Halothane Fluorine-19 Nuclear Magnetic Resonance in Dipalmitoylphosphatidylcholine Liposomes†

Lynda S. Koehler,* Eric T. Fossel,‡ and Karl A. Koehler§

ABSTRACT: The ^{19}F nuclear magnetic resonance (NMR) spectrum of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) in aqueous buffer containing unsonicated dipalmitoylphosphatidylcholine (PC) liposomes appears as a sharp doublet (J = 5 Hz) with a significantly broadened base. At high PC:halothane ratios, the halothane signal is broad and no spin–spin splitting is observed. Thus, the species involved appear to be halothane freely mobile in the aqueous phase and halothane significantly immobilized in association with PC. The exchange rate between these species is slow on the NMR time scale. Gadolinium(III) chloride causes substantial line broadening of the halothane ^{19}F doublet in the presence of phosphatidylcholine liposomes. In the absence of liposomes, gadolinium(III) chloride has no effect on the halothane ^{19}F resonances. These observations indicate that halothane and these paramagnetic metals coexist at the liposome surface and that the halothane molecules in the bulk phase rapidly exchange with the surface oriented anesthetic molecules. As either temperature or halothane concentration is increased, a broadened peak appears in addition to the doublet. Relaxation time (T_1) measurements indicate that the halothane molecules contributing to this signal are considerably less mobile than those contributing to the sharp doublet but more mobile than PC-bound halothane at high PC:halothane ratios. These results are interpreted in terms of an hypothesis involving an effect of halothane/temperature on the environment of halothane in the PC bilayer.

A molecular description of the process of anesthesia must include a description of the interaction of the anesthetic agent with the membrane, the localization of the site of anesthetic action within the central nervous system, and the delineation of the cellular and intercellular processes which are affected by the anesthetic (Woodbury et al., 1975a; Quastel and Linder, 1975). Presently, many different theories have been proposed concerning the primary site of anesthetic action. Jain et al. (1975) believe that the primary process involves drug-induced phase changes in membrane phospholipids with consequences for membrane-associated proteins. This theory is supported by the evidence presented by Miller and Pang (1976) and Shieh et al. (1976) showing that general anesthetics alter the conformation of the phospholipid molecules of membranes, particularly in the region of the head group. A proposal of Eyring et al. (1973) and its subsequent modification (Woodbury et al., 1975b) suggests that anesthetic action involves a direct interaction between the anesthetic and a protein or a lipid-mediated effect as described above. Direct interactions between anesthetics and proteins have recently been discussed (Hsia and Boggs, 1975; Boggs et al., 1976; Brown et al., 1976).

The present report is concerned with the characterization of anesthetic interactions with phospholipid in the simplest of nonperturbed systems and relies on reports arising from the anesthetic itself concerning its environment. These reports are in the form of ^{19}F NMR signals from the trifluoromethyl group of halothane. Measurement of chemical shifts and relaxation parameters allow conclusions to be drawn with regard to the distribution of halothane in unsonicated dipalmitoylphosphatidylcholine suspensions in aqueous media. A dynamic model for the interaction between phospholipid and anesthetic can be developed by inference from these data.

Materials and Methods

Lipids and Chemicals. Crystalline synthetic (grade 1) β-γ-dipalmitoyl-DL-α-phosphatidylcholine (99%) (Sigma Chemical Co.) was applied to an alumina column (activity grade I) containing 50 g of alumina per g of phospholipid. Phospholipid was dissolved in a minimum volume of alcohol-free chloroform and applied to the column. After washing the column with alcohol-free chloroform, the phospholipid was eluted with 30% methanol–70% chloroform and the solvent was flash evaporated. Purity of the phospholipid was verified by thin-layer chromatography employing chloroform–methanol–ammonium hydroxide (60:25:4 by volume). Phospholipid concentration was confirmed by the method of Lowry and Tinsley (1974).

The buffer employed for liposome preparation [2-(*N*-morpholino)ethanesulfonic acid (Mes[1]); Sigma Chemical Co.] was prepared with deionized water and the pH was adjusted to 6.4 by addition of 1 N hydrochloric acid. Sodium chloride was added to make the final buffer solution 0.1 M in sodium chloride.

Halothane free of stabilizers was a gift of Ayerst Laboratories, New York. Sterile light mineral oil (NDC 054-8577)

† From the Anesthesiology Department, University of North Carolina, Division of Health Affairs, Chapel Hill, North Carolina 27514. *Received December 21, 1976.* Supported by clinical funds from the Department of Anesthesiology, University of North Carolina and further supported by National Institutes of Health Grants HL-06350 and HL-18245 and a Grant-in-Aid (to E.T.F.) from the American Heart Association. Purchase of the NMR instrument at UNC was made possible by National Science Foundation Instruments Grants GU-2059, 2059 - Amendment I, and GP-37602, and by National Institutes of Health Grant 5S05RR07072.

‡ Biophysical Laboratory and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. Fellow of the Arthritis Foundation.

§ Departments of Pathology and Biochemistry, University of North Carolina, Division of Health Affairs, Chapel Hill, North Carolina 27514. Established Investigator of the American Heart Association.

[1] Abbreviations used: ^{19}F NMR, fluorine-19 nuclear magnetic resonance; PC, β-γ-dipalmitoyl-DL-α-phosphatidylcholine; halothane, 2-bromo-2-chloro-1,1,1-trifluoroethane; T_1, spin–lattice relaxation time; cP, centipoise; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NOE, nuclear Overhauser enhancement; γ_H, magnetogyric ratio for hydrogen; γ_F, magnetogyric ratio for fluorine.

was a product of Philips Roxan Laboratories, Inc., Columbus, Ohio.

Lanthanide chlorides were obtained from Alpha Products, Ventron Corp., Danvers, Mass. The pH values of the various lanthanide solutions varied between 6.05 and 6.18.

Liposome Preparation. Liposomes were prepared by adding buffer solution to purified dried phospholipid. Glass beads were added to this mixture followed by slow rotation at 50 °C for 3 h. The resulting preparation consists of a stable suspension of concentric multiple bilayer liposomes of somewhat varying size. Unless otherwise noted, the synthetic phosphatidylcholine liposome suspensions typically employed in this study contained either 40 mg of phospholipid/mL (5.4×10^{-2} M) or 250 mg of phospholipid/mL (3.4×10^{-1} M). During the course of these experiments, we have observed no evidence to indicate that we observe separate environments from inner levels of the highly hydrated multilamellar PC structure. The halothane appears to distribute itself over the whole sample.

Viscosity Measurements. Bulk viscosity was measured at room temperature with a LVT Wells-Brookfield micro-(cone-plate) viscometer (kindly provided by Dr. D. A. Gabriel) according to the manufacturer's instructions. The shear rates utilized varied from 230 to 1.15 s^{-1}. Viscosity values are reported in centipoise (cP).

