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Solid State ^{2}H-NMR as a Method for Determining the Orientation of Cannabinoid Analogs in Membranes

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YANG, D.-P., A. BANIJAMALI, A. CHARALAMBOUS, G. MARCINIAK AND A. MAKRIYANNIS. *Solid state ^{2}H-NMR as a method for determining the orientation of cannabinoid analogs in membranes.* PHARMACOL BIOCHEM BEHAV **40**(3) 553–557, 1991.—In order to investigate the correlation between the pharmacological activities of cannabinoids and the geometric features of their interactions with membranes, we have calculated the molecular orientations of five analogs in model membrane bilayers. The studies involved the stereospecific ^{2}H-labeling of each analog in different positions and the use of solid state ^{2}H-NMR. The cannabinoids included in our study are (−)-Δ^9-tetrahydrocannabinol (THC), (−)-Δ^8-THC and its methylated ether analog (−)-O-methyl-Δ^8-THC, as well as two hexahydrocannabinols (HHC) having an additional hydroxyl in the 11-position, (−)-11-OH-9α-HHC and (−)-11-OH-9β-HHC. A new algorithm is used to circumvent the problem of deuterium quadrupolar splitting signs. The method has general applicability for calculating the orientation of a molecule in a anisotropic environment. Our calculations show that the biological inactive O-methyl-Δ^8-THC orients with its long axis parallel to the lipid acyl chains, whereas the psychoactive cannabinoids assume "awkward" orientations in which the hydroxyl groups are pointing towards the bilayer interface, presumably to maximize the amphipathic interaction with the membrane. To produce their biological effects, cannabinoids may need to acquire an appropriate location and orientation in the membrane bilayer so that, through lateral diffusion, they can reach their sites of action and interact productively with these sites.

Solid state NMR Deuterium NMR Cannabinoid Tetrahydrocannabinol Hexahydrocannabinol Molecular orientation Drug-membrane interactions

CANNABINOIDS have a wide range of pharmacological effects including psychotropic activity, bronchodilation, increased heart rate, reduced intraocular pressure and analgesia. Existing evidence has related these effects, at least in part, to their interactions with those lipids that make up the environment of membrane-associated proteins (5). These interactions are governed by strict stereoelectronic requirements and small changes in drug structure can result in dramatic changes in biological activity. In the membrane, each cannabinoid has a specific geometric preference which favors a particular kind of interaction with the lipids and through lateral diffusion it may reach and bind to a protein receptor if it has the proper orientation (4). Solid state ^{2}H-NMR spectroscopy has been used to provide data for the calculation of orientations of some symmetric or pseudosymmetric molecules in the membrane using a method in which ratios of experimental and calculated quadrupolar splittings are compared (3,10). However, for cannabinoids, because of their asymmetric molecular nature, a definitive determination of their orientations in the membrane requires a more rigorous formulation. For this purpose, we have developed an algorithm which involves a complete tensor analysis and a self-consistent screening process, and applied this approach in the determination of the orientation of (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) in dipalmitoylphosphatidylcholine (DPPC) model membranes (6). The experimental part of this work required specific deuteration of the cannabinoid molecules at six different positions on the rigid tricyclic ring system and obtaining ^{2}H-NMR spectra from semi-solid samples of hydrated bilayer preparations. The analytical part of the work involved extending the theoretical framework and devising a computer program that implements the new algorithm. The new scheme overcomes the difficulties in the determination of the quadrupolar splitting signs which are not available directly from experiments. This method has now been applied to a pair of cannabinoid analogs, namely the pharmacologically active (−)-Δ^8-tetrahydrocannabinol (Δ^8-THC) and the inactive (−)-O-methyl-Δ^8-tetrahydrocannabinol (Me-Δ^8-THC). We have also obtained preliminary results on the orientations of two hexahydrocannabinols (HHC) possessing two hydroxyl groups, namely, 11-OH-

