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The perturbation of model membranes by (−)-Δ^9-tetrahydrocannabinol. Studies using solid-state ^{2}H- and ^{13}C-NMR

Alexandros Makriyannis [1,2], De-Ping Yang [1], Robert G. Griffin [2] and Sunil K. Das Gupta [2]

[1] *School of Pharmacy and Institute of Materials Science, University of Connecticut, Storrs, CT and* [2] *Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, MA (U.S.A.)*

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The effects of (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) on model phospholipid membranes were studied using solid-state ^{2}H and ^{13}C nuclear magnetic resonance spectroscopy. Aqueous multilamellar dispersions of dipalmitoylphosphatidylcholine with specific ^{2}H- and ^{13}C-labels as endogenous probes at the $C_{7'}$ methylene and the carbonyl groups, respectively, of the *sn*-2 chain were used to study the conformational and dynamic properties of the bilayer as a function of temperature and drug concentration. The drug molecule decreases the phase transition temperature of the bilayer in a concentration dependent manner up to 20 molar percent when full saturation has occurred. The ^{2}H spectra show that Δ^9-THC broadens the phase transition during which the spectra acquire a characteristic shape of a two-component system exchanging at an intermediate rate ($\approx 10^6$ s^{-1}) with some liquid crystalline features. Such spectra provide information related to the melting of the phospholipid chains. At intermediate temperatures, the ^{13}C spectra show a gel-like and a liquid-crystalline-like exchanging components and provide information about a conformational change at the phospholipid glycerol backbone occurring at or near the pretransition. The spectral composition and rate of exchange are both dependent on drug concentration. We have carried out computer simulations of the ^{13}C spectra and obtained conformational information related to the phase transition process in the bilayer from gel to liquid crystal. Our studies show that Δ^9-THC has a stronger effect on the *sn*-2 carbonyl near the bilayer interface than on the lipid chains and serve to describe the membrane perturbing effects of cannabinoids in molecular terms.

Introduction

(−)-Δ^9-Tetrahydrocannabinol (Δ^9-THC, Fig. 1) is the principal psychoactive constituent of marijuana. Many of its pharmacological actions, including psychotropic properties, bronchodilation, increased heart rate, reduced intraocular pressure and analgesia, can be related to its effects on cellular membranes. Existing evidence indicates that these membrane effects, at least in part, are due to interactions with those lipids that make up the environment of membrane-associated proteins [1,2]. The cannabinoid–membrane interactions presumably induce changes in the functions of a number of these proteins and thus produce a variety of physiological effects. Experimental evidence for a mechanism of action involving cannabinoid–phospholipid interactions came from calorimetric measurements [3–7] and from electron spin resonance experiments [8,9]. Recently, we have used small angle X-ray diffraction techniques to study the location of cannabinoids in phosphatidylcholine model membranes and found that the amphipathic drug molecule resides near the water/hydrocarbon-core interface of the lipid bilayer [10]. However, these experiments provided only limited information on the molecular features of the drug–phospholipid interactions. We have now obtained more detailed information from the solid-state ^{2}H- and ^{13}C-NMR spectra of appropriately ^{2}H- and ^{13}C-labeled dipalmitoylphosphatidylcholine (DPPC, Fig. 1) preparations into which the drug was incorporated.

Solid-state ^{2}H- and ^{13}C-NMR experiments with drug/phospholipid preparations can be carried out by

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; Δ^9-THC, (−)-Δ^9-tetrahydrocannabinol; NMR, nuclear magnetic resonance.

Correspondence: A. Makriyannis, School of Pharmacy and Institute of Materials Science, University of Connecticut, Storrs, CT 06269, U.S.A.

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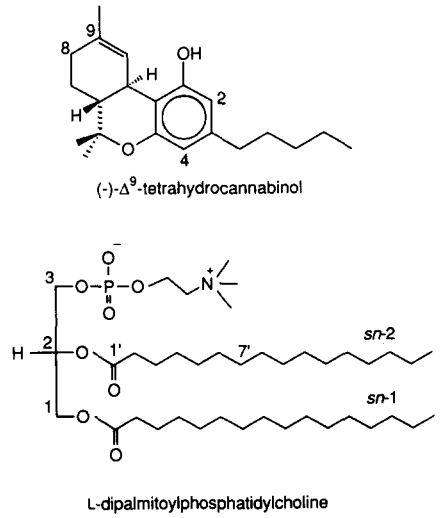

Fig. 1. Structures of Δ^9-THC and DPPC.

observing spectra from either the drug or the phospholipid. In earlier work, we obtained solid-state ^{2}H-NMR spectra from DPPC bilayers containing Δ^9-THC specifically ^{2}H-labeled in six different positions and determined the orientation of Δ^9-THC in the model membrane [11]. In the present communication, we describe and analyze the effects of Δ^9-THC on the ^{2}H- and ^{13}C-NMR spectra obtained from bilayer preparations of DPPC labeled with two ^{2}H nuclei at the $C_{7'}$ methylene group of the *sn*-2 chain and with one ^{13}C nucleus at the carbonyl group of the same chain.

Our choice of DPPC as the model membrane was motivated by the fact that phosphatidylcholines are a major membrane component whose phase properties have been studied extensively. Calorimetric measurements for fully hydrated DPPC show two endothermic transitions; a broad low-enthalpy pretransition at $T_c' = 35.3\,^\circ$C and a main transition at $T_c = 41.3\,^\circ$C [12,13]. Below the pretransition, the phospholipid molecules are arranged in a lamellar gel phase ($L_{\beta'}$), while above the main transition they exist in the liquid crystalline phase (L_α). At temperatures between T_c' and T_c, the ripple phase ($P_{\beta'}$) is composed of coexisting gel and liquid crystalline components [14,15]. Our present study seeks to uncover more detailed molecular information on the interactions of the cannabinoids with the phospholipid bilayer. To that effect, we have systematically obtained solid-state ^{2}H- and ^{13}C-NMR spectra of DPPC/Δ^9-THC with molar ratios 1.00 : 0.00, 0.95 : 0.05, 0.90 : 0.10, 0.80 : 0.20 and 0.60 : 0.40 at temperatures ranging from 17 °C to 50 °C. We have analyzed the temperature and concentration dependences of the lineshapes of both ^{2}H- and ^{13}C-NMR spectra and carried out simulations of all of the ^{13}C spectra. The effects of Δ^9-THC on DPPC bilayers are then discussed in terms of changes in the conformational and dynamic properties of the model membrane.

Materials and Methods

Δ^9-THC was obtained from the National Institute on Drug Abuse while 2-[1′-^{13}C]DPPC and 2-[7′,7′-$^2H_2$]DPPC were synthesized according to literature procedures [14–16].

