

# Nuclear Magnetic Resonance Studies of 1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethene in Aqueous Phospholipid Dispersions†

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**ABSTRACT:** The proton magnetic resonance spectrum of the insecticide *p,p'*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene (II) has been recorded in dispersions of various phospholipids in D<sub>2</sub>O. The phospholipids used were DL- $\alpha$ -lecithin, egg-yolk lecithin, sphingomyelin, phosphatidylserine, and phosphatidylethanolamine. The ring proton spectrum of II showed changes in the line shape as a function of concentration of phospholipid and temperature of the solution. At higher concentrations of phospholipid or at higher temperatures a new peak low field to the original was observed. The appearance of a low-field peak of II has been interpreted in terms of distribution of II in the gel to the liquid crystalline phase of the phospholipid. The in-

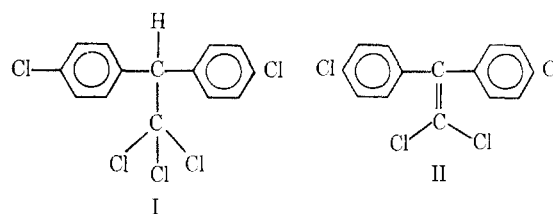
tensities of the two peaks have been used to calculate the partition coefficient as well as the enthalpy changes of the distribution process. Similar studies with 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (I) did not produce such concentration and temperature effects. I gave one set of resonance peaks corresponding to the crystalline phase. This behavior may be explained by the difference in the hydrophobic character as well as configuration of the two chemicals. Since II is the major metabolite of the insecticide I, these data may have significance in relation to the molecular basis of mode of action of such compounds.

The toxicity of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (I) and related compounds usually involves the central nervous system. Perturbations in axonic transmission are manifested by changes in action potential which in turn can be associated with changes in ion efflux, and it has been suggested that I is acting by influencing membrane permeability (Narahashi and Haas, 1968; Hille, 1968). The possibility of membrane involvement would be supported by the observation that I blocks the action of valinomycin on a phospholipid bilayer (Hilton and O'Brien, 1970).

Considering the hydrophobic character of these compounds some interaction with the membrane lipoprotein complex might be expected. Little information is available in this area other than the observations that I will bind to components of cockroach nerve (O'Brien and Matsumura, 1964). We have used nuclear magnetic resonance (nmr) techniques to study the binding of I and related compounds with lipids. In carbon tetrachloride these compounds will bind to lecithin and it is possible to relate binding characteristics to insecticidal activity (Tinsley *et al.*, 1971; Haque *et al.*, 1973).

Binding studies in carbon tetrachloride are of biological significance only if such observations can be extended to systems which approximate a biological environment more closely. To this end proton magnetic resonance (pmr) techniques have been used to explore the behavior of these compounds in aqueous dispersions of phospholipids.

Initial observations have been made with 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene (II) and I. II, the major metabolite of I distributed in the environment, is generally considered to be less toxic than I; however, it has been implicated as a contributing factor in the problem of eggshell thinning and the



decline of populations of raptorial birds (Cade *et al.*, 1971; Ratcliffe, 1970). With the distinctions in biological activity it was of interest to compare the behavior of these compounds in the liposome system.

Both compounds gave pmr spectra in the aqueous phospholipid dispersions. However, the spectra of II in contrast to that of the I showed substantial changes when the concentration of phospholipid or the temperature was varied. These changes are discussed with reference to configuration of the phospholipids. Under such conditions phospholipids form liquid crystals of smectic mesophase (Bangham *et al.*, 1965; Sessa and Weissmann, 1968; Hammes and Tallman, 1970), the structures of which depend upon concentration, temperature, and ionic strength of the medium. Sonication of these dispersions produces liposomes which consist of concentric phospholipid vesicles enclosing an aqueous compartment. These phospholipid bilayer structures possess membrane-like features and provide an excellent model for membrane studies (Papahadjopoulos and Miller, 1967; Papahadjopoulos and Watkins, 1967).