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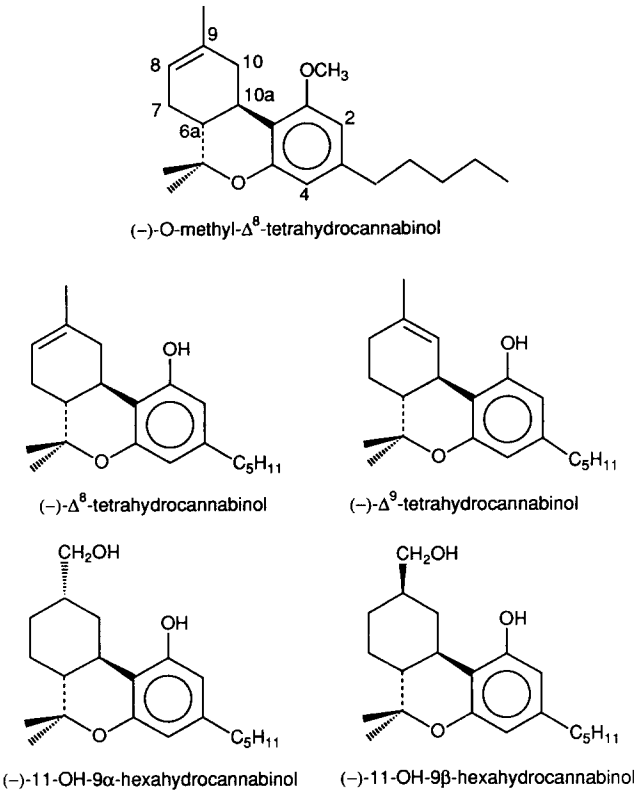

FIG. 1. Chemical structures of cannabinoids; (−)-Me-Δ^8-THC, (−)-Δ^8-THC, (−)-Δ^9-THC, (−)-11-OH-9α-HHC and (−)-11-OH-9β-HHC.

9α-HHC and 11-OH-9β-HHC. The above five molecules (all shown in Fig. 1) fall into three different groups of classical cannabinoids: (a) Me-Δ^8-THC has no free hydroxyl group; (b) each of Δ^8-THC and Δ^9-THC has one free phenolic hydroxyl group; and (c) each of 11-OH-9α-HHC and 11-OH-9β-HHC has two free hydroxyl groups. We felt that the determination of the different orientations of these molecules in the membrane may allow us to gain an insight into the molecular mechanisms of action of cannabinoids. Since the analogs used in this study show a wide spectrum of biological activities, the data on their orientations in the membrane could also provide useful information on the structure activity relationships of cannabinoids.

METHOD

Sample Preparation and NMR Experiments

Deuterium-labeled Me-Δ^8-THC, Δ^8-THC, Δ^9-THC, 11-OH-9α-HHC and 11-OH-9β-HHC were synthesized in our laboratory (1,7). For the THC compounds, a total of six ^{2}H-labels were placed at the 2, 4, 8, 10 and 10a positions on the rigid tricyclic ring system. The HHC compounds were ^{2}H-labeled at the 2, 4 and 9 positions. DPPC was obtained from Sigma Chemical Co. (St. Louis, MO).

For the samples of solid state NMR experiments, DPPC (150 mg) and an appropriate amount of cannabinoid were dissolved in chloroform (70/30 DPPC/cannabinoid molar ratio). The solvent was evaporated by first passing a stream of nitrogen over the solution at 50°C and then placing the residue under vacuum (0.1 mmHg) for 12 h. The DPPC/cannabinoid mixture was subsequently transferred into a 7-mm glass tube and 150 mg of ^{2}H-depleted water was added. The glass tube was sealed under vacuum while the preparation was submerged in liquid nitrogen. Before recording the spectra, the samples were equilibrated at 50°C for 15 min.

