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Geometric Requirements for Membrane Perturbation and Anesthetic Activity

Conformational Analysis of Alphaxalone and Δ^{16}-Alphaxalone and ^2H NMR Studies on Their Interactions with Model Membranes

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SUMMARY

^2H NMR spectra were obtained for dimyristoylphosphatidylcholine multilamellar dispersions, perdeuterated in the fatty acid chains, in the presence and absence of two steroid analogs. The presence of the active anesthetic steroid alphaxalone results in consistently smaller ^2H quadrupolar splittings ($\Delta\nu_Q$) for individual C^2H_2 segments of the fatty acid chains, indicating increased molecular disorder. In contrast, the inactive analog Δ^{16}-alphaxalone causes no significant change in the ^2H spectra of the phospholipid. The conformational analysis of alphaxalone and Δ^{16}-alphaxalone in solution was carried out with the help of ^1H and ^{13}C high resolution NMR spectroscopy and the results were used to propose a molecular model for the interaction of the two steroids with membrane phospholipids. The model correlates the observed differences in the manner in which the two steroids interact with model membranes with differences in their respective conformations and provides a molecular basis for anesthetic steroid activity.

INTRODUCTION

In many classes of anesthetics, there is a good correlation between a compound's anesthetic 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 anesthetic activity (2, 3). To account for this structural specificity, some investigators (4) have suggested that anesthetic steroids act after binding at a distinct site on a target synaptic membrane protein. Others (5) have hypothesized that the sites of action are membrane lipids capable of a high degree of structural discrimination. Evidence for this line of argument was obtained from electron spin resonance experiments (5) with spin-labeled lipid bilayers containing cholesterol.

Our initial studies focused on two structurally related steroids (Fig. 1) with widely different physiological 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 also could be observed in other membrane systems, we tested these two steroids for their ability to inhibit anion transport in human erythrocytes (6). We found that the anesthetic alphaxalone inhibited sulfate transport to a much greater extent than did its nonanesthetic Δ^{16} analog. On the other hand, the two steroids differed only slightly in their oil/water-partitioning properties (7).

Recently, we investigated (8) 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 ^1H, ^{13}C and ^2H high resolution NMR spectroscopy. Our results showed that the two steroids experienced very different motional properties in the lipid bilayer and were interpreted (8) as evidence that the biologically active steroid perturbs the phospholipid bilayer more effectively than its inactive analog.

The present investigation, which examines the effects of alphaxalone and Δ^{16}-alphaxalone on multilamellar phospholipid dispersions as monitored by ^2H NMR, provides evidence supporting our previous interpretation. The studies described here have sought to obtain infor-

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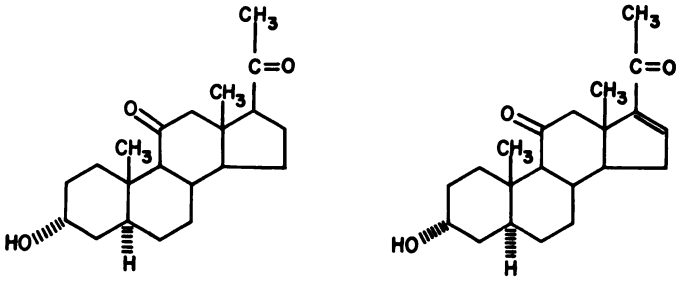

FIG. 1. *Steroid structures*

mation on the relative degree of ordering of the DMPC[2]-perdeuterated fatty acid chains in the presence and absence of the steroids. We also have examined the conformational properties of alphaxalone and Δ^{16}-alphaxalone in solution. Based on these results, we have proposed a molecular model that describes the interaction of the two steroids with membrane phospholipids and attempts to explain their different abilities to perturb model membranes.

