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Amphipathic interactions of cannabinoids with membranes. A comparison between Δ^8-THC and its O-methyl analog using differential scanning calorimetry, X-ray diffraction and solid state ^{2}H-NMR

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The effects of (−)-Δ^8-tetrahydrocannabinol (Δ^8-THC) and its biologically inactive *O*-methyl ether analog on model phospholipid membranes were studied using a combination of differential scanning calorimetry (DSC), small angle X-ray diffraction and solid state ^{2}H-NMR. The focus of this work is on the amphipathic interactions of cannabinoids with membranes and the role of the free phenolic hydroxyl group which is the only structural difference between these two cannabinoids. Identically prepared aqueous multilamellar dispersions of phosphatidylcholines in the absence and presence of cannabinoids were used. The DSC thermograms and X-ray diffraction patterns of these preparations allowed us to detect the strikingly different manners in which these two cannabinoids affect the thermotropic properties and the thickness of the bilayer. In order to study the effects of the cannabinoids on different regions of the bilayer, we used solid state ^{2}H-NMR with four sets of model membranes from dipalmitoylphosphatidylcholine deuterated in different sites, viz., the choline trimethylammonium head group, or one of the following three groups in the acyl chains; the 2′-methylene, 7′-methylene, 16′-methyl groups. Analysis of quadrupolar splittings indicated that Δ^8-THC resides near the bilayer interface and the inactive analog sinks deeper towards the hydrophobic region. The temperature dependence of the solid state ^{2}H-NMR spectra showed that, during the bilayer phase transition, the disordering of the choline head groups is a separate event from the melting of the acyl chains, and that amphipathic interactions between Δ^8-THC and the membrane separate these two events further apart in temperature. The inactive analog lacks the ability to induce such a perturbation.

Introduction

Extensive studies on the structure-function correlation of cannabinoids have shown that the phenolic hydroxyl group has a pivotal role in determining the pharmacological properties of these molecules [1,2]. Indeed, it is well known that removal of the hydroxyl hydrogen by methylation to give the *O*-methyl ether results in analogs devoid of biological activity [3,4]. Similarly, substitution of the OH group with dimethylamino or sulfhydryl groups results in activity loss [5]. Several phenolic cannabinoid esters have been reported to possess biological activity [6,7]. However, these are generally believed to function as pro-drugs for the free phenolic compounds. Although well documented, the role of the phenolic hydroxyl in determining biological activity is not understood. A phenolic proton appears to be required for activity ad substitution of OH with other groups possessing hydrogen-bonding hydrogens results in analogs with no better than partial activity. These facts point to the possible

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; Δ^8-THC, 1-(−)-Δ^8-tetrahydrocannabinol; Me-Δ^8-THC, (−)-*O*-methyl-Δ^8-tetrahydrocannabinol; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

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involvement of the OH, NH or SH groups as hydrogen donors at the site of the action.

According to existing evidence, many biological properties of cannabinoids can be attributed to their respective effects on biological membranes [8–10]. These effects may involve amphipathic interactions with either the membrane lipids or the membrane-associated cannabinoid receptor [11]. Various biophysical methods have been applied to study the interactions between cannabinoids and phospholipids in model membranes. Differential scanning calorimetry (DSC) has been used extensively to study the effects of various cannabinoids having a wide range of biological potencies on the thermotropic properties of phospholipid bilayers [12,13]. Recently, we have applied small angle X-ray diffraction techniques to investigate the topography of cannabinoids in partially hydrated model membranes and found that these amphipathic drug molecules reside near the hydrocarbon-core/water interface of the bilayer [14]. In an earlier work in our laboratory using high resolution ^{1}H-NMR and solid state ^{2}H-NMR, we have determined the conformations of Δ^9-tetrahydrocannabinol (Δ^9-THC) and its inactive analog $\Delta^{9,11}$-THC in solution [15] and the orientation of Δ^9-THC in model membranes [16], respectively. We have also applied solid state ^{2}H- and ^{13}C-NMR to a system in which different concentrations of cannabinoids were incorporated into model membranes consisting of phospholipid with specific isotopic labels [17]. These NMR experiments allowed us to obtain information at the molecular level on the manner in which cannabinoids affect the conformational and dynamic properties of the bilayer. Since DSC, X-ray diffraction and solid state NMR provide complementary results related to the cannabinoid–phospholipid interactions with the membrane, analysis based on the combined data from these three different biophysical methods greatly enhances our ability to outline a molecular mechanism for cannabinoid action.

Our present study compares the effects on model membranes of two cannabinoids; a pharmacologically active one possessing the phenolic hydroxyl group, (−)-Δ^8-tetrahydrocannabinol (Δ^8-THC, Fig. 1) and an inactive analog with a methyl ether group replacing the phenolic hydroxyl group, (−)-*O*-methyl-Δ^8-tetrahydrocannabinol (Me-Δ^8-THC, Fig. 1). Although (−)-Δ^9-tetrahydrocannabinol is recognized as the major biological active constituent in marijuana, its isomer Δ^8-THC is also present in the plant. The two molecules which differ only in the position of the double bond in the C-ring have very similar pharmacological properties and are almost equipotent. However, Δ^8-THC is considerably more stable chemically and, thus, more suitable for our biophysical studies. Our goal was to obtain and analyze the differences in the abilities of these two molecules to perturb the membrane bilayer. Specifically, the different perturbations induced by these two molecules were investigated by observing changes in the thermotropic behavior of the bilayer using DSC, by detecting variations in the bilayer thickness using X-ray diffraction, and by monitoring differences in the dynamic properties in specific regions of the bilayer using solid state ^{2}H-NMR. For our studies, we chose fully hydrated dipalmitoylphosphatidylcholine (DPPC, Fig. 1) and dimyristoylphosphatidylcholine (DMPC) as homogeneous model membranes to probe the interactions of the two cannabinoids with the lipid bilayers. Furthermore, DPPC and DMPC have well-characterized phase behavior and can be isotopically labeled in different sites through chemical synthesis. Phosphatidylcholines are also major membrane components in which they are found as mixture of variable fatty acid composition. Our experiments were limited to cannabinoid concentrations ranging from 0.05 to 0.40 molar fractions, considerably higher than those associated with pharmacological activity since lower drug concentrations produced spectral changes which were not easily observable. However, our primary motivation for these experiments was not to reproduce the physiological effects of these analogs but, rather, to study their interactions with membrane lipids at the molecular level and to compare their effects on the bilayer preparations. Interestingly, DPPC is present as a major constituent in lung surfactant and this study may provide useful information relating to the effects of inhalation of THC in marijuana smoking. The model membranes from the saturated phospholipids (DPPC, DMPC) employed here are very stable and can accommodate large amounts of these lipophilic drugs without losing their bilayer structure. Parallel experiments with

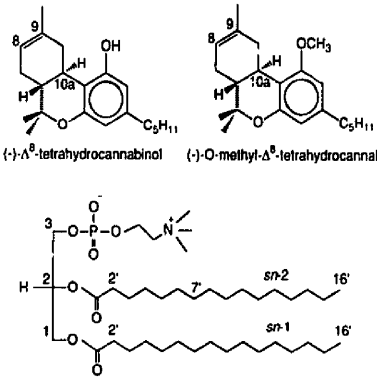

Fig. 1. Structures of (−)-Δ^8-tetrahydrocannabinol (Δ^8-THC), (−)-*O*-methyl-Δ^8-tetrahydrocannabinol (Me-Δ^8-THC), and dipalmitoylphosphatidylcholine (DPPC). The structure of dimyristoylphosphatidylcholine (DMPC) is identical to that of DPPC except for shorter chains by two methylene groups.

