Evidence of Intercalation of *trans*-Diethylstilbestrol and Its Methyl Ether Derivatives in Multibilayers of Egg Phosphatidylcholine by High-Power Deuterium Nuclear Magnetic Resonance (^{2}H-NMR) Spectroscopy

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The mode of incorporation of ^{2}H-labeled *trans*-diethylstilbestrol (DES) (1b) and its methyl ether derivatives (2a, 2b, and 3) into multibilayers of egg phosphatidylcholine was analyzed by means of deuterium nuclear magnetic resonance. A clear distinction was found between DES or its methyl ether derivatives incorporated into lipid bilayers and those precipitated in the aqueous phase, by taking into account the extent of the motionally averaged quadrupole interaction. Thus, it was found that the relative proportion of these compounds incorporated into multibilayers decreased in the following order: DES (1b) > DES monomethyl ether (2a and 2b) > DES dimethyl ether (3). In addition, we demonstrated that the mode of intercalation in the multibilayers differs greatly among these compounds.

Keywords lipid bilayer; multibilayer; ^{2}H-NMR; diethylstilbestrol, egg phosphatidylcholine; microtubule inhibitor; synthetic estrogen

trans-Diethylstilbestrol (DES) (**1a**) is a potent synthetic estrogen capable of eliciting biological responses similar to those induced by the endogenous 17β-estradiol.[1] In recent years, studies[2] in humans and mice have suggested that *in utero* exposure to **1a** induces cancerous lesions of the reproductive tract. The mechanism of these effects of **1a** is unknown, in spite of many studies covering metabolic activation,[3] deoxyribonucleic acid (DNA) binding,[4] radical formation[5] or DNA breaks.[6] We reported that **1a** and its derivatives inhibit microtubule assembly *in vitro*.[7] As a first step in evaluating the significance of such biological activities, it is essential to examine how DES and its analogues are transported through cell membranes. In this connection, it is known that DES inhibits proton adenosine triphosphatase (ATPase) in eukaryotic cells.[8] At high concentration, however, DES damages membrane bilayers and produces a nonspecific effect.[9] Nevertheless, no detailed analysis has been done as to how these chemicals are incorporated into lipid bilayers or cell membranes. Useful information about the mode of binding could be obtained by analysis of the deuterium nuclear magnetic resonance (^{2}H-NMR) spectral pattern of selectively deuterated substances incorporated into multibilayers of egg phosphatidylcholine or other synthetic multibilayers. This technique was successfully employed for local anesthetics,[10] bile acids,[11] tumor promoters[12] or antidepressant drugs.[13] In the present article, we present a ^{2}H-NMR study of selectively deuterated DES and its methyl ethers incorporated into multibilayers of egg phosphatidylcholine.

Results and Discussion

Syntheses of Selectively Deuterated DES, and Mono- and Dimethyl Ethers of DES (Chart 1) Deuterium-labeled DES was obtained from DES (**1a**) by the method of Liehr and Ballatore.[14] After three cycles of deuterium exchange reactions, deuterated DES (**1b**) was obtained in an isotopic purity as high as >98 atom% D as determined by proton NMR (^{1}H-NMR) analysis. One monomethyl ether derivative (**2a**) was prepared by methylation of **1b** with dimethyl sulfate. On the other hand, deuterated mono- and dimethyl ether derivatives (**2b** and **3**) were prepared by methylation of **1a** with dimethyl sulfate-d_6. The isotopic purities of **2b** and **3** were >98 atom% D.