MATERIALS AND METHODS

^2H-depleted ^1H_2O was purchased from Sigma Chemical Co., St. Louis, MO, and DMPC-d_{54} was obtained from Cambridge Isotope Laboratories, Cambridge, MA. 3α-Hydroxy-5β-pregnan-11,20-dione (alphaxalone) and 3α-hydroxy-5β-pregn-16-ene-11,20-dione (Δ^{16}-alphaxalone) were kindly donated by Glaxo Research, Middlesex, England. Lipophilicity was measured using reverse phase thin layer chromatography. For alphaxalone and Δ^{16}-alphaxalone, respectively, R_m = 0.33 ± 0.1 and 0.23 ± 0.1.

^1H and ^{13}C high resolution NMR spectra were obtained on an HX-270 Bruker spectrometer operating at 270 and 67.9 MHz, respectively. Samples were dissolved in $CDCl_3$ and fully degassed as 0.01 (^1H) and 0.25 M (^{13}C) solutions.

^2H NMR spectra were recorded on a Bruker CXP-200 spectrometer operating at 30.7 MHz. The instrument was equipped with a solenoid probe which accepts microcells with a capacity of 0.5 ml. To eliminate the problems of spectrometer dead time and phase distortions, the quadrupole spin-echo pulse sequence (9) was employed using 55- and 50-μsec delays after the first and second 90° pulses (7.3 μsec), respectively, with phase cycling. A sweep width of 100 kHz, 4096 data points, and 0.25-sec recycle time were used. ^2H NMR samples were prepared by dissolving the steroid and phospholipid in chloroform and evaporating the solvent under a stream of nitrogen followed by exposure to high vacuum (0.2 mm Hg) for 6 hr. To the dried sample, ^2H-depleted ^1H_2O was added, and the preparation was mixed with a Vortex mixer for 10 min at 50° and transferred to the microcell.

^1H-^1H nuclear Overhauser enhancement (10, 11) measurements were performed on a Bruker WH-90 spectrometer using $CDCl_3$ solutions (0.01 M) that were degassed to remove all oxygen. Spectra with NOE were run by irradiating the required frequency with low power for a duration of 2 sec prior to accumulation of the free induction decay. A minimum delay of 10 sec between decays was allowed. The power requirements for irradiation were chosen to optimize the NOE effect while still maintaining selectivity. To obtain spectra with no NOE, the oscillator frequency was moved to a frequency greater than 100 Hz from any proton absorption. The area of each frequency of interest was then carefully integrated and NOE values were obtained by comparing individual peak areas from spectra with and without NOE. NOE values were obtained as the average of three determinations.

^1H-^1H-coupling constants were extracted from the corresponding ^1H spectra. ^1H assignments were made from an analysis of the scalar couplings and extensive homonuclear decoupling experiments.

^1H-^{13}C-coupling constants were obtained from ^1H-coupled ^{13}C NMR spectra. ^{13}C assignments were made by analogy from previously assigned ^{13}C spectra of steroids (12) and by off-resonance and single resonance ^1H spin-decoupling experiments.

[2] The abbreviations used are: DMPC, dimyristoylphosphatidylcholine; NOE, nuclear Overhauser enhancement; DMPC-d_{54}, 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphocholine.

RESULTS AND DISCUSSION

Conformational Analysis

Alphaxalone. ^1H-^1H vicinal coupling constants between the 17 and 16α, 16β protons of alphaxalone are shown in Table 1. Based on the observed coupling constants, dihedral angles of 20° [H(17)C(17)C(16)H(16α)] and 140° (H(17)C(17)C(16)H(16β)] were calculated from a modified Karplus equation (13). The results suggest a puckered D-ring conformation. This is in close agreement with the conformation of alphaxalone obtained in the crystalline state (14) in which the D-ring conformation is intermediate between a half-chair and a $C_{1,3}$ envelope (Fig. 2).

