Biochimica et Biophysica Acta, 1027 (1990) 59–64
Elsevier

BBAMEM 74930

Organic pesticides modify lipid-lipid and lipid-protein domains in model membranes. A laser Raman study

Surendra P. Verma and Anjana Rastogi

Department of Community Health, Tufts University School of Medicine, Boston, MA (U.S.A.)

(Received 7 February 1990)
(Revised manuscript received 17 April 1990)

Key words: Hexachlorocyclohexane; Phospholipid; Thermal transition; Raman spectrum

The effect of hexachlorocyclohexane (all isomers) on the thermal transition properties of phospholipid liposomes was determined by Raman spectroscopy. Raman spectra of liposomes with and without the presence of hexachlorocyclohexanes were recorded in the C-H stretching region which shows three major bands around 2850, 2880 and 2930 cm^{-1}. Thermal transition properties were estimated from plots of I_{2880}/I_{2850} and or I_{2930}/I_{2850} vs. temperature, where I represents the intensity of the respective band. Our data on phospholipid liposomes reveal that δ- and γ-hexachlorocyclohexanes drastically reduce and broaden the main thermal transitions of phospholipids at toxic level concentrations. These effects are more pronounced in liposomes containing 18 or more carbon atom long acyl chains. α- and β-isomers at similar concentrations show a minimum effect on the thermal transition properties of phospholipids. Raman analysis of phospholipid liposomes containing melittin, interestingly, reveal that the δ-isomer unlike the γ-isomer strongly alters the transition properties of boundary lipids. These data suggest that the effect of hexachlorocyclohexanes on the thermal transition properties of membranes is stereo specific and that the δ-isomer preferably disrupts the lipid-protein domains. Results are explained on the basis of the dynamic flexibility owing to the equatorial and axial chlorine atoms of various hexachlorocyclohexane isomers.

Introduction

Many organic pesticides and their relatives are soluble in oils and fats but are poorly soluble in water. These pesticides would preferably accumulate in membrane lipids and would conceivably modify their organization as well as membrane fluidity. The change in membrane fluidity, if it occurs, would alter many cell membrane properties. For example, (i) the transport of ions and nutrients is directly related to membrane fluidity [1], and (ii) both membrane fluidity and lipid composition affect the activities of certain membrane-associated enzymes [2] and the function of membrane-receptors [3]. The availability of the mobile surface receptors also depends on the membrane composition and fluidity. This is significant since the primary toxic mode of action of pesticides is believed to be related to neuroreceptors [4].

Chlorinated pesticides have been shown to alter the activities of membrane bound enzymes [5–7] as well as the permeabilities of Ca^{2+} [8] and Na^+ and K^+ ions [9]. Effects of hexachlorocyclohexane (HCCH) isomers on various properties of cells are quite frequently studied [10–16] in their own right and also as models for stereoisomers of inositol. Parries and Hokin-Neaverson [12] have suggested that the inhibition of activities of phosphatidylinositol synthase and other membrane associated enzymes by γ- and δ-isomers of HCCH is due to their insertion into the hydrophobic domains of the enzymes or into the boundary lipids. These authors have further reported that the degree of inhibition by HCCH isomers is in the following order; $\delta > \gamma > \alpha > \beta$, which is the same as their order of membrane saturation capacity [17]. However, the order of relative toxicity of these isomers as estimated by the production of superoxide anion (O^{2-}) and by the release of calcium in human polymorphonuclear leukocytes ($\gamma > \alpha > \delta > \beta$), is unrelated to their membrane unsaturation capacity [15]. It has been further suggested that the involvement

Abbreviations: HCCH, hexachlorocyclohexane; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DAPC, diarachidoylphosphatidylcholine.

Correspondence: S.P. Verma, Department of Community Health, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111, U.S.A.

of membrane perturbation or altered membrane fluidity by HCCH isomers is unlikely to account for their toxicity [15]. Studies on other pesticides, DDT [18], herbicides and several phenylamides [19] have been shown to broaden and shift the phase transition temperature of phospholipid bilayers. Disruption of membrane structure by pesticides is implicated in these studies. However, their site(s) in membranes as well as the underlying molecular mechanism of action, which may also depend on the physical or chemical properties of pesticide molecules, are not well understood.

