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Studies on the interaction of anesthetic steroids with phosphatidylcholine using ^{2}H and ^{13}C solid state NMR

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The effects of the anesthetic steroid alphaxalone and its inactive analog Δ^{16}-alphaxalone on model phospholipid membranes were studied using ^{13}C and ^{2}H solid-state nuclear magnetic resonance spectroscopy. Aqueous multilamellar dispersions of dipalmitoylphosphatidylcholine (DPPC) with specific ^{13}C and ^{2}H labels as endogenous probes at the carbonyl and the C-7 methylene groups, respectively, of the *sn*-2 chain were used to study the conformational and dynamical properties of the bilayer as a function of temperature. There were no significant changes between the ^{13}C and ^{2}H spectra of the DPPC preparation containing the inactive steroid and that of DPPC with no drug However, the physiologically active steroid produces significant spectral ^{2}H and ^{13}C changes. These changes include a reduction of the main phase transition temperature and a broadening of that transition. Alphaxalone also increases the relative number of *gauche* conformers in the liquid-crystalline phase of DPPC and increases the rate of axial diffusion in both the gel and liquid-crystalline phase The thermotropic properties of the above preparations, as monitored by differential scanning calorimetry, were congruent with the spectroscopic data

Introduction

In many classes of anesthetics, there is a good correlation between a compound's potency and its oil/water partition coefficient [1] suggesting that anesthetic activity results from a nonspecific interaction with the membrane lipids However, in the case of the anesthetic steroids, small structural changes with no significant effect on their partitioning properties can lead to large differences in activity [2,3] This has led investigators [4] to postulate specific interactions between the anesthetic steroids and synaptic membranes To account for the structural specificity some investigators [5] have suggested that anesthetic steroids act after binding at a distinct site on a target membrane protein, while others [6] have hypothesized that the sites of action are membrane lipids capable of a high degree of structural discrimination Evidence for this latter line of argument was obtained from electron spin resonance experiments [6] with spin-labeled lipid bilayers containing cholesterol These experiments showed that steroids with anesthetic activity caused a fluidization of the model membrane while structurally related inactive analogs produced much less disorder

Our initial studies focused on two structurally related steroids (Fig 1) with widely different phys-

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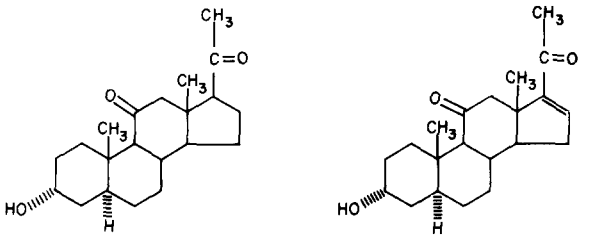

(a) alphaxalone (b) Δ16-alphaxalone

Fig 1 Steroid structures

iological properties One of them, alphaxalone, has potent anesthetic properties and is used clinically as the main active component in the anesthetic, Althesin The other, Δ^{16}-alphaxalone, which differs from alphaxalone only by a double bond in the 16-position, lacks anesthetic activity In an attempt to determine whether the observed differences in activity on the nerve could also be observed in other membrane systems, we tested the above two steroids for their ability to inhibit anion transport in human erythrocytes [7] We found that the anesthetic, alphaxalone, inhibited sulfate transport to a much greater extent than its nonanesthetic, Δ^{16}-analog The two steroids differed only slightly in their oil/water partitioning properties [8]

Recently, we investigated [9] the interactions of alphaxalone and Δ^{16}-alphaxalone with phosphatidylcholine bilayer vesicles that were used as model membranes The steroids were incorporated in the bilayer and the preparations were studied with the help of ^{1}H and ^{13}C high resolution NMR spectroscopy Examination of the spectra suggested that in such preparations alphaxalone is considerably more mobile than Δ^{16}-alphaxalone These findings were corroborated by high resolution ^{2}H-NMR experiments on vesicle preparations containing the same steroids, isotopically labeled with deuterium Our results showed that the two steroids experienced very different motional properties in the lipid bilayer and were interpreted [9] as evidence that the biologically active steroid perturbs the phospholipid bilayer more effectively than its inactive analog

