across Phospholipid Vesicle Membranes†

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ABSTRACT: The effects of small hydrophobic molecules on valinomycin-mediated K^+ transport in small unilamellar soybean phospholipid vesicles have been studied by using a vesicle-entrapped pH-sensitive hydrophilic fluorescence probe to monitor counterion-limited, passive H^+ diffusion into vesicles after an abrupt decrease in external pH [Clement, N. R., & Gould, J. M. (1981) *Biochemistry* (preceding paper in this issue)]. Under conditions where, even in the absence of valinomycin, transmembrane K^+ movement represented the primary and limiting counterion flux, <1 valinomycin molecule/vesicle was sufficient to accelerate the rate of H^+ entry into all of the vesicles. Incorporation of the bulkily substituted molecules butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *p*-di-*tert*-butylbenzene into soybean lipid bilayers had no effect upon K^+ diffusion in the absence of valinomycin. However, the presence of these hydrophobic molecules increased the apparent efficacy for K^+ transport of a given valinomycin concentration by as much as 4–6-fold. The less bulky membrane perturbants *tert*-butyl alcohol, phenol, and heptane showed very much less dramatic effects. While the rate of valinomycin-mediated K^+ transport (in the presence or absence of BHT) was very sensitive to temperature-induced changes in membrane fluidity, the degree of synergistic interaction between valinomycin and BHT was independent of temperature. Furthermore, BHT, BHA, and *p*-di-*tert*-butylbenzene, at levels which alter valinomycin-mediated K^+ transport, did not by themselves induce changes in membrane fluidity. It is postulated that changes in phospholipid head-group packing and/or surface charge density brought about by the presence of bulky perturber molecules leads to changes in partitioning of valinomycin or the valinomycin–K^+ complex between the aqueous and membrane phases.

The rate of protein-mediated solute and ion transport across biological membranes is, in many cases, determined by the phospholipid composition and physical state of the bilayer. Active and passive transport in bacterial cells and derived vesicles (Linden et al., 1973; DeKruyff et al., 1973; McElhaney et al., 1973; Schechter et al., 1974; Thilo et al., 1977), sarcoplasmic reticulum membranes (Inesi et al., 1973), neural membranes (Kimelberg & Papahadjopoulos, 1974), and in reconstituted systems (Melchior & Czech, 1979) characteristically shows increased activity when membrane fluidity is increased by elevated temperatures or by the inclusion of predominantly unsaturated fatty acids in the phospholipids.

Antibiotic ionophores such as valinomycin and the macrotetralide actins have been employed as models for the study of transmembrane transport systems operating by a cage-carrier or shuttle-type mechanism (Szabo et al., 1969; Stark & Benz, 1971) since, like the transport systems cited above for natural membranes, the rate of carrier-mediated ion transport across planar bilayers and artificial vesicle membranes is strongly dependent upon the degree of ordering among the aliphatic chains of the bilayer's hydrocarbon interior (Krasne et al., 1971; Papahadjopoulos et al., 1973; Benz et al., 1973, 1977). The motional freedom experienced by the phospholipid molecules within a bilayer, often referred to as the membrane fluidity, is determined primarily by the fatty acid and phospholipid composition, the temperature, and the presence in the bilayer of other lipid or hydrophobic components (Overath & Trauble, 1973). While the effects of temperature and phospholipid composition are predictable, the effects of small hydrophobic or amphiphilic molecules on the physical properties of phospholipid bilayers are both diverse and complex, depending in part upon the location of the small molecule (perturber) within the vertical dimension of the bilayer (Jain & Wu, 1977) and upon its partitioning between liquid-crystalline and gel phases (Pringle & Miller, 1979). The most widely studied membrane perturber is undoubtedly cholesterol, which alters the bilayer organization in a complex fashion, increasing molecular mobility in the gel state while decreasing molecular mobility in the liquid-crystalline state (Ladbrooke et al., 1968; Oldfield & Chapman, 1971; Darke et al., 1972). Cholesterol decreases the transmembrane mobility of valinomycin and other carrier-type ionophores in planar bilayers (Szabo et al., 1969; Benz et al., 1977; Benz & Cros, 1978; Benz & Gisin, 1978) and in large, multilamellar liposomes while reportedly increasing valinomycin mobility in small, unilamellar vesicles (LaBelle & Racker, 1977).