Samples of model membranes for the solid-state NMR experiments were prepared by dissolving the ^{2}H- or ^{13}C-labeled phospholipid (50 mg) without or with Δ^9-THC in 2 ml of dichloromethane. 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 the drug : phospholipid mixture was subsequently introduced into 7-mm glass tubes appropriately constricted, and ^{2}H-depleted water was added to produce a 50 : 50 (w/w) lipid/water preparation. The preparation was submerged in liquid nitrogen while the tube was being sealed at the constriction under vacuum. The samples were equilibrated before recording the spectra by heating to 50 °C for 15 min.

NMR spectra were obtained on a home-built solid state pulse spectrometer operating at a magnetic field of 6.8 T (73.9 MHz for ^{13}C, 294 MHz for ^{1}H, and 45.1 MHZ for ^{2}H). For the ^{13}C spectra, a cross-polarization experiment was used with a refocusing π pulse [17] and typical parameters used were 4.0 μs for the $\pi/2$ pulse and 2.5–3.5 ms for the mixing time. Sample heating due to the ^{1}H-decoupling was minimized by employing the minimum decoupling power necessary to obtain sharp spectra and by using long recycle delays of 3–5 s. The number of ^{13}C free induction decays accumulated ranged between 1000 and 3000, depending on the sample temperature. ^{2}H spectra were obtained using the quadrupole echo pulse sequence, $[(\pi/2)_x-\tau-(\pi/2)_y]$, which consists of a pair of 90 °-phase-shifted $\pi/2$ pulses separated by a time τ (typically 35–50 μs) longer than the spectrometer recovery time [18]. Spectral simulations were carried out on a VAX computer system using a FORTRAN program described elsewhere [15,19].

Differential scanning calorimetry thermograms were obtained on a Perkin-Elmer DSC-7 instrument. The samples were prepared by dissolving 5 mg of DPPC and the appropriate amount of Δ^9-THC in chloroform. After removing the solvent using a nitrogen stream, the samples were dried under vacuum for 6 h. The preparations were scraped into weighed stainless steel capsules. Distilled and deionized water (50 : 50, w/w) was added and the capsules were sealed. The samples were scanned at a heating rate of 2.5 K/min.

Results and Discussion

Solid-state NMR has now established itself as a powerful method for the study of lipid and membrane dynamics and conformation [17,20]. To obtain informa-

tion on the manner in which Δ^9-THC interacts with the phospholipid components of the membrane, we made use of aqueous dispersions of DPPC into which the drug molecule was incorporated. These preparations exist in the form of multilamellar bilayers and possess dynamic properties resembling those of natural membranes [21]. Because of its highly lipophilic character and as confirmed by our X-ray diffraction studies [10], tetrahydrocannabinol partitions exclusively in the membrane bilayer [22]. Presumably, the drug molecule intercalates between the chains of contiguous DPPC molecules, a location analogous to that of cholesterol in the bilayer.

^{2}H-NMR spectra of 2-[7′,7′-$^2H_2$]DPPC preparations

The deuterium nucleus gives rise to a doublet in the absorption spectrum due to the interaction between its quadrupole moment ($I = 1$) and the surrounding electric field gradient. The separation between the two spectral lines can be observed in an oriented samples and is equal to:

$$\nu_2 - \nu_1 = (3/4)(e^2qQ/h)(3\cos^2\theta - 1)$$

where e^2qQ/h is the ^{2}H quadrupole coupling constant (≈ 170 kHz) and θ is the angle between the magnetic field and the axis of molecular ordering [23]. In a sample of an aqueous phospholipid dispersion, all values of θ are possible and the spectrum from ^{2}H nuclei at one site is a 'powder pattern' in which the two principal peaks correspond to $\theta = 90°$ (perpendicular edges, $\nu_\perp$) and the two shoulders to $\theta = 0°$ (parallel edges, $\nu_\parallel$).

In the liquid crystalline phase, ^{2}H-labeled multilamellar phospholipid bilayers give a characteristic ^{2}H powder pattern spectrum with quadrupolar splittings considerably narrowed from the rigid lattice value (120 kHz) due to rapid axial diffusion and *trans-gauche* isomerization in the chain. We have systematically carried out solid-state ^{2}H-NMR experiments on hydrated preparations of 2-[7′,7′-$^2H_2$]-DPPC/Δ^9-THC at different drug concentrations and various temperatures. Shown in Fig. 2 are representative ^{2}H-NMR spectra from DPPC/Δ^9-THC preparations with molar ratios of 1.00 : 0.00, 0.95 : 0.05, 0.90 : 0.10, 0.80 : 0.20 and 0.60 : 0.40 (or $x = 0.00$, 0.05, 0.10, 0.20 and 0.40) at temperatures ranging from 18 °C to 42 °C.

Our earlier work using differential scanning calorimetry [7] showed that the presence of Δ^9-THC in DPPC bilayers broadens the phase transitions from gel to liquid crystal and lowers the transition temperature. The ending point of the gel to liquid crystal endotherm was regarded as the phase transition temperature at which the bilayers exist entirely in the liquid crystalline phase. These temperatures were measured to be 42.0 °C, 40.5 °C, 39.0 °C, 36.5 °C and 36.5 °C for hydrated DPPC with Δ^9-THC concentrations of $x = 0.00$, 0.05, 0.10, 0.20 and 0.40, respectively. Our ^{2}H-NMR spectra from the different DPPC/Δ^9-THC preparations, respec-