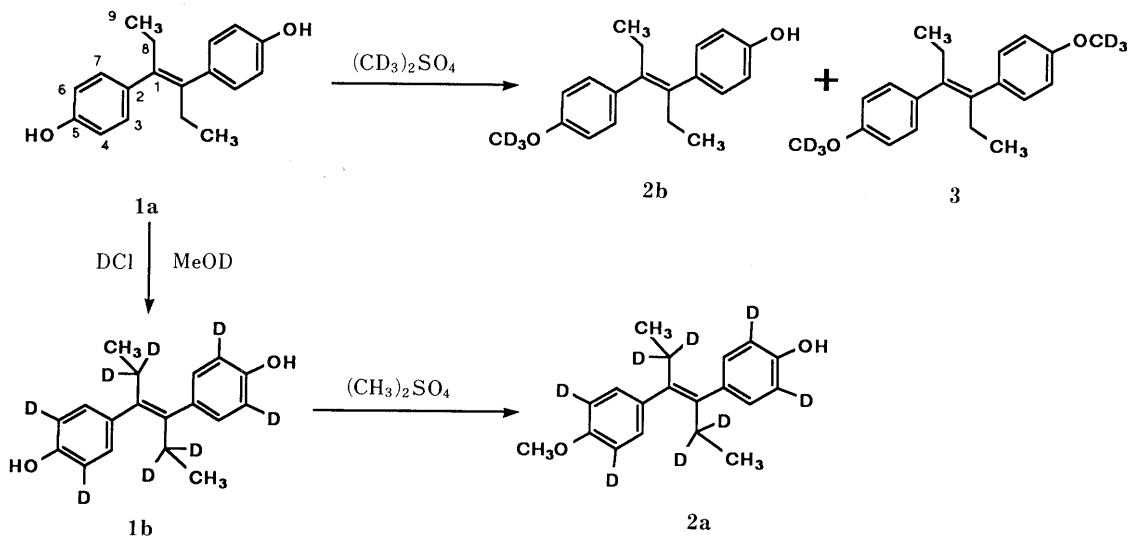

Chart 1

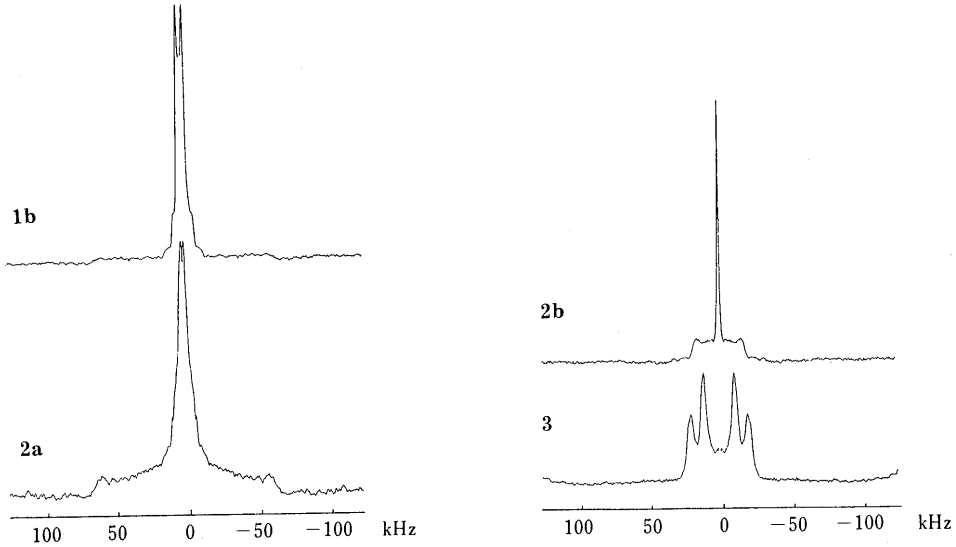

Fig. 1. The 46.06 MHz ^{2}H-NMR Spectra of **1b**, **2a**, **2b** and **3** in the Presence of Multibilayers of Egg PC

Intercalation of Deuterated DES and Deuterated Methyl Ether Derivatives with Multibilayers Figure 1 shows the ^{2}H-NMR spectra of deuterated DES (**1b**) and deuterated mono- and dimethyl ether derivatives (**2a**, **2b** and **3**) bound to the multibilayers of egg phosphatidylcholine (PC). Theoretically, the ^{2}H quadrupole splitting as defined by the splitting of peaks D_q is related to the angle of the principal axis of the electric field gradient tensor (C–^{2}H vector) with respect to the applied magnetic field.[15]

$$D_q=\frac{3}{4}\left(\frac{e^2qQ}{h}\right)(3\cos^2\theta-1) \quad (1)$$

where e^2qQ/h is the quadrupole coupling constant (170 kHz and 180 kHz for aliphatic and aromtic C–^{2}H, respectively). For rigid polycrystalline solids, all values of θ are possible and the resulting peak-splitting D_q in Eq. 1 results in

$$D_q=\frac{3}{4}\left(\frac{e^2qQ}{h}\right)\cong 135\,\text{kHz} \quad (2)$$

in the case of aromatic C–^{2}H. This value is reduced to 128 kHz in the case of aliphatic C–^{2}H but in many instances it is reduced to less than 40 kHz if rotation occurs in the solid as in a methyl group. Thus, the large peak-splittings in **1b** and **2a** which amount to about 130 kHz can definitely be ascribed to polycrystalline solids not incorporated into the multibilayers.[11] It is expected that the quadrupole splitting from ^{2}H-labelled DES or its derivatives would be reduced to some extent, if they are incorporated into the bilayers, depending on the anisotropic motions. Thus, we can estimate roughly the relative proportion of DES or its derivatives incorporated into the multibilayers by comparison of the relative peak-intensities of the broad and narrow components. In fact, **1b** and **2a** are quite well incorporated into the multibilayer, although the amount of the latter is higher than that of the former.