In this study we have examined the effects of several hydrophobic or weakly amphiphilic molecules of low molecular weight on valinomycin-mediated K^+ transport in small unilamellar vesicles. Several of the membrane perturbers studied exhibit an unusual synergistic effect with valinomycin, greatly increasing the apparent effectiveness of the ionophore while having no detectable effect on K^+ permeability in the absence of valinomycin or on the fluidity of the bilayer interior.

Experimental Methods

Preparation of Vesicles. Unilamellar phospholipid vesicles containing the pH-sensitive hydrophilic fluorescence probe 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) entrapped within the internal aqueous phase were prepared from purified (Kagawa & Racker, 1971) soybean phospholipids (asolectin;[1] Associated Concentrates, Woodside, NY) by the sonication

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[1] Abbreviations used: asolectin, purified soybean phospholipids; tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; BHT, 2,6-di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene); BHA, 2(3)-*tert*-butyl-4-methoxyphenol (butylated hydroxyanisole).

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procedure described in the preceding paper (Clement & Gould, 1981a).

Measurement of Ion Transport. The hydrogen ion concentration of the internal aqueous compartment of the vesicles was continuously monitored by recording the fluorescence intensity of entrapped pyranine as described elsewhere (Clement & Gould, 1981a). For these experiments vesicles (50–400 μg of phospholipid) preequilibrated at pH 8.2 were suspended in 3 mL of 0.2 M sucrose, 0.1 M KCl, 5 mM tricine/KOH, and 5 mM Mes/KOH (pH 8.2) in a continuously stirred 1-cm fluorescence cuvette. After thermal equilibration (2 min) the external pH was rapidly dropped to 6.6 by the addition of a small volume of HCl from a microliter syringe. There is an immediate rapid and electrically uncompensated influx of protons into the vesicles, followed by a much slower, counterion-limited proton–counterion exchange until transmembrane pH equilibration has occurred (Clement & Gould, 1981a). The rate of this slower portion of the passive proton influx is accelerated by valinomycin (see Results) or permeant counterions such as SCN^-, indicating that, under these conditions, the rate of proton influx (visualized by changes in pyranine fluorescence) is accurately reflecting the rate of electrically compensating counterion fluxes.

Incorporation of Membrane Perturbers. For most experiments the desired amount of perturber was added in a small volume of ethanol (<10 μL) directly to the vesicle suspension in the cuvette at least 2 min before the HCl pulse. Control experiments in which the perturber in ethanol or chloroform solution was dried down with the phospholipid prior to sonication gave results identical with those obtained when the perturber was added after vesicle formation at the same perturber/phospholipid ratio.

Fluorescence Polarization. The fluidity of the hydrocarbon region of the phospholipid bilayer was estimated from the fluorescence polarization of the hydrophobic membrane probe 1,6-diphenyl-1,3,5-hexatriene (Cogan et al., 1973) by using an Elscint MV-1a polarimeter. For these experiments pyranine was omitted from the vesicle preparation solution.

Reagents. Valinomycin, 2,6-di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, BHT), and 2(3)-*tert*-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA) were purchased from Sigma Chemical Co. BHA was recrystallized twice from petroleum ether before use. *p*-Di-*tert*-butylbenzene and 1,6-diphenyl-1,3,5-hexatriene were obtained from Aldrich Chemical Co. and used without further purification. Laser grade pyranine was obtained from Eastman. All other reagents were of reagent grade.

Results

The kinetics of proton diffusion into unilamellar asolectin vesicles following a rapid decrease in external pH are biphasic (Figure 1a). In an earlier study (Clement & Gould, 1981a) we have shown that the initial fast influx of protons (the fast component of the fluorescence decrease) corresponds to an electrically uncompensated movement along the H^+ concentration gradient. When a sufficient transmembrane electric potential opposing further charge influx has built up, proton influx becomes much slower, and a counterion-limited proton–counterion exchange occurs until the transmembrane proton concentration gradient is eliminated. Thus, valinomycin in the presence of K^+ accelerates the slow phase of proton efflux dramatically but has very little effect upon the extent of the fast component (Figure 1b). Semilog replots of the fluorescence decay yielded straight lines from which an apparent pseudo-first-order rate constant (k') for the fluorescence decrease could be calculated[2] (Figure 1c; see Discussion).

