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DO CLINICAL LEVELS OF GENERAL ANAESTHETICS AFFECT LIPID BILAYERS?

EVIDENCE FROM RAMAN SCATTERING

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We have used Raman spectroscopy to investigate the effects of the general anaesthetics halothane and chloroform on lipid bilayer order. Clinical concentrations of these anaesthetics had no significant effect on the hydrocarbon chain conformation in multilamellar vesicles of dimyristoylphosphatidylcholine/cholesterol. This result was obtained with a technique sufficiently precise to monitor changes in the acyl chain *trans-gauche* population ratio associated with a 1–2 K alteration in temperature. Very high levels of anaesthetics caused a marked disordering of the hydrocarbon chains. The danger of inferring an effect at clinical concentrations from data obtained at much higher levels is illustrated by a statistical analysis of our dose-response curves.

Introduction

Although a variety of theoretical and experimental descriptions of general anaesthesia implicate the lipid bilayer of nerve membranes as the primary site of action, recent studies have thrown doubt on this hypothesis [1–4]. In view of both the importance of the problem and the contradictory nature of the conclusions reached thus far, we felt it worthwhile to reinvestigate this problem using an independent physical technique (Raman scattering) which offers several unique advantages.

Raman spectroscopy has been widely used to study lipid conformations in bilayers (For reviews, see Refs. 5 and 6). In contrast to methods such as electron spin resonance (ESR) and fluorescence spectroscopy, this method does not require the use of probe molecules, which may either perturb the bilayer or interact with the anaesthetics under investigation. Certain features in the Raman spectrum are directly sensitive to *trans-gauche* isomerizations in the phospholipid hydrocarbon chains and thus provide a virtually instantaneous snapshot of chain conformation [7,8]. Because the Raman experiment reports on molecular vibrations in the subpicosecond time range, it monitors events which are averaged over during the 10^{-6}–10^{-9} s periods sampled in nuclear magnetic resonance (NMR) and ESR spectroscopy. Finally, the technique is useful as a supplement to diffraction techniques, in that it can sample a wide variety of bilayer preparations (e.g. films cast on metal surfaces, multilamellar and unilamellar vesicles) without loss of information.

Nerve cell membranes contain high concentrations of cholesterol [9,10]. In this paper, we describe the results of a Raman spectroscopic investigation of the effects of halothane, chloroform,

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and temperature on a simple model for the lipid bilayer portion of these membranes, namely multibilayer vesicles of a mixture of dimyristoylphosphatidylcholine and 40% (molar) cholesterol.

Materials and Methods

Materials

Dimyristoylphosphatidylcholine and cholesterol (chromatography grade) were obtained from Sigma Chemical Co. The phosphatidylcholine showed a single spot with the appropriate R_F value by thin-layer chromatography ($CHCl_3/CH_3OH/H_2O$, 65:25:4, v/v) with I_2 staining and showed a differential-scanning calorimetry curve in good agreement with literature values for both the pretransition and the main endotherm [11]. For the Raman experiments, halothane was obtained from Abbott Laboratories and chloroform (spectroscopic grade) was obtained from Fisher Scientific Co. For the partition coefficient experiments, halothane was obtained as Fluothane from Imperial Chemical Industries Ltd., while chloroform (Analar) was from BDH Chemicals Ltd. [1-^{14}C]Halothane and [^{14}C]chloroform were from New England Nuclear Corp.; both of these radioactive compounds were found to contain impurities which had to be removed (see below) before being used to determine partition coefficients.

Sample preparation

Weighed quantities of lipids were dissolved in chloroform or chloroform/methanol (10:1, v/v). The organic solvent was evaporated under a stream of N_2 gas; complete removal was achieved under high vacuum for several hours. A lipid stock solution was then prepared by hydrating the dried lipid with sodium phosphate buffer (10 mM, pH 7.2) containing 150 mM NaCl. Complete dispersal of the lipid was obtained by extensive mixing by vortex action.

For the Raman experiments, the appropriate amount of anaesthetic was added to aliquots of the lipid stock solution, using Hamilton syringes and either the neat anaesthetic liquid or a solution of this in buffer. Care was taken to minimize the volume of air in contact with all solutions containing anaesthetics, to avoid the inadvertent escape of anaesthetic. The precaution was especially important when using halothane, since its water/air partition coefficient (Ostwald solubility coefficient) is only 1.5 at 25°C [12]. (For example, had a stoppered flask been only half filled with a halothane buffer solution, at equilibrium 40% of the halothane would have been in the gas phase and could have escaped upon unstoppering the flask.)