We used model phospholipid membranes to demonstrate that δ- and γ-isomers of HCCH disrupt drastically the lipid-lipid and lipid-protein phase in contrast to α- and β-isomers and that the membrane-disrupting properties of these isomers could be related to their structural configuration.

Materials and Methods

Materials. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), diarachidoylphosphatidylcholine (DAPC), HCCH (δ- and γ-isomers) and melittin were obtained from Sigma Chem. Co. (St. Louis, MO). α- and β-HCCH were obtained from Aldrich Chem. Co. (Milwaukee, ML). All chemicals were used as supplied except melittin which was purified as in Ref. [20].

Methods. Multibilayer liposomes were prepared as follows: Phospholipids (10 mg) + pesticides (10–70 μg) were mixed in chloroform/methanol (2:1, v/v). The solvent was removed by nitrogen gas followed by drying under vacuum for 2 h. The dried samples were hydrated with water for the lipid-lipid system and with melittin solution (1 mg/ml in phosphate buffer, pH 7.4 + 10 mM EDTA) for the lipid-protein system. The hydrated samples were placed in a water bath at temperatures 2–3 C° higher than the respective thermal transitions of phospholipids for 2 h. Samples were periodically agitated by vortex. The control liposomes without pesticides were treated identically.

Raman spectroscopy. Raman spectra were recorded using a Ramalog 4 spectrometer (Spex Industries, Edison, NJ) modified for Optical Spectrometric Multichannel Analysis (OSMA). We used IRY 700 (visible/blue) detector head, cooled to −20 °C and a ST120 controller (Princeton Instruments, Trenton, NJ) interfaced to an IBM compatible AT computer (Zenith). The samples, sealed in Kimax capillaries (1.2 mm, i.d.) were irradiated by an Ar^+ ion laser (Spectra Physics model 164) tuned at 514.5 nm line (power 500 mW). Sample temperatures were controlled by a flow of temperature-regulated nitrogen gas, by using a Harney-Miller cell. The temperature in the cell was measured continuously with a thermistor placed close to the laser beam. Measured temperatures were 2–3 C° lower than sample temperature, according to calibration curves with pure phospholipids and fatty acids. The transition temperatures reported in the paper are those measured by the thermistor. The exposure time per scan varies depending on the background fluorescence. A minimum of 200 spectra were stored and averaged. Averaged spectra were plotted by a Hewlett Packard plotter (model Color Pro).

Results

C-H stretching and thermal transition

Representative C-H stretching spectra of DPPC with and without the presence of HCCH isomers (δ and γ) are shown in Fig. 1 This region shows bands at 2850, 2880 and 2930 cm^{-1}, assigned to symmetric, asymmetric C-H stretching vibrations in CH_2 and symmetric vibrations in CH_3 groups, respectively [21]. The variation in the intensity of the 2880 cm^{-1} feature has been attributed to Fermi resonance [22]. The intensity of the 2850 cm^{-1} band remains relatively unaltered and has been used as an internal standard. The other phospholipids used also show similar Raman features in this region. We analyzed the effect of pesticides on the