^{13}C-^1H vicinal coupling constants. Due to the lack of protons at the C-20 position, ^1H-^1H vicinal coupling

TABLE 1

^1H-^1H and ^{13}C-^1H vicinal coupling constants (J) and the corresponding dihedral angles (ϕ)

Alphaxalone			Δ^{16}-Alphaxalone		
Dihedral angle	*J*	ϕ	Dihedral angle	*J*	ϕ
	Hz	*degrees*		*Hz*	*degrees*
H_{17}-$H_{16\alpha}$	8.8	20	H_{16}-$H_{15\alpha}$	1.9	64
H_{17}-$H_{16\beta}$	9.4	140	H_{16}-$H_{15\beta}$	3.0	57
C_{21}-H_{17}	≤0.8	90	H_{14}-$H_{15\alpha}$	10.0	0
			H_{14}-$H_{15\beta}$	4.4	122

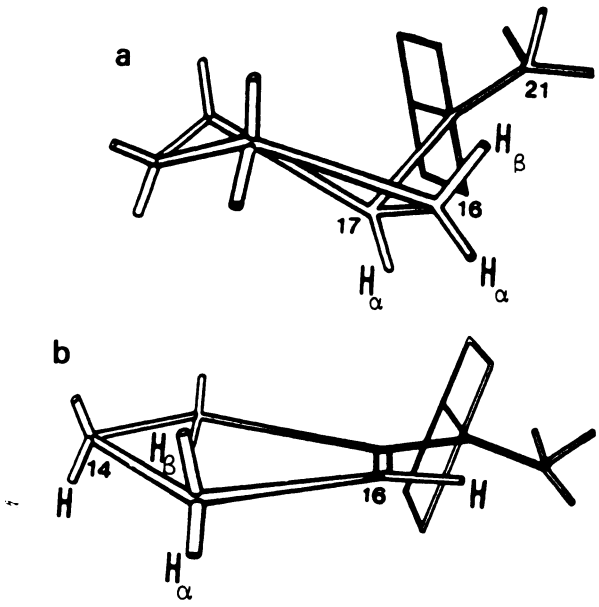

FIG. 2. *The D-ring conformation of alphaxalone (a) and Δ^{16}-alphaxalone (b) in solution as determined by high resolution NMR*

constants could not be used to define the side chain acetyl group conformation of alphaxalone. Instead, we used the ^{13}C-^{1}H-coupling constant between C_{21} and H_{17} to obtain information on the C(21)C(20)C(17)H(17) dihedral angle. These ^{13}C-^{1}H vicinal coupling constants have been shown to have an orientational dependence analogous to that of protons (15). The ^{13}C-^{1}H-coupling constant obtained from the ^{1}H-coupled ^{13}C NMR spectrum was found to be very small (<0.8 Hz), below the limits of resolution under the experimental conditions employed. It suggested that the favored conformation of the side chain acetyl group of alphaxalone is one in which the dihedral angle between C_{21} and H_{17} is approximately 90° (Fig. 2).

Δ^{16}-*Alphaxalone*

^{1}H-^{1}H vicinal coupling constants obtained for the D-ring protons of Δ^{16}-alphaxalone and their corresponding dihedral angles are shown in Table 1. The calculated dihedral angles support a flattened D-ring conformation with all of the carbon atoms of the ring lying in the same plane (Fig. 2).

^{13}C chemical shifts. Information on the side chain acetyl group conformation of Δ^{16}-alphaxalone was obtained from the ^{13}C chemical shift of the C-20 carbonyl carbon which belongs to an α,β-unsaturated ketone system. Such carbonyl groups have chemical shifts that are influenced by the acetyl group conformation (16). Compounds in which the C=O and C=C groups are coplanar with overlapping π-electron clouds have carbonyl chemical shifts between 195 and 198 ppm. However, in α,β-unsaturated ketones in which the carbonyl group is not coplanar with the double bond, ^{13}C=O chemical shifts are above 200 ppm. The ^{13}C spectrum of Δ^{16}-alphaxalone showed a C=O equal to 196 ppm, supporting a planar conformation.

^{1}H-^{1}H nuclear Overhauser enhancement. The ^{13}C chemical shifts did not allow us to distinguish between the two possible *S-cis* or *S-trans* planar conformations (Fig. 3). This type of conformational analysis had previously relied on Raman and ultraviolet spectroscopic analysis. However, these methods gave only ambiguous results with Δ^{16}-alphaxalone. We thus turned to ^{1}H-^{1}H NOE measurements for the resolution of this problem. NOE involves a change in intensity of the resonance signal of a proton when another proton is irradiated with a second radio frequency field. The effect which is transmitted through space and depends on the distance (r^{-6}) between the two protons has been used successfully in structural and conformational determinations of organic molecules (11).