If the lipid/buffer partition coefficient K ($\equiv$ moles of anaesthetic/volume of lipid divided by moles of anaesthetic/volume of buffer) had been constant, the equilibrium concentration c_{buf} of anaesthetic in the aqueous phase of the Raman specimen could have been calculated directly from the formula

$$c_{buf} = \frac{N_{tot}}{KV_{lip} + V_{buf}} \tag{1}$$

where N_{tot} is the total amount of anaesthetic added, V_{buf} is the volume of buffer and V_{lip} is the volume of lipid (calculated from the lipid weight, using a mass density of 1.035 $g \cdot cm^{-3}$ [2,4]) in the specimen. However, since (see below) K was found to vary linearly with c_{buf} in the range studied, i.e.

$$K = K_0 + mc_{buf} \tag{2}$$

where K_0 and m are positive constants at 25°C, it was necessary to solve Eqns. 1 and 2 simultaneously for c_{buf}, yielding

$$c_{buf} = \left\{ -\left(K_0V_{lip} + V_{buf}\right) + \left(\left(K_0V_{lip} + V_{buf}\right)^2 + 4mV_{lip}N_{tot}\right)^{1/2} \right\} \left\{2mV_{lip}\right\}^{-1} \tag{3}$$

Eqn 3 was used to calculate all values of c_{buf} presented in this paper, using the least squares estimates of K_0 and m obtained from our experimental data and reported below.

Portions of this equilibrated solution were injected using Hamilton syringes directly into the 1.5 mm i.d. melting point capillary tubes used for Raman spectroscopic measurements. The capillary tubes were immediately sealed in a flame, and the lipids were packed at one end of the tube using a hematocrit centrifuge.

Raman spectroscopy

Our Raman apparatus consists of a Jarrell-Ash 0.75 m double monochromator interfaced to a Spex Industries data acquisition system (SCAMP). The excitation source is a Spectra-Physics Model 164 Ar^+ laser. Scattered light is detected with an RCA C31034 phototube, cooled to −20°C, and a Spex Industries DPC-2 photon counter.

Raman spectra were obtained using standard digital techniques. The normalized photon counter output was sampled for a given length of time at a given wavenumber setting of the monochromator, usually 1 or 2 s at each wavenumber. The monochromator was then stepped to a new wavenumber setting and the cycle repeated. The spectrum was acquired into one of the 4 'quadrants' (1024 points) available on the SCAMP. Signal averaging was accomplished using multiple scans.

The wavenumber range of interest for each sample was scanned 15–20 times. The resultant summed data was smoothed using a 7-point Savitsky-Golay statistical routine [13]. The intensity ratio I_{1130}/I_{1090} was measured as the ratio of peak heights at the position of maximum intensity above a baseline drawn to connect minima in the spectra near 1035 and 1155 cm^{-1}. It is known that, as conformational order in the bilayer decreases, the frequencies at 1130 and 1090 cm^{-1} actually shift by as much as 5–8 cm^{-1}. The origin of these shifts has been discussed elsewhere [5].

Extensive precautions to insure uniform experimental conditions were necessary in order to obtain intensity data reproducible to 2% or better (duplicate measurements on one set of samples from a given batch of lipid) or 3–4% (samples from different batches of lipids). Laser power was kept at 100–125 mW of 5145 Å excitation. Power levels above 200 mW led to reduced precision, possibly due to local heating of the sample.

The origin of the reduced precision in I_{1130}/I_{1090} with samples from different batches of lipid compared to the precision for duplicate measurements within one batch is probably related to such factors as slight variations in the DMPC/cholesterol ratio, average vesicle size (thereby affecting acyl chain packing characteristics), etc. In order to normalize for comparative purposes data sets obtained from different lipid batches, values of I_{1130}/I_{1090} were multiplied by a single constant factor for each set so that values of the ratio for zero levels of anaesthetic matched between samples. Observed values were never adjusted by more than 3–4%.

Samples, contained in 1 mm i.d. melting point capillaries, were mounted in a massive thermostatically controlled brass block and were examined in the transverse mode. The temperature was regulated to ±0.25°C. The temperature was calibrated by determining the Raman melting behavior of DMPC and DPPC. T_m for the former was found to be 22.5°C (calorimetric value = 24.1°C) and 38°C for the latter (calorimetric value = 41.5°C). A linear calibration curve was established from these data.

Determination of partition coefficients

These were determined by equilibrium dialysis using ^{14}C-labelled halothane and chloroform. A stock solution of the labelled anaesthetic in anaesthetic saturated sodium phosphate buffer (10 mM, pH 7.2) containing 150 mM NaCl was first prepared by dissolving the labelled anaesthetic into buffer containing excess unlabelled liquid anaesthetic. For such experiments, it is essential that the label be confined to the anaesthetic molecules, since the presence of small amounts of impurity having different solubility characteristics can lead to misleading lipid/buffer partition coefficients. For this reason, the purity of the labelled anaesthetic was checked by measuring its partition coefficient between the neat anaesthetic liquid and buffer. Initially, these coefficients were found to be substantially less than expected from the known solubilities in water, indicating the presence of a small percentage of hydrophilic labelled impurity. This impurity was removed by repeatedly washing the neat anaesthetic liquid with fresh buffer, until the anaesthetic/buffer partition coefficients reached limiting values close to the theoretical values.