## Experimental Section

**Materials.** The phospholipids DL- $\alpha$ -lecithin ( $\beta$ , $\gamma$ -dipalmitoyl-DL- $\alpha$ -phosphatidylcholine) and dipalmitoylphosphatidylserine were obtained from Nutritional Biochemicals, while sphingomyelin, dipalmitoylphosphatidylethanolamine, and egg-yolk lecithin were the products of Sigma Chemical Co. The insecticide II was purchased from Aldrich Chemicals.

**Preparation Samples for Nmr Studies.** In view of the extremely low water solubility of II and phospholipids, their

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high-resolution nmr spectra as such in water or  $D_2O$  cannot be obtained. However, ultrasonicated dispersions of phospholipids in  $D_2O$  produce well-defined nmr spectra (Finer *et al.*, 1972). Various procedures were tried in an effort to increase the solubility of II in aqueous phospholipid dispersions. The following resulted in good, high-resolution nmr spectra for both II and the phospholipid.

A known quantity of phospholipid (20–200 mg) and 5 mg of II were dissolved in a test tube in  $CHCl_3$ . Chloroform was evaporated under vacuum and 1 ml of  $D_2O$  was added to the test tube. The sample was sonicated for 10 min on a Biosonik ultrasonicator at a setting of 30. This method produced good homogeneous solution. In some cases the sample was centrifuged to remove any insoluble particulate matter. This procedure has generally been used by other investigators to study the behavior and molecular interactions of phospholipids using nmr technique. The sample was transferred to an nmr tube and the spectrum was recorded on a Varian HA-100 nmr spectrometer, operating at 100 MHz, equipped with standard variable temperature accessories. Chemical shifts are reported with reference to an external tetramethylsilane lock standard.

## Results

The nmr spectrum of a II–phospholipid dispersion showed resonance peaks corresponding to both the compounds. The methylene and methyl phospholipid protons usually showed broad peaks, which became sharper as the temperature of the sample was raised. Choline methyl protons of lecithin and sphingomyelin have a relatively sharp peak. The line shape and the chemical shifts of various protons corresponding to the phospholipid under consideration were in agreement with the earlier findings (Finer *et al.*, 1972) and were not changed to any significant extent by the addition of II. This observation would be consistent with previous studies indicating II did not bind to lecithin (Haque *et al.*, 1973).

The line shape of ring protons of II, however, showed interesting changes. The spectrum of ring protons of II should theoretically correspond to an AA'BB'-type pattern (Keith *et al.*, 1969). In a nonpolar solvent it usually consists of a pair of doublets having chemical shifts of 7.12 and 7.25 ppm with a coupling constant of 9.0 Hz. In the phospholipid dispersions, the ring protons of II gave only a single broad peak. Apparently, this is due to the heterogeneous nature of the dispersion resulting in a decrease in the spin–spin relaxation time which in turn produces line broadening and collapse of the spin–spin splitting. In addition, as either the concentration of lipid was increased or the temperature of the sample was raised, a new peak appeared. This low-field peak was dominant at higher concentrations of lipid and higher temperatures whereas the reverse was true for the high-field peak. Conditions under which these two peaks were observed varied with the nature of the phospholipid.

In a  $D_2O$  dispersion of dipalmitoyllecithin (20 mg/ml), the II resonance spectrum showed only one peak ( $\delta \sim 6.82$  ppm). The line width of the peak was about 8–10 Hz. As the concentration of lecithin was increased a low-field peak ( $\delta \sim 7.16$  ppm) appeared (Figure 1) and increased in intensity with a concomitant decrease in the intensity of the high-field peak. Finally, when the concentration of the lecithin was increased to 150 mg/ml the high-field peak disappeared completely. The line width of both peaks showed no significant concentration dependence. The total separation between the two peaks was about  $35 \pm 2$  Hz and was independent of DL- $\alpha$ -lecithin concentration.

