Kuhlenschmidt, T. B., & Lee, Y. C. (1984) *Biochemistry 23*, 3569-3575.
Kuhlenschmidt, T. B., Kuhlenschmidt, M. S., Roseman, S., & Lee, Y. C. (1984) *Biochemistry 23*, 6437-6444.
Lee, R. T. (1982) *Biochemistry 21*, 1045-1050.
Lee, R. T., & Lee, Y. C. (1974) *Carbohydr. Res. 37*, 193-201.
Lee, R. T., & Lee, Y. C. (1979a) *Carbohydr. Res. 77*, 149-156.
Lee, R. T., & Lee, Y. C. (1979b) *Carbohydr. Res. 77*, 270-274.
Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 156-163.
Lee, R. T., & Lee, Y. C. (1982) *Carbohydr. Res. 101*, 39-47.
Lee, R. T., & Lee, Y. C. (1987) *Glycoconjugate J. 4*, 317-328.
Lee, R. T. & Lee, Y. C. (1988) *Biochem. Biophys. Res. Commun. 155*, 1444-1451.
Lee, R. T., Myers, R. W., & Lee, Y. C. (1982) *Biochemistry 21*, 6292-6298.
Lee, R. T., Lin, P. & Lee, Y. C. (1984) *Biochemistry 23*, 4255-4261.
Lee, R. T., Wong, T. C., & Lee, Y. C. (1986) *J. Carbohydr. Chem. 5*, 343-357.
Lee, R. T., Wong, T. C., Lee, R., Yue, L., & Lee, Y. C. (1989) *Biochemistry 28*, 1856-1861.
Lee, Y. C. (1978) *Carbohydr. Res. 67*, 509-514.
Lee, Y. C. (1989) *Ciba Found. Symp. 145*, 80-95.
Lee, Y. C., & Lee, R. T. (1982) *Glycoconjugates 4*, 57-83.
Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956-3963.
Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M., & Lönn, J. (1983) *J. Biol. Chem. 258*, 199-202.
Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., & Bock, K. (1984) in *Biochemical and Biophysical Studies of Proteins and Nucleic Acids* (Lo, T. B., Lin, T. Y., & Li, C. H., Eds.) pp 349-360, Elsevier, New York.
Lemieux, R. U., & Bock, K. (1983) *Arch. Biochem. Biophys. 221*, 125-134.
Loeb, J. A., & Drickamer, K. (1987) *J. Biol. Chem. 262*, 3022-3029.
Lunney, J., & Ashwell, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 341-343.
Piskarev, V. E., Sepetov, N. F., Likhosherstov, L. M., Galenko, E. L., Derevitskaya, V. A., & Kochetkov, N. K. (1989) *Bioorg. Khim.* (submitted for publication).
Sarkar, M., Liao, J., Kabat, E. A., Tanabe, T., & Ashwell, G. (1979) *J. Biol. Chem. 254*, 3170-3174.
Seglen, P. O. (1976) *Methods Cell Biol. 13*, 29-83.
Townsend, R. R., Hardy, M. R., Wong, T. C., & Lee, Y. C. (1986) *Biochemistry 25*, 5716-5725.
Weigel, P. W., Naoi, M., Roseman, S., & Lee, Y. C. (1979) *Carbohydr. Res. 70*, 83-91.

Effects of Hydrostatic Pressure on the Location of PRODAN in Lipid Bilayers: A FT-IR Study†

Parkson Lee-Gau Chong,*,‡ Sarah Capes,§ and Patrick T. T. Wong§
Department of Biochemistry, Meharry Medical College, Nashville, Tennessee 37208, and Division of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

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ABSTRACT: The effects of hydrostatic pressure on the location of 6-propionyl-2-(dimethylamino)naphthalene (PRODAN), an environmentally sensitive fluorescent probe, in phosphatidylcholine lipid bilayers have been studied by Fourier-transform infrared spectroscopy (FT-IR) over the pressure range of 0.001-25 kbar. The results derived from the PRODAN C=O stretching band, the correlation field splitting of the methylene scissoring mode, and the methylene symmetric stretching mode as well as the absorption of the naphthalene ring show that in the sample of 4% (w/w) PRODAN in dimyristoyl-L-α-phosphatidylcholine (DMPC) at pH 6.8, most of the PRODAN molecules are embedded in the bilayers. In contrast, at pH 3.0, PRODAN was found to reside either on the membrane surface or dispersed in water. Compared to DMPC, egg yolk phosphatidylcholine (egg PC), which contains a substantial amount of unsaturated fatty acyl chains, is more susceptible to PRODAN permeation. The present study shows that the pressure dependence of the location of PRODAN in lipid membranes is different from that of tetracaine, a local anesthetic, in lipid bilayers. The model regarding the PRODAN location in lipid bilayers derived from the present infrared data has been compared with that obtained with previous fluorescence studies.

