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A PROTON NUCLEAR MAGNETIC RESONANCE STUDY ON THE RELEASE OF BOUND WATER BY INHALATION ANESTHETIC IN WATER-IN-OIL EMULSION

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Water-in-oil emulsion was prepared from glycerol-α-monooleate, *n*-decane and water, and was used to analyze the behavior of bound water molecules in response to the addition of an inhalation anesthetic, enflurane. The motion of water molecules is monitored by proton nuclear magnetic resonance spectroscopy. To the first approximation, the half-height width of the proton signal of dispersed water is related to the spin-spin relaxation time and represents the motion of the water molecule. It appears that one of the two OH moieties of glycerol-α-monooleate forms a hydrogen bond with the water molecule in average. The half-height width of the dispersed water proton showed a maximal value when the glycerol α-monooleate/*n*-decane mole ratio was $4 \cdot 10^{-2}$. The cause of this maximum is not immediately clear, but it is suggested that the assembly mode of glycerol-α-monooleate may be different between the lower and higher concentration range. Enflurane decreased the half-height width of the dispersed water, indicating an increase in the motion of water molecules. This results demonstrates that the anesthetic weakened the hydrogen bond between water and glycerol-α-monooleate molecules, and released bound interfacial water. It is postulated that dehydration of the interface, as shown by the release of bound water, would interfere with the transport of current-carrying hydrated ions through membranes and may constitute a molecular mechanism of anesthesia.

Introduction

A misconception about anesthesia research is the frequently raised criticism that the observed effects in model systems may be caused equally by organic solvents other than general anesthetics. It is probably relevant to stress here that all lipophilic membrane perturbants, including gases, hydrocarbons, halogenated hydrocarbons, ethers, halogenated ethers and other organic solvents, are anesthetics when given sufficiently high partial pressures. The anesthetic potency of xenon was firmly established when Cullen and Gross [1] successfully anesthetized a patient solely with xenon gas for a surgical procedure. The anesthetic effect of nitrogen gas became evident in scuba diving; at about 30 atmospheric pressure, nitrogen gas is a potent anesthetic. Carbon dioxide anesthesia has been recognized as 'CO_2 narcosis' among pulmonary-crippled patients, and Eisele et al. [2] estimated that the MAC (minimal alveolar concentration of vapors that anesthetize 50% of the patients) value was 245 mmHg or 0.3 atm in dogs. These gases are obviously unsuitable for clinical application because of the high cost of xenon, the high pressure required for nitrogen gas, and the

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pH disturbance caused by carbon dioxide. A myriad of organic solvents are precluded from clinical uses, not from the lack of anesthetic potency but because of toxicity to vital organs. The only exception is helium; the partial pressure required for anesthesia is so high that the ordering effect of pressure counterbalances the disordering effect of the gas; hence, anesthesia does not occur.

Another important feature of anesthetics is that weakly dipolar hydrophobic molecules are stronger anesthetics compared to the apolar counterparts. This is obvious when one compares methane or carbon tetrachloride against chloroform, ethane against ethanol or halothane ($CF_3CHClBr$), and ethylene against trichlorethylene. (This solvent was one of the standard clinical anesthetic agents.) This feature was used in the drug design for the development of the first inflammable modern agent, halothane [3]. This property suggests (but does not prove) that the action site of inhalation anesthetics may be the surface of membranes or macromolecules rather than the hydrophobic core.

We have reported [4–13] the interfacial preference in the action of modern inhalation anesthetics. When monitored by proton nuclear magnetic resonance spectroscopy, these weakly dipolar anesthetics induced phase transition in the interfacial choline protons of dipalmitoylphosphatidylcholine vesicle membranes at lower concentrations compared with the lipid-core protons, whereas thermally induced phase transition occurred simultaneously in both regions [5]. A proton nuclear magnetic resonance study also showed that the single proton of the halogenated end of an inhalation anesthetic, methoxyflurane $CH_3 \cdot O \cdot CF_2CCl_2H$, did not lose contact with the aqueous phase when bound to a surfactant micelle at clinical concentrations [6]. From the dependency of electrical capacitance and conductance of a giant planar lipid bilayer (BLM) on the anesthetic concentrations, we have shown that these modern anesthetics are adsorbed to the interface at clinical concentrations without penetrating into the hydrophobic core [12,13].