NMR Measurements. NMR tubes (10 mm) were cleaned in chromic acid solution prior to each use, rinsed with deionized water, soaked in aqueous disodium ethylenediaminetetraacetate, and thoroughly rinsed with deionized water. ^{19}F NMR spectra were obtained on two spectrometers: (1) a Varian XL-100 spectrometer operating at 23.5 kG and employing a sweep width of 1000 Hz and (2) a JEOL FX 60 NMR spectrometer employing a field strength of 14.2 kG and a sweep width of 1000 Hz. All spectra obtained on the Varian instrument are reported relative to a coaxial external standard of hexafluoroacetone in D_2O (1%). Chemical shifts are reported relative to this standard. Those spectra obtained on the JEOL instruments are reported relative to the same external standard run separately. T_1 values were calculated manually from the FT spectra at varying τ values. Typically 10τ values were employed and a sequence repetition rate was at least $5T_1$. NOE values were determined by gated decoupling of the halothane proton. Samples were not degassed.

Results

^{19}F NMR Spectra of Halothane and Solvent Effects. The ^{19}F NMR spectrum of halothane in aqueous Mes-sodium chloride buffer solution consists of a single doublet centered at 6.62 ppm (J = 5 Hz) which collapses to a narrow singlet upon decoupling the proton on the adjacent carbon (Figure 1). The ^{19}F NMR spectrum of halothane in aqueous buffer over the pH range 2 to 8 or over the temperature range of 25 to 45 °C shows no alteration in chemical shift or line shape. Amounts of halothane greater than 4.7×10^{-2} M exceed the solubility of halothane in water and the halothane forms a separate layer in the bottom of the NMR tube. The excess halothane may be suspended by sonication. The ^{19}F NMR spectrum of such a sample shows a sharp doublet centered at 6.6 ppm and a second sharp doublet centered at 5.1 ppm (approximately the chemical shift of neat halothane). Halothane, in amounts which yield 9.41×10^{-3} M to 2.36×10^{-1} M final concentrations, was added to both octane and pentane. The halothane did not appear to exceed its solubility in the alkanes. The ^{19}F NMR spectra of these samples showed a single sharp doublet at 4.54 ppm in pentane and at 5.14 ppm in octane.

Association of Halothane with Phospholipid. The ^{19}F NMR spectrum of 4.7×10^{-2} M halothane in aqueous buffer

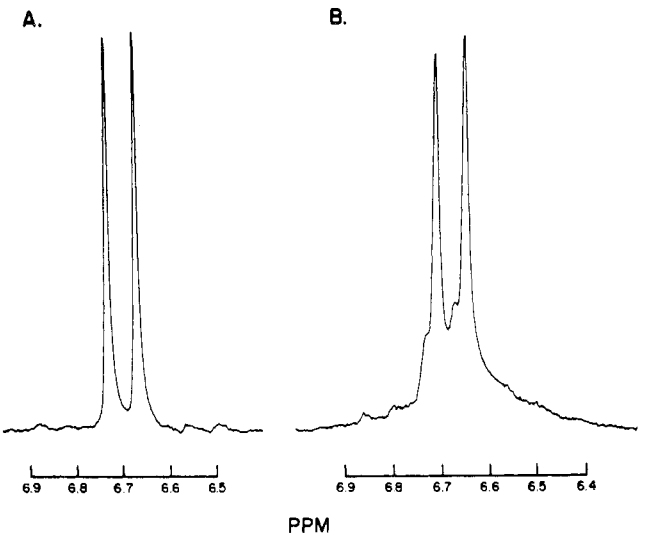

FIGURE 1: ^{19}F NMR spectra of halothane in aqueous buffer (A) and in aqueous buffer containing dipalmitoylphosphatidylcholine (B). (A) Halothane (4.7×10^{-2} M) in 5×10^{-3} M Mes-0.1 M sodium chloride, pH 6.3. (B) Halothane (4.7×10^{-2} M) in 5.4×10^{-2} M PC in 5×10^{-3} M Mes-0.1 M sodium chloride, pH 6.3. Observed splitting in A is 5.61 Hz; in B it is 5.62 Hz.

containing 5.4×10^{-2} M unsonicated liposomes prepared from synthetic dipalmitoylphosphatidylcholine consists of a sharp doublet with a broadened base, both centered at 6.5 ppm (Figure 1). When a concentrated solution of PC unsonicated liposomes was added to NMR tubes containing halothane (to yield a final concentration of 4.7×10^{-2} M halothane) giving final concentration of phospholipid of 6.8×10^{-2}, 2.7×10^{-2}, 6.8×10^{-2}, and 14×10^{-2} M, the ^{19}F NMR doublet signal decreased and the broad base increased as the final concentration of phospholipid increased. No chemical-shift change was apparent. If the amount of phospholipid is increased to 0.34 M, no doublet is observed; only a broad signal remains.

Employing a PC concentration of 5.4×10^{-2} M at 25 °C and increasing halothane concentrations from 9.41×10^{-3} M to 9.41×10^{-2} M resulted in ^{19}F NMR spectra showing a doublet at approximately 6.5 ppm downfield from the standard with a slight (less than 0.1 ppm), but definite, upfield shift with increasing halothane concentration. At halothane concentrations of 1.41×10^{-1} to 2.36×10^{-1} M, a shoulder which does not show the 5-Hz splitting appears near the doublet at approximately 0.2 ppm upfield (Figure 2). This shoulder shows a small concentration-dependent upfield shift. At 2.36×10^{-1} M halothane, the shoulder has shifted 0.34 ppm upfield relative to the standard at 25 °C. The portion of the total fluorine signal contained in the shoulder compared with that in the doublet increases as the amount of halothane increases.

At a halothane concentration of 4.7×10^{-2} M, the full width at half maximum intensity at 94.1 MHz for the halothane ^{19}F signal increases linearly from 5.6 to 14 Hz as the phospholipid concentration is increased to 8.5×10^{-2} M. A second experiment at 56.2 MHz employing the same halothane concentration and varying phospholipid concentration resulted in line width increases which were linear from 0 (8.1 Hz) to 1.7×10^{-1} M (25 Hz) dipalmitoylphosphatidylcholine and which appear to level off at approximately 3.4×10^{-1} M phospholipid (31 Hz).

