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Effects of Anesthetic and Nonanesthetic Steroids on Dipalmitoylphosphatidylcholine Liposomes: A Calorimetric and Raman Spectroscopic Investigation†

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ABSTRACT: The effects of anesthetic and nonanesthetic steroids on dipalmitoylphosphatidylcholine liposomes were studied by use of high sensitivity scanning calorimetry and Raman spectroscopy. Calorimetric measurements indicated that both anesthetic and nonanesthetic steroids depressed and broadened the gel to liquid-crystalline phase transition. There was no correlation between the perturbations by the steroids on the primary gel to liquid-crystalline phase transition temperature and anesthetic potency. The magnitudes of the steroid-induced transition broadening and lowering of the pretransition temperature, however, correlated well with anesthetic potency. This effect appeared to arise from the projection from the plane of the D ring of substituents at the C(17) position of the steroid nucleus. Raman spectroscopic measurements demonstrated that the steroid molecule is localized within the acyl region of the bilayer and that effects of the steroid do not extend to either the head-group or interface regions of the lamellae. The data are consistent with unitary hypotheses relating general anesthesia to lipid perturbations. For model systems, perturbations to the subtle structural and dynamical properties of the bilayer pretransition may provide a more sensitive marker than the main phase transition in assessing the significance of lipid mediation in inducing anesthetic action.

General anesthesia is widely believed (Roth, 1979, 1980; Janoff & Miller, 1982; Mountcastle et al., 1978; Trudell, 1977; Seeman, 1972) to result from a lipid-mediated perturbation of protein function in excitable membranes. Evidence supporting this hypothesis consists primarily of numerous correlations between anesthetic potency, lipid solubility, and lipid effects (Janoff & Miller, 1982; Meyer, 1899; Overton, 1901; Janoff et al., 1981; Seeman, 1966, 1972). Various investigators (Janoff & Miller, 1982; Trudell et al., 1973a,b; Mountcastle et al., 1978; Hill, 1978) have shown that general anesthetics lower and broaden the main bilayer phase transition of phosphatidylcholine liposomes and that these effects, like general anesthesia, may be reversed by pressure. General anesthesia both in mice and in tadpoles results from inhalation anesthetics at concentrations that induce approximately 1 °C decrease in the phase transition temperatures of dipalmitoylphosphatidylcholine (DPPC) liposomes (Janoff & Miller, 1982); this corresponds to anesthetic concentrations in the DPPC bilayers of about 4 mol % (Hill, 1978). Despite

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the large body of evidence supporting the concept of a lipid-mediated mechanism of anesthetic action, various authors [see, for example, Franks & Lieb (1978, 1982)] maintain that general anesthetics directly perturb proteins, citing evidence that clinical concentrations of anesthetics produce no demonstrable structural changes in lipid bilayer systems investigated by X-ray and neutron diffraction [Franks & Lieb (1978, 1982) and references cited therein] and by Raman spectroscopy (Lieb et al., 1982).