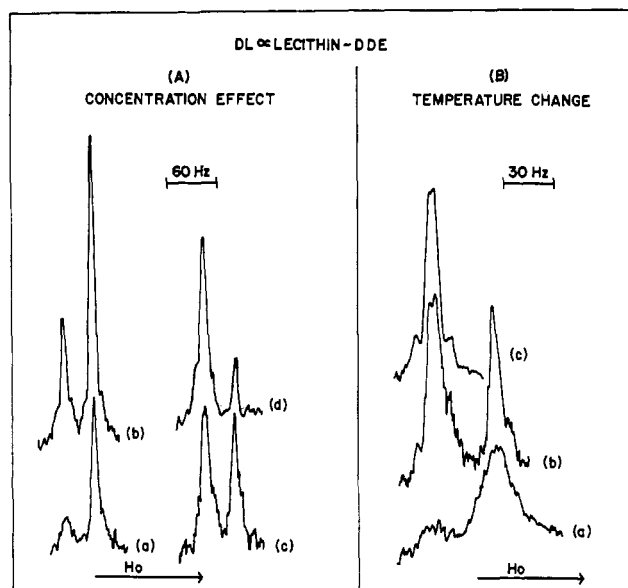


FIGURE 1: (A) Nmr spectra of II (5 mg/ml) ring protons in a  $D_2O$  dispersion in the presence of varying concentrations (mg/ml) of DL- $\alpha$ -lecithin: (a) 20; (b) 35; (c) 45; (d) 100, at room temperature. (B) Nmr spectra of II (5 mg/ml) ring protons in a DL- $\alpha$ -lecithin (45 mg/ml)- $D_2O$  dispersion at various temperatures: (a) 20°; (b) 35°; (c) 44°.

A concentration of 45 mg/ml of DL- $\alpha$ -lecithin was chosen for the study of temperature dependence since at this concentration the two peaks of II were of almost equal intensity. As the temperature of the sample was raised, the intensity of the low-field peak increased while the intensity of the high-field peak diminished and finally at 45° disappeared completely (Figure 2). The behavior of egg-yolk lecithin differed from that of dipalmitoyllecithin. II always showed one peak ( $\delta \sim 7.18$  ppm) probably corresponding to the low-field peak (Figure 1). Attempts to observe the high-field peak by lowering the temperature of the sample were unsuccessful. Apparently a decrease in temperature causes a substantial decrease in the solubility of II in the  $D_2O$ -lipid dispersion.

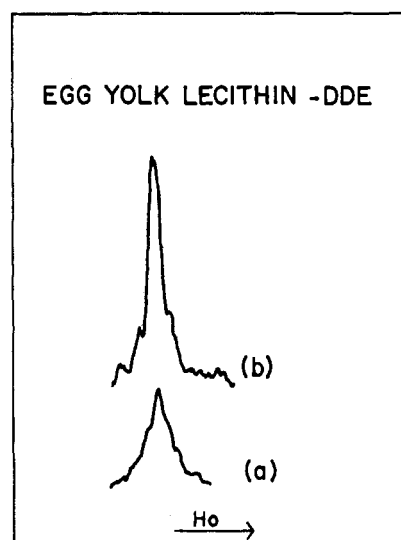


FIGURE 2: Nmr spectra of II (5 mg/ml) ring protons in egg-yolk lecithin- $D_2O$  dispersions. Egg-yolk lecithin concentration (mg/ml): (a) 20; (b) 50.