The fact that the stable formation of lipid membranes requires the very existence of water molecules indicates that the water phase is not a simple supporting matrix for the membrane but that interaction between water and lipid molecules is essential. It is generally accepted that there are clusters of bound water molecules at the macromolecular surface which show different characteristics from free bulk water molecules. The physical properties of these bound water molecules may affect the structure and stability of the membranes, and may participate in the membrane function, such as ion permeability and selectivity. The interfacial adsorption of anesthetic molecules, which have a relative electrical permittivity of about 4 to 6 [12], is expected to decrease the relative electrical permittivity of the interface and decrease the interacting force of the hydrophilic domain to water molecules, leading to changes in the membrane structure and function.

The dispersed water molecules in w/o (water in oil) emulsion interact strongly with the hydrophilic moiety of the surfactants dissolved in the oil phase (see, for instance, Refs. 14–17). Generally, in the lipid vesicle or planar bilayer systems, the number of bound water molecules are much less than the number of free water molecules. In the w/o emulsion system, however, most of the water molecules are in the bound form. In this communication, we used w/o emulsion as a model for bound water and the motion of water molecules was monitored by proton nuclear magnetic resonance spectroscopy. The effect of an inhalation anesthetic, enflurane $CHF_2 \cdot O \cdot CF_2CHFCl$, on proton movement in a w/o emulsion prepared from water in glycerol-α-monooleate and *n*-decane is reported.

Experimental

Glycerol-α-monooleate $C_{17}H_{33}COOCH_2CH(OH)CH_2(OH)$ was obtained from Sigma and its purity was confirmed by thin-layer chromatography on a silica-gel plate (Merck) to show a single spot. *n*-Decane was obtained from Tokyo Kasei (Tokyo) and was purified by passing through an aluminum oxide column (Wako Pure Chemical, Tokyo). Enflurane was a gift from Japan Abbott (Tokyo) and was used as received. All other chemicals were the highest grade available. Water was twice-distilled in all-glass stills.

Glycerol-α-monooleate was dissolved in *n*-decane in mole ratios between $1.8 \cdot 10^{-2}$ and $7.7 \cdot 10^{-2}$. A small amount of water was added by a microsyringe to the oil phase. Water-in-oil (w/o)

emulsion was prepared by ultrasonic irradiation using a Branson Model 185 Sonifier (Danbury, CT, U.S.A.). Enflurane was added to the mixture by a microsyringe. The emulsion was immediately transferred into an NMR tube (inner diameter 5 mm), tightly sealed and let stand for at least 30 min before the measurement. Tetramethylsilane was sealed in a capillary tube and placed in the sample tube.

^{1}H-NMR spectra of the sample were obtained by a Hitachi Perkin-Elmer R-20-B spectrometer 60 MHz. The sample temperature was maintained at 31.5 ± 0.5°C. Because of the difficulty in improving the small water signal against the large -CH_2- signal from the oil phase by the pulse-FT method using a 200 MHz spectrometer, the continuous-wave method was used. To ensure the accuracy of estimating the half-height width of the water signal, the stability of the spectrometer was checked by the constancy of the half-height width of the reference tetramethylsilane; the scatter of the width was confirmed to be within ± 0.2 Hz during the whole experiment. Three samples of identical composition were prepared and each sample was scanned three times. The chemical shift of the water proton was estimated from the tetramethylsilane signal. The sweep time was 200 s and the sweep width was 300 Hz.

Infrared spectra of glycerol-α-monooleate were measured by a Japan Spectroscopic Model IRA-1 (Tokyo) in a 0.5 cm lightpath cuvette with single crystal NaCl windows. Glycerol-α-monooleate was dissolved in carbontetrachloride.