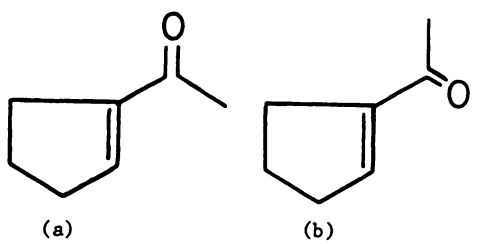

FIG. 3. *The two possible planar conformations for the acetyl group of Δ^{16}-alphaxalone: S-trans (a) and S-cis (b)*

The experiment was carried out by irradiating the C-21 methyl protons and observing the NOE effects on the C-12β, and C-16 vinylic protons. Each of these two protons exists in close proximity to the irradiated acetyl protons when this group exists respectively in the *S-cis* or the *S-trans* conformation (Fig. 3). In each conformation, a significant NOE value is expected for only one of the two protons. Calculation of the expected NOE values presents a great deal of uncertainty because the C(=O)CH_3 protons can experience rotation around two single bonds (C_{17}—C=O and C_{20}—C=O) so that their distances from neighboring protons are not fixed. However, the measured NOE values lend themselves to a satisfactory semiquantitative interpretation. In our experiment, no NOE effect was observed for the C-12 proton, while the C-16 proton showed a significant NOE effect of 8%. These results indicate that the acetyl group exists predominantly in the *S-trans* conformation (Fig. 3).

Comparison of alphaxalone and Δ^{16}-alphaxalone conformations. The NMR data discussed above were interpreted in terms of a single conformation although the possibility of several interconverting conformers for each molecule cannot be ruled out. The data show subtle but significant geometrical differences between the two steroids in solution. In the anesthetic steroid alphaxalone (Fig. 5b), the D-ring is puckered, and the 17-acetyl group protrudes from the β-face of the steroid. In contrast, the inactive Δ^{16} analog (Fig. 5a) has the D-ring almost flat while the acetyl side chain is in the *S-trans* conformation and does not protrude significantly above the plane of the steroid ring.

Steroid-Phospholipid Interactions

Wideline ^{2}H NMR has proven to be one of the most fruitful tools for obtaining information on the structural and dynamic properties of lipids in biological membranes (17, 18). The method also can be extended to study lipid interactions with other molecules such as proteins, cholesterol (19), or local anesthetics (20, 21).

In a system undergoing anisotropic motions as in the case of ^{2}H-labeled membranes or multilamellar bilayer vesicles in the liquid-crystalline phase, each deuteron in the molecule gives rise to a "powder spectrum." In the spectrum, a maximum intensity doublet corresponds to those orientations in which the bilayer normal is perpendicular to the magnetic field. The doublet spacing or quadrupolar splitting ($\Delta\nu_Q$), which is obtained for each deuteron in the system, will depend on the degree of anisotropy and on the orientation of the C-^{2}H bond with respect to the molecular symmetry axis and is equal to: $\Delta\nu_Q = (3/4)\,(e^2qQ/h)S_{CD}$. In this equation, e^2qQ/h is the static quadrupole coupling constant which for paraffinic C-D bonds in phospholipids is 167 kHz (18). S_{CD} is the order parameter that can be used to describe the amount of motional averaging of the C-D bond vector with respect to a fixed symmetry axis (director).

In the liquid crystalline phase, the fatty acid chains of multilamellar DMPC bilayers exhibit fast long axis rotation and *trans:gauche* isomerization. As a result of this, the powder patterns due to each of the C-D groups in the chain are axially symmetric. In such a system, an

increase in the number of *gauche* segments in the chain will contribute to a reduction of the quadrupolar splitting and the order parameter.