Interactions of small molecules with membranes are important issues in membrane biology [reviewed by Gennis (1989)]. Understanding the role of small molecules in modulating the structure and function of biological membranes requires knowledge of (1) the location of small molecules in membranes and (2) the degree of perturbation of membranes caused by these molecules. Many aspects of this subject have been investigated by physical techniques in model membrane systems. While most investigations were conducted at atmospheric pressure, a few were done at high pressures. Knowledge regarding the effects of pressure on the interaction between small molecules and membranes, particularly the aspect of pressure dependence of the location of small molecules in lipid bilayers, is fundamentally important for understanding membrane events at high pressures [reviewed in MacDonald (1984) and Wong (1984)]. In addition, pressure

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* Correspondence should be addressed to this author.
‡ Meharry Medical College.
§ National Research Council of Canada.

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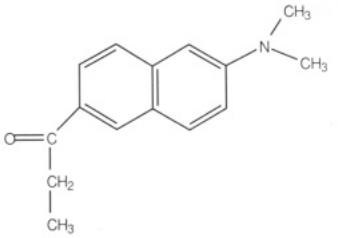

FIGURE 1: Structure of PRODAN.

is a useful tool to study intermolecular interactions since pressure can decrease the intermolecular distance and thus can enhance the intermolecular interactions.

6-Propionyl-2-(dimethylamino)naphthalene (PRODAN)[1] is a synthetic fluorescent probe (Figure 1). Its emission spectra are extremely sensitive to changes in environmental polarity because it has a large dipole moment in the lowest singlet excited state. This property has been utilized in many studies of PRODAN and its derivatives on local environments in membranes (Lakowicz et al., 1984; Massey et al., 1985; Parasassi et al., 1986; Morrison & Weber, 1987) and proteins (Macgregor & Weber, 1981, 1986). Because of the high sensitivity to environmental changes, PRODAN and its derivatives can be used to explore solute permeability in membranes since a lipid bilayer possesses a high polarity at the water–lipid interface and a low polarity in the hydrocarbon core. Recently, in an attempt to understand the location of small molecules in membranes at elevated pressures, the location of PRODAN in phosphatidylcholine lipid bilayers and in goldfish brain synaptic membranes has been investigated by fluorometric approaches over the pressure range of 0.001–2 kbar (Chong, 1988). On the basis of the fluorescence emission spectrum, it was concluded that although PRODAN remains more or less at the interfacial region, it undergoes a pressure-induced relocation from the "polar" disposition to the "less polar" disposition. The model suggests that the PRODAN carbonyl group remains on the membrane surface in the pressure range of 0.001–2 kbar whereas the naphthalene ring and the dimethylamino group can either flip into the bilayer to adopt a less polar disposition or reside in the interfacial region to adopt a polar disposition [see Figure 9 in Chong (1988)].

In the present study, we use Fourier-transform infrared spectroscopy (FT-IR) to test the previously proposed model and to obtain more detailed information about probe location in membranes at high pressures. We present infrared data obtained with PRODAN in dimyristoyl-L-α-phosphatidylcholine (DMPC) and PRODAN in egg yolk phosphatidylcholine (egg PC) lipid bilayers at different pHs and PRODAN concentrations in the pressure range of 0.001–25 kbar.

Experimental Procedures

Preparation of Lipid Bilayers. Dimyristoyl-L-α-phosphatidylcholine (DMPC) and egg yolk phosphatidylcholine (egg PC) were purchased from Avanti (Birmingham, AL). PRODAN was obtained from Molecular Probes (Eugene, OR). Lipids and PRODAN were dissolved in chloroform in an Eppendorf vial, subsequently dried with nitrogen, and lyophilized overnight. The dry lipid mixture was dispersed in a Tris buffer made with D_2O. The concentrations of PRODAN and phospholipid were calculated from weight determinations.

Measurements of the IR Spectrum at High Pressures. Small amounts of the well-mixed lipid–PRODAN dispersions were placed, together with powdered α-quartz, in a 0.37-mm-diameter hole in a 0.23-mm-thick stainless-steel gasket mounted on a diamond anvil cell, as described previously (Wong et al., 1985). Pressure was determined from the 695 cm^{-1} phonon band of α-quartz. The frequency of this band shifts as the pressure varies. The pressures were calculated according to the equation $P = 1.2062\Delta\nu + 0.015164\Delta\nu^2$ where $\Delta\nu$ is the frequency shift (Wong et al., 1985; Siminovitch et al., 1987). Infrared spectra were measured at 28 °C on a Bomem Model DA3.02 Fourier-transform spectrophotometer with a liquid nitrogen cooled mercury–cadmium telluride detector. The infrared beam was condensed by a sodium chloride lens system onto the pinhole of the diamond anvil cell. For each spectrum, 512 scans were performed at a spectral resolution of 4 cm^{-1}.

