Anesthetic Steroid Mobility in Model Membrane Preparations As Examined by High-Resolution ^1H and ^2H NMR Spectroscopy

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A series of structurally related pregnane analogues which exhibit a wide range of anesthetic potencies were incorporated into unilamellar egg lecithin vesicles and their relative mobilities examined with ^1H and ^2H high-resolution NMR spectroscopy. The data from this study reveal a trend suggesting a relationship between the motional properties of a steroid and its anesthetic potency. The data are congruent with the idea that anesthetic activity is associated with perturbation of the membrane bilayer by the steroid molecule; the degree to which the membrane is perturbed is apparently dependent upon the specific structural and stereochemical features of the steroid. This study supports the hypothesis that lipid bilayers are capable of a high degree of structural discrimination.

Introduction

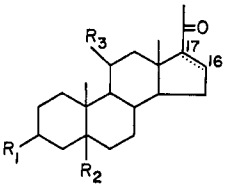
Many classes of anesthetics show good correlation between anesthetic activity and oil/water partitioning ability,[1] which suggests that anesthetic activity arises from nonspecific interaction with membrane lipids. The potency of an anesthetic steroid, however, is highly dependent on minor stereochemical and structural changes which have little effect on their partition coefficient.[2] It has been postulated that these drugs produce their effects by interacting with a specific site on a target membrane protein at the neuronal synapse.[3,4] Other investigators hypothesize that it is not necessary to invoke the idea of a specific receptor protein in order to account for the structural specificity of anesthetic steroids.[5] Using ESR techniques and nitroxide-labeled dipalmitoyllecithin as a molecular probe, Lawrence and Gill reported that steroids exhibiting anesthetic activity caused fluidization of the lipid bilayer.[5] Structurally related inactive analogues were shown to produce much less disorder of the spin-labeled liposomes. These results demonstrate that phospholipid bilayers are capable of a high degree of structural discrimination.

Our initial studies focused on studying the effects of two anesthetic steroids on membrane preparations. One of the drugs, 3α-hydroxy-5α-pregnane-11,20-dione (1 in Table I; alphaxalone), is a potent anesthetic and is used clinically as the main active component in the anesthetic Althesin.[6] The other, 3α-hydroxy-5α-preg-16-ene-11,20-dione (7 in Table I; Δ^{16}-alphaxalone), which differs structurally from alphaxalone by a double bond in position 16–17, completely lacks anesthetic activity. An early report from our laboratory describes the effects of 1 and 7 on a membrane preparation not involved in the anesthetic response.[7] We found that anesthetically active alphaxalone (1) inhibited anion transport in human erythrocytes much more effectively than its inactive analogue (7). These results indicate that the differences in activity between 1 and 7 are not restricted to interactions with nerve membrane. We also investigated the interactions of alphaxalone and Δ^{16}-alphaxalone with phosphatidylcholine bilayer vesicles that were used as model membrane systems. The steroids were incorporated into the bilayer and their relative mobilities were examined with ^1H, ^{13}C, and ^2H high-resolution NMR spectroscopy.[8] Our investigation showed that the anesthetic alphaxalone was much more mobile than its inactive analogue. These results were interpreted as evidence that the biologically active steroid perturbs the phospholipid bilayer much more effectively than the inactive steroid.

A subsequent study, in which the effects of 1 and 7 on model phospholipid membranes were examined by ^2H and ^{13}C solid-state NMR, provided evidence to support our previous interpretation.[9,10] These studies showed that alphaxalone perturbs the model membranes while the inactive steroid has no significant effect on these preparations.

