Rotational Relaxation Times of 1,6-Diphenyl-1,3,5-hexatriene in Phospholipids Isolated from LM Cell Membranes. Effects of Phospholipid Polar Head-Group and Fatty Acid Composition†

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ABSTRACT: Phospholipids were isolated from mitochondrial, microsomal, and plasma membranes of LM cells and fractionated into individual phospholipid classes on silicic acid columns. The fatty acid composition and the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene (DPH) were determined for each phospholipid class. Sphingomyelin was the only phospholipid isolated from LM cell membranes that showed a phase transition within the temperature range investigated, 5–40 °C. The rotational relaxation times for DPH were lowest in phosphatidylcholine in all the membrane fractions. Phosphatidylcholine isolated from the three membrane fractions of choline-supplemented cells had similar rotational relaxation times and phosphatidylcholine isolated from microsomal membranes of linoleate-supplemented cells had lower rotational relaxation times. The results indicate that the differences in the rotational relaxation times of DPH between mitochondrial, microsomal, and plasma membrane phospholipids could be explained primarily by differences in the polar head-group composition, while differences in the fatty acid composition had only a minor effect. This provides a basis for understanding how the different lipid components in these cells contribute to membrane fluidity.

In the preceding paper the fluidity of mitochondrial, microsomal, and plasma membranes was shown to be markedly different as determined by the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] (Gilmore et al., 1979). The differences in the rotational relaxation time were due to differences in the desmosterol content and the phospholipid composition of the membranes. Supplementation of the growth medium with ethanolamine or linoleate indicated that changes in both the phospholipid polar head-group and fatty acid composition could affect the rotational relaxation time of DPH in LM cell membranes.

Many investigators have studied the physical properties of phospholipids in recent years using a variety of techniques. Systematic studies have been carried out on synthetic phospholipids, such as the disaturated phosphatidylcholines, which show sharp gel to liquid-crystalline phase transitions. Several studies have resulted in phase diagrams for two component mixtures of phospholipids in which lateral phase separations occur (Phillips et al., 1972; Shimshick & McConnell, 1973; Lee, 1977). DPH is very sensitive to phase transitions (Shinitzky & Barenholz, 1974; Barenholz et al., 1976; Suurkuusk et al., 1976; Lentz et al., 1976a) and has been used to construct phase diagrams (Andrich & Vanderkooi, 1976; Lentz et al., 1976b). In addition, fluorescence polarization measurements of DPH provide direct information about molecular motion which is important for understanding the function of animal cell membranes.

The lipid composition of animal cells is very complex. There are six major phospholipid classes in LM cells, for example, which occur in different proportions in different subcellular membrane fractions. In addition, each membrane contains different proportions of saturated and unsaturated fatty acids that vary in chain length from 14 to 24 carbons. Although physical studies have been done on individual phospholipids isolated from selected natural sources, no studies have been carried out on all the different phospholipid classes derived from a single membrane in order to determine how each component contributes to the overall membrane properties.

In order to determine which phospholipid components were responsible for the changes in the rotational relaxation times of DPH between the different membrane fractions of LM cells, the following membranes were chosen for further phospholipid fractionation in this study: mitochondrial, microsomal, and plasma membranes from choline-supplemented cells and microsomal membranes from linoleate-supplemented cells. The rotational relaxation times of DPH and the fatty acid composition for the individual phospholipids were determined.

Materials and Methods

General Methods. The procedures used for cell culture, membrane preparation, lipid analysis, and fluorescence measurements were described in the preceding paper (Gilmore et al., 1979).

Analysis of Sphingosine and Dihydrosphingosine. Sphingomyelin was extracted from LM cells grown on choline-containing medium. The phospholipids were separated by one-dimensional thin-layer chromatography on silica gel G plates (250 μm, Analtech, Inc.) using $CHCl_3$:CH_3OH:H_2O (65:25:4) as the solvent. The sphingosine-free bases were prepared by the method of Shinitzky & Barenholz (1974) and analyzed by gas–liquid chromatography of the trimethylsilylated derivatives (Carter & Gaver, 1967) on a 1.5-m column of 3% SE-30 on 80–100 Gas Chrom Q (Supelco, Inc.) at 220 °C.

Identification of Lysobisphosphatidic Acid. Lysobisphosphatidic acid was identified by two-dimensional thin-layer chromatography. It migrated to the same position as described by Rouser et al. (1968) in both two-dimensional solvent systems. It was isolated by two-dimensional thin-layer chromatography on Redi-coat-2D plates (250 μm, Supelco, Inc.) using $CHCl_3$–CH_3OH–NH_4OH (65:25:5) in the first dimension and $CHCl_3$–acetone–CH_3OH–acetic acid–H_2O (3:4:1:1:0.5) in the second dimension. Lysobisphosphatidic

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[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin, CL, cardiolipin.