In summary, at a given temperature in the presence of a fixed amount of phospholipid and a low concentration of halothane, a doublet with a broadened base is observed. As the concentration of halothane is increased, a broad upfield peak

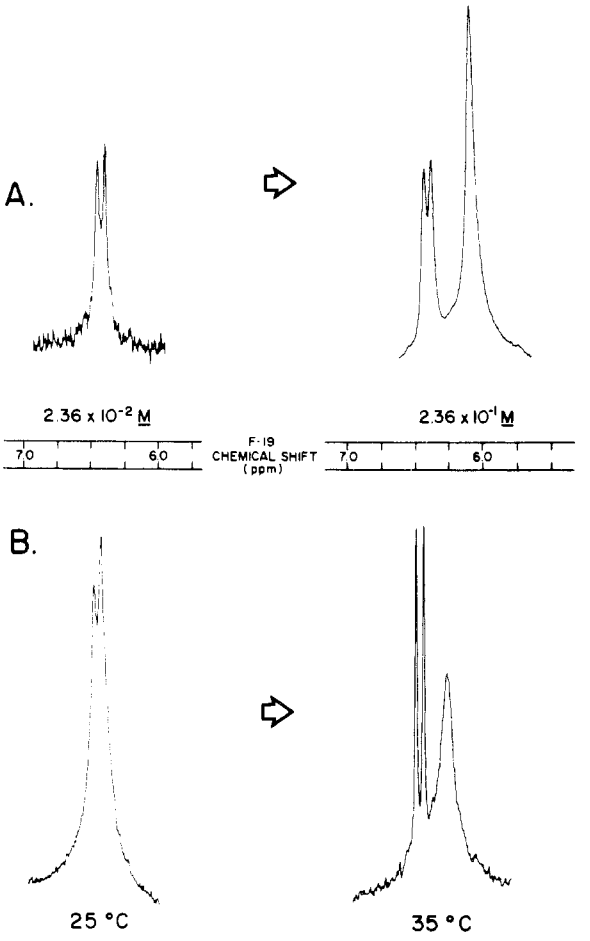

FIGURE 2: Effects of halothane concentration and temperature on the ^{19}F NMR spectrum of halothane in aqueous Mes buffer containing 5.4 $\times 10^{-2}$ M PC. At 25 °C increasing the halothane:phospholipid ratio from 0.44 to 4.4 leads to the change indicated in part A. In part B a system containing approximately a 1:1 halothane:phospholipid ratio is heated from 25 to 35 °C with the noted change in spectrum morphology. See text for details.

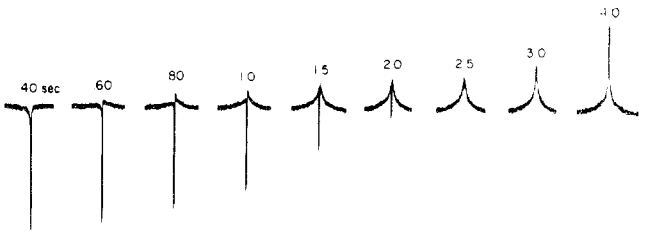

FIGURE 3: Partially relaxed Fourier transform ^{19}F NMR spectra of 4.7 $\times 10^{-2}$ M halothane in an aqueous suspension of 5.4 $\times 10^{-2}$ M PC at 25 °C. Numbers above spectra indicate delay time before data acquisition.

appears. The area of this broad peak increases relative to the doublet as the halothane concentration increases. Ultimately, the area of the broad upfield peak at high halothane concentrations is 3- to 10-fold that of the doublet signal.

Temperature Effects. As the temperature is increased through 41 °C, the hydrated PC used in these experiments undergoes a structural reorganization in passing from a gel to a liquid crystalline state. ^{19}F NMR spectra of mixtures of PC liposomes with various concentrations of halothane were obtained over a variety of temperature ranges. Samples containing from 9.4 $\times 10^{-3}$ to 1.9 $\times 10^{-1}$ M halothane in 5.4 $\times 10^{-2}$ M PC liposomes were subjected to temperatures ranging from 25 to 45 °C. As the temperature is increased, samples which showed only doublet and broad base develop a shoulder on the upfield side of the doublet at 6.3 ppm, the area of which increases as the temperature is increased (Figure 2). For example, a sample containing 5.4 $\times 10^{-2}$ M PC in Mes buffer and 4.7 $\times 10^{-2}$ M halothane was subjected to temperatures of from 25 to 40 °C. The room temperature ^{19}F NMR spectrum showed a doublet centered at approximately 6.55 ppm. At 37 °C, a shoulder appeared on the upfield side of the doublet. As the temperature was further increased, the shoulder increased relative to the doublet. There is a small but consistent upfield shift of the doublet and a slightly greater upfield shift of the shoulder with increasing temperature. Samples in which the shoulder appeared as temperature was increased showed an increase in the ratio of fluorine signal in the shoulder to that in the doublet as the temperature was further increased. Decreasing temperature from 45 °C reverses the sequence noted above: the shoulder disappeared at 37 °C and the doublet only was observed at 25 °C. A plot of the temperature and halothane concentration at the appearance of the shoulder extrapolated to zero halothane concentration intersects at 41 °C, the transition temperature of the phospholipid.

Two halothane samples containing 5.4 $\times 10^{-2}$ M PC were studied at a series of temperatures from 0 to 80 °C: (1) 4.71 $\times 10^{-2}$ M halothane, which shows only a doublet at ambient temperature (25 °C), and (2) 1.87 $\times 10^{-1}$ M halothane, which shows a doublet and shoulder at ambient temperature. All temperatures investigated appeared grossly to be compatible with stable phospholipid liposomes. In both samples, as the temperature increased, the ratio of fluorine integrated intensity of the fluorine signal in the shoulder to the doublet increased. At 80 °C, the shoulder began to show the appearance of the characteristic 5-Hz halothane splitting. Spectrum morphology observed as a function of temperature can be described as follows: At low temperature (approximately 0 °C), a single broad peak with a broad base is observed. As temperature is increased to 12 °C, the broad base becomes less distinct, the peak broadens, and at 19 °C it appears that two broad peaks exist. The signal narrows near 25 °C but appears to consist of two peaks again at 40 °C, followed by narrowing to a single peak at 80 °C. In a sample comprised of 6.7 $\times 10^{-2}$ M of halothane in 0.34 M PC in a volume of 1 mL run at 94.1 MHz, the peak center moves from 6.56 ppm at 10 °C to 6.4 ppm at 25 °C and to 6.2 at 80 °C (relative to an internally run external standard). In a second sample comprised of 4.7 $\times 10^{-2}$ M halothane in 1 mL of 0.34 M PC at 30 °C run at 56.2 MHz having a peak centered at 6.95 ppm (70 Hz, line width), at 50 °C, the peak had narrowed and shifted to 7.42 ppm (44-Hz line width).

