SC-300 superconducting spectrometer equipped with a Varian 620L/100 computer. The probe temperature was calibrated by the chemical shift of ethylene glycol. Experiments were carried out within a few hours after the vesicles were freshly prepared.

Electron Spin Resonance Measurements. Electron spin resonance measurements of the spin-label probes Tempo and C_5- and C_{12}-doxylstearic acid were carried out with a Varian E-line century series spectrometer (E109). Signals were observed over a 100 to 40 G range at room temperature and variable temperatures. The modulation amplitude was kept low to avoid saturation of the signals. The probe temperature was measured directly using digital thermometer equipped with a thermocouple.

Calculation of Order Parameter S. The electron spin resonance signals of the nitroxide group of C_5- and C_{12}-doxylstearic acid change with the mobility of the hydrocarbon region to which it is attached. Following the theoretical treatment by Hubbell and McConnell[6] and Gaffney,[7] the order parameter was obtained according to the following equation:

$$S = \frac{T_\parallel' - [T_\perp' \text{ (ca.)} + C]}{T_\parallel' + 2[T_\perp' \text{ (ca.)} + C]} \times 1.66$$

where $C = \{4.06 - 0.053[T_\parallel' - T_\perp' \text{ (ca.)}]\}$ MHz and $T_\parallel'$ and $T_\perp'$ (ca.) = one-half the separation of the outer and inner extreme of the spectrum shown in Figure 3b.

Calculation of DOPC Vesicle Concentration. Since the average size of the ultrasonically prepared DOPC vesicles is about 250 Å, the total number of DOPC lipid molecules in an averaged sized vesicle can be calculated using 60 Å^2 as the area of each lipid head group and 60 Å as the thickness of the bilayer membrane. The average number of the DOPC molecules per vesicle is calculated to be about 4000. The concentration of the DOPC vesicle is therefore $^1/_{4000}$ the concentration of the DOPC lipid.

Data Analysis of ^{1}H NMR Spectra. The proton transverse relaxation rate of the drug molecules was used to study sulindac sulfide–membrane interaction. The transverse relaxation rate ($1/T_2$) of a proton is related to its half-height line width ($\Delta\nu$) of $1/T_2 = \pi\Delta\nu$. Using similar treatment for the substrate and macromolecule binding[9,17] and assuming n binding sites of each vesicle, the binding equilibrium between a vesicle, V, and a drug molecule S can be expressed as

$$S + nV \rightleftharpoons SV$$

$$K_d = \frac{[S]_f\, n[V]_f}{[SV]_b} \quad (1)$$

where K_d is the dissociation constant and b and f refer to the binding and free state of the vesicles and drugs.

The fraction of drugs bound to vesicles is expressed in eq 2,

$$F = \frac{[SV]_b}{[S]_0} \quad (2)$$

with $[S]_0$ being the total drug concentration.

Since the total transverse relaxation rate of the specific drug proton is an averaged property of free and membrane-bound molecules, eq 3 is derived based on the assumption:

$$\frac{1}{T_2} = \frac{1-F}{T_{20}} + \frac{F}{T_{2b}+\tau} \simeq \frac{1}{T_{20}} + \frac{F}{T_{2b}+\tau} \quad (3)$$

$$F \ll 1$$

In eq 3, $1/T_2$ is the measured relaxation rate of the drug protons, $1/T_{20}$ is the relaxation rate of the drug protons in the absence of vesicles, $1/T_{2b}$ is the relaxtion rate of the drug protons when bound to vesicles, and τ is the exchange lifetime of free and membrane-bound drug molecules. Combination of eq 1–3 yields eq 4, where $[V]_0$ is the total concentration of the vesicles. Equation

$$\frac{1}{T_2} = \frac{1}{T_{20}} + \frac{n[V]_0}{(K_d + [S]_0)(T_{2b}+\tau)} \quad (4)$$

$$[S]_0 \gg [V]_0$$

4 predicts that a plot of $1/T_2$ against $[V]_0$ should give a straight line with a slope of

$$\frac{n}{(K_d + [S]_0)(T_{2b}+\tau)}$$

and an intercept of $1/T_{20}$.

Acknowledgment. We thank Drs. Eugene H. Cordes, Sunney I. Chan, and Byron H. Arison for their helpful discussions concerning this study.

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Membrane Effects of Antiinflammatory Agents. 2. Interaction of Nonsteroidal Antiinflammatory Drugs with Liposome and Purple Membranes

San-Bao Hwang* and T. Y. Shen

Membrane & Arthritis Research, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065.
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The interaction of the nonsteroidal antiinflammatory drugs (NSAIDS) indomethacin, diflunisal, and flurbiprofen and the active sulfide metabolite of sulindac with phosphatidylcholine (PC) liposomes was investigated using differential scanning calorimetry (DSC). These biologically active structures decrease the phase transition temperature and broaden the transition peak with increasing concentration, but without affecting the enthalpy change for the transition on the thermal scan. A comparison with the effects of the prodrug sulindac and its inactive sulfone metabolite suggests that the main action of NSAIDS on membranes is a reduction of the cooperative interaction between phospholipid molecules. The probable positions of these compounds in the bilayer are inferred from similar DSC effects of several reference compounds whose mode of binding to the PC bilayer have previously been described. The active antiinflammatory structures appear to insert deeply into the hydrocarbon region of the bilayer, whereas the inactive compounds probably bind mainly to the carbonyl region near the surface. Using purple membrane as a model to study the drug effect on protein–protein interaction in this membrane system, low concentrations of active NSAIDS effectively dissociate the bacteriorhodopsin lattice. These results suggest that the active NSAIDS studied here are able to partition deeply into the hydrocarbon region of the bilayer and interact with a membrane protein imbedded inside the bilayer. The prodrug sulindac per se is devoid of any significant membrane effects.

The interaction of various membrane affectors, such as local anesthetics and aralkylamines, with natural and artificial membranes has been examined extensively by several biophysical techniques.[1,2] Some changes of mem-

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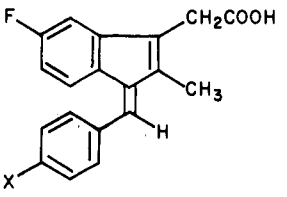
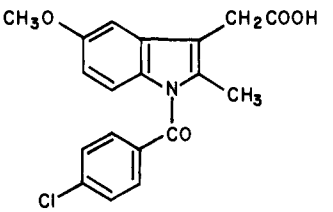
brane characteristics, such as increasing permeability to positive and negative ions, shifting melting profile of phospholipid vesicles, and increasing fluidity of the bilayer, are induced by these agents. However, similar biophysical studies with biologically active aralkyl acids have not been reported. A notable group of these acidic molecules are a large family of nonsteroidal antiinflammatory drugs (NSAIDS)[3] developed in the past 2 decades. The biochemical mechanism of action of many NSAIDS, e.g., indomethacin, sulindac (via its active sulfide metabolite), and most arylpropionic acid derivatives, have been attributed mainly to their inhibition of fatty acid cyclooxygenase which converts arachidonic acid to prostaglandins.[4] Nevertheless, at higher concentrations NSAIDS also exert some membrane effects, such as the stabilization of erythrocyte and lysosomal membranes. A membrane-associated phospholipase A_2, which liberates arachidonic acid from membrane phospholipids, was recently shown to be inhibited by indomethacin at a 0.1 mM concentration in vitro.[5] Many inflammatory responses, e.g., the activation of arachidonic acid and complement cascades, chemotaxis, and platelet aggregation, are also intimately related to the physical states of biomembranes. Presumably, more potent and selective membrane regulators may possess novel antiarthritic actions. Furthermore, in current quantitative structure–activity relationship studies only the physical properties of pharmacologically active molecules per se are available for analysis. Obviously it would be of interest to have a better understanding of drug effects on macromolecular targets such as membranes. From these considerations a series of biophysical studies to elucidate the possible effects of NSAIDS on biomembrane systems was initiated in our laboratories.

