Effect of Halothane on the Natural-Abundance ^{13}C NMR Spectra of Excised Rat Brain†

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ABSTRACT: Halothane increases the intensity of the 30.5- and 129-ppm resonances in ^{13}C nuclear magnetic resonance spectra of excised rat brain, of phospholipid vesicles prepared from chloroform–methanol extract of rat brain, and of brain excised from rats anesthetized with halothane. The ^{13}C spin–lattice relaxation times of the 30.5- and 129-ppm resonances are increased in excised brain, or phospholipid vesicles, upon addition of halothane, and they are also increased in brain excised from rats anesthetized with halothane. Excised brain and its membrane-rich subcellular fractions interact with [^{14}C]halothane reversibly. The interaction is virtually abolished when the phospholipids are extracted from the brain. The [^{14}C]halothane content of the brain membranes is correlated with the halothane-induced increase in the integral of the 129-ppm resonance. From this correlation and from the phospholipid content of the membranes, a halothane concentration of 3.34 mM and a partition of 0.057 mol of halothane/mol of phospholipid may be calculated in the brain of anesthetized rats.

The molecular mechanism of anesthesia has been studied by a large variety of biological and physicochemical techniques (Fink, 1975, 1980; Koblin & Eger, 1979; Tiengo & Cousins, 1983). Nuclear magnetic resonance (NMR)[1] investigations focused on the effect of anesthetics on lipid bilayers using several nuclei: ^{1}H (Shieh et al., 1975; Hunt & Jones, 1983), ^{2}H (Boulanger et al., 1980, 1981; Koehler et al., 1980; Kelusky & Smith, 1984), ^{13}C (Yeagle et al., 1977; Koehler et al., 1980), ^{14}N (Siminovitch et al., 1984), ^{19}F (Koehler et al., 1977a,b, 1978), and ^{31}P (Yeagle et al., 1977; Cullis et al., 1980; Koehler et al., 1980; Hornby & Cullis, 1981; Boulanger et al., 1981; Siminovitch et al., 1984). Wyrwicz and collaborators (Wyrwicz et al., 1983a) pioneered in observing fluorinated anesthetics in intact tissues with ^{19}F NMR spectroscopy. Moreover, they recorded ^{19}F NMR spectra in brain of anesthetized live rabbits (Wyrwicz et al., 1983b). Burt and collaborators (Burt et al., 1983, 1984) further extended the potential of halothane and related anesthetics for monitoring membrane alterations in tumors by ^{19}F NMR.

Recently, we recorded high-resolution natural-abundance ^{13}C NMR spectra of intact brains and assigned over 50 resonances tentatively (Bárány et al., 1985a). In this paper, we have applied this ^{13}C NMR spectroscopy for studying membrane alterations in brains of halothane-anesthetized rats. We describe the increased intensities and increased T_1 relaxation times of methylene and polyunsaturated carbon resonances in phospholipids of brains excised from rats anesthetized with halothane. A correlation was found in vitro between the halothane-induced increase in intensity of the polyunsaturated carbon resonance and the partitioning of ^{14}C-labeled halothane into brain membrane phospholipids. From such a calibration curve, we calculated that in vivo during anesthesia of rats 0.057 mol of halothane is partitioned per mol of phospholipid into brain membranes. Some of our preliminary findings have been presented (Bárány et al., 1985b).

Experimental Procedures

Halothane Treatment of Excised Brain. Brains were dissected from rats killed by decapitation. Aliquots of 4 g of brain were placed in screw-capped glass vials and incubated with various amounts of halothane (Ayerst Laboratories, New York, NY) in a bath of 40 °C for 10 or 30 min. Brains incubated concurrently without halothane served as controls. The samples were transferred to 12-mm NMR tubes with 0.5 mL of 0.15 M NaCl, dissolved in $^{2}H_2O$ (uncorrected pH meter reading of 6.53).

Partitioning of [^{14}C]Halothane. [1-^{14}C]Halothane was purchased from the Biomedical Products Department of E. I. du Pont de Nemours and Co., Boston, MA. The 2.51-μL sample was diluted with 2.5 mL of nonradioactive halothane to have a specific activity of 9477 dpm/μmol in a Delta 300 liquid scintillation counter (TM Analytic, Inc., Elk Grove Village, IL).

For the determination of halothane partitioning, about 8-g samples of rat brain in glass vials, kept in ice, were mixed with various amounts of [^{14}C]halothane and incubated in a water bath at 40 °C for 30 min. The halothane-treated brain was chilled in ice and homogenized at 0 °C with a solution of 0.15 M NaCl, pH 7.5, by using a loose-fitting Teflon-glass homogenizer. The volume of the 0.15 M NaCl solution used was calculated to give 10 mM final halothane concentration. The homogenate was centrifuged in sealed tubes in the TI 60 head of the Beckman Model L5-75 ultracentrifuge at 40 000 rpm (161100*g*), 4 °C, for 75 min, and both the pellet and supernatant were saved. A 0.3-g aliquot of the pellet was weighed into a small Teflon–glass homogenizer, and it was homogenized with 1.0 mL of distilled water; this corresponds to a 4.33-fold dilution of the pellet. Triplicate aliquots, each of 0.1 mL, were taken from the pellet homogenate both for liquid scintillation counting and for protein determination by the biuret method (Bárány & Bárány, 1959). The turbidity which developed upon addition of the biuret reagent was removed from the samples by centrifugation at 18 000 rpm. Triplicate aliquots, each of 0.1 mL, were also taken from the 161100*g* supernatant for counting. The partitioning of [^{14}C]halothane was calculated from the counts in the pellet, corrected for the occluded counts from the supernatant in the pellet, and from the known specific activity of [^{14}C]halothane. The fractional occluded

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[1] Abbreviations: NMR, nuclear magnetic resonance; T_1, spin–lattice relaxation time.

