Esko, J. D., Gilmore, J. R., & Glaser, M. (1977) *Biochemistry 16*, 1881.
Ferguson, K. A., Glaser, M., Bayer, W. H., & Vagelos, P. R. (1975) *Biochemistry 14*, 146.
Gaffney, B. J., Willingham, G. L., & Schepp, R. S. (1983) *Biochemistry 22*, 881.
Gilmore, J. R. (1980) Ph.D. Thesis, University of Illinois, Urbana, IL.
Gilmore, R., Cohn, N., & Glaser, M. (1979) *Biochemistry 18*, 1042.
Glaser, M., Ferguson, K. A., & Vagelos, P. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4072.
Hale, A. H., Pessin, J. R., Palmer, F., Weber, M. J., & Glaser, M. (1977) *J. Biol. Chem. 252*, 6190.
Heiniger, H.-J., Kandutsch, A. A., & Chen, H. W. (1976) *Nature (London) 263*, 515.
Jameson, D. M., Williams, J. F. & Wehrly, J. A. (1977) *Anal. Biochem. 79*, 623.
Kates, M. (1972) *Techniques in Lipidology*, pp 335–336, American Elsevier, New York.
Lakowicz, J. R., & Hogen, D. (1977) in *Membrane Toxicity* (Miller, M. W., & Shamoo, A. E., Eds.) pp 509–546, Plenum Press, New York.
Lakowicz, J. R., & Hogen, D. (1980) *Chem. Phys. Lipids 26*, 1.
Lakowicz, J. R., Hogen, D., & Omann, G. (1977) *Biochim. Biophys. Acta 471*, 401.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Omann, G. M., & Lakowicz, J. R. (1981) *Pestic. Biochem. Physiol. 16*, 231.
Omann, G. M., & Lakowicz, J. R. (1982) *Biochim. Biophys. Acta 684*, 83.
Pessin, J. E., Salter, D. W., & Glaser, M. (1978) *Biochemistry 17*, 1997.
Saito, Y., Chou, S.-M., & Silbert, D. F. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3730.
Selinger, Z., & Lipidot, Y. (1966) *J. Lipid Res. 7*, 174.
Shinitzky, M., & Rivnay, B. (1977) *Biochemistry 16*, 982.
Smolochowski, M. (1917) *Z. Phys. Chem., Abt. A 92*, 129.
Spector, A. A., & Hoak, J. C. (1969) *Anal. Biochem. 32*, 277.
Spencer, R. D., & Weber, G. (1969) *Ann. N.Y. Acad. Sci. 158*, 361.
Stern, O., & Volmer, M. (1919) *Phys. Z. 20*, 183.
Wehrly, J., Williams, J., Jameson, D., & Kolb, D. (1976) *Anal. Chem. 48*, 1424.
Welti, R., Rintoul, D. A., Goodsaid-Zalduonodo, F., Felder, S., & Silbert, D. F. (1981) *J. Biol. Chem. 256*, 7528.
Williams, R. E., Wisnieski, B. J., Rittenhouse, H. G., & Fox, C. F. (1974) *Biochemistry 13*, 1969.
Wisnieski, B. J., Williams, R. E., & Fox, C. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3669.
Yang, S. F., Freer, S., & Berson, A. A. (1967) *J. Biol. Chem. 242*, 477.

Influence of Phospholipid Peroxidation on the Phase Behavior of Phosphatidylcholine and Phosphatidylethanolamine in Aqueous Dispersions†

Gert van Duijn,* Arie J. Verkleij, and Ben de Kruijff

ABSTRACT: The influence of oxygen-induced phospholipid peroxidation on the phase behavior of aqueous dispersions of both egg phosphatidylcholine (egg-PC) and egg phosphatidylethanolamine (egg-PE) has been investigated. Phospholipid peroxidation was followed via malondialdehyde formation and analyses of acyl chain compositions. ^{13}C nuclear magnetic resonance spectroscopy (NMR) and the amino-indicating probe trinitrobenzenesulfonic acid were used to study the effect of peroxidation on the chemical structure of hydrated egg-PE. The macroscopic organization of the phospholipids was monitored by ^{31}P NMR and small-angle X-ray diffraction. Differential scanning calorimetry was employed to study the influence of peroxidation on the thermotropic behavior of egg-PE. The results show that egg-PE is more sensitive to the effects of peroxidation than egg-PC. In the latter, no changes in the macromolecular organization were observed. However, peroxidation strongly influenced the polymorphic phase behavior of PE. Initial peroxidation stabilized hydrated egg-PE in a lamellar system up to 70 °C, presumably by modification of the head group. Such modifications were confirmed by ^{13}C NMR experiments, which indicated the formation of Schiff bases between PE head groups and aldehydes. Furthermore, quantitative analyses of trinitrobenzenesulfonic acid reactable egg-PE and the corresponding fatty acid compositions revealed the presence of cross-links between the ethanolamine head groups, likely involving the bifunctional malondialdehyde. Prolonged peroxidation of egg-PE resulted in a loss of order in the system, possibly by the formation of intermediate nonbilayer structures.

Lipid peroxidation in biological membranes and its consequences for different cellular processes are areas of growing interest [for a recent review, see Ramasarma (1982)]. The complexity of the various peroxidation mechanisms and the resulting physiological changes, which often appear to involve changes in membrane function, has resulted during the last decade in intensive studies on the peroxidation of phospholipids in more simple, model membrane systems.

Considerable progress has been made concerning the oxygen radical initiation and the mechanism of peroxidative reactions (Frankel, 1980; Porter et al., 1980a). The chemical structure of different products derived from peroxidized phosphati-

† From the Institute of Molecular Biology, State University of Utrecht, Transitorium 3, Padualaan 8, 3508 TB Utrecht, The Netherlands. *Received January 26, 1984.*

0006-2960/84/0423-4969$01.50/0

dylcholines (PC's)[1] with various fatty acid compositions has been elucidated (Porter et al., 1980b; Wu et al., 1982). Apparently, there is a dramatic shift from the cis–cis configuration to the trans–cis configuration and, after β-scission, to the trans–trans configuration in the lipid hydroperoxides (Porter & Lehman, 1982) of phospholipids from natural sources.

The rate of lipid peroxidation is dependent on the degree of unsaturation in the acyl chains. Corliss & Dugan (1970) have found that the addition of one extra double bond in a fatty acid doubles the rate of peroxidation. Furthermore, the same authors noticed that PE is more sensitive to peroxidation than PC and shows a decreased reactivity toward an amino-indicating probe like trinitrobenzenesulfonic acid during lipid peroxidation. It has been proposed that decomposition products of lipid hydroperoxides (malondialdehyde and other thiobarbituric acid positive products) may form Schiff bases with amino groups (Gutteridge et al., 1982) and induce cross-linking between aminophospholipids, proteins, and nucleic acids (Kergonou et al., 1982).

The effects of lipid peroxidation on the functional properties of the membrane have also been investigated. First, the transbilayer movement of phospholipids in sonicated PC vesicles was found to be enhanced during lipid peroxidation (Shaw & Thompson, 1982). It has been suggested that products of lipid peroxidation promote phospholipid flip-flop via the formation of nonbilayer structures (Barsukov et al., 1980). Second, Gast et al. (1982) discussed the role of PC hydroperoxides as fusogens. During autoxidation they measured an increase in the mean diameter of PC vesicles using quasi-elastic light scattering and electron microscopy.