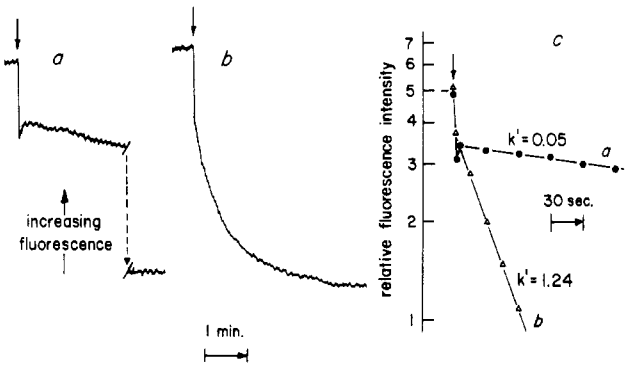

FIGURE 1: Effect of valinomycin on rate of proton diffusion into unilamellar asolectin vesicles following a rapid drop in external pH. The hydrogen ion concentration of the vesicles internal aqueous compartment was determined at 25 °C from the fluorescence intensity of entrapped pyranine as described by Clement & Gould (1981a). A decrease in fluorescence intensity represents an increase in internal hydrogen ion concentration. The reaction mixture (3 mL) contained 0.2 M sucrose, 0.1 M KCl, 5 mM tricine/KOH, 5 mM Mes/KOH (pH 7.8), and vesicles containing 364 μg of phospholipid. The downward arrows indicate the addition of 12 μL of 0.1 N HCl, which immediately lowered the external pH to 6.2. (Trace a) Vesicles only; (trace b) 1.6 μg of valinomycin was added to the suspension 2 min prior to the HCl pulse; (trace c) semilogarithmic replot of the fluorescence decreases shown in traces a and b. Note that the apparent first-order-rate constant (k') for the counterion-limited H^+ influx is greater in the presence of valinomycin.

Because hydrogen ion influx and therefore changes in pyranine fluorescence are counterion-limited under these conditions, changes in the rate constant for the slow component of the fluorescence decrease were taken to represent net changes in transmembrane counterion permeability. Even in the absence of valinomycin, the primary counterion flux accompanying H^+ influx appears to be K^+ efflux since the rates of H^+ influx for vesicles in KCl and K_2HPO_4 (0.1 M K^+) media were about the same and were much greater than the rate of H^+ influx for vesicles in choline chloride medium (Figure 2).

The relationship between valinomycin concentration, phospholipid concentration, and the rate of transmembrane K^+ movement is shown in detail in Figure 3. The apparent rate constant for valinomycin-mediated K^+ efflux increased linearly with increasing valinomycin concentration, although the rate of increase depended upon the amount of phospholipid present in the sample. In other words, the actual increase in the apparent rate constant for K^+ transport depended upon the valinomycin/phospholipid ratio (Figure 3b), indicating that valinomycin partitions strongly into asolectin membranes and that the partitioning coefficient between the membrane and aqueous phases remains constant over the ranges of valinomycin and phospholipid concentrations used in this experiment. Interestingly, the relationship between k' and the valinomycin/phospholipid ratio was unchanged even when there was <1 valinomycin molecule/vesicle (assuming ~3000 phospholipid molecules/vesicle; Watts et al., 1978), indicating that an individual valinomycin molecule must be able to act upon more than one vesicle (Johnson & Bangham, 1969; Kornberg et al., 1972). This is in contrast to the effects of the trans-

[2] Care was taken to determine the apparent rate constant for the fluorescence decrease accompanying counterion-limited proton influx when the internal pH, determined from pyranine fluorescence, was between 7.5 and 7.0. Plots of molar hydrogen ion concentration vs. fluorescence intensity were approximately linear over this range, so that the observed apparent rate constants for the fluorescence change can be accurately related to rate constants for transmembrane hydrogen ion or counterion fluxes.