The present study examines the dynamic properties of seven structurally related pregnane analogues (1–7 in Table I) in lecithin vesicles using ^1H and ^2H high-resolution NMR spectroscopy. The relative mobilities of the drugs in the

Table I. Steroid Structure and Corresponding Anesthetic Activity for the Pregnane Analogues 1–7



steroid	R_2	R_2	R_3	anesthetic activity,[a,b] mg/kg
1	3α-OH	5α-H	=O	3.1
2	3α-OH	5α-H	H_2	3.1
3	3α-OH	5β-H	=O	6.3
4	3α-OH	5β-H	H_2	3.1
5	3β-OH	5α-H	H_2	100
6	3β-OH	5β-H	H_2	25
7[d]	3α-OH	5α-H	=O	inactive[c]

[a] Lowest dose (mg/kg) producing loss of righting reflex. [b] Data taken from ref 11. [c] Data taken from ref 12. [d] Double bond present at position 16, 17.

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membrane preparations are examined and compared to their reported anesthetic potency[11,12] in an attempt to establish a relationship between membrane-perturbing ability and anesthetic activity.

Results and Discussion

The group of 3-hydroxypregnane analogues used in this study have structural differences which include the stereochemistry of the 3-OH group, the stereochemistry of the A/B ring juncture, the presence or absence of a carbonyl group at the 11-position of the C ring, and the presence or absence of a double bond in the 16–17-position of the D ring. Although these analogues are closely related in structure, they exhibit a wide range of anesthetic activity (Table I).[11,12] Previous testing of these compounds for anesthetic potency showed clear correlation between structure and function.[11,12] As can be seen by comparing the data in Table I, the stereochemistry of the 3-hydroxyl group plays an important role in determining anesthetic activity. Steroids which possess a 3β-hydroxy group (5 and 6) are much less active than those possessing a 3α-hydroxy group (1–4). Changes in the stereochemistry at the 5-position have a smaller effect on anesthetic activity. Steroids with either a cis or trans A/B ring juncture may be active anesthetics, depending on the stereochemistry of the 3-OH group. The presence or absence of a carbonyl group at the C ring appears to have little effect on anesthetic potency (e.g., 1 and 2), while the presence of a double bond at position 16–17 (7) completely abolishes anesthetic activity.

We first examined the ^{1}H high-resolution NMR spectra of drugs 1–7 in order to obtain information regarding the mobility of these drugs in bilayer preparations. NMR line width ($\nu_{1/2}$) measurements can provide information on the slower components of motion in anisotropic systems through the relationship of line width to effective transverse relaxation time (T_2^*) according to the following equation:

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

Line widths have been shown to vary with vesicle size due to differences in isotropic tumbling rates.[13] All lecithin vesicles used in this study were examined by electron microscopy;[14] we observed no differences in average vesicle size, nor in the range of vesicle sizes, at the drug concentrations used for the various steroid preparations.[15] This suggests that observed differences in NMR line width among the steroid/membrane preparations arise from differences in local motion and/or anisotropic behavior of the steroid bilayer rather than from differences in overall tumbling of the lipid vesicles.

Steroid molecules for which motion in the bilayer is restricted are expected to exhibit broad line widths and short T_2^* relaxation times. For example, in a study of cholesterol incorporated into lecithin bilayers, only very broad ^{1}H resonances corresponding to cholesterol were observed,[16] reflecting the highly restricted motion of cholesterol in the bilayer.[16-18]