In the case of the methoxy group-deuterated derivatives (**2b** and **3**), maximal quadrupole splitting as large as 42 kHz is seen,[16] as observed in polycrystalline solids, since C_3 rotation of methyl groups occurs even in the solid state of **2b** and **3**. Thus, it is concluded that the proportions of these compounds in the multibilayers decrease in the following order: **1b**>**2a** and **2b**>**3**. This order can be easily understood because introduction of the methoxy group in DES results in a reduction of the amphiphilic character which is essential for incorporation of the molecule into lipid bilayers.

TABLE I. The Quadrupole Splittings of **1b**, **2a**, **2b**, and **3**

	Intercalated (kHz)		Precipitated (kHz)
1b	3.6	13.0	130
2a		3.0	130
2b	0.72	35.0	42
3		22.0	42

When DES or its derivatives undergo anisotropic motions in the multibilayers, the quadrupole splittings are reduced to

$$D_q=\frac{3}{4}\left(\frac{e^2qQ}{h}\right)\left(\frac{3\cos^2\alpha-1}{2}\right)\left(\frac{3\cos^2\beta-1}{2}\right) \quad (3)$$

where α and β stand for the angle of the C–^{2}H vector under consideration with respect to the director (the axis of fast rotational diffusion) and the angle between the director and the normal of the bilayers, respectively. The doublet pattern arises from ^{2}H-NMR signals of both methylene and aromatic deuteriums. The aromatic ring of **1b** is considered to be free to rotate about the axis connecting the C_2 and C_5 carbons, because the quadrupole splitting of the inner pair (see Table I) is less than the value of 17 kHz which was expected for this free rotation based on the Eq. 3.[17] It is likely, in the multibilayers, that this sort of internal motion could be coupled with an overall rotational diffusion about an axis connecting the two aromatic rings. Nevertheless, it is interesting that an additional plausible rotational motion of methylene groups cannot completely average out the quadrupole interaction of the methylene group.

The inner peaks of **2b** and **2a** are split by 0.72 and 3.0 kHz, respectively (see Table I). It is interesting to note that the quadrupolar interaction of the former is approximately one-third of the latter. Therefore, the presence of species with such smaller splitting can be ascribed to DES

highly solubilized by egg PC.

The quadrupole splitting of the wider component of **2b** is about 36 kHz, corresponding to 3/4 of 42 kHz, which indicates that the angle between the director of **2b** and the normal of the bilayer is about 0°. This type of orientation within the bilayer is reasonable, because the hydrophilic hydroxyl group is near the head groups of the lipid bilayers while the methoxyl end could be located in the hydrophobic interior. Interestingly, the quadrupole splitting of the inner doublet in **3** is 22 kHz, which corresponds to 1/2 of 42 kHz, and can be explained in terms of specific orientation of the director vector at 90° with respect to the bilayer normal on the basis of Eq. 3. This type of orientation is again plausible because there is no hydrophilic end in **3** and embedding within the interior of bilayers may be stabilized. It is emphasized that the outer component of the quadrupole coupling as large as 42 kHz can be ascribed to the precipitate of **3**.

In conclusion, a ^{2}H-NMR study allows one to analyze the mode of interaction of DES or its derivatives with lipid bilayers. In particular, it is possible to estimate the relative amount of drug not bound to the bilayers. This sort of experiment is not possible by measurements of radioactivity of isotope-labeled drugs. In addition, it is also possible to infer the relative orientation of the foreign moleceules within the bilayers.