Although the vast majority of studies directed toward elucidating lipid–anesthetic interactions have utilized either inhalation agents or the anesthetic alcohols, such systems are not ideal for this type of investigation because of the difficulty in determining the extent of anesthetic partitioning into the lipid bilayer. Furthermore, these agents interact nonspecifically with both proteins and lipids (Franks & Lieb, 1982), making it difficult to draw conclusions regarding the relative importance of lipid-mediated and direct protein effects. In contrast, the anesthetic steroids (Gyermek & Soyka, 1975; Selze, 1941, 1942; Seeman, 1966; Lawrence & Gill, 1975; Lee, 1979; Figdor et al., 1957) do not display either of these limitations. Their very low water solubilities [e.g., 156 μmol/L for testosterone (Lundberg, 1979)], high lipid–water partition coefficients [e.g., 3268 for progesterone in egg phosphatidylcholine (Heap et al., 1971)], and moderate lipid solubilities [e.g., 109 mmol of progesterone/mol of egg phosphatidylcholine (Heap et al., 1971)] ensure that virtually all the steroid colyophilized with lipid in a model membrane dispersion remains in the lipid phase (Lawrence & Gill, 1975). In addition, while many steroids interact with specific binding and transport proteins, they are not known to induce nonspecific perturbations of protein structure. These agents, some of whose structures are given in Figure 1, display pronounced variations in anesthetic potency as a consequence of relatively minor structural changes. Thus, while Δ^{16}-alphaxalone is inactive as an anesthetic, alphaxalone is the most potent of the anesthetic steroids (Phillips, 1974; Child et al., 1971). Despite these interesting and useful characteristics, the steroid anesthetics have not been extensively investigated from a biochemical perspective. Connor et al. (1974) reported that 10 mol % concentrations of alphaxalone lowered the melting temperature of DPPC liposomes, by up to 8 °C; details of this scanning calorimetric work, however, were not reported. Several authors have noted that anesthetics abolish the bilayer pretransition (Lee, 1979; Mountcastle et al., 1978). Lawrence & Gill (1975) determined that, for spin-labeled liposomes, alphaxalone decreased the acyl chain order parameter more than anesthetically inactive steroids. These results present a confusing picture of the membrane effects of steroids.

In order to elucidate possible structure–activity relationships manifest by steroids in model membranes, we apply the techniques of high sensitivity scanning calorimetry and Raman spectroscopy. The latter yields conformational information for the head-group, interface, and acyl chain regions of the bilayer without the introduction of perturbing probes (Levin, 1984). Raman spectroscopy provides both direct and indirect information on the membrane location of perturbing molecules by monitoring membrane conformational changes and by identifying specific spectral characteristics representative of the perturbant (Bunow & Levin, 1978).

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC), obtained from Sigma Chemical Co., was recrystallized from ethanol and lyophilized from chloroform. Progesterone, pregnanedione, testosterone, and testosterone acetate were also obtained from Sigma Chemical Co. and used without further purification. Alphaxalone and Δ^{16}-alphaxalone were gifts of Glaxo Group Research, Ltd., and were also used without further purification. Samples were prepared by colyophilizing DPPC with the steroid from a chloroform solution in a lipid to steroid mole ratio of 9:1 and then rehydrating to approximately 75% water. The resulting liposomes were cycled above (50 °C) and below the gel to liquid-crystalline transition temperature four times and mechanically agitated following each heating. Highly concentrated phospholipid dispersions (25% w/w) were used in this work to minimize the possibility that any significant quantity of steroid would enter the aqueous phase and to carry out both the spectroscopic and calorimetric measurements under identical conditions for the liposomes. Samples were allowed to equilibrate at room temperature for at least 10 days.

The heat flow scanning calorimeter described by Ross & Goldberg (1974) was used in this work. In a typical calorimetric experiment, about 20 mg of the liposomal preparation (25% DPPC) was loaded into the calorimetric cell, with the tare cell remaining unfilled, and heated from 9.7 to 47 °C at a scanning rate of 14.9 K h^{-1}. The solvent contribution to the heat capacity was determined in a separate loading of the sample cell and subtracted from the phospholipid run. The differential thermopile voltage was measured with a Keithley Model 181 nanovoltmeter and recorded at 15-s intervals by an HP85 computer. The differential voltage signal was converted to power absorbed W (watts) by the Tian equation

$$W = \epsilon(V + \tau\, dV/dt)$$

where ϵ is the instrument calibration constant (watts per volt) and τ is the instrumental time constant (seconds) (Randzio & Suurkuusk, 1980).