In another study [10] we used a multilamellar bilayer dispersion of dimyristoylphosphatidylcholine with perdeuterated acyl chains (DMPC-d_{54}) as a model membrane We showed that alphaxalone consistently decreased the quadrupolar splittings of DMPC-d_{54} in the liquid-crystalline phase, an observation which was explained as an increase in the number of *gauche* segments of the acyl chains of DMPC The inactive Δ^{16}-alphaxalone did not produce this effect These preferential effects of alphaxalone in the bilayer were attributed to the observed differences in the conformations of the two steroids

Our present study was motivated by a desire to obtain more detailed molecular information on the interactions of the steroids with the phospholipid bilayer We used dipalmitoylphosphatidylcholine (DPPC) with specific ^{13}C and ^{2}H labels as endogenous probes in the phospholipid bilayer The ^{13}C label was placed in the carbonyl group of the *sn*-2 chains while the ^{2}H label was placed on the methylene group of C-7 in the same chain We have now obtained ^{13}C- and ^{2}H-NMR spectra as a function of temperature that shed some light on the conformational and dynamical changes that occur in the phospholipid bilayers as a result of their interactions with the drug molecules

Materials and Methods

5α-Pregnan-3α-ol-11,20-dione (alphaxalone) and 5α-pregn-16-ene-3α-ol-11,20-dione (Δ^{16}-alphaxalone) were kindly donated by Glaxo Research, Middlesex, U K 2[1′-^{13}C]DPPC and 2-[7′,7′-$^{2}H_2$]DPPC were synthesized according to literature procedures [11]

Samples for NMR experiments were prepared by dissolving the phospholipid (50 mg) with and without the steroid in 2 ml CH_2Cl_2 The solvent was then evaporated by passing a stream of nitrogen over the solution at 50°C and the residue was placed under vacuum (0 1 mmHg) for 12 h The phospholipid or drug/phospholipid (molar ratio 2 10) mixtures were subsequently introduced in 7 mm glass tubes appropriately constricted and ^{2}H-depleted water (Sigma) was added to produce 4 6 (w/w) lipid/water preparations The samples were sealed under high vacuum and equilibrated at 5–10 K above the phase-transition temperature for one hour before recording the spectra

^{13}C-NMR spectra were obtained on a home-

built solid state pulse spectrometer operating at 6 8 T (73 9 MHz for ^{13}C and 294 MHz for ^{1}H) A cross-polarization experiment was used with a 180° refocusing pulse [12] Typical parameters used were 4 0 μs for the $\pi/2$ ^{13}C pulses and 2 5–3 5 ms for the mixing time Sample heating due to ^{1}H decoupling was minimized by employing the minimum decoupling power necessary to obtain sharp spectra and by using long recycle delays (3–5 s) The number of ^{13}C free-induction decays accumulated, ranged between 1000 and 3000, depending on the sample temperature ^{2}H-NMR spectra were obtained (at 45 3 MHz) using a 90°_{x}-τ-90°_{y} quadrupole echo sequence [13] The ^{2}H $\pi/2$ pulse width was 2 1–2 4 μs, the 90° pulse separation (τ) was 40 μs while dwell times were 2 μs and 5 μs for gel and liquid-crystalline spectra, respectively Recycle delays were 0 2 s and 4000–16 000 echos were accumulated for each spectrum Differential scanning calorimetry (DSC) traces were obtained on a Perkin-Elmer DSC-2 Samples consisted of 5–10 mg of lipid and the scanning rates were 1 25 K/min