^{2}H-NMR spectra were obtained on a home-built solid state pulse spectrometer operating at 8.456T (55.2 MHz for ^{2}H and 360 MHz for ^{1}H) using the quadrupole echo pulse sequence (2), which consists of a pair of 90°-phase-shifted π/2 pulses separated by a time $\tau(\approx 30\ \mu s)$, $(\pi/2)_x - \tau - (\pi/2)_y$.

Theoretical Aspects and Computational Methods

Solid state ^{2}H-NMR spectra are dominated by the nuclear electric quadrupole interaction which is generally anisotropic, i.e., orientation dependent. When a rigid structure with deuterium labels is incorporated in the uniaxial liquid crystalline model membrane, each ^{2}H-label results in a different residual quadrupolar splitting, $\Delta\nu_Q$. Each splitting can be expressed as

$$\Delta\upsilon_Q = \frac{3}{4} A_Q |S_{C-D}| \qquad (1)$$

where A_Q is the deuterium quadrupolar coupling constant (≈170 kHz) and $|S_{C\text{-}D}|$ is the absolute value of the C-D bond order parameter. Because of the symmetric nature of solid state ^{2}H-NMR spectra, the sign of $S_{C\text{-}D}$ cannot be determined directly from the experiments. The value of $S_{C\text{-}D}$ varies from one C-D bond to another because different C-D bonds have different orientations with respect to a director axis which is parallel to the lipid chains of the membrane bilayer. The rigid tricyclic structure of the cannabinoid undergoes certain fluctuational motions about this axis. The effect of this motion is described by a traceless, symmetric, second-order tensor, S, which has a maximum of five independent components. In a chosen Cartesian coordinate system attached to the rigid structure, the components of S are related to $S_{C\text{-}D}$ by (3,10).

$$S_{C-D} = \sum_{ij} S_{ij} \cos\alpha_i \cos\alpha_j, \quad i = x,y,z;\ j = x,y,z, \qquad (2)$$

where $\cos\alpha_x$ $\cos\alpha_y$ and $\cos\alpha_z$ are C-D bond direction cosines, which can be calculated from X-ray crystallographic data of the molecular structure. There are five independent components (S_{xx}, S_{xy}, S_{xz}, S_{yy}, S_{yz}) and they can be calculated, in principle, if we have five experimentally determined values of $S_{C\text{-}D}$. However the unavailability of the signs of $S_{C\text{-}D}$ complicates the analysis. To overcome this problem, we have used a sixth ^{2}H-label and developed the following self-consistent algorithm.

We start by choosing any five ^{2}H-labels out of six. Each $S_{C\text{-}D}$ can be either positive or negative, resulting in a total of 32 possible sign combinations for five $|S_{C\text{-}D}|$ values. All sign combinations are tried and, in each case, the tensor components S_{ij} are calculated. The solutions of S_{ij} are fed back into Eq. [2] to predict the sixth $S_{C\text{-}D}$, whose absolute value is then compared with the experimentally observed $|S_{C\text{-}D}|$. Those sign combinations that give small deviations are considered candidates and others are discarded. Next, we choose a different set of five $|S_{C\text{-}D}|$ values from the six observed ones and use the same screening method. This procedure can be followed repeatedly six times and the number of candidates is further reduced after every time. Finally, the actual sign combination stands out among the 32 possible ones, because it always predicts the correct values and

