procedure used has been described previously.[3,5,15] Calf uterine cytosol (final concentration of protein 5 mg/mL), the dextran-coated charcoal (DCC) method, and the plotting method according to Lineweaver and Burk[16] were applied.

Estrogen and Antiestrogen Assays. Estrogenic and antiestrogenic activities were determined by stimulation of the uterine growth[17] and the inhibition of the uterine growth stimulated by estrone,[18] respectively, using immature NMRI mice as described previously.[3,5] Twenty-day-old female mice (weight 14.5 ± 1.2 g, mean ± SD) were randomly distributed into groups of 10 animals. They were subcutaneously injected daily for 3 days with 0.1 mL of olive oil solutions containing the test compound. The uteri were removed 24 h after the last injection. Furthermore, the estrogenic activity was assayed using the vaginal cornification test. The methods applied were similar to that described in the literature.[9] Female NMRI mice weighing about 15 g were ovariectomized. The completeness of castration was checked 10 days after ovariectomy (mainly leucocytes). The animals were primed with a single injection of estradiol 3-benzoate (0.2 μg). Only animals responding with vaginal estrus (mainly cornified cells) were used. The animals were randomly distributed into groups of three and were subcutaneously injected daily for 3 days with 0.1 mL of the test compound dissolved in olive oil. Vaginal smears were taken 6, 24, 30, 48, and 54 h after the last injection by flushing the vagina with 10 μL of physiological saline. The smears were evaluated according to the conventional Allen–Doisy test.[19]

Breast Tumor Cell Proliferation Inhibition Test. A rapidly growing culture of MCF-7 cells which had been cultivated in McCoy's 5a medium supplemented with fetal bovine serum was used. The serum had been stripped of endogenous estrogens by treatment with DCC for 0.5 h at 45 °C. The cells were washed with phosphate-buffered saline (PBS) and were dispersed with 0.25% trypsin–EDTA solution, counted, and diluted to 10^6 cells/mL in serum-free medium. Test compounds were dissolved in ethanol and serially diluted with complete medium to obtain the appropriate concentrations. The final concentration of ethanol in the assay plates did not exceed 0.5%. The cell culture medium containing the appropriate concentration of test compound was inoculated with cell suspension, yielding 10^4 cells/mL. This mixture was placed into 60-mm culture plates at 5 mL/plate, yielding a final inoculum of 5×10^4 cells/plate. On days 3, 5, 7, 10, and 13, three replicate plates for each test or control group were washed with PBS, and the cells were dispersed with trypsin–EDTA and were counted on the Coulter counter, three counts per plate. In a second experiment the compounds were retested at an inhibitory concentration of 10^{-7} M without and with estradiol (10^{-7} and 10^{-8} M) added. The same procedure was used as described above.

Mammary Tumor Growth Inhibition Test. The methods used have been described previously.[3,5] The tumor-inhibiting effect was determined using the DMBA-induced, hormone-dependent mammary adenocarcinoma of the SD rat. Animals bearing at least one tumor greater than 140 mm^2 were classified in groups of ten. Compounds were dissolved in olive oil and applied sc. Measurement of tumor size and determination of body weight were made twice weekly. The therapy was continued for 28 days.

Acknowledgment. Thanks are due to the Deutsche Forschungsgemeinschaft and to the Verband der Chemischen Industrie, Fonds der Chemischen Industrie, who supported this work by grants.

(15) S. G. Korenman, *J. Clin. Endocrinol. Metab.*, **28**, 127 (1968).
(16) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
(17) B. L. Rubin, A. S. Dorfman, L. Black, and R. J. Dorfman, *Endocrinology*, **49**, 429 (1951).
(18) R. A. Edgren and D. W. Calhoun, *Proc. Soc. Exp. Biol. Med.*, **94**, 537 (1957).
(19) E. Allen and E. A. Doisy, *J. Am. Med. Assoc.*, **81**, 819 (1923).