Molecular interpretation of these results is greatly hampered by the lack of knowledge of the effect of peroxidation on the molecular dynamics of phospholipids in model membranes. During lipid peroxidation in aqueous dispersions of the polar lipids of broad bean leaves, Galanopoulou et al. (1982) noticed a considerable restriction of the motion of the probe in the hydrocarbon phase when using fluorescence polarization. This was explained by the presence of cross-links between the peroxidized lipids. Bruch & Thayer (1983), using electron spin resonance, also determined a differential decrease in the bilayer fluidity of soybean phospholipid vesicles. The most predominant increase in order parameter, registered in the case of a spin-label at the 12-position of a fatty acid, was interpreted by these authors as a consequence of the loss of unsaturation in this region of the acyl chains during lipid peroxidation. Despite these findings, virtually nothing is known about the influence of lipid peroxidation on the macroscopic organization of membrane phospholipids. This question becomes even more pertinent in relation to lipid polymorphism and the effects of peroxidation on the functional aspects of membranes. It has been demonstrated that under physiological conditions many membrane lipids can adopt nonbilayer lipid structures such as the hexagonal (H_{II}) phase. PE is a good example of such a lipid; it can undergo a reversible transition from lamellar to hexagonal (H_{II}) phase depending on pH and temperature. At physiological temperatures, hydrated unsaturated PE's typically prefer the hexagonal (H_{II}) phase (Cullis & de Kruijff, 1978a; Rand et al., 1971). The possible role of nonbilayer structures in events such as the transbilayer movement of phospholipids and membrane fusion has been discussed in detail (Cullis & de Kruijff, 1979; de Kruijff et al., 1980).

The purpose of this study was, therefore, to investigate the influence of oxygen-induced phospholipid peroxidation on the phase behavior of aqueous dispersions of both PC and PE. The structural organization and thermotropic behavior of the phospholipids were monitored by ^{31}P NMR, small-angle X-ray diffraction, and differential scanning calorimetry as a function of lipid peroxidation. Peroxidation was followed via the rate of formation of malondialdehyde and analyses of the fatty acid composition. The combination of ^{31}P NMR and X-ray diffraction can give reliable information about the polymorphism in phospholipid–water systems (Cullis & de Kruijff, 1978b; Reiss-Husson, 1967; Luzzati et al., 1966). The effect of autoxidation on the chemical structure of hydrated PE was monitored by ^{13}C NMR and by a determination of the percentage of free amino groups.

Materials and Methods

Purification of Phospholipids. PC and PE were purified from hen egg yolk. After precipitation of protein and lipid with acetone according to Pangborn (1951), the total lipid was extracted three times with chloroform/methanol (2:1 by volume). The crude extract was evaporated under reduced pressure and the residual lipids were separated by partition chromatography on silica (Polygosil, 63–100-μm particle size, purchased from Mackerey, Nagel Duren) in $CHCl_3$/MeOH/H_2O/NH_3 (40:60:2:2 by volume) according to Dekker et al. (1983). Finally, the crude PC and PE fractions were purified by high-performance liquid chromatography using a 500 × 50 mm column filled with Polygosil (5–20-μm particle size) with a mobile phase of $CHCl_3$/MeOH/H_2O/NH_3 (65:35:4:1 by volume in the case of PC and 68:28:2:2 by volume in the case of PE).

All purification steps were carried out in a nitrogen atmosphere. In addition, hydroquinone (purchased from Sigma) was added as an antioxidant to an amount of 0.1% by weight of the lipid. The purified lipids were stored as stock solutions of approximately 50 mM in hexane/ethanol (1:1 by volume) at −20 °C under nitrogen. Before the phospholipids were used, the antioxidant was removed via adsorption chromatography on silica in $CHCl_3$/MeOH (9:1 by volume) and the phospholipids were eluted in $CHCl_3$/MeOH (7:3 by volume) under a stream of nitrogen.

All lipids were chromatographically pure as judged by thin-layer chromatography using HPTLC plates with $CHCl_3$/MeOH/H_2O/NH_3 (68:28:2:2 by volume) as the eluent.

Liposome Preparation. Lipids, dissolved in $CHCl_3$/MeOH (7:3 by volume), were dried by evaporation under reduced pressure. Residual solvent was removed under high vacuum over 16 h. The lipids were dispersed by vortexing at 4 °C under nitrogen in a buffer containing 100 mM NaCl and 10 mM Pipes/NaOH (pH 7.0). The lipid concentration was approximately 70 mM, determined by the method of Böttcher et al. (1961).

Lipid Peroxidation Assay. Peroxidation was initiated at 40 °C in a shaking water bath by passing a continuous flow of oxygen (0.3 kgf/cm^2) through a glass tube (10 × 1.5 cm) into the phospholipid dispersions (70 mM). In control experiments the phospholipid dispersions were incubated under nitrogen in a closed glass tube.

At various times aliquots were removed and used for different analytical and physicochemical measurements. Lipid peroxidation was assayed by the thiobarbituric acid method (Fukuzawa et al., 1982). Absorbance values measured at 532 nm were used for the calculation of the malondialdehyde

[1] Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; NMR, nuclear magnetic resonance; HPTLC, high-performance thin-layer chromatography; Pipes, 1,4-piperazinediethanesulfonic acid; DSC, differential scanning calorimetry.

Table I: Fatty Acid Compositions of Egg-PC and Egg-PE and Time-Dependent Decrease of Different Fatty Acids (mol %) during Incubation at 40 °C under Oxygen or Nitrogen Atmosphere[a]

	egg-PC			egg-PE				
		decrease (mol %)			decrease (mol %)			
					O_2 incubation			N_2 incubation,
fatty acid	composition (mol %)	O_2 incubation, 24 h	N_2 incubation, 24 h	composition (mol %)	4 h	6 h	24 h	24 h
C16:0	37.3 ± 1.6	0	0	21.0 ± 1.2	0	0	0	0
C18:0	11.4 ± 1.3	0	−0.3	27.5 ± 1.2	0	0	0	0
C18:1	33.9 ± 1.5	1.4	0.3	20.1 ± 2.3	0.1	0.2	1.3	−0.5
C18:2	14.6 ± 0.8	1.5	0	10.4 ± 0.7	0.1	0.9	1.6	0.2
C20:4	2.8 ± 0.3	0.9	0	13.9 ± 0.6	0.2	2.4	5.2	−0.3
C22:5				3.0 ± 0.6	1.1	0.5	0.9	0.1
C22:6				4.3 ± 0.8	0.8	1.3	0.6	0.3

[a] The changes in fatty acid composition are related to the amount of palmitic acid, which was found to be constant during the incubation. The means and standard deviations of at least three experiments are shown.

formation with a molar absorption coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ (Buege & Aust, 1978). The amount of free amino groups in peroxidation experiments involving PE was determined with trinitrobenzenesulfonic acid according to Siakotos (1967) with the modification that 50 μL of the phospholipid dispersion was first diluted with 450 μL of $CHCl_3$/MeOH (2:1 by volume). To 50 μL of this solution (containing 0.2–0.5 μmol of lipid) was added 950 μL of methanol.