The Raman spectrometer has been described previously (Huang et al., 1982). Raman spectra were acquired with an instrumental resolution of ~4–5 cm^{-1} and, depending on the spectral region, were signal averaged for 1–400 scans at a scan rate of 1 cm^{-1}/s. Samples were equilibrated for 5–10 min at each temperature prior to acquiring spectra. Temperature profiles in the C–H (2800–3100-cm^{-1}) and C–C stretching (1000–1200-cm^{-1}) mode regions were obtained at 1 °C intervals for all steroids listed above. Spectral subtractions of the steroid contributions to the lipid signals were not required in order to obtain reliable lipid $I_{2935cm^{-1}}/I_{2980cm^{-1}}$ and $I_{1090cm^{-1}}/I_{1100cm^{-1}}$ peak height intensity ratios, since relatively low steroid concentrations are involved in the liposomal preparations. Spectra were obtained for anhydrous DPPC–steroid samples over the 675–1525-cm^{-1} and 1600–1800-cm^{-1} intervals. Spectra in these regions were also obtained for hydrated samples of DPPC–alphaxalone, DPPC–progesterone, and DPPC–pregnanedione.

Results and Discussion

Calorimetric scans for pure DPPC and for DPPC–steroid multilamellar dispersions are shown in Figures 1 and 2. The main lipid bilayer phase transition temperatures and enthalpies for DPPC were 314.63 ± 0.03 K and 8480 ± 1000 cal/mol (average of 15 determinations), in excellent agreement with the results of Albon & Sturtevant (1978) for highly purified material. While our transition temperature determinations were very precise, our enthalpy determinations exhibited a range of values that we attribute to difficulties associated with the preparation and handling of these highly concentrated phospholipid suspensions. Difficulties in preparing lipid suspensions (at much higher dilutions) that give reproducible enthalpy determinations have been noted recently by Chen & Sturtevant (1981). Our calorimetrically determined enthalpies