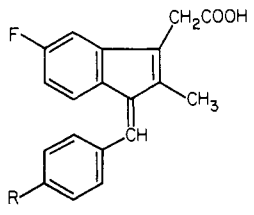
Membrane Effects of Antiinflammatory Agents. 1. Interaction of Sulindac and Its Metabolites with Phospholipid Membrane, a Magnetic Resonance Study

Sophie S. Fan* and T. Y. Shen

Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065. *Received October 22, 1980*

High-resolution proton NMR and spin-label ESR spectroscopies have been applied to examine the interaction of the nonsteroidal antiinflammatory drug sulindac (1) and its active sulfide metabolite (2) and inactive sulfone metabolite (3) with phospholipid membranes. Only weak interactions were observed with 1 and 3, but a strong interaction with 2 was indicated both by specific changes in the proton transverse relaxation rate ($1/T_2$) of different substituents in 2 and by a unique shift in membrane transition temperature in the presence of 2 as measured by the ESR technique. Since the structural differences of these compounds are confined to a single polar substituent, i.e., the oxidation state of the sulfur atom, the strong interaction of the sulfide metabolite (2) with the neutral phospholipid membrane is ascribed to its high partition coefficient in the lipid membrane and its ability to penetrate into the lipid bilayer with the carboxyl group remaining at the polar membrane surface. As evidenced from the ESR spectra of two spin-labels, C_5- and C_{12}-doxylstearic acid, no significant change of the membrane fluidity was induced by the interaction of 2 with phospholipid vesicles.

The antiinflammatory agent sulindac (1) is a new type



1 (sulindac), R = S(=O)CH_3
2 (active metabolite), R = SCH_3
3 (inactive metabolite), R = S(=O)$_2CH_3$

of reversible prodrug.[1] In vivo it is reversibly reduced to an active sulfide metabolite (2) and irreversibly oxidized to an inactive sulfone metabolite (3). Like many antiinflammatory agents, sulindac sulfide (2) inhibits the biosynthesis of prostaglandins by a membrane-associated enzyme, the arachidonic acid cyclooxygenase.[2] Since many inflammatory processes are cell-surface phenomena, the possible effects of these structures on model membrane systems were investigated in our laboratories.[3]

(1) T. Y. Shen, *Handb. Exp. Pharmacol.*, **50**(2), 305–347 (1978).
(2) G. J. Roth, N. Stanford, and P. W. Majerus, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3073 (1975).
(3) A preliminary communication of this study has been presented. See Sophie S. Fan, M. Driscoll, and T. Y. Shen, in "Abstracts of Papers", 179th National Meeting of the American Chemical Society, Houston, TX, Mar 23–28, 1980, American Chemical Society, washington, DC, 1980, Abstr MEDI 039.

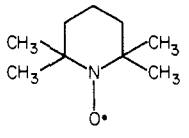
0022-2623/81/1824-1197$01.25/0

Table I. Incorporation of Sulindac (1) and Its Active Sulfide Metabolite (2) in DOPC Vesicles, Measured at Room Temperature[a]

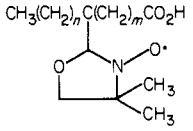
drug/lipid mol ratio	% in vesicles: sulindac sulfide	% in vesicles: sulindac
0.10 (cosonication)	74	7
2 (cosonication)	41	5
3.5 (mixing)	25	

[a] Results are an average of at least two measurements.

Sulindac and its metabolites are amphiphilic compounds which possess identical stereochemical configuration, differing only in the oxidation state of the sulfur atom. However, the polarity of the substituent, CH_3S vs. $CH_3S \rightarrow O$ vs. CH_3SO_2, varies considerably. Marked differences in aqueous solubility and lipid/water partition of these compounds have also been observed.[3] The model membrane systems used in this study include ultrasonically dispersed vesicles and unsonicated multilamellar liposomes of dioleoyl-L-α-phosphatidylcholine (DOPC) and dipalmitoyl-L-α-phosphatidylcholine (DPPC). The transition temperature of DOPC and DPPC are −22 and 41–42 °C, respectively. Using two model membranes with different transition temperatures, we could correlate the spectral change with the membrane physical parameters more accurately. High-resolution proton magnetic resonance spectroscopy was used to examine the mode of interaction of these compounds with the model membranes. This method has previously been used to study the location of small fluorescence probes in phospholipid vesicles.[4] Analogous to the NMR analysis of substrate–protein interaction, transverse relaxation rate of drug molecules was used to characterize the membrane binding of these compounds. The pertubation of the motional state of the lipid bilayer induced by drug interaction was monitored by three spin-label probes, i.e., 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo, 4)[5] and C_5- and C_{12}-doxylstearic acid (5 and 6).[6,7] Data from these two different approaches complement each other regarding the mode of membrane interaction of sulindac and its metabolites.