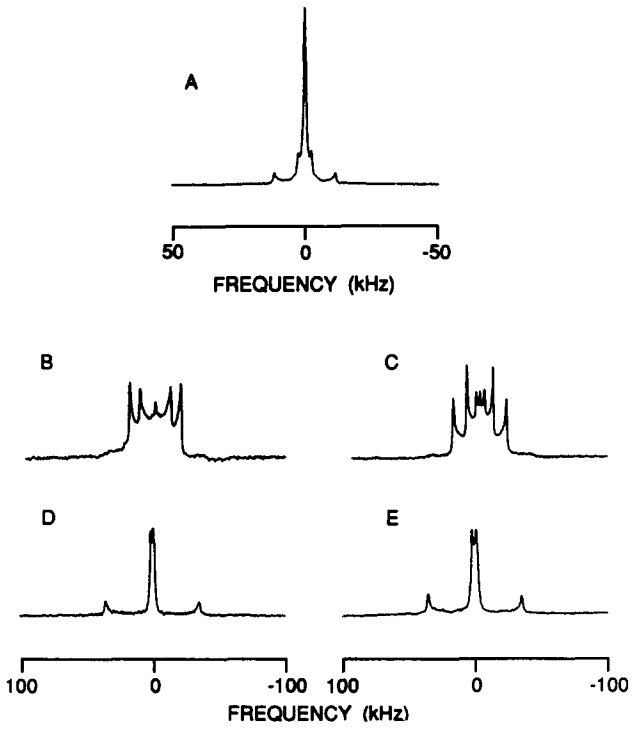

FIG. 2. Representative solid state ^{2}H-NMR spectra from DPPC model membranes (42°C) containing the five cannabinoids each having two deuterium labels at 2 and 4-positions: 2,4-d_2-Me-Δ^8-THC(A), 2,4-d_2-Δ^8-THC(B), 2,4,-d_2-Δ^9-THC(C), 2,4-d_2-11-OH-9α-HHC(D) and 2,4-d_2-11-OH-9β-HHC(E).

signs of the six $\Delta\nu_Q$ as well as consistent S_{ij} values in all six rounds of calculations.

The matrix containing the tensor components S_{ij} is then diagonalized using the standard eigenvalue analysis, which gives the principal components S_{XX}, S_{YY} and S_{ZZ}, the asymmetry parameter $\eta = (S_{YY} - S_{XX})/S_{ZZ}$, and the Euler angles β and γ. These two

TABLE 1

OBSERVED QUADRUPOLAR SPLITTING VALUES ($\Delta\nu_Q$, IN kHz) FOR SIX ^{2}H-LABELED POSITIONS OF THE THC COMPOUNDS AND THREE POSITIONS OF THE HHC COMPOUNDS [THE QUADRUPOLAR COUPLING CONSTANTS (A_Q, IN kHz) ARE ALSO LISTED]

		$\Delta\nu_Q$				
Label	A_Q	Me-Δ^8-THC	Δ^8-THC	Δ^9-THC	11-OH-9α-HHC	11-OH-9β-HHC
2	180	4.9	23.0	20.8	2.5	3.5
4	180	22.8	37.6	43.0	70.7	70.4
8	175	9.3	5.5	—	—	—
8α	170	—	—	5.1	—	—
8β	170	—	—	12.2	—	—
9α	170	—	—	—	—	1.95
9β	170	—	—	—	21.1	—
10	175	—	—	16.7	—	—
10α	170	5.4	33.8	—	—	—
10β	170	16.9	19.0	—	—	—
10a	170	17.9	16.4	14.9	—	—

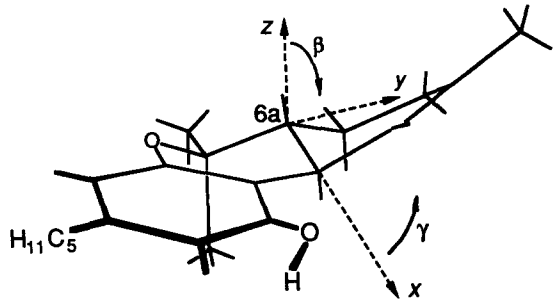

FIG. 3. The molecular-fixed axis system x, y and z with the definition of the Euler angles β and γ for the orientation of the motional director.

angles indicate the orientation of the principal axes X, Y, and Z with respect to the molecular-fixed axes x, y and z. The principal Z-axis represents the direction of the largest principal component and thus the most ordered axis. The principle component S_{ZZ} is commonly referred to as the molecular order parameter S_{mol}. The Z-axis is the director of the fluctuational motion and coincides with the normal of the bilayer plane. Therefore, the orientation of the drug in membrane is determined by positioning the molecule in the bilayer such that the principal Z-axis is parallel to the lipid acyl chains.