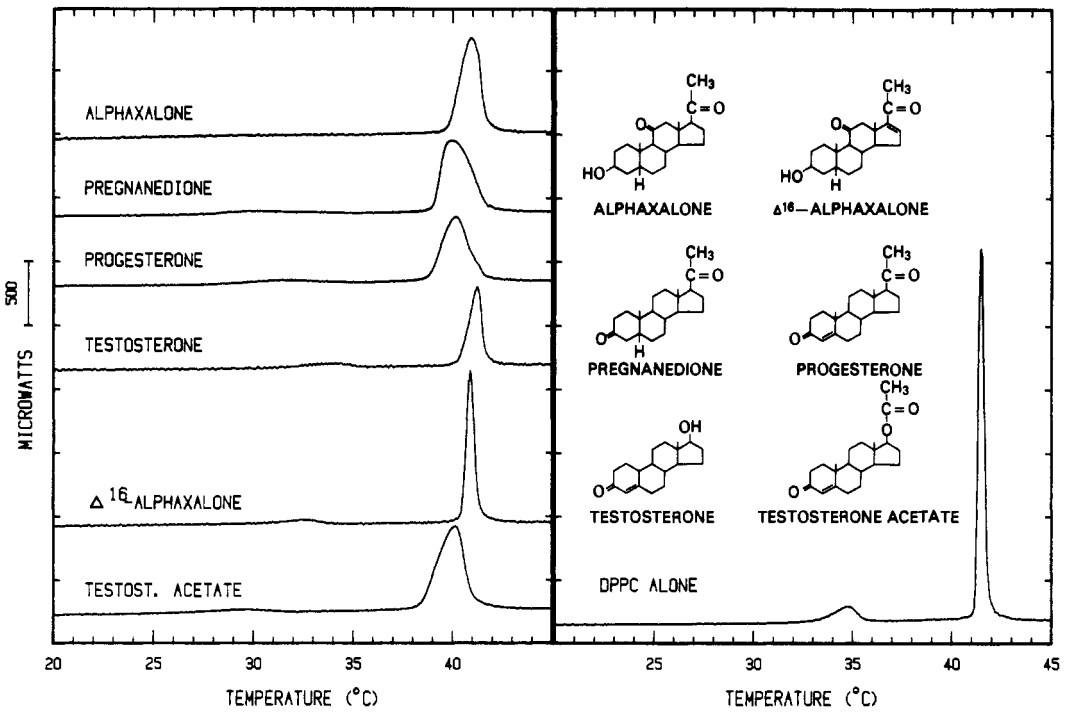

FIGURE 1: Representative calorimetric tracings for DPPC–steroid (9:1 mole fraction) liposomes (left) and for pure DPPC liposomes (right). Both the bilayer pretransitions and the primary gel to liquid-crystalline phase transitions are decreased by all steroids. Sample weights are 15–23 mg. The insert contains the chemical structures of the steroids investigated in this study. Alphaxalone, pregnanedione, progesterone, and testosterone are all anesthetic (listed in order of decreasing potency). Δ^{16}-Alphaxalone is nonanesthetic. The possible activity of testosterone acetate is unknown.

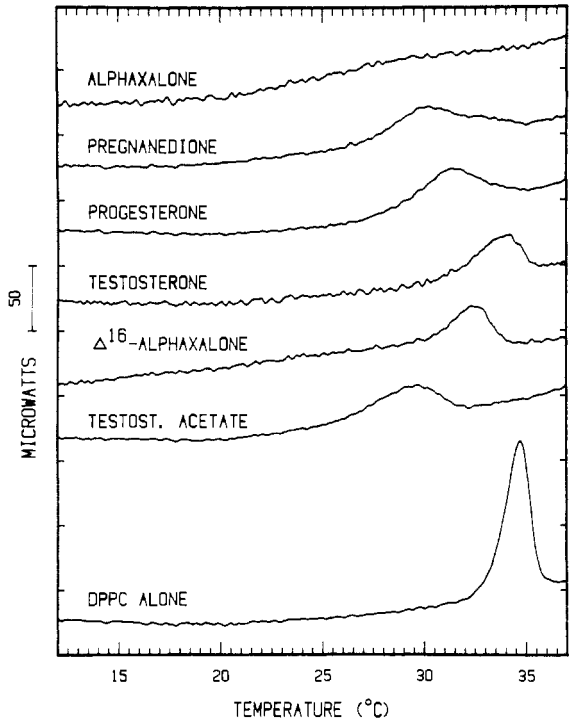

FIGURE 2: Representative calorimetric tracings (samples as in Figure 1) for the pretransition region of DPPC–steroid (9:1 mole fraction) liposomes and for pure DPPC liposomes. The broadening and lowering in temperature of the pretransition feature are greatest for the more potent anesthetics. The breadth of the pretransition feature for DPPC–alphaxalone liposomes nearly precludes its detection.

of melting for the 10 mol % steroid–DPPC bilayers were within two standard deviations of the value we obtain for pure DPPC bilayers, and since only a limited number of samples were measured, these values are not included in this report. All steroids investigated caused significant depression and broadening of both the lipid pretransition and the gel to liquid-crystalline transition (Table I; Figures 1 and 2); for alphaxalone–DPPC bilayers the breadth of the pretransition nearly precludes its observation. For all preparations, except testosterone acetate–DPPC bilayers, the end point of the main transition corresponds very nearly to that for pure DPPC. This type of broadening of the main phase transition may be observed for systems in which ideal solutions are formed in both the gel and liquid-crystalline phases with no decrease in phase transition cooperativity (Sturtevant, 1982). Such behavior is also compatible with the segregation of membrane components into pure DPPC and DPPC–steroid regions. Aggregation of spin-labeled steroids into domains has previously been reported for steroid concentrations lower than those employed here (Träuble & Sackmann, 1972a,b). Our results, however, cannot discriminate between these possibilities. Calorimetric melting profiles exhibiting broadened transition and lowered transition temperatures, in comparison to profiles of pure DPPC liposomes, have been interpreted to be characteristic of bilayers modified by the addition of membrane components into the C(1)–C(18) portion of the acyl chain region (Jain & Wu, 1977).