4



5, $n = 12$; $m = 3$
6, $n = 5$; $m = 10$

Results and Discussion

Interaction of Drug Molecules with DOPC Vesicles. The interaction of sulindac (1) and its sulfide metabolite (2) with DOPC vesicles was measured with radiolabeled samples by two different procedures. In the first experiment, [^{14}C]sulindac and [^{3}H]sulfide were mixed separately with sonicated DOPC vesicles. In the second experiment, labeled 1 and 2 were cosonicated separately with DOPC to form vesicles. In each case the free drug molecules were separated by Bio-Gel chromatography, and the radioactivity associated with the vesicles were determined. It should be pointed out that the slight dilution of the aqueous phase during gel filtration might have lowered the membrane drug concentration to a small extent. Therefore, the percentages of drug incorporation in DOPC vesicles shown in Table I are probably somewhat less than the actual values. Nevertheless, it seems clear that only th sulfide 2 can partition into the vesicles in large quantity. The extent of incorporation is dependent upon the ratio of drug/lipid concentration, approaching a maximum of 1:1 drug/lipid mixture in the vesicle. Interestingly, as shown in freeze–fracture electron micrographs,[8] the bilayer membrane structure of the vesicles remains largely intact even at such a high concentration of 2 in the vesicle.

Table II. Slopes of the Aliphatic Protons of Sulindac Sulfide as Calculated from Figure 2

protons	slope,[a] s^{-1} mM^{-1} vesicle
CH_2	6.0×10^4
CH_3	2.9×10^4
SCH_3	1.1×10^4

[a] The definition of this slope is expressed in eq 4.

^{1}H NMR of Drug Interaction with DOPC Vesicles. The interaction between sulindac and its metabolites with DOPC vesicles was investigated by the change of their proton NMR signals as shown in Figure 1a–c. It is noticed that CH_3SO_2 protons of 3 are labile and exchanging rapidly with the deuterium in D_2O solution under alkaline conditions (Figure 1c). While the aliphatic protons of all three compounds show clearly separated resonances for each group, the aromatic proton resonances are less well resolved. For simplicity, the resonance peaks of the aliphatic protons in the methylene and two methyl groups are used as selective markers. Since these groups are located at different positions of the molecule, the individual linewidth changes are indicative of the changes in the microenvironment inside the lipid bilayer.

The proton NMR spectra of the three compounds when mixed with dioleoylphosphatidylcholine vesicles are shown in Figure 1d–f. The signal of the CH_3SO_2 protons in Figure 1f is totally diminished by deuterium exchange. At a maximum drug/lipid concentration ratio of 3, only signals of sulindac sulfide (2) show obviously large line-width changes. The changes in 1 and 3 are much less. Since no chemical-shift changes were observed under our experimental conditions, the contribution of chemical-shift differences between free and bound compounds 1 to 3 can be ignored. The changes in the transverse relaxation rate ($1/T_2$) of the various protons of 2 as a function of the vesicle concentrations are shown in Figure 2. A linear relationship between the relaxation rate and the vesicle concentration is clearly shown to fit eq 4 (see Experimental Section). The slopes obtained from Figure 2 are listed in Table II.

The distinct values of the slope related to various protons in the same molecule indicate a highly specific interaction of 2 with the membrane, which are not observed in the control experiments (see Experimental Section). In eq 4, K_d, S_0, n and τ are all constants for various spin systems within one molecule; therefore, the difference in slope is directly related to the difference in the relaxation rate of drug protons when bound to the vesicles, $1/T_{2b}$.

(4) R. W. Barker, K. Barrett-Bee, J. A. Berden, C. E. McColl, and G. K. Radda, in "Dynamics of Energy-Transducing Membranes", L. Ernster, R. W. Estabrook, and E. C. Slater, Eds., Elsevier, Amsterdam, 1974.
(5) H. M. McConnell, *Biochem. Biophys. Res. Commun.*, 47, 273 (1972).
(6) W. L. Hubbell and H. M. McConnell, *J. Am. Chem. Soc.*, 93, 314 (1971).
(7) B. J. Gaffney, in "Spin Labeling—Theory and Applications", Lawrence J. Borliner, Ed., Academic Press, New York, 1974, p 567.
(8) S. B. Hwang and T. Y. Shen, following paper in this issue.