Equilibrium dialysis was performed using Perspex (Lucite) cells having a capacity of about 1 ml in each chamber. Twelve cells were rotated together at 25°C in a dark, temperature-controlled room until the control cells had reached equilibrium (6–24 h). Each chamber was separated by a single thickness of Visking dialysis tubing, which had previously been cleaned by boiling for 1–2 h

in first 1% $NaHCO_3$, then 10^{-4} M EDTA, and then distilled water, followed by repeated washing at room temperature in the dialysis buffer. One chamber of each dialysis cell contained the lipid stock solution (3–4% lipid by weight), while the opposite chamber contained appropriate dilutions of the radioactive stock buffer. To obtain the highest equilibrium anaesthetic concentrations, in some cells the radioactive buffer was removed from its chamber after a few hours of dialysis and replaced with fresh radioactive stock buffer.

After equilibrium had been achieved, aliquots of the paired lipid and buffer solutions from each cell were rapidly transferred using glass (Pasteur) pipettes to prepared and preweighed glass scintillation vials; the exact amount of solution was then determined, to an accuracy of about 0.1%, by weighing the loaded vials. The vials had been prepared by adding a fixed volume of scintillation fluid and an appropriate volume of either unlabelled buffer or unlabelled lipid solution to ensure that each vial would have the same quench correction. The scintillation fluid was made up by dissolving 12.1 g of Permablend III (Packard Instrument Co.) in 1375 ml of toluene (A.R. grade) and then mixing in 500 ml of Triton X-100 (BDH Chemicals Ltd.; scintillation grade).

The total activity of each vial was measured in either a Packard Tri-Carb 2660 or a Nuclear Chicago Mark II scintillation counter. For each dialysis cell, then, the activities per unit volume $c^{\star}_{sus}$ and $c^{\star}_{buf}$ for the lipid suspension and buffer chambers, respectively, could be calculated directly. Denoting the ratio of these by

$$R \equiv c^{\star}_{sus}/c^{\star}_{buf} \tag{4}$$

the partition coefficient K (concentration of anaesthetic per unit volume of membrane divided by concentration per unit volume of buffer) was determined using the formula

$$K = R + (V_{buf}/V_{lip})(R-1) \tag{5}$$

where V_{buf}/V_{lip} is the ratio of buffer to lipid volumes in the lipid suspension. Finally, the total concentration c_{buf} of anaesthetic in the buffer of both dialysis chambers was found using

$$c_{buf} = c_{sat}(c^{\star}_{buf}/c^{\star}_{sat}) \tag{6}$$

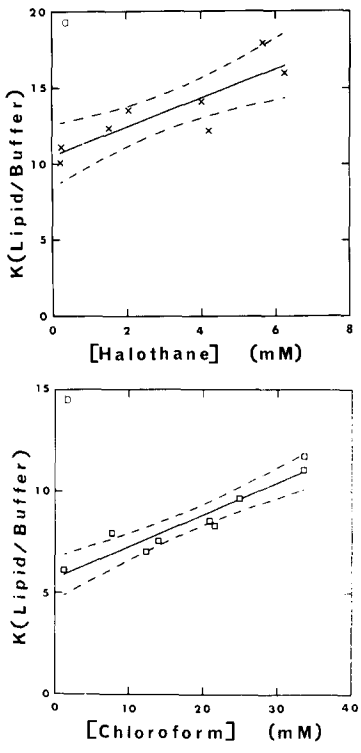

Fig. 1. Partition coefficients of halothane (a, ×) and chloroform (b, □) as a function of the equilibrium concentration of anaesthetic in the aqueous phase. The solid lines are the least-squares straight lines; the dashed lines are the 95% confidence envelopes. The least squares lines are (a) $K = 10.5 + 0.954\ c_{buf}$ for halothane and (b) $K = 5.68 + 0.157\ c_{buf}$ for chloroform, where K is the partition coefficient (moles of anaesthetic/litre of membrane divided by moles of anaesthetic/litre of buffer) for multi-bilayer dispersions of dimyristoylphosphatidylcholine and 40% (molar) cholesterol, and c_{buf} is the concentration of anaesthetic in mmol/litre of buffer.

where c_{sat} and $c^{\star}_{sat}$ are the total concentration of anaesthetic and the activity per unit volume, respectively, of the saturated radioactive stock buffer solution.