DPPC and DMPC model membrane preparations containing Δ^8-THC and Me-Δ^8-THC gave direct evidence that Δ^8-THC exerts strong effects on the bulk and microscopic properties of model membranes whereas Me-Δ^8-THC perturbs the bilayer only marginally. Our results point to the requirement of the phenolic hydroxyl group for cannabinoid function in membranes.

Materials and Methods

Δ^8-THC and Me-Δ^8-THC were synthesized in our laboratory using known procedures. ^{2}H-labelled cannabinoids were synthesized according to procedures which we described elsewhere [18,19]. DPPC and DMPC were obtained from Avanti Polar Lipids, Birmingham, AL. For the solid state ^{2}H-NMR experiments, we used four different ^{2}H-labelled DPPC lipid molecules, namely, N(C^2H_3)$_3$-DPPC, 1,2[2′,2′-$^2H_2$]-DPPC, 2[7′,7′-$^2H_2$]-DPPC and 1,2[16′,16′,16′-$^2H_3$]-DPPC. The first three compounds were synthesized in this laboratory using known procedures [20] and the last one was obtained from Serdary Research Laboratories, London, Ontario, Canada.

Sample preparation

Sample preparation procedures were identical for DSC, X-ray diffraction and solid state ^{2}H-NMR experiments. Appropriate amounts of phospholipid and cannabinoid were dissolved in CH_2Cl_2. The solvent was then evaporated by passing a stream of O_2-free nitrogen over the solution at 50°C and the residue was placed under vacuum (0.1 mmHg) for 12 h. Distilled and de-ionized water (^{2}H-depleted water in the case of solid state ^{2}H-NMR samples) was added to the dried samples to produce a 50% (w/w) lipid/water preparation. For DSC experiments, the hydrated sample ($\approx$ 5 mg) was placed in a stainless steel capsule (Perkin Elmer) and sealed hermetically. For small angle X-ray diffraction experiments, the sample ($\approx$ 2 mg) was drawn into a thin-walled capillary tube (inner diameter 0.5 mm) which was then sealed at both ends with wax. For the solid state ^{2}H-NMR, the sample ($\approx$ 100 mg) was introduced into a 7-mm glass tube appropriately constricted. The samples were then sealed under vacuum and equilibrated at 5–10 K above the phase transition temperature for 15 min before recording the spectra.

Differential scanning calorimetry

Differential scanning calorimetry was carried out using a Perkin Elmer DSC-7 instrument. Prior to scanning, samples were held at a temperature above their phase transition for 15 min to ensure complete equilibrium. Each sample was scanned at least twice until identical thermograms were obtained. Heating and cooling scans gave similar results. The thermograms presented here were obtained with a heating scanning rate of 2.5 K/min and the temperature scale of the calorimeter was calibrated using both fully hydrated DPPC and pure indium as standard samples.

Small angle X-ray diffraction

Small angle X-ray diffraction experiments were carried out with an Elliott GX18 generator supplying CuKα radiation (λ = 1.54 Å) equipped with a camera utilizing a single vertical Franks' mirror. Sample temperature could be controlled and varied from 7°C to 70°C. Diffraction patterns were collected using a Braun position sensitive proportional counting gas flow detector with 29.27 digital channels per mm in the direction of diffraction. A small beam-stop was used to prevent the main beam from reaching the detector and, for a fully hydrated sample in a capillary tube, diffraction orders on both sides of the main beam were recorded. The sample-to-detector distance was 200 mm.

Solid state ^{2}H-NMR

Solid state ^{2}H-NMR spectra were obtained on a home-built pulse spectrometer operating at a magnetic field of 8.75 T (55.2 MHz for ^{2}H and 360 MHz for ^{1}H) 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 μs) which is longer than the spectrometer recovery time [21]. The $\pi/2$ pulse width for ^{2}H was 2.5–4.5 μs while the dwell time was 3.5 μs. Recycle delays were 0.2 s and 20000 to 50000 echoes were accumulated for each spectrum.

Results and Discussion

Differential scanning calorimetry

Hydrated DPPC or DMPC preparations exist in the form of multilamellar bilayers and possess dynamic properties resembling those of natural membranes [22]. Calorimetric measurements with hydrated DPPC and DMPC preparations show two endothermic transitions, namely, a somewhat broad low-enthalpy pretransition and a sharp main transition with peaks at temperatures designated by T_c' and T_c, respectively. Highly sensitive DSC measurements showed that DPPC preparations have T_c' = 34.0°C and T_c = 41.75°C while DMPC preparations have T_c' = 13.5°C and T_c = 23.70°C [23]. At temperatures below the pretransition, the phospholipid molecules are arranged in a lamellar gel phase ($L_{\beta'}$) in which the chains are tilted with respect to the plane of the bilayer and exist in an all-*trans* conformation. At temperatures above the main transition, the phospholipid bilayers exist in the liquid crystalline phase (L_α) with the chains perpendicular to the plane of the bilayer and their methylene segments in two interconverting *gauche* and *trans* populations. At temperatures

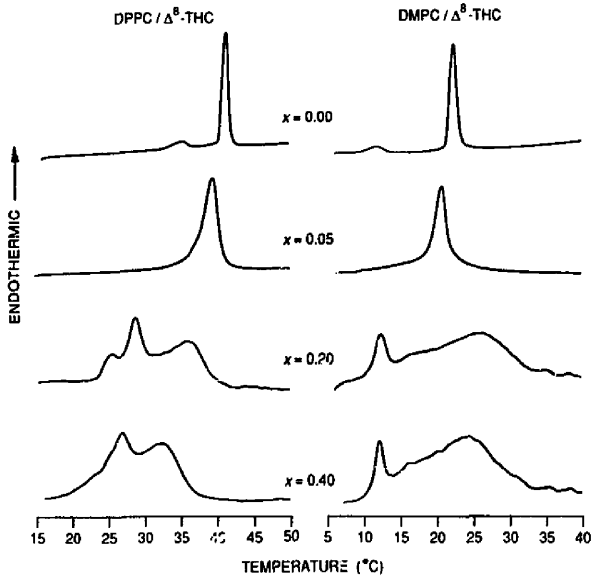

Fig. 2. Thermograms of model membrane preparations of DMPC and DPPC containing Δ^8-THC with increasing concentrations. The molar fraction of Δ^8-THC is indicated next to each thermogram.

between T_c' and T_c, the preparations exist in the ripple phase ($P_{\beta'}$), which is believed to be composed of coexisting gel and liquid crystalline components [17,24].