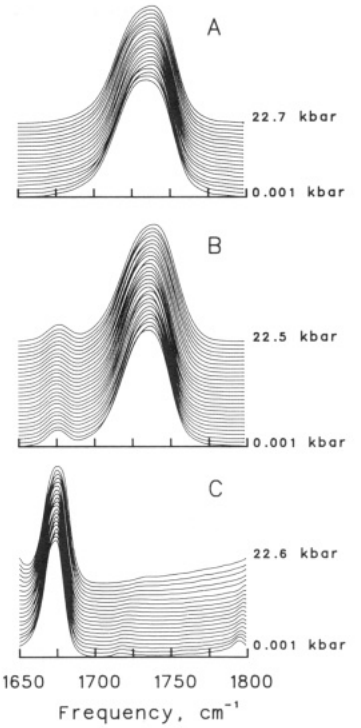

FIGURE 2: Stacked contour plots of the infrared spectra in the 1650–1800 cm^{-1} region. (A) Pure DMPC in D_2O; (B) 10% (w/w) PRODAN in DMPC at pH 6.8; (C) pure PRODAN in D_2O.

Results and Discussion

C=O Stretching Band. Figure 2A shows the peak position of the ester C=O stretching mode band of pure DMPC in water (D_2O) at different pressures. The ester C=O stretching band consists of two overlapping C=O stretching bands of the *sn*-1 and *sn*-2 carbonyl groups. The pressure-induced frequency shift is very small for these two C=O stretching bands due to the hydrogen bonding with water molecules (Wong & Mantsch, 1988). Figure 2C shows the peak position of the C=O band of PRODAN in D_2O. As in the case of DMPC in water, the frequency of the PRODAN C=O stretching band (near 1674 cm^{-1}) is more or less independent of pressure [also see Figure 4 (○)]. Therefore, there must be hydrogen bonding between the C=O group of PRODAN and water since this frequency would increase with increasing pressure due to the compression of the C=O bond, whereas at the same time it would decrease with increasing pressure due to the pressure-induced strengthening of the hydrogen bond.

Figures 2B and 3A,B show the effects of pressure on the C=O stretching bands of both PRODAN and DMPC in the

[1] Abbreviations: DMPC, dimyristoyl-L-α-phosphatidylcholine; egg PC, egg yolk phosphatidylcholine; FT-IR, Fourier-transform infrared spectroscopy; PRODAN, 6-propionyl-2-(dimethylamino)naphthalene.

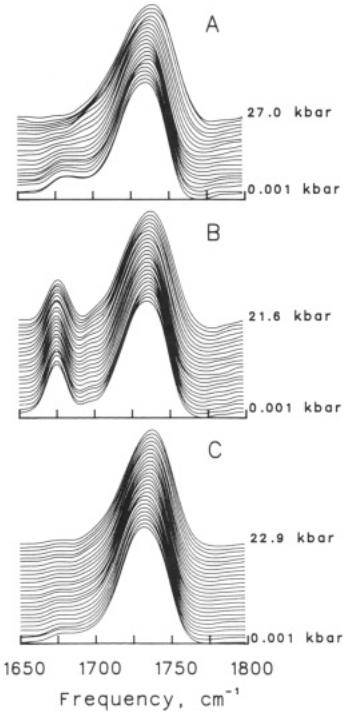

FIGURE 3: Stacked contour plots of the infrared spectra in the 1650–1800 cm^{-1} region. (A) 4% (w/w) PRODAN in DMPC at pH 6.8; (B) 4% (w/w) PRODAN in DMPC at pH 3; (C) 4% (w/w) PRODAN in egg PC at pH 6.8.

PRODAN/DMPC mixtures. The frequency of the PRODAN C═O stretching band in the sample of 10% (w/w) PRODAN, pH 6.8 (Figure 2B), is similar to that of pure PRODAN (Figure 2C). Consequently, the PRODAN C═O stretching band observed in this sample is mainly from those extra PRODAN molecules which are outside the lipid matrix, either on the surface of the lipid bilayers or dispersed in water. Further, the frequency of the PRODAN C═O stretching band does not seem to change significantly with pressure. This suggests that in the sample of 10% PRODAN in DMPC at pH 6.8 pressure does not induce large changes in the partition of PRODAN between water and lipid bilayers.