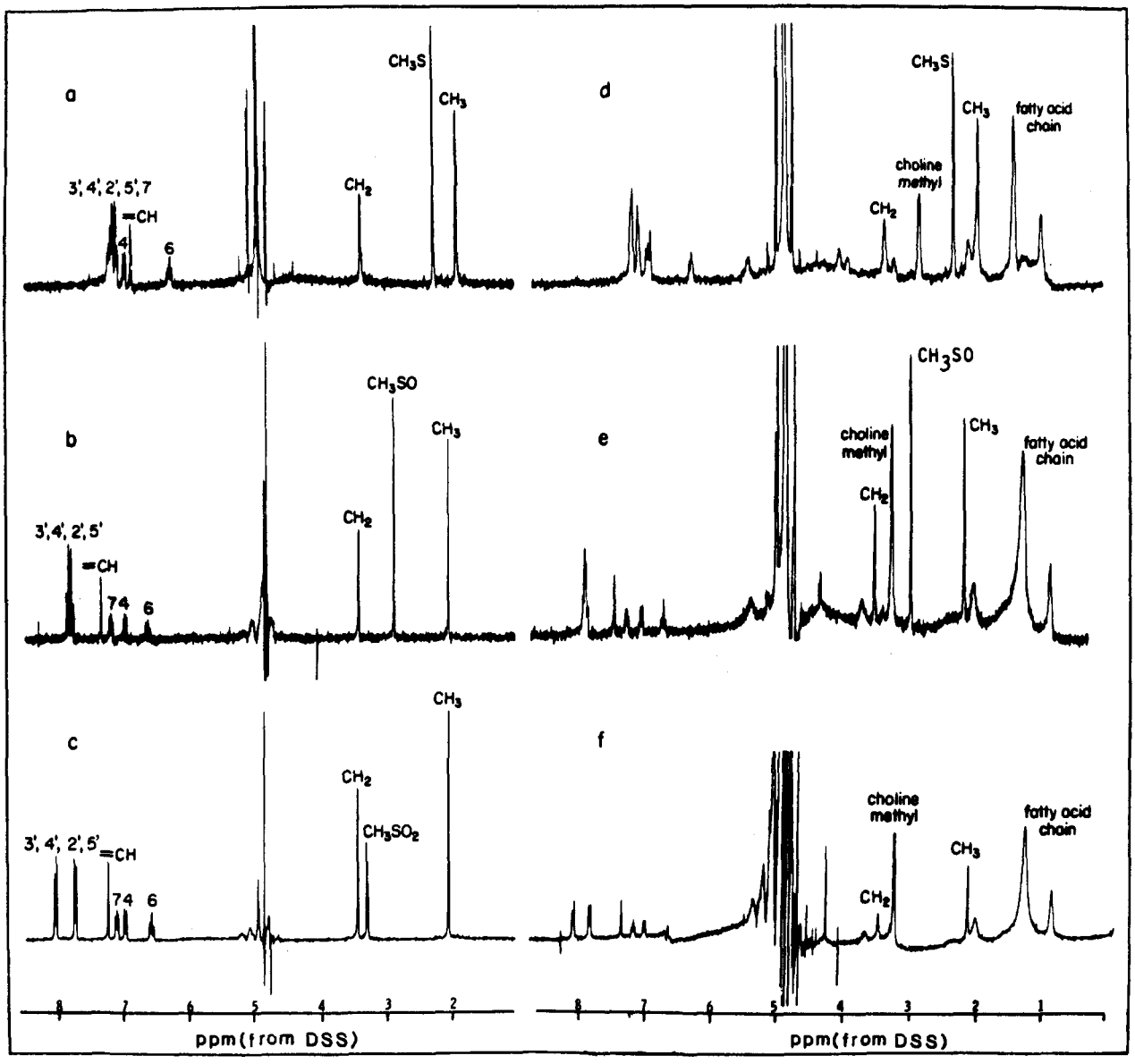


Figure 1. Proton magnetic resonance spectra of sulindac and its metabolites in the absence and presence of DOPC vesicles. The drug concentrations are 8.5–9.5 mM, and the vesicle concentration is 7.5×10^{-4} mM (or lipid concentration of 3 mM). The vertical scales in the different spectra are not the same. The spectra were recorded at 25 ± 1 °C: (a) sulindac sulfide; (b) sulindac; (c) sulindac sulfone; (d–f) mixture of DOPC vesicles with sulindac sulfide, sulindac, and sulindac sulfone, respectively.