The resulting plots of K versus c_{buf} are shown in Fig. 1a for halothane and in Fig. 1b for chloroform. Notice that, for both anaesthetics, the partition coefficient shows a significant (at the 95% level) increase with increasing concentration of anaesthetic.

Results

Typical Raman data in the region 1020–1150 cm^{-1} are shown in Fig. 2 for DMPC-cholesterol

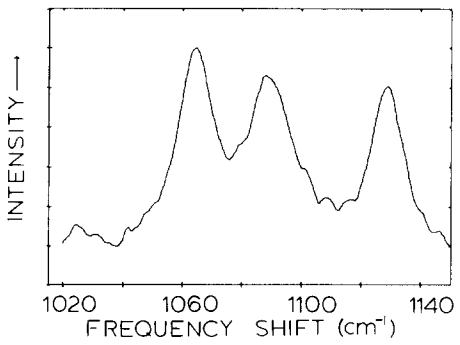

Fig. 2. Typical Raman data. The sample was DMPC/cholesterol in multilamellar vesicle form containing no added anaesthetic. 100 mW of 5145 Å was used for excitation. 20 scans were added together, smoothed as described [13], and flattened to produce a horizontal baseline.

(60:40, mol/mol) at 26°C. The three major peaks near 1060, 1090 and 1130 cm^{-1} arise primarily from C-C stretching modes of the phospholipid hydrocarbon chains, [7,14] although the band at 1090 cm^{-1} includes a small contribution from the O = P = O symmetric stretching mode [15]. The contribution of cholesterol to this spectral region has been shown to be negligible [7]. The band at 1130 cm^{-1} arises from skeletal C–C stretching vibrations from chain segments in the all-*trans* conformation, while the band at 1090 cm^{-1} arises from chain segments containing *gauche* rotamers [14]. The ratio of the peak intensities of these two bands (I_{1130}/I_{1090}) is thus a convenient measure of lipid conformation and has been widely used in this role [5,6]. Quantitative correlation of this ratio with the exact number of *gauche* rotamers is difficult because the intensity ratio varies non-linearly with the number and with the location along the chains of the *gauche* rotamers [16]. Nevertheless, it is a very useful indicator of lipid fluidity/order which can be measured with great precision (S.E. ≈ 1–2%) using computer averaging of repeated scans.

Lack of an effect at low concentrations of anaesthetics

In Fig. 3a we plot this intensity ratio as a function of halothane concentration in the aqueous phase for relatively low concentrations of halothane. Also indicated on the figure is the minimum aqueous concentration of halothane

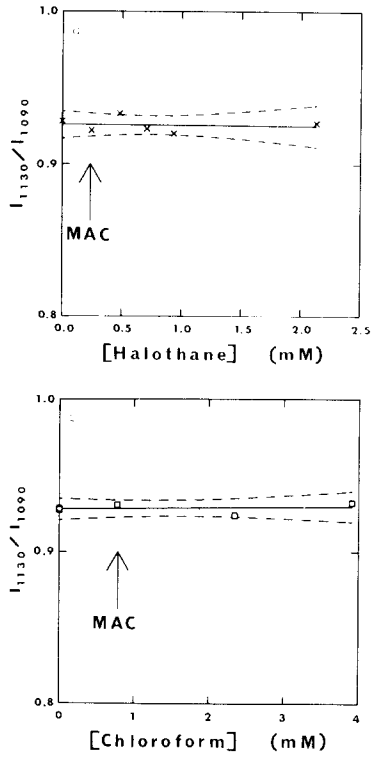

Fig. 3. Lack of an effect of clinical concentrations of general anaesthetics upon the Raman spectrum. The Raman intensity ratio I_{1130}/I_{1090} is plotted against the equilibrium concentration of anaesthetic in the aqueous phase, for halothane (a, ×) and for chloroform (b, □). The solid lines are the least squares straight lines, while the dashed curves are the 95% confidence envelopes. The slopes (±S.E.) of the straight lines are (a) −0.34 (±3.16) M^{-1} for halothane and (b) 0.41 (±1.02) M^{-1} for chloroform. The arrows indicate the minimum alveolar concentrations (MAC) for man.

needed to maintain general anaesthesia in man. (This concentration was calculated from the known partial pressure P(atm) needed to maintain general anaesthesia (commonly called the minimum alveolar concentration or MAC) using this formula [1]:

$$c_{buf} = \frac{P\lambda}{RT} \tag{7}$$

where λ is the water/gas partition coefficient (Ostwald solubility coefficient) for the anaesthetic vapour, R is the gas constant (0.082 litre · atm · deg^{-1} · mol^{-1}), and T is the absolute temperature in Kelvin. For halothane, using the values [17] for man of a minimum alveolar concentration partial

pressure $P = 0.0075$ atm and an Ostwald solubility coefficient $\lambda = 0.8$ at body temperature ($T = 310$ K), Eqn. 7 yields $c_{buf} = 0.24$ mM halothane as the human minimum alveolar concentration value in the aqueous phase. The least squares line through the data points of Fig. 3a has a slope which is not statistically different from zero.