Fig. 4a shows the ^2H NMR spectrum of 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphocholine as a multilamellar dispersion in the liquid crystalline phase. It is a typically complex spectrum consisting of a number of overlapping axially symmetric quadrupolar powder patterns, one for each deuteron of the hydrocarbon chains. The complexity of the spectrum is increased because of the inequivalence of the two phospholipid chains and the inequivalence of the C-2 deuterons in chain 2. However, several of these peaks can be resolved and tentatively identified by comparison with spectra of selectively deuterated samples (17, 19). The outermost ^2H resonances ($\Delta\nu_Q$ = 22.2–25.4 kHz) in the spectrum correspond to deuteria attached to the C(3)-C(8) carbons of both chains as well as those of C-3 in chain 2 and the C-2 in chain 1. This is a region of relatively constant segmental motion and represents the "plateau" in the plot of quadrupolar splittings versus chain position. The ^2H signals approaching the center of the spectrum correspond to segments of the acyl chains with decreasing molecular order and are assigned to respective chain methylene units with increasing numbers. The only exception to this general rule are the C-2 deuterons of chain 2 (Fig. 4). This methylene segment is situated on the bend that characterizes chain 2 of phospholipids. The group has reduced flexibility and a geometry different from that of the other methylene segments of the two chains. As a result, the corresponding C-^2H bonds have different average orientations with respect to the bilayer normal and unlike those of the other C-^2H bonds in the chains.

The distribution of quadrupolar splittings in the ^2H spectra was used as an indicator for changes in the dynamic and conformational behavior of the fatty acid chains in the phospholipid preparations. Fig. 4b depicts the ^2H NMR spectrum of a multilamellar dispersion of DMPC-d_{54} into which Δ^{16}-alphaxalone was incorporated. The spectrum is virtually identical to that of the pure phospholipid with no change in the quadrupolar splittings. In contrast, the corresponding ^2H spectrum for the multilamellar dispersion containing alphaxalone (Fig. 4c) has consistently smaller quadrupolar splittings (Table 2). These results indicate that alphaxalone reduces the degree of order for the entire length of the fatty acid chains, whereas the inactive Δ^{16}-alphaxalone causes no such perturbation.

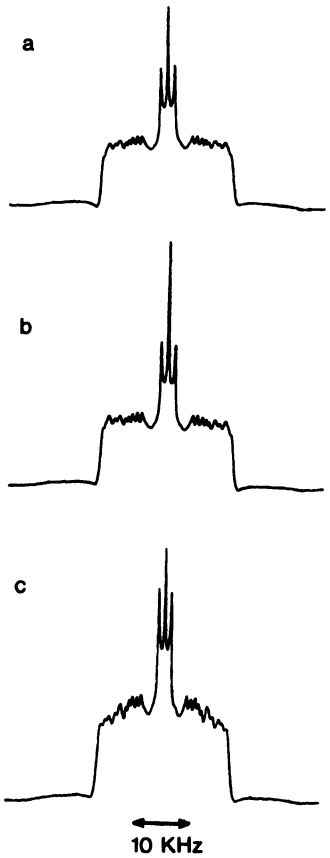

FIG. 4. *^2H powder-type spectra of DMPC-d_{54} multilamellar vesicle preparations: (a) no steroid; (b) +Δ^{16}-alphaxalone; (c) + alphaxalone*

Samples *b* and *c* were 16.7% molar in steroid concentrations with respect to DMPC while the phospholipid concentration in all three samples was 10% (w/v) in ^1H_2O. The spectra were obtained at 40° using the quadrupolar echo sequence (9) and 2000 accumulations.

Conclusions

Although alphaxalone and Δ^{16}-alphaxalone have very similar structures, they have very different anesthetic properties. Previously, we have found that the anesthetic alphaxalone inhibited anion transport in the human erythrocyte while the inactive Δ^{16}-alphaxalone inhibited anion transport only modestly (7). Since both steroids were found to be incorporated into the red cell in equal amounts (8), the differences in biological activity cannot be attributed to a preferential penetration of the steroid into the membrane. Most likely, they are due to a differ-

TABLE 2

Quadrupolar splittings ($\Delta\nu_Q$ in kilohertz) and order parameters (S_{CD}) of individual deuterons in DMPC-d_{54} multilamellar vesicles in the presence and absence of steroids