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acid and all the other major phospholipids were eluted from the plates with $CHCl_3$–CH_3OH–acetic acid–H_2O (5:5:1:1). Mild alkaline hydrolysis products were prepared from the compounds by the method of Dittmer & Wells (1969) and separated by one-dimensional thin-layer chromatography on Polygram CEL 300 (100 μm, Brinkman Instruments, Inc.). The hydrolysis product of lysobisphophatidic acid comigrated with glycerophosphorylglycerol in two solvent systems. The first solvent system consisted of isopropyl alcohol–NH_4OH–H_2O (7:1:2) and the second solvent system consisted of phenol saturated with water–acetic acid–ethanol (50:5:6) (Dawson, 1967).

Phospholipid Fractionation. The phospholipid fractionation was a modification of the procedure of Newman et al. (1961). Mallinckrodt 100-mesh silicic acid was prepared for chromatography by successive washes with chloroform and methanol followed by heating at 110 °C for 12 h. The activated silicic acid was stored in a desiccator until needed. Phospholipids labeled with [^{32}P]phosphate were separated on a 1 × 9 cm glass column. Activated silicic acid (2.5 g) was poured into the column in a chloroform slurry and washed with 50 mL of chloroform prior to the application of the sample. Between 2 and 5 μmol of phospholipids in chloroform was applied to the column. The phospholipids were eluted by five stepwise increases in the concentration of methanol in chloroform: 20 mL of 9% (v/v), 25 mL of 18.5%, 20 mL of 25%, 60 mL of 40%, and 25 mL of 100%. The column was run under nitrogen, and the flow rate was maintained at approximately 15 mL/h by regulating the pressure. Fractions of 5.0 mL were collected. Extraction of the original membranes in phosphate-buffered saline was essential for obtaining a reproducible separation.

For some experiments sphingomyelin was further purified to remove small amounts of phosphatidylcholine using the procedure of Rouser et al. (1961). This method utilizes Unisil silicic acid–silicate–water chromatography with elution by controlling the water content of the eluting solvent. Phosphatidylcholine was eluted with 1.5% water in chloroform–methanol (4:1, v/v) and sphingomyelin was eluted with 100% methanol.

After fractionation of the phospholipids extracted from the membranes on silicic acid columns, peaks containing individual phospholipids were pooled. The phospholipid pools were absorbed onto 5-cm Unisil columns and washed extensively with $CHCl_3$. They were eluted with 70% CH_3OH–$CHCl_3$ (v/v), dried under nitrogen, suspended in 1.0 mL of $CHCl_3$, and stored under argon at −20 °C until used for fluorescence measurements. This last column procedure was used to eliminate any contaminants introduced during the chromatographic separations. This did not result in a loss of phospholipids as monitored by ^{32}P counts, with the exception of eliminating the small quantity of lysophosphatidylcholine (approximately 0.5%) present in the original phospholipid extracts.

Results

Fractionation of the Phospholipids Extracted from Different Membranes. Phospholipids extracted from mitochondrial, microsomal, and plasma membranes from choline-supplemented cells and microsomal membranes from linoleate-supplemented cells were fractionated on silicic acid columns. The separations were done on silicic acid columns rather than on thin-layer chromatography plates for several reasons. Oxidation of unsaturated fatty acids and hydrolysis of phospholipids were greatly reduced on columns as compared with thin-layer chromatography plates. The visualization and

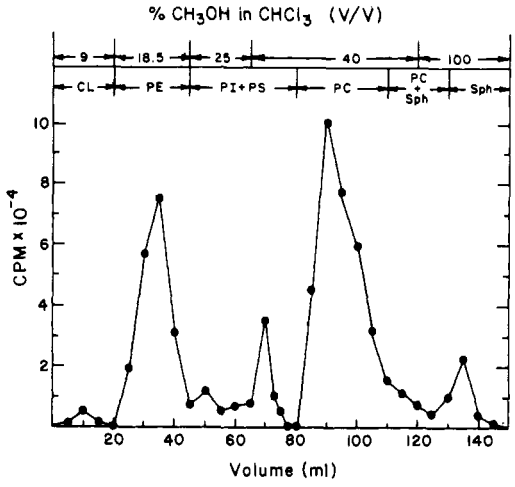

FIGURE 1: The elution profile of silicic acid chromatography of phospholipids isolated from microsomal membranes of choline-supplemented cells. Phospholipids (3.6 μmol) labeled with [^{32}P]-phosphate were applied to the column and eluted as described in Materials and Methods. The elution solvents and the pools that were collected are indicated on the upper horizontal axis.

elution of lipids from thin-layer chromatography plates may result in phospholipid modification, loss of minor components, and the contamination of the sample with visualization reagents. The production of approximately 4% lysophosphatidylethanolamine during the elution of thin-layer chromatography plates resulted in a greatly altered fluorescence polarization of the phospholipid dispersions. Column separations resulted in the complete recovery of the phospholipids applied to the column and no hydrolysis products were detected.

A typical fractionation is shown in Figure 1. The first peak to come off the column was eluted with 9% methanol in chloroform and it consisted of cardiolipin. Practically all the cardiolipin in LM cells was located in mitochondria (for the phospholipid composition of the different membrane fractions, see Gilmore et al., 1979). When the fractions under the first peak were pooled for the sample from mitochondrial membranes, cardiolipin made up over 95% of the phospholipids.