The fatty acid composition of the phospholipids was determined after formation of the fatty acyl methyl esters. Phospholipid dispersions (50 μL) were incubated with 15 mL of 5% sulfuric acid in methanol for 2 h at 70 °C. Methyl esters were extracted with hexane and separated by gas chromatography on a Packard Becker 419 gas chromatograph equipped with a 1.8-m column (2 mm in diameter) filled with 12% poly(ethylene glycol) adipate on Gaschrom Q, at a temperature of 190 °C. Quantitative changes in acyl chain compositions were calculated with myristic acid as an internal standard. This was added as dimyristoyl-PC to the phospholipid dispersion in a 1:4 molar ratio prior to transesterification.

Nuclear Magnetic Resonance (NMR). Proton noise decoupled (18-W input power) ^{31}P NMR spectra were obtained with a Bruker WH-90 spectrometer operating at 36.4 MHz. Free induction decays were accumulated from 10000 to 20000 transients by employing a 9-μs 40° radio-frequency pulse, a 12-kHz sweep width, and a 0.17-s interpulse delay. An exponential multiplication corresponding to 50-Hz line broadening was applied to the accumulated free induction decays prior to Fourier transformation.

Samples for the ^{31}P NMR experiments were prepared by mixing 800 μL of the phospholipid dispersion with 200 μL of a buffer containing 100 mM NaCl and 10 mM Pipes/NaOH in D_2O (pH 7.0). The tubes were hermetically sealed under nitrogen. Natural abundance ^{13}C NMR spectra were recorded at 30 °C on a Bruker WP-200 spectrometer operating at 50.3 MHz. Accumulated free induction decays were obtained from 15000 to 25000 transients; a spectral width of 10 kHz, a 90° radio-frequency pulse (18 μs), and a 0.9-s interpulse time were used in the presence of continuous broad-band proton decoupling (2-W input power). The signal to noise ratio of the spectrum was increased by an exponential multiplication of the accumulated free induction decays, resulting in a 5-Hz line broadening.

The phospholipids for the ^{13}C NMR measurements were extracted from the liposomes according to Bligh & Dyer (1959) and were subsequently dissolved in deuterated chloroform (70 mM).

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry was carried out on a Perkin-Elmer DSC-2 calorimeter as described by Van Dijck et al. (1976). Heating scans were recorded at a scanning rate of 5 °C/min. The enthalpy of the gel to liquid-crystalline transition of PE was calculated by integrating the peaks obtained with a Hewlett-Packard digitizer (type 9864A) and by using dipalmitoyl-PC (8.0 kcal/mol of DPPC) as a reference (Petri et al., 1980).

X-ray Diffraction. Small-angle X-ray diffraction profiles were obtained by using a Kratky camera with a 10 × 0.2 mm Cu Kα beam (40 kV, 20 mA) equipped with a position-sensitive detector (LETI). An entrance slit of 100 μm was used. The exposure time for each measurement was 5–15 min. Hydrated phospholipid samples were mounted in a temperature-controlled slit (16 × 1.5 × 1.5 mm) between two sheets of cellophane.

Chemicals. Thiobarbituric acid, trinitrobenzenesulfonic acid, and methylbenzethonium hydroxide were obtained from Sigma. Chloroform and methanol were distilled before used. Other reagents were of analytical grade.

Results

Phospholipid Peroxidation Measurements. Oxygen-initiated peroxidation of phospholipids was measured first via the formation of thiobarbituric acid reactive material. Both egg-PC and egg-PE showed a considerable amount of thiobarbituric acid reactive material immediately after dispersing the lipids in buffer. This is most likely caused by the presence of endogenous hydroperoxides in the phospholipids and not due to an artifact in the assay since freshly prepared dipalmitoyl-PC and dioleoyl-PC dispersions did not show any significant thiobarbituric acid positive reaction (data not shown). In control experiments (phospholipid dispersions stored under nitrogen at 40 °C), there was no increase in malondialdehyde formation during 24 h (Figure 1). In contrast, there was a substantial increase in malondialdehyde formation for both phospholipids in the presence of oxygen. This reached maximal levels after 6–8 h of incubation (Figure 1). PE is apparently more sensitive to peroxidation than PC, as demonstrated by the difference in maximal malondialdehyde formation.

Alternatively, the peroxidation of phospholipids was followed by comparing the fatty acid composition at different time points during incubation with oxygen. The absolute amount of palmitic acid was found to be constant during 24 h of peroxidation incubation (data not shown). In Table I the disappearances of unsaturated fatty acids are expressed relative to the amount of palmitic acid. After 24 h of lipid peroxidation the total disappearance of unsaturated fatty acids was 3.8% in aqueous dispersions of PC and 9.6% in PE. In control experiments under nitrogen, no significant changes in the fatty

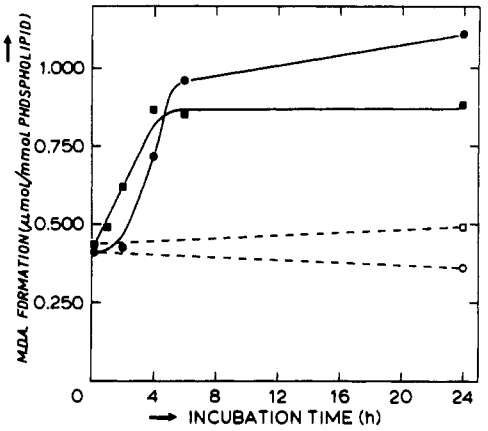

FIGURE 1: Time-dependent malondialdehyde formation in aqueous dispersions of egg-PC and egg-PE at 40 °C. The points plotted are averages of duplicate determinations in PC and of at least three measurements in the case of PE. Egg-PC under O_2 (■); egg-PC under N_2 (□); egg-PE under O_2 (●); egg-PE under N_2 (○).

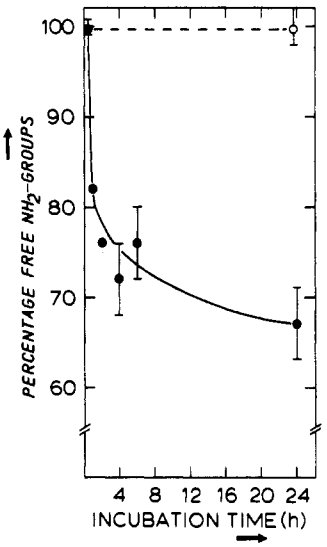

FIGURE 2: Time-dependent reactivity of egg-PE toward trinitrobenzenesulfonic acid (TNBS) during incubation at 40 °C under an oxygen (●) or a nitrogen atmosphere (○). The error bars indicate the standard deviations.

acid compositions were measurable (Table I).

The relative loss of oleic acid, linoleic acid, and arachidonic acid in PE dispersions after a 24-h incubation amounted to 6.5%, 15.4%, and 37.4%, respectively. In PC liposomes, the values were comparable (4.1%, 10.3%, and 32.1%, respectively). Therefore, the decrease in absolute amounts of the different unsaturated fatty acids during lipid peroxidation was proportional to the degree of unsaturation and is in agreement with previous published results (Wu et al., 1982).

Effect of Lipid Peroxidation on Head Group Structure in PE. The ethanolamine head group of PE has also been shown to be very sensitive toward lipid peroxidation (Corliss & Dugan, 1970). Figure 2 shows the time-dependent decrease of trinitrobenzenesulfonic acid reactive head groups in PE model membranes during lipid peroxidation. After 24 h of incubation under oxygen at 40 °C, about 33% of the ethanolamine head groups were modified in such a way that they were no longer reactive toward the amino-indicating probe. However, the most dramatic disappearance of free amino groups was monitored during the first 2 h of lipid peroxidation. In control experiments (PE stored under nitrogen at 40 °C for 24 h), all of the PE head groups remained trinitrobenzenesulfonic acid positive.