Deuterium positions[a]	DMPC-d_{54}[b]	DMPC-d_{54} + alphaxalone[b,c]	DMPC-d_{54} + Δ^{16}-alphaxalone[b,c]
14	2.7 (0.022)	2.4 (0.019)	2.7 (0.022)
13_a	9.6 (0.077)	8.7 (0.070)	9.7 (0.077)
13_b, 2_{b_2}	11.5 (0.092)	10.4 (0.083)	11.5 (0.092)
12_a	13.3 (0.106)	12.1 (0.097)	13.4 (0.107)
12_b	14.7 (0.117)	13.3 (0.106)	14.8 (0.118)
11_a	15.9 (0.127)	14.4 (0.115)	16.0 (0.128)
11_b, 10_a, 2_{b_1}	18.2 (0.145)	16.5 (0.132)	18.1 (0.145)
10_b, 9_a	20.1 (0.161)	18.7 (0.149)	20.0 (0.160)
9_b, 8_a	22.2 (0.177)	21.0 (0.168)	22.3 (0.178)
8_b, 7_a, 7_b	23.4 (0.187)	22.5 (0.179)	23.1 (0.184)
2_a, 3–6	25.4 (0.203)	24.5 (0.195)	25.4 (0.203)

[a] The numbers denote the individual carbon atoms of the fatty acid chains to which the deuteron is attached. The subscripts *a* and *b* refer to chain 1 or chain 2, respectively. 2_{b_2} and 2_{b_1} are respectively the 2*S* and 2*R* deuterons of chain 2. Tentative assignments were made by analogy (17, 19).

[b] Phospholipid concentration was 10% (w/v) in H_2O. Measurements were made at 40°. Order parameters (S_{CD}) listed in parentheses were calculated from the equation listed in the text.

[c] Molar ratio of steroid:phospholipid was 0.167.