The effects of proton decoupling on the appearance of the halothane ^{19}F spectra employing 6.7 $\times 10^{-2}$ M halothane in 0.34 M PC in a volume of 1 mL were investigated. At 10 °C, the single 48-Hz peak is resolved by decoupling into two relatively sharp peaks: the largest at 6.61 ppm (18-Hz line width) and a narrower peak at 6.42 ppm (4-Hz line width). At 25 °C, the broad somewhat asymmetric peak with resolved sharp resonances at 6.4 ppm narrows with proton decoupling to about 67 Hz (from 94 Hz). The peak at 80 °C, centered at 6.22 ppm (43 Hz), narrows with proton decoupling to a highly assymmetric peak which appears to be composed of at least four components, the major one of which is at 6.18 ppm (22 Hz).

Relaxation Studies. Spin-lattice (T_1) relaxation times were obtained for neat halothane, several different concentrations of halothane in synthetic PC multilayer liposome suspensions, halothane in aqueous buffer, mineral oil, and hexadecane. The ^{19}F NMR spectra obtained at a variety of delay times on a sample of 4.7 $\times 10^{-2}$ M halothane in 5.4 $\times 10^{-2}$ M synthetic

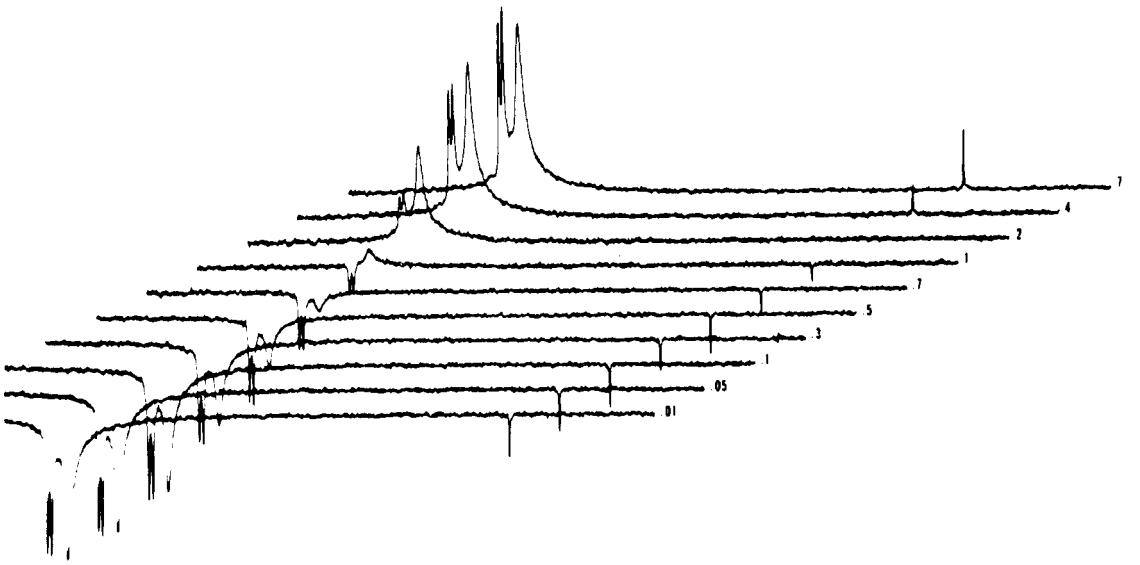

FIGURE 4: Stack plot of partially relaxed Fourier transform ^{19}F NMR spectra of 2.1×10^{-1} M halothane in an aqueous suspension of 5.4×10^{-2} M PC. Run carried out at 25 °C. Signal at far right is the external standard, hexafluoroacetone in deuterium oxide. Delay times in seconds are indicated at the right of each spectrum.

TABLE I: ^{19}F, T_1, and NOE Values for Halothane in Membrane and Solvent Systems.

System	Temp (°C)	T_1 (s)[a] Doublet	Shoulder	Base	NOE[e] Doublet	Shoulder	Bulk viscosity[c]
Neat halothane	25	3.22					8.0
4.7×10^{-2} M halothane							
In buffer	25	4.34			1.27		4.34
In hexadecane	25	2.41					2.41
In mineral oil	25	1.09					21.0
In 40 mg/mL PC[b]	25	2.18					
2.4×10^{-1} M halothane							
In 40 mg/mL PC	25	2.83	1.25				
In 40 mg/mL PC	80	2.22	2.08				
1.9×10^{-1} M halothane	25	2.97	1.31	0.49	1.16	1.11	33.8, 7.5[d]

System	Temp (°C)	T_1 (s) Proton decoupled spectra Broad signal	Sharp signal	Undecoupled signal	NOE[f] Entire signal
5.4×10^{-2} M halothane					
In 250 mg/mL PC	10	0.10	2.39	0.75	0.56
In 250 mg/mL PC	25	0.56	1.19	1.67	0.81
In 250 mg/mL PC	80		1.78	2.49	0.72
7.05×10^{-2} M halothane					
In 250 mg/mL PC	25	0.72			

[a] Errors in T_1 measurements are estimated to be 10%. [b] Synthetic dipalmitoylphosphatidylcholine. [c] Viscosity determinations were carried out at 21 °C. [d] The phospholipid suspension containing 1.9×10^{-1} M halothane exhibited non-Newtonian behavior. At low shear rates, the viscosity approaches 33.8 cP, while at higher shear rates (>100 s^{-1}) the viscosity approaches 7.5 cP. All other systems measured showed Newtonian behavior. [e] Calculated using integrated intensities and gated decoupling. [f] Calculated by cutting out peaks and weighing.

PC liposomes (Figure 3) suggest the presence of at least two distinct populations of halothane molecules with chemical shifts quite close to that observed for the fluorine doublet of halothane in aqueous buffer. A T_1 value of 0.5 s can be estimated for the signal observed at the base of the doublet. The series of partially relaxed halothane ^{19}F NMR spectra obtained on a sample of 9.4×10^{-2} M halothane in 5.4×10^{-2} M synthetic PC liposomes is plotted in Figure 4. These results indicate the existence of different relaxation times and, consequently, different mobilities, for the doublet and shoulder (Table I). Experiments employing 5.4×10^{-2} M halothane in the presence of 0.34 M PC but in the absence of proton decoupling reveal a broad ^{19}F spectrum with the apparent T_1 values recorded in Table I (undecoupled). Proton decoupling allows resolution of a sharp signal distinct from the broader signal and the corresponding T_1 values are recorded in Table I.