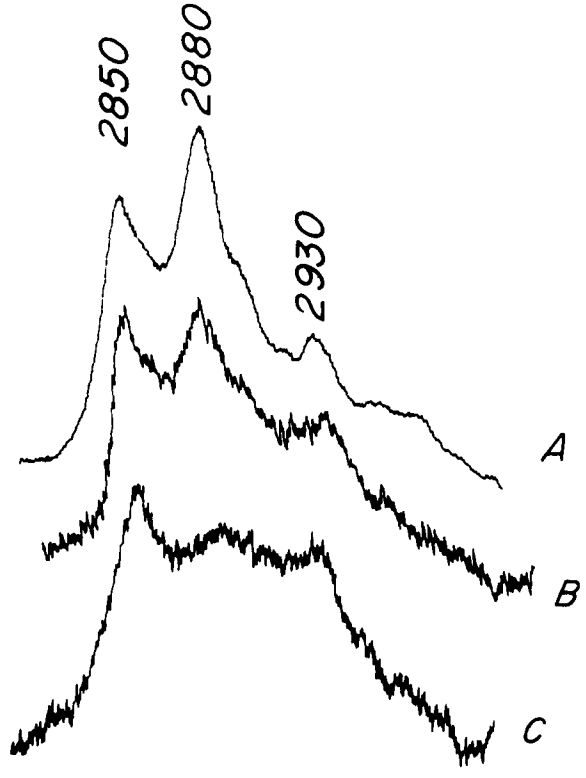

Fig. 1. Raman spectra of DPPC liposomes +/− hexachlorocyclohexanes in the C-H stretching region recorded at room temperature. A, DPPC; B, DPPC + δ-hexachlorocyclohexane (1:0.04, w/w); C, DPPC + γ-hexachlorocyclohexane (1:0.04, w/w). Spectra recording conditions: excitation line 514.5 nm; power 500 mW; entrance slit 400 μm; exposure time 0.33 s/scan; total scans 100.

interchain interactions and on the thermal transition properties of lipid and lipid-protein domains from I_{2880}/I_{2850} vs. temperature plots [21], where I is the intensity of respective bands.

Thermal transitions of phospholipids in the presence of HCCHs

(A) DMPC. The main transition of DMPC occurs at 23° C. In the presence of δ-HCCH (1 : 0.002, w/w; DMPC : δ-HCCH), the main transition although remains cooperative but shifts to 20° C (Fig. 2A). γ-HCCH (1 : 0.002, w/w; DMPC : γ-HCCH) broadens (transition width 6 C°) and shifts the main transition to 14° C (Fig. 3A).

(B) DPPC. DPPC liposomes yield a main transition near 41° C. Liposomes containing δ-HCCH (1 : 0.002, w/w; DPPC : δ-HCCH) show a broad transition around 35° C (midpoint) (Fig. 2B). At this concentration, the broadening of the transition of DMPC by δ-HCCH is less. The transition of DPPC, in the presence of γHCCH, exhibits the width of approx. 6 C° and shifts to 37° C (midpoint (Fig. 3B).

In the presence of α- and β-HCCH (1 : 0.02, w/w; lipid/α-HCCH), the main transition of DPPC almost remains cooperative and unaltered. However, at 1-times higher weight ratio, the transition is still cooperative but shifts to 37° C. The ratio I_{2880}/I_{2850} continuously increases at temperatures from 36° C to 20° C (data not shown).

(C) DSPC. DSPC yields a cooperative transition at 50° C. δ-HCCH drastically alters the transition properties of DSPC liposomes. At weight ratio of 1 : 0.002 (DSPC/δ-HCCH), the transition is considerably broad

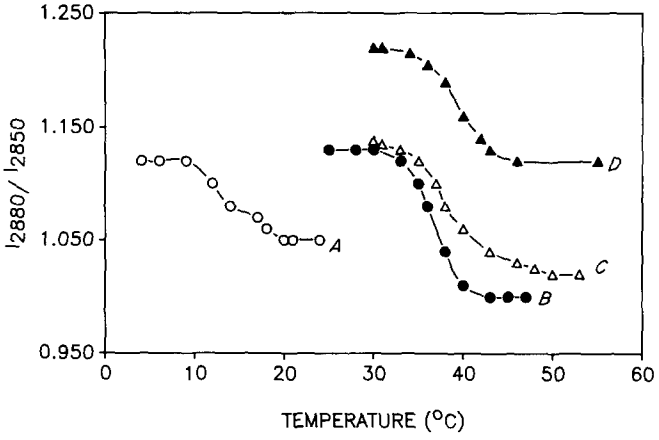

Fig. 3. Variation of the ratio [I_{2880}/I_{2850}] of phospholipids + γ-hexachlorocyclohexane (1 : 0.002, w/w) liposomes as a function temperature. A, DMPC; B, DPPC; C, DSPC; D, DAPC. Recording conditions were the same as in Fig. 1. Curves are drawn by Sigma-Plot (Jandel Scientific). The maximum error in each data point is ±0.05 on average.

and shift to about 40° C (midpoint) (Fig. 2C). In the presence of γ-HCCH, the main transition of DSPC shifts to 38° C (midpoint) and exhibits a width of 3 C° (Fig. 3C).