As an initial model, the chemically defined bilayer membrane system, liposomes, was used. A small group of NSAIDS previously synthesized in our laboratories,[4] sulindac (1) and its metabolites 2 and 3, indomethacin (4), diflunisal (5), and flurbiprofen (6), were selected as typical examples. All of these are hydrophobic molecules with strong protein-binding properties.[6] The first four compounds possess similar stereochemistry. Indomethacin (4) and sulindac sulfide (2) are also isosteric electronically. Sulindac (1) differs from its metabolites only in the oxidation stage of the methyl sulfide group, although only the sulfide metabolite (2) is biologically active. Flurbiprofen (6) is a typical antiinflammatory arylpropionic acid possessing a hydrophobic biphenyl moiety and an aliphatic acid side chain. Diflunisal (5) also has a biphenyl structure but with an *o*-hydroxy carboxylic acid as its polar function. All active compounds are reversible inhibitors of the fatty acid cyclooxygenase.

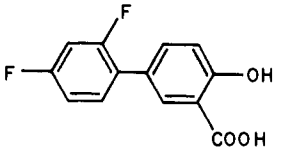
When pure phospholipids are mechanically agitated vigorously in water, they readily form liposome structures



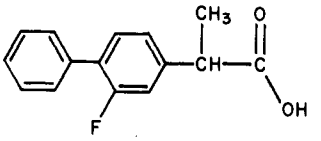
1 (sulindac), X = $S(=O)CH_3$
2 (sulindac sulfide), X = SCH_3
3 (sulindac sulfone), X = $S(=O)_2CH_3$



4 (indomethacin)



5 (diflunisal)



6 (flurbiprofen)

composed of many concentric bilayer sheets of various sizes interspersed with water. Thermal scans of the phospholipid multilamellar dispersions show highly cooperative reversible phase transitions at well-defined temperatures. Two reversible phase transitions have been found in the phosphatidylcholine (PC) liposomes.[7] The main transition, the gel to liquid crystal transition, involves a change in the lipid hydrocarbon chains within the bilayer from an ordered array at low temperatures to a liquid-crystalline-like structure. A minor pretransition is probably related to the reorganization of the phospholipid.[8] The endothermic transition heat and the cooperativity between phospholipids at the transition temperature are important parameters that can be directly measured by DSC. With a high degree of sensitivity the shape of the transition peak faithfully reflects the extent of the transition as a function of temperature.

It is commonly believed that the permeability of a given class of molecules through biological membranes is correlated with their solubility in an organic liquid. However, it should be recognized that phospholipids and some other biomembrane constituents are amphipathic molecules. The polar head groups and a few methylene groups down the lipid chain constitute a hydrophilic region which shields the hydrophobic center of the bilayer.[9] Therefore, the hydrophobicity of a drug molecule alone does not assure solubility in a bilayer, and it is likely that the partition coefficient of a drug molecule is a function of its position in the bilayer.[10] Our measurements showed that several organic molecules, e.g., TPP^+, TPB^-, and hexane, which are known to locate in different regions in the bilayer, induce distinct changes on the thermal behavior of phospholipid multilamellar dispersions. These reagents were then used as reference markers in our DSC study to indicate the position of NSAIDS in the bilayer membrane.

To extend our study to a more complex model membrane system containing both lipid and protein constituents, we have chosen the well-characterized purple membrane from halophilic bacteria. Bacteriorhodopsin molecules in purple membrane have a high percentage of protein (70% by weight). They aggregate and crystallize into a two-dimensional hexagonal lattice.[11] Exciton interaction

(1) P. Seeman, *Pharmacol. Rev.*, **24**, 583–655 (1972).
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(3) Abbreviations used here are as follows: NSAIDS, nonsteroidal antiinflammatory drugs; DSC, differential scanning calorimetry; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; TPP^+, tetraphenylphosphonium; TPB^-, tetraphenylboron; $TPMP^+$, triphenylmethylphosphonium; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
(4) T. Y. Shen, *Handb. Exp. Pharmacol.*, **50**(2), 305–347 (1978).
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Table I. Transition Properties of Pure PC

substance	pretransition temp, °C	main transition temp, °C	main transition enthalpy, kcal/mol	cooperative unit, molecules
DMPC	14.2	24	5.9	275
DPPC	35.5	41.5	8.4	215
DSPC	51.5	55	12.1	100

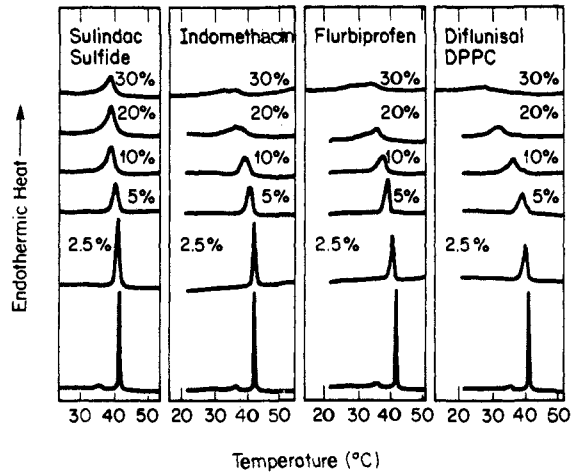

Figure 1. Differential scanning calorimetric curves of DPPC liposomes with sulindac sulfide, indomethacin, flurbiprofen, and diflunisal at various concentrations. The concentrations are (from bottom to top) 0, 2.5, 5, 10, 20, and 30% mole ratios (NSAIDS/DPPC), respectively, in 50 mM phosphate buffer, pH 7.0.

between the chromophores gives rise to the negative band in the visible CD spectrum of purple membrane which disappears when the purple membrane is dissociated to monomers. This exciton band splitting afforded us a sensitive indicator for the dissociation of bacteriorhodopsin lattice in the membrane as induced by antiinflammatory drugs. As described below, highly specific behaviors of drug molecules in these model systems were indeed observed.

Results

Thermotropic Behavior of Pure DMPC, DPPC, and DSPC. Calorimetric scans for pure DMPC, DPPC, and DSPC are repeatedly shown in Figures 1, 4, 6, 9, and 11 as control. The temperature, enthalpy, and the size of the cooperativity of the main transition and the pretransition temperature are listed in Table I for comparison. They are in good agreement with values previously obtained.[7]

It should be noted that Bangham liposomes used here are usually contaminated with some small vesicles as seen in the electron microscope.[12] Large onion-type liposomes and small vesicles have markedly different thermotropic behaviors.[13] In this study, since only one transition peak near 37 °C was observed for DPPC and the enthalpy change at 42 °C was very close to the published value, the contribution due to small vesicle appears to be negligible.

Thermotropic Behavior of NSAIDS/DPPC Mixtures. The differential thermal scans of various mixtures of sulindac sulfide (2), indomethacin (4), flurbiprofen (5), and diflunisal (6) with DPPC (Figure 1) show several

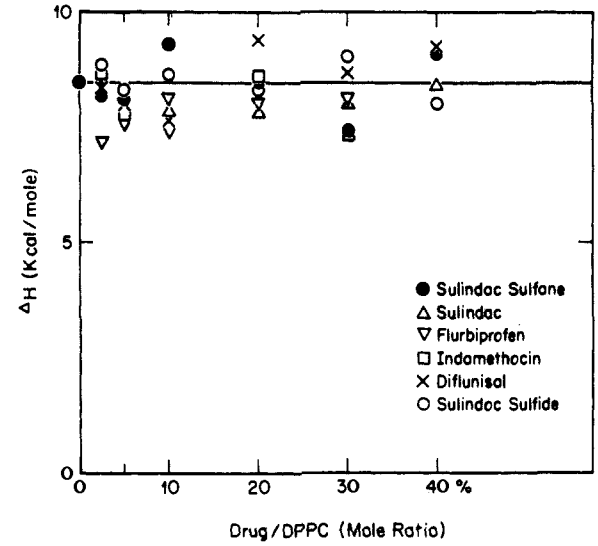

Figure 2. The transition heat for the gel to liquid-crystalline main transition for multilamellar DPPC dispersion with and without sulindac, sulindac sulfide, sulindac sulfone, indomethacin, diflunisal, and flurbiprofen at various concentrations in 50 mM phosphate buffer, pH 7.0.