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supernatant volume was calculated from the 18.84% dry weight of the pellet, taking into account the 4.33-fold dilution of the pellet: $(1.0000 - 0.1884)/4.33 = 0.1874$.

For the experiments the results of which are shown on Figure 4, the partitioning of [^{14}C]halothane was expressed as moles of halothane per mole of phospholipid in the 161100g brain pellet, on the basis of the protein content of the pellet used for counting and from the phospholipid content of the pellet which was determined to be 45.75 mol of phospholipid/10^5 g of protein:

$$\frac{\text{mol of halothane}}{\text{mol of phospholipid}} = \frac{[\text{dpm/mL pellet} - (0.1874)\text{dpm/mL supernatant}]/9477}{0.4575(\text{mg of protein/mL of pellet})}$$

$$\frac{\text{mol of halothane}}{\text{mol of phospholipid}} = \frac{\text{dpm/mL pellet} - (0.1874)\text{dpm/mL supernatant}}{4206(\text{mg of protein/mL of pellet})}$$

To the remainder of the pellet was added ^2H_2O to 25% final concentration, and the ^{13}C NMR spectrum was recorded at 20 °C. At the end of the spectroscopy, the radioactivity of the pellet was redetermined: in general, this was in agreement with the radioactivity measured before spectroscopy.

For the experiments the results of which are shown in Table II, the [^{14}C]halothane partitioning was determined by the same method as described before. The partitioning was expressed as moles of halothane per 10^5 g of protein in order to compare the partitioning of [^{14}C]halothane among the various subfractions of the brain.

Phospholipid Quantitation. The 161100g pellet from about 15 g of brain, containing 1396 mg of protein, was freeze-dried. The powder was extracted with 50 volumes (v/w) of chloroform/methanol (2/1 v/v) overnight, and after separation of the supernatant from the residue by centrifugation at 7700g, the residue was reextracted with 10 volumes of chloroform/methanol. The combined supernatants were equilibrated with $^1/_4$th volume of 0.1 M KCl for 1 h. The bottom chloroform layer was separated from the aqueous layer, and 50-μL aliquots were removed for determination of the phospholipid content by digestion with perchloric acid and subsequent inorganic phosphate determination (Kates, 1972).

The same procedure was used for the determination of the phospholipid content of the intact brain.

Phospholipid Vesicles. These were prepared from freeze-dried brain. The powder was extracted with chloroform/methanol (2/1 v/v) containing 0.3 mM butylated hydroxytoluene, and the extract was partitioned with 0.1 M KCl as described before. Aliquots were removed from the chloroform layer for determination of the phospholipid content, then the chloroform extract was evaporated under a stream of nitrogen at room temperature, and the last traces of chloroform were removed with nitrogen between 40 and 50 °C. The residue was suspended in a solution containing 0.15 M NaCl and 1 mM $NaHCO_3$ in 25% ^2H_2O, and it was sonicated under a nitrogen atmosphere with the microtip of the Branson Model 185 sonifier at set 7 (100–120 W) for 1 h (4 × 15 min). During sonication, the temperature was kept at 40–50 °C, and the pH was kept at 7.8–8.0. The final suspension was ultracentrifuged at 150000g, 25 °C, for 30 min, and the supernatant was used for NMR studies.

^{13}C NMR Spectroscopy. Natural-abundance ^{13}C NMR spectra were recorded with a 12-mm probe at 90.8 MHz in a Nicolet NMC-360 narrow-bore spectrometer. Spectra were acquired with quadruature detection, a 40° pulse angle, a sweep width of ±10 204 Hz, and a recycling time of 1.20 s, and 8K data points were collected. Two-level broad-band ^{1}H decoupling was employed with 5-W decoupling power during the pulsing and data acquisition time and 0.5 W during the delay period. Spectra were recorded either at 20 ± 1 or at 37 ± 1 °C. Varying the recycling time from 1.2 to 5.2 s showed that, under these conditions, the saturated and unsaturated phospholipid carbons of brain were fully relaxed. The homogeneity of the magnetic field in the tissues was optimized by shimming on the ^2H_2O added to the samples. Dioxane, 4.4% in ^2H_2O, was placed in a coaxial capillary and inserted into the samples to serve as a chemical shift reference of 67.40 ppm. Spectra were recorded without spinning the samples.

T_1 relaxation measurements of the methylene and unsaturated carbons of brain and phospholipid vesicles were carried out at 20 °C. The spectrometer frequency was placed at 40 ppm for the methylene carbons and at 120 ppm for the unsaturated carbons, and the T_1 values were determined by using a 180°–τ–90° pulse sequence (Vold et al., 1968) with 11 τ values between 0.02 and 3.0 s and a recycling time of 3.5 s. T_1 values were calculated by the three-parameter fit (Levy & Peat, 1975) program (T13IR) of the Nicolet 1280 computer. The uncertainty for T_1 values is given with ±1 standard deviation as calculated from the χ^2 of the fitted curve by the 1280 computer.