$1/T_{2b}$ is a characteristic function of its environmental motional states, and its dependence on the molecular motion is quite complex. However, it is known that in an enzyme–inhibitor system, sharply decreased molecular mobility of the inhibitor can lead to a considerable line broadening.[9] In this case, presumably the large changes in the proton relaxation rate of the sulfide (2) may also be attributed to its slow molecular motion when bound with the vesicle. The microenvironment inside the vesicle lipid region changes along the hydrocarbon chain. From studies with fatty acids with a spin-label attached at various positions of the hydrocarbon chain, it has been demonstrated that the isotropic motion of fatty acid chains in a phospholipid bilayer increases in the direction of the center of the bilayer.[6,7] Also, the result of a ^{13}C NMR longitudinal relaxation time (T_1) measurement of dipalmitoyllecithin vesicles (DPPC) indicates that the center of the lipid bilayer is more mobile than the polar surface.[10,11] Putting all these evidences together, it seems resonable to suggest that the decrease in slope of $CH_2 > CH_3 > SCH_3$ seen in Table II can be related to the higher mobility of the corresponding protons. Thus, we feel that it is likely that sulindac sulfide is submerged inside the membrane with the ionized carboxyl group positioned near the polar surface and its SCH_3 group in the lipid bilayer. This conclusion is further supported by a differential scanning calorimetry study and freeze–fracture electron micrographs measured in our laboratories.[8]

^1H NMR of Sonicated Drug–DPPC-d_{31} Vesicles. The transition temperature of DPPC vesicles (42 °C) enabled us to study vesicle–drug interactions in both the gel and liquid–crystalline states. DPPC containing deuterated palmitoyl chains was used in order to reduce the complexity of the aliphatic proton spectrum observed in the DOPC system. Unfortunately, signals from undeuterated aliphatic protons in the glyceryl and choline moieties still prevent direct measurement of aliphatic protons in com-

(9) R. A. Dwek, "Nuclear Magnetic Resonance in Biochemistry", Clarendon Press, Oxford, 1975, pp 138–143.

(10) Y. K. Levine, P. Partington, G. C. K. Roberts, N. J. H. Birdsall, A. G. Lee, and J. C. Metcalfe, *FEBS Lett.*, **23**, 203 (1972).

(11) Y. K. Levine, N. J. H. Birdsall, A. G. Lee, and J. C. Metcalfe, *Biochemistry*, **11**, 1416 (1972).

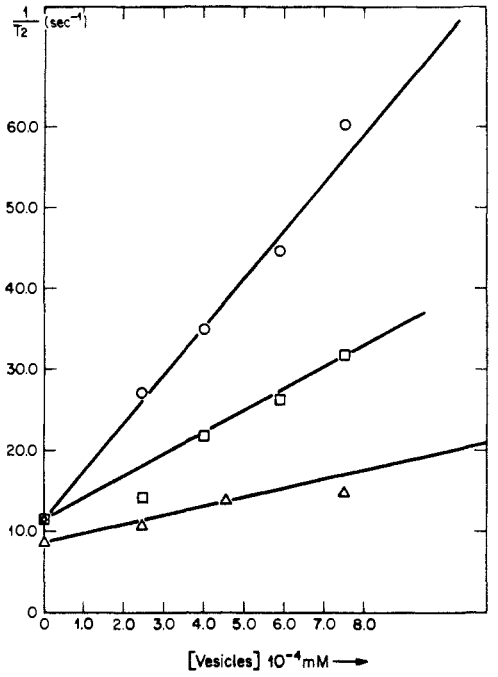


Figure 2. Change of proton relaxation rates of sulindac sulfide as a function of the DOPC vesicle concentrations: (○) CH_2; (□) CH_3 protons; (△) SCH_3 protons. The measurements were carried out at 23 ± 1 °C.