In order to reduce the contribution from PRODAN in water to the infrared absorption, it is necessary to lower the concentration of PRODAN. Figure 3A shows that the PRODAN C═O stretching band in the sample of 4% PRODAN in DMPC at pH 6.8 is very broad and weak and shifts to a higher frequency [about 1680 cm^{-1}; Figure 4 (Δ)] in the pressure region of 0.001–3 kbar. Above 3 kbar, the C═O stretching band becomes indiscernible (Figures 3A and 4) (Δ)]. The spectral shift and broadening can be taken to indicate [e.g., see Auger et al. (1987)] that when the concentration of PRODAN is lowered from 10% to 4%, almost all of the PRODAN molecules become embedded in the DMPC bilayers. The disappearance of the PRODAN C═O absorption at pressures >3 kbar strongly suggests that the bilayer is disordered by PRODAN at elevated pressures.

Figure 3B shows that the frequency of the PRODAN C═O stretching band in the sample of 4% (w/w) PRODAN at pH 3 is similar to that of 10% PRODAN at pH 6.8 (Figure 2B), indicating that PRODAN resides either on the surface of the lipid bilayers or dispersed in water at pH 3.0. This is somewhat expected since the pK_a of the amino group in PRODAN is about 4.5. At pH 3, PRODAN should carry a partially positive charge which makes it difficult to penetrate into the lipid bilayer. Pressure does not significantly change the frequency of the C═O stretching band [Figure 4 (+)], which

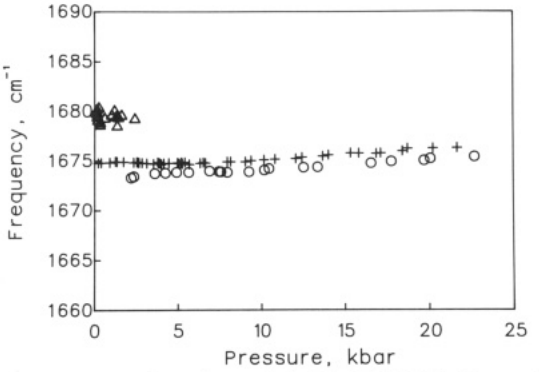

FIGURE 4: Pressure dependence of the PRODAN C═O stretching band in the sample of pure PRODAN in D_2O (○), 4% PRODAN in DMPC at pH 3.0 (+), and 4% PRODAN in DMPC at pH 6.8 (Δ).

indicates that elevated pressure does not push the positively charged PRODAN molecules into the interior of the bilayer. Similar phenomena have been previously observed by Auger et al. (1987) and by Boulanger et al. (1981) for the case of tetracaine in DMPC. The difference is that the charged tetracaine is embedded in the membrane at ambient pressure and squeezed out of the membrane at pressures >4.6 kbar (Auger et al., 1987) whereas the charged PRODAN cannot penetrate into the bilayer over the entire pressure range examined.

In order to examine the effect of fatty acid unsaturation on the permeability of PRODAN in lipid bilayers, egg yolk phosphatidylcholine (egg PC), which typically contains >50% unsaturated fatty acyl chains [for example, see Chong (1988)], was used to replace DMPC. Figure 3C shows the infrared spectra of 4% (w/w) PRODAN in egg PC at pH 6.8. The results are similar to those of 4% PRODAN in DMPC at pH 6.8 (Figure 3A) in that the C═O stretching band of PRODAN is broad and weak and that the band is pressure dependent. However, the intensity ratio of the PRODAN C═O band over the lipid C═O band is different in these two lipid systems. At atmospheric pressure, the ratio is about 0.09 in the case of egg PC (Figure 3C) whereas the ratio for DMPC is about 0.18 (Figure 3A). This suggests that PRODAN molecules can penetrate deeper into lipid bilayers when the lipid membrane contains more unsaturated lipids. It is known that unsaturated lipids are less tightly packed. Thus, compared to saturated lipids such as DMPC, egg PC is more susceptible to PRODAN permeation. Similar effects of lipid unsaturation on PRODAN location have been previously suggested in fluorescence studies (Chong, 1988).

Correlation Field Splitting of the Methylene Scissoring Mode. The pressure-induced correlation field splitting of the methylene scissoring mode is very useful for the characterization of interchain packing (Boerio & Koenig, 1970; Wong, 1984). At atmospheric pressure, a single methylene scissoring band (1470 cm^{-1}) is observed usually due to the fact that the orientation of the methylene chains is highly disordered as a result of the large angle reorientational fluctuations. Increasing pressure increases the molecular order of the acyl chains and increases interchain interactions, which gives rise to the correlation field splitting. Figure 5A–C shows the methylene scissoring mode of pure DMPC, 4% PRODAN in DMPC (pH 6.8), and 4% PRODAN in DMPC (pH 3), respectively. It is clearly shown that pressure induces the correlation field splitting of the methylene scissoring mode in lipid systems (Figure 5A–C). Figure 6 compares the correlation field splitting of DMPC in the absence vs the presence of PRODAN. The splitting for pure DMPC at pH 6.8 takes place