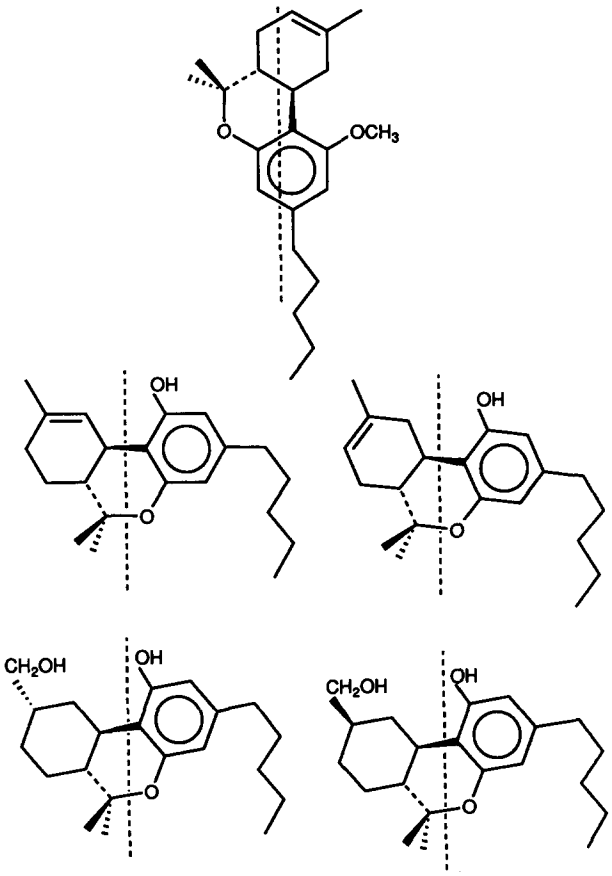

FIG. 4. Orientations of Me-Δ^8-THC (top), Δ^8-THC and Δ^9-THC (middle), 11-OH-9α-HHC and 11-OH-9β-HHC (bottom) in hydrated DPPC bilayers. The dashed lines represent the direction of the lipid acyl chains.

TABLE 2

RESULTS OF SELF-CONSISTENT CALCULATIONS FOR THE THC COMPOUNDS, INCLUDING $S_{C\text{-}D}$ USED IN INPUT, VALUES OF $S_{C\text{-}D}$ PREDICTED, AND CALCULATED ORDER PARAMETER TENSOR COMPONENTS IN TWO COORDINATE SYSTEMS AS WELL AS THE EULER ANGLES

		Δ^9-THC		Δ^8-THC		Me-Δ^8-THC	
$S_{C\text{-}D}$ used		2	+0.154	2	−0.170	2	−0.037
		4	+0.319	4	+0.279	4	−0.169
		8α	−0.040	8	−0.042	8	−0.071
		8β	+0.096	10α	+0.265	10α	−0.043
		10	+0.127	10β	+0.149	10β	−0.132
		10a	−0.117	10a	+0.129	10a	+0.140
$S_{C\text{-}D}$ predicted		2	+0.156	2	−0.181	2	−0.039
		4	+0.333	4	+0.267	4	−0.172
		8α	−0.099	8	−0.062	8	−0.077
		8β	+0.091	10α	+0.281	10α	−0.038
		10	+0.125	10β	+0.203	10β	−0.117
		10a	−0.112	10a	+0.120	10a	+0.138
Average tensor components	S_{xx}	0.282		0.034		−0.008	
	S_{xy}	0.255		0.196		−0.086	
	S_{xz}	−0.090		−0.331		−0.069	
	S_{yy}	−0.014		0.123		−0.127	
	S_{yz}	−0.075		−0.359		0.358	
	S_{zz}	−0.268		−0.157		0.135	
	S_{XX}	−0.293		−0.475		−0.381	
	S_{YY}	−0.155		−0.120		−0.032	
	S_{ZZ}	0.448		0.595		0.412	
	η	0.307		0.597		0.845	
Euler angles	β	−80.8°		−57.0°		−37.9°	
	γ	30.3°		49.6°		−66.5°	