We have obtained thermograms of hydrated DPPC and DMPC preparations containing increasing concentrations of Δ^8-THC and Me-Δ^8-THC in a temperature range of 5°C to 50°C. Fig. 2 shows a set of normalized thermograms of PC preparations containing the pharmacologically active Δ^8-THC with phospholipid/THC molar ratios 1.00:0.00, 0.95:0.05, 0.80:0.20, and

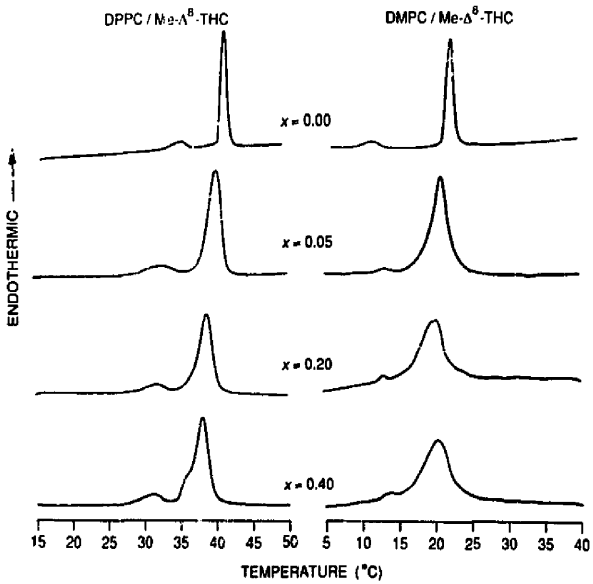

Fig. 3. Thermograms of model membrane preparations of DMPC and DPPC containing Me-Δ^8-THC with increasing concentrations. The molar fraction of Me-Δ^8-THC is indicated next to each thermogram.

0.60 : 0.40 (or $x = 0.00$, 0.05, 0.20 and 0.40). The drug-free DPPC and DMPC ($x = 0.00$) show the pretransition and the main transition at the expected temperatures. When Δ^8-THC is incorporated, the thermograms of both DPPC and DMPC change significantly in a similar concentration-dependent manner. With a Δ^8-THC concentration of $x = 0.05$ in either DPPC or DMPC, the most drastic effect is the disappearence of the pretransition peak accompanied by a broadening and shifting of the main peak to a lower temperature by about 2 K. At a drug concentration of $x = 0.20$, the main transition broadens significantly and a new peak emerges at 31°C and 12°C for DPPC and DMPC preparations, respectively. In both instances, this new peak appears at a temperature about 12 K below the T_c of the drug-free PC bilayer. A further increase in Δ^8-THC concentration from $x = 0.20$ to $x = 0.40$ does not significantly change the thermograms, indicating a state of saturation beyond $x = 0.20$. We have observed that Δ^8-THC induces more broadening in the phase transition of the DMPC model membrane than in DPPC. It thus appears that Δ^8-THC disrupts the chain cooperativity in the DMPC preparation more effectively than in DPPC, presumably because DMPC has shorter acyl chains than DPPC.

Fig. 3 depicts a set of normalized thermograms obtained from parallel experiments using PC preparations containing the pharmacologically inactive *O*-methyl analog with the same molar ratios. Contrary to the case of Δ^8-THC, the incorporation of Me-Δ^8-THC into the bilayers produces only small effects on the thermotropic behavior of the PC bilayers. Here, the endothermic pretransition peak can still be clearly seen while no significant change in the main transition of DPPC or DMPC is produced regardless of the Me-Δ^8-THC concentration. At a concentration of $x = 0.20$, the temperature shift is only about 2.5 K. Again, DMPC bilayers seem to be affected more by the presence of Me-Δ^8-THC than DPPC bilayers; nevertheless, the general features of the thermal properties of either bilayer preparation remain unchanged. Increasing Me-Δ^8-THC concentration from $x = 0.20$ to $x = 0.40$ does not change the shape of the thermogram in either DPPC or DMPC preparations.

A comparison of the above two sets of thermograms shows that the two structurally-related cannabinoids affect the thermotropic behavior of model membranes differently. The biologically active compound is able to perturb the membrane much more effectively than its inactive analog. The significant broadening of the main transition in the calorimetric data indicates that Δ^8-THC disorders the membrane by lowering its cooperativity while the temperature shift in the main transition points to the fluidizing effect of Δ^8-THC on the bilayer. Conversely, the thermograms of the PC bilayers containing Me-Δ^8-THC clearly show that, when the phenolic hydroxyl group of Δ^8-THC is methylated, the cannabinoid does not perturb the membrane significantly.

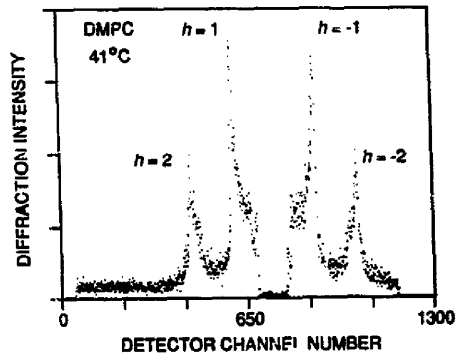

Fig. 4. Representative small angle X-ray diffraction patterns from a fully hydrated DMPC bilayer preparation at 41°C. The pattern was recorded using a position-sensitive detector which has a total of 1300 channels and 29.72 channels per millimeter. The distance from the sample to the detector was 201 mm.

Small angle X-ray diffraction

Small angle X-ray diffraction patterns from fully hydrated bilayer preparations of both DPPC and DMPC without and with Δ^8-THC or Me-Δ^8-THC at various temperatures were obtained and analyzed. A representative diffraction pattern from DMPC bilayer at 41°C is shown in Fig. 4. These peaks were indexed by $h = 1$ and $h = 2$ on the left and $h = -1$ and $h = -2$ on the right according to Bragg's law, $2d\sin\theta = h\lambda$, where d is the total period repeat distance (d-spacing) and θ is the diffraction angle. By measuring the exact locations of the peaks in the diffraction pattern, we calculated $\theta = 0.7°$ and 1.4° for the first and second orders, respectively, and $d = 62.2$ Å at this particular temperature.