For the difference spectra between halothane-treated and untreateed brain samples, an equal number of transients were recorded for each type of sample, and the same dioxane capillary was used for both samples. The difference spectrum was prepared from the difference of the free induction decays of the samples.

Anesthetization of Rats. Two rats were placed in a thick-wall, glass, rectangular chromatography jar, 27 cm long, 7.6 cm wide, and 21.5 cm deep (Brinkmann). A piece of cheesecloth was attached on the inner side of the glass cover, and 0.6 mL of halothane was placed on the cloth in the hood with a glass pipet. The cover was placed on the jar so that at the upper right and lower left corners there was a space to allow air to enter. Under these conditions, the rats remained anesthetized for 20 min; then a fresh 0.3-mL halothane sample was placed on the cloth to allow a 20-min additional anesthesia. The rats were killed after 30 min of total anesthesia by decapitation; the brains were dissected and placed into NMR tubes which were kept in ice.

This empirical anesthetic procedure was well reproducible, and the rats readily recovered from the anesthesia. The procedure is validated by the similar halothane concentration in the brain of our anesthetized rats as that in the experiments of Burt et al. (1984), who used a Fluotec vaporizer with 1% halothane in a 94% O_2/5% CO_2 mixture. This point will be further elaborated under Discussion.

Results

Halothane Treatment of Excised Brain and Phospholipid Vesicles. When rat brain is incubated with a high concentration of halothane (100 μmol of halothane/g of brain) at 37 °C for 10 min, marked changes are observed in the brain ^{13}C NMR spectrum (Figure 1). In the control brain (bottom part of Figure 1), the 30.5-ppm peak is minor, whereas in the halothane-treated brain (upper part of Figure 1), the height of the 30.5-ppm peak exceeds that of the 67.40-ppm dioxane reference peak. Along with the 30.48-ppm peak, several other resonances show increases in the aliphatic envelope: the terminal methyl carbon ($-CH_3$) at 15.11 ppm, the methylene

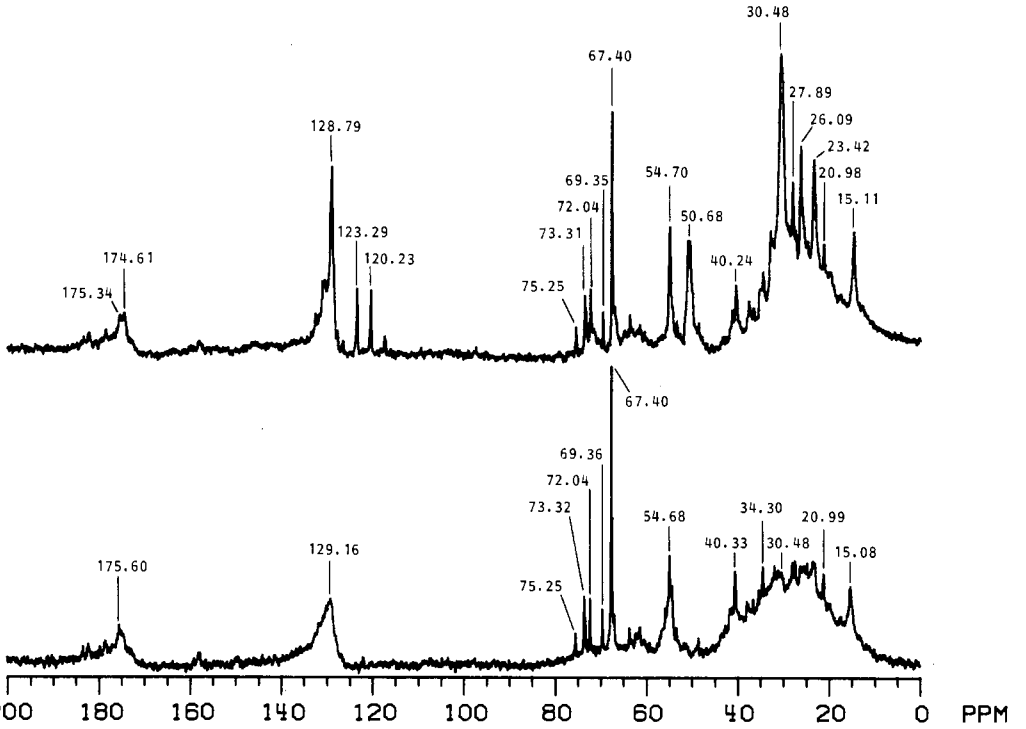

FIGURE 1: Comparison of ^{13}C NMR spectra of rat brains treated with high concentrations of halothane (upper trace) and untreated (lower trace). Transients, 2096 and 2100, respectively, were collected at 20 °C, and a 10-Hz line broadening was used in both cases. The signal at 67.40 ppm in both spectra is from dioxane in a capillary. See text for peak assignments and the halothane concentration.