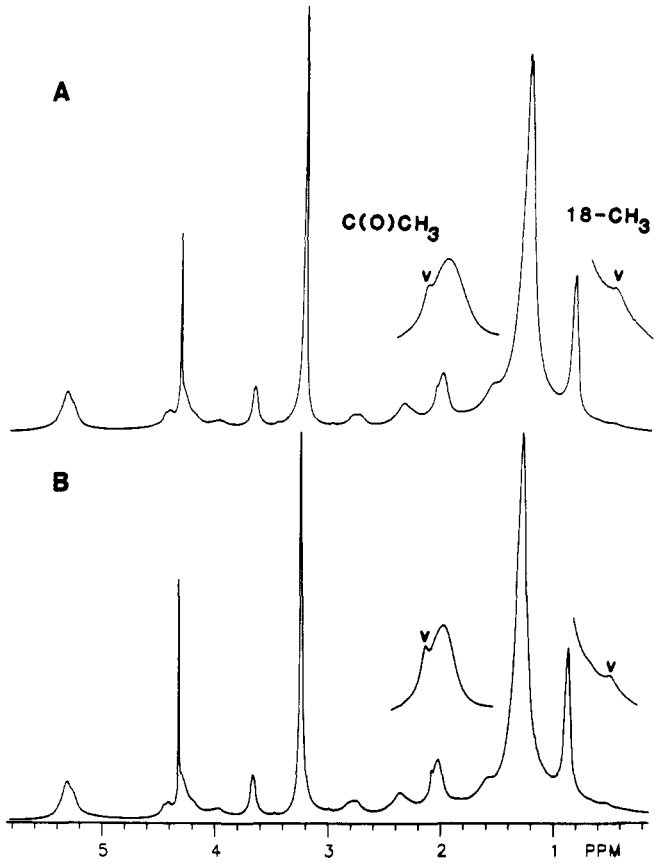

Figure 1. ^{1}H NMR spectra, acquired at 70 °C, of two phosphatidylcholine vesicle preparations (40 mM) containing (A) 4 and (B) 8 mM of alphaxalone. The insets above the spectra indicate the proton resonances corresponding to the C(O)CH_3 and 18-CH_3 methyl protons of the steroid.

Table II. ^{1}H NMR Line Widths ($\nu_{1/2}$) of Resonances Corresponding to the C(O)CH_3 Protons of Steroids Incorporated in Lecithin Vesicles

steroid	$\nu_{1/2}$, Hz 37 °C	50 °C	70°C	anesthetic activity,[a] mg/kg
1	8.0	5.0	2.5	3.1
2	8.0	4.0	2.5	3.1
3	8.0	5.0	2.5	6.3
4	5.5	4.0	2.5	3.1
5	*b*	*b*	*b*	100
6	*b*	*b*	*b*	25
7	*b*	*b*	*b*	inactive

[a] As defined in Table I. [b] Resonance not detected.

We have previously reported that no ^{1}H NMR signals due to Δ^{16}-alphaxalone are detected in the ^{1}H NMR spectrum of lecithin vesicles containing the steroid.[8] In contrast, ^{1}H NMR signals due to the C(O)CH_3 and 18-CH_3 protons of alphaxalone are observed in the corresponding vesicle preparation.[8] Figure 1 depicts two recently obtained ^{1}H NMR spectra of phosphatidylcholine vesicles (40 mM) containing either 4 (Figure 1a) or 8 mM (Figure 1b) of alphaxalone, which were obtained at 70 °C. As illustrated by the insets above each spectrum, the acetyl methyl protons (2.1 ppm) and C-18 methyl protons (0.5 ppm) of alphaxalone can be readily observed. The results show that for alphaxalone, different nuclei located in different positions of the molecule have consistently longer T_2^* values than in the Δ^{16}-analogue. This suggests that

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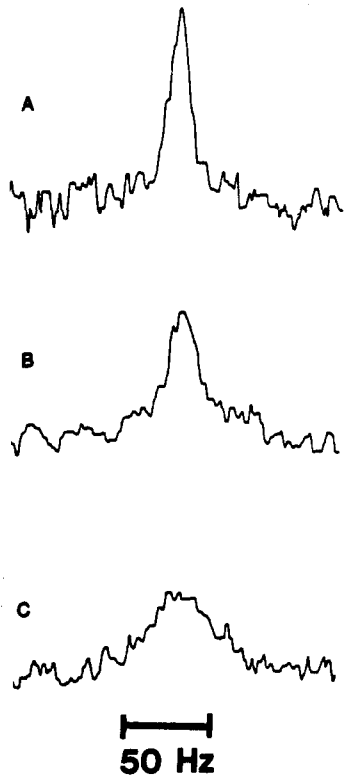

Figure 2. ^2H NMR spectra of alphaxalone deuterated in the acetyl methyl position (95%) incorporated in lecithin bilayers. The spectra, recorded at (A) 70, (B) 50, and (C) 37 °C, show the $C(O)CD_3$ methyl resonances corresponding to the steroid.

alphaxalone is considerably more mobile in the bilayer than is Δ^{16}-alphaxalone.