The 18.5% methanol pool contained between 86% and 94% phosphatidylethanolamine for all four membrane fractions. The remaining phospholipids in this pool were largely phosphatidylglycerol (approximately 25%) and lysobisphosphatidic acid (approximately 75%) which is a positional isomer of phosphatidylglycerol. Lysobisphosphatidic acid has been identified in BHK cells (Brotherus et al., 1974) and in some mammalian tissues (Rouser et al., 1968; Wherrett & Huterer, 1972; Body & Gray, 1967), but has not been identified previously in LM cells.

The peak eluting with 25% methanol was phosphatidylserine and the next peak was phosphatidylinositol. These two peaks were combined so that enough material could be obtained for fluorescence lifetime analysis. The complete separation of phosphatidylserine and phosphatidylinositol from other phospholipids was difficult to achieve and it depended on a number of factors including the counterion, the amount applied to the column, and the moisture content of the silicic acid (Rouser et al., 1961). The phosphatidylserine and phosphatidylinositol pool isolated from microsomal and plasma membranes of choline-supplemented cells was 90% pure, while the pool isolated from microsomal membranes of linoleate-supplemented cells was 84% pure. The remainder consisted of phosphatidylglycerol. The separation from mitochondrial membranes was particularly poor and the phosphatidylserine

Table I: Fatty Acid Composition of the Phospholipid Pools Obtained from the Silicic Acid Column[a]

pool	membrane fraction	fatty acid composition (wt %)								% saturated fatty acids
		14:0	16:0	16:1	18:0	18:1	18:2	odd chain[c]	>C-18[d]	
PC	PM	3.0	18.9	3.7	12.0	50.5		8.8	5.6	39.8
PC	MicM	2.1	15.6	3.8	8.4	58.6		8.8	4.9	30.5
PC	MitM	2.0	14.2	5.1	7.4	61.3		8.7	3.9	27.7
PC	MicM (18:2)[b]	1.3	14.2	2.4	7.6	26.1	37.8	3.7	7.5	27.2
PE	PM	0.8	5.7	1.7	8.1	60.9		9.3	19.6	21.4
PE	MicM	0.4	4.2	1.8	8.8	64.6		9.1	16.8	19.4
PE	MitM	0.4	3.2	2.3	14.9	65.4		9.0	9.1	24.4
PE	MicM (18:2)[b]	0.6	3.6	1.8	11.9	40.3	23.3	5.9	16.1	20.4
PS + PI	PM	0.1	2.7	0.5	24.5	52.6		9.7	15.8	35.6
PS + PI	MicM	0.1	2.9		22.2	58.5		9.5	12.4	32.0
PS + PI	MitM	0.5	8.0	1.6	13.5	61.0		9.4	10.2	30.3
PS + PI	MicM (18:2)[b]	0.1	2.0	0.2	32.5	26.7	18.0	5.4	18.2	38.8
Sph	whole cell	1.6	52.5	3.0	2.2	1.8		6.5	33.3[e]	61.9
Sph	PM	1.2	42.9	3.2	3.9	6.1		9.7	38.3[e]	61.7
Sph	MicM	1.4	38.8	6.2	7.1	5.7		15.7	34.8[e]	54.0
Sph	MicM (18:2)[b]	1.2	39.3	3.0	2.4	3.8	2.7	11.5	43.6[e]	55.3
CL	MitM	0.8	16.6	2.4	5.0	60.0		12.9	7.6	29.3

[a] Phospholipids were isolated from mitochondrial (MitM), microsomal (MicM), and plasma membranes (PM) from cells supplemented with choline and linoleate and fractionated on silicic acid columns as described in Materials and Methods. Fatty acid methyl esters were prepared by the $NaOH:CH_3OH$ method except for the fatty acid methyl esters of sphingomyelin that were prepared by the $HCl:CH_3OH$ method. The exact fatty acid composition depended on the separation achieved on the silicic acid column. The composition and fluorescence data are given for the same sample. [b] The phospholipids from these membranes were isolated from cells supplemented with linoleate. The rest of the phospholipids were isolated from membranes obtained from normal choline-supplemented cells. [c] Includes odd chain fatty acids up to C-23. No fatty acids >C-24 were found. No polyunsaturated fatty acids were found except on supplementation with linoleate. [d] Includes fatty acids from C-19 to C-24. [e] Over 80% of the long-chain fatty acids in sphingomyelin consisted of 24:1.

and phosphatidylinositol pool also contained 22% cardiolipin and 16% phosphatidylethanolamine.

Phosphatidylcholine was eluted with 40% methanol and the phosphatidylcholine pool was greater than 99.5% pure for all four membrane fractions. The phosphatidylcholine peak tailed and the next pool consisted of a mixture of phosphatidylcholine and sphingomyelin. This pool represented 3% to 4% of the total and was not analyzed further except for the experiments where all pools were recombined to give the original phospholipid mixture.