The interactions involved in the gel to liquid-crystalline phase change of the acyl chains in phospholipid assemblies were examined as a function of temperature by monitoring the Raman spectral changes in the 2800–3100-cm^{-1} hydrocarbon chain C–H stretching mode region. Since the observed spectral features and their complex changes as functions of either temperature or the introduction of a bilayer perturber have been discussed in detail [see, for example, Levin (1984) and Huang et al. (1982)], we simply note here that temperature profiles based upon the empirical $I_{2935cm^{-1}}/I_{2880cm^{-1}}$ peak height intensity ratios have been demonstrated to be a sensitive measure of both interchain and intrachain order–disorder processes in the bilayer acyl chains. In contrast, the C–C stretching mode region in the 1050–1150-cm^{-1} spectral interval directly reflects intramolecular trans/gauche conformational

Table I: Calorimetric Melting Temperature and Half-Widths for DPPC and DPPC–Steroid (10 mol %) Multilayers

	$T_{p,1/2}$[a] (°C)	$\Delta T_{p,1/2}$[b] (°C)	T_m[c] (°C)	$\Delta T_{m,1/2}$[d] (°C)	ED_{50}[e] (mg/kg)
DPPC–alphaxalone	29.5	6.0	40.92	1.21	1.79[f]
DPPC–pregnanedione	30.8	3.7	39.90	1.76	12[f]
DPPC–progesterone	31.8	3.2	40.21	1.52	89[f]
DPPC–testosterone	33.4	2.6	41.25	0.74	150[g]
DPPC–Δ^{16}-alphaxalone	32.4	2.0	40.88	0.47	>200[h]
DPPC–testosterone acetate	29.6	3.4	40.15	1.57	
DPPC	34.8 ± 0.2	1.5 ± 0.1	41.47 ± 0.03	0.30 ± 0.02	

[a] Peak temperature for the pretransition. [b] Full width at half-maximum for the pretransition. [c] Peak temperature at the gel to liquid-crystalline phase transition. [d] Full width at half-maximum for the gel to liquid-crystalline transition. [e] Dose of steroid at which 50% of mice are anesthetized. The correlation between log ED_{50} of the four anesthetic steroids and both T_p and $\Delta T_{p,1/2}$ is significant at the $p < 0.05$ level. [f] From Gyermek & Soyka (1975). [g] From Seeman (1966). [h] From Phillips (1974).