Results and Discussion

The peak of the proton signal of bound water appeared at about 4.7 ppm (Fig. 1). When the composition of the oil phase was maintained at a constant value of mole ratio $7.4 \cdot 10^{-2}$ (glycerol-α-monooleate versus *n*-decane) and the water content was increased, the area under the peak of bound water was linearly increased. The area is expressed relative to the area under the peak of the two protons attached to the double bonds of glycerol-α-monooleate. Fig. 2 shows the linearity of the increase in the relative area of bound water as a function of the amount of water added to the oil phase. When extrapolated to zero water content, however, the line did not intersect the relative area axis at the zero point. This is caused by the overlapping of the proton signals of hydroxyl moiety of glycerol-α-monooleate and water. Because the unit relative area represents two protons and the plot at zero water content intersected at 0.55, this value is equivalent to about 1 proton;

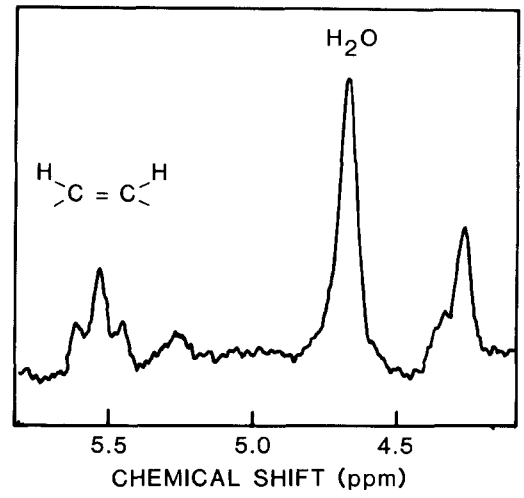

Fig. 1. Proton nuclear magnetic resonance spectrum of the water in oil emulsion. The oil phase consists of glycerol-α-monooleate dissolved in *n*-decane. Relevant peak assignments are shown in the figure.

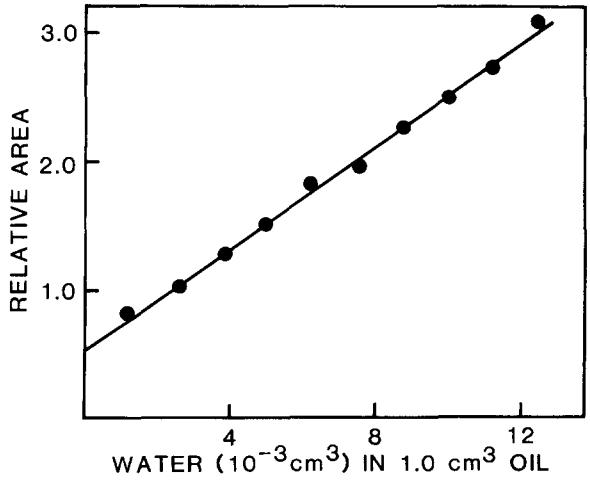

Fig. 2. The area under the H_2O proton peak as a function of water content. The area is expressed relative to the area under the signal of two protons attached to the double bond in oleate moiety.

among the two OH moieties present in glycerol-α-monooleate, one proton appears to form hydrogen bonding with water molecules in average.

Infrared spectra of glycerol-α-monooleate, dissolved in carbontetrachloride, showed two absorbance peaks of the O-H stretching band at 3580 cm^{-1} and 3480 cm^{-1}. The peak near 3600 cm^{-1} represents the free OH in stretching vibrational motion whereas that close to 3400 cm^{-1} indicates the hydrogen-bonded OH [18]. When the glycerol-α-monooleate concentration was increased, the logarithm of the absorbance ratio, $\ln(I_o/I)$, where I_o is the absorbance in the absence of glycerol-α-monooleate, increased linearly with the increase of the glycerol-α-monooleate concentration until about $2 \cdot 10^{-2}$ M, in accordance with the Lambert-Beer law. This indicates that the hydrogen bond is intramolecular. When the glycerol-α-monooleate concentration exceeds $2 \cdot 10^{-2}$ M, the values of $\ln(I_o/I)$ of both peaks deviate from the straight line, indicating that the proportion of the number of the free OH relative to the hydrogen-bonded OH started to decrease. In other words, intermolecular hydrogen bonds started to increase in exchange for the relative decrease of intramolecular hydrogen bonds. From these findings, it may be concluded that at least one of the two OH moieties of the glycerol-α-monooleate molecule forms intra- or intermolecular hydrogen bonding.