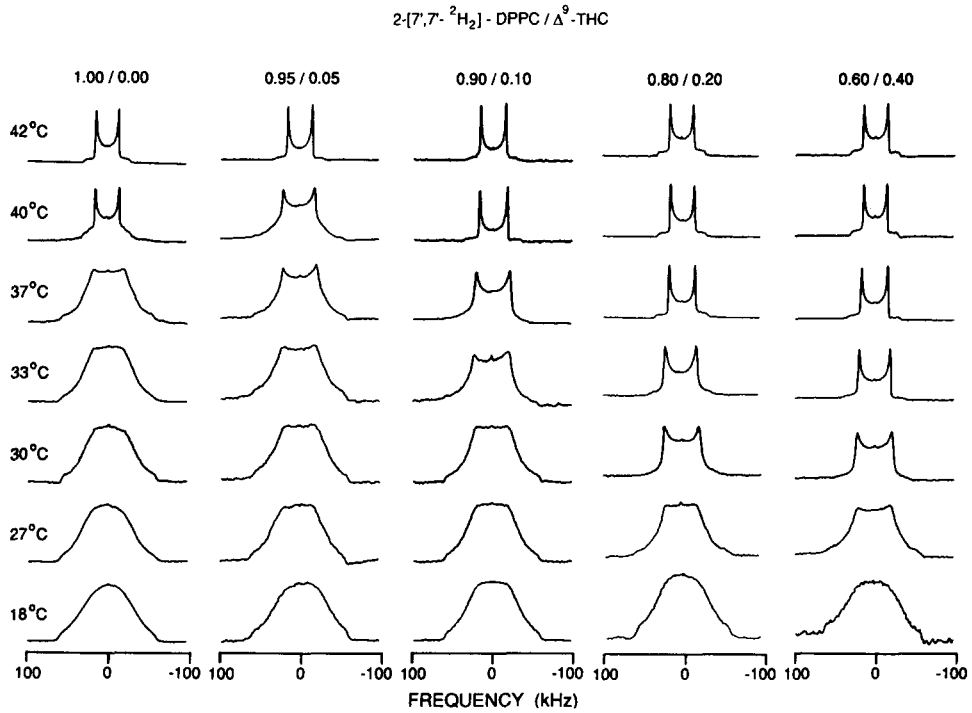

Fig. 2. Solid-state ^{2}H-NMR spectra obtained from aqueous dispersions of 2-[7′,7′-$^2H_2$]-DPPC/Δ^9-THC with molar ratios of 1.00 : 0.00, 0.95 : 0.05, 0.90 : 0.10, 0.80 : 0.20 and 0.60 : 0.40 at temperatures between 18 °C and 42 °C. To obtain each spectrum, 8000 to 16000 echoes were signal-averaged.

tively, show almost exactly the same phase transition temperatures.

At 42° C, preparations with all concentrations are in the liquid crystalline phase (L_α) and the spectra are qualitatively similar, exhibiting the characteristic fast-limit axially symmetric powder pattern. The sharp perpendicular and parallel edges in the spectra indicate that the phospholipid molecules are now undergoing fast axial diffusion and *gauche-trans* isomerization with motional rates greater than 10^7 s^{-1}. The residual quadrupolar splitting ($\Delta\nu_Q$) as measured directly from the separation of the two 90° edges in the spectrum was 28.1 kHz for pure DPPC at 42° C. Addition of Δ^9-THC produced a small increase in the splitting indicating a slight increase in the *trans:gauche* ratio of methylene chain conformers. A comparison between the first ^2H spectra to show the characteristic L_α lineshape in each of the series of the drug-containing preparations shows that this increase in the residual quadrupolar splitting is concentration dependent. The first L_α-like ^2H spectra for each of the four drug-containing preparations were observed at 41° C, 39° C, 36° C and 36° C, and had respective $\Delta\nu_Q$ values equal to 30.8, 31.9, 32.4 and 33.1 kHz for increasing Δ^9-THC concentrations.

At temperatures just below the main phase transition, the spectra of pure hydrated DPPC do not display the sharp 90° and 0° edges that characterize the fast-limit spectra. As can be seen, between 40° C and 33° C the spectra acquire a flat top appearance with the 90° and 0° edges still discernible, but severely broadened. This broadening continues as the temperature is further lowered. Below the pretransition, all the liquid-crystalline spectral features disappear. At 18° C, the ^2H spectrum has an almost conical shape with a rounded top. The overall width of the gel-phase spectra is about 120 kHz, only one-half of that expected for the rigid lattice values and is evidence that, even at 18° C, the DPPC molecules continue to undergo long axis diffusion.

The most dramatic effects of Δ^9-THC on the ^2H-NMR lineshapes of 2-[7′,7′-$^2H_2$]DPPC were produced during the transition from gel to liquid crystalline phase. A general feature of this transition with the different preparations is that it becomes progressively more gradual with increasing drug concentrations and the spectra retain more liquid-crystalline features. Characteristically, the spectra from the DPPC/Δ^9-THC ($x = 0.20$) preparation at 35° C show a gradual broadening of the 0° edges while the center of the spectrum starts to fill. However, the 90° edges maintain their sharp vertical features and the splitting is quite visible until 27° C at which temperature the spectrum acquires the flat top appearance. This particular spectral shape can also be found in the corresponding spectra of pure DPPC at 37° C as well as in the spectra of other drug-containing preparations of $x = 0.05$, 0.10 and 0.40 at 33° C, 30° C and 27° C, respectively. Interestingly, spectra for $x = 0.20$ and $x = 0.40$ are virtually identical at all temperatures, indicating a saturation of Δ^9-THC at concentrations greater than $x = 0.20$.

The gradual transition in the spectral features described above is accompanied by a correspondingly gradual change in the quadrupolar splitting value which was obtained by measuring the separation between the two 90° edges. When a spectrum does not have the features of a Pake pattern and the 90° edges are not sharp but still discernible, the quadrupolar splitting value was measured between the two highest points in the spectrum. As the temperature is raised, the quadrupolar splitting from each preparation decreases, a direct indication that the ratio of *gauche:trans* conformers increases with temperature. Fig. 3 shows quantitatively the temperature dependence of quadrupolar splittings (open circles) in the L_α-type spectra for two of the preparations, DPPC and DPPC + Δ^9-THC ($x = 0.20$). A very sharp decrease in the quadrupolar splitting is observed for DPPC as it goes through the phase transition temperature while DPPC + Δ^9-THC shows a gradual change in $\Delta\nu_Q$ over a temperature span

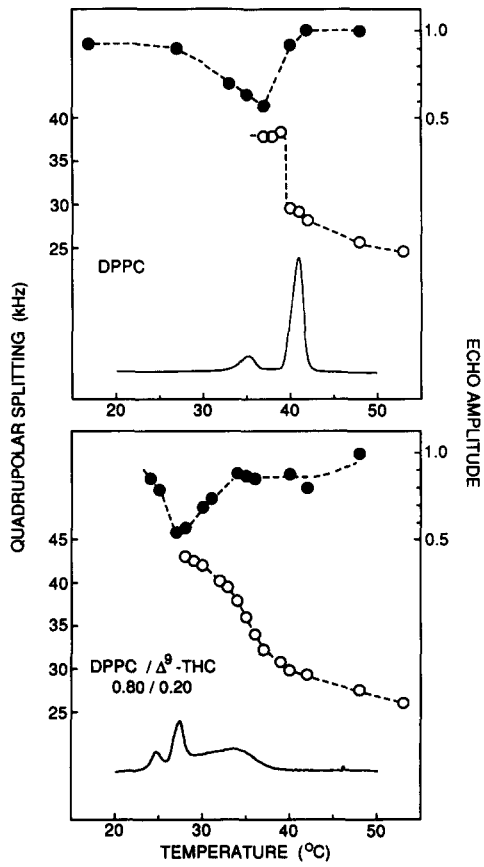

Fig. 3. Temperature dependence of the ^2H quadrupolar splitting (○) in the solid-state ^2H-NMR spectra of the 2-[7′,7′-$^2H_2$] segment of DPPC and relative echo amplitudes (●). Top: DPPC; Bottom: DPPC/Δ^9-THC with molar ratio 0.80:0.20. The thermogram included in each graph was obtained from an identically prepared sample using differential scanning calorimetry.

of more than 10 K. This is in very good agreement with the thermograms (included at the bottom of each graph in Fig. 3) from our differential scanning calorimetric measurements [7], where we observe a sharp main transition for DPPC and a broad transition for DPPC + Δ^9-THC. In addition, the temperature dependence of $\Delta\nu_Q$ parallels the results of our small angle X-ray diffraction experiments [10], where we observed a very similar temperature dependence for the total period repeat distance (d-spacing) of the bilayer.