The corresponding results for low levels of chloroform are given in Fig. 3b, where we have plotted the intensity ratio I_{1130}/I_{1090} versus the chloroform concentration in the aqueous phase from 0 to about 5-times the minimum alveolar concentration. (A minimum alveolar concentration value of $c_{buf} = 0.79$ mM chloroform was calculated using Eqn. 7 and the values [17] for man of $P = 0.005$ atm and $\lambda = 4.0$ at the body temperature $T = 310$ K.) Once again, the least squares line through these data points had a slope which was not statistically different from zero.

Effects of very high concentrations of anaesthetics

Although we found no significant effects of halothane or chloroform at clinical or up to at least five times clinical concentrations, the intensity ratio I_{1130}/I_{1090} does decrease significantly at very high concentrations of anaesthetic. This is shown for halothane in Fig. 4a and for chloroform in Fig. 5a, where the intensity ratio has been plotted for anaesthetic concentrations up to about 50-times the minimum alveolar concentration for man. Raman spectra for zero and about 37-times

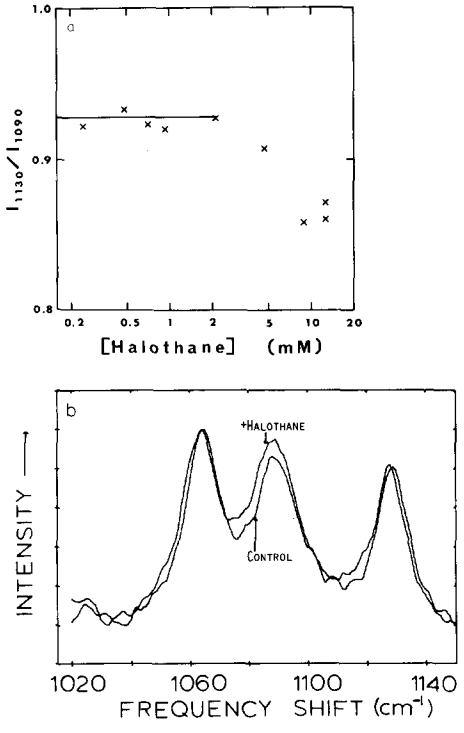

Fig. 4. Effects of very high concentrations of halothane upon the Raman spectrum. (a) I_{1130}/I_{1090} versus the equilibrium concentration of halothane in the aqueous phase. Notice that the concentration is on a logarithmic scale. The height of the horizontal line drawn to 2.1 mM halothane (about 9-times the human minimum alveolar concentration) gives the intensity ratio in the absence of halothane. (b) Raman spectra in the absence of anaesthetic (Control) and in the presence of 8.84 mM halothane (about 37-times the human minimum alveolar concentration).

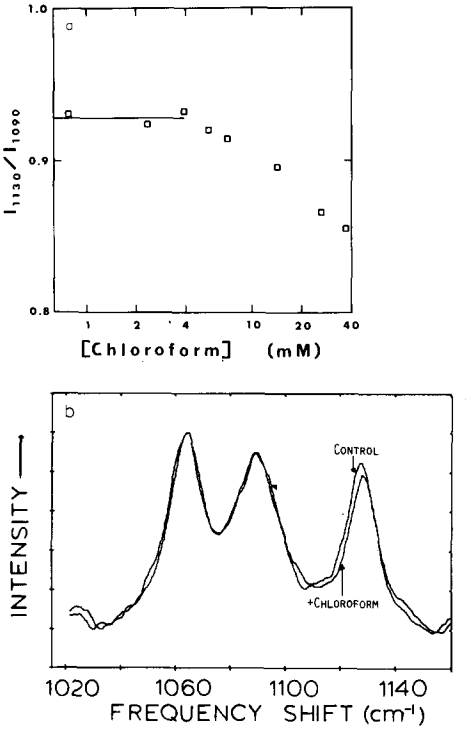

Fig. 5. Effects of very high concentrations of chloroform upon the Raman spectrum. (a) I_{1130}/I_{1090} versus the equilibrium concentration of chloroform in the aqueous phase. Notice that the concentration is on a logarithmic scale. The height of the horizontal line drawn to 3.9 mM chloroform (about 5-times the human minimum alveolar concentration) gives the intensity ratio in the absence of anaesthetic. (b) Raman spectra in the absence of anaesthetic (Control) and in the presence of 26.2 mM chloroform (about 33-times the human minimum alveolar concentration).

the minimum alveolar concentration are shown in Fig. 4b for halothane, while data for zero and about 33-times the minimum alveolar concentration are shown in Fig. 5b for chloroform.