(D) DAPC. The main transition of DAPC at 63° C drastically broaden and shifts to 45° C in the presence of delta-HCCH (1 : 0.002; w/w; DAPC : δ-HCCH) (Fig. 2D). The effect of γ-HCCH on the thermal transition of DAPC is shown in Fig. 3D. The main transition shifts to 41° C and the width is about 2–3 C°. However, the ratio I_{2880}/I_{2850} rises continuously at temperatures below 41° C.

(E) DPPC + melittin. The phospholipid-melittin liposomes have been used as a model for lipid–protein interaction [20,23]. Transition properties of the lipid-

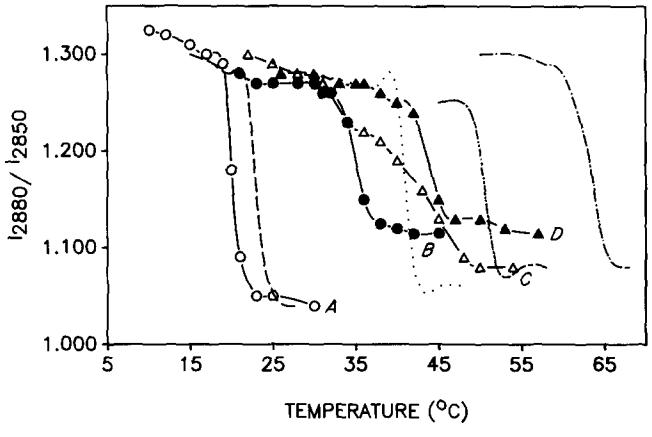

Fig. 2. Variation of the ratio [I_{2880}/I_{2850}] of phospholipids + δ-hexachlorocyclohexane (1 : 0.002, w/w) liposomes as a function of temperature. A, DMPC; B, DPPC; C, DSPC; D, DAPC. Recording conditions were the same as in Fig. 1. The maximum error in each data point is ±0.05 on average. Transition curves of control phospholipids are shown without symbols. Curves are drawn by Sigma-Plot (Jandel Scientific).

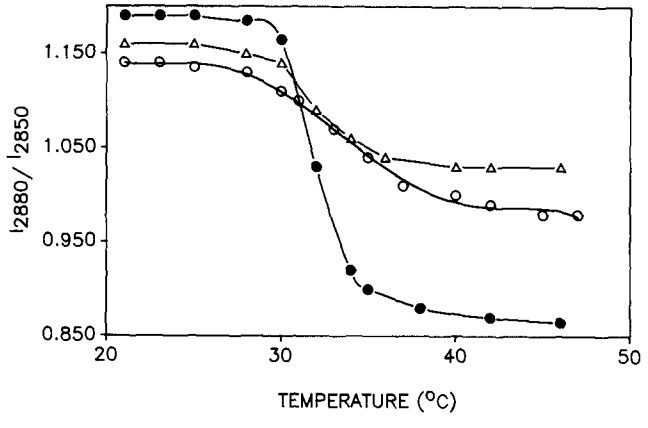

Fig. 4. Variation of the ratio [I_{2880}/I_{2850}] of DPPC + melittin +/− γ- and δ-hexachlorocyclohexane (1 : 0.1 : 0.002, w/w) liposomes as a function of temperature. DPPC + melittin: DPPC + melittin + γ-hexachlorocyclohexane; DPPC + melittin + δ-hexachlorocyclohexane. Recording conditions were the same as in Fig. 1. Curves are drawn by Sigma-Plot (Jandel Scientific). The maximum error in each data point is ±0.05 on average.

melittin phase have been established [20,23] and this simple system appears suitable to study the effects of HCCHs as a model for the lipid-protein phase. Fig. 4 shows the temperature profiles of DPPC + melittin +/− δ- and γ-HCCH (1 : 0.1 : 0.002, w/w/w; DPPC/melittin/HCCH). The transition of DPPC-melittin complex is broad (width approx. 8 C°) and occurs around 32°C (midpoint). The ratio I_{2880}/I_{2850} difference between above and below thermal transition is about 0.15 (average value). In the presence of γ-HCCH, the transition is more cooperative and shifts to 30°C (midpoint). The ratio difference between gel and liquid-crystalline state is about 0.10. Interestingly, δ-HCCH affects more drastically the amplitude of the DPPC-melittin transition. The ratio I_{2880}/I_{2850} decreases from about 1.17 to about 0.88, a difference of 0.29, which is about 3-times higher than that shown by γ-HCCH. The thermal transition is cooperative (width about 2°C) and centers near 32°C.