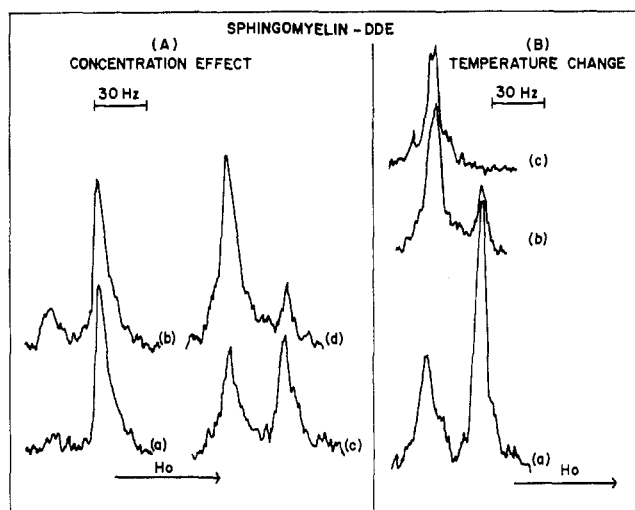


FIGURE 3: (A) Nmr spectra of II (5 mg/ml) ring protons in sphingomyelin- $D_2O$  dispersion at room temperature. Sphingomyelin concentration (mg/ml): (a) 25; (b) 50; (c) 100; (d) 200. (B) Nmr spectra of II (5 mg/ml) ring protons in sphingomyelin (50 mg/ml)- $D_2O$  dispersion at (a) 40°; (b) 60°; (c) 80°.

Sphingomyelin gave a response similar to the dipalmitoyllecithin (Figure 3). A single peak corresponding to the high-field resonance signal of II ( $\delta \sim 6.81$  ppm) was observed at a sphingomyelin concentration of 25 mg/ml. The low-field peak of II ( $\delta \sim 7.06$  ppm) appeared at 50 mg/ml and increased in intensity with further increases in the concentration of sphingomyelin. At 50 mg/ml the intensities of both peaks of II were comparable, and an increase in the temperature of the sample produced an increase in the intensity of the low-field peak. Finally, at 80° the low-field peak was dominant and the high-

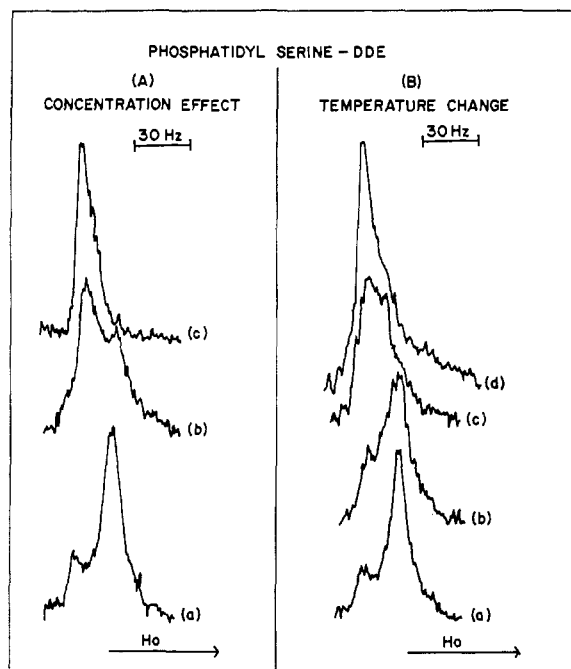


FIGURE 4: (A) Nmr spectra of II (5 mg/ml) ring protons in phosphatidylserine- $D_2O$  dispersion at room temperature. Serine concentration (mg/ml): (a) 20; (b) 35; (c) 50. (B) Nmr spectra of II (5 mg/ml) ring protons in phosphatidylserine (20 mg/ml)- $D_2O$  dispersion at: (a) 40°; (b) 60°; (c) 80°; (d) 90°.