carbon adjacent to the terminal methyl ($-CH_2CH_3$) at 23.42 ppm, the methylene carbon β to the carboxyl terminal ($-CO-CH_2CH_2$) at 26.09 ppm, and the methylene carbon adjacent to the allylic carbon ($-CH_2CH=$) at 27.89 ppm. A major increase is seen in the height of the resonance of the polyunsaturated carbons at 128.79 ppm. On the other hand, no significant changes are noted in the following peak heights: methyl and alcoholic carbons of lactic acid at 20.98 and 69.35 ppm, respectively; total carbons from the methyl group of creatine and the C2 carbons of *N*-acetylaspartate, glutamate, and glutamine at 54.70 ppm; inositol carbons at 72.04, 73.31, and 75.25 ppm; and carbonyl carbons at 174.61–175.34 ppm (Bárány et al., 1985a). Free halothane is visible in the upper spectrum of Figure 1; it produces the peak at 50.68 ppm ($-CHBrCl$) and a quartet with center peaks at 120.23 and 123.29 ppm (CF_3-). When rat brain was incubated with a low halothane concentration (4 μmol/g of brain) at 37 °C for 30 min, small, but qualitatively the same, changes were observed in the spectrum similar to those observed with high halothane concentration.

When rat brain phospholipid vesicles are incubated with 0.4 mol of halothane/mol of phospholipid at 37 °C for 10 min, changes are observed in the spectrum (Figure 2) similar to those in the spectrum of halothane-treated brain (Figure 1). The heights of the 15-, 23-, 26-, 28-, 30.5-, and 129-ppm peaks are markedly increased. Free halothane is also present in the phospholipid spectrum.

Effect of Halothane on Brain of Anesthetized Rats. We recorded the ^{13}C spectrum of brain excised from rats anesthetized with halothane for 30 min (Figure 3). The difference spectrum between anesthetized and control rats shows an increase for the peaks characteristic for the membranes, such as 15, 30.5, and 129 ppm, and decreases for peaks corresponding to metabolites such as 34, 55, 63, 72–73, and 182 ppm. These latter peaks may be tentatively assigned (Bárány et al., 1985a) to glutamate C4 (34.30 ppm), to the methyl groups of creatine, choline, and choline derivatives (54.67 ppm), to glutamate C2 (55.70 ppm), to the C1 and C3 carbons of a glycerol derivative (63.48 ppm), to inositol carbons (72.05–73.34 ppm), and to glutamate C5 (181.98 ppm).

Table I: Values for the 30.5- and 129-ppm Carbon Resonances in Rat Brain and Phospholipid Vesicles with and without Halothane

peak (ppm)	sample	T_1 (s)[a]
30.5	brain	0.44 ± 0.01
30.5	brain + halothane[b]	0.55 ± 0.01
30.5	brain from halothane-anesthetized rats	0.50 ± 0.01
30.5	vesicles	0.43 ± 0.01
30.5	vesicles + halothane[c]	0.52 ± 0.02
129	brain	0.71 ± 0.01
129	brain + halothane	0.88 ± 0.02
129	brain from halothane-anesthetized rats	0.83 ± 0.01
129	vesicles	0.57 ± 0.03
129	vesicles + halothane	0.85 ± 0.02

[a] T_1 Values for vesicles and vesicles + halothane are the result of a single experiment; all other T_1 values are the average of duplicates in different samples, with ± the largest standard deviation. [b] 30 μmol of halothane/g of brain. [c] 0.1 mol of halothane/mol of phospholipid.

Changes in the T_1 Relaxation Times of Fatty Acyl Carbons of Excised Brain and Phospholipid Vesicles in the Presence of Halothane. Table I shows that the T_1 values of the 30.5- and 129-ppm carbon resonances in excised brain are increased upon addition of halothane from 0.44 to 0.55 s and from 0.71 to 0.88 s, respectively. The T_1 values of these resonances are also increased in brain excised from rat anesthetized with halothane, to 0.50 and 0.83 s, respectively. Similar increases are observed when brain phospholipid vesicles are treated with halothane; T_1 increases from 0.43 to 0.52 s for the 30.5-ppm peak and from 0.57 to 0.85 s for the 129-ppm peak.

Characterization of Halothane Partitioning into Brain. Excised brains or brain fractions were treated with the same amount of [^{14}C]halothane, and the partitioning of halothane per 10^5 g of protein was compared (Table II). When halothane-treated brain is fractionated into a 17500*g* pellet and subsequently into a 161100*g* pellet, the first subcellular fraction contains twice as much halothane (6.75 mol) as the second