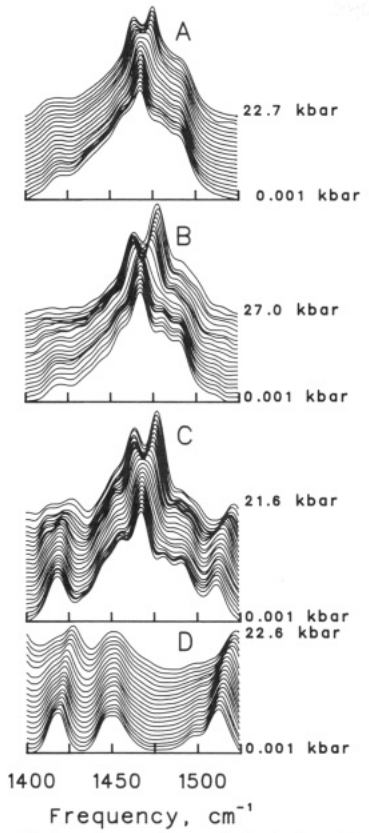

FIGURE 5: Stacked contour plots of the infrared spectra in the 1400–1525 cm^{-1} region. (A) Pure DMPC in D_2O; (B) 4% (w/w) PRODAN in DMPC at pH 6.8; (C) 4% (w/w) PRODAN in DMPC at pH 3.0; (D) pure PRODAN in D_2O.

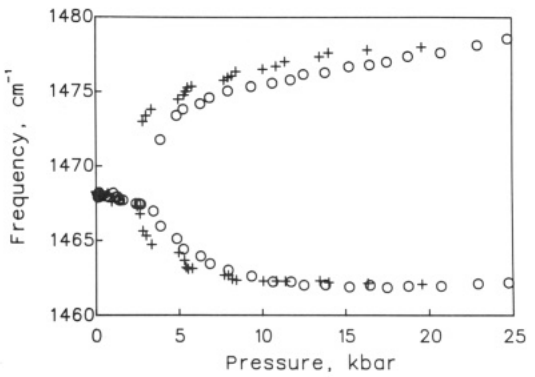

FIGURE 6: Pressure-induced correlation field splitting of the methylene scissoring mode in the sample of pure DMPC in D_2O (+) and 4% (w/w) PRODAN in DMPC at pH 6.8 (O).

at 3.2 kbar while the splitting for DMPC containing 4% PRODAN at the same pH occurs at 3.9 kbar. Figure 7 shows the case for egg PC. The splitting pressure for egg PC at pH 6.8 is 8.2 kbar whereas the splitting pressure for egg PC plus 4% PRODAN at the same pH is 9.2 kbar.

The correlation field splitting pressure increases in the presence of PRODAN, indicating that PRODAN molecules insert into both DMPC and egg PC lipid bilayers. It also indicates that the presence of PRODAN in the bilayers induces more orientational disorder of the acyl chains so that a higher pressure is required to completely damp the reorientational fluctuations (correlation field splitting occurs only when the reorientational fluctuations are completely damped). By comparing the splitting pressures for egg PC and egg PC plus PRODAN (Figure 7) with those for DMPC and DMPC plus PRODAN (Figure 6), it can be concluded that the lipid packing in egg PC, in the presence and absence of PRODAN,

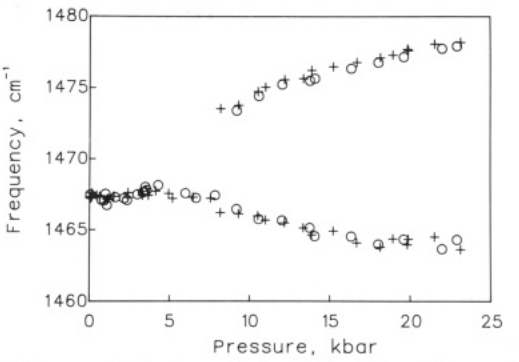

FIGURE 7: Pressure-induced correlation field splitting of the methylene scissoring mode in the sample of egg PC (+) and in 4% (w/w) PRODAN in egg PC at pH 6.8 (O).

is much less tight than the packing in DMPC. The implication, similar to that derived from the C=O stretching mode data, is that egg PC can accommodate more PRODAN molecules.

The change in the shape of the correlation field component bands of the methylene mode in the presence of PRODAN (Figure 5B,C) also indicates that PRODAN molecules are located in the bilayers under those experimental conditions. The relative intensity between the two correlation field component bands is larger in the presence of PRODAN (Figure 5B,C vs Figure 5A). Therefore, the angle between the zig-zag planes of neighboring fatty acyl chains becomes smaller in the presence of PRODAN.