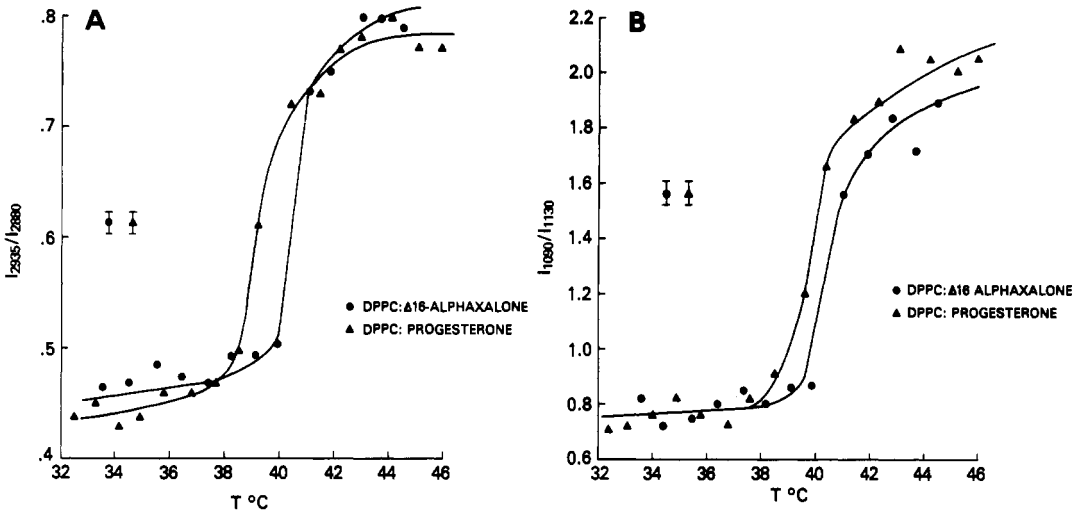

FIGURE 3: Temperature profiles for 9:1 DPPC–progesterone and DPPC–Δ^{16}-alphaxalone liposomes using (A) $I_{2935cm^{-1}}/I_{2880cm^{-1}}$ peak height intensity ratios as indices and $I_{1090cm^{-1}}/I_{1130cm^{-1}}$ peak height intensity ratios as indices. Although the transition temperature is lower for DPPC–progesterone liposomes than that for pure DPPC multilayers, the peak height ratios above and below the transition are comparable to those of pure DPPC liposomes.

changes within the hydrocarbon chain region of the lipid matrix. As discussed in earlier references [Levin (1984) and references cited within], temperature profiles derived from the $I_{1090cm^{-1}}/I_{1130cm^{-1}}$, I_{gauche}/I_{trans}, peak height intensity ratios are also useful in comparing bilayer order–disorder characteristics between similar liposomes. For DPPC–Δ^{16}-alphaxalone and DPPC–progesterone (9:1 lipid:anesthetic mole ratios), typical melting profiles obtained by Raman spectroscopy from spectral parameters observed in the acyl chain C–H stretching and C–C stretching mode regions are shown in Figure 3. Except in the region of the phase transition, profiles reflecting the respective gel and liquid-crystalline regions and constructed from the Raman peak height intensity $I_{2935cm^{-1}}/I_{2880cm^{-1}}$ ratios do not differ significantly from the profiles of pure DPPC multilayers. This indicates that the entropy of melting is not greatly different from that of pure DPPC (Huang et al., 1982), in agreement with our calorimetric results and those reported for DPPC multilayers in the presence of the inhalation anesthetics halothane and enfluorane (Mountcastle et al., 1978). The temperature profiles derived from Raman spectroscopic parameters reflecting both intermolecular (Figure 3A) and intramolecular (Figure 3B) changes in the bilayer region indicate that progesterone broadens and lowers the DPPC bilayer phase transition (~2 °C) to a greater extent than Δ^{16}-alphaxalone (~1 °C). This agrees with the calorimetric results.

Raman spectra of both anhydrous (spectra not shown) and hydrated (Figure 4) bilayers displayed no perturbations originating from the introduction of the steroids on either the head-group choline totally symmetric C–N stretching fundamental observed at 711 (anhydrous form) to 717 cm^{-1} (hydrated form) or in the carbonyl stretching mode region at 1721 and 1740 cm^{-1} (anhydrous form) and at 1736 cm^{-1} (hydrated form). These results indicate that these agents do not significantly perturb either the head-group or the glycerol backbone regions of the bilayers at the concentrations used in this study. Although spectral changes in the acyl chain CH_2 deformation modes at ~1440 cm^{-1} are characteristic of interchain interactions, interpretation of the expected effects of anesthetics in this spectral interval of "anhydrous" bilayers is complicated by the dramatic effects produced by even trace amounts of water (1–2 molecules per lipid molecule) (Bush et al., 1980a). No spectral effects were discerned, however, in the CH_2 deformation region of hydrated bilayers as a result of steroid introduction.