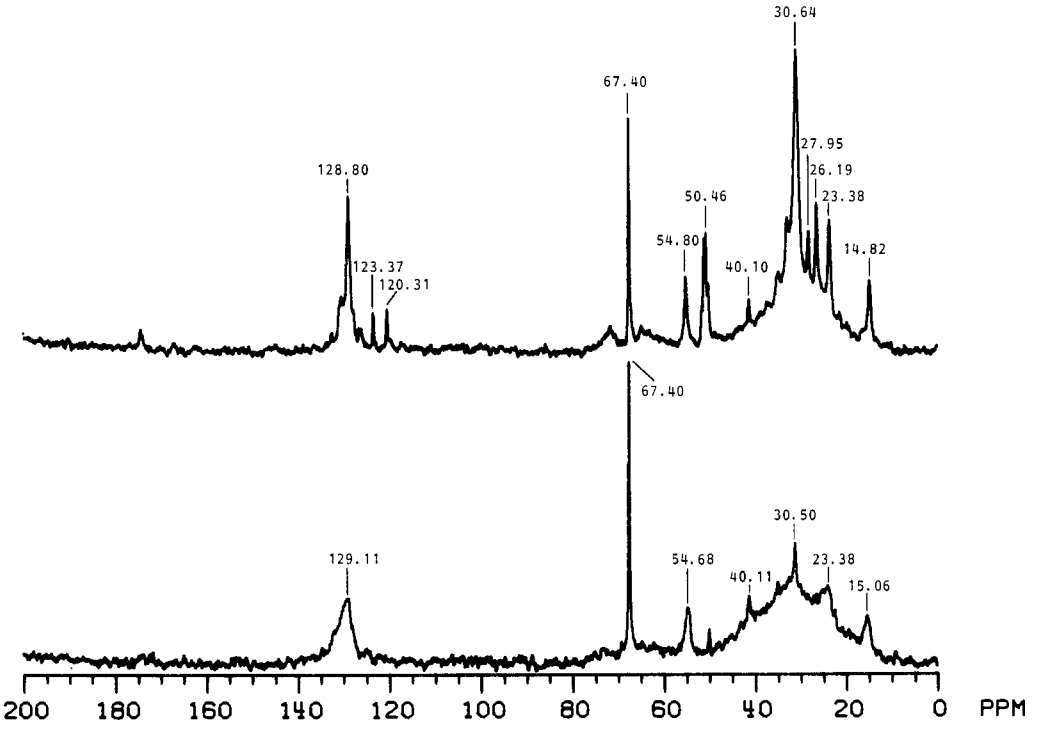

FIGURE 2: Comparison of ^{13}C NMR spectra of brain phospholipid vesicles treated with high concentrations of halothane (upper trace) and untreated (lower trace). For each spectrum, 2000 transients were collected at 20 °C, and a 20-Hz line broadening was used. The signal at 49.86–49.99 ppm is from traces of methanol remaining in the vesicles.

Table II: Partitioning of Halothane into Excised Rat Brain and Its Fractions[a]

expt	sample	mol of halothane/10^5 g of protein
1	17500*g* pellet of halothane-treated brain	6.75
	161100*g* pellet of same halothane-treated brain obtained from supernatant of 17500*g* pellet	2.97
1	17500*g* pellet of halothane-treated brain, washed twice	1.39
	161100*g* pellet of same halothane-treated brain obtained from supernatant of 17500*g* pellet, washed twice	0.61
2	17500*g* pellet of brain, treated with halothane	9.23
2	161100*g* pellet of brain obtained from supernatant of 17500*g* pellet, treated with halothane	3.32
3	chloroform–methanol-extracted residue of brain, treated with halothane, and pelleted at 161100*g*	0.64
3	161100*g* pellet of halothane-treated brain obtained from homogenate directly	7.13

[a] Excised brains were treated with 57 μmol of ^{14}C-labeled halothane per gram of fresh brain weight and incubated in a water bath of 40 °C for 30 min. In the case of the 17500*g* or 161000*g* pellet of brain, or its chloroform–methanol-extracted residue, 57 μmol of halothane was added per 130 mg of protein, which is equivalent to the amount of protein present in 1.0 g of brain. At the end of incubation, brains or fractions were homogenized in 5 volumes of 0.15 M NaCl, pH 7.0, at 0 °C. Washing of the pellets refers to the original 5 volumes of 0.15 M NaCl. The pellets not isolated at 161100*g* were recentrifuged at this gravitational force before the partitioning of halothane was measured.

fraction (2.97 mol). When isolated 17500*g* pellet is treated with [^{14}C]halothane, more halothane is partitioned in this pellet (9.23 mol) than in the [^{14}C]halothane-treated 161100*g* pellet (3.32 mol), which is obtained from the supernatant of the 17500*g* pellet. These data indicate that the 17500*g* membrane fraction interacts with more halothane than the 16100*g* fraction.

Table II also shows that the interaction of halothane with the brain membranes is reversible; two washings decreased the halothane content of the 17500*g* pellet from 6.75 to 1.39 mol of halothane/10^5 g of protein and that of the 161000*g* pellet from 2.97 to 0.61 mol of halothane/10^5 g of proteins.

Finally, Table II illustrates that after removal of the phospholipids from the brain with chloroform–methanol, the remaining residue (dried and homogenized in 0.15 M NaCl) interacts only with a negligible amount of halothane, 0.64 mol, compared with the 7.13 mol in the 161100*g* pellet, obtained directly from the homogenate of the halothane-treated brain. This result was well reproducible.

Simultaneously with the halothane partitioning determinations, we recorded the ^{13}C NMR spectra of the 17500*g* and 161100*g* membrane fractions and the 161100*g* supernatant fraction of the brain. Highly elevated 30.5- and 129-ppm peaks were found in both the 17500*g* and 161100*g* fractions, as compared to the same fractions prepared from untreated brains. However, no difference was found in the spectra between supernatant fractions of halothane-treated and untreated brains.