When the composition of the oil phase was kept constant (mole ratio $7.4 \cdot 10^{-2}$ glycerol-α-monooleate/*n*-decane) and the water content was increased, the half-height width ($\nu_{1/2}$) of the water peak started to decrease and approached a limiting value. This $\nu_{1/2}$ is related to the relaxation time according to the following equation.

$$\nu_{1/2} = 1/(2\pi T_2^*) \qquad (1)$$

where T_2^* is the spin-spin relaxation time, including contributions from magnetic field inhomogeneity. To the first approximation, this T_2^* equals the true T_2 and provides information about the overall motion of H_2O molecules, i.e., when the motion is increased, T_2^* increases and $\nu_{1/2}$ becomes smaller.

The increase of water mass would increase the number of hydrogen-bonded water molecules. It would also increase the number of free water molecules in the bulk water phase contained in the reversed micelles, since the number of water molecules that can bind to one glycerol-α-monooleate molecule is limited. Because the H_2O molecules in the water phase exchange rapidly with those hydrogen-bonded to glycerol-α-monooleate molecules, the averaged motion of H_2O molecules increases when compared with low water concentrations where entire water molecules are hydrogen-bonded to glycerol-α-monooleate. Hence, addition of water to the oil phase in excess of a certain limit leads to the increase in the number of free water molecules and the averaged motion of water molecules approaches that of unbound free water. This corresponds to the decrease of $\nu_{1/2}$ according to Eqn. 1 and matches the experimental data.

The $\nu_{1/2}$ values of the dispersed water also varied according to the composition of the oil phase as shown in Fig. 3. In this case, the water concentration was maintained at a constant value ($8 \cdot 10^{-3}$ cm^3 water in 1.0 cm^3 oil phase). When the mole ratio (glycerol-α-monooleate/*n*-decane) was about $4 \cdot 10^{-2}$, the $\nu_{1/2}$ value showed a maximal value. The cause of this maximum is not immediately apparent, but based on the analogy of the report on the phosphatidylcholine-hexane system [19], it may be assumed that the assembly

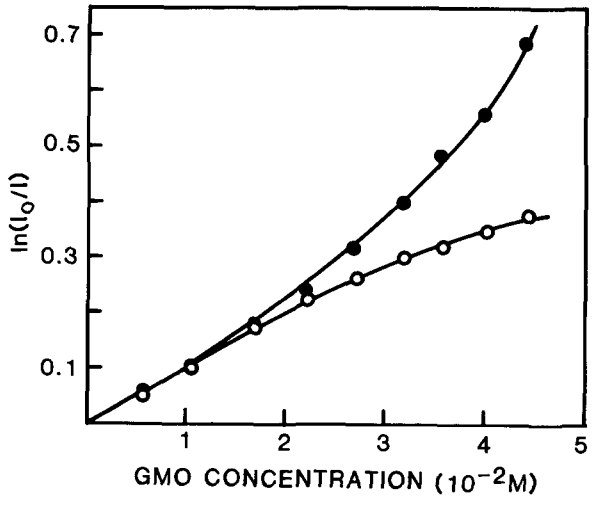

Fig. 3. Glycerol-α-monooleate (GMO) concentration versus $\ln(I_o/I)$ where I_o is the infrared absorbance in the absence of glycerol-α-monooleate . Open circles are absorbance at 3580 cm^{-1}, and filled circles are at 3480 cm^{-1}. At high glycerol-α-monooleate concentration, the 3580 cm^{-1} absorbance started to decrease and the 3480 cm^{-1} absorbance started to increase.

mode of glycerol-α-monooleate molecules is different between the low and high glycerol-α-monooleate concentrations and is separated at the mole ratio $4 \cdot 10^{-2}$: for instance, spherical reverse micelle and cylindrical reverse micelle.