Small changes in the nature of the immediate environments of the steroid molecules are seen spectroscopically as one compares the polycrystalline steroid material to the DPPC–steroid multilayers. Increases in the frequency of the two C=O stretching modes of progesterone from 1665 and 1703 cm^{-1} to 1667 and 1706 cm^{-1}, respectively, for the polycrystalline steroid and the steroid–DPPC multilayers suggest that the steroid is in a slightly more hydrophobic environment in the DPPC multilayer than in the pure steroid crystal (Bush et al., 1980b). This observation, which would be expected of a steroid occupying a position in the acyl chain region of the bilayer, is consistent with both our calorimetric and our spectroscopic results, which suggest that the hydrocarbon core of the bilayer is the most likely location of the anesthetic

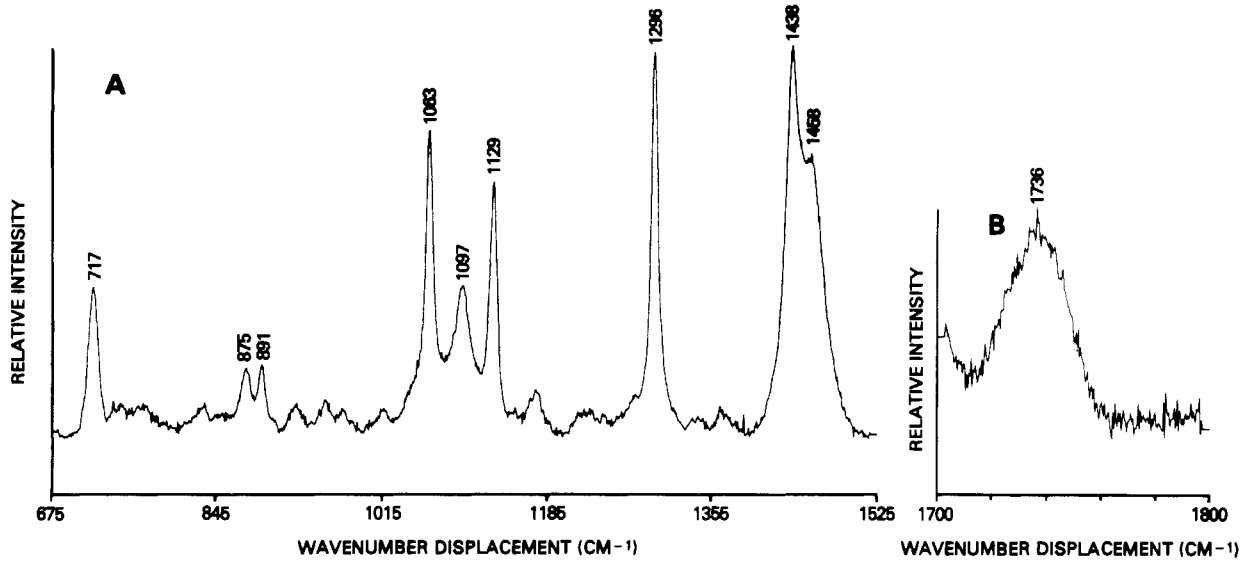

FIGURE 4: (A) Spectrum of hydrated DPPC–alphaxalone multilayers at 0 °C from 675 to 1525 cm^{-1}. (b) Spectrum of hydrated DPPC–alphaxalone multilayers at 0 °C in the 1700–1800-cm^{-1} C=O stretching mode region. Both spectra are nearly identical with those of hydrated DPPC liposomes.

perturber. In particular, this conclusion, which agrees with the structural analysis of Willmer (1961) and with the results of Träuble & Sackmann (1972a,b) on spin-labeled steroids, contradicts an earlier conclusion drawn from film balance studies that steroids are not incorporated within the membrane (Gershfeld & Heftmann, 1963; Munck, 1957; Cleary & Zatz, 1977; Khaiat et al., 1975). The latter studies do not provide direct information on steroid localization. The frequency of the carbonyl stretching modes, however, is a sensitive indication of the dielectric constant (ϵ) of the surrounding medium and, as such, is capable of distinguishing the local environment of the steroid nucleus. For example, the C=O stretching mode for *N*-methylurethane found at 1744 cm^{-1} in hexane (ϵ = 1.89) drops to 1698 cm^{-1} in aniline (ϵ = 6.89) (Davies, 1963). Thus, it appears quite likely that a change from the relatively nonpolar acyl chain environment for the steroid in the colyophilized crystal to a highly polar aqueous phase in the liposome would result in a significantly higher frequency for the carbonyl stretching mode of the steroid than that spectroscopically observed.