Quantification of the Halothane-Induced Changes in the Brain Spectrum. We treated a large number of excised brains with increasing concentrations of ^{14}C-labeled halothane and prepared directly the 161100*g* pellets from the brain homogenates. This pellet contains both halothane-interacting components of the brain, the major one which is the 17500*g* pellet and the minor one which is pelleted from the 17500*g* supernatant at 161000*g*. After determining the [^{14}C]halothane content of the pellets, we recorded their ^{13}C spectra and compared the spectra with those of the pellets which were prepared from untreated brains for each set of experiment. We have used the 129-ppm resonance in the difference spectrum to correlate halothane-induced changes in the spectrum of the 161100*g* pellet with the halothane content of

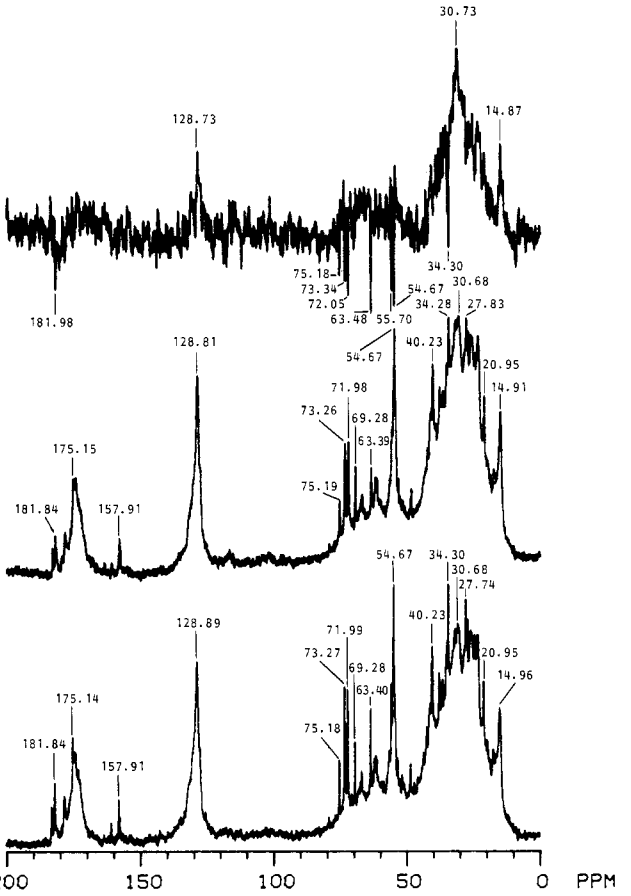

FIGURE 3: Comparison of ^{13}C NMR spectra of brains excised from untreated rats (lower trace), rats anesthetized with halothane (middle trace), and the difference between halothane-anesthetized and control rats (upper trace). For each of the lower and middle spectra, 6260 transients were collected at 37 °C, and a 10-Hz line broadening was used. For the difference spectrum, the line broadening was 20 Hz. The signal at 54.67 ppm from creatine was used as a reference.

the pellet: The dioxane reference peak, which was from the same capillary in both samples, was integrated, and its value was set to 100. Next the 129-ppm peak in the difference spectrum was integrated, and its value was expressed relative to the 100 value of the dioxane.

We prepared 13 difference spectra between the 161100*g* pellets from [^{14}C]halothane-treated excised brains and the corresponding untreated brains. All spectra were recorded at 20 °C. The [^{14}C]halothane content of the pellets was determined and was expressed in terms of moles of halothane per mole of phospholipid in the pellet, as described under Experimental Procedures. In Figure 4, the relative integral values of the 129-ppm peak in the difference spectrum are plotted vs. the moles of halothane per mole of phospholipid in the pellets. A straight line is obtained which is described by the equation:

$$Y = 283.98X + 4.63$$

and the correlation coefficient is 0.968.

On the basis of the correlation shown in Figure 4, we have estimated the amount of halothane partitioned into the 161100*g* brain fraction of rats anesthetized with halothane. An example is shown in Figure 5. Comparison of the spectrum from anesthetized rat (middle) with that of the control rat (bottom) shows increased phospholipid carbon resonances in both the aliphatic and aromatic regions. These are well demonstrated in the difference spectrum (top). Relative integration of the 129-ppm peak in the difference spectrum gave

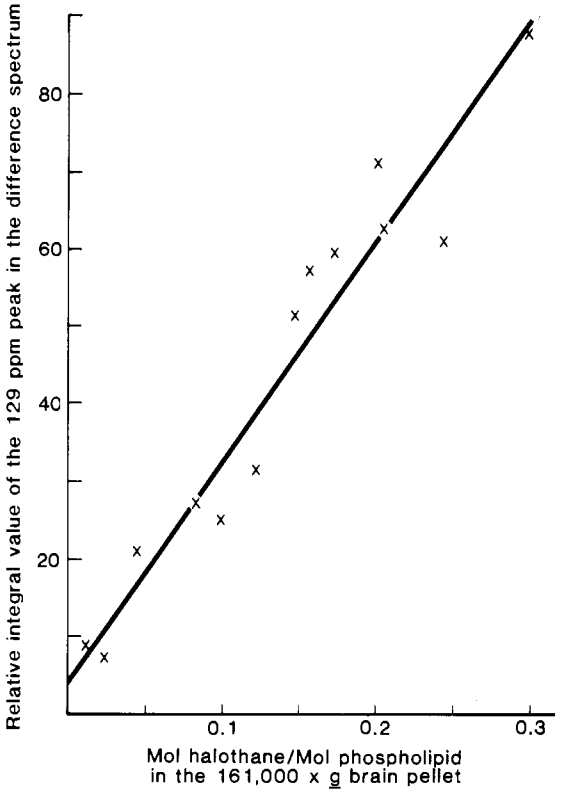

FIGURE 4: Relationship between the relative integral value of the 129-ppm peak in the difference spectrum of the 161100*g* pellets from [^{14}C]halothane-treated excised brain and untreated brain vs. the halothane content of the pellet.