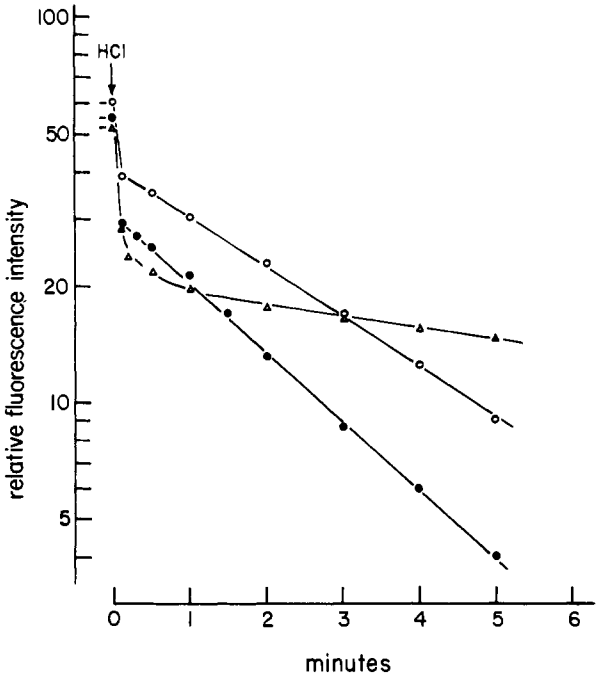

FIGURE 2: Ion dependence for counterion-limited proton influx in unilamellar asolectin vesicles. Reaction conditions were essentially as described in the legend to Figure 1a. Vesicles were prepared and suspended in media containing 1 mM tricine, 1 mM Mes, and either 0.1 M KCl (○), 48 mM K_2HPO_4 plus 4.8 mM KH_2PO_4 (●), or 0.1 M choline chloride (△). The buffers were adjusted with KOH (KCl and K_2HPO_4) or NaOH (choline chloride) to pH 8.2.

membrane channel gramicidin, which apparently does not rapidly exchange between individual vesicles (Clement & Gould, 1981b).

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are widely used commercial food additives which have been found to partition strongly into natural and artificial phospholipid membranes (Clement & Gould, 1980). Neither BHT, BHA, nor the deoxy analogue *p*-di-*tert*-butylbenzene had any detectable effect upon K^+ permeability in asolectin vesicles in the absence of valinomycin (Figure 4). However, in the presence of a single fixed concentration of valinomycin, increasing levels of BHT, BHA, and *p*-di-*tert*-butylbenzene resulted in an increasing rate of valinomycin-mediated K^+ transport. This unusual synergistic effect between BHT, BHA, or *p*-di-*tert*-butylbenzene and valinomycin was dependent upon the concentrations of both the membrane perturber and valinomycin. BHT, BHA, and *p*-di-*tert*-butylbenzene appeared to be about equally effective at stimulating transmembrane valinomycin–K^+ fluxes, although these data do not consider possible differences in membrane–solvent partitioning. Very much smaller synergistic effects were observed for the less bulky hydrophobic molecules *tert*-butyl alcohol, phenol, and heptane (Figure 5), while no synergism at all was observed with similar levels of benzene (not shown).

The stimulations of transmembrane valinomycin–K^+ transport by BHT, BHA, and *p*-di-*tert*-butylbenzene appear not to be the result of alterations in the fluidity of the hydrocarbon region of the bilayer (Figure 6). The absolute rates of K^+ transport in the presence or absence of valinomycin or valinomycin plus BHT were strongly dependent upon membrane fluidity, however (Figure 7), although the relative stimulation resulting from a given concentration of BHT was independent of temperature (Figure 7, inset).