Also in Fig. 3, we have plotted echo amplitudes representing changes in the absolute ^{2}H spectral intensities (solid circles) as a function of temperature. In the case of DPPC alone, we observed a gradual loss of intensity as the temperature was increased and a sudden recovery just before the main transition. The Δ^9-THC-containing sample gave spectra in which there was also a gradual lowering of the spectral intensity as the temperature was raised. After going through a minimum, the spectral intensity recovered but, unlike the pure DPPC spectra, it did so gradually and formed a maximum when it reached the liquid crystalline phase. This phenomenon has also been observed in our earlier work involving the interactions of anesthetic steroids with DPPC [24].

The decrease in total intensity of the ^{2}H-NMR signal is because of very short T_{2e} relaxation time values at those temperatures. Under such circumstances, a significant amount of magnetization is lost during the interval (τ) between the two $\pi/2$ pulses of the quadrupole echo pulse sequence and is not refocused by the second $\pi/2$ pulse. An additional spectral features that accompanies the overall loss in spectral intensity is that different portions of the powder pattern exhibit different T_{2e} values. The net outcome of this are lineshape distortions which are very sensitive to the value of τ. Fig. 4 shows a representative set of ^{2}H-NMR spectra from a DPPC/Δ^9-THC ($x = 0.20$) preparation at 30 °C with six systematically varied τ-values. These spectra are plotted with their absolute intensities on the left and with scaled intensities on the right to show the details of the lineshapes. As can be seen, the overall intensity of the spectrum decreases as the τ-value increases from 50 μs to 190 μs. Also, the drop in the perpendicular ($\theta = 90°$) and parallel ($\theta = 0°$) edges of the powder pattern is more rapid than that in the center ($\theta = 54°$) of the spectrum, resulting in drastic changes in the spectral lineshapes.

Losses in spectral intensity and the lineshape distortion are most pronounced when the C-^{2}H bond orientations are characterized by motional rates of the order of the quadrupole interaction ($\approx 10^5$ s^{-1}). Phenomena similar to the one observed here have been attributed to an inter- or intramolecular exchange process with an intermediate rate [23]. In this instance, the intermolecular process could be an exchange between the gel and

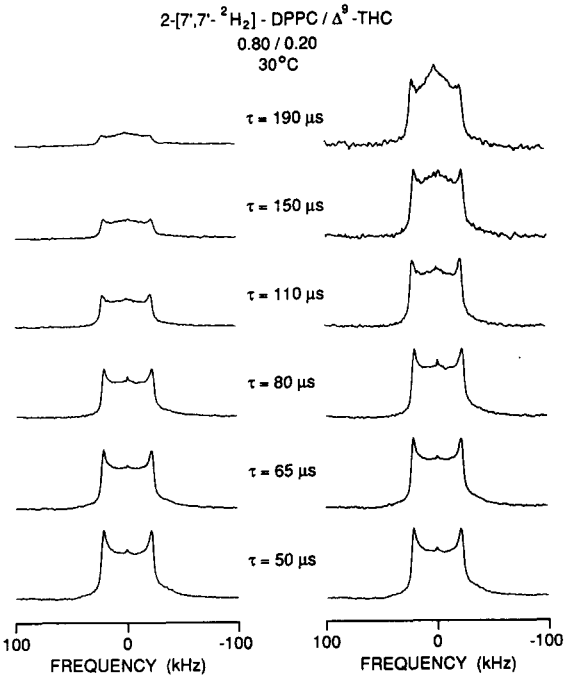

Fig. 4. Solid-state ^{2}H-NMR spectra of 2-[7',7'-$^2H_2$]DPPC/Δ^9-THC (molar ratio 0.80:0.20) at 30 °C obtained using a quadrupolar echo sequence with variable interval τ between the two $\pi/2$ pulses. On the left are spectra plotted with absolute intensity while on the right are the same ones scaled up to show the details of the lineshape distortions.

liquid crystal in a two-component DPPC phase mixture (presumably $P_{\beta'}$), while the intramolecular process may result from an exchange between *gauche* and *trans* conformers in the phospholipid acyl chains.

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

The ^{13}C-enriched carbonyl in the phospholipid *sn*-2 chain of the membrane gives rise to a powder pattern spectrum due to the chemical shift anisotropy, i.e., the chemical shift value depends on the angle θ between the magnetic field and the molecular ordering axis. If the molecular motions about this axis are in fast limit on the ^{13}C-NMR time scale and are axially symmetric, the spectrum shows a characteristic axially symmetric powder pattern which consists of a distribution of intensities between a perpendicular edge at one chemical shift value ($\sigma_\perp$) and a parallel edge at another value ($\sigma_\parallel$).

Shown in Fig. 5 is a set of proton-decoupled solid state ^{13}C-NMR spectra of 2-[1'-^{13}C]DPPC/Δ^9-THC preparations of identical molar ratios as those used in the ^{2}H-NMR experiments and at temperatures ranging from 17 °C to 40 °C. In each spectrum, the envelope of resonances extending from 65 to 146 ppm on the far right and the smaller signal to the left of it are due to the natural abundance ^{13}C of the protonated acyl chains and those of the head group and glycerol carbons, respectively. The dominant powder pattern in the spec-