a value of 23.92 from which the halothane content of the pellet was calculated to be 0.068 mol/mol of phospholipid. In three other experiments, the relative integration values were 18.33, 20.67, and 20.82, corresponding to 0.048, 0.056, and 0.057 mol of halothane/mol of phospholipid in the pellets, respectively. Thus, on the average, 0.057 ± 0.007 mol of halothane was partitioned per mole of phospholipid in brain membranes of halothane-anesthetized rats.

Discussion

High-resolution, natural-abundance ^{13}C NMR of intact rat brain is a new approach for studying the molecular mechanism of halothane-induced anesthesia. Halothane treatment of excised brain or halothane anesthesia of the living rat increases the intensity of the 30.5- and 129-ppm resonances (corresponding to the repeating methylene and polyunsaturated carbons in the fatty acid acyl chains of membrane-bound phospholipids) in the in vitro spectrum of brain as compared with that of the control brain. The extent of increase depends on the halothane concentration (Figure 4). Phospholipid vesicles, prepared from a chloroform–methanol extract of rat brain, also show increases in the intensity of the 30.5- and 129-ppm resonances upon halothane treatment similarly to those seen in the intact brain (cf. Figures 2 and 1); this suggests that halothane affects the phospholipid bilayer in the brain membrane. Halothane-induced changes in lecithin bilayers were shown previously by ^{1}H and ^{13}C NMR (Shieh et al., 1975; Koehler et al., 1980).

The increased intensity of the 30.5- and 129-ppm resonances in ^{13}C NMR spectra of brain excised from rats anesthetized with halothane, and in spectra of brain or phospholipid vesicles treated with halothane, is observed under experimental conditions when the carbons responsible for these resonances are fully relaxed. Therefore, the increased intensity is caused by

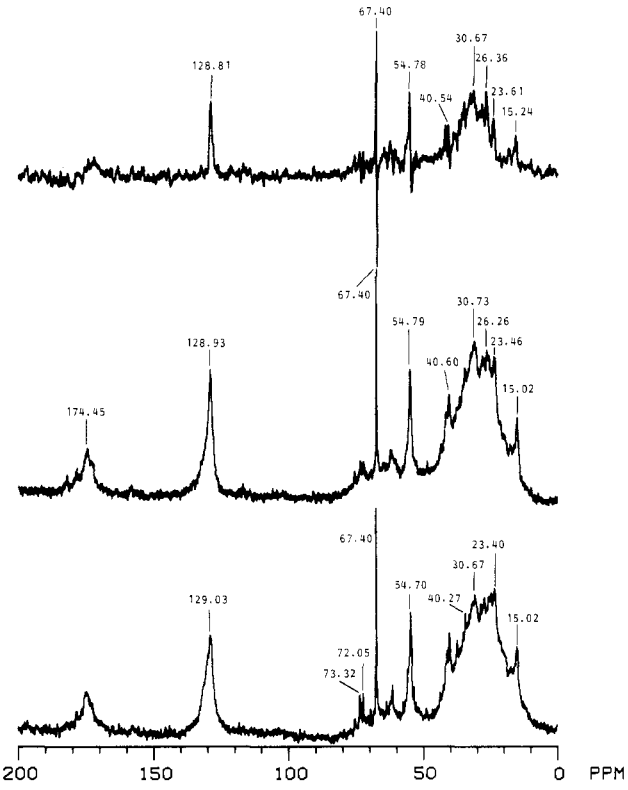

FIGURE 5: Comparison of ^{13}C NMR spectra of the 161100g pellets from brains of untreated rats (lower trace), rats anesthetized with halothane (middle trace), and the difference between halothane-anesthetized and untreated rats (upper trace). For each of the lower and middle spectra, 5000 transients were collected at 20 °C and a 10-Hz line broadening was used. For the difference spectrum, the line broadening was 20 Hz.

a new population of mobile phospholipids as a result of halothane intercalation in the brain membrane.

Halothane also increases the T_1 relaxation time of the 30.5- and 129-ppm peaks in brains excised from halothane-anesthetized rats, halothane-treated brains, or halothane-treated phospholipid vesicles, with respect to controls (Table I). Apparently, the interaction with halothane changes the mobility of the entire fatty acyl chain. Several authors have shown that the T_1 relaxation time of phospholipid vesicles is sensitive to the conformation of the fatty acyl chains (Levine et al., 1972; Barton & Gunstone, 1975; Fuson & Prestegard, 1983).