common features induced by these active antiinflammatory drugs. The disappearance of pretransition, even at the lowest drug concentrations, indicates that the hexagonal packing of DPPC and the angle of tilt of hydrocarbon chains are distorted. The shift of the main transition temperature of pure DPPC to a lower temperature suggests that the packing of the hydrocarbon chains is destabilized. The broadened transition peak demonstrates that the cooperativity between phospholipids during the process of transition is weak. The heat of transition (ΔH_{cal}) for the gel to liquid crystal transition remains roughly a constant (Figure 2) within the experimental error, indicating that the proportion of the phospholipids in the membrane that are capable of melting remains in the same level. The transition peak is usually found to be asymmetric—the onset of the transition tends to be gradual in comparison to the completion of the transition. Multitransitions appearing as separated peaks or as a shoulder of the main transition peak are observed at the higher concentration of these drugs. In the cases of flurbiprofen and diflunisal, multitransitions appear even at concentrations as low as 5% mole ratio (agent/DPPC).

pH Dependence of Drug-Induced Changes of the Thermotropic Behavior of DPPC. Most NSAIDS are moderately acidic molecules, which exist as an equilibrium mixture of charged and uncharged species at the physiological pH. These two species can induce different membrane effects. The thermal scan at pH 4.5 (Figure 3, inset A) shows a transition peak with half-width of 2.1 °C, which is broader than the transition peak at pH 10.0 with a half-width of 1.1 °C (Figure 3, inset B). However, the transition temperature at pH 4.5 (40.2 °C) is higher than that at pH 10.0 (39 °C). Following the analysis of Hill,[14] the eq 1 can be derived, where V_w and V_m are the volume

$$\frac{C_T}{\Delta T_m} = \frac{C_m'}{P} + C_m'\left(\frac{V_m}{V_w}\right) \quad (1)$$

of solvent or water and lipid bilayer, respectively, in the liposome preparation, C_m' is the concentration of drug needed to produce a 1 degree depression, ΔT_m is the depression of the phase transition temperature in comparison

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(12) S.-B. Hwang and T. Y. Shen, unpublished results.
(13) J. M. Sturtevant, *Annu. Rev. Biophys. Bioeng.*, **3**, 35–51 (1974).
(14) M. W. Hill, *Biochim. Biophys. Acta*, **356**, 117–124 (1974).

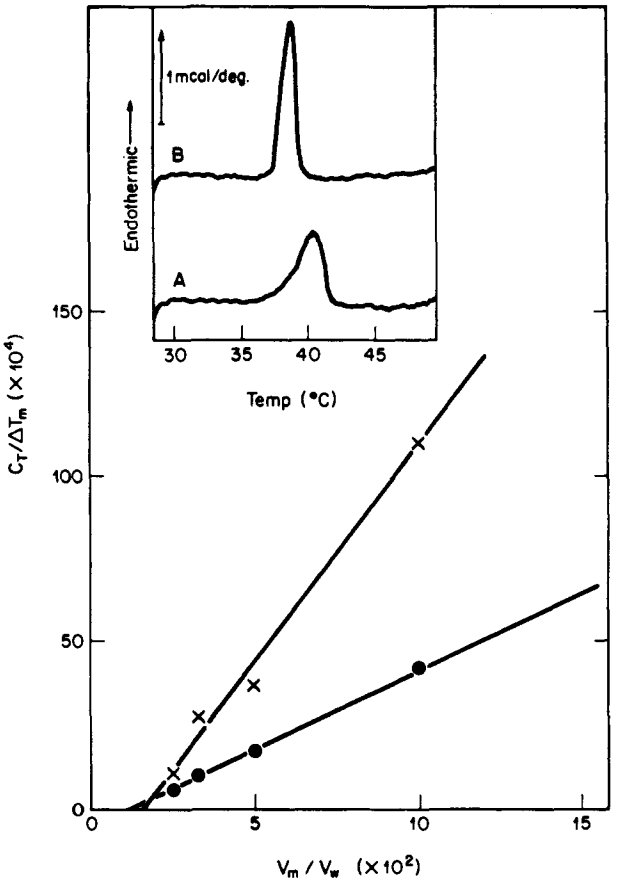

Figure 3. The change in $C_T/\Delta T_m$ as a function of V_m/V_w for sulindac sulfide at pH 4.5 (X) and at pH 10.0 (●). The lipid concentration was prepared at 100 mg/mL with a 5% mole ratio of sulindac sulfide. After the differential scans, the samples were diluted with the same buffer solution and equilibrated for at least 16 h at room temperature and then the thermal scans were repeated on the diluted samples. The volume of the membrane phase was calculated by assuming the density of the hydrated lecithin to be 1.014[32] and the density of the buffer solution to be 1.0. The McIlvaine's citric acid–phosphate buffer at pH 4.5 and sodium bicarbonate buffer at pH 10.0 were used as buffer solutions.[33] *Inset*: The thermal scan of DPPC with a 5% mole ratio of sulindac sulfide in DPPC liposome at a concentration of 25 mg DPPC/mL: A, thermal scan at pH 4.5; B, thermal scan at pH 10.0.

with pure DPPC, C_T is the aqueous concentration of drug assuming no partition loss, and P is the partition coefficient given by C_m/C_w, with C_w and C_m as the concentration of NSAID in the water and in the lipid phase, respectively. The experimental data give a straight line by graphing $C_T/\Delta T_m$ against V_m/V_w as shown in Figure 3. The slope is equal to C_m', and the partition coefficient P is calculated from the intercept. The results are listed in Table II. The pK of sulindac sulfide has been determined in our laboratory to be 4.7. Using eq 2 where C_{A^-W} and C_{AHW} are the

$$\frac{C_{A^-W}}{C_{AHW}} = \frac{[A^-]}{[AH]} = 10^{pH-pK} \quad (2)$$

concentration of charged and uncharged species in the aqueous solution respectively, at pH 10.0, the concentration of the uncharged species, [AH], is negligible and the effects on the thermotropic behavior of DPPC must be induced by the charged species. The partition coefficient of the charged species must be equal to that measured at pH 10.0 or as shown in eq 3, where C_{A^-M} is the concen-

$$P[A^-] = \frac{C_{A^-M}}{C_{A^-W}} = P(\text{pH } 10) = 72 \quad (3)$$

Table II. pH Dependence of the Partition Coefficient of Sulindac Sulfide and the Concentration Required to Depress 1 Degree

pH	C_m',[a] M	P[b]
10	4.692×10^{-2}	72.06
4.5	1.319×10^{-1}	60.06

[a] C_m' = concentration of sulindac sulfide to produce a 1-degree depression. [b] P = partition coefficient of sulindac sulfide.

tration of charged species in the membrane phase. The partition coefficient calculated here is very close to that obtained from the equilibrium dialysis technique[12] (see also Discussion). Similarily, the concentration of charged species required to produce a 1 degree depression must be equal to that at pH 10.0 (eq 4). In contrast, both charged

$$C_m'[A^-] = C_m'(\text{pH } 10.0) = 4.7 \times 10^{-2} \text{ M} \quad (4)$$

and uncharged species are present at pH 4.5. From eq 2 one gets eq 5 and the partition coefficient at pH 4.5 is as

$$\frac{[A^-]}{[AH]} = \frac{C_{A^-W}}{C_{AHW}} = 0.63 \quad (5)$$

shown in eq 6, where C_{AHM} is the concentration of un-

$$P(\text{pH} = 4.5) = \frac{C_{A^-M} + C_{AHM}}{C_{A^-W} + C_{AHW}} = 60 \quad (6)$$

charged species in the membrane phase. Through a manipulation of eq 4–6 we can calculate the partition coefficient of sulindac sulfide in the un-ionized form, P[AH], at pH 4.5

$$P[AH] = \frac{C_{AHM}}{C_{AHW}} = 52$$

Also, by assuming that both charged (A^-) and neutral species (AH) depress the phase transition temperature

$$C_M'\Delta T_M = C_{A^-M} + C_{AHM}$$

(ΔT_M = 1.3 °C from Figure 3, inset A), we obtained the depressed phase transition temperature due to the charged species, ΔT_{A^-M},

$$\Delta T_{A^-M} = \frac{C_{A^-M}}{C_m'(\text{pH } 10.0)} = 1.6 \text{ °C}$$

and the depressed transition temperature contributed by the neutral form of sulindac sulfide, ΔT_{AHM} = −0.3 °C. From the above data analysis, we conclude that the transition temperature depression in the presence of sulindac sulfide is mainly due to the charged species. However, the uncharged species has a more significant contribution to the broadening of the transition peak (see Figure 3 inset).