Table II: Numbering and Positions of ^{13}C NMR Resonances Obtained from Egg-PE[a]

$CH_3CH_2CH_2CH_2(CH_2)_m(CH_2CH_2CH=CHCH_2CH_2)(CH_2)_nCH_2CH_2CH_2CO_2-$

ω-1 ω-3 γ α β n 3 1

ω ω-2 m β α γ 4 2

^{13}C resonance assignments		^{13}C resonance assignments	
resonance	position	resonance	position
1	1 (*sn*-1; *sn*-2)	10	2
2	α	11	ω - 2
3	CH=CHCH$_2$CH=CH	12	m; n
		13	4; ω - 3
4	$CDCl_3$	14	β
5	CHOCOR	15	CH=CHCH$_2$CH=CH
6	POCH$_2$CH	16	3
7	CH$_2$OCOR	17	ω - 1
8	CH$_2$CH$_2$N	18	ω
9	CH$_2$N		

[a] The positions of the fatty acid carbon atoms are given for oleic acid as an example.

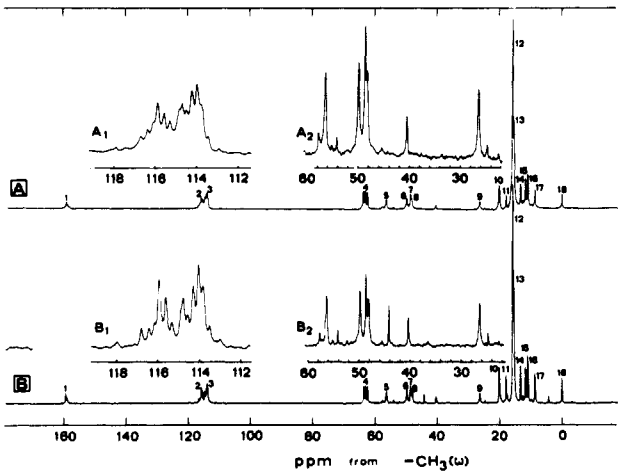

FIGURE 3: Proton-decoupled 50.3-MHz natural abundance ^{13}C NMR spectra obtained from egg-PE in $CDCl_3$ before (A) and after (B) 24 h of lipid peroxidation at 40 °C in aqueous dispersions. A-1 and B-1, expanded ^{13}C NMR spectra of the olefinic region of egg-PE in $CDCl_3$ before (A-1) and after (B-1) 24 h of incubation under oxygen; A-2 and B-2, expanded ^{13}C NMR spectra of the glycerophosphatidylethanolamine head group region of egg-PE in $CDCl_3$ before (A-2) and after (B-2) lipid peroxidation (24 h). (For experimental details, see Materials and Methods.) The resonances were assigned according to Birdsall et al. (1975) (see also Table II) using the correction $\delta(CH_3) = \delta(Me_4Si) + 14.26$ and are expressed in parts per million downfield from the terminal ($-CH_3$) methyl carbon atom. The origin of the resonance at 40.6 ppm is not known.

To gain insight into the nature of the chemical modification of the ethanolamine head group of PE during lipid peroxidation, ^{13}C NMR experiments were performed. The natural abundance ^{13}C NMR spectrum of egg-PE in deuterated chloroform was recorded after extraction of the phospholipid from a freshly prepared aqueous dispersion (Figure 3A). The different resonances are assigned in Table II. The most interesting parts of this spectrum, the olefinic region and the glycerophosphoethanolamine region, are expanded in parts A-1 and A-2 of Figure 3, respectively.

Figure 3B shows the ^{13}C NMR spectrum of egg-PE in deuterated chloroform, extracted from the hydrated sample after 24 h of lipid peroxidation. Apparently, two new resonances are present in this spectrum, one at a position 4.1 ppm and the other at a position 44.2 ppm downfield from the terminal methyl group C resonance (Figure 3B-2). Changes in the intensity of the signal derived from the α-carbon atom in the ethanolamine head group as a result of lipid peroxidation were quantitated relative to the glycerol C-2 atom resonance

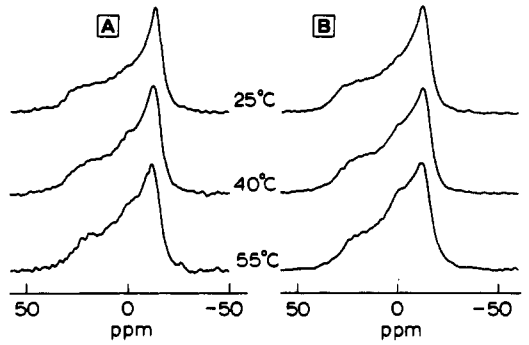

FIGURE 4: Proton-decoupled 36.4-MHz ^{31}P NMR spectra of an aqueous dispersion of egg-PC before (A) and after (B) 24 h of lipid peroxidation.

intensity (Figure 3, peak positions 9 and 5), assuming that the latter was not affected by peroxidation. After 24 h of incubation under oxygen, the resonance intensity of the ethanolamine α-carbon atom was lowered to 73% of the initial signal strength. The new carbon resonance peak at 44.2 ppm reached an intensity of 27% as related to the glycerol C-2 atom resonance. This peak position (44.2 ppm) is typical of a —C—N= carbon atom in a Schiff base (Martin et al., 1976). These results, together with the decrease of reactivity of the ethanolamine head group toward trinitrobenzenesulfonic acid, strongly suggest the formation of Schiff bases between about 30% of the PE molecules and aldehydes (e.g., malondialdehyde) derived from the peroxidized acyl chains.

The origin of the second new resonance at 4.1 ppm is not known. The olefinic region of the peroxidized PE spectrum (expanded in Figure 3B-1) shows a 10% decrease in relative signal intensity compared with the corresponding region in the nonperoxidized PE spectrum, with the glycerol C-2 atom resonance as a reference.

After 24 h of peroxidation, the phospholipids were analyzed by thin-layer chromatography. The peroxidized phospholipids showed an elongated spot on HPTLC plates. When the spectrum was visualized with 20% sulfuric acid, with $CHCl_3/MeOH/NH_3/H_2O$ (68:28:2:2 by volume) as the eluent, no lyso compounds were detected (data not shown).

Phospholipid Organization. Freshly prepared egg-PC liposomes are organized in extended bilayers over a large temperature range, as evidenced by the low-field shoulder and high-field peak (Cullis et al., 1976) separated by approximately 40 ppm (Figure 4A) in the ^{31}P NMR spectrum. Figure 4B shows the ^{31}P NMR spectra of a PC dispersion after 24 h of lipid peroxidation. As shown in Figure 4, the peroxidation did not affect the ^{31}P NMR characteristics of the lamellar PC system during the 24-h incubation. Only a very small isotropic peak was visible in the ^{31}P NMR spectra obtained from the peroxidized PC liposomes (Figure 4B). This might be due to the formation of some smaller vesicles caused by the mechanical agitation of the dispersions during the incubation. In addition, the effective residual chemical shift anisotropy, which is the distance between the low-field shoulder and the high-field peak and which is a measure of the local order of the phosphate region (Seelig, 1978), was not affected by the peroxidation process.