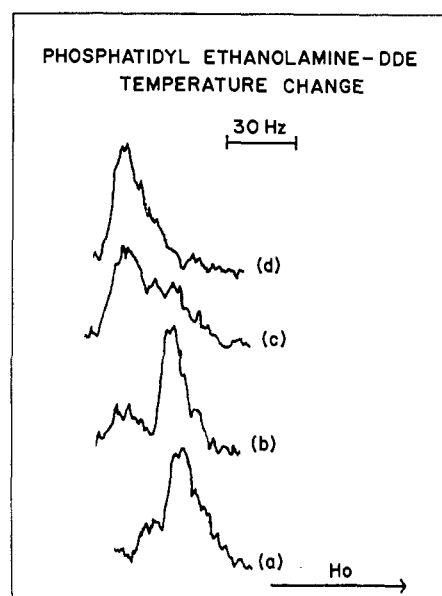


FIGURE 5: Nmr spectra of II (5 mg/ml) ring protons in phosphatidylethanolamine (32 mg/ml)- $D_2O$  dispersion at: (a) 31°; (b) 40°; (c) 50°; (d) 70°.

field peak disappeared. The separation between the two peaks was  $29 \pm 3$  Hz and was independent of sphingomyelin concentration and temperature.

With the cephalins (phosphatidylserine and phosphatidylethanolamine) the two peaks were poorly resolved and the chemical-shift differences between the two peaks were smaller. Higher temperatures were needed to observe the low-field peak. For phosphatidylserine, the two peaks were observed at a concentration of 20 mg/ml of the phospholipid. Again, with increasing concentration of lipid, the high-field peak ( $\delta \sim 6.93$  ppm) intensity diminished and the low-field peak ( $\delta \sim 7.0$  ppm) intensity increased, and finally at 100 mg/ml of phosphatidylserine, the high-field peak vanished (Figure 4). The separation between the two peaks was about  $17 \pm 2$  Hz. For a concentration of 25 mg/ml of phosphatidylserine a temperature of 90° was needed for the low-field peak to become dominant.

In the case of phosphatidylethanolamine, it was difficult to obtain a good homogeneous sample for nmr study. However, in the presence of 20 mg/ml of the lipid, one broad peak of II at 6.86 ppm was observed. Increasing the concentration of phosphatidylethanolamine did not produce a good nmr spectrum. Increasing the temperature of the 20-mg/ml lipid sample produced a new peak corresponding to the low-field peak and the intensity of this peak increased with a further increase in the temperature and finally at 70° the high-field peak disappeared and the low-field peak ( $\delta \sim 7.05$  ppm) was dominant (Figure 5). The separation between the two peaks was about 20 Hz.

## Discussion

The observation of two peaks corresponding to the ring protons of II indicates that the electronic environment of the compound is changing as a function of the concentration of the phospholipid and the temperature of the sample. The rate of exchange of protons of II in the two electronic environments is slow enough that the two peaks are observable. These changes in the line shape of the resonance peak of II could result from such processes as the binding of II to phospho-

lipid vesicles, the stacking or aggregation of II in the suspension, and the distribution of II between the phospholipid bilayer and other phases of the phospholipid dispersion. Since the addition of II did not produce any change in either the chemical shift or the line width of the phospholipid protons it is concluded that II has little effect on either the molecular motion or the electronic environment of the phospholipid. This behavior could be compared with the work of Hauser *et al.* (1969) and Finer *et al.* (1969) who observed no changes in either the line width or chemical shift of phospholipid protons with the addition of the drug Gramicidine S dihydrochloride to egg-yolk lecithin or with the addition of anesthetics to phosphatidylserine.

Interpretation of these data would thus seem to involve the various phases of phospholipids attainable in aqueous dispersion. The addition of water to phospholipids results in the formation of aggregates with a mol wt in the order to  $10^8$  (Robinson, 1960; Bangham, 1963). According to a true definition, these aggregates cannot be termed as micelles (McBain, 1913). Upon sonication, the aggregates are usually reduced in size giving a mol wt of the order of  $10^6$ . At this point, the dispersion becomes clear and such a solution contains both micelles as well as liquid crystalline mesophases (Small, 1967; Saunders, 1966). Although the most common structural species of lecithin dispersed in water are liposomes, the size of the liposomes depends upon the concentration of the phospholipid and temperature as well as upon the ultrasonication procedures. Other structures of these aggregates include hexagonal middle phase and the lamellar type. Luzzati and Husson have described other structures intermediate between the middle phase and the lamellar liquid crystalline phase (Luzzati and Husson, 1962).