Discussion

Bulkily substituted small molecules such as BHT, BHA, and

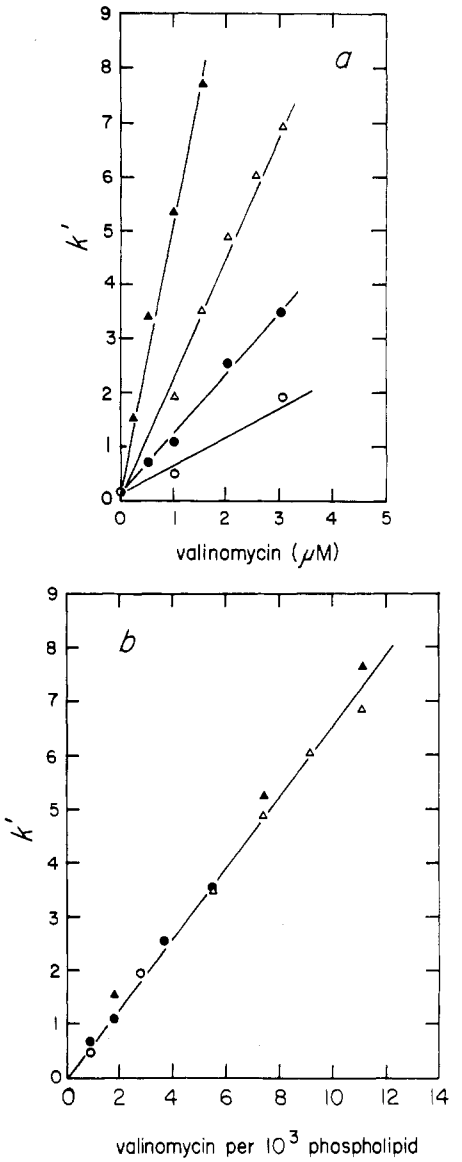

FIGURE 3: Relationship between valinomycin concentration, phospholipid concentration, and rate of counterion-limited proton influx in unilamellar asolectin vesicles. Reaction conditions were as described in the legend to Figure 1, except that the concentrations of vesicles and valinomycin were varied as indicated. (Panel a) Stimulation of counterion-limited proton influx by valinomycin for samples with vesicle concentrations equivalent to 813 (○), 407 (●), 203 (△), and 102 (▲) μg of phospholipid/mL. (Panel b) Data from panel a, normalized for the valinomycin/phospholipid ratio.

p-di-*tert*-butylbenzene were found to exhibit an unusual synergistic enhancement of transmembrane K^+ diffusion with the cation carrier valinomycin. These molecules had no effect upon K^+ diffusion by themselves. However, their presence in the membrane markedly enhanced the efficacy of a given concentration of valinomycin to catalyze transmembrane K^+ diffusion. Less bulkily substituted molecules were much less effective, suggesting that these effects may be related to alterations in molecular packing within the bilayer. BHT, BHA, and *p*-di-*tert*-butylbenzene, at levels which significantly enhanced valinomycin-mediated K^+ transport, had no measurable effect on the fluidity of the hydrocarbon interior of asolectin vesicle membranes, however, indicating that changes in molecular organization may be localized in the region of the membrane–water interface (see below).

The synergistic effects of these small molecules on valinomycin-mediated K^+ diffusion may be explained within the context of the carrier hypothesis by which cyclodepsipeptides

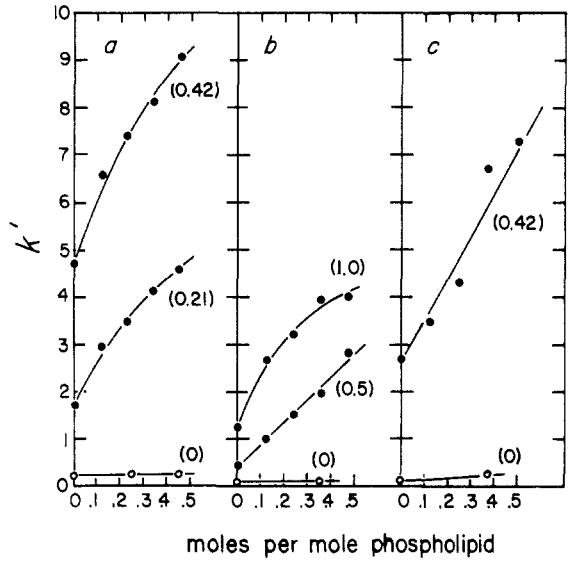

FIGURE 4: Effects of BHT (panel a), BHA (panel b), and *p*-di-*tert*-butylbenzene (panel c) on counterion-limited proton influx into asolectin vesicles in the presence and absence of valinomycin. Reaction conditions were as described in the legend to Figure 1a. Vesicle concentrations for the 3-mL samples were 59 (panel a), 203 (panel b), and 57 (panel c) μg of phospholipid/mL. Valinomycin concentrations (μM) are given in parentheses. Note the strong synergistic effect of each of these compounds with valinomycin.