Fully hydrated egg-PE molecules showed a polymorphic phase behavior, as monitored by ^{31}P NMR and small-angle X-ray diffraction. Figure 5 shows the temperature-dependent shift from the ^{31}P NMR characteristics of a lamellar system to line shapes with a reversed asymmetry and a reduced line width, typical of phospholipid molecules organized in a hexagonal (H_{II}) phase (Cullis & de Kruijff, 1976). A freshly

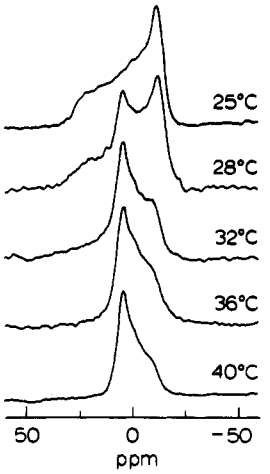

FIGURE 5: ^{31}P NMR spectra of fully hydrated egg-PE as a function of temperature. The phospholipids were dispersed at 4 °C under nitrogen.

prepared egg-PE dispersion shows this phase transition in the temperature region of 25–40 °C, which is in agreement with previous data (Cullis & de Kruijff, 1978a). Small-angle X-ray diffraction measurements of the PE dispersion revealed one diffraction band with a d value of 51.7 Å at 20 °C (Figure 6A), indicating a repeat distance in this lamellar system of 51.7 Å. At 30 °C the small-angle X-ray diffraction profiles of the PE system showed four diffraction bands (Figure 6B) at 62.2, 47.2, 34.7, and 30.1 Å, respectively. At this temperature a lamellar phase and a hexagonal (H_{II}) phase coexist, as has also been revealed by ^{31}P NMR (Figure 5). The 47.2-Å reflection corresponds to the lamellar phase. The other three d values show a $1:(1/\sqrt{3}):0.5$ relationship and represent the first-order and higher order diffractions of a hexagonally organized system. The distance between the hexagonal (H_{II}) tubes is 69.4 Å. Figure 6C shows three diffraction bands with d values of 58.3, 33.9, and 29.4 Å, obtained from a PE dispersion at 40 °C. The distance between tubes in this pure hexagonally organized phospholipid system is 67.8 Å.

In PE dispersions undergoing peroxidation, rather dramatic changes were monitored during phospholipid peroxidation by ^{31}P NMR and X-ray diffraction. Figure 7 shows the peroxidation time-dependent changes in the ^{31}P NMR spectra of a PE dispersion. Before the incubation under oxygen was begun, all PE molecules were organized in bilayers at 25 °C and in a hexagonal (H_{II}) phase at 40 °C. After 2 h of peroxidation, a ^{31}P NMR signal was observed at 40 °C, which is typical of a mixture of a lamellar and a hexagonal phase. After a pure H_{II}-phase spectrum (Figure 8C) was subtracted from this "mixed-phase" spectrum (Figure 8A), the contribution of the bilayer-forming phospholipids was calculated by an integration of the residual spectrum (Figure 8B). At this level of peroxidation about 25% of the PE molecules were still oriented in a hexagonal (H_{II}) phase at 40 °C. The bilayer-stabilizing effect of lipid peroxidation in PE was complete after 6 h of incubation (Figure 7). ^{31}P NMR spectra typical of a hexagonal II organization were not observed at temperatures lower than 70 °C (data not shown). In control experiments the hexagonal (H_{II}) phase was still present at 40 °C after 24 h of incubation under nitrogen (Figure 7).

Both at 25 °C and at 40 °C, the ^{31}P NMR spectra, typical of phospholipid molecules organized in a lamellar system, contained a small isotropic peak. This isotropic signal became more dominant after 24 h of peroxidation.

In the small-angle X-ray diffraction profile of the (24-h) peroxidized PE no defined diffraction bands could be observed

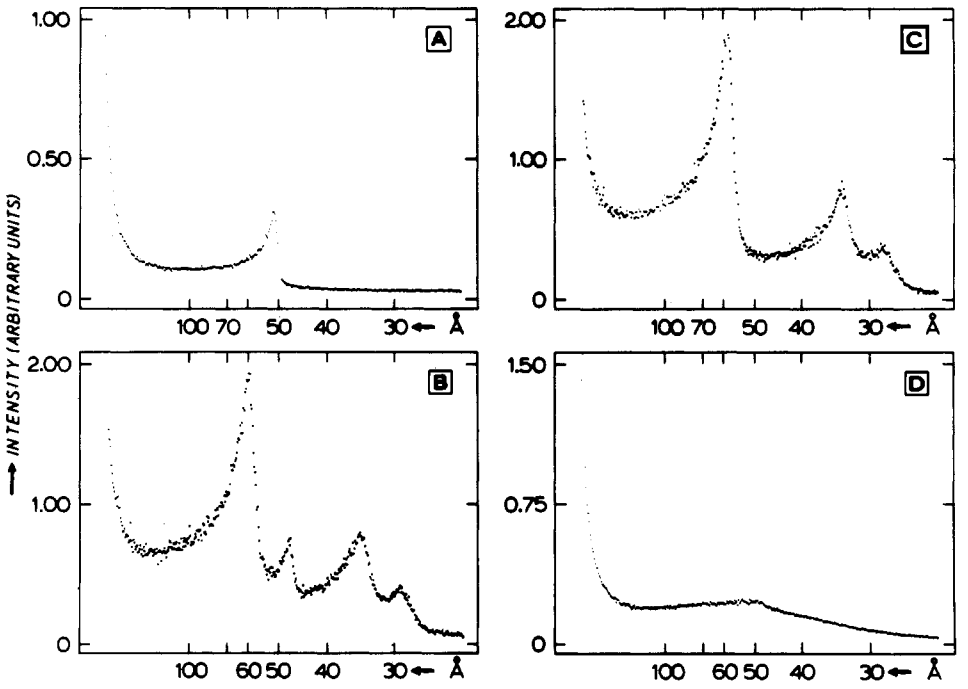

FIGURE 6: Small-angle X-ray diffraction profiles of hydrated egg-PE as a function of temperature (A–C) and after lipid peroxidation (D). A, egg-PE at 20 °C; B, egg-PE at 30 °C; C, egg-PE at 40 °C; D, egg-PE at 40 °C after 24 h of incubation under oxygen.

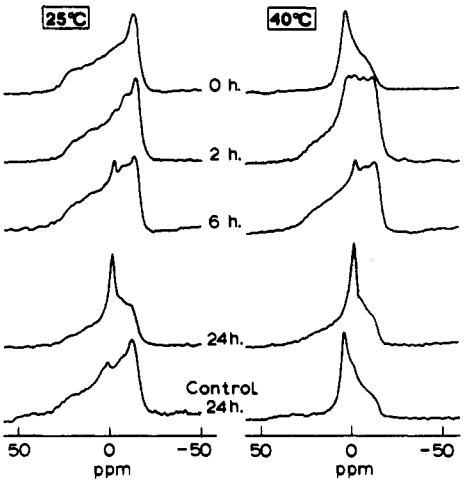

FIGURE 7: Temperature-dependent proton-decoupled 36.4-MHz ^{31}P NMR spectra obtained from aqueous dispersions of egg-PE as a function of the time the lipid dispersion was incubated under an oxygen atmosphere. The sample for the control measurements was stored under nitrogen for 24 h at 40 °C.