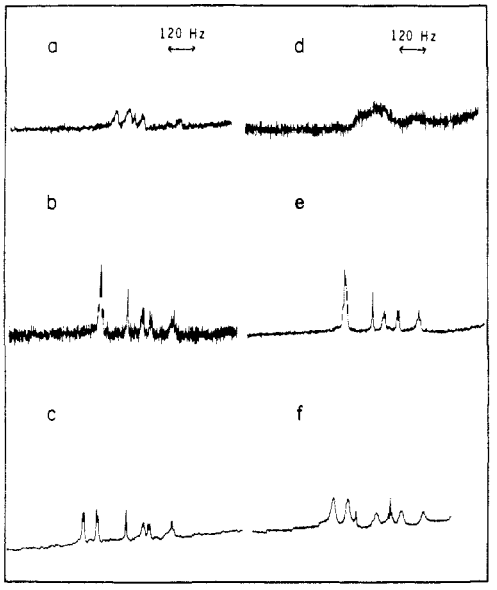


Figure 3. Aromatic proton resonance signals of sulindac and its metabolites when cosonicated wiih DPPC vesicles. The DPPC lipid concentration is 10 mg/mL, and the drug concentration is 20 mol % with respect to the lipid. The vertical scales in the different spectra are not the same. The chemical shifts are in the region higher than 5 ppm with respect to DSS (internal standard): (a–c) spectra of sulindac sulfide, sulindac, and sulindac sulfone measured at temperature of 65 °C, respectively; (d–f) spectra of sulindac sulfide, sulindac, and sulindac sulfone measured at temperature of 25 °C, respectively.

pounds 1–3. Figures 3a–c shows aromatic proton NMR signals of sulindac and its metabolites when cosonicated with DPPC. Again, a large line-width change is observed with the sulfide (2), and only slight changes occur with sulindac (1) and sulfone (3) at temperatures above the lipid phase transition temperature (42 °C). When mixtures of drug and DPPC vesicles were cooled to 25 °C, below the lipid phase transition temperature, a marked change in relaxation rate was observed with 2 but not with 1 and 3. Since the sharp line-width difference between Figure 3a

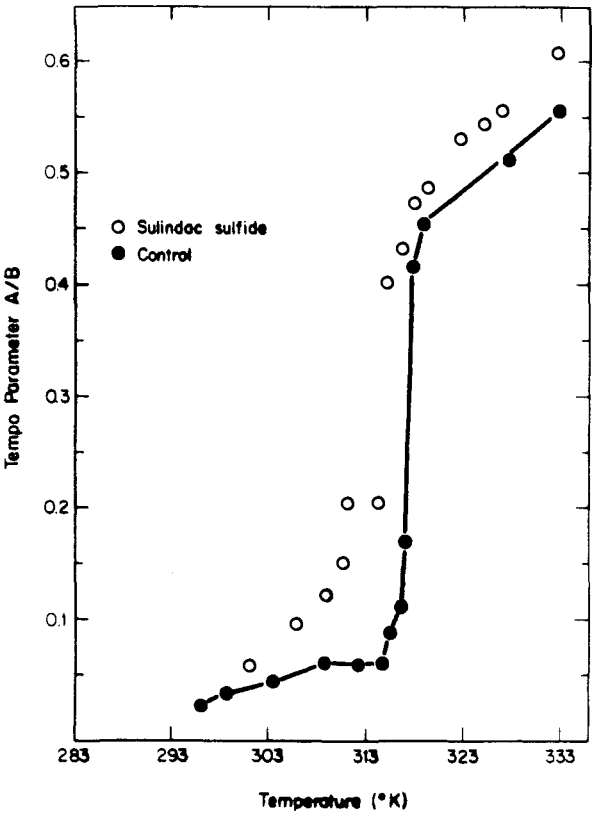


Figure 4. Change of Tempo spectral parameter in DPPC liposomes as a function of temperature. The lipid concentration is 10 mg/mL and the Tempo concentration is 1 mol %: (○) sulindac sulfide; (●) control experiment.

and 3d is induced only by temperature change, it is very likely that the molecular motion of 2 is strongly influenced by the physical state of the lipid bilayer. It is also well known that the line width of methylene proton signals of dipalmitoyllecithin vesicles is significantly broadened below the transition temperature,[12] when lipid bilayers are very rigid in a gel phase. This implies that the sulfide 2 must be located in the lipid bilayer region in order to be strongly influenced by the physical state of the lipid. Comparison of Figures 3c and 3f shows that although the relaxation rate of aromatic protons of sulfone (3) is also increased at the temperature below lipid phase transition, the change is not typically like that induced by strong immobilization of the sulfone in a liquid–gel phase. These observations lend further support to our previous conclusion that sulindac sulfide is embedded inside the membrane bilayer phase, whereas sulindac and the sulfone metabolite are unable to penetrate into the DPPC membrane.