Fig. 5 shows d-spacings as functions of temperature. For preparations of drug-free DPPC and DMPC, our measured d-spacing values and their temperature dependence were consistent with the results reported previously [25]. For both lipids, the d-spacing increases with increasing temperature, reaches a maximum near the respective phase transition, and decreases after passing the chain melting temperature. A plot of d-spacing vs. temperature thus shows a peak corresponding to that observed in the thermograms. The temperature dependence of the d-spacing, thus, provides information on the phase transition which is complementary to that obtained from DSC. Incorporation of the pharmacologically active Δ^8-THC ($x = 0.20$) increases the d-spacing of DPPC bilayer by 8 Å at temperatures below the phase transition and, in addition, broadens and lowers the peak which now occurs at 32°C, 9 K

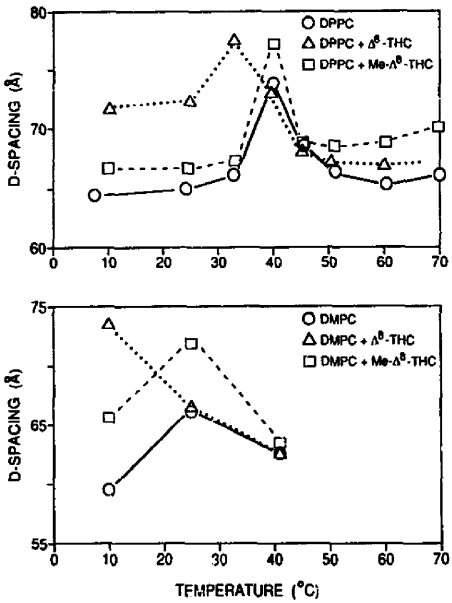

Fig. 5. Temperature dependence of *d*-spacing of fully hydrated DPPC and DMPC bilayer preparations in the absence and presence of Δ^8-THC ($x = 0.20$) and Me-Δ^8-THC ($x = 0.20$).

below that of the pure phospholipid membrane. The *d*-spacing values of DPPC + Δ^8-THC preparation in the gel phase are larger than those of DPPC preparation, probably due to the drug-induced alteration of the lipid tilting angle of the lipid molecules with respect to the bilayer plane. On the other hand, when Me-Δ^8-THC is incorporated, the *d*-spacing peak location and peak width are identical to those observed from pure DPPC. The only noticeable difference is that the *d*-spacing values of DPPC + Me-Δ^8-THC at all temperatures are consistently higher than those of DPPC by 2 to 3 Å. This increase in the *d*-spacing values induced by Me-Δ^8-THC may be originating from the presence of Me-Δ^8-THC molecules deeper in the bilayer near the terminal methyl groups of the acyl chains.

As shown in the lower graph in Fig. 5, the *d*-spacing values for DMPC preparations at three temperatures (10°C, 25°C and 41°C) show good resemblance with the *d*-spacing behavior of DPPC preparations (32°C, 40°C and 45°C). However, the peaks in *d*-spacing curves of the DMPC preparations are broader than those in the corresponding DPPC preparations. This is consistent with the fact that the endothermic peaks in the DMPC thermograms are broader than those of DPPC.

Solid state ^{2}H-NMR spectroscopy

Solid state ^{2}H-NMR experiments can be carried out in fully hydrated multilamellar bilayers by observing spectra of deuterium labels on either the phospholipid or the drug molecules. In the present work, we have obtained spectra from drug-containing or drug-free lipid bilayer preparations of DPPC ^{2}H-labeled at four different sites. The ^{2}H-labels were specifically placed; (1) at the 7'-methylene group of the *sn*-2 chain (2[7',7'-$^2H_2$]-DPPC); (2) at the methylene groups alpha to the carbonyls of both acyl chains (1,2[2',2'-$^2H_2$]-DPPC); (3) at the terminal methyl groups of both acyl chains (1,2[16',16',16'-$^2H_3$]-DPPC); and (4) at the trimethylammonium choline group (N(C^2H_3)$_3$-DPPC).

Because of the interaction between the electric quadrupole moment and the electric field gradient at the nuclear site, the deuterium nucleus gives rise to a doublet in the spectrum of an oriented sample. The anisotropic nature of this interaction gives two spectral lines with a frequency difference of $\nu_1 - \nu_2 = (3/4)A_Q$ $(3\cos^2\theta - 1)$, which depends on the angle θ between the magnetic field and the axis of molecular ordering. In an aqueous phospholipid dispersion sample in the liquid crystalline phase, all values of θ are possible and the spectrum from ^{2}H nuclei at one site in the molecule is a 'powder pattern' in which the two principal peaks correspond to $\theta = 90°$ (perpendicular edges) and the two shoulders to $\theta = 0°$ (parallel edges). The separation between the two 90°-edges is referred to as the quadrupolar splitting ($\Delta\nu_Q$). At temperatures below the main phase transition, the axial diffusion rate is intermediate or slow, and one observes a broad, conical or rounded spectrum which does not show any sharp features. By studying the spectral shape as a function of temperature and the changes in $\Delta\nu_Q$ due to the presence of drug, we can obtain information on the dynamic properties of the lipid bilayer in the particular region where the ^{2}H-label is placed.

Spectra from 2[7',7'-$^2H_2$]-DPPC preparations. Fig. 6 shows representative solid state ^{2}H-NMR spectra from bilayers of fully hydrated DPPC having two equivalent ^{2}H-labels at the 7'-methylene group of the *sn*-2 chain. The drug-free DPPC preparation at 27°C shows a rounded-top spectrum. The overall width of this gel phase spectrum is about 120 kHz, only one half of that expected for the rigid lattice value. This is evidence that, even though in the gel phase, the DPPC molecules undergo diffusion along their long axis. As the temperature is increased towards 37°C where the DPPC bilayer turns into the $P_{\beta'}$ phase, the spectrum acquires a flat top appearance and the 90°- and 0°-edges start to be discernible. At 40°C, the spectrum shows a superposition of a sharp liquid crystalline Pake pattern and a broad gel-phase line shape, which indicates the co-existence of $L_{\beta'}$ and L_α phases in DPPC bilayers. When the temperature is above T_c, the spectrum of DPPC is completely liquid crystalline type, exhibiting the characteristic fast-limit axially symmetric powder pattern. The residual quadrupolar splitting as measured directly

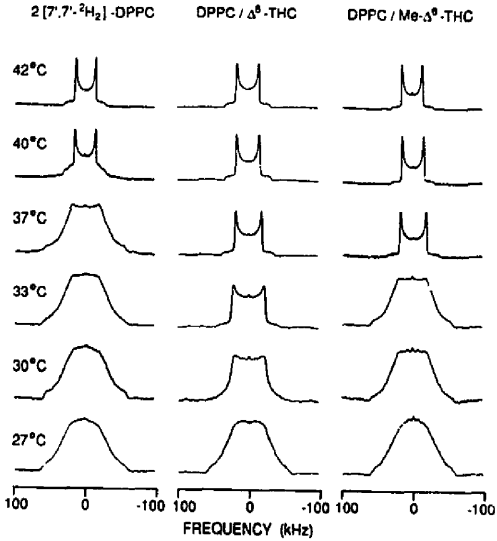

Fig. 6. Temperature dependence of solid state ^{2}H-NMR spectra due to 2[7',7'-$^2H_2$] segment of DPPC bilayer preparations in the absence and presence of Δ^8-THC ($x = 0.20$) and Me-Δ^8-THC ($x = 0.20$).

from the separation of the two 90°-edges in the spectrum of DPPC at 42°C is 28.6 kHz.