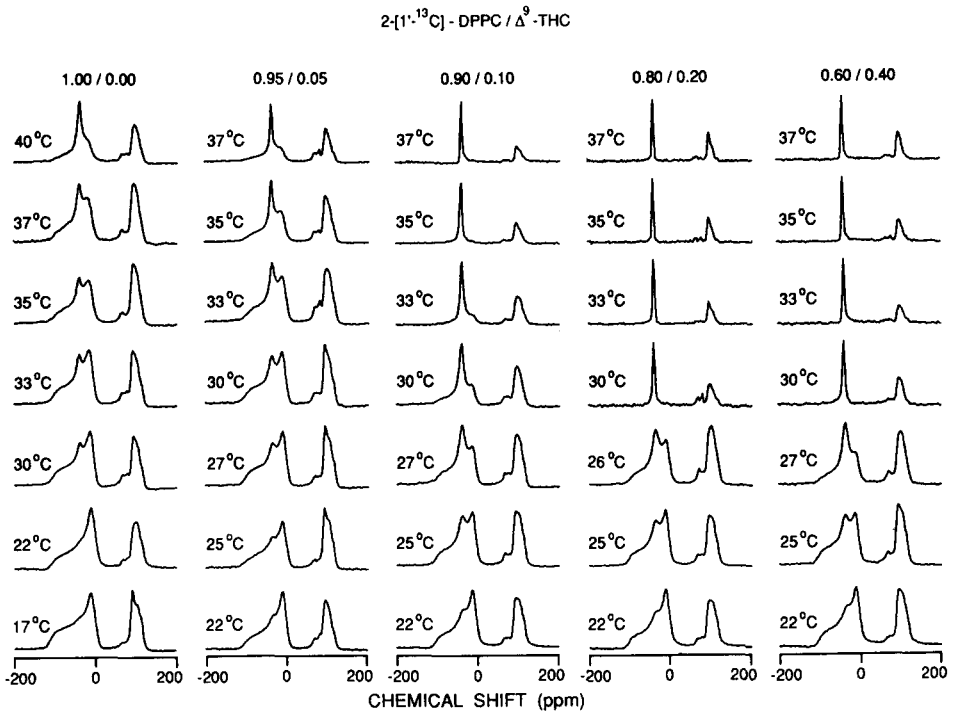

Fig. 5. Proton-decoupled solid-state ^{13}C-NMR spectra obtained from aqueous dispersions of 2-[1'-^{13}C]-DPPC/Δ^9-THC with molar ratios of 1.00:0.00, 0.95:0.05, 0.90:0.10, 0.80:0.20 and 0.60:0.40 at temperatures between 17°C and 40°C. 1000 to 3000 free induction decays were signal-averaged to obtain each spectrum.

tral region between −120 and 0 ppm is due to the ^{13}C-enriched *sn*-2 carbonyl group.

The details of the temperature dependence of ^{13}C-NMR spectra of a pure 2-[1'-^{13}C]DPPC model membrane preparation and a corresponding dry lipid preparation have been discussed elsewhere [14]. For our hydrated 2-[1'-^{13}C]DPPC dispersion preparation at 17°C, the spectral component due to the $^{13}C=O$ group shows an axially symmetric powder pattern with $\sigma_\perp = -7$ ppm, $\sigma_\parallel = -119$ ppm and a residual anisotropy $\langle\Delta\sigma\rangle = \sigma_\parallel - \sigma_\perp = -112$ ppm. The breadth of the spectrum is thus reduced when compared to that of the dry lipid ($\Delta\sigma = -148$ ppm) indicating that, in hydrated lipid bilayers, the lipid molecules undergo some anisotropic motion faster than $\gamma H_0 \Delta\sigma \approx 10^4$ s^{-1}. At 30°C, the spectrum shows the presence of a new symmetric line of width ≈ 1 kHz. The chemical shift of this line, $\langle\sigma\rangle = -44$ ppm, is equal to that expected if the tensor values of the powder pattern are completely averaged, i.e., $\langle\sigma\rangle = (2\sigma_\perp + \sigma_\parallel)/3$. As the sample temperature is further increased, the narrow component increases in intensity and becomes the only component at temperatures above the main phase transition temperature (T_c = 41.3°C). The effective line width of this narrow component at $T > T_c$ is about 500 Hz, which includes a substantial instrumental contribution.

With preparations containing Δ^9-THC, the spectral band due to the 2-[1'-^{13}C]DPPC carbonyl group shows the same two components observed in the pure DPPC preparation. There is no discernible difference in the shape of each of the components. However, there are distinct differences in their relative intensities. In these spectra, the narrow component appears and the broad powder pattern diminishes at progressively lower temperatures as the Δ^9-THC concentration increases. For example, in the spectra from DPPC/Δ^9-THC of concentration $x = 0.20$, the narrow component is already apparent at 17°C and increases gradually in intensity so that at 30°C the ^{13}C-enriched spectral band is a sharp peak of linewidth less than 1 kHz. In fact, we observe the complete disappearance of the broad Pake pattern at 42°C, 40°C, 36°C, 32°C and 32°C for Δ^9-THC concentrations of $x = 0.00$, 0.05, 0.10, 0.20 and 0.40, respectively. Fig. 5 shows that we can obtain virtually identical ^{13}C spectra from all of the drug-containing preparations, although at different temperatures. For example, the ^{13}C spectra for pure DPPC at 33°C is identical to those of DPPC/THC $x = 0.05$ at 30°C and $x = 0.10$ at 25°C. Characteristically, and as we observed with the ^{2}H-NMR spectra, the ^{13}C spectra for Δ^9-THC concentrations $x = 0.20$ and $x = 0.40$ are virtually identical at the same temperatures indicating again that the effect of the drug at $x = 0.20$ has already reached a saturation point.

The two-component ^{13}C-NMR lineshape of the 2-[1'-^{13}C] segment of several phospholipids at temperatures just below their main transition was observed and studied in earlier work [14,15]. After considering differ-

ent explanations, the authors carried out spectral simulations to exclude unreasonable models. They explained the lineshape as being due to the coexistence of gel-like and liquid-crystal-like molecules in the bilayer, resulting in the $L_{\beta'}$-like broad pattern and the L_α-like sharp peak, respectively. The temperature-dependent superposition of the two spectral components was simulated by assuming two types of axially symmetric $^{13}C=O$ tensors with the unique tensor component $\sigma_{\parallel}$ (along the $^{13}C=O$ bond) making an angle $\theta_{\beta'} \approx 28°$ with respect to the diffusion axis for the $L_{\beta'}$-like pattern and $\theta_\alpha = 54°$ for the L_α-like line. Both tensors were allowed to execute rapid 3-fold jumps ($\approx 10^5$ s^{-1}) about the diffusion axis and, in addition, there was a chemical exchange between the two tensor orientations. Very good simulations of the spectra were obtained by adjusting the exchange rate, k_{ex}, and the fraction of lipid in the L_α form, f_α. The authors concluded that the ripple $P_{\beta'}$ phase possesses properties of both $L_{\beta'}$ and L_α phases with a locally varying microscopic structure and that the collapse of the powder pattern to the sharp line is due to a conformational change at the *sn*-2 carbonyl which is associated with the $L_{\beta'}$-to-L_α phase transition.

We have observed this two-component lineshape in the ^{13}C-NMR spectra from all of the THC-containing DPPC preparations. Although the presence of the cannabinoid appears to abolish the endothermic peak associated with the pretransition in the thermograms of fully hydrated DPPC preparations, the ^{13}C spectra provide a different picture. Indeed, the presence of $L_{\beta'}$-like and L_α-like features in spectra obtained at temperatures about 12 K below the respective main transition temperatures of the DPPC/THC preparations indicates that the $P_{\beta'}$ phase is till present and that the microscopic process of the $L_{\beta'}$-to-L_α transition is qualitatively similar to that of pure DPPC. However, the transition occurs at progressively lower temperatures with increasing drug concentrations. Furthermore, the lineshapes show some subtle differences when compared to those obtained from the pure DPPC preparation. In order to quantitatively study the effects of Δ^9-THC on the dynamic behavior of the DPPC bilayer, we have adopted the model described above and carried out ^{13}C-NMR spectral simulations for that part of the spectrum originating from the 2-[1′-^{13}C] segment of DPPC in the absence and presence of Δ^9-THC for all of the spectra.