Observation of the two resonance peaks with II could involve the manner in which the compound is distributed between the crystalline gel configuration and the liquid crystalline form of the phospholipid. The predominance of the low-field peak at high temperatures would correspond to the situation where II is freely dispersed in the hydrophobic "liquid" environment of the bilayer. A progressive transition with temperature would suggest that the gel to liquid-crystal transition is occurring over a range possibly as a result of the presence of II.

At low temperatures and low concentration of phospholipid the high-field peak predominates. The phospholipid would exist primarily in the crystalline form and dispersion of II in the bilayer would be limited. With a pure phospholipid the transition from the gel to the liquid-crystal configuration occurs over a narrow temperature range. Incorporation of other components into the bilayer tends to broaden the temperature range over which the transition occurs. The change of the peak of II from high to low field with increasing concentration of phospholipid may involve such a possibility. At lower concentrations of phospholipids in the system the II: phospholipid ratio is close to unity. If the transition occurs over a broader temperature range it is possible that regions of the phospholipid might exist in the liquid-crystalline form below the transition temperature of the pure phospholipid. II dispersed in these regions would give the low-field peak. Increasing concentrations of phospholipid may reduce the II: phospholipid ratio somewhat but at the same time provide an increase in the amount of liquid-crystalline environment in which II can be dispersed—hence, the increase in the low-field peak.

We would suggest that the high-field peak may result from the aggregation of II and the following would tend to support

TABLE I: Partition Coefficient  $K$  as a Function of Phospholipid Concentration.

DL- $\alpha$ -Lecithin		Sphingomyelin		Phosphatidylserine	
Concn (M)	$K$	Concn (M)	$K$	Concn (M)	$K$
20	0.3	25	0	20	0.263
35	0.57	50	0.25	25	0.533
45	1.02	100	1.04	35	1.09
100	6.4	150	1.30	50	
		200	5.03		

such a hypothesis. A high-field shift has been observed with the stacking of purine nucleosides (Broom *et al.*, 1967). More recently Biros (1972)<sup>1</sup> has observed that the chemical shift of the aromatic protons of 2,2-bis(*p*-chlorophenyl)acetic acid in aqueous solution moved to high field with increasing concentration. The phenomenon is interpreted as resulting from the association of 2,2-bis(*p*-chlorophenyl)acetic acid in water.

The line width of the resonance of II in the two forms is approximately the same and relatively sharp. Molecules of II would thus have considerable rotational freedom as aggregates compared to the relatively rigid phospholipid.

The behavior of II in the two phospholipid environments may be expressed as



If  $C_1$  and  $C_2$  are the respective concentrations of II in the two environments and  $C$  is the original concentration of II then

$$C_1 + C_2 = C \quad (2)$$

A partition coefficient  $K$  for this process may be calculated from the intensities  $I_1$  and  $I_2$  of the two peaks from eq 3. The

$$K = I_2/I_1 = C_2/C_1 \quad (3)$$

calculated values of  $K$  as a function of lipid concentration are given in Table I. The temperature dependence of  $K$  may be used to calculate the value of enthalpy change  $\Delta H$  for this transition. An Arrhenius plot of  $1/T$  vs.  $\log K$  is shown in Figure 6 and the  $\Delta H$  values obtained in such a manner are given in Table II.

It is interesting to note that the temperature at which the low-field peak becomes dominant corresponds roughly to the transition temperature ( $45^\circ$  for dipalmitoyllecithin). With egg-yolk lecithin the low-field peak becomes dominant even below room temperature, consistent with the lower transition temperature. The higher temperatures required to obtain only one peak corresponding to protons of II in sphingomyelin, phosphatidylethanolamine, and phosphatidylserine are consistent with higher transition temperatures for these phospholipids (Ladbrooke and Chapman, 1969). The plot of  $\log K$  vs.  $1/T$  for phosphatidylserine gave a distinctive break showing a temperature dependence of  $\Delta H$ . This behavior may involve several characteristic properties of dipalmitoylserine such as its inability to form lyotropic mesophases in water and ob-

<sup>1</sup> Biros, F. J. (1972), private communication.