Effects of temperature

Our negative findings at low anaesthetic concentrations might conceivably been due to a lack of sensitivity of the Raman technique. We therefore tested the sensitivity by measuring the intensity ratio I_{1130}/I_{1090} over the temperature range 5 to 45°C. The results are shown in Fig. 6a, and spectra at three temperatures are shown in Fig. 6b. The slope (±S.E.) of the least squares straight line (Fig. 6a) relating the intensity ratio to temperature is −0.0122 (±0.0004) K^{-1}, which corresponds to a change of 1.4% per degree at the temperature at which the anaesthetic experiments were performed. Since the 95% confidence envelopes for the intensity ratio in the anaesthetic plots of Figs. 3a and 3b differ from the least squares lines by at most 1.5%, this means that, had clinical concentrations of anaesthetic produced an effect on the bilayer similar to that produced by a change in temperature of only 1–2 K, this effect would have been observed.

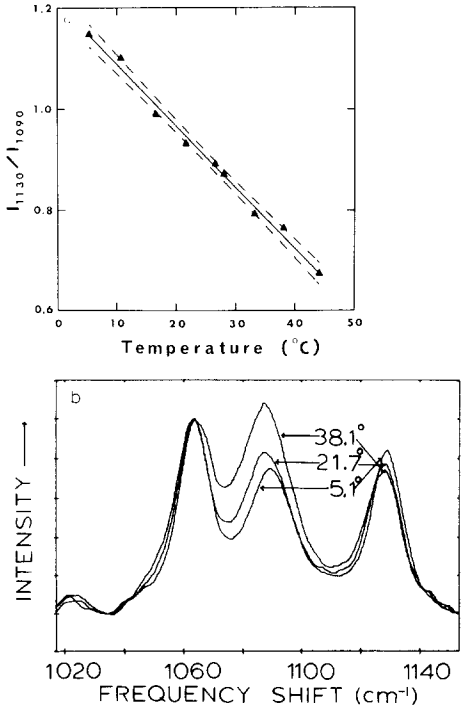

Fig. 6. Effect of temperature upon the Raman spectrum. (a) I_{1130}/I_{1090} versus temperature. The solid line is the least-squares straight line, with a slope (±S.E.) of −0.0122 (±0.0004) K^{-1}). The dashed curves are the 95% confidence envelopes. (b) Raman spectral data at various temperatures.

Discussion

The most important result of our work is that surgical concentrations of the inhalational general anaesthetics halothane and chloroform do not induce significant conformational changes in the phospholipid hydrocarbon chains of phosphatidylcholine/cholesterol bilayers. It is unlikely, therefore, that the lipid bilayer per se is the primary site of action in general anaesthesia.

Our negative results cannot be attributed to any lack of sensitivity in the Raman technique. Of course, when an anaesthetic enters a bilayer, the packing of lipid molecules next to the anaesthetic molecules must be somewhat altered, and it is reasonable to suppose that some technique might be capable of sensing this. The fact that a given technique, such as Raman scattering, does not detect this does not necessarily mean that nothing happens. What is needed is some type of yardstick against which to calibrate the observed responses

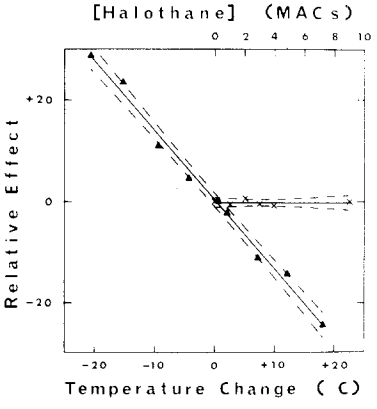

Fig. 7. Comparison of the effects of temperature and low concentrations of halothane. Relative values of the Raman intensity ratio I_{1130}/I_{1090} are plotted against the change in temperature (▲) from 26°C in the absence of anaesthetic and against halothane concentration (×) (expressed as multiples of the human minimum alveolar concentration (MAC)) at a fixed temperature of 26°C. The solid lines are least squares straight lines, while the dashed lines are the 95% confidence envelopes.

against those in situations where conformational order is known to be disturbed. We chose temperature for this purpose. In fig. 7 we have plotted, on the same graph, the relative effects on the Raman intensity parameter I_{1130}/I_{1090} of changes in both temperature and low concentrations of halothane. It is clear that, even at the 95% confidence level, we could easily detect effects due to a temperature change of only a few degrees, whereas halothane at up to 9-times its surgical concentration produced no significant effect. Since the body temperature of many animals fluctuates over a much larger range than this without causing general anaesthesia, any possible slight effect of halothane undetected by our technique seems most unlikely to be relevant to the production of general anaesthesia.