The strong 129-ppm resonance in the brain spectrum arises from the high content of unsaturated fatty acids in the membrane phospholipids. Docosahexaenoic acid (22:6) is the major polyunsaturated fatty acid of the rat brain phospholipids. It comprises as much as 14% of the total fatty acids (Cotman et al., 1969), and it assumes an approximate helical conformation (Stubbs & Smith, 1984). Multiple cis double bonds in this and other unsaturated fatty acyl chains may produce a lipid conformation that can be recognized by a small molecule such as halothane. This hypothesis fits the finding that general anesthetics can selectively perturb lipid bilayer membranes (Miller & Pang, 1976).

Halothane is preferentially partitioned into the 17500g pellet fraction of the brain (Table II) corresponding to the synaptosomes (Eichberg et al., 1964). This is in agreement with the generally accepted view that clinical anesthesia is the result of interference with the central synaptic transmission (Mullins, 1975). In addition to the synaptosomes, halothane is also partitioned into the microsomes (the brain fraction sedimenting between 17500g and 161000g). However, less than 10% of the maximal halothane partitioning remains after extraction of the phospholipids from the brain (Table II). Our results favor the theory of a lipid-mediated mechanism of anesthesia (Miller & Pang, 1976; Pang et al., 1979; Boulanger et al., 1980), which may involve lipoproteins (Woodbury et al., 1975) or the lipid annulus which surrounds the sodium channel (Lee, 1976, 1977). Our data are consistent with the ^{19}F NMR studies of Wyrwicz et al. (1983a,b) and Burt et al. (1984) which show that halothane is partitioned into hydrophobic environments of cell membranes. Previously, Koehler et al. (1977a) showed by ^{19}F NMR that halothane is largely distributed along the methylene carbons of artificial phospholipid membranes. Nevertheless, our data do not exclude a protein-mediated mechanism of halothane action (Hsia & Boggs, 1975; Hulands et al.; 1975; Franks & Lieb, 1978, 1984; Brown & Halsey, 1980; Smith et al., 1984) in any way.

[^{14}C]Halothane labeling of intact brain led to quantitation of the amount of halothane partitioned into the brain phospholipids at the time of halothane-induced anesthesia in live rats. [^{14}C]Halothane-labeled brain membranes, prepared from brains treated with [^{14}C]halothane in vitro, exhibit increases in the 30.5- and 129-ppm resonance intensities as compared with membranes from untreated brains. In the difference spectrum, the 129-ppm peak is especially useful because it resides over a flat base line; thus, the peak area can be integrated easily. We have found that the halothane-induced increase in the integral of the 129-ppm peak in the ^{13}C NMR spectra of the membranes is correlated with the partitioning of [^{14}C]halothane into the membrane phospholipids (Figure 4). With aid of such a calibration curve, it could be calculated that the increment in the 129-ppm peak in membranes of rats anesthetized with halothane (Figure 5) corresponds to an average partitioning of 57 mmol of halothane/mol of phospholipid. Mastrangelo et al. (1978) exposed a model nerve membrane bilayer, 66 mol % phosphatidylcholine and 33 mol % cholesterol, to clinical concentrations of halothane and measured the resultant concentration of halothane in the phospholipid bilayer by gas chromatography. A value of 30 mmol of halothane/mol of phospholipid–cholesterol was obtained. This value may be compared with ours as follows: Our phospholipid determination in adult rat brain, 58.56 μmol/g, agrees with the value of Cuzner & Davison (1968), 59.82 μmol/g. Therefore, we may use the cholesterol content, 48.27 μmol/g, of the brain determined by Cuzner & Davison. This reduces the 57 mmol halothane/mol of phospholipid in anesthetized rat brain to 31 mmol of halothane/mol of phospholipid–cholesterol, in a good agreement with the 30-mmol halothane content of the phospholipid–cholesterol bilayer system.

From the 57 mmol of halothane partitioned per mol of phospholipid in brain membranes of anesthetized rats and from the 58.56 μmol of phospholipid content per gram of rat brain, one can calcuate a halothane concentration of 3.34 mM in the brain during anesthesia. Burt et al. (1984) estimated, from ^{19}F NMR spectra, 1.7 mM halothane in brains of rats anesthetized for 30 min with a halothane vaporizer. Their halothane concentration is in the same range as ours obtained with an empirical method of anesthesia. The 2-fold difference observed may also be caused by the difference in the reference standards, [^{14}C]halothane in our case or internal trifluoroacetic acid in the experiments of Burt et al. The millimolar halothane concentration necessary for anesthesia indicates that the affinity of halothane to the brain membranes is low as would be expected from a process which must be reversed rapidly. Indeed, washing of halothane-loaded membranes with 0.15 M

NaCl removes most of the partitioned halothane (Table II). Koehler et al. (1977b) showed that halothane bound to dipalmitoylphosphatidylcholine liposomes exchanges with halothane free in the aqueous phase. Furthermore, Wyrwicz et al. (1983a) observed a slow exchange between halothane bound to tissues and its environment by ^{19}F NMR and have also found a slow elimination of halothane from the brain of live rabbits (Wyrwicz et al., 1983b).

This work illustrates the potential of natural-abundance ^{13}C NMR as an intrinsic probe for the investigation of anesthetic action in intact brain. The ability of natural-abundance ^{13}C NMR to detect perturbations in unsaturated phospholipid carbons may be of general use for studying drug-induced membrane perturbations in various neural tissues or in kidney.

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