Sphingomyelin was eluted with 100% methanol and was the last phospholipid to come off the column. The sphingomyelin pool contained between 90% and 95% sphingomyelin for the samples isolated from microsomal and plasma membranes. The mitochondrial membrane fraction contained only trace amounts of sphingomyelin.

Fatty Acid Composition of the Phospholipid Pools. The fatty acid composition of phosphatidylcholine isolated from mitochondrial and microsomal membranes from choline-supplemented cells was very similar (Table I). The phosphatidylcholine isolated from the plasma membrane of these cells had a significant increase in the amount of saturated fatty acids which was mainly due to a reduction in 18:1 and an increase in 18:0. When the cells were supplemented with linoleate, the largest change was in the phosphatidylcholine pool which incorporated 37.8% linoleate primarily at the expense of oleate. The total microsomal phospholipids contained 31.2% linoleate.

The phosphatidylethanolamine pool contained more long chain fatty acids and less saturated fatty acids than the phosphatidylcholines. Differences in the fatty acid composition of phosphatidylcholines and phosphatidylethanolamines in LM cells have been observed previously (Ferguson et al., 1975). The phosphatidylserine plus phosphatidylinositol pools contained a greater amount of long chain fatty acids and saturated fatty acids than the phosphatidylcholine pools. The sphingomyelin pools contained only a few fatty acids, mainly 16:0 and 24:1, and did not incorporate significant amounts of linoleate when linoleate was added to the cells for 18 h. Sphingomyelin contained approximately 70% C_{18}-sphingosine and 30% C_{18}-dihydrosphingosine. The fatty acid composition of cardiolipin resembled mitochondrial phosphatidylcholine except it contained slightly more odd chain and long chain fatty acids.

Rotational Relaxation Times of DPH in the Different Phospholipid Pools. In order to determine how individual phospholipid classes contributed to the overall membrane fluidity, the fluorescence polarization and lifetime of DPH were measured in the phospholipid pools as a function of temperature. Rotational relaxation times were calculated from these measurements and the results are shown in Figure 2. Sphingomyelin from microsomal and plasma membranes was the only phospholipid that showed a phase transition within the temperature range investigated and it had the highest rotational relaxation times as compared with the other phospholipids. The rotational relaxation times of DPH in sphingomyelin closely resembled those for dimyristoylphosphatidylcholine except that the transition for sphingomyelin was centered around 30 °C, while for dimyristoylphosphatidylcholine it was centered around 24 °C (data not shown). The sphingomyelin transition was also broader which reflected its fatty acid heterogeneity and the small amount of contamination with phosphatidylcholine (see below).

In mitochondria, the rotational relaxation times of DPH were the highest in cardiolipin since mitochondria did not contain sphingomyelin. All the other phospholipid pools isolated from mitochondria except phosphatidylcholine had lower rotational relaxation times as compared with the corresponding pools from microsomal and plasma membranes. In all membranes phosphatidylcholine had the lowest rotational relaxation times, while the phosphatidylethanolamine and the phosphatidylinositol plus phosphatidylserine pools were intermediate.

A comparison of the phosphatidylcholine pools isolated from the different membranes is shown in Figure 3. Since these pools contained only phosphatidylcholine, the differences between the pools were due solely to the differences in the fatty acid composition. The rotational relaxation times of DPH in