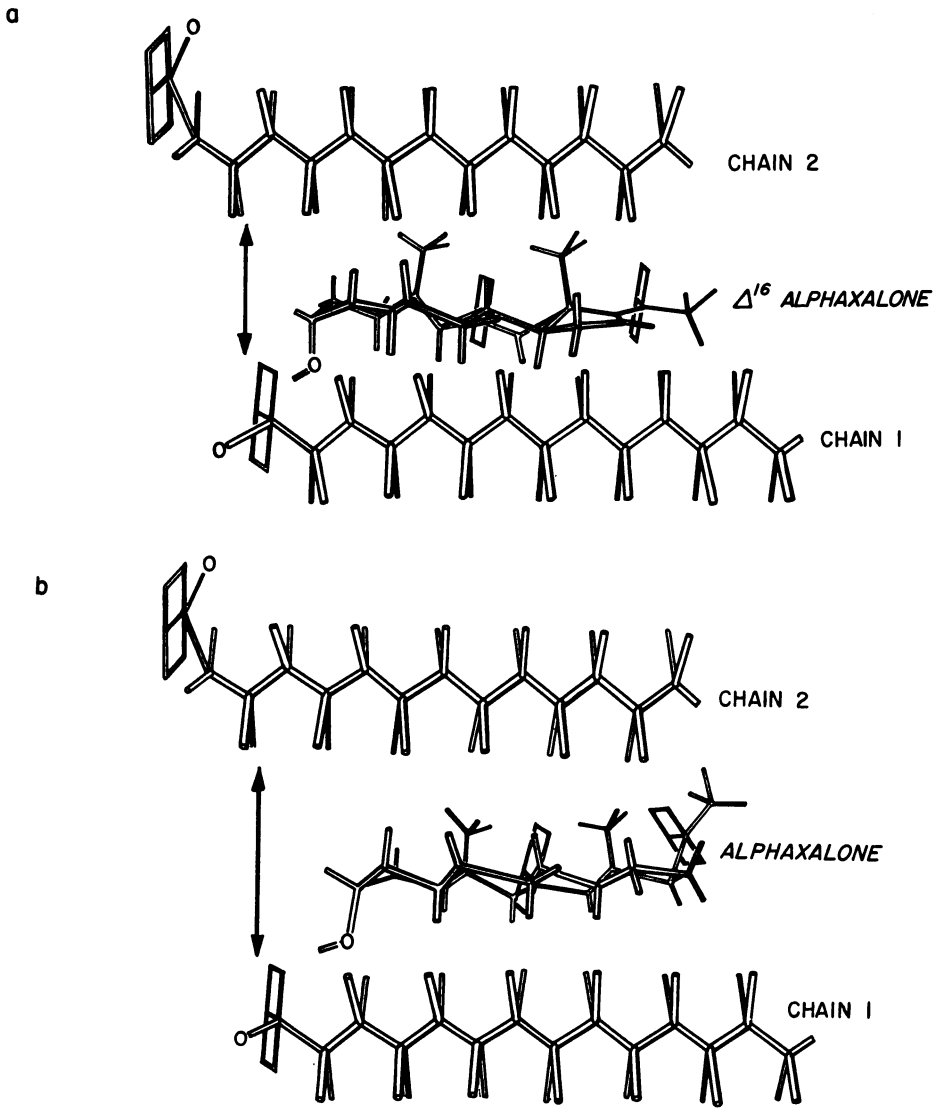

FIG. 5. *Molecular models for steroid:phospholipid chain interactions*

Each of the two steroids is placed between chain 2 (top) and chain 1 (bottom) of two adjacent DMPC molecules. The steroids are represented as being all-*trans*. The model shows that Δ^{16}-alphaxalone (a) accommodates easily between the two chains while alphaxalone (b) requires more space.

ence in the mode with which the two molecules interact with the membrane.

Our previous high resolution NMR studies provided evidence that, when incorporated in bilayer vesicles, alphaxalone undergoes faster motions than Δ^{16}-alphaxalone (8). On the other hand, the ^2H NMR data presented here indicate that, in multilamellar phospholipid dispersions containing alphaxalone, the fatty acid chains are more "disordered" than in the corresponding Δ^{16}-alphaxalone preparations. Both results are congruent with the interpretation that the two steroids differ in the manner with which they interact with phospholipid bilayers.

Based on the data presented here, we propose a model for the DMPC:steroid interactions, depicted in Fig. 5. The figure represents chain 1 and chain 2 of two adjacent phospholipid molecules separated by a space normally occupied by cholesterol. The inactive steroid Δ^{16}-alphaxalone (Fig. 5a) has a flat conformation and is easily accommodated between the fatty acid chains. However, the anesthetic alphaxalone, because of its protruding D-ring, occupies more space (Fig. 5b) and produces conformational alterations in the phospholipid bilayer. The changes probably involve an increase in the ratio of *gauche:trans* segments in the chain, which is reflected in a decrease in the order parameter for all the chain segments. This explanation is compatible with the observed decrease in quadrupolar splittings for the C^2H_3 and all C^2H_2 groups of the fatty acid chains in the multilamellar bilayer preparation when the anesthetic steroid is incorporated.

The above results are congruent with data we have recently obtained using differential scanning calorimetry on preparations of hydrated dipalmitoylphosphatidylcholine. We found that incorporation of 0.17% molar alphaxalone into the phospholipid depressed its phase transition temperature by 6.5°, from T_c = 41.5 to T_c =

35°. On the other hand, the inactive Δ^{16}-alphaxalone depressed the phase transition temperature by only 0.5°. Recent preliminary solid state NMR studies using phospholipids deuterated in one fatty acid chain segment corroborate the calorimetric results.[3]

It has been argued (22) that perturbations produced in the lipid region of the membrane could be transmitted to the membrane-associated proteins, resulting in a modification of their functions. Such a model has been used to explain the mechanism of anesthetic action (23). Our experiments provide evidence that the perturbation of membrane lipids by anesthetic steroids is governed by specific geometric requirements and can be used to explain the differences in anesthetic activity between alphaxalone and Δ^{16}-alphaxalone.

Currently, we are seeking to identify in more detail the specific conformational and dynamic changes in the phospholipid bilayers that are associated with membrane perturbation.

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