Table II lists the ^1H NMR line widths for resonances corresponding to the acetyl methyl protons in steroids 1–7 after incorporation into lecithin vesicles. The spectra were recorded at 37, 50, and 70 °C. At 37 °C, resonances with relatively narrow line widths were observed for the active anesthetics 1–4. As the temperature was raised, the resonances narrowed, consistent with increased mobility of the $C(O)CH_3$ moiety at higher temperature. For membrane preparations containing the inactive steroids 5–7, no resonances corresponding to the steroids could be detected at any temperature. These results suggest that ^1H resonances of the inactive analogues are quite line-broadened due to motional restriction of the steroids in the phospholipid bilayer.[16] The biologically active steroids are apparently not experiencing such restricted motion.

Only relatively narrow ^1H NMR resonances due to the steroid $C(O)CH_3$ group can be observed in the presence of the overwhelmingly large lecithin signals. In order to observe only steroid signals in the lecithin bilayer preparations, the steroids were selectively deuterated (>95%) in the acetyl methyl position and the ^2H NMR spectra of the steroids incorporated into lecithin vesicles were recorded. Previous examination of the ^2H spectra from deuterated alphaxalone and Δ^{16}-alphaxalone show that the line width of the $C(O)CD_3$ resonance for the active steroid is much narrower than that of the inactive analogue.[8] Figure 2 illustrates the ^2H spectra of a deuterated alphaxalone/vesicle preparation recorded at 37, 50, and 70 °C. The ^2H resonances of the $C(O)CD_3$ group become progressively broader as the temperature is lowered, clearly reflecting decreased mobility of the $C(O)CD_3$ segment in the bilayer.

Figure 3 compares the ^2H NMR spectra of steroids 4 (Figure 3A–C) and 6 (Figure 3D–F) collected at 37, 50, and

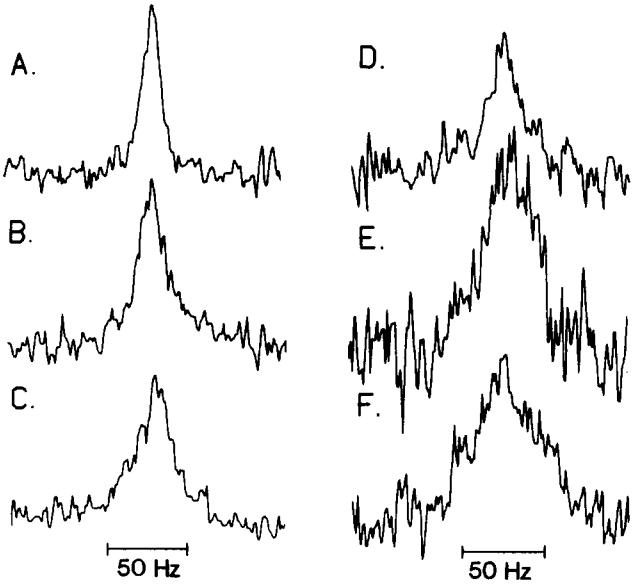

Figure 3. ^2H NMR spectra of 4 (A–C) and 6 (D–F) deuterated in the acetyl methyl position recorded at 70 (A and D), 50 (B and E) and 37 °C (C and F).

Table III. ^2H NMR Line Widths ($\nu_{1/2}$) for $C(O)CD_3$ Group for Steroids Incorporated in Lecithin Vesicles[a]

steroid	$\nu_{1/2}$, Hz 37°C	50 °C	70 °C	anesthetic activity,[c] mg/kg
1	40	17	12	3.1
3	47	35	20	6.3
4	26	19	14	3.1
5	*b*	*b*	30	100
6	60	42	27	25
7	*b*	*b*	50	inactive

[a] ^2H line widths were not measured for steroid 2; error in line width measurement ±6 Hz. [b] Resonances not detected. [c] As defined in Table I.