Hydrocarbon Chain Length Dependence of the Drug-Induced Thermal Behavior. The van der Waals interactions between acyl chains and, thus, the chain packing efficiency increase with the chain length of the phospholipid. Consequently, the phase transition temperature also increases with chain length. As demonstrated in Figure 4, sulindac sulfide (2) significantly depresses the phase transition temperature and broadens the transition peak of the DMPC liposomes in aqueous dispersion. On the other hand, in both cases of DPPC and DSPC, the transition peak is still pronounced even at 30 or 40% of sulindac sulfide concentration.

Nonrandom Distribution of NSAIDS in the Bilayer. Bilayers composed of phosphatidylcholines with fatty

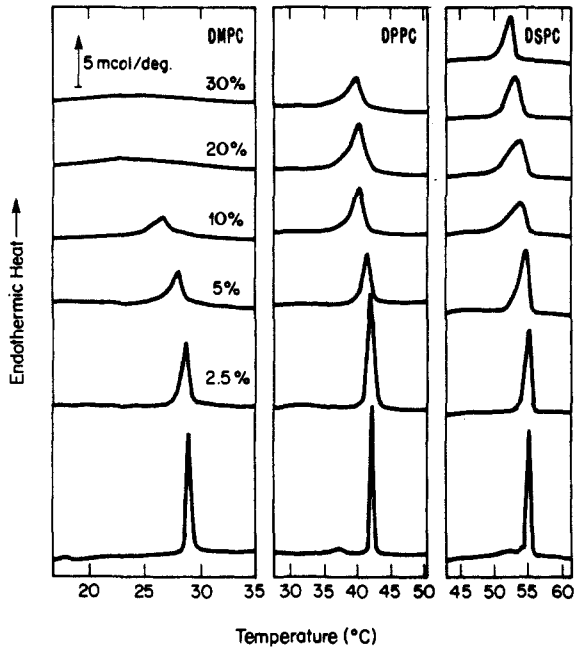

Figure 4. Differential scanning colorimetric curves of DMPC (left), DPPC (center), and DSPC (right) with and without sulindac sulfide. The sulindac sulfide concentration is varied from 0, 2.5, 5, 10, 20, 30, and 40% mole ratio (sulfide/DPPC) from bottom to top for each column of the figure, 50 mM phosphate buffer, pH 7.0, was used.

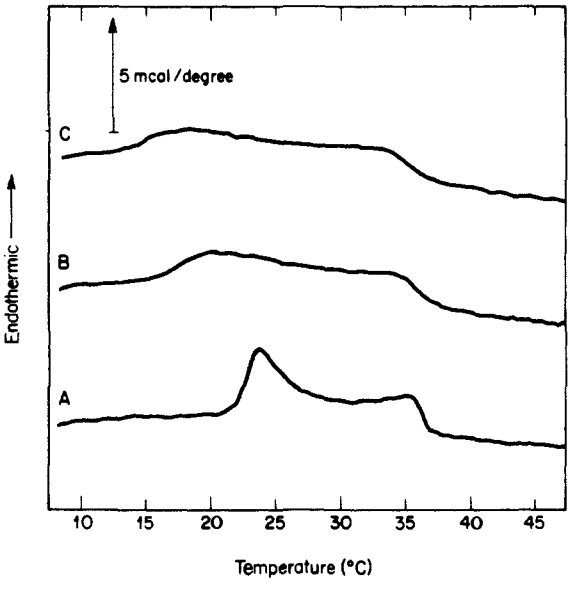

Figure 5. Differential scans of mixtures of DMPC and DSPC (50:50 weight ratio) without NSAIDS (A), with 10% indomethacin (B), and with 10% sulindac sulfide (C) [NSAIDS/(DMPC + DSPC) mole ratio] in 50 mM phosphate buffer, pH 7.0.

acid chains differing at least four carbon atoms show phase separation.[15,16] Figure 5 shows the thermal scans of a DMPC–DSPC mixture with and without NSAID. Without NSAID, the phase separation is indicated by two transitions (Figure 5A). However, in the presence of 10% indomethacin (4) (Figure 5B) and sulindac sulfide (2) (Figure 5C), the peak is significantly reduced and broadened at the lower temperature but not at the higher temperature. These results indicate that NSAIDS show a preference for the species with lower transition temperature in a phase-separated PC mixture.

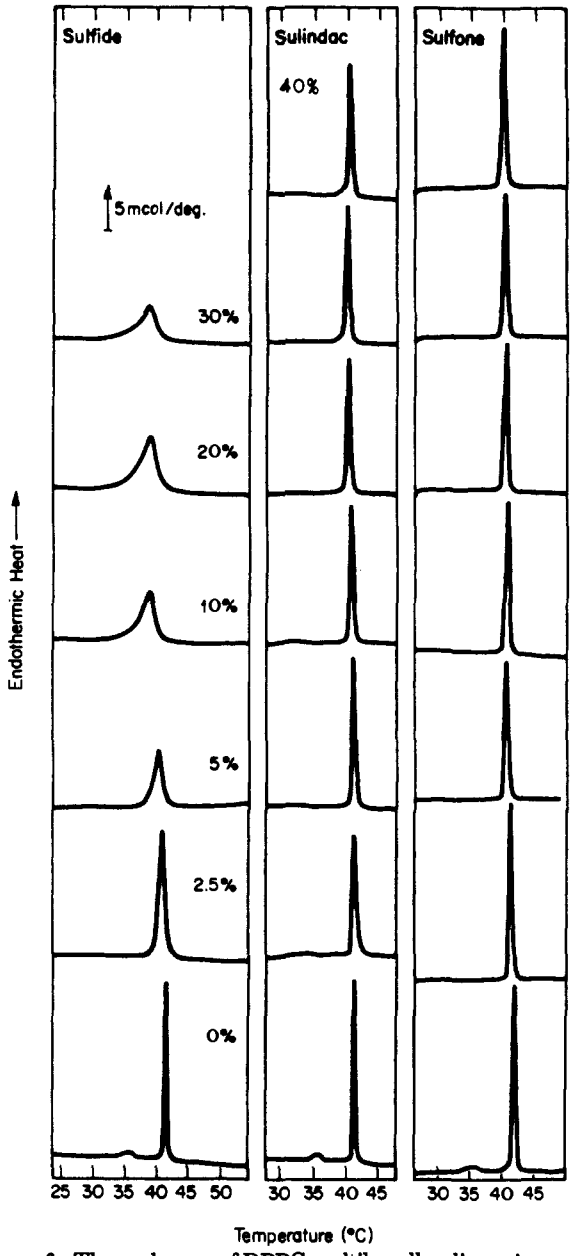

Figure 6. Thermal scans of DPPC multilamellar dispersions with various mole ratios of sulindac sulfide (left), sulindac (center), and sulindac sulfone (right column). The concentration of sulindac and its metabolites are (from bottom to top) 0, 2.5, 5, 10, 20, 30, and 40% mole ratios; 50 mM phosphate buffer, pH 7.0, was used.