Naphthalene Ring. The naphthalene ring of PRODAN can also be easily detected from the infrared absorption. Figure 5D shows the infrared spectra of pure PRODAN in D_2O. A strong naphthalene band appears at about 1510–1520 cm^{-1}. This band shifts to higher frequencies at elevated pressures. Figure 5A shows no naphthalene absorption in the same frequency region for pure DMPC in water. For the mixture of 4% PRODAN in DMPC at pH 6.8, the naphthalene band is essentially undetectable (Figure 5B), suggesting that the PRODAN naphthalene ring is embedded in lipid bilayers, which gives rise to the broadening of the naphthalene band due to the disordered environment in the interior of lipid bilayers. When the pH is lowered to 3.0, the naphthalene absorption band becomes detectable over the entire pressure range examined (Figure 5C), indicating that at pH 3, PRODAN molecules are excluded from the bilayer. These conclusions are consistent with those derived from the C=O stretching mode data and the correlation field splitting results mentioned earlier.

Pressure-Induced Phase Transition. The frequency of the methylene symmetric stretching mode (2850–2852 cm^{-1}) undergoes a dramatic change during the phase transition of lipids. Thus, pressure-induced liquid-crystal to gel-phase transitions of lipids can be monitored by the changes in frequencies in the range of 2850–2852 cm^{-1}. The phase transition starts at 0.2, 0.4, and 1.3 kbar for pure DMPC (Figure 8A), DMPC + 4% PRODAN at pH 6.8 (Figure 8B), and DMPC + 4% PRODAN at pH 3 (Figure 8C), respectively. The transition ends at 0.8 kbar for pure DMPC (Figure 8A), 1.4 kbar for DMPC plus 4% PRODAN at pH 6.8 (Figure 8B), and 1.7 kbar for DMPC plus 4% PRODAN at pH 3 (Figure 8C). Note that the phase transition of DMPC in the presence of PRODAN can also be monitored by the ratio of the fluorescence intensity at 435 nm to that at 510 nm (Chong, 1988). The onset and completion pressures for the phase transition of pure DMPC at 28 °C determined by the infrared absorption (Figure 8A) were found to be comparable to those

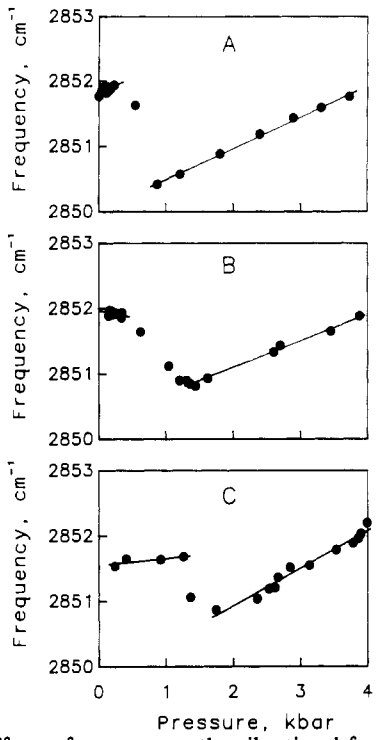

FIGURE 8: Effects of pressure on the vibrational frequency of the methylene symmetric stretching mode. (A) Pure DMPC; (B) 4% PRODAN in DMPC at pH 6.8; (C) 4% PRODAN in DMPC at pH 3.0.

obtained with the PRODAN fluorescence in the sample of DMPC containing a trace amount of PRODAN at 32 °C [Figure 2 in Chong (1988)].

The transition of DMPC in the presence of 4% PRODAN at pH 6.8 is broader (Figure 8B) than that of pure DMPC, indicating a decrease of cooperativity at the phase transition as a result of less tightly packed lipid bilayers disturbed by PRODAN. This would happen if PRODAN is embedded in the hydrophobic bilayer region at pH 6.8. In contrast, the transition of DMPC in the presence of 4% PRODAN at pH 3.0 is not so broad as the case at pH 6.8 (Figure 8C). This is taken to indicate that PRODAN is indeed excluded from the hydrophobic bilayer region at pH 3.0. Note that the infrared data of the C=O stretching mode (Figures 2 and 3), the correlation field splitting (Figures 5–7), and the naphthalene ring (Figure 5) have led to a similar conclusion. Moreover, the presence of PRODAN in lipid bilayers increases the transition pressure, which indicates that the presence of PRODAN also induces conformational disorder in individual acyl chains. In the sample of 4% PRODAN in DMPC at pH 3, most PRODAN molecules are supposed to be out of the lipid bilayers and located in the interfacial region (discussed earlier). However, the transition pressure becomes much higher (1.3 kbar), which indicates that the conformation of the acyl chains is extremely disordered and is much more disordered than that in the samples of pure DMPC (Figure 8A) and DMPC plus 4% PRODAN at pH 6.8 (Figure 8B). This can be interpreted as follows: PRODAN molecules are located between head groups of neighboring lipid molecules, which increases the space between the intermolecular acyl chains. Consequently, more gauche bonds are formed which result in an enlargement of the apparent diameter of the acyl chain. Thus, higher pressures are needed in order to convert all gauche bonds to trans configuration.