70 °C. While these steroids differ structurally only in the stereochemistry of the 3-hydroxy group, 4 exhibits an 8-fold higher anesthetic potency compared to that of 6. These two compounds also show marked differences in the observed $C(O)CD_3$ line widths in their respective ^2H NMR spectra (Figure 3). Although the $C(O)CD_3$ resonance broadens with decreasing temperature for both steroid preparations, resonances due to 4 are substantially narrower than those due to 6 at all three temperatures.

The $C(O)CD_3$ ^2H NMR line widths at 37, 50, and 70 °C for the steroids in lecithin vesicles are compiled in Table III. As the data in the table reveal, the line widths corresponding to the active steroids (1–4) are consistently narrower than those corresponding to the inactive analogues (5–7), reflecting the faster mobility of the former in the bilayer. Interestingly, within this small drug sample, the narrowness of the $C(O)CD_3$ ^2H NMR line width appears to parallel anesthetic potency.

We have also examined the ^1H NMR spectra of steroids 1–7 incorporated into dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC). Egg lecithin (PC) is a mixture of phospholipids in which palmitoyloleoylphosphatidylcholine is the principal component. DPPC and DOPC are pure phospholipids which differ in the structure of their hydrophobic fatty acid chains. The ^1H NMR line widths, which were recorded at 70 °C, for resonances corresponding to the $C(O)CH_3$ protons of steroids incorporated into the three different model membranes are compiled in Table IV. The ^1H NMR line widths were first estimated by assuming sym-

Table IV. Comparison of ^1H NMR Line Widths for Resonances Corresponding to $C(O)CH_3$ Protons of Steroids Incorporated into PC, DPPC, and DOPC Vesicles[a]

steroid	$\nu_{1/2}$, Hz PC	DPPC	DOPC	anesthetic activity,[b] mg/kg
1	2.5	4.1	3.5	3.1
2	2.5	6.0	4.0	3.1
3	2.5	4.7	4.5	6.3
4	2.5	5.0	4.5	3.1
5	c	13.0	c	100
6	c	9.0	c	25
7	c	>15.0	c	inactive

[a] Abbreviations defined within the text. [b] As defined in Table I. [c] Resonance not detected.

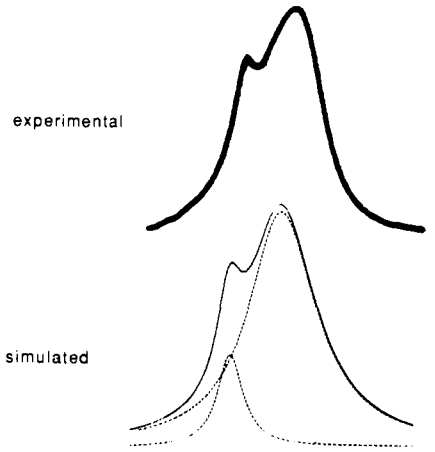

Figure 4. The experimental and simulated ^1H spectra of the acetyl methyl region of alphaxalone (4 mM) in phosphatidylcholine vesicles (40 mM) at 70 °C.

metric peaks and then refined by simulating the overlapping signals as symmetric Lorentzian lines. Figure 4 shows the experimental acetyl methyl region of the ^1H spectrum of alphaxalone (4 mM) in phosphatidylcholine vesicles (40 mM) at 70 °C and its simulation. As the data in Table IV reveals, the ^1H NMR line widths for the active anesthetics are significantly narrower than the inactive analogues in all three membrane preparations. Our data also seem to indicate that egg lecithin, which contains naturally occurring phosphatidylcholine, best discriminates between active and inactive steroids while the saturated DPPC is least discriminating.