Interaction of Inactive Derivatives of Antiinflammatory Drugs with the Bilayer. To investigate the specificity of drug interactions with the PC bilayer, two biologically inert compounds, sulindac (1) and sulindac sulfone (3), were also studied. Figure 6 shows the thermal scans of DPPC multilamellar dispersions with various mole ratios of 1–3. Unlike the sulfide analogue (2) and other active NSAIDS (see Figure 1), neither 1 nor 3 has any effect on the transition peak, which remains almost as sharp as that of the pure DPPC bilayer (Figure 7B). However, all three compounds induce similar reductions in the transition temperature (Figure 7A). This suggests that changes of the phase transition temperature induced by these compounds are nonspecific. On the other hand, the unique changes in the degree of cooperative interaction between lipid molecules induced by active drugs are

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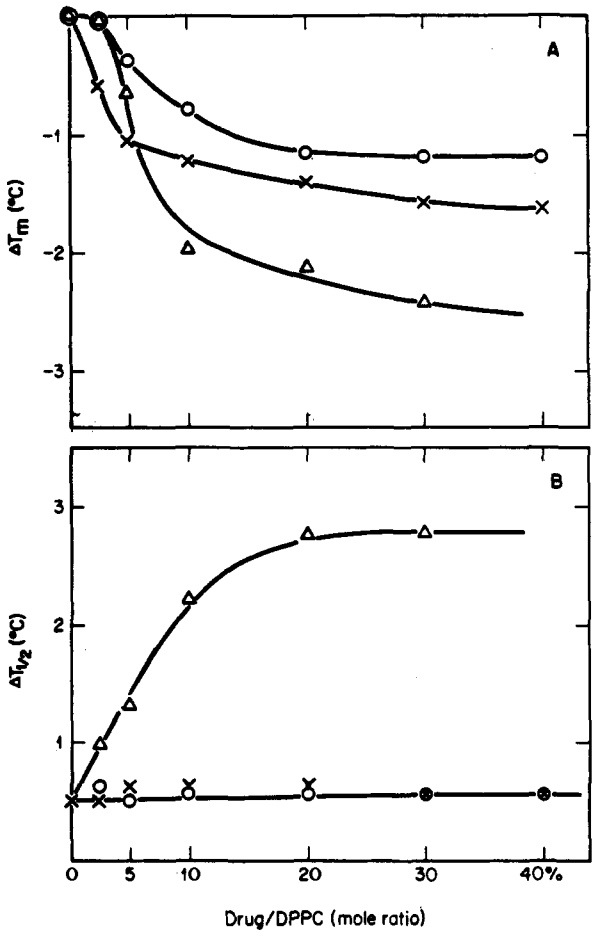
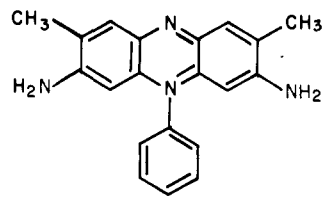


Figure 7. (A) Changes of the temperature at the phase transition peak as a function of sulindac (O), sulindac sulfone (X), and sulindac sulfide (Δ). (B) Changes of the half-width of the phase transition peak as a function of sulindac (O), sulindac sulfone (X), and sulindac sulfide (Δ).

probably important for the function of antiinflammatory agents.

Interaction of NSAIDS with Purple Membrane. The drug-induced dissociation of the bacteriorhodopsin lattice in purple membrane was monitored with changes of ellipticity of the negative CD band at 590 nm. Figure 8A shows a dose-dependent dissociation of the bacteriorhodopsin lattice. The ellipticity of the negative CD band decreases linearly with increasing concentration of antiinflammatory agents. The varying slopes correlate roughly with the antiinflammatory potency of these drugs. With the addition of sulindac sulfide at 20 μg/mL to a purple membrane suspension, the negative CD band is completely diminished (Figure 8B), indicating that the protein lattice is fully dissociated.

Interaction of Membrane Markers with the Bilayer. To localize the possible position of NSAIDS in the bilayer, several membrane reagents with known binding sites in the PC bilayer were employed as reference markers. Tetraphenylboron (TPB^-) interacts strongly with the choline head-group region of the PC molecules in the bilayer.[17,18] Hydrophobic ions, such as tetraphenylphosphonium, (TPP^+), triphenylmethylphosphonium ($TPMP^+$), and safranine O (7) appear to bind preferentially to the boundary region of the bilayer which is located



7 (safranine O)

beneath the level of the zwitterionic PC head group, near the carbonyl region of the bilayer but not at the membrane solution interface.[17,19] Nonbranched alkanes (hexane and decane) which partition readily into the hydrocarbon region of the bilayer[20-22] were also used.

Figure 9 shows the thermal scans of DPPC bilayers mixed with TPP^+, TPB^-, and hexane. The depressed phase transition temperature induced by these molecules are plotted in Figure 10A. Clearly all three types of molecules with different binding sites in the PC bilayer show specific features in DSC. TPP^+ produces only small effects on the transition temperature and cooperativity between phospholipid molecules during the transition. Even up to an 80% mole ratio of TPP^+ (Figure 9E), the half-width of the transition peak is about 0.6 °C, comparable to that of pure DPPC (about 0.4 °C). The pretransition is detectable but broadened and shifted to a lower temperature with increasing TPP^+ concentration. Similar results are obtained with the addition of safranine O (not shown). However, in the case of TPB^- (Figure 9F-J), the thermal behaviors are very different. At a 10% mole ratio (TPB^-/DPPC), distinct transition peaks are clearly temperature resolved with peaks at 33.4, 35.3, and 37.5 °C (Figure 9G). As the concentration of TPB^- is increased to a 20% mole ratio (TPB/DPPC), a stable TPB^-/DPPC complex is formed. The transition temperature of this stable complex is at 33.1 °C and the half-width of the transition peak is 0.4 °C, as narrow as pure DPPC (Figure 9H). At a higher concentration of TPB^-, the transition peak is broadened but the shift of the transition peak is minimized. This could result from a disruption of the bilayer structure, since the separation of some amorphous material from the solution was noticed at higher concentrations. The pretransition is diminished even at the lowest concentration (5% mole ratio) tested here.

Vaughan and Keough[23] have studied phase transitions of modified dipalmitoyl lipids and reported an inverse linear relationship with the extent of methylation of the choline head group. An extrapolation of the plot of the transition temperature vs. the number of head group methylations shows that the transition temperature of a stable DPPC-TPB^- complex corresponds with that of dipalmitoyl lipid with a tetramethyl head group (Figure 10B). This interesting result provides further evidence to support the conclusion that increased bulk (either by methylation or by complexing with TPB^-) in the head group of phospholipids allows for a decreased packing density of hydrocarbon chains and thus lowers the transition temperatures.[24]

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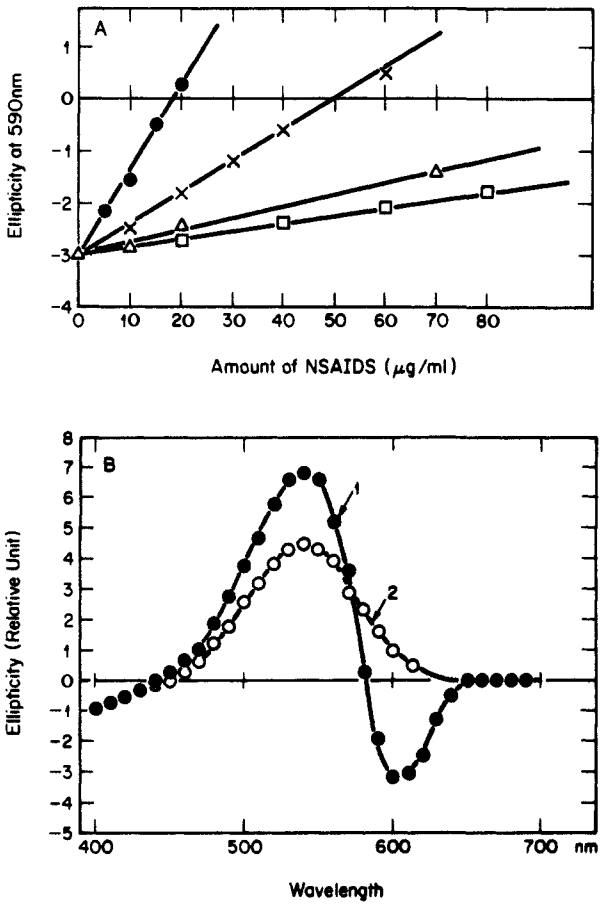

Figure 8. (A) NSAIDS-induced changes on ellipticity of the negative CD band at 590 nm of purple membrane at a concentration of 0.35 mg of bacteriorhodopsin/mL in pH 4.0 buffer: (●) with sulindac sulfide; (X) with indomethacin; (△) with sulindac sulfone; (□) with sulindac. (B) CD spectra of purple membrane (0.35 mg of bacteriorhodopsin/mL) without (1) and with (2) 20 μg of sulindac sulfide/mL, pH 4.0.