Effect of Drugs on the Tempo Spectral Parameter. In addition to NMR measurements, the spin-label probe (Tempo) was used to detect a drug-induced membrane change of unsonicated DPPC liposomes. As shown in Figure 4, in the presence of 8 mol % of sulindac sulfide, the transition temperature of the DPPC vesicle is shifted from 42 to 40 °C. A similar concentration of sulindac and the sulfone metabolite produce no detectable changes of the transition temperature. These data again suggest that sulindac sulfide penetrates into the lipid bilayer and perturbes the transition temperature of the lipid.

The above results are consistant with observations in scanning differential calorimetric experiments;[8] sulindac

(12) J. Ulmius, H. Wennerstrom, G. Lindblom, and G. Arvidson, *Biochim. Biophys. Acta*, **389**, 192 (1975).

Table III. Order Parameter S of C_5-Doxylstearic Acid in DOPC Vesicles or Liposomes in the Presence of Sulindac and Its Metabolites[a]

content	order parameter S (pH 7.0) unilamellar vesicles (8 mol % content)	multilamellar vesicles (30 mol % content)
control	0.53	0.58
sulindac	0.53	0.55
sulindac sulfide	0.54	0.56
sulindac sulfone	0.55	0.55

[a] The lipid concentration is 10 mg/mL and the spin-label molecule used is 1 mol %.

sulfide (2) decreases the phase transition temperature and broadens the transition peak in the differential thermal scans, but sulindac (1) or its sulfone (3) do not.

Effect of Drug on DOPC Membrane Fluidity. To determine the effect of sulindac sulfide on membrane fluidity, order parameters of both C_5- and C_{12}-doxylstearic acid incorporated in the lipid membrane were measured. Order parameter S is a measurement of the anisotropy or randomness of the spin probe motion in the lipid phase,[6,7] which, in turn, is related to the local fluidity of the lipid phase. Results with C_5-doxylstearic acid as the probe under two different experimental conditions are summarized in Table III. Using unsonicated multilamellar DOPC liposomes, the order parameter decreases only slightly in the presence of 30 mol % of all three compounds. However, with ultrasonically dispersed vesicles of DOPC lipid mixed with 8 mol % of these compounds, the order parameter is either unchanged or slightly increased. Similar results were obtained with C_{12}-doxylstearic acid. It would seem that none of these antiinflammatory agents has any significant effect on membrane fluidity. In distinction to local anesthetics,[13,14] induction of a membrane fluidity change is probably not a critical factor in the primary action of the active sulfide metabolite of sulindac.

In conclusion, the membrane interaction of the nonsteroidal antiinflammatory drug sulindac and its metabolites has been studied with radiolabeled samples and NMR and ESR spectroscopy using unilamellar vesicles and multilamellar phospholipid liposomes as model membrane systems. Qualitatively different interactions between these arylacetic acid derivatives and lipid membranes are discernable. Evidences described above enable us to distinguish two classes of compounds, those which can penetrate into the lipid bilayer and those which can not. The active metabolite of sulindac (2), which possesses a hydrophobic sulfide substituent with a smaller dipole moment[15] than the sulfoxide (1) or sulfone (3), can penetrate into the lipid bilayer and leave the ionized carboxyl group near the polar surface of the membrane. On the other hand, the prodrug sulindac (1) and its inactive sulfone metabolite (3), which have the same hydrophobic benzylidenylindene structure and stereochemical configuration, as well as similar protein-binding properties as 2, behave in a very different manner. From the structure–activity point of view, this is an interesting differentiation of the reversible produrg sulindac and its active metabolite 2 at the membrane level. It also raises the question whether a strong hydrophobic interaction with the inner region of the lipid membrane is a desirable property for antiinflammatory agents. Further correlation of nonsteroidal antiinflammatory structures with alterations of other membrane parameters is discussed in the following paper in this issue.