Raman spectra of HCCH isomers

Raman spectra of α-, β-, γ-, and δ-isomers (solid powder) in the 400 to 1600 cm^{-1} region are shown in Fig. 5. The band positions are marked in the figure. β-HCCH has all six chlorine atoms in the equatorial position [24] and shows two very strong bands at 848 and 742 cm^{-1}, which are tentatively assigned to ring 'breathing' and C-Cl stretching (equatorial) vibrations,

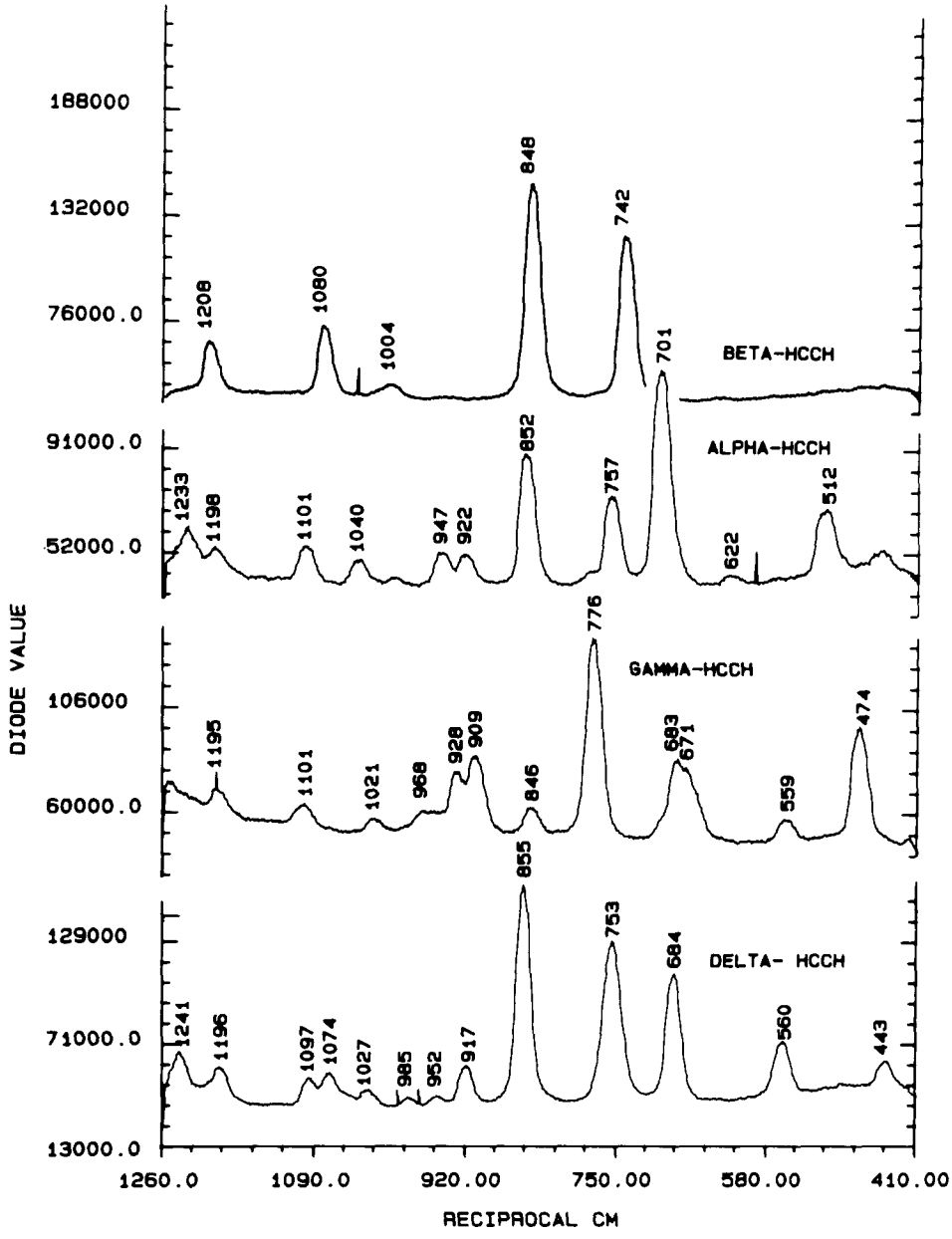

Fig. 5. Raman spectra of the isomers of hexachlorocyclohexane (powder) in the 400–1600 cm^{-1} region. Recording conditions: excitation line 514.5 nm; power 10 mW; exposure 1 s; slit 400 μm; total scans 10.

respectively [25]. The spectrum of δ-HCCH, which has one axial and five equatorial chlorine atoms [24], is quite different when compared to the spectrum of β-HCCH. The strong band at 855 cm^{-1} could be the ring 'breathing' vibration while the two medium strong bands at 753 and 684 cm^{-1} could be tentatively assigned to C-Cl stretching of equatorial and axial states, respectively. γ-HCCH which has equal numbers of equatorial and axial (three of each) chlorine atoms [24], shows a similar banding pattern but their intensity and positions vary. The strong band occurs at 776 cm^{-1} and the position is closer to the position of the tentatively assigned C-Cl stretching of equatorial chlorine atoms. Upon comparing the positions of the tentatively assigned bands for equatorial chlorine atoms in the four isomers, it seems that as the number of chlorine atoms in the equatorial positions decreases ($\beta > \delta > \alpha > \gamma$) the C-Cl stretching vibration shifts towards higher frequencies. Interestingly, the intensity of the ring 'breathing' vibration of γ-HCCH at 846 cm^{-1} is relatively very weak.

Discussion