Nuclear Overhauser Enhancements. In order to appropriately interpret changes in ^{19}F T_1 relaxation times, it is necessary to deduce that the contribution of dipolar process is predominant. For this reason nuclear Overhauser enhancements (NOE) were determined for samples containing 4.7×10^{-2} M halothane in aqueous buffer, 1.9×10^{-1} M halothane in 5.4×10^{-2} M PC and for 5.4×10^{-2} M halothane in 0.34 M PC at 10, 25, and 80 °C. The results of these experiments are recorded in Table I. The NOE values varied from 0.56 to 0.72.

Effect of Added Paramagnetic Metal Ions. When the lanthanide chlorides, Tb, Eu, Dy, Nd, or Gd at concentrations of 5×10^{-3} M, were added to halothane in aqueous buffer, no

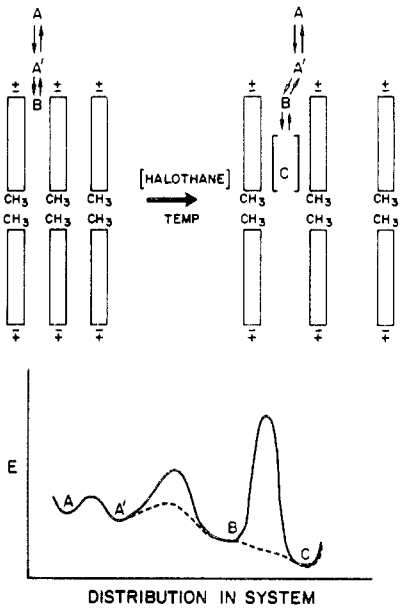

FIGURE 5: (Above) Hypothesis relating the effects of temperature and halothane concentration to the distribution of halothane in the system. The letters have the following meanings: A, halothane molecules in the bulk aqueous phase; A', halothane molecules associated with the aqueous phase:phospholipid interface; B, halothane molecules near the polar head groups of the phospholipid, but in the hydrocarbon chain; C, halothane molecules in the hydrocarbon chain closer to the terminal methyl group. (Below) Plot illustrating hypothesized free energy changes associated with the distribution of halothane in dipalmitoylphosphatidylcholine liposomes. Solid line represents the system below the lipid gel to liquid crystalline transition temperature. Dashed line represents the system above the lipid gel to liquid crystalline transition temperature.

broadening of the ^{19}F NMR doublet (observed line width at half maximum approximately 10–12 Hz) was observed. This is also true of the ions Cu and Mn. If, however, gadolinium is added to halothane in the presence of PC liposomes in aqueous buffer, substantial broadening to approximately a 34-Hz half width is observed. At these halothane and phospholipid concentrations, the observed line width varies from 14 Hz at 10^{-4} M gadolinium(III) to 82 Hz at 10^{-2} M gadolinium(III). Resolution of the doublet is lost when the gadolinium(III) ion concentration reaches approximately 10^{-3} M. Manganese sulfate (5×10^{-3} M) broadens the signal from 4.71×10^{-2} M halothane in the presence of 5.4×10^{-2} M PC to 25 Hz, which is somewhat less than the broadening resulting from a corresponding gadolinium concentration.

Discussion

The effects of general anesthetics on aspects of the molecular organization of different membranes have been examined intensively in recent years. Perturbations in membrane structure as a result of the incorporation of inhalation anesthetic molecules into the bilayer phospholipid assembly have been inferred from a variety of studies. Introduction of anesthetic-like molecules into dipalmitoylphosphatidylcholine smectic mesophases and sonicated dimyristoylphosphatidylcholine preparations leads to a lowering of the phase transition temperature (Hill, 1974, 1975; Jain et al., 1975; Vanderkooi et al., 1977). The membrane phase transition directly reflects many organizational properties of phospholipids in aggregate states. Simon et al. (1975) have discussed the effect of phase separations in a calcium–phosphatidylserine bilayer system on cyclopropane partitioning and on phase transition temperatures. Neal et al. (1976) have observed effects of anesthetics on calcium ion phospholipid interactions in spin-labeled multibilayer membranes. Also employing spin-label methods, Paterson et al. (1972) have observed a parallel between alcohol concentrations required to produce increases in membrane fluidity and to cause anesthesia in tadpoles. Employing aqueous sonicated egg yolk phosphatidylcholine labeled with spin-labeled phosphatidylcholine molecules, Trudell et al. (1973a,b) reported a generalized fluidization of the bilayer membrane as a result of the introduction of methoxyfluorane (1,1-difluoro-2,2-dichloroethyl methyl ether) and halothane at anesthetic concentrations to lipid ratios of up to 0.6:1.0. Similar results were observed whether the spin label was located near the polar head groups or closer to the center of the bilayer. The apparent polarity of the 7,8 spin-labeled bilayers showed a discontinuity at an anesthetic to lipid mole ratio of 0.1:1.0 which may be a result of a change in the membrane localization of the label. On the other hand, employing unsonicated phosphatidylcholine–cholesterol liposomes, Boggs et al. (1976) have observed considerably greater fluidization as a function of halothane concentration when the reporting label is 12-doxylstearate compared with 8-doxylstearate. This may suggest that the halothane disruptive effect is of greatest magnitude closer to the center of the lipid bilayer and that this effect is to some extent dampened nearer the head groups and further suggests that some important anisotropic determinants may be lost upon sonication. Boggs et al. (1976) report possible nonlinearity in plots of S, order parameter, vs. halothane. Rosenberg et al. (1975) have obtained spin-label data suggesting a membrane organizing role for anesthetics such as halothane at low drug:membrane ratios and a disorganizing effect at higher drug:membrane ratios. Finally, differential scanning calorimetric measurements reported by Jain et al. (1975) also suggest a response of dipalmitoylphosphatidylcholine membranes to halothane which varies with anesthetic concentration. The results reported here have focused on the consequences of membrane structure, and its perturbation, on the halothane molecule itself.

Our data are most simply explained by the following specific hypothesis regarding halothane–membrane interactions. At temperatures below the phospholipid gel to liquid crystalline phase transition, halothane exists in three distinct environments in equilibrium with one another. Halothane in the bulk aqueous buffer phase (A) (Figure 5) is in equilibrium with those molecules located at the polar head group–bulk phase interface (A'). These interfacial halothane molecules are in equilibrium with halothane molecules located in the phospholipid methylene chain near the polar head groups (B). As a result of membrane disruptive events, similar to the normal gel to liquid crystalline transition, a further environment is created for halothane (C). Environment C may be located toward the terminal methyl end of the hydrocarbon chains of the phospholipid, near the center of the bilayer. This area of the bilayer seems, by deuterium NMR and spin-labeling experiments, to be the least ordered of the regions in the membrane (Seelig and Niederberger, 1974; Seelig and Seelig, 1974). In our model the gel to liquid crystalline phase transition has a primary consequence in the relative partitioning of the halothane into subsites B and C. Once associated with phospholipid, the halothane apparently can choose among a number of distinct environments in the bilayer. Its choice will depend on (1) the energy of interaction at that region of the bilayer and (2) the energy barrier of access to each region of the bilayer. We propose that the halothane molecule is in a state of dynamic equilibrium, not only between bulk solution and the liposome, but also with respect to its occupation of environments within the bilayer.