The most dramatic effects of Δ^8-THC on the spectral lineshapes are produced during the transition between the gel phase and the liquid crystalline phase. At 27°C, DPPC + Δ^8-THC ($x = 0.20$) shows a gel type spectrum with a flat top, similar to that observed from DPPC at 33°C. As the temperature increases, however, the breadth of the spectrum diminishes while the corners of the flat top turn into the 90°-edges and the center of the spectrum develops a gradually deepening depression. The spectral shapes from DPPC + Δ^8-THC at 30°C and 33°C were never observed in pure DPPC at any temperature and are characteristic of two components exchanging at an intermediate rate [26]. When the temperature is 37°C or higher, the spectrum shows the sharp features of liquid crystalline type lineshapes. The transition from gel to liquid crystal as manifested by the lineshape change in the spectrum of DPPC + Δ^8-THC spanned a temperature range from 27°C to 37°C, in good agreement with the broad endothermic peak observed in the corresponding thermogram. Conversely, the spectra from DPPC + Me-Δ^8-THC ($x = 0.20$) indicate a sharp transition from gel to liquid crystal between 33°C and 37°C. The presence of Me-Δ^8-THC in DPPC does not have any effect on the spectral shape except that the corresponding spectra appear at lower temperatures by about 4 K. For example, the spectrum of DPPC + Me-Δ^8-THC at 33°C has the same shape as the one from pure DPPC at 37°C.

Spectra from 1,2[2',2'-$^2H_2$]-DPPC preparations. To obtain more detailed molecular information on the DPPC–cannabinoid interactions, we have examined the effects of the drugs on a different site in the DPPC bilayer, namely, the first methylene segment in each of the two phospholipid chains. Shown in Fig. 7 are solid state ^{2}H-NMR spectra of three 1,2[2',2'-$^2H_2$]-DPPC preparations, DPPC, DPPC + Δ^8-THC ($x = 0.20$) and DPPC + Me-Δ^8-THC ($x = 0.20$). In the liquid crystalline phase (42°C), the spectrum of DPPC shows three visible components represented by three quadrupolar splittings, $\Delta\nu_Q$ = 12.1, 18.7 and 28.0 kHz. According to an earlier assignment [27] the largest splitting is attributed to the two deuterons at the 2'-position of the *sn*-1 chain and the other two splittings to the corresponding deuterons of the *sn*-2 chain. The difference in $\Delta\nu_Q$ values is due to the fact that the *sn*-1 chain is almost perpendicular to the bilayer surface with its two α-methylene deuterons at equivalent angles with respect to the axis of chain rotation. On the other hand, the *sn*-2 chain has an initial bend which places the carbonyl–α-methylene bond nearly parallel to the bilayer surface with each of the two deuterons having different average angle with respect to the chain axis. Assignment of the individual methylene deuterons was based on an earlier study using specific deuteration [28] which revealed that the splitting of 18.7 kHz was due to 2[2'*R*-^{2}H] and that the splitting of 12.1 kHz was due to 2[2'*S*-^{2}H]. These findings were supported by our calculations of the angles between the C–^{2}H

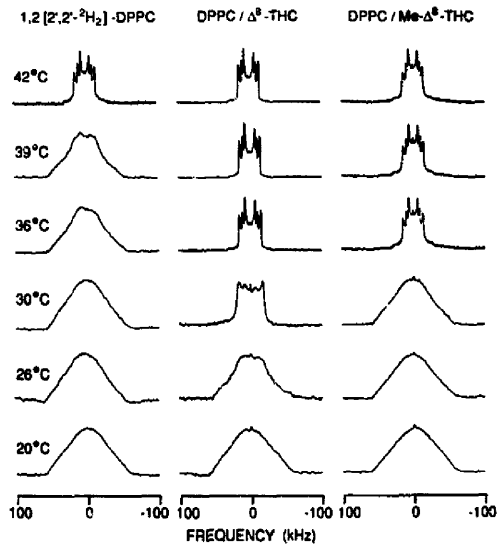

Fig. 7. Temperature dependence of solid state ^{2}H-NMR spectra due to 1,2[2',2'-$^2H_2$] segments of DPPC bilayer preparations in the absence and presence of Δ^8-THC ($x = 0.20$) and Me-Δ^8-THC ($x = 0.20$).

bonds and the lipid chain direction based on X-ray crystallographic data [29]. Indeed, the crystal structure showed that in 2'-methylene segment of *sn*-1 chain, both C–^{2}H bonds are almost perpendicular to the lipid chains, whereas in the *sn*-2 chains the two C–^{2}H bonds have angles of 76° and 45° with the lipid chain direction. Consequently, these three different orientations of the C–^{2}H bonds in the bilayer are expected to be responsible for the different $\Delta\nu_Q$ values.

At 20°C, the spectra from the drug-free and drug-containing 1,2[2',2'-$^2H_2$]-DPPC preparations are virtually identical, each having a rounded conical shape, which is almost identical to the gel-phase spectra from 2[7',7'-$^2H_2$]-DPPC preparations. As the temperature is raised to 33°C, the spectra from DPPC and DPPC + Me-Δ^8-THC show no change in the gel type lineshapes, whereas the spectra of DPPC + Δ^8-THC show very sharp 90°-edges but still no 0°-edges. At 36°C, while DPPC alone is still in the gel phase, the spectrum from DPPC + Δ^8-THC is now completely of liquid crystalline type and the one from DPPC + Me-Δ^8-THC shows mostly liquid crystalline characteristics with some gel phase features remaining. Finally, the spectra from these three preparations at 42°C show very sharp liquid crystalline lineshapes, each consisting of three overlapping Pake patterns. The above observed differences between the effects of Δ^8-THC and Me-Δ^8-THC on the spectra of 1,2[2',2'-$^2H_2$]-DPPC preparations point again to the significance of the phenolic hydroxyl group in cannabinoids during their interaction with membranes.