Results

^{13}C spectra of 2[1'-^{13}C]DPPC

Three sets of proton-decoupled ^{13}C spectra as a function of temperature are shown in Fig 2 The third column (C) includes spectra of 2[1'-^{13}C]DPPC preparation dispersed in H_2O and containing no drug molecules, while columns (A) and (B) show spectra obtained from similar DPPC preparations containing 0 1 M alphaxalone (A) and 0 1 M Δ^{16}-alphaxalone (B), respectively

The broad band (65–146 ppm) on the far right of each spectrum is due to the natural-abundance ^{13}C of the protonated acyl chain carbons To the left of it are smaller signals due to natural-abundance ^{13}C of the headgroup and the glycerol carbons The most dominant feature in the spectra is the powder pattern on the far left, due to the ^{13}C-enriched *sn*-2 carbonyl group (from −100 to 0 ppm) The ^{13}C spectra of pure dispersed 2[1'-^{13}C]DPPC have been discussed in detail elsewhere [11]

At 18°C the component of the spectrum due to the $^{13}C{=}O$ group shows an axially symmetric powder pattern with a residual anisotropy $\langle \Delta\sigma \rangle$ = $\sigma_{\parallel} - \sigma_{\perp} = -112$ ppm This represents a reduction in the breadth of the spectrum when compared to that of the dry lipid ($\Delta\sigma = -148$ ppm) and indicates that the dispersed lipid molecules undergo some anisotropic motion faster than γH_0 $\Delta\sigma \approx 10^4$ s^{-1} At 27°C the spectrum begins to show the presence of two overlapping components The predominant component is the axially symmetric powder pattern observed at lower temperatures while the minor component consists of a symmetric line of width approximately 1 kHz with a chemical shift $\langle \sigma \rangle = -44$ ppm, a value equal to the average tensor values from the spectrum of the dry lipid As the temperature is increased, the narrow component increases in intensity and becomes the only component present after the temperature exceeds the principal phase transition temperature (T_c = 41 3°C)

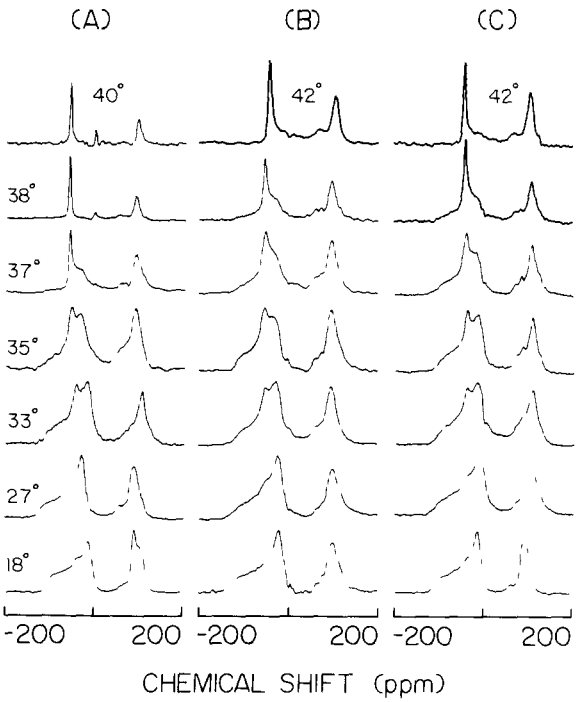

Fig 2 Temperature dependence of the ^{13}C-NMR spectra due to the 2[1'-^{13}C]carbonyl group of DPPC Spectra were obtained using a cross-polarization experiment and 1000–3000 scans were signal-averaged for each spectrum (A) DPPC + 0 167 M alphaxalone, (B) DPPC + 0 167 M Δ^{16}-alphaxalone, (C) DPPC