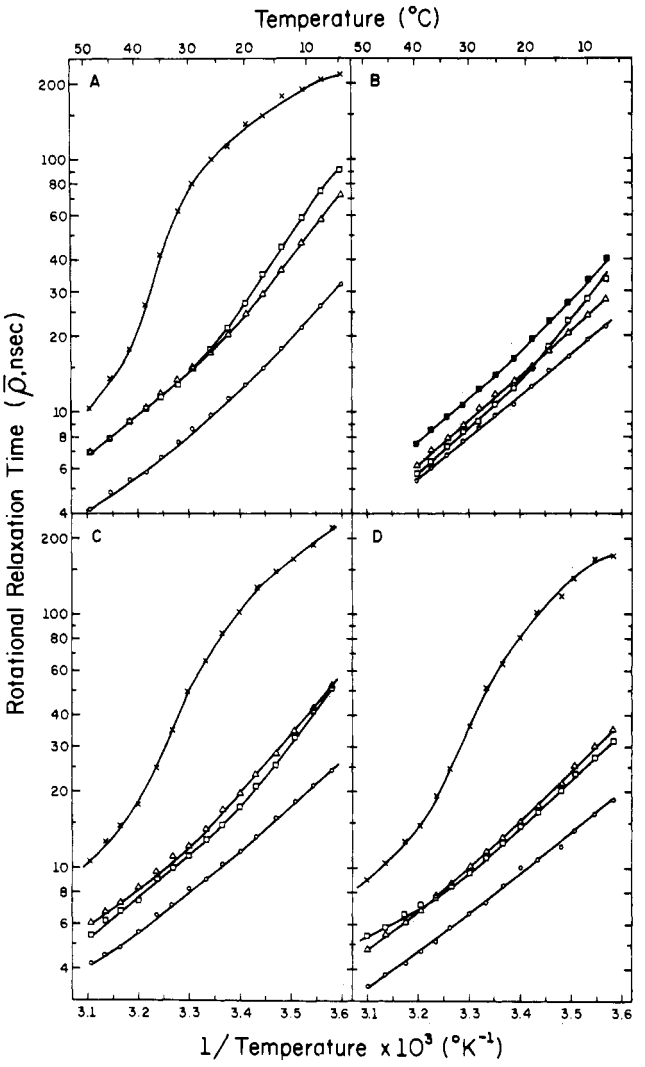

FIGURE 2: The temperature dependence of the rotational relaxation times of DPH in phospholipid fractions isolated from (A) plasma membranes, (B) mitochondrial membranes, (C) microsomal membranes from choline-supplemented cells, and (D) microsomal membranes from linoleate-supplemented cells. Isolated phospholipid pools are: (O—O) phosphatidylcholine; (Δ—Δ) phosphatidylserine and phosphatidylinositol; (□—□) phosphatidylethanolamine; (×—×) sphingomyelin; and (■—■) cardiolipin. The procedures used for phospholipid purification are described in Materials and Methods.

phosphatidylcholine from the three membrane fractions from choline-supplemented cells were remarkably similar. The curves deviated only below 20 °C where the rotational relaxation times of DPH in plasma membrane phosphatidylcholine were slightly higher. This could be attributed to the increased content of saturated fatty acids in this fraction. The rotational relaxation times of DPH were decreased in phosphatidylcholine isolated from microsomal membranes of linoleate-supplemented cells. Comparison of the other phospholipid pools between the various membrane fractions showed differences, but the analysis of the effects of the fatty acid composition was complicated by the varying degrees of purity of the pools.

Rotational Relaxation Times of DPH in Mixtures of Sphingomyelin and Phosphatidylcholine. Sphingomyelin comprised approximately 8% of microsomal membranes and 11% of plasma membranes from LM cells. Since this pool showed a distinct phase transition, it was of interest to determine the behavior of pure sphingomyelin and of mixtures of sphingomyelin at the levels found in membranes. Sphingomyelin that was isolated by silicic acid chromatography

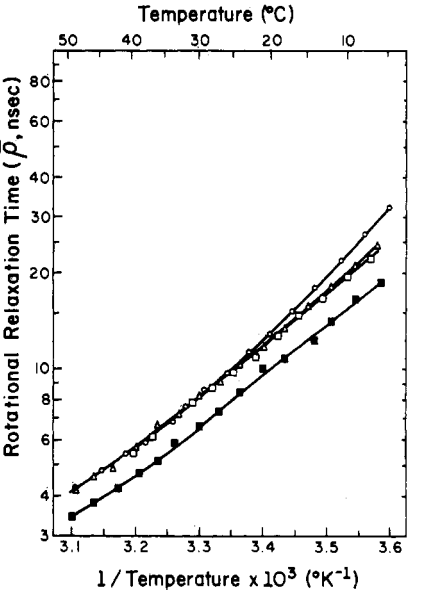

FIGURE 3: The temperature dependence of the rotational relaxation times of DPH in phosphatidylcholine. Phospholipids were isolated from: (O—O) plasma membranes; (Δ—Δ) microsomal membranes; (□—□) mitochondrial membranes from choline-supplemented cells; and (■—■) microsomal membranes from linoleate-supplemented cells.

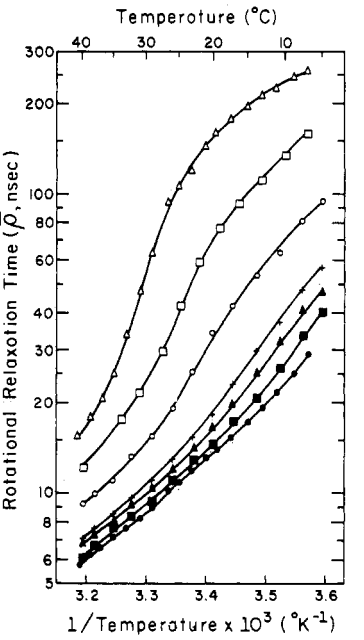

FIGURE 4: The temperature dependence of the rotational relaxation times of DPH in mixtures of sphingomyelin and phosphatidylcholine. Sphingomyelin and phosphatidylcholine were isolated from whole LM cells as described in Materials and Methods. The mole fractions of sphingomyelin in phosphatidylcholine were: (●—●) 0.00 Sph; (■—■) 0.1 Sph; (▲—▲) 0.18 Sph; (+—+) 0.25 Sph; (O—O) 0.50 Sph; (□—□) 0.75 Sph; and (Δ—Δ) 1.00 Sph.

contained several percent phosphatidylcholine. The sphingomyelin pool from whole cell phospholipids that was isolated on the silicic acid column was further purified by silicic acid–silicate–water chromatography to obtain pure sphingomyelin. The additional purification of sphingomyelin did not significantly change the fatty acid composition and resulted in only a slightly sharper transition (Figure 4). When the sphingomyelin was mixed with phosphatidylcholine, the major phospholipid species in LM cells, the transition became broader and shifted to lower temperatures. When the concentration of sphingomyelin was reduced to the levels found in mem-