Spectra from 1,2[16',16',16'-$^2H_3$]-DPPC preparations. With this set of preparations, we have used deuterium probes at the terminal methyl groups of the acyl chains to detect the effects of Δ^8-THC and Me-Δ^8-THC on the membrane region that corresponds to the center of the bilayer. The six deuterons on each DPPC molecule are magnetically equivalent and give rise to ^{2}H-NMR spectra which are much narrower than those from the methylene segments because of the three-site hop reorientational averaging [30]. Fig. 8 shows the spectra due to the deuterated terminal methyl groups in hydrated preparations of DPPC, DPPC + Δ^8-THC ($x = 0.20$) and DPPC + Me-Δ^8-THC ($x = 0.20$) at temperatures from 27°C to 45°C. The spectra from DPPC and DPPC + Me-Δ^8-THC at 27°C are almost identical and contain a gel-type lineshape having 20 kHz in width. However, the spectrum from DPPC + Δ^8-THC at the same temperature has a smaller breadth (< 15 kHz) and starts to show some sharp features which gradually narrow and turn into two 90°-edges when the temperature increases. The phase transition in the DPPC preparation is observed between 38°C and 41°C, during which temperature range the spectra show superpositions of a gel-type lineshape and a well defined powder pattern characteristic of the liquid crystalline

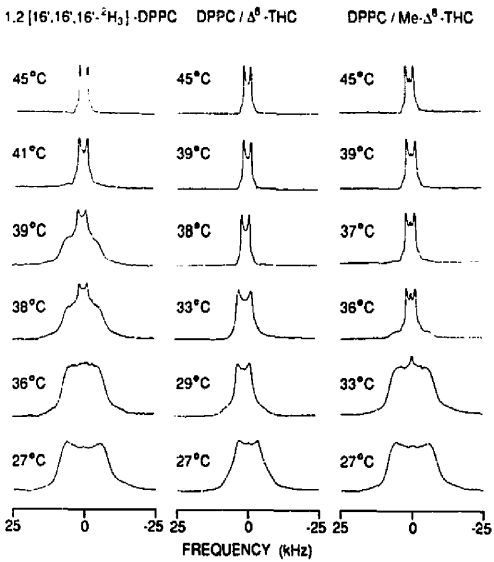

Fig. 8. Temperature dependence of solid state ^{2}H-NMR spectra due to 1,2[16',16',16'-$^2H_3$] segments of DPPC bilayer preparations in the absence and presence of Δ^8-THC ($x = 0.20$) and Me-Δ^8-THC ($x = 0.20$).

phase. At 38°C, the L_α-like spectral features start to emerge from the broad gel-type lineshape. Based on the relative intensities of these two components, we estimate that 20% of the lipid molecules are in the liquid crystalline phase while the rest of the lipid molecules still remain in the gel phase. This observation provides another piece of direct evidence for the co-existence of the $L_{\beta'}$ and L_α phases in the phosphatidylcholine model membrane preparations at temperatures between the pretransition and the main transition ($P_{\beta'}$ phase), as we have observed and discussed previously in a study using solid state ^{13}C-NMR [17]. At 39°C, the relative intensity of the liquid crystalline component in the spectrum of DPPC has increased to about 35% of the total intensity, indicating a new equilibrium between these two components. At 41°C, the spectrum shows mainly the powder pattern from the liquid crystalline component with only very small portion of surviving gel-type component, while at temperatures above 43°C the spectrum is purely liquid crystalline type. The quadrupolar splitting value decreases continuously with increasing temperature. Similar superpositions of the $L_{\beta'}$ and L_α components are also observed in the spectra of DPPC + Me-Δ^8-THC at 36°C and 37°C, about 4 K lower than in the spectra of drug-free DPPC. In contrast, the spectra of DPPC + Δ^8-THC during the phase transition do not show the co-existence of the gel and liquid crystalline components. Instead, the gel phase lineshape gradually narrows, sharpens and transforms into the liquid crys-

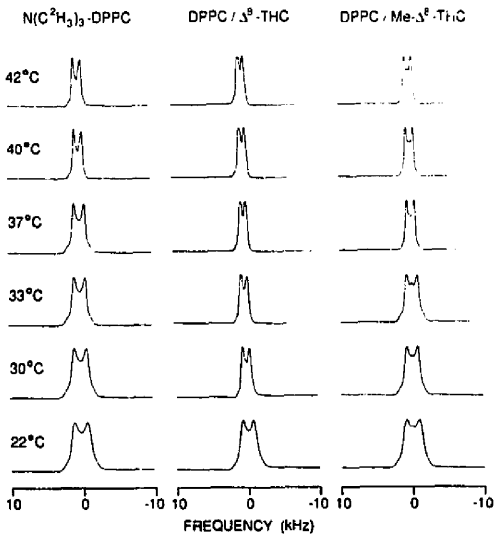

Fig. 9. Temperature dependence of solid state ^{2}H-NMR spectra due to $N(C^2H_3)_3$ segment of DPPC bilayer preparations in the absence and presence of Δ^8-THC ($x = 0.20$) and Me-Δ^8-THC ($x = 0.20$).

talline pattern. This phenomenon can be compared with what we observe in the spectra from 1,2[2′,2′-$^2H_2$]-DPPC and 2[7′,7′-$^2H_2$]-DPPC containing Δ^8-THC. At intermediate temepratures between the gel and liquid crystalline phases, Δ^8-THC-containing preparations of DPPC with different labels in the acyl chains give rise to unusual spectra which differ from those obtained from the DPPC or DPPC + Me-Δ^8-THC preparations. The spectral lineshapes observed here resemble those obtained from ^{2}H-NMR lineshape simulations in which the chains had intermediate rates of both axial hopping ($\approx 10^5$ s^{-1}) and *trans-gauche* isomerization ($\approx 10^4$ s^{-1}) on the ^{2}H-NMR time scale [17,26] or rapid torsional oscilations ($\approx 10^{11}$ s^{-1}) about the chain axis [31].

Spectra from $N(C^2H_3)_3$-DPPC preparations. Shown in Fig. 9 is a set of representative solid state ^{2}H-NMR spectra due to ^{2}H-labels at the choline trimethylammonium group in fully hydrated bilayer preparations of pure DPPC, DPPC + Δ^8-THC ($x = 0.20$) and DPPC + Me-Δ^8-THC ($x = 0.20$) at temperatures from 22°C to 42°C. The nine deuterons are magnetically equivalent and give rise to a single powder pattern with a very narrow quadrupolar splitting, reflecting extensive averaging from the three-site hops of the trimethyl groups and the reorientation of the choline group about the C–N bond as studied previously [32]. In Fig. 9, the spectrum from the preparation of the drug-free DPPC at 22°C shows a lineshape which consists of a doublet with rounded 90°- and 0°-edges. When the temperature is raised to 37°C, the spectrum undergoes gradual narrowing and sharpening. The center of the spectrum becomes progressively deeper and the 90°-edges appear more vertical. At temperatures between 37°C and 40°C, the spectrum turns abruptly into a very sharp Pake pattern accompanied with a sudden narrowing of the quadrupolar splitting, as measured from the separation between the two 90°-edge peaks in the spectra. Further increase in temperature has very little effect on the spectral shape except that the value of the quadrupolar splitting becomes gradually smaller.