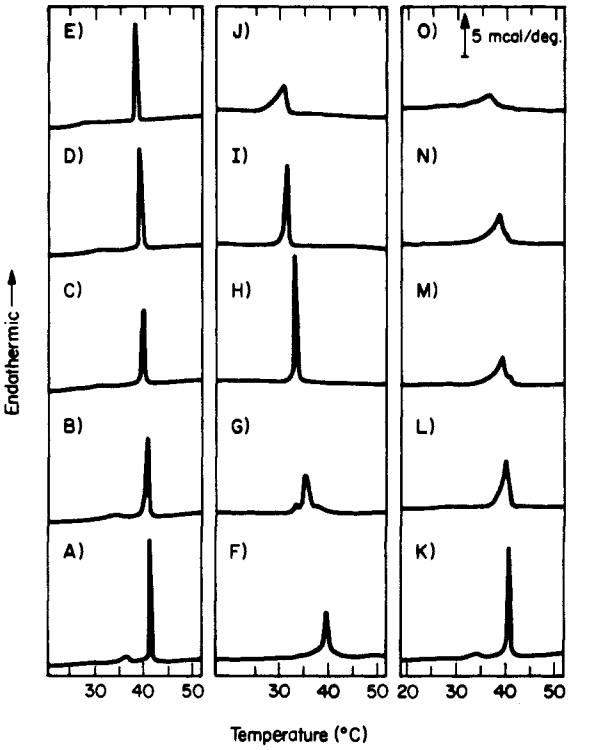

Figure 9. Differential thermal scans of pure DPPC liposome (A) and DPPC liposome mixed with TPP^+ (B–E), TPB^- (F–J), and hexane (K–O) at 5 (TPP^+/DPPC) (B), 20 (TPP^+/DPPC) (C), 40 (TPP^+/DPPC) (D), 80 (TPP^+/DPPC) (E), 5 (TPB^-/DPPC) (F), 10 (TPB^-/DPPC) (G), 20 (TPB^-/DPPC) (H), 40 (TPB^-/DPPC) (I), 80 (TPB^-/DPPC) (J), 25 (hexane/DPPC) (K), 50 (hexane/DPPC) (L), 100 (hexane/DPPC) (M), 150 (hexane/DPPC) (N), and 200% mole ratio (hexane/DPPC) (O); 50 mM phosphate buffer, pH 7.0, was used.

For hexane which partitions deeply into the center region of the bilayer, the transition peak is significantly broadened as the concentration is increased (Figure 9K–O). It also causes a decrease in the transition temperature which is smaller than that of TPB^- and is comparable to that of TPP^+. The pretransition is also abolished except at a concentration of 25% mole ratio (Figure 9K). The shoulder near the transition temperature of pure DPPC may be due to an imperfect mixing of DPPC with hexane, because the hexane was added after the DPPC multilamellar dispersions were formed. Similar results with broadened transition peaks were obtained with decane and cholest-5-ene, which are also principally located in the middle of the bilayer (not shown).

In contrast to that of TPP^+, the presence of triphenylmethylphosphonium ($TPMP^+$) induces, as shown in Figure 11, two transition peaks which are clearly temperature resolved between 40 and 80% mole ratio ($TPMP^+$/DPPC). This suggests that some interaction of $TPMP^+$ with phosphate in the head-group region may occur at high concentration even though $TPMP^+$ has long been assumed to be localized in the boundary region. Some nuclear magnetic resonance results[17] are in accordance with this interpretation.

Discussion

We have studied the interaction of several antiinflammatory agents with a phospholipid model system by DSC and employed membrane markers to estimate their possible locations in the bilayer. These membrane markers are comparable to NSAIDS in molecular weight, and their specific locations in the PC bilayer are known. Fortunately, we found that they also induce specific changes of the thermotropic behavior of bilayer membranes which can be correlated with their mode of interaction with the PC molecule. Thus, we were able to use DSC as a convenient method to investigate the membrane interaction of NSAIDS. A comparison of DSC curves of phospholipids interacting with membrane markers and NSAIDS shows that only a small decrease in the phase transition temperature (<3 °C) is induced by inactive structures, e.g., the prodrug sulindac and its sulfone metabolite, and the transition peak remains about as narrow as pure PC. These are similar to those induced by TPP^+ and safranine O, which bind preferentially to the boundary region of the bilayer. In contrast, DSC curves of PC liposomes interacting with active antiinflammatory agents, e.g., the active sulfide metabolite of sulindac, indomethacin, diflunisal, and flurbiprofen, all show a broadened transition peak of PC liposomes. Similar DSC curves are induced by hexane or decane, which partitions into the inner hydrocarbon region of the bilayer. These results suggest that all active drugs investigated can insert deeply into the hydrocarbon region, whereas two inactive structures (1 and 3) interact only with the boundary region or carbonyl group in the PC bilayer and are unable to partition into the hydrocarbon region of the phospholipid bilayers.

This conclusion is corroborated by results from NMR studies,[25] from studies using freeze–fracture technique,[12] by the nonrandom distribution of NSAIDS in a mixture

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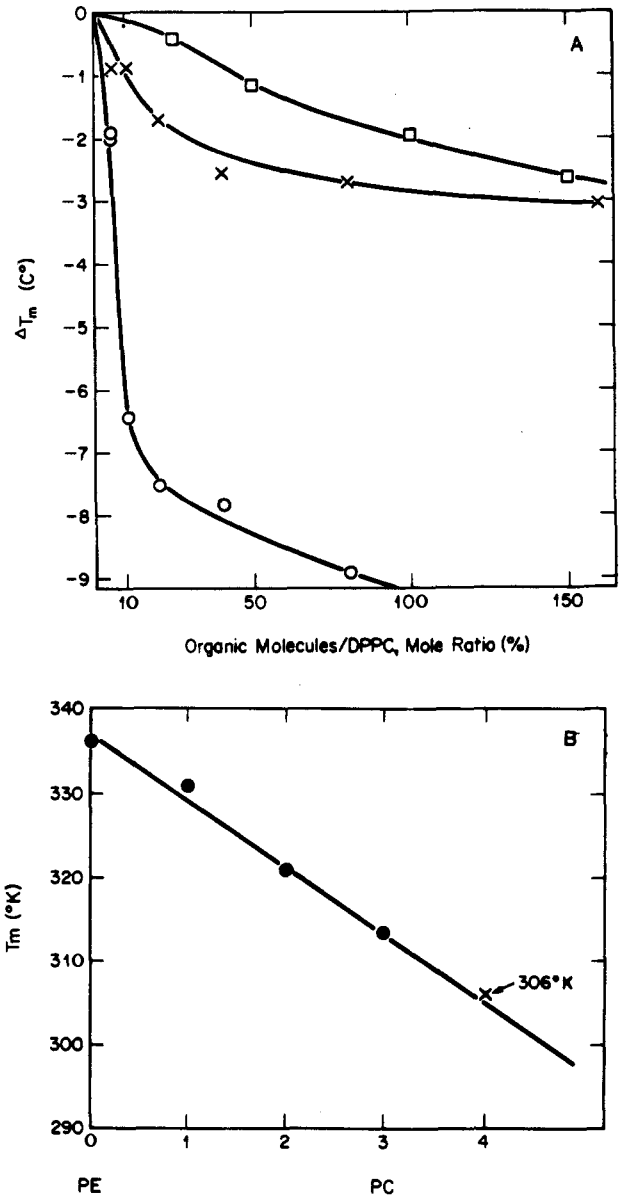

Figure 10. (A) Changes of temperature at the maximum transition peak of DPPC liposomes induced by TPP^+ (X), TPB (O), and hexane (□); 50 mM phosphate buffer, pH 7.0, was used. (B) Variation of the phase transition temperature of dipalmitoyl lipids with different degrees of methylation in the nitrogen of the head group. The data points for dipalmitoyl-, *N*-methyldipalmitoyl-[(NCH_3)DPPE], and *N,N*-dimethyldipalmitoyl phosphatidylethanolamine [$NN(CH_3)_2$DPPE] and DPPC were taken from Figure 2 of Vaughn and Keough[22] and the point (X) at 306 K was the temperature transition peak of a 20% mole mixture of TPB^- to DPPC.