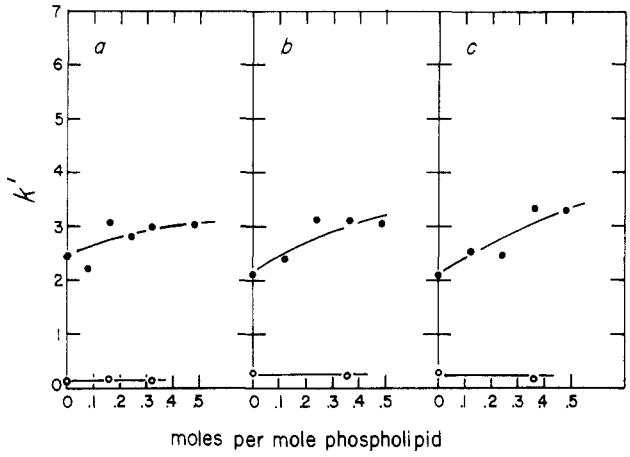

FIGURE 5: Effects of *tert*-butyl alcohol (panel a), phenol (panel b), and heptane (panel c) on counterion-limited proton influx into asolectin vesicles in the presence and absence of valinomycin. Reaction conditions were as described in the legend to Figure 1a. Vesicle concentrations for the 3-mL samples were equivalent to 53 (panel a) and 60 (panels b and c) μg of phospholipid/mL. Valinomycin was 0.42 μM when added (closed circles, ●).

such as valinomycin are thought to function. Complexation of monovalent cations by these compounds is accompanied by a conformational transition resulting in a more hydrophobic surface for the carrier molecule and an effective shielding of the cation charge from the solvent (Schemyakin et al., 1969). The hydrophobic cation–carrier complex can then freely diffuse across the hydrophobic bilayer to the other side, where the complex dissociates, releasing the cation. The rate (v) of valinomycin-mediated K^+ transport across a membrane at any given total valinomycin concentration (in the presence of excess K^+) can be represented as

$$v = k[\text{val}\cdot K^+_m]$$

where k is the pseudo-first-order rate constant and $[\text{val}\cdot K^+_m]$ is the concentration of the valinomycin–K^+ complex *in the membrane*. In the absence of specific data for (1) the partitioning of the valinomycin–K^+ complex between the membrane and aqueous phases and (2) the relative proportion of membrane-bound valinomycin complexed with K^+ under the

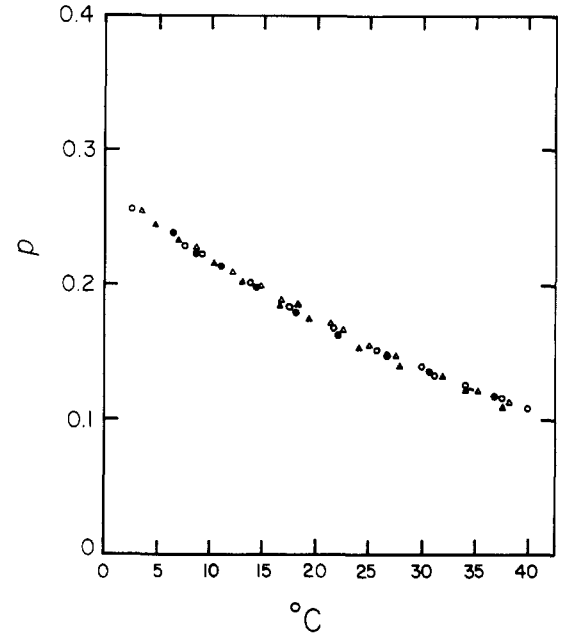

FIGURE 6: Temperature dependence of fluorescence polarization of the probe 1,6-diphenyl-1,3,5-hexatriene incorporated into unilamellar asolectin vesicles in the presence of BHT, BHA, or *p*-di-*tert*-butylbenzene. Vesicles were prepared by sonication (see Experimental Methods) of 325 μg of asolectin phospholipid containing 10 nmol of 1,6-diphenyl-1,3,5-hexatriene in 0.5 mL of 0.2 M sucrose, 0.1 M KCl, 5 mM tricine/KOH, and 5 mM Mes/KOH (pH 8.2). BHT (●), BHA (▲), or *p*-di-*tert*-butylbenzene (▲) were added in a small volume (<10 μL) of ethanol to the vesicle suspension at a final ratio of perturber/phospholipid of 0.4 and allowed to incubate at room temperature for 5 min before the polarization was determined. The open circles (○) are for a sample of asolectin vesicles with no added perturber.