RESULTS AND DISCUSSION

Figure 2 shows representative solid state ^{2}H-NMR spectra from DPPC model membranes containing the five cannabinoids each having two deuterium labels at 2- and 4-positions, namely, 2,4-d_2-Me-Δ^8-THC, 2,4-d_2-Δ^8-THC, 2,4-d_2-Δ^9-THC, 2,4-d_2-11-OH-9α-HHC and 2,4-d_2-11-OH-9β-HHC. The quadrupolar splitting values $\Delta\nu_Q$ were measured from the first derivatives of the spectra and are listed in Table 1. The quadrupolar coupling constants (A_Q) were taken to be 170 kHz, 175 kHz and 180 kHz for sp^3, sp^2 and aromatic sp^2 C-D bonds, respectively (10).

The Cartesian coordinate axes associated with the rigid tricyclic system are shown in Fig. 3. For every cannabinoid molecule, the origin of the axes is chosen at C(6a). The *x*-axis is along the C(6a)–C(10a) bond and the *z*-axis is in the plane of atoms C(6a), C(10a) and H(6a). The definitions of the two Euler angles β and γ for the orientation of the principal *Z*-axis are also indicated in the figure. In the calculations, direction cosines for the six C-D bonds were derived from X-ray crystallographic data of (−)-Δ^9-tetrahydrocannabinolic acid B as a model for the atom positions in the tricyclic ring system (9).

Table 2 lists the results from calculations using the self-consistent algorithm for Me-Δ^8-THC, Δ^8-THC and Δ^9-THC. In the first part are experimentally measured $S_{C\text{-}D}$ values along with their signs. This particular sign combination, chosen from 32 possible combinations, is the one that gives the correct prediction and is consistent in all of the six rounds of calculations. The second part of the table contains the predicted $S_{C\text{-}D}$ values, each of which is calculated when five of the six $S_{C\text{-}D}$ values in the first part are used as input parameters. Note that they have exactly the same sign combinations as those listed in the first part of the table. The predicted $S_{C\text{-}D}$ values are very close to the experimentally observed ones, the deviation being generally within 0.02. The third part tabulates the order parameter tensor components S_{ij} in the molecular-fixed *x*-*y*-*z*-system and S_{XX}, S_{YY} and S_{ZZ} in the principal *X*-*Y*-*Z*-system, as well as the asymmetry parameter η. These are average values over the six rounds of calculations and represent the self-consistent solution to the linear equation system. The last part in the table lists the Euler angles which provide quantitative description of the orientation of the most ordered axis with respect to the molecular-fixed axes. From these β and γ values, we have determined the orientations of Me-Δ^8-THC, Δ^8-THC and Δ^9-THC in the membrane (Fig. 4).

Our calculations show that Δ^8-THC and Δ^9-THC have similar orientations in the bilayer, with their motional director nearly in the plane of the tricyclic ring system and almost at a right angle with respect to the long axis of the molecule. Such an orientation places the phenolic hydroxyl group of the cannabinoid at the bilayer water/hydrocarbon-core interface while the hydrophobic moiety extends into the lipid acyl chain region. This amphipathic cannabinoid-membrane interaction has a primary incentive to direct the polar and hydrophobic components of the drug molecule towards the respective sites in the amphipathic bilayer. In the cases of Δ^8-THC and Δ^9-THC, the phenolic OH group serves as an anchoring point, orienting these psychoactive analogs so that the long axis of their tricyclic components are almost orthogonal to the bilayer chains. On the other hand, the calculated results for Me-Δ^8-THC show that this bio-