The effects of the steroid molecules on the broadening and T_m values for the DPPC phase transition, given in Table I, while significant, are much smaller than the calorimetric results of Connor et al. (1974). This difference may arise as a result of variations in the experimental procedure; we have observed somewhat lower bilayer "melting" temperature in systems that were not allowed to equilibrate for the long periods employed in this work. While the lowering of the main bilayer phase transition temperature was greater for anesthetic than for nonanesthetic steroids, as shown in Table I, neither the melting temperature T_m nor the "half-widths" of the calorimetric melting profile demonstrated a correspondence with anesthetic potency. A lack of correlation between anesthetic potency and effects on the main DPPC phase transition temperature has previously been noted by Pringle & Miller (1978).

A significant correlation is observed between anesthetic potency and the effects of steroids on the calorimetrically determined DPPC pretransition (see Table I). The bilayer pretransition is characterized by the formation of ripples in the bilayer (Janiak et al., 1976). Although the bilayer change is accompanied by a small amount of trans/gauche isomerization in the acyl chains (Levin & Bush, 1981), the pretransition represents primarily a lattice rearrangement of the acyl chains. Previous investigators have shown that this structural rearrangement occurs relatively slowly and in calorimetric data may give rise to perturbations arising from kinetic effects (Lentz et al., 1978). The thermodynamic parameters that we determined for pure DPPC were self-consistent and in agreement with results reported by others (Lentz et al., 1978; Mabrey & Sturtevant, 1976). On the basis of our present data, however, we are unable to assess whether the observed pretransition effects originate from only thermodynamic factors or also reflect kinetic effects. Nevertheless, the observation for the present series of steroids of a correlation between the extent of an anesthetic-induced lipid perturbation and anesthetic potency both strongly implicates the lipid bilayer as having a major role in anesthetic action and demonstrates the practical utility of using the subtle structural alterations reflected in the pretransition as a probe of membrane reorganizations.

A clear correspondence between the substituent at the steroid C(17) position and the effects on the bilayer phase transitions is also apparent. Compounds with bulky substituents projecting from the plane of the steroid at the C(17) position (see Figure 1) perturb the bilayer more effectively (Table I) than those with compact groups (testosterone) or groups that do not project out of the plane of the D ring (Δ^{16}-alphaxalone) at this position. On the basis of these considerations and the calorimetric results, we predict that testosterone acetate should manifest anesthetic properties.

Although we examined only DPPC–steroid interactions, we expect similar lipid effects for other symmetric chain phosphatidylcholines, such as dimyristoylphosphatidylcholine or distearoylphosphatidylcholine. Similar relative ordering of membrane effects by various anesthetic agents has previously been observed in different phosphatidylcholines for both inhalation anesthetics (Janoff & Miller, 1982) and charged local anesthetics (Singer & Jain, 1980). In general, the magnitude of the lipid perturbation is greatest for short-chain bilayers and decreases monotonically with increasing chain length (Janoff & Miller, 1982).

In summary, this investigation provides strong support for the concept of a lipid-mediated mechanism for anesthetic action by establishing a structure–activity relationship between clinical potency and lipid perturbation for several anesthetic and nonanesthetic steroids that are not expected to perturb

protein conformation as a result of nonspecific binding to protein. The bilayer perturbation results from the insertion of the steroid into the acyl chain region of the membrane, rather than into the head-group or interface regions of the bilayer. Furthermore, the efficacy of a steroid in perturbing the lipid membrane is highly dependent on the projection and bulk of substituents at the steroid C(17) position.

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