C-H stretching features are used to probe the interchain interactions and thermotropic properties of lipid and lipid-protein domains in the presence of organic pesticides. This region sensitively reflects vibrational interactions between membrane lipid acyl chains, as well as perturbations of lateral effects. The spectral analysis of this region has been used to define the configurational states of lipid-lipid and lipid-protein/polypeptide domains as a function of temperature (thermal transitions) and other perturbants [21]. Lipids can occur in gel (ordered) or in liquid-crystalline (disordered) phases. The temperature at which these states are in equilibrium is known as transition temperature (T_c). The analysis of the thermal transition properties is a sensitive parameter for biologically more relevant perturbations [26]. We have evaluated the thermal transition properties of phospholipids and phospholipid-melittin (models for lipid-lipid and lipid-protein domains, respectively) liposomes with and without the presence of HCCHs.

δ- and γ-HCCHs decrease and broaden the main thermal transition of phospholipids and these effects are more prominent for DSPC and DAPC liposomes. The transitions of DMPC, DPPC, DSPC and DAPC are shifted by 3, 6, 10 and 18 C°, respectively, in the presence of δ-HCCH (1 : 0.002, w/w; lipid/δ-HCCH). The effect of γ-HCCH on the transitions of phospholipids is somewhat more pronounced in comparison to the δ-HCCH. γ-HCCH shifts the transitions of DMPC, DPPC, DSPC and DAPC by about 9, 5, 12 and 22 C°, respectively. These data suggest that both δ- and γ-HCCH accumulate in acyl chains and influence the interchain interactions as well as the order–disorder thermal transitions.

HCCH isomers do not have a planer structure [24]. These isomers are stable in the 'chair' configuration [26], and their insertion would create space between acyl chains which in turn would reduce interchain interactions and would influence the thermal transition properties of lipids. Our thermal transition data show that γ-HCCH maximally reduces the transition temperatures of phospholipids. This effect could be related to the equatorial and axial positions of chlorine atoms in the γ-HCCH molecule. Probably these positions of chlorine atoms make this isomer dynamically more flexible which may allow more of these molecules to partition into the acyl chains.