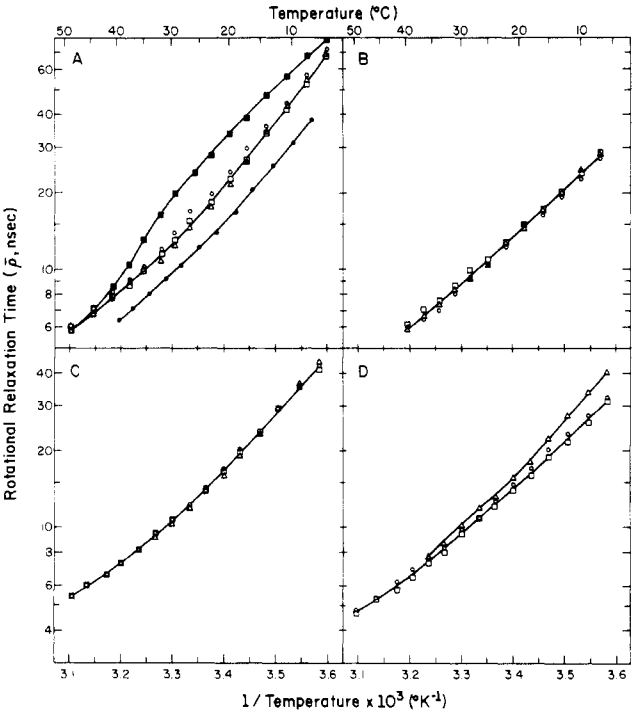

FIGURE 5: The temperature dependence of the observed and calculated rotational relaxation times of DPH in phospholipids from (A) plasma membranes, (B) mitochondrial membranes, (C) microsomal membrane from choline-supplemented cells, and (D) microsomal membranes from linoleate-supplemented cells. Measured values of the rotational relaxation times in the (O—O) original phospholipid dispersions and in the (□—□) recombined phospholipid dispersions after the silicic acid column. (Δ—Δ) The calculated values of the rotational relaxation times using the observed rotational relaxation times for the individual phospholipid pools weighted by the size of the pool. The contribution of sphingomyelin was obtained from phosphatidylcholine–sphingomyelin mixtures. (■—■) The same calculation for plasma membrane phospholipids using separate phosphatidylcholine and sphingomyelin pools. (●—●) The calculated values of the rotational relaxation times for plasma membrane phospholipids using the rotational relaxation times of DPH in the phosphatidylethanolamine and phosphatidylserine plus phosphatidylinositol pools from mitochondria, and the phosphatidylcholine–sphingomyelin mixture from plasma membranes. See the text for further details.

branes, the rotational relaxation times were only slightly higher than those in pure phosphatidylcholine, especially at high temperatures.

Recombination of the Isolated Phospholipid Pools. To ensure that the silicic acid chromatography did not cause an alteration in the physical properties of the phospholipids through oxidation, hydrolysis, or selective loss of individual compounds, the isolated phospholipid pools were recombined using an equal fraction of each pool obtained during the purification. The rotational relaxation times of DPH were determined for the recombined mixtures and compared with the original phospholipid dispersions before separation (Figure 5). No significant deviations were observed between the recombined samples and the original lipid mixture. The phospholipid and fatty acid composition of the recombined mixtures were also identical with the original mixtures within experimental error (data not shown). No phospholipid hydrolysis products were detected in these samples by two-dimensional thin-layer chromatography where the lower limit of detection was approximately 0.3%.

Discussion

The rotational relaxation times of DPH in mitochondrial, microsomal, and plasma membranes showed distinct differences which could be attributed to differences in the desmosterol content and phospholipid composition (Gilmore et al., 1979). In order to further evaluate the effects of phospholipid polar head groups and fatty acids, the rotational relaxation times of DPH were determined in individual phospholipid classes isolated from the total membrane phospholipids on a silicic acid column.

Sphingomyelin was the only phospholipid that showed a phase transition in the temperature range investigated, 5–40 °C. The fatty acid compositions of the sphingomyelins isolated from microsomal and plasma membranes were similar and the phase transitions were all centered around 30 °C. When sphingomyelin was mixed with phosphatidylcholine, the transition became broader and was shifted to a lower temperature. This is consistent with a broad region of lateral phase separations which extended considerably below 0 °C. At 10% sphingomyelin, which is approximately the concentration found in microsomal and plasma membranes, the rotational relaxation time of DPH at 37 °C was only slightly higher than pure phosphatidylcholine. As the temperature was decreased, the deviation became larger. Untracht & Shipley (1977) have determined the phase diagram for mixtures of brain sphingomyelin and egg yolk phosphatidylcholine. They found evidence for a peritectic molecular compound (67% phosphatidylcholine, 33% sphingomyelin) which was not detected with the phospholipids used in this study.

The LM cell membranes that contain sphingomyelin also have a high content of desmosterol. Cholesterol has been shown to abolish sphingomyelin phase transitions (Oldfied & Chapman, 1972; Demel et al., 1977). Consequently, desmosterol would be expected to abolish any sphingomyelin transition that would occur in intact membranes or whole lipid dispersions.