Conclusion

This study has shown that the ^1H and the ^2H NMR line widths due to the $C(O)CH_3$ and $C(O)CD_3$ resonances, respectively, are significantly narrower for the active anesthetics than for the inactive analogues, reflecting a greater mobility of the former in the phospholipid bilayer. The trends in NMR line width described in this study suggest that anesthetic activity has a direct relationship to the mobility of the steroid in the bilayer. The observed differences in mobility between the active and inactive steroids may arise from differential phospholipid–steroid interactions due to variation of steroid geometry. The biologically active steroids interact with the phospholipid in such a way as to disrupt the bilayer geometry. This disruption is evidenced by the greater mobility of the incorporated anesthetics as measured by NMR line width. In contrast, the inactive analogues interact with the membrane in such a manner that the most stable bilayer geometry is maintained and the steroid is partially immobilized. With this hypothesis in mind, it is interesting to note that 7, which has a double bond in position 16–17, shows a complete lack of anesthetic activity. Introduction of a double bond in this position generates an α,β unsaturated ketone which results in a planar five-membered D ring. This planarity may allow steroid 7 to intercalate into the bilayer without causing significant disruption to the phospholipid.

We are currently studying the conformational properties of the individual steroids as well as their relative orientation with respect to the membrane bilayer. Such information will be helpful in developing a molecular model to describe membrane perturbation.

Experimental Section

Steroid Preparation. All of the nondeuterated steroids used in this study were kindly supplied to us by Glaxo Research, LTD (Research Triangle, NC). Deuterated steroids were prepared from their nondeuterated analogues by using a base ($NaOCH_3$) catalyzed exchange reaction in dioxane/D_2O.[10] Typically, the reaction mixture was heated to 50 °C in an N_2 atmosphere for 20 h. After cooling, the solution was acidified (1 N HCl) and extracted three times with $CHCl_3$. After drying (anhydrous Na_2SO_4), the combined $CHCl_3$ extracts were removed and the remaining residue recrystallized from a suitable solvent. Nearly quantitative yields (>95%) were obtained for all the steroids. The ^1H NMR chemical shifts and the melting points of the deuterated steroids were essentially identical with those of the corresponding nondeuterated analogues. By integration of the ^1H spectra, the steroids were found to have >95% ^2H in the acetyl methyl position.

Vesicle Preparation. Phospholipid vesicles containing the steroids were prepared by dissolving both the steroid and purified phosphatidylcholine from egg yolk (Type VII-E; Sigma Chemical Co.) in $CHCl_3$ and then removing the solvent under high vacuum for 12 h. The residue was then dissolved in D_2O or D_2O-depleted H_2O (for the ^2H NMR experiments; Sigma Chemical Co.). Translucent preparations were obtained by sonicating the suspension for 15 min at 0 °C with a Branson Model W-200 probe sonicator (microtip) with 50% pulse power. Vesicle sizes were determined with a Hitachi electron microscope at a magnification of 172500. Electron micrographs were taken within 1 h of vesicle preparation and average vesicle size and range of vesicle sizes for all steroid/phopholipid preparations were ca. 250 and 225–275 Å, respectively.

NMR Measurements. ^1H NMR spectra were recorded on a HX-270 Bruker NMR spectrometer and a GN300 General Electric spectrometer, operating at 270 and 300.1 MHz, respectively, which were equipped with variable-temperature NMR probes. The spectra were collected with a sweepwidth of 1800 Hz and a 5-s delay between scans, and were recorded after 100 accumulations. ^1H chemical shifts were measured relative to 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$ (TSP) which was used as an internal standard. Refinement of the ^1H line widths by simulating the overlapping acetyl methyl and PC resonances treated each signal as a symmetric Lorentzian line. Parameters used in simulation included chemical shift, line width at half height and relative intensity. The ^2H NMR spectra were recorded on a Bruker HX-270 NMR spectrometer which operated at 41.4 MHz and was equipped with a variable temperature NMR probe. Spectra were acquired under unlocked conditions in 10-mm NMR tubes fitted with vortex plugs. A sweepwidth of 1000 Hz and a 2-s repitition rate was used with 0.5–2 h of accumulation time. Broad-band ^1H decoupling was employed for all ^2H measurements. All NMR experiments were repeated three times under identical conditions to ensure reproducibility of results. Nondetectable ^1H and ^2H resonances have line widths >15 and >62 Hz, respectively.

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