There is considerable evidence that in the two-component spectra the powder pattern is con-

tributed by the gel phase ($L_{\beta'}$) of DPPC while the narrow component is from a liquid-crystalline (L_α) phase The $L_{\beta'}$ and L_α components coexist for the temperature range corresponding to the monoclinic $P_{\beta'}$ phase of hydrated phosphatidylcholines The ^{13}C spectra that characterize this phase are made up of a gel superimposed on a liquid-crystalline-like component and the two components exchange at a rate which is intermediate on the ^{13}C time scale ($\approx 10^3$ s^{-1}) The striking difference in the lineshapes of the two spectral components has been explained by invoking a conformational change at the *sn*-2 carbonyl group This involves a realignment of the C=O bond from $\theta \simeq 24°$ in the $L_{\beta'}$ phase to $\theta \simeq 54°44'$ (magic angle) in the L_α phase, where θ is the angle between the unique shielding tensor of the C=O group and the axis of motion This interpretation is congruent with computer simulations for the *sn*-2 carbonyl portion of the spectrum that compare well with the experimentally obtained spectra The simulations assume two orientations of the symmetry axis of the carbonyl tensor, one tilted at approximately 24° with respect to the diffusion axis, $\hat{D}$, the other tilted at 54° Both tensors are allowed to execute rapid 3-fold jumps about $\hat{D}$ and to exchange with each other

The ^{13}C spectra of the DPPC dispersion containing the physiologically inactive steroid Δ^{16}-alphaxalone, are virtually identical to those of the pure DPPC sample On the other hand, the preparation containing the active steroid alphaxalone gave spectra that were significantly different Most striking are the differences in the spectra above 33°C (a) The spectra become isotropic-like with a sharp line for the ^{13}C=O component, at 38°C, approximately 3 K below the corresponding temperature of pure DPPC, (b) In the two-component spectra, the L_α-type component is larger than in the corresponding spectra of DPPC at the same temperature It thus appears that the presence of the steroid in the bilayer decreases the main transition temperature for DPPC At the same time, it enhances the L_α-like component among the two components that make up the $P_{\beta'}$ phase

^2H Spectra of 2[7′,7′-$^2H_2$]DPPC

Fig 3 includes a selection from three sets of ^{2}H-NMR spectra of 2[7′,7′-$^2H_2$]DPPC obtained

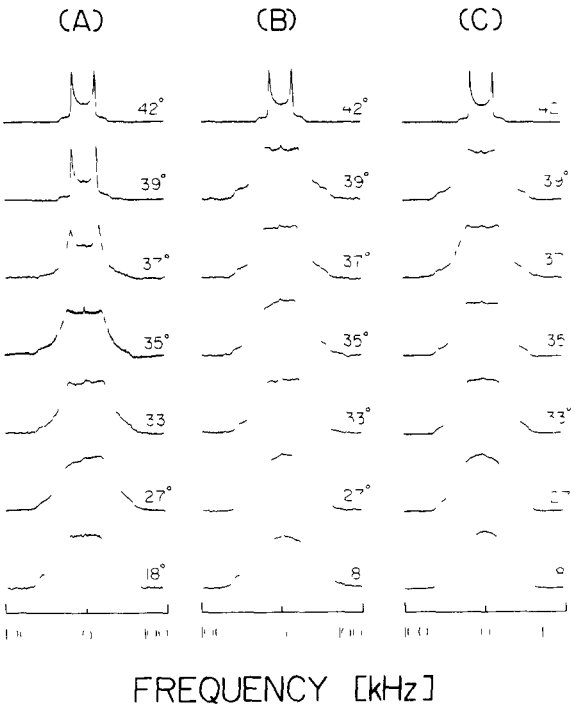

Fig 3 Temperature dependence of the ^{2}H-NMR spectra due to the 2(7′ 7′-C$^2H_2$) segment of DPPC 4000–16000 echos were signal-averaged to obtain each spectrum (A) DPPC + 0 167 M alphaxalone, (B) DPPC + 0 167 M Δ^{16}-alphaxalone, (C) DPPC

as a function of temperature, from 18 to 53°C As with the corresponding ^{13}C spectra the third row (C) has spectra obtained from a pure hydrated DPPC preparation while the other two rows show spectra of hydrated DPPC into which 0 167 M alphaxalone (A) and 0 167 M Δ^{16}-alphaxalone (B) were incorporated, respectively