Phosphatidylcholine was the major phospholipid class in LM cell membranes. The rotational relaxation times of DPH in the phosphatidylcholines isolated from the three membrane fractions of choline-supplemented cells were identical within experimental error above 20 °C. Below 20 °C, the rotational relaxation times for phosphatidylcholine from plasma membranes were higher by only a small extent which correlated with approximately an 11% decrease in monounsaturated fatty acids. Thus in choline-supplemented cells the differences in the fatty acid composition resulted in minor differences in the observed rotational relaxation times of DPH in the purified phosphatidylcholines. Phosphatidylcholine isolated from microsomal membranes of linoleate-supplemented cells had reduced rotational relaxation times. There was extensive incorporation of linoleate which primarily replaced oleate and increased the number of double bonds per fatty acid chain.

The short rotational relaxation times of DPH in the phosphatidylcholines were similar to those observed for a pure homogeneous phosphatidylcholine above the gel to liquid-crystalline phase transition. The slight upward curvature of the Arrhenius plot at low temperatures for plasma membrane phosphatidylcholine, as well as for some of the other phospholipid pools, may indicate that it was approaching a transition below 0 °C.

Examination of the fatty acid composition of the phosphatidylcholines indicates that most of the phosphatidylcholine molecules should have transition temperatures below 0 °C. Microsomal phosphatidylcholine, for example, contained 70% unsaturated fatty acids. If one assumes that the 2 position on the glycerol backbone is only esterified to an unsaturated fatty acid, then there were 60% monounsaturated and 40% diunsaturated molecules. The phase transition temperatures

for the two major species, 1-palmitoyl-2-oleylphosphatidylcholine and dioleylphosphatidylcholine, are −5 °C (De Kruijff et al., 1973) and −22 °C (Phillips et al., 1972), respectively. The other phospholipid classes also contain a high percentage of unsaturated fatty acids and the majority of the molecular species in these phospholipids would be expected to have low transition temperatures. Although saturated phosphatidylethanolamines have higher transition temperatures than corresponding phosphatidylcholines, the cis-unsaturated phosphatidylethanolamines have similar transition temperatures as compared with the corresponding phosphatidylcholines (Van Dijck et al., 1976).

Small differences in the fatty acid composition of the other phospholipid pools were observed between the different membrane fractions. Since the pools, with the exception of the phosphatidylcholine pool, contained more than one phospholipid species, it is difficult to determine the effect of small changes in the fatty acid composition upon the rotational relaxation time of DPH in these pools. These phospholipids were less abundant in the membranes than phosphatidylcholine so the small differences in the fatty acid composition could not be responsible for the differences in the rotational relaxation times observed between the mitochondrial, microsomal, and plasma membranes.

The rotational relaxation times of DPH in the original phospholipid extract from a membrane and in the recombined phospholipid pools after the silicic acid column gave identical values within experimental error. This demonstrated that the isolation methods did not produce any degradation of the phospholipids or introduce any contaminating compounds that interfered with the measurements. Attempts were made to calculate the rotational relaxation times for the original mixture by averaging the values for the individual phospholipid pools. The contribution for each pool was weighted by its mole fraction in the mixture. (This is the same as weighting each individual phospholipid by its mole fraction except that some pools contained more than one phospholipid.) This calculation assumes that mixing the individual phospholipids will result in a mixture of intermediate properties. When this was done for mitochondrial phospholipid pools, the agreement with the experimental data was excellent (Figure 5B). The calculated values gave poor results, however, for the microsomal and plasma membrane phospholipids which contained sphingomyelin (Figure 5A). This was due to the transition that occurred in pure sphingomyelin. In order to damp out the transition, which is what occurs at low sphingomyelin concentrations, the rotational relaxation times for the appropriate mixtures of sphingomyelin and phosphatidylcholine were determined as in Figure 4. These rotational relaxation times were used to give the combined contribution of the two phospholipids. The results for microsomal and plasma membrane phospholipids from choline-supplemented cells now gave excellent agreement with the experimental data (Figures 5A and 5C). The calculated values of the rotational relaxation times for the microsomal phospholipids from linoleate-supplemented cells gave higher values than the experimental data (Figure 5D). This was because the contribution of sphingomyelin was calculated using the sphingomyelin–phosphatidylcholine mixture from choline-supplemented cells. If the data from linoleate-supplemented cells, which was not available, had been used, the agreement should have been better. Likewise, calculating the rotational relaxation times for the phospholipid from one membrane using the values of the rotational relaxation time determined on the phospholipid pools from another membrane (Figure 5A) did not give quantitative agreement because of the difficulty in calculating the contribution of sphingomyelin and the differences in the phospholipid and fatty acid composition of the pools from the different membranes. If more data were available on pure phospholipids, it may be possible to calculate the rotational relaxation times for the different membranes.