Our present findings extend to the subpicosecond time domain prior negative results obtained with lipid bilayers using X-ray and neutron diffraction [1,4], ESR [2] and ^{2}H-NMR [18]. However, there are innumerable other reports which suggest that perturbations of the lipid bilayer are involved in the induction of general anaesthesia. How do we account for these? As has been previously pointed out [4], most studies which report positive effects have relied on working at higher than surgical concentrations of general anaesthetics and interpolating back to clinical levels. Some of the difficulties of this approach can be seen using Fig. 8. Here we have plotted the intensity ratio I_{1130}/I_{1090} versus chloroform concentration in the range 0 to about 50-times the human minimum alveolar concentration, omitting data points at low concentrations. Had we relied only on these data at high concentrations and assumed a linear relationship between the intensity ratio and concentration, we might have erroneously inferred a real decrease in the parameter (and hence an increase in the number of *gauche* rotamers) at the human minimum alveolar concentration. This would have been clearly incorrect. As is evident from the size of the 95% confidence intervals at 1 MAC, there is no significant effect at surgical concentrations of chloroform. Thus it is not valid to simply assume that because one detects a real effect at high anaesthetic concentrations that a reduced yet nonetheless real effect would occur at surgical concentrations. It is striking that those studies [1,2,4] which have shown no effects on lipid bilayers at surgical concentrations did in fact utilize clinical concentrations, whereas most studies which have reported positive effects are based on experimental results at higher concentrations.

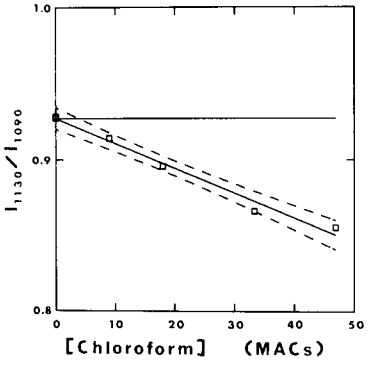

Fig. 8. The interpolation fallacy. We have plotted the Raman intensity ratio I_{1130}/I_{1090} versus chloroform concentration, purposely omitting data points at low concentrations. The chloroform concentrations are on a linear scale, expressed as multiples of the human minimum alveolar concentration. The downwards sloping solid line is the least-squares straight line; it has a slope of -0.18% per MAC, when expressed in terms of the intercept at 0 MAC. The dashed lines are the 95% confidence envelopes. The horizontal straight line, having the ordinate value of the intercept, is the line of 'no effect'.

There is, nonetheless, one effect of clinical concentrations of general anaesthetics upon lipid bilayers which is well-documented [19,20]. This is the lowering of the gel to liquid crystal phase transition temperature (T_m) of pure phospholipid bilayers. However, it is most doubtful that this effect has any relevance to anaesthesia (see Refs. 1, 3 and 4 for discussions). Among other reasons, this is because such pure phospholipid systems, with highly cooperative phase transitions, are not good models for nerve plasma membranes. The latter contain high concentrations of cholesterol (similar to those used in our investigation), which are known to abolish the cooperative nature of the phase transition.

An important feature of our experiments was that we corrected for the amount of anaesthetic taken up by the lipid membranes, in order to obtain true equilibrium buffer concentrations of anaesthetic using Eqn. 3. This allowed us to compare our anaesthetic concentrations with known clinical concentrations. To perform these correc-

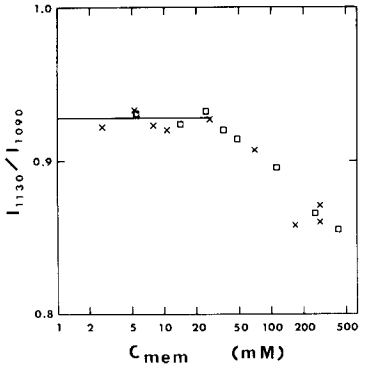

Fig. 9. Comparison of the effects of halothane (×) and of chloroform (□) as a function of their concentrations in the membrane. The horizontal straight line drawn from 1 to about 30 mmol/litre of membrane gives the average value of the intensity ratio I_{1130}/I_{1090} at zero anaesthetic concentration. Notice that concentration is plotted on a logarithmic scale.

tions, we first had to determine the membrane/buffer partition coefficients for both halothane and chloroform. As can be seen from Fig. 1, there is a very significant increase in partition coefficient with anaesthetic concentration, so that it is not permissible to obtain and use partition coefficients determined at only one (usually limitingly low) concentration. From solubility values, one can calculate that the partition coefficient between neat liquid anaesthetic and water is about 500 for halothane and 180 for chloroform at room temperature. These values are much higher than those between membranes and water, so that it is not surprising that as more and more anaesthetic partitions into the membrane and anaesthetic-anaesthetic interactions in the membrane phase become more important, the membrane/water partition coefficients increase.