The second column in Fig. 9 shows the temperature dependence of spectra of DPPC + Δ^8-THC. The spectrum at 20°C has generally the same shape as the one from drug-free DPPC but the quadrupolar splitting corresponds to the one observed in drug-free DPPC bilayer preparation at the much higher temperature of 36°C. This is an indication of the disordering effect of Δ^8-THC on the lipid thus affecting the cooperativity of the bilayer in the gel phase. This disordering effect of Δ^8-THC is accompanied by the lowering and broadening of the phase transition temperature of the bilayer, as evidenced by the more gradual decrease of $\Delta\nu_Q$ at about 30°C and in agreement with the broader thermal transition in our DSC results. Conversely, incorporation of the inactive cannabinoid Me-Δ^8-THC in the bilayer produces only small changes in the corresponding spectra, shown in the third column of Fig. 9. In the temperature range from 18°C to 33°C, not only are the spectral shapes of DPPC + Me-Δ^8-THC almost identical to those of pure DPPC but also the temperature dependence of $\Delta\nu_Q$ is virtually identical to that of pure DPPC. In the presence of Me-Δ^8-THC, the abrupt decrease of $\Delta\nu_Q$ occurs between 33°C and 37°C, about 3 K lower than in the case of drug-free DPPC bilayer, and the $\Delta\nu_Q$ values from DPPC + Me-Δ^8-THC are generally smaller by 0.1 kHz than those of DPPC at the same temperatures in both the gel and the liquid crystalline phases.

The above qualitative comparisons between the ^{2}H-NMR spectral shapes from the four sets of DPPC bilayer preparations with deuterium labels in different parts of the lipid molecule clearly indicate that the active cannabinoid Δ^8-THC induces significant changes in the dynamic and conformational properties of the bilayer, whereas the inactive analog Me-Δ^8-THC lacks this ability.

Changes in quadrupolar splittings and transition temperatures. Unlike DSC or X-ray methods which are suitable for recognizing disordering in the bulk phase, solid state ^{2}H-NMR has the distinctive advantage of detecting local perturbations through the use of specific deuterium labels as 'native' probes. The $\Delta\nu_Q$ data from our experiments indicate that the dynamic changes induced by Δ^8-THC are not uniform throughout the different sites of the bilayer. Table I lists the $\Delta\nu_Q$

TABLE I

Quadrupolar splitting values at 45°C and transition temperature values measured from solid state ^{2}H-NMR spectra of four sets of ^{2}H-labelled DPPC preparations in the absence and presence of Δ^8-THC and Me-Δ^8-THC

A = DPPC, B = DPPC + Δ^8-THC, and C = DPPC + Me-Δ^8-THC.

^{2}H-label positions	Quadrupolar splitting $\Delta\nu_Q$ (kHz)			Transition temperature (°C)		
	A	B	C	A	B	C
N(C^2H_3)$_3$	0.98	0.70	0.91	39	29	35
1[2′,2′-$^2H_2$]	26.14	27.06	25.74	41	37	37
2[2′*S*-^{2}H]	11.93	14.09	12.21	41	37	37
2[2′*R*-^{2}H]	17.37	18.91	17.23	41	37	37
2[7′,7′-$^2H_2$]	26.85	28.40	27.83	41	37	37
1,2[16′,16′,16′-$^2H_3$]	2.74	2.34	2.63	41	37	37

values from the solid state ^{2}H-NMR spectra of those four sets of differently deuterated DPPC bilayer preparations at 45°C, a temperature at which all the preparations exist in the liquid crystalline phase. The presence of Δ^8-THC in the bilayer decreases the $\Delta\nu_Q$ values from the choline trimethylammonium head group and terminal methyl groups of the acyl chains by 0.28 kHz (30%) and 0.40 kHz (15%), respectively. This spectral narrowing is an indication of increased motional averaging at the head group and the bilayer center resulting from the presence of Δ^8-THC. However, Δ^8-THC has an opposite effect on the $\Delta\nu_Q$ values due to the 1,2[2′,2′-$^2H_2$] and 2[7′,7′-$^2H_2$] segments of DPPC. The drug increases the $\Delta\nu_Q$ value of the *sn*-1 α-methylene deuterons by $\approx$ 1 kHz and those of the *sn*-2 chain by 2 kHz. This increase in $\Delta\nu_Q$ could result from a combination of motional restrictions and orientational changes in the 2′-methylene segments. In this particular case, the possibility of any significant orientational change is unlikely because of the following argument. For the two deuterons of the *sn*-1 chain, the angle (θ) between each of the C–^{2}H bonds and the chain axis is approximately 90°. Changes in chain orientation and consequent changes in the C–^{2}H bond angles would therefore be expected to result in a lowering of the $\Delta\nu_Q$ values. Conversely, for the two deuterons of the *sn*-2 chain, the θ angles (76° and 45°) are on the opposite sides of 54.7°, the magic angle. Any changes in bond orientations would then increase one $\Delta\nu_Q$ and decrease the other. The observation that both $\Delta\nu_Q$ values increase by the same amount in the presence of Δ^8-THC favors the interpretation of motional restrictions on the 2′-methylene segments rather than one involving orientational changes. The motion is presumably restricted because Δ^8-THC interacts with this part of the bilayer. This confirms the conclusion based on our X-ray diffraction experiments that Δ^8-THC resides near the interface of the bilayer [14]. It also provides supporting evidence to the speculation that Δ^8-THC orients in the bilayer by anchoring itself at one of the carbonyl groups through hydrogen bonding [16]. We notice that the increase in $\Delta\nu_Q$ value is more significant with the two spectral components due to the *sn*-2 chain. This effect may be interpreted to indicate a closer interaction of the drug molecule with the *sn*-2 chain than with the *sn*-1 chain. Our results also show that Δ^8-THC produces an increase in the $\Delta\nu_Q$ value of the spectra due to the 2[7′,7′-$^2H_2$] segment of DPPC. This increase of 1.5 kHz can be explained by our drug-membrane model according to which the tricyclic ring system of Δ^8-THC is located between the C-2′ and C-7′ methylene segments of the lipid acyl chains and thus imposes a motional restriction on the upper part of those chains. The outcome of this motional restriction is the increased *trans:gauche* conformer ratio in these chain segments giving rise to the increased $\Delta\nu_Q$ values. In summary, the observation of decreased $\Delta\nu_Q$ values from deuterium labels in the head group and center of bilayer and increased $\Delta\nu_Q$ values from those in the upper part of the acyl chains provides supporting evidence that Δ^8-THC is located in the region between C-2′ and C-7′ in the bilayer.

Compared with Δ^8-THC, the biologically inactive analog Me-Δ^8-THC has only small effects on the $\Delta\nu_Q$ values from ^{2}H-labels at the four sites on the DPPC molecule. The most prominent of these changes is in the spectra due to the 7′-methylene group of DPPC where an increase of approximately 1 kHz is observed. This result is consistent with our findings from small angle X-ray diffraction experiments [33] which indicate that the preferred average location of Me-Δ^8-THC is between the C-7′ methylene and the terminal methyl groups in the bilayer.