The differences in the rotational relaxation times of DPH between the phospholipids of mitochondrial, microsomal, and plasma membranes (Gilmore et al., 1979) can be explained by the differences in the polar head-group composition. Plasma membrane phospholipids had the lowest concentration of phosphatidylcholine and the highest concentrations of sphingomyelin, phosphatidylethanolamine, and phosphatidylserine. Of all the phospholipids isolated in this study, a change in the concentration of sphingomyelin in the membrane caused the greatest change in the rotational relaxation times. The rotational relaxation times of DPH in phosphatidylcholine were the lowest of all the phospholipids. This should give a membrane with the highest rotational relaxation times which was the observed result. The rotational relaxation times of DPH in mitochondrial phospholipids were the lowest and these phospholipids contained only trace amounts of sphingomyelin and phosphatidylserine and the highest amounts of phosphatidylcholine and cardiolipin. The high rotational relaxation times of DPH in cardiolipin would be expected to compensate for the absence of sphingomyelin to a small extent. It is likely that the rotational relaxation times of DPH in phosphatidylserine were high which would be consistent with the observed results. The rotational relaxation times of DPH in microsomal membranes were intermediate and so was the phospholipid composition. When cells were supplemented with ethanolamine in place of choline, there were increases in the rotational relaxation times in the phospholipids of all three membrane fractions. This was consistent with the increase in phosphatidylethanolamine and sphingomyelin and the decrease in phosphatidylcholine which occurred in all the membranes.

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Apoproteins of Avian Very Low Density Lipoprotein: Demonstration of a Single High Molecular Weight Apoprotein†

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ABSTRACT: The high molecular weight apoproteins of very low density lipoprotein (VLDL) were compared after preparation of VLDL from plasma and sera of diethylstilbestrol-treated roosters. When prepared from plasma with adequate control of endogenous proteolytic activity, VLDL contained a single high molecular weight apoprotein (apo-VLDL-B) as judged by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Serum VLDL contained multiple apoprotein species, the largest of which corresponded to apo-VLDL-B. Immunological analyses showed that the multiple apoproteins of serum VLDL were quantitatively and qualitatively indistinguishable from plasma apo-VLDL-B. These data indicate that apo-VLDL-B can be cleaved during VLDL isolation to produce an apparent heterogeneity of high molecular weight apoproteins. The molecular weight of plasma apo-VLDL-B was estimated to be 350 000. This protein was stable to reduction and S-carboxymethylation and showed no association with apo-VLDL-II [Chan, L., Jackson, R. L., O'Malley, B. W., & Means, A. R. (1976) *J. Clin. Invest. 58*, 368] through disulfide linkage. Apo-VLDL-B and apo-VLDL-II represented 54% and 46%, respectively, of the total VLDL protein recovered following gel filtration chromatography in sodium dodecyl sulfate. Protein recovery in the chromatographic analyses (92%) was sufficient to conclude that apo-VLDL-B and apo-VLDL-II are the major and possibly the only apoproteins of chicken VLDL. The molar ratio of the apo-VLDL-II monomer to apo-VLDL-B was estimated to be 32.

The association between hyperlipoproteinemic states and the development of atherosclerotic disease in man (Goldstein & Brown, 1977) has focused considerable attention on the structure and metabolic regulation of VLDL.[1] In this regard the chicken has frequently been employed for studies of VLDL. Blood levels of VLDL increase greatly with the onset of egg laying in the hen (Schjeide, 1954) or after estrogen administration to the rooster (Hillyard et al., 1956). Large quantities of chicken VLDL can be readily obtained for structural studies, and the hormonal regulation of VLDL synthesis can be studied in vivo (Luskey et al., 1974; Chan et al., 1976) or in cultured chick liver cells (Tarlow et al., 1977).

Studies of this nature require adequate knowledge of the individual protein moieties of VLDL. Until recently, however, little has been known about the number of apoproteins in VLDL or the characteristics of the individual protein species. There is general agreement that VLDL apoproteins fall into two very different molecular weight categories on the basis of gel-filtration chromatography in the presence of detergent (Hearn & Bensadoun, 1975; Chan et al., 1976; Chapman et al., 1977). A low molecular weight apoprotein, apo-VLDL-II, has recently been isolated, characterized, and sequenced (Chan et al., 1976; Jackson et al., 1977). This protein contains 82 amino acid residues, constitutes 40–50% of the total VLDL protein, and is probably the sole low molecular weight VLDL apoprotein. With regard to the high molecular weight apoproteins, however, there is little agreement as to the number of protein species in VLDL. In a recent study of hen VLDL (Chapman et al., 1977), $NaDodSO_4$ gel electrophoresis showed seven or more protein bands ranging in molecular weight from approximately 140 000 to over 300 000. Similar extents of

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[1] Abbreviations used: VLDL, very low density lipoprotein; apo-VLDL-B, apoprotein B of VLDL; apo-VLDL-II, apoprotein II of VLDL; DES, diethylstilbestrol; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; PSP, 0.02 M sodium phosphate (pH 7), 0.15 M NaCl, 100 μg/mL $PhCH_2SO_2F$; $NaDodSO_4$ sodium dodecyl sulfate.

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