An advantage of knowing the membrane/buffer partition coefficients as functions of the buffer anaesthetic concentrations was that we were able to determine the variation of the Raman intensity parameter I_{1130}/I_{1090} with the concentration of anaesthetic in the membrane. The combined results for halothane (×) and for chloroform (□) are given in Fig. 9. A number of important conclusions follow from this figure. First, the intensity ratio follows the same relationship for both halothane and chloroform when expressed in terms of membrane anaesthetic concentrations. This means that a molecule of halothane has much the same effect as a molecule of chloroform in affecting or not affecting the number of *gauche* rotamers in the phospholipid hydrocarbon chains. Secondly, neither halothane nor chloroform has any significant effect until its membrane concentration exceeds about 30 mmol per litre of membrane. Above this concentration, both halothane and chloroform begin to cause a significant increase in the number of *gauche* rotamers.

We note in passing that Seeman [21] has reported that the propagation of action potentials along frog sciatic nerves is blocked by buffer concentrations of 5 mM halothane and 5 mM chloroform, whereas Larrabee and Posternak [22] reported that 13 mM chloroform is required to reduce by 50% the compound action potential in nerve fibres passing directly through the cat stellate ganglion. It can be seen from Figs. 4a and 5a that at these concentrations we begin to see an effect of these anaesthetics on the Raman intensity parameter I_{1130}/I_{1090}. It is thus just possible that the blockage of axonal conduction by these inhalational anaesthetics may involve direct effects upon the lipid bilayer portion of nerve plasma membranes.

The remainder of this discussion will be devoted to the effects of very high (but not saturating) concentrations of halothane and chloroform. Although these effects are not relevant to the problem of general anaesthesia, they are nonetheless interesting from the viewpoint of membrane structure and membrane physical chemistry. At these high concentrations, there is a very significant reduction in the Raman intensity ratio I_{1130}/I_{1090}, indicating an increase in the number of *gauche* rotamers (see Figs. 4 and 5). Further, as mentioned above, Fig. 9 shows that at equal membrane concentrations, halothane and chloroform are equally effective.

We first compare our Raman results with earlier X-ray and neutron diffraction results. Consistent with our present findings, Franks and Lieb [1,4] found, using phosphatidylcholine/cholesterol bilayers, no measurable change in bilayer structure at clinical concentrations of halothane, nitrous oxide and cyclopropane. At very high concentrations of halothane, however, they did find a significant disordering of the membranes [4]. Interest-

ingly, this disordering was not accompanied by any measurable change in bilayer thickness, at up to 50-times the human minimum aveolar concentration. Rand and Pangborn [23] measured the repeat spacings of lamellar phases of egg lecithin/cholesterol multilayers as a function of temperature and found a significant decrease with increasing temperature. Since the water content of their specimens was kept constant, it is reasonable to assume (as did the authors) that changes in the repeat spacing represented changes in the bilayer thickness. Thus it appears that increasing temperature causes phosphatidylcholine/cholesterol bilayers to thin. Further, our Raman results (Fig. 6) show that the number of *gauche* rotamers increases significantly with temperature.

Thus, increasing temperature appears to both disorder and thin phosphatidylcholine/cholesterol bilayers, whereas high concentrations of halothane only disorder the bilayers. The temperature results are easy to understand, since an increase in the number of *gauche* rotamers will decrease the effective length of each hydrocarbon chain. The results with high concentrations of halothane, however, are more difficult to interpret. How can the hydrocarbon chains become more disordered without the bilayer thinning? The answer may be that halothane is located mainly near the center of the membrane and thus has a tendency to increase membrane thickness merely because of the volume it occupies. On the other hand, the formation of *gauche* rotamers by halothane tends to thin the membrane. The net result of these two opposing tendencies could be to leave the thickness unchanged. This speculation is consistent with the X-ray diffraction evidence: Franks and Lieb [4] found that the depth of the central trough in the electron density profiles of phosphatidylcholine/cholesterol bilayers appeared to decrease progressively as the halothane concentration was raised from about 20–50-times the minimum alveolar concentration. Furthermore, both X-ray diffraction [24] and electrical capacitance measurements [25] indicate that saturated solutions of the lower alkanes, which have been shown [26] to reside mainly in the center of the bilayer, can actually thicken lipid bilayers. An interesting corollary to this discussion is that spectroscopic techniques which measure disorder (e.g. ESR, NMR and Raman spectroscopy) cannot automatically be used to infer thickness changes [27,28].

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