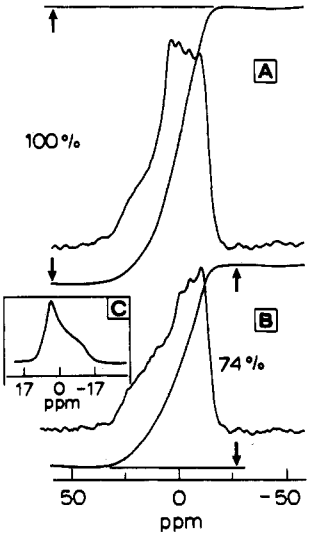

FIGURE 8: (A) Proton-decoupled and integrated 36.4-MHz ^{31}P NMR spectrum recorded at 40 °C, obtained from an aqueous dispersion of egg-PE after 2 h of lipid peroxidation. (C) "Pure hexagonal" type ^{31}P NMR spectrum obtained from a freshly prepared egg-PE dispersion at 40 °C. (B) 36.4-MHz ^{31}P NMR spectrum obtained from an aqueous dispersion of egg-PE after 2 h of lipid peroxidation and subsequent subtraction of the "pure hexagonal" type ^{31}P NMR spectrum obtained from a freshly prepared egg-PE dispersion at 40 °C.

at 40 °C but, instead, a broad (*d* value ranging from 40 to 100 Å) diffuse scattering profile was observed (Figure 6D).

Calorimetric Measurements. The calorimetric properties of aqueous dispersions of egg-PE are illustrated in Figure 9A. The gel to liquid-crystalline transition was observed over a broad temperature range (−4 to 18.5 °C), which is in agreement with previous published results (Cullis & de Kruijff, 1978a), and had a heat content of 4.9 ± 0.9 kcal/mol of phospholipid. Figure 9B shows the heating scan (5 °C/min) of PE after 24 h of incubation.

During lipid peroxidation both the transition temperature range and the endothermic enthalpy of the gel to liquid-crystalline transition changed. The broad transition shifted to a higher temperature range (3–23 °C), and the associated enthalpy change decreased to 2.0 ± 0.4 kcal/mol of phospholipid.

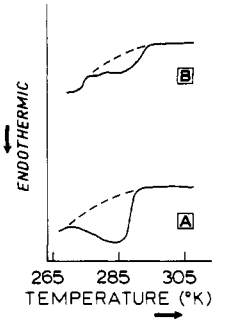

FIGURE 9: DSC heating curves of an aqueous dispersion of egg-PE before (A) and after (B) 24 h of lipid peroxidation.

Discussion

The aim of the present study was to establish whether

peroxidation of aqueous dispersions of egg-PC and egg-PE leads to changes in macroscopic structure and thermotropic behavior of the phospholipids.

Quantitative measurements of peroxidation in phospholipid dispersions revealed that egg-PE is more sensitive to peroxidation than egg-PC. The higher level of malondialdehyde in PE dispersions during lipid peroxidation is most likely due to the higher degree of unsaturation in the acyl chains. In addition, egg-PE showed a decreased reactivity toward the amino-indicating probe trinitrobenzenesulfonic acid during lipid peroxidation such that, after 24 h of peroxidation, only 67% of the PE head groups reacted with trinitrobenzenesulfonic acid. This result is in agreement with previously published data (Corliss & Dugan, 1970) and indicates structural modifications in the ethanolamine head group. This view is supported by ^{13}C NMR experiments that revealed a 27% decrease in intensity of the resonance derived from the α-carbon atom of the ethanolamine head group and the concomitant appearance of a new resonance at a position indicative of the formation of a Schiff base involving the amino group of PE. Assuming that each peroxidized polyunsaturated acyl chain can give rise to the formation of one dialdehyde, the decreased amount of polyunsaturated acyl chains in PE after 24 h of peroxidation (8.3%) would correspond with a 16.6% decrease in reactivity of the phospholipid toward trinitrobenzenesulfonic acid if all malondialdehydes form Schiff bases with ethanolamine head groups. However, the 33% loss of reactivity of PE toward the amino-indicating probe, as measured after 24 h of incubation under oxygen, indicates cross-linking between PE head groups via the bifunctional malondialdehyde.

Phospholipid peroxidation did not significantly affect the molecular organization of egg-PC molecules in liposomes during 24 h, as demonstrated by ^{31}P NMR (Figure 4). The ^{31}P NMR line shape, typical of phospholipid molecules experiencing restricted anisotropic motion in bilayers, changed little during lipid peroxidation. The very small (maximal 2%) isotropic signal, obtained after 24 h of incubation under oxygen, is probably caused by the formation of some smaller vesicles as a result of prolonged shaking of the sample. It has to be realized, however, that such isotropic ^{31}P NMR signals alone cannot give unambiguous information about the macromolecular structure of phospholipids. Therefore, the possible existence of small amounts of intermediate nonbilayer structures (e.g., inverted micelles), which have been proposed previously to be responsible for the enhanced phospholipid transbilayer movement in PC liposomes after peroxidation (Barsukov et al., 1980), cannot be excluded.

In contrast to the results obtained with egg-PC, phospholipid peroxidation strongly influenced the polymorphic phase behavior of egg-PE in aqueous dispersions. In agreement with previously published data (Cullis & de Kruijff, 1978a; Rand et al., 1971) both ^{31}P NMR and small-angle X-ray diffraction measurements revealed the transition from a lamellar organization at 25 °C to a hexagonal (H_{II}) phase at 40 °C in a freshly prepared egg-PE dispersion. After 2 h of incubation under oxygen about 75% of the PE molecules were organized in bilayers at 40 °C. At this level of peroxidation 20% of the ethanolamine head groups have been modified or cross-linked with malondialdehyde. After 6 h of peroxidation, the bilayer stabilization in the PE system below 70 °C was complete, while 74% of all PE molecules still contained a free ethanolamine head group.

The structural modifications can be related to the polymorphic phase behavior of egg-PE in terms of the shape–structure model of Cullis & de Kruijff (1979). This model relates the overall dynamic molecular shape of a phospholipid to the macroscopic structure adopted by this lipid in aggregates in aqueous media. With respect to the peroxidation experiments, it is useful to consider the dynamic shape of the PE molecule at two levels. First, with regard to the polar region, it can be noticed that the small size of the ethanolamine head group, its low hydration, and the possibility of intermolecular hydrogen bonding will tend to reduce the area of the molecules at the lipid–water interface, resulting in cone-shaped molecules compatible with an organization in a hexagonal H_{II} phase. Second, unsaturation will increase the cross-sectional area of the acyl chains, thereby enhancing the cone character of the molecule. It is now well documented that increasing chain unsaturation will lower the bilayer–hexagonal (H_{II}) phase transition temperature (Dekker et al., 1983). The bilayer-stabilizing effect of peroxidation in PE dispersions during the first 6 h of incubation can be explained on the basis of the molecular shape concept. Peroxidation of PE will result in a decreased amount of polyunsaturated acyl chains and will give rise to the appearance of trans–cis and trans–trans configurations in the acyl chains (Porter & Lehman, 1982), thereby decreasing the cross-sectional area of the apolar part of the molecule. However, from the differences in transition temperatures of cis- and trans-unsaturated PE (Tilcock & Cullis, 1982) and the effect of polyunsaturated acyl chains on the bilayer to hexagonal transition of mixed-acid PE species (Dekker et al., 1983), it can be estimated that this will not result in an increase in transition temperature of at least 40 °C at this level of peroxidation. Therefore, this effect alone cannot explain the more dramatic bilayer stabilization of egg-PE to 70 °C after 6 h of incubation. Most likely the structural modifications in the ethanolamine head group play a quantitatively more important role with respect to the changed phase behavior. The formation of Schiff bases or cross-links in the PE head group region will increase the molecular area of the lipid–water interface and will disrupt the possible intermolecular hydrogen bonds, giving rise to a more cylindrical shape of the molecule. Apparently, the presence of 25% head group modified molecules in the PE dispersions is sufficient to stabilize all phospholipid molecules in the lamellar system after 6 h of peroxidation (Figure 7). In view of this strong effect of lipid peroxidation on the phase behavior of PE, it has to be considered that the differences between published bilayer to hexagonal (H_{II}) phase transition temperatures of egg-PE could have been caused by variable levels of phospholipid peroxidation [for a comparison see Cullis & de Kruijff (1978a), Hardman (1982), Mantsch et al. (1981), and Boggs et al. (1981)].