The weight of our data suggests that halothane partitions

into the phospholipid structures present in this membrane system. In particular, estimated halothane:membrane partition coefficients (Seeman, 1972; Koehler et al., 1977), the observed line broadening of the halothane signal as a function of added phospholipid, and the observed spin-lattice relaxation times suggest that the halothane is distributed at equilibrium between aqueous and lipid phases.

Significant line broadening effects due to the addition of gadolinium ion which are not observed in the absence of phospholipid are evidence for the existence of an exchange between interfacially bound and bulk phase halothane molecules which is fast on the NMR time scale. Thus, the chemical shifts and T_1 values observed are weighted averages of these species. The ^{19}F NMR signal due to the bulk-interface exchange of halothane molecules is evident in Figure 1B. On the other hand, observation of distinct membrane-bound (B) and bulk-interfacial (A′) species (Figures 1B and 3) suggests that the exchange of halothane molecules between site B and site A′ is slow on the NMR time scale. Thus, in Figure 5 (bottom), transport of halothane from site A′ to B occurs at the expense of activation energy. Below the phospholipid phase transition temperature partitioning of halothane into environment C is apparently thermodynamically unfavorable. Above the transition temperature partitioning of halothane into this environment is favored. Halothane molecules in site C appear as the unsplit shoulder in Figure 2.

It is apparent from the ^{19}F chemical shifts observed for free and interfacial halothane and for halothane completely partitioned into PC considered with the asymmetric relationship of the sharp doublet signal to the broad signal of halothane bound to PC (Figure 3) that, although similar, the chemical shifts of halothane fluorine nuclei are distinct in environments A and A′ and B. The apparent similarity of chemical shifts for these species is presumably due to hydrogen-bonding interactions between anesthetic and solvent in the bulk aqueous phase (Koehler et al., 1977). At higher halothane concentrations or at increased temperatures, the ^{19}F chemical shift of the broad ^{19}F resonance moves upfield suggesting that halothane molecules are moving into environments somewhat different from B. The quite emphatic ^{19}F chemical shift change toward the reference is similar to that produced by temperature changes at low halothane:membrane ratios. Although we hypothesize that a change in location of halothane causes this chemical-shift change, altered methylene chain packing in the bilayer could conceivably lead to such an effect in the absence of a repositioning of the anesthetic.

Further evidence for multiple and distinct aqueous and membrane environments for halothane comes from relaxation time and line width measurements. Arguments involving the use of spin-lattice relaxation times as a means of estimating molecular motion require that dipole-dipole interactions constitute the principal relaxation process contributing to the observed T_1.

The maximum nuclear Overhauser enhancement expected upon observing the ^{19}F nucleus and decoupling ^1H can be calculated to be equal to $1 + \gamma_H/2\gamma_F = 1.53$, assuming that the fluorine nucleus is relaxed entirely by a ^{19}F:^1H dipolar mechanism and that the extreme narrowing condition is obeyed. For the fluorine nuclei of halothane in aqueous buffer, we obtain an NOE value of 1.27 (Table I). Since oxygen was not excluded from these samples, this represents a minimum enhancement. We may then conclude that approximately 85% of the relaxation processes contributing to the observed T_1 values in Table I are due to dipole-dipole relaxation. Occasionally we have run ^{19}F T_1 experiments in which the system was deoxygenated by bubbling argon through the lipid suspension in the NMR tube prior to addition of halothane. This treatment had no apparent effect on the T_1 values obtained compared with similar runs without deoxygenation. Further consistent with the conclusion that ^{19}F T_1 results reliably indicate halothane motion in the experiments reported here are the observed effects of viscosity on halothane relaxation rates. It is apparent from Table I that halothane T_1 values correlate reasonably well with bulk viscosity when mineral oil, neat halothane, and aqueous buffer are considered. ^{19}F relaxation times in phospholipid suspensions require further comment. Bulk viscosity determined at shear rates greater than 100 s^{-1} for the PC liposomes approach a viscosity of 7.5 cP which is not in agreement with the T_1 value observed for lipid-bound halothane assuming that bulk viscosity is a dominant determinant of the correlation time of the halothane molecule. On the other hand, the viscosity observed at low shear rates (11 s^{-1}), 34 cP, is consistent with the correlation of T_1 with bulk viscosity. These results indicate that (1) halothane molecules in environments A and A′ are experiencing a microviscosity considerably less than the observed bulk viscosity and (2) at low sheer rates halothane molecules bound to the lipid experience a microviscosity consistent with the observed bulk viscosity. At high shear rates the aggregate properties of the liposomes are apparently affected. Finally our deductions in this discussion employ observed T_1 values in a qualitative manner. No attempt is made to specify exact correlation times for the halothane molecule.

It is clear from Table I that the halothane doublet T_1 observed in the presence of liposomes is significantly different (65% smaller) from that observed in aqueous buffer, a result consistent with the hypothesized A $\rightleftarrows$ A′ equilibrium where the mobility of interfacially oriented halothane molecules A′ is decreased relative to halothane dissolved in the bulk aqueous phase. Furthermore, this observation and the fact that significant line-broadening effects due to the addition of gadolinium ion are not observed in the absence of phospholipid suggest that halothane molecules located in the bulk aqueous phase are in rapid equilibrium with halothane molecules associated in some manner with the liposomes. The membrane associated population of halothane molecules could be located in the proximal region of the phospholipid hydrocarbon chains or at the polar head group-bulk solution interface. Examples of both types of interaction have been observed by others. Such interfacial orientation may be consistent with observations on molecules such as benzene, nitrobenzene, and substituted fluorobenzenes in the presence of micellar phases (Arrington et al., 1970). Binding of benzyl alcohol to PC head group regions has been inferred by Colley and Metcalfe (1972).