The α-, β-, γ-, and δ-HCCH molecules, respectively, possess four equatorial and two axial, all six equatorial, three equatorial and three axial and five equatorial and one axial chlorine atoms in the cyclohexane ring. The most stable and preferred position with a minimum interference with other atoms on the ring, is an equatorial one [27]. According to this model β-HCCH should be the most stable or dynamically the most 'rigid' molecule followed by the δ-, α- and γ-isomer. β-HCCH yield a single C-Cl stretching band since their average environments are identical. This is not true in other isomers and they show multiple bands for C-Cl stretching.

The equatorial/axial hypothesis can also explain the order of toxicity ($\gamma > \alpha > \delta > \beta$) as reported by Kuhns et al. [15] and could also be accounted for the lesser solubility of β-HCCH in DPPC liposomes [17]. However, the equatorial/axial hypothesis does not satisfactorily explain the higher solubility of δ-HCCH in DPPC liposomes [17]. Examination of Raman spectra of HCCH isomers (Fig. 5) could provide some of the answers to these questions. Both δ- and γ-HCCH show C-Cl stretching bands at 684 and 683 cm^{-1}, respectively (tentatively assigned to axial positions) and show comparable effects on the thermal transition properties of phospholipids. In contrast, α-HCCH although has two axial Cl atoms, does not show C-Cl stretching band around these positions. Importantly, α-HCCH has also shown a lesser membrane perturbing effect. This may have some relationship with the observed effects of HCCH isomers on thermal transitions but extensive studies are needed to correlate the states of axial/equatorial groups with the HCCH-induced membrane disorganization effect.

Biological membranes are heterogeneous systems and contain several symmetric and asymmetric lipid-lipid and lipid-protein domains. HCCH molecules, due to their stereochemical structure, could unequally distribute among these domains. This has been indicated by the influence of these isomers on the thermal transition properties of DPPC-melittin liposomes. Our data show

that δ-HCCH unlike the γ-HCCH isomer drastically alters the I_{2880}/I_{2850} ratio when liposomes are cycled through the DPPC-melittin transition temperature. Interestingly, δ-HCCH drastically lowers the I_{2880}/I_{2850} ratio which may indicate that this isomer further fluidizes the lipids and influences the lipid-melittin association. Considering the effect of δ-HCCH on the transitions of DPPC and the DPPC-melittin liposomes, these events are possible if δ-HCCH (i) preferably partition into the DPPC associated with melittin (boundary lipids) and/or (ii) occupies the hydrophobic site/s of melittin. The possibility (ii) could modify the structure or insertion of melittin in liposomes which in turn could alter the transition properties of lipids. In case δ-HCCH directly interacts with melittin then one would expect a change in the secondary structure of melittin. However, this possibility is difficult to verify in membranes since the secondary structure of melittin in model liposomes has been shown to be modified by its interaction with lipids [28]. If the organization of acyl chains change upon the insertion of δ-HCCH then this would lead to changes in the lipid-peptide association and possibly to changes in the secondary structure of melittin. In any event both (i) and (ii) possibilities would modify the lipid–protein interaction. At present our data cannot distinguish between (i) and (ii). However, our quantitative analysis of the secondary structure of melittin in liposomes containing δ- and γ-HCCH by curve fitting analysis of the amide I bands [29] indicates that δ-HCCH may bind with melittin (will be published elsewhere).

The observation that γ-HCCH is comparatively less sensitive to modify the transition properties of melittin-influenced lipids could not be accounted due to the difference in the axial and equatorial positions of chlorine atoms in these isomers. γ-HCCH is relatively more flexible and this molecular property would allow this isomer to partition more into the hydrophobic sites of lipids and melittin.

In conclusion, our Raman transition data on phospholipid liposomes show that chlorinated organic pesticides affected the organization of lipids and lipid-protein domains. γ-HCCH is more disruptive to the lipid-lipid domains while δ-HCCH is found to be relatively most disruptive to the lipid-protein domains.