Again, the model involves two different types of long-lived conformations for the *sn*-2 carbonyl group; one in which the $^{13}C=O$ bond has a variable angle $\theta_{\beta'}$ with respect to the diffusion axis (presumably along the bilayer normal) and the other with the $^{13}C=O$ bond vector near the magic angle $\theta_\alpha = 54°$. We were able to account for the slight narrowing of the $L_{\beta'}$-like pattern with increasing temperatures by adjusting the $\theta_{\beta'}$-value, generally within 7°. According to our simulations, increases in temperature and/or Δ^9-THC concentration result in progressive increases in the value of the $^{13}C=O$ tilting angle. Both conformations were allowed to undergo rapid three-fold jumps about the diffusion axis at a rate of $5 \cdot 10^6$ s^{-1} which is in fast limit for ^{13}C. Since

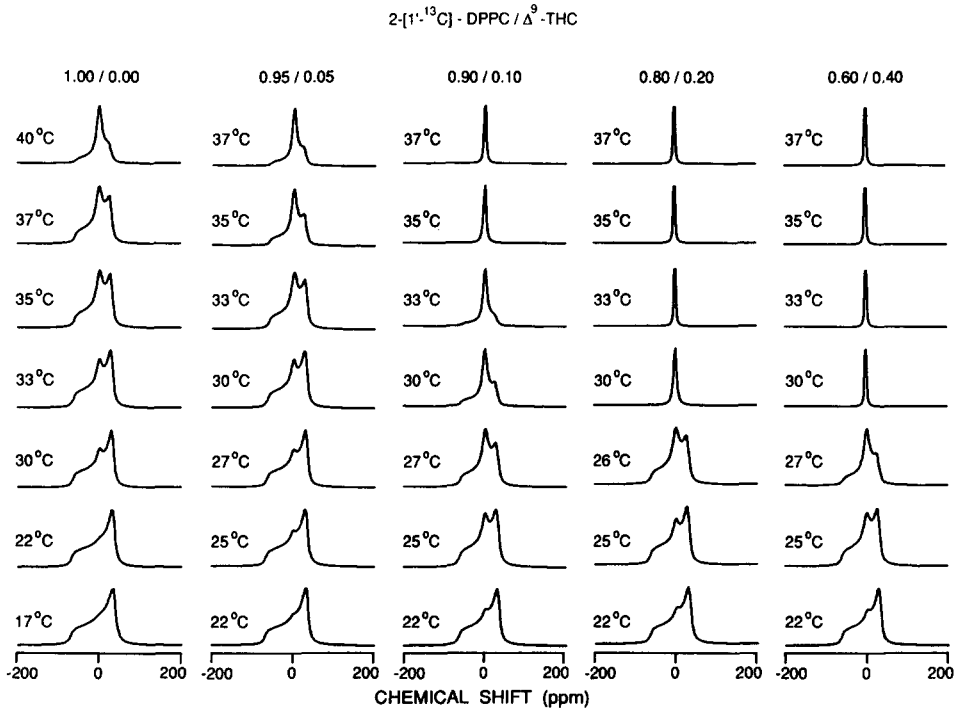

Fig. 6. Computer simulations of the spectral region due to 2-[1′-^{13}C] segment of DPPC in the corresponding solid-state ^{13}C-NMR spectra in Fig. 5. Each simulated spectrum contains 128 points in the 400-ppm chemical shift span.

TABLE I

Temperature and composition dependences of the ^{13}C spectral simulation parameters: $\theta_{\beta'}$, angle between the gel-like $^{13}C = O$ bond and the diffusion axis; f_α, fraction of lipid molecules in the liquid crystalline form; k_{ex}, exchange rate between the two conformations

Parameter [a]	Temp. (°C)	Molar ratios of DPPC/Δ^9-THC				
		1.00:0.00	0.95:0.05	0.90:0.10	0.80:0.20	0.60:0.40
$\theta_{\beta'}$(°)	17	25	25	26	27	28
	22	26	26	27	28	29
	25		27	28	29	30
	27		28	29	30 [b]	31
	30	28	29	31	33	33
	33	29	30	32	34	–
	35	30	31	33	–	–
	37	31	32	34	–	–
	40	32	33	–	–	–
f_α	17	0.01	0.01	0.02	0.02	0.02
	22	0.01	0.02	0.05	0.05	0.05
	25		0.05	0.14	0.10	0.15
	27		0.07	0.24	0.21 [b]	0.33
	30	0.08	0.12	0.39	0.85	0.99
	33	0.12	0.21	0.55	0.99	1.00
	35	0.19	0.33	0.90	1.00	1.00
	37	0.22	0.45	0.96	1.00	1.00
	40	0.42	0.95	1.00	1.00	1.00
k_{ex} (s^{-1})	17	800	800	800	1000	1000
	22	800	800	1000	1300	1300
	25		1000	1300	1700 [b]	1700
	27		1300	1600	2000	2000
	30	1000	1600	1800	2000	2000
	33	1300	1800	2000	2000	–
	35	1600	2000	2000	–	–
	37	2000	2000	2000	–	–
	40	2000	2000	–	–	–

[a] The estimated uncertainties in the values are ±0.5° for $\theta_{\beta'}$, ±0.01 for f_α and ±200 s^{-1} for k_{ex}.
[b] This spectrum was obtained at 26 °C.

higher axial diffusion rates produce no change in the ^{13}C lineshapes, the value used in our calculations represents only a lower limit. Our simulations show that both k_{ex}, the rate of exchange between the two components, and f_α, the fraction of lipid molecules in the L_α form, increase with increasing temperatures. A complete set of simulated spectra are shown in Fig. 6. For each simulated spectrum, 128 points were calculated for the 400-ppm chemical shift span. Each of these points was obtained by averaging over 128 values of the polar angles θ' and ϕ' in the powder pattern calculation. It is evident that the simulations using this model reproduce the experimental spectra quite well. The final values of the simulation parameters are listed in Table I. The estimated uncertainties in these values are ±0.5° for $\theta_{\beta'}$, ±0.01 for f_α and ±200 s^{-1} for k_{ex}.