Experimental Section

Materials. Dioleoyl-L-α-phosphatidylcholine (DOPC) and dipalmitoyl-L-α-phosphatidylcholine (DPPC) were purchased either from Supelco or Sigma. Labeled DPPC with deuterated fatty acid chain was from Avanti Polar Lipids, Inc. Tempo and C_5- and C_{12}-doxylstearic acid were obtained from Syva Research Chemicals. Deuterium oxide (99.8%) was from Bio-Rad. Sulindac and its sulfide metabolite and inactive sulfone metabolite were previously synthesized in our laboratory.

Preparation of Membrane Vesicles. A chloroform solution of 10 mg of DOPC in the absence and presence of 8.0 mol % of drug or metabolite per mol of phospholipid was concentrated in vacuo in a rotary evaporator, and the remaining thin film was dried under high vacuum. The residue was suspended in 10 mL of 50 mM, pH 7.0 phosphate buffer in deuterium oxide and then sonicated in a bath-type sonicator from Laboratory Supplies Co. at room temperature for 30–40 min. In the case of DPPC vesicles, the suspension of lipid and drugs (8 mol %) in buffer solution was dispersed by heating the sample in a 60 °C hot water bath and then mixed by vortex for 2 min prior to sonication. The sonication was performed at a water bath temperature of 55 °C. It is known that preparing vesicles at temperature below the lipid phase transition temperature may result in imperfect ones.[16] For the ^1H NMR study, the DOPC vesicles were prepared in the absence of drugs. Vesicles prepared under these conditions remained in clear solution with an average diameter of 250 Å as estimated from an electron micrograph obtained with a Carl Zeiss EM 95-2, using a negative stain of the uranic acetate of phosphotungstic acid.

Control Experiments. For ^1H NMR study of sulindac and its metabolites with membrane vesicles, 8.5–9.5 mM compounds in D_2O solution of pD 12 were used in control experiments (pD = pH + 0.4). pD 12 was used to increase the solubility of sulindac sulfide in D_2O. A small amount of the 10 mg/mL DOPC vesicles described above was added to the drug solution in NMR tubes directly and mixed by vortex agitation.

The possible intermolecular association between molecules of 1 to 3 was checked before adding the vesicles. The proton relaxation rate of these compounds was measured as a function of increasing concentration. Under the experimental conditions used in this study, no change in proton relaxation rate was observed (1–10 mM). The temperature effect on the proton line width of drug molecules prior to adding the vesicles was also examined between 25 and 65 °C. Only negligible effect was observed. A ^1H NMR study of a $CDCl_3$ solution of DOPC and 1 to 3 also showed no strong intermolecular interactions.

In order to ascertain the specific nature of the interaction between sulindac sulfide (2) and the DOPC vesicle, the interactions of compounds 1–3 with the DOPC molecule in deuterated chloroform solution were also investigated. The proton relaxation rate of these molecules was measured as a function of DOPC lipid concentration. The drug concentrations used were 4–6 mM, and the lipid concentrations were up to 10 mM. The proton relaxtion rate of three aliphatic spin systems in all three compounds gave an identical and slight increase, presumably due to the viscosity change of drug solutions in the presence of DOPC molecules.

Membrane Vesicles or Liposomes Containing Spin-Label Probes. Tempo or C_5- and C_{12}-doxylstearic acid in chloroform was added to the drug–lipid mixture in chloroform with the final spin-label concentration of 1 mol %. Careful mixing before untrasonication was critical in obtaining reproducible results.

Nuclear Magnetic Resonance Measurements. Proton magnetic resonance (^1H NMR) spectra were measured in a Varian

(13) K. W. Miller and K. Y. Pang, *Nature (London)*, **263**, 253 (1976).

(14) D. K. Lawrence and E. W. Gill, *Mol. Pharmacol.*, 11, 595 (1975).

(15) Dipole moments of CH_3SCH_3, $CH_3S(=O)CH_3$ and $CH_3S(=O)_2CH_3$ are 1.50, 3.96, and 4.49 D, respectively. The above values are quoted from "Handbook of Chemistry and Physics", Chemical Rubber Co., Cleveland, OH, 1960, p E63.

(16) Rudiger Lawaczeck, Masatsume Kainosho, Jean-Luc Girardet, and Sunney L. Chan, *Nature (London)*, **256**, 584 (1975).

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.

(17) A Lanir and G. Navon, *Biochemistry*, **10**, 1024 (1971).

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.
Received October 22, 1980

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-

0022-2623/81/1824-1202$01.25/0