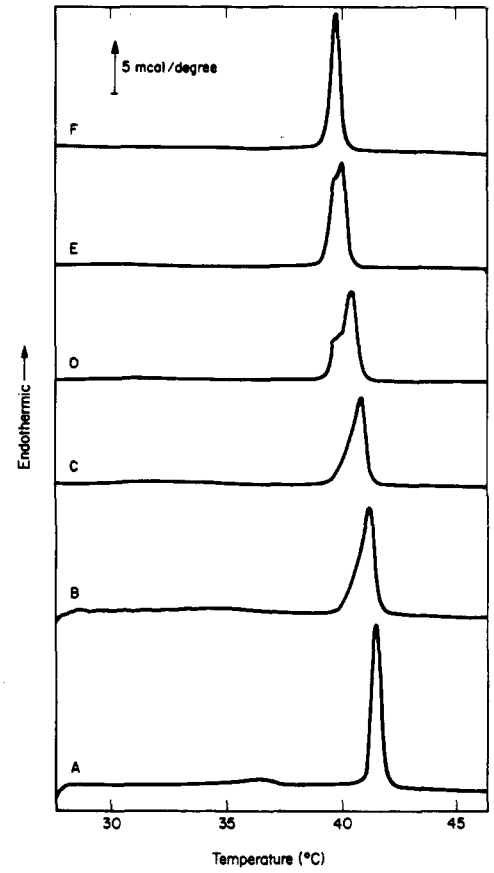

Figure 11. Differential scanning calorimetric curves of DPPC liposomes with $TPMP^+$ at a 0 (A), 10 (B), 20 (C), 40 (D), 80 (E), and 160% (F) mole ratio ($TPMP^+$/DPPC) in 50 mM phosphate buffer, pH 7.0.

of PC with different hydrocarbon chain length, and by their efficiency in dissociating a membrane protein, the bacteriorhodopsin lattice in an isolated purple membrane reported here. The tendency of antiinflammatory agents to interact more strongly with DMPC than DSPC in a bilayer mixture further indicates that they can reach and sense fluidity difference in the inner region of hydrocarbon chains.

Many NSAIDS are acidic molecules which dissociate into carboxylic anions at neutral pH. Our measurements at different pHs indicate that both charged and uncharged species of NSAIDS can broaden the phase transition peak. Regarding the molecular orientation of NSAIDS in the bilayer, it would seem unreasonable to place an ionized carboxyl group deep in the center of the bilayer because of the large Born free energy induced by the charge group localized in the low dielectric constant region.[26] Instead, one may visualize a NSAID molecule interdigitating between the hydrocarbon chains of the phospholipid with its charged group facing the aqueous solution. Such an orientation is also favored by our NMR experiments.[25]

The reasons for such a distinct differentiation of the thermotropic behavior of phospholipid iiposomes induced by these compounds are not only because active drugs and inactive analogues interact with different parts of the phospholipid molecules in the bilayer, but also because active drugs can effectively partition into the membrane phase. Using an equilibrium dialysis technique with radioactive compounds, more than 90% of the added sulindac sulfide partitions into the membrane phase, but only 15% of the prodrug sulindac binds to the membrane phase under the experimental conditions used here for DSC studies.[12] The partition coefficient of sulindac sulfide calculated is about 71, whereas for the prodrug sulindac it is only about 1.8. Therefore, the active metabolite sulfide interacts with the phospholipid bilayer much more strongly than the prodrug sulindac. Furthermore, the partition coefficient calculated from equilibrium dialysis data agrees very well with that obtained from Hill plots reported here. Since the gel to liquid-crystal transition of PC liposomes is a true isothermal first-order transition,[27] the use of Hill plots to calculate partition coefficients and the concentrations required to depress 1 degree of the DPPC phase transition temperature induced by charged and uncharged

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species of sulindac sulfide seems appropriate.

The high concentration of NSAIDS used here was necessitated by the low sensitivity of the DSC-2 instrument. As shown in Figure 1, marked changes of the transition peak were observed at a 2.5–5% mole ratio of active antiinflammatory drugs (corresponding approximately to 3.4–6.8 mM). These changes increased in a dose-related manner up to 20–30% mole ratios. At lower concentrations only a significant depression of the enthalpy change of the DPPC pretransition was detectable at a 0.1% mole ratio.[12]

Interestingly, some significant differences in the membrane behavior of an active drug and an inactive prodrug became observable at much lower concentrations once a well-characterized membrane protein, bacteriorhodopsin, was incorporated into the phospholipid bilayer. Like many membrane proteins, bacteriorhodopsin has a coat of hydrophobic amino acids which forms an interface with the outside lipid environment and shields the charged and hydrophilic groups inside.[28] Conceivably, the hydrophobic antiinflammatory drugs may partition into the lipophilic interface to interact with the membrane protein. Indeed, it was found that active antiinflammatory drugs are able to dissociate the bacteriorhodopsin lattice very efficiently even at low concentrations (>20 μg/mL). It has been reported that the bacteriorhodopsin lattice remains intact even after more than 80% of the original phospholipid was removed with an ionic detergent,[29] presumably due to strong protein–protein interactions. In general, the dissociation of the bacteriorhodopsin lattice can only be induced by drastic structural changes, such as detergent solubilization or removal of the chromophore.[11] Thus, it is of interest to note that the addition of active antiinflammatory drugs affords a simple and gentle way to dissociate the bacteriorhodopsin lattice. Under this condition, the purple membrane is not destroyed and remains in the membrane sheet form as seen in the electron microscope. It shows a similar absorption spectrum and an equivalent photocyclic time as monitored by the photo-induced transient decay of the photointermediate M_{412}.[12] On the other hand, the inactive sulindac and sulfone metabolite do not show any significant effect up to very high concentrations, even though they can bind human serum albumin as strongly as many active NSAIDS.[6] The aqueous solubility of indomethacin, sulindac sulfide, sulindac, and the sulfone metabolite at pH 7 are ca. 0.4, 0.03, 2.7, and 0.5 mg/mL, respectively. Obviously, physical parameters measured in a solution phase related to protein binding and hydrophobicity do not have any simple correlation with activities of compounds in a phospholipid membrane. Further understanding of factors influencing the behavior of a drug molecule in a membrane environment is obviously needed to facilitate the future design of selective membrane regulators.

Finally, the membrane reagents used here are not only useful in localizing drugs in the PC bilayer but also valuable in understanding the endothermic properties of the multilamellar phospholipid liposomes. The combination of a TPB^- molecule with the phosphorylcholine head group substantially expands the head-group region in the bilayer, prevents full utilization of the van der Waals attraction between the hydrocarbon chains of the lipids, and decreases the transition temperature of DPPC. In other words, an increase of crowding in the head-group region is an effective way to lower the phase transition temperature of the bilayer. One may also conclude that, since only molecules interacting with the hydrocarbon chain region of the phospholipid can broaden the transition peak or reduce the cooperative phenomena of the phospholipid bilayer, the cooperativity between phospholipid molecules during the phase transition may mainly depend on the extent of the interaction between the hydrocarbon chains but not the other part of phospholipid molecules.