In the case for which it was convenient to estimate the T_1 of the base of the doublet at relatively high halothane:lipid ratios (1.9×10^{-1} M, halothane in 5.4×10^{-2} M PC) and under conditions of relatively low halothane:lipid ratios (5.4×10^{-2} M halothane in 0.34 M PC), a value of approximately 0.5 s is obtained. Since, in the extreme narrowing limit, for the dipolar case, T_1 is inversely related to τ_c (the overall correlation time of the molecule), we may conclude that the halothane molecules partitioned into environment B in Figure 5 are moving considerably less freely than those in regions A and A′. The weighted average T_1 values for the undecoupled spectra of 5.4×10^{-2} M halothane in 0.34 M DPC increase as temperature increases, consistent with increasing mobility of the halothane molecules. The fact that proton decoupling (Table I) allows a relatively sharp signal to be characterized with a T_1 of 2.4 s suggests that a contribution due to halothane molecules in environments A and A′ is present. The longer correlation time of halothane in site B results in shorter T_2 values

and, therefore, in the line broadening seen at the base of the doublet. This result would be expected if these halothane molecules are intimately associated with phospholipid liposomes, which have very long correlation times. It is conceivable that the observed line widths of membrane-associated halothane molecules are the result of exchange of halothane between two or more membrane environments with somewhat different chemical shifts (compared with the halothane fluorine:proton coupling constant). The possibility that halothane molecules associated with interlamellar water molecules might exhibit unusual spectral characteristics, resulting in the observed broadened base observed in the ^{19}F NMR spectrum of halothane in the presence of PC (Figure 1B), must be logically entertained. The interpretation of the data presented here assumes that interlamellar halothane molecules experience an environment substantially the same as that experienced by halothane in the bulk aqueous phase. Evidence has not been forthcoming to indicate immobilization or to attribute other special properties to interlamellar water in multilayer lecithin liposomes of the sort employed in this study. Utilizing deuterium NMR methods, Finer (1973) and Finer and Darke (1974) have estimated the mobility of water in various environs of egg yolk lecithin. They conclude that water trapped between lipid lamellae has the motional characteristics of bulk water. The most tightly bound water in the system is that involved in the hydration of the phospholipid head groups. As a result of these considerations, we may conclude that the line broadening observed in Figure 1B is due to restriction of the motion of phospholipid-associated halothane.

Measurement of ^{19}F T_1 values for the shoulder (Figure 2A and Table I) suggests that those halothane molecules occupying environment C (Figure 5) possess a mobility intermediate between those in sites B and A'. Increasing the temperature to 80 °C increases the motion in environment C. Consistent with this observation is the appearance of splitting in the undecoupled shoulder signal at this high temperature.

A plot of temperature vs. the halothane concentration at which the shoulder appears in the ^{19}F NMR spectrum extrapolates back to 41 °C at zero halothane concentration. Since the transition temperature of the PC is 41 °C, we may speculate that those processes involved in the generation of the ^{19}F shoulder are related to membrane processes similar to those occurring during phase transitions. The reversibility of the B $\rightleftarrows$ C equilibrium as a function of temperature further suggests that the typically observed membrane phase transition results in the appearance of a halothane binding region in the membrane. In summary, the alteration of ^{19}F NMR spectra in the phospholipid with temperature is an effect of changes in halothane partitioning and environments. Two-fold increases have been observed by Colley and Metcalfe (1972) for the partitioning of benzyl alcohol into sonicated dipalmitoyllecithin suspensions as the transition temperature is crossed. As expected, such behavior is not observed for egg lecithin over the temperature range 20–50 °C.

In a recent report by Trudell and Hubbel (1976) halothane ^{19}F NMR spectra obtained in sonicated egg lecithin vesicles are interpreted by the authors to indicate that there is, on the NMR time scale, rapid free exchange between the interior of the bilayer and the aqueous medium. Egg lecithin at ambient temperature is above its phase transition temperature. Evidently in this case the energy barrier between bulk solution and the bilayer interior is low and the interfacial population (A' in Figure 5) appears to be negligible. This conclusion is reinforced by experiments with "natural" phospholipids above their phase transition temperature. The halothane ^{19}F NMR signal in multilayer vesicles constituted from natural lipids such as bovine heart or egg lecithin at 25 °C reveals significant broadening and absence of a "doublet" resonance, indicating that the halothane molecules more readily partition into the interior of the bilayer in these lipids (Koehler et al., 1977).

From the nature of the model, proposed here, it is clear that phospholipid–anesthetic interactions are phenomena which deserve close attention in the quest to define the mechanism of general anesthesia (Metcalfe et al., 1974). Such membrane–drug interactions may be crucial to the transmission of anesthetic-induced perturbations of the membrane to membrane proteins and other membrane components resulting, through an unknown mechanism, in unconsciousness. Since anesthetic-related perturbations may occur via membrane phospholipid based transitions, direct physical coupling between the site(s) of action of the anesthetic and the molecular processes leading to unconsciousness may not even exist. Therefore, the nature of the "unconsciousness site" may not necessarily be inferred from observations relating to anesthetic binding sites.

Acknowledgments

The authors express their appreciation to Drs. P. D. Ellis, W. Freemen, M. K. Jain, C. N. Reilley, A. K. Solomon, and K. Sugioka for encouragement and helpful criticism, and to Dr. D. Harris for running samples on the XL-100 spectrometer at U.N.C.

References

Arrington, P. A., Clouse, A., Doddrell, D., Dunlap, R. B., and Cordes, E. H. (1970), *J. Phys. Chem. 74,* 665–668, and references therein.

Boggs, J. M., Young, T., and Hsia, J. C. (1976), *Mol. Pharmacol. 12,* 127–135.

Brown, F. F., Halsey, M. J., and Richards, R. E. (1976), *Proc. R. Soc. London Ser. B 193,* 387–411.

Colley, C. M., and Metcalfe, J. C. (1972), *FEBS Lett. 74,* 241–246.

Eyring, H., Woodbury, J. W., and D'Arrigo, J. S. (1973), *Anesthesiology 38,* 415–424.

Finer, E. G. (1973), *J. Chem. Soc., Faraday Trans. 2 69,* 1590–1600.

Finer, E. G., and Darke, A. (1974), *Chem. Phys. Lipids 12,* 1–16.

Hill, M. W. (1974), *Biochim. Biophys. Acta 356,* 117–124.

Hill, M. W. (1975), *Biochem. Soc. Trans. 3,* 149–152.

Hsia, J. C., and Boggs, J. M. (1975), in Molecular Mechanisms of Anesthesia, Fink, B. R., Ed., New York, N.Y., Raven Press, pp 327–338.

Jain, M. K., Wu, N. M., and Wray, I. V. (1975), *Nature (London) 255,* 494–496.

Koehler, L. S., Curley, W., and Koehler, K. A. (1977), *Mol. Pharmacol. 13,* 113–121.

Lowry, R. R., and Tinsley, I. J. (1974), *Lipids 9,* 491–492.

Metcalfe, J. C., Hoult, J. R. S., and Colley, C. M. (1974), in Molecular Mechanisms in General Anesthesia, Halsey, M. J., Millar, R. A., and Sutton, J. A., Ed., Edinburgh, Churchill Livingstone, pp 145–162.