The most sensitive parameters in the simulations are the tilting angle θ_β, and the fraction f_α of the L_α-like lipid component. In Fig. 7, we have plotted the temperature- and concentration-dependence of the values of these two parameters obtained from the simulations. These curves show higher f_α values with increasing temperatures and concentrations accompanied by slight increases in the values of $\theta_{\beta'}$. The curves corresponding to the two highest concentrations have a very similar temperature dependence, indicating saturation of the DPPC bilayer at a Δ^9-THC concentration $x = 0.20$. One interesting effect of increasing Δ^9-THC concentrations is that the f_α curves become progressively steeper indicating that the drug-containing DPPC bilayers exhibit progressively smaller temperature ranges in which the $L_{\beta'}$-like and L_α-like spectral components coexist. This seemingly contradicts the observation that Δ^9-THC broadens the main phase transition of DPPC bilayers, but can be explained by relating the appearance of the two-component ^{13}C spectra with the onset of the pretransition as has also been suggested in the earlier work with different phosphatidylcholine preparations [15]. The endothermic peak associated with the pretransition in the thermogram of hydrated DPPC is no longer observable when Δ^9-THC is present. A likely explanation is that the pretransition peak gradually broadens with increasing drug concentrations and/or is masked by the stronger peak of the main transition which

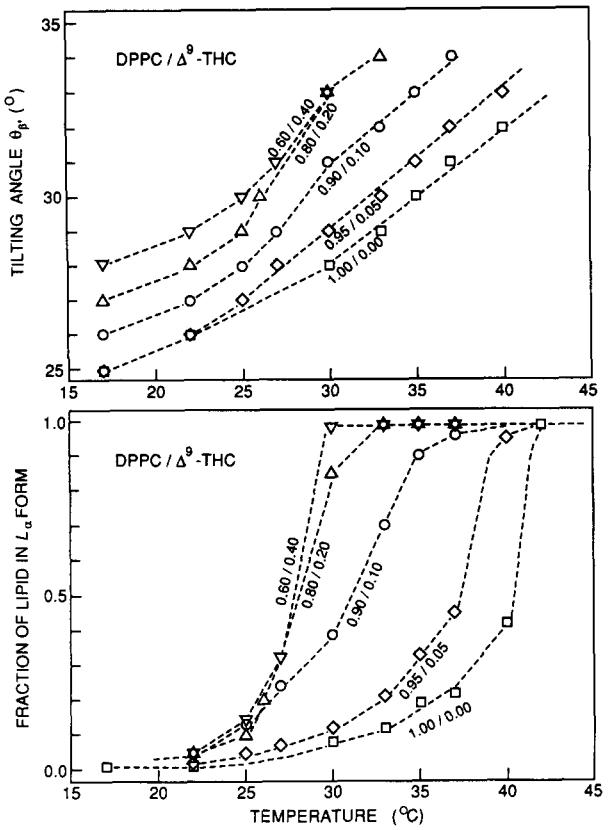

Fig. 7. Temperature and concentration dependences of two of the parameters used in the computer simulations of the solid-state ^{13}C-NMR spectra of 2-[1'-^{13}C]-DPPC/Δ^9-THC with molar ratios of 1.00:0.00 (□), 0.95:0.05 (◇), 0.90:0.10 (○), 0.80:0.20 (△) and 0.60:0.40 (▽). The top graph contains the tilting angle $\theta_{\beta'}$ ($\pm 0.5°$) that the $L_{\beta'}$-like $^{13}C=O$ bond makes with the diffusion axis and the bottom one shows the fraction f_α (± 0.01) of lipid molecules in the L_α form. The uncertainty in the temperature values is ± 0.5 K.

undergoes serious broadening and occurs at progressively lower temperatures. This interpretation would mean that Δ^9-THC, when introduced in a DPPC bilayer preparation, lowers the main phase transition temperature and extends its temperature range while at the same time the drug has a smaller effect on the onset temperature for the pretransition.

In our spectral simulations, we found the exchange rate k_{ex} between the two components to be a less sensitive parameter (Table I). We have varied k_{ex} from 800 to 200 s^{-1} to refine the simulated spectral shapes and found that the presence of Δ^9-THC in DPPC bilayers increases the rate of this exchange in a concentration dependent manner. The exchange between the $L_{\beta'}$ and L_α components can be either inter- or intramolecular. The intermolecular model would involve a coexistence of the $L_{\beta'}$ and L_α phases as separate lipid domains. The domains can be regularly arranged as suggested by the ripple structure observed by X-ray diffraction [25] and the exchange process between two lipid molecules in different phases would take place at the domain boundaries. This in effect moves the boundary while keeping the number of molecules of each type constant for a given temperature. An exchange rate of $\approx 10^3$ s^{-1} implies that there must be fast dynamic movement of the boundaries sweeping through the domains. Direct evidence for the existence of such domains was obtained by a selected reflection dark-field micrograph of a hydrated DPPC single bilayer at 17 °C [26]. On the other hand, the intramolecular model would involve an equilibrium between two lipid conformers, representing the $L_{\beta'}$ and L_α phases, exchanging at a rate of $\approx 10^3$ s^{-1}.

Composition dependence of phase properties in the DPPC /Δ^9-THC system

The combination of solid state ^2H- and ^{13}C-NMR spectra of DPPC/Δ^9-THC preparations can be utilized to characterize the composition dependence of the phase transition properties. Arguably, data from calorimetric measurements may provide the necessary information for this characterization. However, such results would only reflect on the macroscopic phase changes and bulk thermotropic properties of the bilayer, but would provide no information at the molecular level. In this regard, different chemical groups in the phospholipid molecule may respond differently to temperature changes and thus be associated with different stages of the phase transition.

For phosphatidylcholines, there is substantial evidence that the main phase transition and the pretransition can be associated with conformational changes in two separate groups of the lipid molecule. X-ray diffraction [27], electron spin resonance [28,29] and ^{31}P-NMR [30] experiments on saturated phosphatidylcholines show that a conformational change occurs at a temperature near that of the pretransition. Also, in a ^{13}C-NMR study of three saturated phosphatidylcholines, dimyristoyl-, dipalmitoyl-, and distearoyl-phosphatidylcholine, the temperature at which the L_α-like line first appears was found to correlate consistently with the temperature of the pretransition in the calorimetric measurements [15]. In contrast, phosphatidylethanolamines which do not exhibit a pretransition show only the $L_{\beta'}$ component in the *sn*-2 carbonyl ^{13}C-NMR spectra [31]. Collectively, the emergence of the L_α-like line in the ^{13}C spectra is believed to result from a conformational change in the backbone region of the lipid molecule and this change gives rise to the observed pretransition endotherm in the thermograms.

^2H spectra due to labeled methylene segments of saturated phosphatidylcholines acquire the liquid crystalline appearance of a motionally narrowed Pake pattern at the main transition temperature which essentially corresponds with the melting of the lipid acyl chains. As previously reported [24,32], we also found by comparing the ^2H- and ^{13}C-NMR spectra of DPPC that

the conformational change at the *sn*-2 $^{13}C = O$ precedes the melting of the acyl chains.

The combined solid-state NMR data from two different labels in the DPPC molecule provide evidence that the pretransition and the main transition are caused by two separate events, i.e., a conformational change in the glycerol backbone region and melting of the acyl chain. To monitor the effects of Δ^9-THC on the phase transition properties of the DPPC model membrane at the molecular level, we have combined earlier data from differential scanning calorimetry with our present solid-state NMR results. The data are used to construct temperature-composition phase plots in a manner used earlier with the dipalmitoylphosphatidylethanolamine/cholesterol system [33].