Above T_c, all the ^{2}H spectra of DPPC are axially symmetric powder patterns with sharp parallel and perpendicular edges These spectra have residual quadrupolar splittings ($\Delta\nu_Q$) below 40 KHz, an indication that they are fast-limit ($> 10^7$ s^{-1}) and that the molecules undergo fast axial diffusion coupled with rapid *gauche-trans* isomerization in the acyl chains As expected, the value of $\Delta\nu_Q$ decreases as the temperature increases because of an increase in the *gauche-trans* conformer ratio As the temperature is lowered below T_c, the spectra of pure DPPC assume the broadened features of a gel-type spectrum At temperatures just below T_c and through the pre-

transition temperature range ($P_{\beta'}$) the spectra acquire a flat-top appearance However, the parallel and perpendicular edges are still discernible but severely broadened In the gel phase the spectra of pure DPPC become rounder with no sharpness in their features At 18°C the ^2H spectrum has a conical shape with a rounded top

The DPPC preparation containing the inactive steroid Δ^{16}-alphaxalone again gives spectra virtually identical with those obtained from pure DPPC However, as with the corresponding ^{13}C spectra, the anesthetic steroid alphaxalone produces significant changes in the ^2H spectra (a) Fast limit purely liquid crystalline-type spectra appear at 39°C, approximately 2 K below the corresponding temperature for pure DPPC (b) The quadrupolar splittings are slightly smaller (0 5–1 5 kHz) than those of pure DPPC indicating a slightly increased *gauche* population in the acyl chain segments (c) The spectra between 35 and 38°C show considerable more 'liquid-crystalline' character compared to the corresponding spectra from pure DPPC Unlike the flat-top appearance of the corresponding pure DPPC spectra, the alphaxalone-containing spectra show a depression in the center and have clearly discernible perpendicular and parallel edges The increased character of the L_α phase in these spectra is compatible with the ^{13}C observations of a corresponding increase of the L_α isotropic-like component Differences between the two sets of ^2H spectra can also be observed in the $L_{\beta'}$ phase where the alphaxalone containing preparation gives spectra with somewhat sharper edges and flatter tops presumably because of the faster motional rates of the phospholipid molecules

In Fig 4 we have plotted $\Delta\nu_Q$ for the L_α-type spectra We have also included the corresponding values for spectra that are not purely L_α but are two-component spectra in which the fast motion component is easily recognizable As can be seen, the pure DPPC preparation and that containing the inactive steroid show a sharp main phase transition However, the alphaxalone-containing preparation has a broader phase transition These spectroscopic observations are corroborated by the corresponding calorimetric data (Fig 5)

Fig 4 also includes graphs representing changes in the absolute ^2H spectra intensities as a function