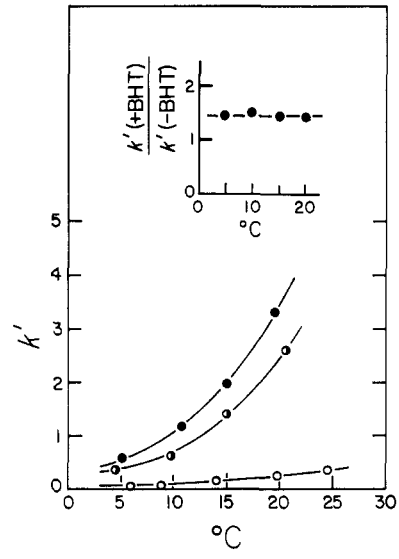

FIGURE 7: Temperature dependence of apparent rate constant (k') for counterion-limited proton influx into asolectin vesicles in the presence and absence of valinomycin and BHT. Reaction conditions were as described in the legend to Figure 1a, with the temperature varied as indicated. The 3-mL samples contained vesicles equivalent to 50 μg of phospholipid/mL (○) and either 0.66 μM valinomycin (◑) or 0.66 μM valinomycin plus 0.25 mol of BHT/mol of phospholipid (●). The inset shows the effect of temperature on the ratio of the apparent rate constants (determined in the presence of valinomycin) with and without BHT. Values for k' (±BHT) were corrected for the contribution to k' from counterion fluxes not dependent upon valinomycin (○).

conditions of this study, the *apparent* rate constant (k') observed in these experiments is actually derived from

$$v = k'[\text{val}_t]$$

where $[\text{val}_t]$ is the total (bulk) valinomycin concentration. Thus, what appears as a change in k' is, in reality, very likely a change in the ratio $[\text{val}\cdot K^+_m]/[\text{val}_t]$, since

$$v = k'[\text{val}_t]\frac{[\text{val}\cdot K^+_m]}{[\text{val}_t]} = k[\text{val}\cdot K^+_m]$$

In other words, any change in the concentration of the valinomycin–K^+ complex in the membrane will appear, under our experimental conditions, as an increase in k'.

Such changes in valinomycin partitioning into the membrane could well result from changes in packing of the phospholipid molecules, particularly in the region of the polar head groups at the membrane–water interface. Because the conformational transition which valinomycin undergoes during K^+ complexation must occur in a relatively nonpolar environment (Schemyakin et al., 1969), such as the membrane–water interface (Stark & Benz, 1971; Benz & Gisin, 1978), changes in surface charge density brought about by perturber molecules such as BHT intercalated in the phospholipid bilayer could serve to facilitate formation of the valinomycin–K^+ complex by allowing greater access of membrane-bound valinomycin to aqueous K^+ ions. Alternatively, molecular packing defects induced within the bilayer by BHT, etc., may simply allow a greater concentration of valinomycin within the membrane, which, at a given K^+ concentration, could result in a greater concentration of the valinomycin–K^+ complex in the membrane as well.

The conclusion that BHT and BHA disturb the molecular packing primarily near the head-group region of the bilayer is suggested by the findings of Singer & Wan (1977), who found that in multilamellar liposomes, the inhibition of passive Na^+ diffusion by BHT and a variety of structural analogues was dependent upon the presence of the relatively hydrophilic phenolic hydroxyl group and upon the bulkiness of the other ring substituents. Their conclusion is consistent with the data presented here, with the exception that *p*-di-*tert*-butylbenzene, which was an effective perturber in our study, lacks a phenolic hydroxyl group. However, the bulkiness of the two *tert*-butyl groups may still allow this molecule to disrupt the molecular organization of the interface region even though it resides primarily in the more hydrophobic interior of the bilayer.

Acknowledgments

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