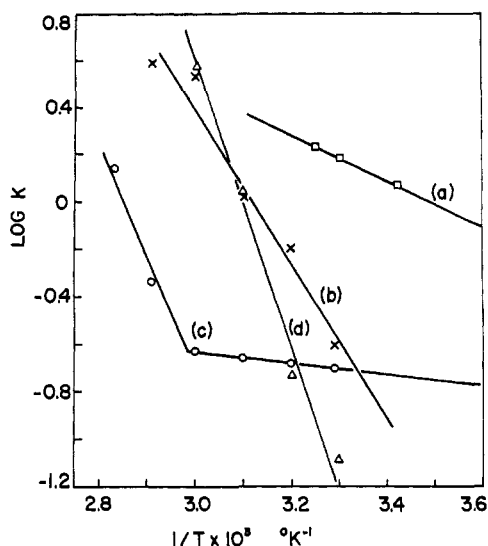


FIGURE 6: Arrhenius plot of  $1/T$  vs.  $\log K$  for various phospholipid-II systems: (a) dipalmitoyllecithin; (b) sphingomyelin; (c) phosphatidylserine; (d) phosphatidylethanolamine.

servation of two transitions for aqueous dispersion of this phospholipid (Ladbrooke and Chapman, 1969). The  $\Delta H$  observed for these lipids follows the series ethanolamine > sphingomyelin > serine > lecithin.

The difference in chemical shift between the two peaks ranges from an average of 17–21 Hz for phosphatidylserine and phosphatidylethanolamine and 30–35 Hz for lecithin and sphingomyelin. This difference may be related to the tendency of the latter two lipids to form widely spaced bimolecular leaflets (Palmer and Schmitt, 1941). The magnitude of the difference in chemical shift between the two peaks of II may involve (Broom *et al.*, 1967) polar, anisotropic, and ring current effects. At the present time, the exact contribution of each effect is difficult to estimate.

Similar experiments when carried out with II produced only one set of resonance peaks corresponding to the liquid-crystalline phase of the phospholipid. A high-field resonance peak was not observable. The difference in behavior of I com-

pared to II may be explained by such factors as hydrophobic character and molecular configuration. Although I and II have comparable lipid solubility, II is considerably more soluble in water than I. For this reason a greater amount of phospholipid is required to solubilize enough I in the  $D_2O$  dispersion for nmr study. The high concentration of phospholipid required to solubilize I for the nmr study may thus always favor an environment corresponding to the low-field resonance peak. On a structural basis II possesses higher symmetry than I and therefore it may be possible that the configuration of the former assist stacking or aggregation. The additional chlorine atom present in I in the trichloroethane moiety may hinder such stacking or association. This difference in the behavior of I and II may be indirectly related to the marked difference in biological activity of these two compounds.

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TABLE II: Chemical-Shift Difference ( $\Delta\delta$ ) between the Two Peaks of II, Transition Temperature for Disappearance of High-Field Peak, and Enthalpy Changes ( $\Delta H$ ) for Various Phospholipids.

Phospholipid	Trans Temp (°C)	$\Delta\delta$ (Hz)	$\Delta H$ (kcal mol <sup>-1</sup> )
DL- $\alpha$ -Lecithin	~44	35 $\pm$ 2	4.8
Egg-yolk lecithin	<25		
Sphingomyelin	~80	29 $\pm$ 3	15.9
Phosphatidylserine	~80	17 $\pm$ 2	22.8, 1.2
Phosphatidylethanolamine	~70	21	29.6