Miller, K. W., and Pang, K. Y. (1976), *Nature (London) 263,* 254–255.

Neal, M. J., Butler, K. W., Polnaszek, C. F., and Smith, I. C. P. (1976), *Mol. Pharmacol. 12,* 144–155.

Paterson, S. J., Butler, K. W., Huang, P., Labelle, J., Smith, I. C. P., and Schneider, H. (1972), *Biochim. Biophys. Acta 266,* 597–602.

Quastel, D. M. J., and Linder, T. M. (1975), Molecular

Mechanisms of Anesthesia, Fink, B. R., Ed., New York, N.Y., Raven Press, pp 157-165.
Rosenberg, P. H., Eibl, H., and Stier, A. (1975), *Mol. Pharmacol. 11*, 879-882.
Schneider, H. (1972), *Biochim. Biophys. Acta 266*, 597-602.
Seelig, A., and Seelig, J. (1974), *Biochemistry 13*, 4839-4845.
Seelig, J., and Niederberger, W. (1974), *Biochemistry 13*, 1585-1588.
Seeman, P. (1972), *Pharm. Rev. 24*, 583-655.
Shieh, D. D., Ueda, I., Lin, H., and Eyring, H. (1976), *Proc. Natl. Acad. Sci., U.S.A. 73*, 3999-4002.
Simon, S. A., MacDonald, R. C., and Bennett, P. B. (1975), *Biochem. Biophys. Res. Commun. 67*, 988-994.
Trudell, J. R., and Hubbel, W. L. (1976), *Anesthesiology, 44*, 202-205.
Trudell, J. R., Hubbel, W. L., and Cohen, E. N. (1973a), *Biochim. Biophys. Acta 291*, 321-327.
Trudell, J. R., Hubell, W. L., and Cohen, E. N. (1973b), *Biochim. Biophys. Acta 291*, 328-332.
Vanderkooi, J. M., Landesberg, R., Selick, H., II, and McDonald, G. G. (1977), *Biochim. Biophys. Acta 464*, 1-16.
Woodbury, J. W., D'Arrigo, J. S., and Eyring, H. (1975a), in Molecular Mechanisms of Anesthesia, Fink B. R., Ed., New York, N.Y., Raven Press, pp 53-87.
Woodbury, J. W., D'Arrigo, J. S., and Eyring, H. (1975b), in Molecular Mechanisms of Anesthesia, Fink, B. R., Ed., New York, N.Y., Raven Press, pp 253-275.

Analysis of Thyroid Hormone Binding to Human Serum Prealbumin by 8-Anilinonaphthalene-1-sulfonate Fluorescence†

Sheue-yann Cheng, Robert A. Pages,‡ Harry A. Saroff, Harold Edelhoch, and Jacob Robbins*

ABSTRACT: Upon binding of 8-anilinonaphthalene-1-sulfonate (ANS) to prealbumin (PA), the absorption maximum is red shifted by 21 nm and the molar extinction coefficient is enhanced by 25%; the fluorescence emission peak of ANS is shifted from 515 to 465 nm with a large increase in quantum yield. At pH 7.4, 2 mol of ANS bind per mol of PA with affinity constants of 3.3×10^5 and 1.0×10^5 M^{-1} as determined by difference absorption spectroscopy and fluorescence enhancement, respectively. Since ANS competes for the thyroxine (T_4) binding sites on PA, the quenching of ANS fluorescence was used to analyze the binding of thyroid hormones and their analogues. Two models were developed to analyze the data. In model I two molecules of T_4 (or analogue) displace two molecules of ANS with quenching only resulting from displacement. By this analysis, the following log K_1 and log K_2 values were obtained: 8.5, 6.7 for T_4; 7.4, 6.1 for 3,5,3′-triiodo-L-thyronine; 7.2, 6.4 for *N*-acetyl-L-thyroxine; 8.2, 6.8 for 4-hydroxy-3,5-diiodobenzaldehyde. These values agree very well with those obtained by equilibrium dialysis. However, in the case of several desamino analogues, model II was employed to analyze the data since it appears that ANS fluorescence is quenched not only by competitive displacement, but also when the analogue occupies the second site. At pH 7.4, the log K_1 values obtained were: 8.2 for 3-(4-hydroxy-3,5-diiodophenyl)propionic acid; 8.4 for 3,5,3′,5′-tetraiodothyroacetic acid; 8.6 for 3,5,3′,5′-tetraiodothyropropionic acid; and 7.7 for 3,5,3′,5′-tetraiodothyrobutyric acid. These values are in accord with those from equilibrium dialysis.

ANS[1] has been widely used to explore the hydrophobic surfaces of proteins (Stryer, 1965; Daniel and Weber, 1966; Edelman and McClure, 1968), as a probe to follow the interactions of an enzyme with a substrate or other cofactors (Brand and Gohlke, 1972; Tu and Hastings, 1975; Seery and Anderson, 1972) and as a label with a long lifetime to measure the rotational behavior of proteins (Weber and Daniel, 1966; Witholt and Brand, 1970). The quenching of ANS fluorescence by competitive displacement has been used to determine the affinities of the thyroid hormones for the single binding site on thyroxine binding globulin (Nilsson and Peterson, 1975a). In the present work with PA, the study of competitive binding has been extended to apply to the case where two molecules of hormones are bound with negative cooperativity.

The structure and number of binding sites of PA have been established recently. X-ray crystallography (Blake et al., 1974) and amino acid sequencing (Kanda et al., 1974) have clearly established that PA consists of four identical subunits. Two identical binding sites for T_4 are located in a central channel formed by a tetrahedral association of the four monomers.

The equilibria for the binding of T_4 have been interpreted recently in terms of two identical sites on PA with negative cooperativity (Ferguson et al., 1975). The binding parameters may also be expressed in terms of two independent sites with different affinities. It was shown by equilibrium dialysis that two molecules of ANS are bound to PA and are competitively displaced by T_4. It was reported earlier by Branch et al. (1971) that the binding of T_4 and its analogues could be followed by the quenching of ANS fluorescence. However, attempts to calculate the binding constants failed since there was no knowledge of the second site for T_4 at that time. With the discovery of the second site (Ferguson et al., 1975), we have

† From the Clinical Endocrinology Branch and the Laboratory of Biophysical Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014.
‡ Present address: Philip Morris Research Center, Richmond, Virginia 23261.

[1] Abbreviations used: PA, human serum prealbumin; T_4, L-thyroxine; T_3, 3,5,3′-triiodo-L-thyronine; ANS, 8-anilinonaphthalene-1-sulfonate; DIPA, 3-(4-hydroxy-3,5-diiodophenyl)propionic acid; TLC, thin-layer chromatography; Tris, tris(hydroymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.