TABLE 3

RESULTS OF RATIO METHOD CALCULATIONS FOR THE HHC COMPOUNDS, INCLUDING THE SIGNS OF VALUES OF $S_{C\text{-}D}$, MOLECULAR ORDER PARAMETER S_{ZZ} (or S_{mol}), AND THE CORRESPONDING EULER ANGLES

		11-OH-9α-HHC		11-OH-9β-HHC	
		2	−0.019	2	−0.026
$S_{C\text{-}D}$		4	+0.524	4	+0.521
		9β	+0.165	9α	−0.015
S_{ZZ} or S_{mol}			0.56		0.69
Euler	β		86°		−78°
angles	γ		49°		43°

logically inactive analog has an orientation in which the long axis of the tricyclic system is almost parallel with the lipid chain direction. Although Me-Δ^8-THC differs from Δ^8-THC only by a methyl ether group replacing the phenolic hydroxyl group, the two molecules assume very different orientations indicating that they undergo different interactions with the membrane bilayer.

The above results provide a possible explanation for the differences in biological activity between the two molecules and for the requirements of free phenolic hydroxyl as a cannabinoid pharmacophore. We can postulate that for Δ^8-THC to produce its physiological effect it first assumes a proper orientation in the bilayer which allows it to diffuse laterally in the membrane and interact productively with its active site on the cannabinoid receptor. In contrast, the different orientation of Me-Δ^8-THC may prohibit or drastically retard its interaction with the active site, resulting in a lack of activity. In an earlier work using small angle X-ray diffraction (8), we have shown that Δ^8-THC resides preferentially near the interface of the membrane bilayer. If the lateral diffusion mechanism is the main channel for Δ^8-THC to reach a receptor, then the location of this receptor site relative to the bilayer must be also near the interface.

Since the hydroxyl group seems to play a pivotal role in the orientation and activity of the cannabinoids, we extended our orientation calculations to 11-OH-9α-HHC and 11-OH-9β-HHC, two cannabinoid analogs each having two hydroxyl groups. At the present time, three deuterated positions on the rigid part of each molecule are available (see Table 1) and we have used the ratio method (3,10) to obtain preliminary results (see Table 3). The calculated orientations are shown in Fig. 4. As can be seen, the two molecules assume orientations similar to those of Δ^8-THC and Δ^9-THC in which both hydroxyl groups interact with the polar component of the bilayer.

CONCLUSIONS

We have shown that solid state ^{2}H-NMR can be used to determine the orientation of molecules in an anisotropic environment. To that effect, we have developed a self-consistent algorithm which finds the correct quadrupolar splitting signs not available from experiments alone. The calculation can determine the orientation of the molecule in the membrane with respect to the bilayer normal. The results reported here allow us to correlate the geometric preferences of cannabinoids in the membranes with their respective pharmacological properties.

The data show that Δ^9-THC and Δ^8-THC, each having one hydroxyl group, orient with their long axis perpendicular to the normal of the bilayer. This "awkward" orientation of the biologically active cannabinoids in the membrane presumably occurs in order to permit the phenolic hydroxyl group to direct itself towards the polar bilayer interface and maximize the amphipathic interaction. On the other hand, the biologically inactive analog Me-Δ^8-THC, devoid of the hydroxyl group, has a natural orientation with its long axis parallel to the lipid acyl chains. This points out the significance of the phenolic OH in determining the orientation of cannabinoids in the membrane. Our findings also show that cannabinoids with more than one hydroxyl group appear to orient in the membrane so that both OH groups participate in the amphipathic interaction.

Based on our data we can postulate a two-step mechanism for the interaction of a cannabinoid with its site(s) of action. First, the molecule partitions preferentially in the bilayer and assumes an orientation determined by the relative stereochemistry of its polar and nonpolar pharmacophores. The molecule then diffuses laterally in the bilayer to reach its active site. To engage in a productive interaction the cannabinoid must fulfill two requirements: (a) It must interact with the membrane in a manner which allows it to reach the active site in a favorable orientation and conformation; (b) It must possess the appropriate pharmacophores and stereochemistry for such an interaction.

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