In conclusion, the probable localization of several NSAIDS and their inactive congeners in phospholipid bilayers has been elucidated by the DSC technique. In agreement with results from NMR and ESR studies, all active NSAIDS studied are able to partition deeply into the bilayer. It was also found that potent NSAIDS can interact directly with a model membrane protein and dissociate the subunits effectively at low drug concentrations. The prodrug sulindac and its inactive sulfone metabolite interact only with the boundary region of the bilayer and are devoid of any significant membrane effects. These methods are potentially useful in characterizing the specific mode of interaction of other membrane regulators.

Experimental Section

Materials. Purple membranes from *H. halobium* R_1 were isolated and purified according to Oesterhelt and Stoeckenius.[30] The bacteriorhodopsin concentrations in the purple membrane phase were determined from the absorbance at 560 nm, assuming a molar extinction coefficient for dark-adapted bacteriorhodopsin of 51 000.[11] The purified fragments were stored at 4 °C in basal salt solution. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC) were obtained from Calbiochem. The homogeneity was checked by thin-layer chromatogrphy, and the compounds were used without further purification. The purity of the phospholipids used was also checked by their expected DSC curves. They all showed sharp transition peaks at the expected transition temperature (see Results). Sulindac and its metabolites (sulindac sulfide and sulindac sulfone), indomethacin, diflunisal, and flurbiprofen were previously synthesized in our laboratories. Glass-distilled hexane and chloroform were obtained from Burdick and Jackson Laboratories Inc. (Muskegon, MI) and used as received. Tetraphenylphosphonium (TPP^+) bromide was purchased from Alfa Products (Thiokol/Ventron Division, Danver, MA), triphenylmethylphosphonium ($TPMP^+$) bromide and cholest-5-ene were from Pfaltz and Bauer, Inc. (Stamford, CT), tetraphenylboron (TPB^-) sodium salt was from Sigma Chemical Co. (St. Louis, MO), and safranine O was from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Methods. Preparation of Liposomes with NSAIDS and Small Organic Molecules. PC was dissolved in glass-distilled chloroform (50 mg/mL), NSAIDS were in chloroform/methanol (1:1) (10 mg/mL), and TPP^+, $TPMP^+$, safranine O and TPB^-, were in ethanol (10 mg/mL). Various mole ratios of agents to phospholipid were mixed, and the organic solvents were removed by running a stream of nitrogen over the samples to form a thin film and then under vacuum overnight to remove any residual organic solvents. To the dried film, prepared solution was added to give a final PC concentration of 100 mg/mL unless otherwise specified. The mixture was then heated to 15 °C above the phase transition temperature of the phospholipid, and the multilamellar phospholipid liposomes, with or without agents in the aqueous suspensions, were prepared by vigorously shaking with a vortex mixer (Model S8220, Scientific Products) at the highest speed for about 1 min. The heating and shaking processes were repeated several times. Since liposomes may change with time during storage, only the freshly prepared batches were used. The volatile hexane and decane were added right after the liposomes were prepared.

Differential Scanning Calorimetric Measurements. The endothermic transition curves of the phospholipid liposomes, in

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the presence or absence of agents in the aqueous phase, were recorded on a differential scanning calorimetric (Model DSC-2, Perkin Elmer) at a heating rate of 2.5 °C/min. A 10-μL sample (1 mg of phospholipid) was appled and encapsulated in an aluminum sample pan. Both abscissa (temperature) and ordinate (transition energy) were calibrated with indium and lead as standards. The calorimetric enthalpy changes (ΔH_{cal}) were measured from the area under the peak. The standard van't Hoff enthalpy changes (ΔH_{vH}) were calculated by eq 7 where T_m is the

$$H_{vH} = 6.9 \frac{T_m^2}{\Delta T_{1/2}} \quad (7)$$

phase transition of the phospholipid and $\Delta T_{1/2}$ is the half-width of the transition peak, and the cooperativity of the transition process of the phospholipid liposome was followed by the definition of Hinz and Sturtevant (eq 8).[31]

$$n = \frac{\Delta H_{vH}}{\Delta H_{cal}} \quad (8)$$

Circular Dichroism Measurements. A Jasco Model ORD/CD5 with Sproul Scientific SS 10 modification was used for circular dichroism (CD) measurments. To minimize the scattering effect, the cell was positioned as close as possible to the photomultiplier.

Acknowledgment. We thank Dr. T. R. Beattie and Ms. C. S. Tripp for initiating this DSC work, to Dr. W. Stoeckenius for providing purple membrane and laboratory facilities to carry out some experiments in this study, and to Drs. J. M. Sturtevant, P. Kroon, J. C. Robbins, and T. W. Doebber for helpful discussions.

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β-Adrenergic Blocking Agents. α- and γ-Methyl(aryloxy)propanolamines

Thomas L. Lemke,* Michael B. Cramer, Stanley W. Adamski,

College of Pharmacy, University of Houston, Houston, Texas 77004

Carolyn A. Pedone, and Gary Brooker

Department of Pharmacology, School of Medicine, University of Virginia, Charlottesville, Virginia 22908.
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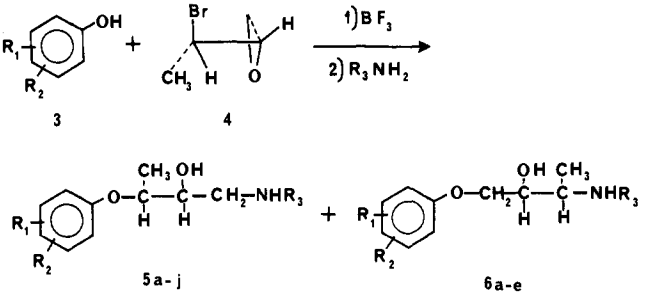
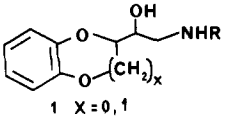
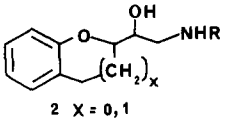
A series of γ-methyl- (5) and α-methyl(aryloxy)propanolamines (6) were synthesized and evaluated for β-adrenergic blocking action. The binding constant (K_D) was determined for compounds **5a–j** on cultured C6-2B astrocytoma cells. Compounds **5a–j** and **6a–e** were evaluated for β_1-antagonist action on guinea pig atria and β_2-antagonist action on guinea pig tracheal strips. Several γ-methyl(aryloxy)propanolamines showed affinity for β receptors and a possible preference for β_2 receptors.

The realization that β-adrenoceptors are not homogeneous but instead can be subdivided into at least two subclasses (β_1 and β_2) has resulted in the publication of numerous articles dealing with β_1- and β_2-adrenoceptor agonists and antagonists. Several years ago we were asked to prepare a series of α-methyl(aryloxy)propanolamines which were under active consideration as β_2 selective antagonists.[1] Since then, studies have shown that introduction of a methyl group into the α position of the nonselective β-adrenoceptor antagonist propranolol does enhance the selectivity of propranolol toward the β_2-adrenoceptors.[2,3] While investigating a general synthetic method for synthesis of the α-methyl(aryloxy)propanolamines, we discovered a novel method for preparing γ-methyl(aryloxy)propanolamines.[4] The only γ-methyl(aryloxy)propanolamine that had been reported previous to this was the erythro/threo mixture of propranolol (**5i**) reported by Howe.[5] While Howe's study suggested that introduction of the methyl group into the side chain gave compounds which are less potent antagonists, the study only considered the effects of the compounds on the heart and, thus, β_1 adrenoceptors. Subsequent studies by Howe and co-workers[6,7] have shown that semirigid γ-alkyl(aryloxy)propanolamines (**1** and **2**) are very potent β_1-adrenoceptor antagonists. We now report our studies directed toward elucidating the structural relationship of

Scheme I







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