From the ^{2}H-NMR spectra of the different DPPC/Δ^9-THC preparation, we have identified the temperatures at which the spectrum transforms completely into a well defined Pake pattern with sharp perpendicular and parallel edges. The ^{13}C spectra allowed us to extract two temperature values for each THC-containing preparation. One value corresponds to the temperature at which the L_α-like line first becomes visible and the other to the temperature at which the $L_{\beta'}$-like pattern disappears completely leaving only the L_α-like line in the spectrum. In a parallel manner, we also measured two temperature values in the respective thermograms corresponding to the starting and ending points of the gel to liquid crystal endotherm. In the case of pure DPPC, the lower temperature corresponds with the onset of the pretransition while the higher temperature is equal to the end of the main transition. These two endotherms are no longer clearly separable in the drug-containing preparations and no attempt was made to separate the individual components representing the phase transformation from the gel to liquid crystal.

Shown in Fig. 8 are five plots of temperature versus THC concentration. The group of two curves at lower temperatures traces out the dependence of pretransition temperature on drug concentration. Specifically, the ^{13}C-NMR data show the temperatures at which the local conformational changes occur while the calorimetric data reflect changes in the bulk properties. It is interesting to notice that the former changes precede the latter in temperature by about 4 to 9 K. The group of three curves at higher temperatures characterizes the dependence of the main transition temperature on the DPPC/Δ^9-THC concentration. Although they show the same downward trend with increasing drug concentrations and are confined within a temperature band of less than 4 K, the three curves do exhibit noticeable differences. The ^{2}H-NMR results closely correspond to the calorimetric data and describe the effect of the different drug concentrations on the melting of the bilayer chains. In contrast, the curve from the ^{13}C-NMR data represents points at which the conformational

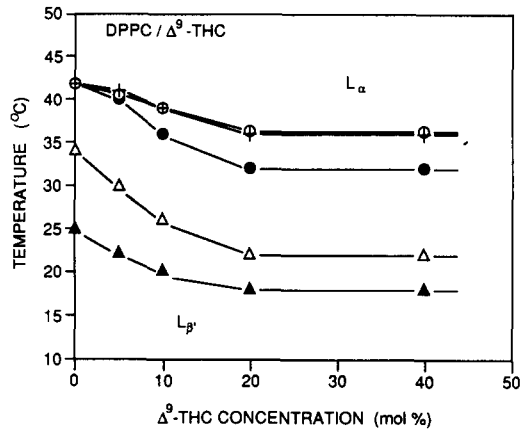

Fig. 8. Composition dependence of phase transition properties for the DPPC/Δ^9-THC system using transition temperature values obtained from ^{2}H-NMR spectroscopy (+), ^{13}C-NMR spectroscopy (● and ▲) and differential scanning calorimetry (○ and △).

change in the glycerol backbone has been completed. In pure DPPC the points from the above three curves coincide, indicating that both conformational events are complete at the same temperature ($\approx 42°$ C). However, in the presence of THC, the full conversion into an L_α-type ^{13}C-spectrum occurs at progressively lower temperatures than its ^{2}H counterpart with increasing drug concentration. The above data provide further evidence that in hydrated DPPC preparations the conformational change at the *sn*-2 carbonyl group is a separate event from the melting of the chains. Although Δ^9-THC influences both events, its effects on the carbonyl site are apparently more intense than those on the chains. This is in accord with other data from our laboratory showing that the cannabinoid resides near the membrane interface and is well positioned to interact directly with the acyl carbonyl groups [10,11].

Conclusions

Our systematic studies on the temperature and concentration dependences of the solid-state ^{2}H- and ^{13}C-NMR spectra show that Δ^9-THC produces drastic effects on the phase properties of DPPC bilayers that are manifested as changes in the bulk properties of the hydrated phospholipid as well as changes in the dynamic and conformational properties of the bilayer.

Changes in the phase transition temperatures observed in our previous differential scanning calorimetric study of the DPPC/Δ^9-THC system [7] are also reflected in the solid-state ^{2}H- and ^{13}C-NMR spectra presented here. Our NMR studies show that the presence of Δ^9-THC in DPPC bilayers induces the formation of the liquid-crystalline phase component at lower temperatures and over a wider temperature range. This effect is intensified with increasing drug concentrations up to $x = 0.20$ when full saturation has occurred.

Changes in the dynamic properties of acyl chains in the DPPC bilayers due to the presence of Δ^9-THC are best seen in the temperature dependence of the quadrupolar splittings and echo amplitudes as measured from the ^2H spectra. The spectral results suggest that Δ^9-THC reduces the cooperativity among the phospholipid chains providing useful insights into the mechanism of membrane perturbation by the cannabinoids. The ^{13}C spectra and the corresponding computer simulations reveal that the equilibrium population between the $L_{\beta'}$ and L_α phase components is drastically altered by the incorporation of the Δ^9-THC into the DPPC bilayers. In effect the presence of Δ^9-THC causes the DPPC bilayers to appear as if the membrane were at a higher temperature.

Regarding the conformational properties of DPPC bilayers, both ^2H and ^{13}C spectra provide evidence of changes induced by Δ^9-THC. The slight increase in the quadrupolar splittings that we observed in the ^2H spectra is a piece of evidence that Δ^9-THC increases the *trans : gauche* conformer ratio in the region from $C_{1'}$ to $C_{7'}$ of the lipid chain when compared to pure DPPC. However, it does this much less effectively than cholesterol. At 42°C, Δ^9-THC ($x = 0.20$) increases $\Delta\nu_Q$ by 1 kHz whereas we have observed an increase of 11 kHz by cholesterol ($x = 0.20$) in DPPC preparations at the same temperature. If we assume that one molecule of Δ^9-THC displaces one molecule of cholesterol in the mammalian cell membrane, one could argue that the net effect of Δ^9-THC is to increase the relative number of *gauche* conformers in the bilayer.

Regarding the ^{13}C-NMR results, these seem to indicate that Δ^9-THC initially affects the bilayer at the interface between the polar and hydrophobic components of the phospholipid and most likely at the carbonyl region. This is in accord with our previous findings on the orientation [11] and topography [10] of Δ^9-THC in the membrane. The effects on the phospholipid chains appear to follow this interaction. Higher drug concentrations produce a wider separation between the two conformational events. This thermal behavior of the bilayer in the presence of Δ^9-THC contrasts the observed properties of pure hydrated DPPC where the thermally induced conformational events at the carbonyl and the chain components of phospholipid proceed concurrently.

Our present data on the interactions of Δ^9-THC with phosphatidylcholine bilayers can be used as a model which describes the molecular events leading to membrane perturbation by amphipathic molecules. These include; (a) interaction of the amphipath with the bilayer at the interface; (b) decreased cooperativity at the bilayer chains represented by a broadening of the phase transition which results from a separation between the conformational events at the interface and those at the bilayer chains; and (c) an overall increase in the ratio of *gauche : trans* conformers in cholesterol containing bilayers.

Acknowledgments

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