Implications and Concluding Remarks. It is demonstrated in this study that high-pressure infrared spectroscopy is a useful tool for identifying the location of fluorescent probes in membranes. A previous model (Chong, 1988) suggested that the PRODAN carbonyl group remains on the membrane surface in the pressure range of 0.001–2 kbar whereas the naphthalene ring and the dimethylamino group could either flip into the bilayer to adopt a "less polar" disposition or reside in the interfacial region to adopt a "polar" disposition. However, the present infrared data suggest that at pH 6.8, the entire PRODAN molecule, including the carbonyl group, penetrates into the lipid bilayer. The discrepancy may result from higher concentrations of PRODAN used in the infrared study. In spite of this discrepancy, both the fluorescence (Chong, 1988) and infrared studies show a similar result, that is, that PRODAN favors a more hydrophobic environment at elevated pressures and it is not squeezed out of lipid bilayers by high pressures.

The present study shows that the pressure dependence of the location of the charged PRODAN is distinctly different from that of the charged tetracaine (a local anesthetic) in lipid membranes (Auger, 1987). PRODAN, in its charged form, tends to stay outside the bilayers at all pressures while the charged tetracaine is squeezed out of the membrane by pressure (Auger et al., 1987). In spite of some structural differences, PRODAN and tetracaine bear similarities: an aromatic ring at the center, a methylated amino group on one side of the aromatic ring, and a carbonyl bond on the other side. Both compounds are lipid-miscible. It is then of interest to address in the future why tetracaine can be squeezed out of the membrane by pressure whereas PRODAN responds differently.

The apparent difference in the pressure dependence of the location in membranes between PRODAN and tetracaine may be linked to the nature of anesthetic–lipid interactions. Anesthetics can induce physiological effects on cells or organisms via interactions with membrane lipids (Trudell et al., 1973). Their effects depend on whether they can penetrate into the lipid bilayer (Seeman, 1972; Kamaya et al., 1981). On the other hand, pressure is known to be a universal antagonist against general (Roth, 1979) or local (Halsey & Wardley-Smith, 1975; Kendig & Cohen, 1977) anesthesia. The apparent phenomenon has been interpreted as the exclusion of anesthetics from membranes by pressure (Franks & Lieb, 1982). Recently this assertion has been substantiated by a high-pressure infrared study of a local anesthetic, tetracaine (Auger et al., 1987). However, to this end, it is not clear how pressure would affect the location of nonanesthetic hydrophobic molecules in membranes. Anesthetics (e.g., alphaxalone) and nonanesthetics (e.g., Δ^{16}-alphaxalone) interact differently with membrane lipids (Makriyannis et al., 1986). If there is a distinct difference in the pressure dependence of the location between anesthetics and nonanesthetics in lipid vesicles, the difference may be a useful diagnostic tool for screening anesthetic drugs and will also help us to understand the interactions between lipids and anesthetics. The present study clearly indicates a difference in the pressure dependence of membrane permeability between PRODAN (presumably a nonanesthetic) and tetracaine (an anesthetic) (Auger et al., 1987). However, more examples are needed in order to generalize the difference between the anesthetics and nonanesthetics.

REFERENCES

Auger, M., Jarrell, H. C., Smith, I. C. P., Wong, P. T. T., Siminovitch, D. J., & Mantsch, H. H. (1987) *Biochemistry 26*, 8513–8516.

Boerio, F. J., & Koenig, J. L. (1970) *J. Chem. Phys. 52*, 3425–3431.