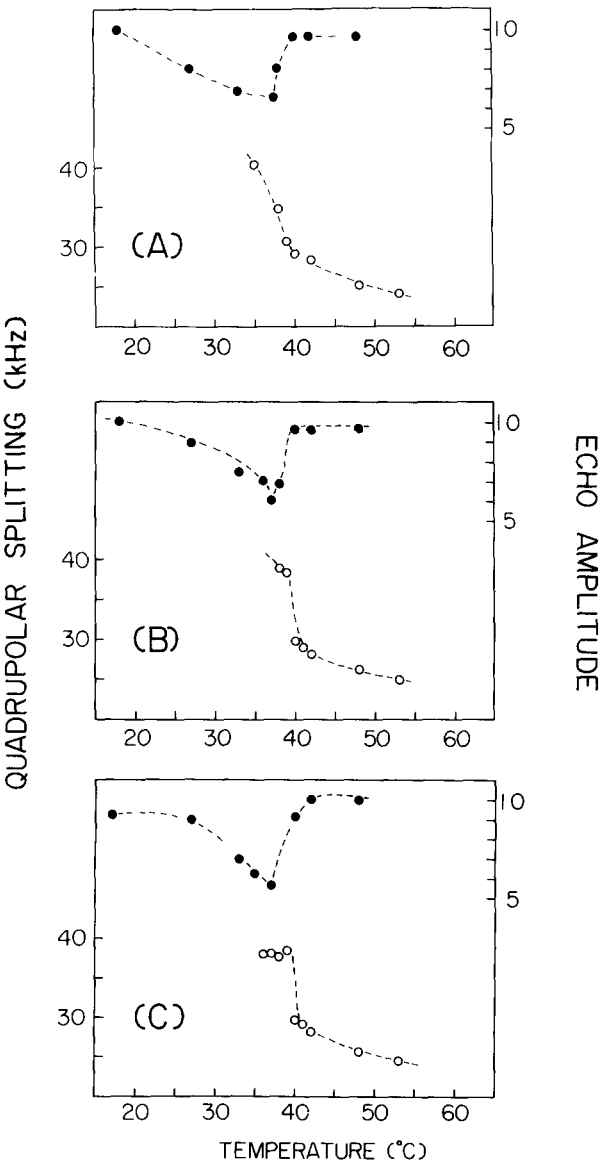

Fig 4 Temperature dependence of the ^2H quadrupolar splittings (○) in the ^2H-NMR spectra of the 2(7′,7′-C^2H_2) segment of DPPC and relative echo amplitudes (●) (A) DPPC + 0 167 M alphaxalone, (B) DPPC + 0 167 M Δ^{16}-alphaxalone, (C) DPPC

of temperature These measurements are a reflection of the effective T_2 values If the T_2 value is very short, the magnetization from the first $\pi/2$ pulse is not totally refocused by the second $\pi/2$ pulse resulting in a loss of spectral intensity In all three DPPC samples, there is a gradual loss of intensity during the pretransition temperature

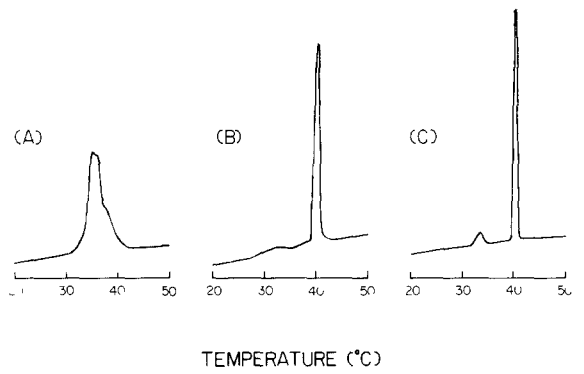

Fig 5 Differential scanning calorimetry traces of hydrated DPPC (60% wt H_2O) samples using a scanning rate of 1 25 K/min (A) DPPC+0 167 M alphaxalone, (B) DPPC+0 167 M Δ^{16}-alphaxalone, (C) DPPC

range and a sudden recovery just before the main transition The drop in spectral intensity which seems to characterize the DPPC pretransition is indicative of an exchange process either inter- or intramolecular

Differential scanning calorimetry

The DSC traces obtained from preparations identical with those used in the NMR experiments are shown in Fig 5 Clearly seen are the two endothermic transitions characteristic of pure DPPC, a broad low enthalpy pretransition (T_c' = 35 3°C) and a sharp higher enthalpy main transition (T_c = 41 3°C) The inactive steroid Δ^{16}-alphaxalone broadens the pretransition but does not alter T_c' and T_c In contrast, the anesthetic steroid alphaxalone produces a distinct lowering of T_c by 4–5 K Also, the presence of the steroid results in a significantly broader transition which seems to be made up of at least two overlapping components, while the low enthalpy pretransition is not discernible The broadening is an indication of decreased cooperativity at the phase transition These DSC results confirm the spectroscopic evidence that alphaxalone lowers T_c and broadens the range of the main transition