Experimental

Apparatus All melting points were obtained on a Shimadzu MM2 micro-melting point apparatus, and are uncorrected. ^{1}H-NMR spectra were obtained at 270 MHz on a JEOL JNM-GX FT NMR spectrometer. All ^{1}H-NMR data were recorded in deuteroacetone and chemical shifts are reported as parts per million downfield from Me_4Si ($\delta=0$). Abbreviations used: s = singlet, d = doublet, br = broad, m = multiplet, dd = doublet of doublets, q = quartet. High-power ^{2}H-NMR spectra in the presence of lipid bilayers of deuterated DES and its derivatives were recorded on a Bruker CXP-300 spectrometer at 46.06 MHz. A quadrupole echo pulse sequence, $90°_{\pm x}$–t_1–$90°_y$–t_2–T, was used to preserve the inhomogeneously broadened portion of the ^{2}H-NMR signal, which is normally lost through spectrometer dead time, where T is the repetition time. The phase of the first 90° pulse was shifted by 180° on alternate scans to reduce artifacts due to coil ringing and imperfect 90° pulses. The 90° pulse was 6 μs long, and the repetition time was 1 s, with $t_1=t_2=50$ μs. The number of data points was 2K and the sweep width was 125 kHz × 2. Spectra were accumulated over 50000 times. Column chromatography was performed with Kanto Kagaku silica gel (100 mesh). Spots on thin layer chromatography (TLC) plates (precoated TLC plates, Silica Gel 60F-254, Merck) were visualized under ultraviolet (UV) light and/or by spraying with concentrated H_2SO_4 and heating on an electric heater.

Materials Egg PC was purchased from Seikagaku Kogyo Co., Ltd., Tokyo. Deuterium-depleted water was purchased from Aldrich Chemical Co., Inc., USA.

Preparation of Multibilayers of Egg PC The deuterated compound (0.1 mmol) dissolved in an acetone was added to egg PC solution (0.2 mmol), and the solvent was evaporated off under a nitrogen stream. Final traces were removed by placing the sample in a high vacuum. Lipid films thus obtained were fully hydrated with deuterium-depleted water. Freezing and thawing were repeated several times together with vortexing of the sample to achieve a homogeneous aqueous suspension of multibilayers.

Preparation of Deuterated DES (1b) and the Monomethyl Ether (2a) Deuterated DES (**1b**) was prepared with some modification by the method of Liehr and Ballatore.[14] DES (1.0 g) was dissolved in a mixture of 15 g of methanol-*d* (99.8 atom% D) and 6 g of deuterium chloride (20 wt, % solution in D_2O). The mixture was kept at 85 °C for 3 d in a sealed glass tube, then the solvent was removed under nitrogen gas. The above reaction was repeated twice more. Recrystallization of the product from MeOH gave [4,4′,6,6′,8,8′-$^2H_8$]DES (**1b**) as colorless needles, mp 173—174 °C. ^{1}H-NMR δ (ppm): 0.74 (6H, br s, 9,9′-CH_3), 7.05 (4H, br s, 3,3′,7,7′-H), 8.20 (2H, br s, 5,5′-OH). The monomethyl ether (**2a**) was prepared as described in the previous paper.[18] The product was recrystallized from MeOH as colorless needles, mp 116—117 °C. ^{1}H-NMR δ (ppm): 0.76 (6H, br s, 9,9′-CH_3), 3.82 (3H, s, 5-OCH_3), 7.06 (2H, br s, 3′,7′-H), 7.14 (2H, br s, 3,7-H), 8.25 (1H, br s, 5′-OH).

Preparation of [5-Methyl-$^2H_3$]mono- and [5,5′-Dimethyl-$^2H_6$]dimethyl Ether (2b and 3) [5-Methyl-$^2H_3$]mono- and [5,5′-dimethyl-$^2H_6$]dimethyl ether (**2b** and **3**) were prepared as described previously[18] except that dimethyl sulfate-d_6 was used as the reagent. 1) Recrystallization of the product from MeOH gave [5-methyl-$^2H_3$]5-monomethyl ether (**2**) as colorless needles, mp 115 °C. ^{1}H-NMR δ (ppm): 0.76 (6H, t, J = 7.3 Hz, 9,9′-CH_3), 2.16 (2H, q, J = 7.3 Hz, 8-H), 2.13 (2H, q, J = 7.3 Hz, 8′-H), 6.86 (2H, br d, J = 8.6 Hz, 4′,6′-H), 6.94 (2H, br d, J = 8.9 Hz, 4,6-H), 7.06 (2H, br d, J = 8.6 Hz, 3′,7′-H), 7.14 (2H, br d, J = 8.9 Hz, 3,7-H), 8.25 (1H, br s, 5′-OH). 2) Recrystallization of the product from MeOH gave [5,5′-dimethyl-$^2H_6$]dimethyl ether (**3**) as colorless needles, mp 123 °C. ^{1}H-NMR δ (ppm): 0.76 (6H, t, J = 7.3 Hz, 9,9′-CH_3), 2.15 (4H, q, J = 7.3 Hz, 8,8′-H), 6.94 (4H, br d, J = 8.9 Hz, 4,4′,6,6′-H), 7.15 (4H, br d, J = 8.9 Hz, 3,3′,7,7′-H).

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