After prolonged (6–24-h) peroxidation of egg-PE dispersions a second change in the phase behavior occurred. After 6 h of incubation under oxygen, ^{31}P NMR measurements revealed the appearance of an isotropic signal superposed on the typical bilayer spectrum at both 25 and 40 °C. This isotropic peak became more dominant after 24 h of peroxidation and was not due to the formation of lyso compounds. Another explanation could be the formation of smaller vesicles; however, freeze–fracture electron microscopy did not support this possibility. When the samples were quenched from 25 °C by fast-freezing methods without cryoprotectants, only large stacked bilayers were observed with no evidence for nonbilayer lipid structures such as lipidic particles (data not shown). However, it should be realized that despite the fast freezing rate, structural transitions could have occurred during the freezing process. This is particularly relevant for egg-PE for which it has not

yet been possible to preserve the hexagonal (H_{II}) phase by freeze–fracturing (A. J. Verkleij, unpublished observations). Therefore, the existence of intermediate structures cannot be excluded as an explanation for the isotropic ^{31}P NMR signal. Another possible explanation may be the occurrence of irregular interwoven bilayers as documented previously in cardiolipin systems (de Kruijff et al., 1982). Indeed, small-angle X-ray diffraction measurements revealed a broad diffuse scattering profile, indicating a loss of order in the system after 24 h of peroxidation. Molecular interpretation of these results is hampered by a lack of understanding of the detailed structure of this "isotropic" phase, but it is clear that the additional effect of continued peroxidation of the acyl chains leads to changes in the macroscopic organization of the lipids.

To distinguish between possible effects of oxygen-induced modifications in acyl chain composition and head group structure on the phase behavior of egg-PE, some preliminary experiments were carried out on PE repurified by high-performance liquid chromatography after 24 h of peroxidation of the lipid dispersion. The repurified phospholipid was representative of PE with peroxidative effects in the acyl chains only, since the thiobarbituric acid assay revealed malondialdehyde formation (1.174 μmol/mmol of phospholipid) comparable with the level after 24 h of peroxidation (Figure 1), while the ethanolamine head groups showed the expected 100% reactivity toward trinitrobenzenesulfonic acid. Freeze–fracture electron microscopy visualized circles of lipidic particles associated with the bilayers in a hydrated sample of the repurified PE (unpublished data). These lipidic particles may be interpreted as inverted micelles, associated with certain stages of membrane fusion [for a recent review, see Verkleij (1984)]. The broad isotropic signals, obtained by ^{31}P NMR over a temperature range between 10 and 70 °C (data not shown), could be caused by isotropic motional averaging of the phospholipid molecules within the strongly curved bilayers as a result of local fusion events.

The role of nonbilayer structures (e.g., inverted micelles), occurring in model membranes made of a mixture of bilayer and hexagonal (H_{II}) phase preferring phospholipids, in biologically important events like phospholipid transbilayer movement and membrane fusion has been investigated and discussed intensively (Verkleij et al., 1980; Noordam et al., 1980; Cullis & de Kruijff, 1979). The effect of phospholipid peroxidation on these events is not predictable because of the dualistic behavior of PE during peroxidation. The bilayer-stabilizing effect of PE head group cross-links would suggest an inhibition of events mediated by nonbilayer structures; however, prolonged peroxidation, inducing isotropic signals in ^{31}P NMR spectra, could support phospholipid transbilayer movement and membrane fusion via the formation of intermediate nonbilayer structures. A discrimination between these possibilities awaits detailed studies on mixed phospholipid systems.

Calorimetric measurements in egg-PE dispersions showed a small increase in the temperature range (from −4 to 18 °C to 3–23 °C) and a concomitant reduction of the heat content of the gel to liquid-crystalline transition as a result of a 24-h incubation under oxygen. The changes were smaller than expected; however, peroxidation will affect the thermotropic behavior of PE in two opposite ways. First, the formation of conjugated and trans double bonds as a result of lipid peroxidation, as documented by Porter & Lehman (1982), will result in a more rigid bilayer. Such behavior leads to an increase in the gel to liquid-crystalline transition temperature, as proposed recently in a PC system (Coolbear & Keough, 1983). The decrease in the bilayer fluidity as a result of lipid peroxidation has also been noticed in experiments using fluorescence polarization and electron spin resonance (Galanopoulou et al., 1982; Bruch & Thayer, 1983). Second, since PE's in general have a higher gel to liquid-crystalline transition temperature than the corresponding PC's (Van Dijck et al., 1976), structural modifications in the ethanolamine head group during peroxidation could result in a lower transition temperature. The formation of inter- or intramolecular cross-links between peroxidized acyl chains tends to decrease the gel to liquid-crystalline transition temperature, comparable with the effect of polymerization of diacetylene-containing PC molecules as has been published recently (Leaver et al., 1983). The same authors also reported a decreased enthalpy of the main endothermic transition in their system as a result of polymerization. Therefore, this interpretation is consistent with the large (60%) reduction of the enthalpy of the gel to liquid-crystalline transition in the PE dispersion during lipid peroxidation.

In summary, the results presented in this paper lead to the following conclusions: First, egg-PE is more sensitive to phospholipid peroxidation than egg-PC. Second, the molecular organization of egg-PC molecules is not affected by peroxidation. Third, initial peroxidation stabilizes PE dispersions in bilayer organizations, presumably by cross-linking between the ethanolamine head groups. Fourth, prolonged peroxidation of hydrated PE gives rise to a loss of order in the system, possibly by the formation of intermediate nonbilayer structures. Finally, peroxidation of PE decreases the heat content of the gel to liquid-crystalline transition.

Acknowledgments

We gratefully acknowledge the technical assistance of Dr. Pathmamanoharan in the X-ray experiments. We thank J. Leunissen-Bijvelt for the performance of the electron microscopy experiments and Dr. P. Child for correcting the English.

References

Barsukov, L. J., Victorov, A. V., Vasilenko, J. A., Evstigneeva, R. P., & Bergelson, L. D. (1980) *Biochim. Biophys. Acta 598*, 153–168.

Birdsall, N. J. M., Ellar, D. J., Lee, A. G., Metcalfe, J. C., & Warren, G. B. (1975) *Biochim. Biophys. Acta 380*, 344–354.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–913.

Boggs, J. M., Stamp, D., Hughes, D. W., & Deben, C. M. (1981) *Biochemistry 20*, 5728–5735.