Discussion

The data described here are congruent with our previously reported work and provide additional evidence that alphaxalone and Δ^{16}-alphaxalone interact differently with membrane bilayers Furthermore, our present results provide us with more information on changes in the conformational and dynamical properties of the bilayer that accompany the phospholipid-steroid interactions The changes are summarized below

(a) The physiologically active steroid, alphaxalone, reduces the main phase transition temperature by approximately 5 K On the other hand, the inactive Δ^{16}-analog fails to do so, but only broadens the pretransition The differences in the thermotropic properties between the two steroid-containing preparations are closely reflected in the corresponding spectroscopic data In general, alphaxalone produces significant spectral (^2H and ^{13}C) changes in the DPPC preparations, while the Δ^{16}-alphaxalone/DPPC preparation gives spectra virtually identical to those of pure DPPC

(b) Alphaxalone broadens the main phase transition During that temperature range both ^{13}C and ^2H spectra show more L_α character with faster motional rates in the phospholipid molecules

(c) Alphaxalone increases the relative number of *gauche* conformers in the acyl chain in the L_α phase as indicated by the decrease of $\Delta\nu_Q$ of the ^2H spectra This behavior sharply contrasts that of cholesterol which produces a considerable increase in $\Delta\nu_Q$ due to a decrease of *gauche* conformers in the phospholipid chain (Cholesterol 0 15 M in 2[7′,7′-^{2}H]DPPC increases $\Delta\nu_Q$ by 10 3 kHz at 42°C [14])

Some tentative conclusions can be drawn about the manner in which anesthetic steroids affect biological membranes based on our present data with the model membranes Work described in the literature using solid state ^2H-NMR has already established that lipid orientational order and chain dynamics in model membrane systems closely mirrors the behavior of biological membranes [15] It is also well established that the dynamic and phase properties of the membrane lipid phase can significantly affect the function of membrane-associated proteins (Ref 15 and references therein) The anesthetic properties of the biologically active steroid can be ascribed to its ability to 'perturb' the lipid membrane component Based on our present data, it is tempting to associate the 'perturbing' effects of the anesthetic steroid with its

ability to induce faster axial diffusion accompanied by increased *gauche* contributions in the phospholipid chains

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References

1 Seeman, P (1972) Pharmacol Revf 24, 583–655
2 Atkinson, R M , Davis, B , Pratt, M A , Sharpe, H M and Tomich, E G (1965) J Med Chem 8, 426–432
3 Phillips, G H (1975) J Steroid Biochem 6, 607–612
4 Richards, C D and Hesketh, T R (1975) Nature 256, 179–182
5 Richards, C D , Martin, K , Gregory, S , Keightley, C A , Hesketh, T R , Smith, G A , Warren, G B and Metcalfe, J C (1978) Nature 276, 775–779
6 Lawrence, D K and Gill, E W (1975) Mol Pharmacol 11, 280–286
7 Makriyannis, A and Fesik, S W (1980) J Neurosci Res 5, 25–33
8 Fesik S W (1981) Ph D Thesis, University Connecticut, Storrs, CT
9 Makriyannis, A and Fesik, S W (1982) J Med Chem 26, 463–465
10 Makriyannis, A and Fesik, S W (1985) Mol Pharmacol 27 624–629
11 Wittebort, R J , Schmidt, C F and Griffin, R G (1981) Biochemistry 20, 4223–4228
12 Griffin, R G (1981) Methods Enzymol 72, 108–174
13 Davis, J H , Jeffrey, K R , Bloom, M , Valic, M J and Higgs, T P (1976) Chem Phys Lett 42, 390–394
14 Makriyannis, A and Griffin, R G , unpublished data
15 Davis, J H (1983) Biochim Biophys Acta 737, 117–171