When enflurane was added to the emulsion, the $\nu_{1/2}$ value decreased. At $8 \cdot 10^{-5}$ M enflurane concentration, the decrement was about 1.0 Hz (Fig. 4). According to Eqn. 1, this result indicates that T_2^* is increased, i.e., the motion of H_2O molecules is enhanced. Enflurane appears to weaken the hydrogen bond between H_2O and glycerol-α-monooleate molecules. Because the action was evident in the entire range of glycerol-α-monooleate concentrations in the oil phase, the anesthetic appears to release bound water molecules irrespective of the assembly mode of glycerol-α-monooleate molecules.

Hydrogen-bond breaking activity of inhalation anesthetics has been extensively studied by Sandorfy and associates [20–23] using infrared spectrophotometry. They have shown that an excellent correlation exists between the anesthetic potency and the hydrogen-bond breaking activity. We have shown that modern inhalation anesthetics

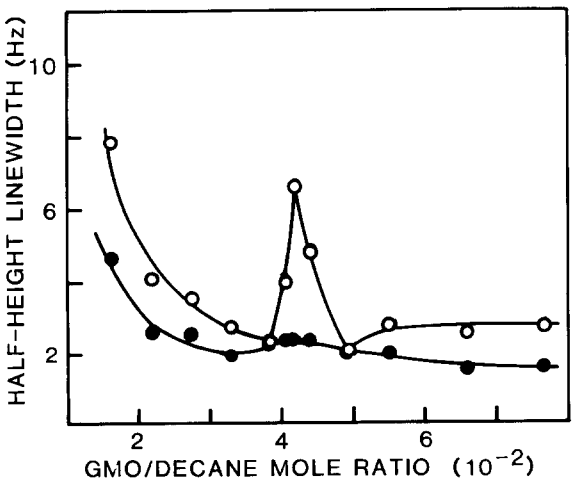

Fig. 4. Half-height linewidth of water proton plotted against the mole ratio between glycerol-α-monooleate and *n*-decane. Water content was constant at $8 \cdot 10^{-3}$ cm ^{3} in 1.0 cm^3 of the oil phase. Open circles are the control without enflurane, and filled circles are in the presence of enflurane $8 \cdot 10^{-2}$ mM.

tend to accumulate at the membrane-water interface and decrease the relative electrical permittivity of the interface [12,13], as discussed in the introduction. This would weaken the effective electrical field of the interface (whether it is membranes or proteins) interacting with the H_2O dipole and release bound water molecules. We postulate that the increased hydrophobicity of the water/macromolecule interface, evidenced by the release of bound water molecules, may become a barrier to the transport of hydrated ions through the membrane and constitutes a molecular mechanism of anesthesia.

In contrast to our theory, lipid-tail disorder or the so-called fluidity increase of membranes, often expressed by fluorescence anisotropy or electron paramagnetic resonance hyperfine splitting of hydrophobic probe molecules incoporated into the membrane, has been generally advocated for the mechanism of anesthesia. But this does not explain why apolar molecules, such as propane, butane, cyclopropane, etc., that preferentially localize in the center of lipid bilayers [24] and are primarily lipid-tail perturbants, are a weaker anesthetic than slightly polar molecules such as chloroform and modern inhalation anesthetics that tend to saturate the membrane/water interface before penetrating into the membrane core.

Perturbation of the lipid core causes lateral expansion of the membrane and decreases surface density of the hydrophilic moiety of membrane molecules. It is entirely possibly to explain the weaker anesthetic potency of apolar molecules on the ground that the action is not directly oriented to the interface: secondary to their effect upon the order of the lipid tail, decreasing hydrophilicity of the membrane interface. The so-called disordering or fluidizing effects of anesthetics may not be directly related to the molecular mechanism of anesthesia.

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