Boulanger, Y., Schreier, S., & Smith, I. C. P. (1981) *Biochemistry 20*, 6824-6830.
Chong, P. L.-G. (1988) *Biochemistry 27*, 399-404.
Chong, P. L.-G., & Weber, G. (1983) *Biochemistry 22*, 5544-5550.
Franks, N. P., & Lieb, W. R. (1982) *Nature (London) 300*, 487-493.
Gennis, R. B. (1989) in *Biomembranes: molecular structure and function*, Chapter 7, pp 235-269, Springer-Verlag, New York.
Halsey, M. J., & Wardley-Smith, B. (1975) *Nature (London) 257*, 811-813.
Kamaya, H., Suezaki, Y., Ueda, I., & Eyring, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3572-3575.
Lakowicz, J. R., Cherek, H., Laczko, G., & Gratton, E. (1984) *Biochim. Biophys. Acta 777*, 183-193.
MacDonald, A. G. (1984) *Philos. Trans. R. Soc. London, B 304*, 47-68.
Macgregor, R. B., & Weber, G. (1981) *Ann. N.Y. Acad. Sci. 366*, 140-154.
Macgregor, R. B., & Weber, G. (1986) *Nature (London) 319*, 70-73.
Makriyannis, A., Siminovitch, D. J., Das Gupta, S. K., & Griffin, R. G. (1986) *Biochim. Biophys. Acta 859*, 49-55.
Massey, J. B., She, H. S., & Pownall, H. J. (1985) *Biochemistry 24*, 6973-6978.
Morrison, L. E., & Weber, G. (1987) *Biophys. J. 52*, 367-379.
Mushayakarara, E. C., Wong, P. T. T., & Mantsch, H. H. (1986) *Biochim. Biophys. Acta 857*, 259-264.
Parasassi, T., Conti, F., & Gratton, E. (1986) *Cell. Mol. Biol. 32*, 103-108.
Roth, S. H. (1979) *Annu. Rev. Pharmacol. Toxicol. 19*, 159-178.
Seeman, P. (1972) *Pharmacol. Rev. 24*, 583-655.
Siminovitch, D. J., Wong, P. T. T., & Mantsch, H. H. (1987) *Biophys. J. 51*, 465-473.
Snyder, R. G. (1961) *J. Mol. Spectrosc. 7*, 116-141.
Trudell, J. R., Hubbel, W. L., Cohen, E. N., & Kendig, J. J. (1973) *Anesthesiology 38*, 207-211.
Weber, G., & Farris, F. J. (1979) *Biochemistry 18*, 3075-3078.
Wong, P. T. T. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 1-24.
Wong, P. T. T. (1987a) in *High Pressure Chemistry and Biochemistry* (van Eldik, R., & Jonas, J., Eds.) pp 381-400, D. Reidel, Dordrecht, The Netherlands.
Wong, P. T. T. (1987b) *Vib. Spectra Struct. 16*, 357-445.
Wong, P. T. T., & Mantsch, H. H. (1988) *Chem. Phys. Lipids 46*, 213-224.
Wong, P. T. T., Moffatt, D. J., & Baudais, F. L. (1985) *Appl. Spectrosc. 39*, 733-735.

Formation of Free Radical Metabolites in the Reaction between Soybean Lipoxygenase and Its Inhibitors. An ESR Study

Jolanda Van der Zee, Thomas E. Eling, and Ronald P. Mason*
Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, North Carolina 27709

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ABSTRACT: Recent studies showed that soybean lipoxygenase inhibitors like phenidone and nordihydroguaiaretic acid (NDGA) reduce the catalytically active ferric lipoxygenase to its inactive ferrous form. Addition of 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD) regenerated the active ferric form. In this paper, it is shown that in such a system the inhibitors are oxidized to free-radical metabolites. Incubation of soybean lipoxygenase and linoleic acid with *p*-aminophenol, catechol, hydroquinone, NDGA, or phenidone resulted in the formation of the one-electron oxidation products of these compounds. Free-radical formation depended upon the presence of the lipoxygenase and 13-HPOD. The free radicals were detected by ESR spectroscopy, and their structure was confirmed by analysis of the spectra, using a computer correlation technique. These data support the proposed mechanism for the inhibition of lipoxygenase by phenolic antioxidants.

Lipoxygenases are dioxygenases that catalyze the hydroperoxidation of polyunsaturated lipids containing a *cis*-1,*cis*-4-pentadiene moiety (Vliegenthart & Veldink, 1982). For example, linoleic acid is converted by molecular oxygen and soybean lipoxygenase to 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD).[1] Interest in the mechanism and inhibition of lipoxygenases has been stimulated by the observation that 5-lipoxygenase has a pivotal role in the biosynthesis of leukotrienes. Leukotrienes play an important role in inflammation and immediate hypersensitivity reactions (Samuelsson, 1983).

Recent studies showed that incubation with NDGA, one of the most efficient inhibitors of lipoxygenases, reduces the catalytically active ferric soybean lipoxygenase to the inactive ferrous form (Kemal et al., 1987). Ferric lipoxygenase exhibits a characteristic ESR signal near $g = 6$, while the ferrous form of the enzyme is ESR silent (Slappendel et al., 1982). Treatment of the enzyme with NDGA resulted in the disappearance of the ESR signal, indicating that the iron is converted to its inactive ferrous state. This reaction, however, was reversible as addition of 13-HPOD to the reaction mixture regenerated the ferric state and restored catalytic activity. The oxidation of ferrous to ferric iron by 13-HPOD results in the

* Author to whom correspondence should be addressed.

[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; NDGA, nordihydroguaiaretic acid; DETAPAC, diethylenetriaminepentaacetic acid; BW 755C, 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline; phenidone, 1-phenyl-3-pyrazolidinone.