Böttcher, C. J. F., Van Gent, C. M., & Pries, C. (1961) *Anal. Chim. Acta 24*, 203.

Bruch, R. C., & Thayer, W. S. (1983) *Biochim. Biophys. Acta 733*, 216–222.

Buege, J. A., & Aust, S. D. (1978) *Methods Enzymol. 52*, 302–310.

Coolbear, K. P., & Keough, K. M. W. (1983) *Biochim. Biophys. Acta 732*, 531–540.

Corliss, G. A., & Dugan, L. R., Jr. (1970) *Lipids 5*, 846–853.

Cullis, P. R., & de Kruijff, B. (1976) *Biochim. Biophys. Acta 436*, 523–540.

Cullis, P. R., & de Kruijff, B. (1978a) *Biochim. Biophys. Acta 513*, 31–42.

Cullis, P. R., & de Kruijff, B. (1978b) *Biochim. Biophys. Acta 507*, 207–218.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Cullis, P. R., de Kruijff, B., & Richards, R. E. (1976) *Biochim. Biophys. Acta 426*, 433–446.
Dekker, C. J., Geurts Van Kessel, W. S. M., Klomp, J. P. S., Pieters, J., & de Kruijff, B. (1983) *Chem. Phys. Lipids 33*, 93–106.
de Kruijff, B., Cullis, P. R., & Verkleij, A. J. (1980) *Trends Biochem. Sci. (Pers. Ed.) 5*, 79–81.
de Kruijff, B., Verkleij, A. J., Leunissen-Bijvelt, J., Van Echteld, C. J. A., Hille, J., & Rijnbout, H. (1982) *Biochim. Biophys. Acta 693*, 1–12.
Frankel, E. N. (1980) *Prog. Lipid Res. 19*, 1–22.
Fukuzawa, K., Tokumura, A., Ouchi, S., & Tsukatani, H. (1982) *Lipids 17*, 511–513.
Galanopoulou, D., Williams, W. P., & Quinn, P. J. (1982) *Biochim. Biophys. Acta 713*, 315–323.
Gast, K., Zirwer, D., Ladhoff, A. M., Schreiber, J., Koelsch, R., Kretschmer, K., & Lasch, J. (1982) *Biochim. Biophys. Acta 686*, 99–109.
Gutteridge, J. M. C., Kerry, P. J., & Armstrong, D. (1982) *Biochim. Biophys. Acta 711*, 460–465.
Hardman, P. D. (1982) *Eur. J. Biochem. 124*, 95–101.
Kergonou, J. F., Bernard, P., Braquet, M., & Rocquets, G. (1982) *Biochem. Int. 5*, 193–199.
Leaver, J., Alonso, A., Durrani, A. A., & Chapman, D. (1983) *Biochim. Biophys. Acta 732*, 210–218.
Luzzati, V., Reiss-Husson, F., Rivas, E., & Gulik-Krzywicki, T. (1966) *Ann. N.Y. Acad. Sci. 137*, 409–413.
Mantsch, H. H., Martin, A., & Cameron, D. G. (1981) *Biochemistry 20*, 3138–3145.
Martin, M. L., Ricolleau, G., Poignant, S., & Martin, G. J. (1976) *J. Chem. Soc., Perkin Trans 2*, 182–186.
Noordam, P. C., Van Echteld, C. J. A., de Kruijff, B., Verkleij, A. J., & De Gier, J. (1980) *Chem. Phys. Lipids 27*, 221–232.
Pangborn, M. C. (1951) *J. Biol. Chem. 188*, 471–476.
Petri, W. A., Jr., Estep, T. N., Pal, R., Thompson, T. E., Bittonen, R. L., & Wagner, R. R. (1980) *Biochemistry 19*, 3088–3091.
Porter, N. A., & Lehman, L. S. (1982) *J. Am. Chem. Soc. 104*, 4731–4732.
Porter, N. A., Weber, B. A., Weenen, H., & Khan, J. A. (1980a) *J. Am. Chem. Soc. 102*, 5597–5601.
Porter, N. A., Wolf, R. A., & Weenen, H. (1980b) *Lipids 15*, 163–167.
Ramasarma, T. (1982) *Biochim. Biophys. Acta 694*, 69–93.
Rand, R. P., Tinker, D. O., & Fast, P. G. (1971) *Chem. Phys. Lipids 6*, 333–342.
Reiss-Husson, F. (1967) *J. Mol. Biol. 25*, 363–381.
Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.
Shaw, J. M., & Thompson, T. E. (1982) *Biochemistry 21*, 920–927.
Siakotos, A. N. (1967) *Lipids 2*, 87–88.
Tilcock, C. P. S., & Cullis, P. R. (1982) *Biochim. Biophys. Acta 684*, 212–218.
Van Dijck, P. W. M., de Kruijff, B., Van Deenen, L. L. M., De Gier, J., & Demel, R. A. (1976) *Biochim. Biophys. Acta 455*, 576–587.
Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43–64.
Verkleij, A. J., Van Echteld, C. J. A., Gerritsen, W. J., Cullis, P. R., & de Kruijff, B. (1980) *Biochim. Biophys. Acta 600*, 620–624.
Wu, G. S., Stein, R. A., & Mead, J. F. (1982) *Lipids 17*, 403–413.

Transmembrane Movement of Phosphatidylglycerol and Diacylglycerol Sulfhydryl Analogues†

Barry R. Ganong and Robert M. Bell*

ABSTRACT: Transmembrane movement of phospholipids is a fundamental step in the process of biological membrane assembly and intracellular lipid sorting. To facilitate study of transmembrane movement, we have synthesized analogues of phosphatidylglycerol and diacylglycerol in which a sulfhydryl group replaces a hydroxyl group in the polar head group. A rapid, continuous assay for the movement of phospholipids across single-walled lipid vesicles was developed that exploits the reactivity of these analogues toward 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a nonpenetrating, colorimetric, sulfhydryl reagent. In the reaction of DTNB with vesicles containing phosphatidylthioglycerol, a phosphatidylglycerol analogue, two kinetic phases were seen, which represent the reaction of DTNB with phosphatidylthioglycerol in the outer and inner leaflets of the bilayer. Analysis of the slow second phase indicated that the half-time for phosphatidylthioglycerol transbilayer movement was in excess of 8 days. In a similar experiment using dioleoylthioglycerol, a diacylglycerol analogue, the reaction was complete within 15 s. The large difference in translocation rates between these two lipids indicates that the primary barrier to transmembrane movement is the polar head group and implies that phospholipid translocation events in biological membranes may not be unlike those for molecules similar to the polar head groups alone.

In mammalian cells, the endoplasmic reticulum is the primary site of phospholipid synthesis. Efforts are currently being made in a number of laboratories to elucidate the molecular mechanisms by which phospholipids synthesized in this membrane are distributed among the various membrane systems in the cell. Phospholipid assembly occurs on the cytoplasmic face of the endoplasmic reticulum (Bell et al., 1981). A fundamental step in the process of intracellular lipid sorting is the movement of phospholipids across the membrane to the lumenal surface.

The rate of phospholipid movement across biological membranes varies over several orders of magnitude depending on the membrane under investigation. Values of half-times range

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received March 16, 1984.* Supported by U.S. Public Health Service Grants AM20205, GM20015, and AM07020.

0006-2960/84/0423-4977$01.50/0