The sets of ^{2}H-NMR spectra from each preparation at different temperatures allowed us to identify the transition temperature at which the bilayer first shows spectra possessing liquid crystalline features (Figs. 6–9). These include a narrowing in the breadth of the spectrum and a sharpening of the 90°-edges. Because the different spectral sets are obtained from ^{2}H-labels in different sites of the DPPC bilayer, the temperatures shown in Table I represent 'local' rather than 'bulk' transition temperatures. Comparisons of the temperature effects on each of the differently labeled sites in the bilayer in the presence and absence of cannabinoid analogs allow us to gain insights into the molecular features of the bilayer phase transition and the effects of the drug molecules on different regions of the bilayer. In all of the DPPC preparations, the three labels in the acyl chains show identical phase transition temperatures that can be associated with the melting of the chains. Conversely, the trimethylammonium label shows a different phase transition profile, which

describes the conformational and/or cooperativity changes at the phospholipid head group. For example, the drug-free DPPC preparation (A) shows a transition temperature of 39°C in the head group and 41°C in the chain region. When Δ^8-THC ($x = 0.20$) is present in the bilayer, the transition temperature in the acyl chains decreases by 4 K while that in the head group decreases by 10 K. It thus appears that the presence of the biologically active Δ^8-THC in the bilayer results in a further separation of the two phase transition events (8 K), an effect which can be ascribed to the amphipathic interaction between the cannabinoid and the membrane. Our results here confirm earlier data from our laboratory in which solid state NMR was used with ^{13}C- and ^2H-labeled DPPC to study spectral changes as functions of temperature and concentration of Δ^9-THC [17].

The corresponding results from DPPC preparations containing Me-Δ^8-THC, a molecule which does not have the OH group of Δ^8-THC, show that all phase transition temperatures are uniformly lowered by 4 K. However, the head group phase transition is only 2 K lower than that of the acyl chains so that, in this regard, this membrane preparation with the inactive cannabinoid resembles that from pure DPPC.

The above is also consistent with the DSC results where the thermogram of DPPC + Δ^8-THC ($x = 0.20$) shows an endothermic peak at 29°C superimposed on a broad peak centered at 37°C (Fig. 2). Interestingly, these two temperatures correspond with the transition temperatures observed in the solid state ^2H-NMR spectra (Table I) and it is tempting to associate the peak at 29°C with the phase transition at the bilayer head group and the peak at 37°C with the transition due to the melting of the chains. Conversely, the thermogram of DPPC + Me-Δ^8-THC ($x = 0.20$) does not show significant additional broadening when compared to that from pure DPPC (Fig. 3), while the endothermic peak appearing at 38°C is very near the phase transition temperature observed in the corresponding NMR spectra.

Conclusions

Comparative studies using a combination of differential scanning calorimetry, X-ray diffraction and solid state ^2H-NMR have provided evidence, at the molecular level, for the requirement of a phenolic hydroxyl group in interactions of cannabinoids with membranes. Our DSC thermograms show the striking differences between the manner in which Δ^8-THC and Me-Δ^8-THC affect the thermotropic properties of DPPC model membrane. Δ^8-THC, an amphipathic molecule with a polar phenolic hydroxyl group, reduces the cooperativity between the phospholipid molecules in the bilayer. This disordering effect is almost entirely absent when the hydroxyl is substituted with the much less hydrophilic *O*-methyl group. Our X-ray diffraction experiments have shown that Δ^8-THC induces structural changes in the membrane especially when the bilayer undergoes the gel to liquid crystalline phase transition, whereas Me-Δ^8-THC produces no changes in the temperature dependence of *d*-spacing except for increasing the the *d*-spacing uniformly by 2 to 3 Å.

Comparisons between the solid state ^2H-NMR spectra from hydrated drug-free and drug-containing DPPC preparations demonstrate that Δ^8-THC dramatically changes the spectral lineshapes of DPPC while Me-Δ^8-THC has only marginal effects on the spectra. Unusual lineshapes in the DPPC/Δ^8-THC spectra indicate an exchange at intermediate rates and are congruent with a drug–membrane interaction in which the cannabinoid affects the dynamic properties of the bilayer by anchoring at the interface through the phenolic hydroxyl. Comparisons of the quadrupolar splitting values at temperatures above the phase transition provide evidence that, while the presence of Δ^8-THC generally induces motional disordering in the membrane lipid, it restricts the motion of the methylene segments from C-2′ to C-7′, a site which corresponds to the average preferred location of the drug in the bilayer. The effect of Me-Δ^8-THC on the quadrupolar splitting values is marginal and the location of Me-Δ^8-THC seems to be between C-7′ and C-16′.

The transition temperature values obtained from solid state ^2H-NMR spectra due to different deuterated sites on DPPC molecules show that the polar head group and the hydrophobic acyl chains undergo distinctive phase transitions. The conformational change in the head group precedes the chain melting temperature by 2 K in both drug-free and Me-Δ^8-THC-containing ($x = 0.20$) DPPC preparations. On the other hand, the amphipathic interactions between Δ^8-THC and the membrane interface separate these two events further apart in temperature. The ^2H-NMR data can be used to tentatively assign the endothermic peaks in the thermograms of DPPC + Δ^8-THC.

Δ^8-THC and its methyl ether are weakly polar molecules and our data show that they both fit in the membrane bilayer. Because of its phenolic hydroxyl, Δ^8-THC anchors itself at the membrane interface, presumably through bonding with the phospholipid ether groups or with water molecules present at the interface. In this location the drug molecule can induce bilayer perturbations most effectively. Coversely, Me-Δ^8-THC locates itself deeper in the bilayer away from the interface, a property which acounts for its decreased ability to perturb the membrane.

At higher concentrations ($x > 0.05$), Me-Δ^8-THC appears to phase-separate in the interior of the bilayer as 'micro-droplets' where it has no significant effect on the dynamic and phase properties of the model mem-

brane. There may, thus, be regions where the bilayer has expanded to accommodate these phase-separated pools of Me-Δ^8-THC. This interpretation is congruent with our X-ray measurements which show small increase in the average thickness of the bilayer. It also accounts for the lack of any significant thermotropic effects by this molecule at concentrations above $x = 0.05$.

Each of the three methods used in this study has provided information on a different aspect of the interaction of the two cannabinoid analogs with membranes. In combination, the data provide a clear picture of these interactions at the molecular level. The presence of the highly polar phenolic group in the cannabinoid interacting with one or more corresponding polar groups at the bilayer interface is the pivotal event involved in membrane perturbation.

It is very likely that the molecular principles involved in the amphipathic interactions of cannabinoids with membranes apply to similar interactions involving other classes of membrane-active drugs or other endogeneous molecules such as hormones and neurotransmitters. Preliminary studies in our laboratory using purified synaptosomal plasma membranes [34] and bilayer preparations of total lipid extracts obtained from the same membranes confirm that, generally, the molecular features of the interactions of cannabinoids with model membranes also apply to biological membranes.

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