Acknowledgements

These studies are supported by US EPA under assistance agreement No. CR-813-481-01-1 to the Center for Environmental Management, Tufts University, Medford, MA. These studies do not reflect the view of EPA and no official endorsement should be inferred. We sincerely thank Dr. Carl F. Blackman, EPA, Research Triangle Park, NC, and Dr. Mark Boyer, Department of Community Health, Tufts University School of Medicine, Boston, MA, for many helpful discussions and advice during this work.

References

1 Wiley, J.S. and Cooper, R.A. (1975) Biochim. Biophys. Acta 413, 425–431.
2 McMurchie, E.J. and Raison, J.K. (1979) Biochim. Biophys. Acta 554, 364–374.
3 Ginsbery, B.H., Jabour, J. and Spector, A.A. (1982) Biochim. Biophys. Acta 690, 157–164.
4 Matsmura, F. (1975) Toxicology of Insecticides, Plenum Press, New York.
5 Doherty, J.D. (1979) Pharmacol. Ther. 7, 123–151.
6 Jones, O.T., Froud, R.J. and Lee, A.G. (1985) Biochim. Biophys. Acta 812, 740–751.
7 Jones, O.T. and Lee, A.G. (1985) Biochim. Biophys. Acta 812, 731–739.
8 Publicover, S.J. and Duncan, O.J. (1979) Naunyn-Schmiedeberg's Arch. Pharmacol. 308, 179–182.
9 Narahashi, T. and Hass, H.G. (1968) J. Gen. Physiol. 51, 177–198.
10 Hokin, M.R. and Brown, D.F. (1969) J. Neurochem. 16, 475–483.
11 Fisher, D.P. and Mueller, G.C. (1971) Biochem. Pharmacol. 20, 2515–2518.
12 Parries, G.S. and Hokin-Neaverson, M. (1985) J. Biol. Chem. 260, 2687–2693.
13 Mead, C.J., Harvey, J., Boot, J.R., Turner, G.A. and Bateman, P.E. (1984) Biochem. Pharmacol. 33, 289–293.
14 Holian, A., Marchiarullo, M.A. and Stiekle, D.F. (1984) FEBS Lett. 176, 151–154.
15 Kuhns, D.P., Kaplan, S.S. and Basford, R.E. (1986) Blood 68, 535–540.
16 Stark, Joy, R.M. and Hollinger, M.A. (1987) Exp. Neurol. 98, 276–284.
17 Omann, G.M. and Lakowicz, J.R. (1982) Biochim. Biophys. Acta 684, 83–95.
18 Chefurka, W., Chatelier, R.C. and Sawyer, W.H. (1987) Biochim. Biophys. Acta 896, 181–186.
19 Stidham, M.A., Siedow, J.N., McIntosh, T.J., Porter, N.A. and Moreland, D.E. (1985) Biochim. Biophys. Acta 812, 721–730.
20 Dasseux, J., Faucon, J., Lafleur, M., Pezolet, M. and Dufourcq, J. (1984) Biochim. Biophys. Acta 775, 37–50.
21 Verma, S.P. and Wallach, D.F.H. (1983) in Biomembrane Structure and Function (Chapman, D., ed.), pp. 167–192, McMillan Press, London.
22 Snyder, R.G., Hsu, F.L. and Krimm, S. (1978) Spectrochim. Acta, Part A 34, 395–406.
23 Lafleur, M., DAsseux, J.-L., Pigeon, M., Dufourcq, J. and Pezolet, M. (1987) Biochemistry 26, 1173–1179.
24 Hornstein, I. (1955) Science 121, 206–207.
25 Dollish, F.R., Fateley, W.G. and Bentley, F.F. (1974) Characteristic Raman Frequencies of Organic Compounds, pp. 23, John Wiley and Sons, New York.
26 Bach, D. (1983) in Biomembrane Structure and Function (Chapman, D., ed.), pp. 1–41, McMillan Press, London.
27 Petrucci, R. (1985) General Chemistry, Principles and Modern Applications, 4th Edn., pp. 804–806.
28 Vogel, H. and Jahnig, F. (1986) Biophys. J. 50, 573–582.
29 Williams, R.W. (1983) J. Mol. Biol. 166, 581–603.