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Paramagnetic Isoprenoid Carrier Lipids. 1. Chemical Synthesis and Incorporation into Model Membranes†

Michael A. McCloskey‡ and Frederic A. Troy*

ABSTRACT: The synthesis, purification, and characterization of two types of spin-labeled glycosyl carrier lipids and shorter chain isoprenols are described. As models for phosphorylated lipid intermediates, phosphodiesters of tempol and the prenols dolichol, ficaprenol, solanesol, phytol, and farnesol were prepared. For analogues of neutral species each prenol was esterified with a pyrrolidinecarboxylic acid based label. Tripropylbenzenesulfonyl chloride was used as the condensing agent in both cases. Phosphodiester yields ranged from 36% for the 55-carbon compound to >66% for the 95-carbon prenol. Both types of probes were incorporated into phospholipid bilayers, where each became oriented with the artificial head group at, or very close to, the water–hydrocarbon interface. Electron spin resonance spectra of the phosphodiesters are matrix dependent, indicating rapid isotropic tumbling in chloroform but highly anisotropic reorientation in unsaturated phosphatidylcholine (PC) hosts. Rotation or large amplitude oscillation about either or both the tempo C_4–O linkage and the P–O (chain) bond as well as whole molecule rotation within the bilayer could account for the observed *x*-axis anisotropy. Segmental motion within the polyprene chain does not appear to be a determinant.

Dolichyl phosphate and undecaprenyl phosphate are poly-cis-isoprenoid lipids which function as chemical carriers of saccharide units during the membrane-directed assembly of mammalian glycoproteins and a variety of bacterial surface polysaccharides. During the past 10 years a substantial amount of information concerning their biochemistry has been amassed (Hemming, 1974; Lennarz, 1975; Waechter & Lennarz, 1976; Parodi & Leloir, 1979). Reactions leading to their biosynthesis and acylation as well as the detailed sequences of transfer reactions they mediate are being mapped out. Subcellular locations, nucleotide and polyisoprenoid specificities, antibiotic sensitivities, and detergent and metal effects for the complementary transferases are continually being defined. Singularly lacking, however, is an understanding of the organizational and dynamic parameters of the lipid carriers and transferases within either model or biological membranes. To our knowledge only one group has attempted to directly probe the immediate membrane environment of a "lipid-linked sugar" (Johnston & Neuhaus, 1975; Weppner & Neuhaus, 1978). After studying the fluorescence spectra of an endogenous dansylated intermediate of peptidoglycan synthesis in *Staphylococcus aureus*, they concluded that the dansyl moiety was located near the membrane surface and that the intermediate was immobilized within the membrane.

It is conceivable that the length and poly-cis geometry of these compounds could endow them with some unique physicochemical properties that are important to their biological function(s). For the purpose of investigating certain of these characteristics in model systems, we have chemically synthesized two groups of spin-labeled polyisoprenols. One group was designed to mimic the behavior of phosphorylated intermediates, and a second series of carboxylate esters was intended to model properties of the abundant neutral species found in vivo (Keenan et al., 1977a; Bohnenberger & Sandermann, 1976). Human dolichol and plant undecaprenol were chosen as the primary starting materials. In order to compare cis/trans and chain-length effects, analogues from farnesol, phytol, and solanesol were also synthesized. This report outlines details of the synthesis and purification of these probes and also gives a preliminary account of their spectral properties in various systems.

Experimental Section

General Methods and Materials. Anhydrous solvents were prepared as described elsewhere[1] and dispensed via a repipet with an attached drying tube. In condensation reactions where anhydrous conditions were required, the separate or combined reagents were dried in a small (5–50 mL) round-bottom flask

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* Address correspondence to this author. He is the recipient of U.S. Public Health Service Career Development Award CA 00095.
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[1] The details of these experiments are described fully in McCloskey (1979).

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by repetitive azeotropic distillation with either pyridine or toluene. A Büchi roto-vap with an oil-pump vacuum (≤10 torr) was employed. Water bath temperatures ranged from 25 to 40 °C. Between evaporations the pressure was equilibrated by filling the system with oil-pumped nitrogen or argon gas.

2,4,6-Triisopropylbenzenesulfonyl chloride (Aldrich) was recrystallized three times from isopentane to a melting range of 98.5–99.8 °C (lit. mp 97–98 °C: Newton, 1943). Egg phosphatidylcholine (egg PC)[2] was isolated from hen's eggs as described by Singleton et al. (1965).

Analytical thin-layer chromatography (TLC) was performed on precoated plates (250 μm × 10 cm × 20 cm) of silica gel F254 (Merck). Preparative thick-layer chromatography (PLC) was on precoated plates (2 mm × 20 cm × 20 cm) of silica gel F254 (Merck) or silica gel G (Analtech). Solvent systems employed were as follows: (A) $CHCl_3$–CH_3OH–H_2O (65:25:4 v/v); (B) $CHCl_3$–CH_3OH–NH_4OH (14.8 M)–H_2O (70:30:4:1); (C) $CHCl_3$–MeOH (5:1); (D) toluene–ethyl acetate (95:5); (E) $CHCl_3$–MeOH–H_2O (10:10:3). Descending paper chromatography was on Whatman 3MM paper (20 × 10 in.) using as solvent 2-propanol–NH_4OH (concentrate)–H_2O (7:1:2 v/v). Nitroxide-containing spots were visualized on paper and silica by their orange color and absorption of UV light (254 nm). Phosphate esters were detected on paper with the Wade–Morgan (1953) dip and on silica with the reagent of Vaskovsky & Kostetsky (1968). General lipid stains for TLC were iodine and sulfuric–dichromic acid/heat. Polyterpene was visualized with the anisaldehyde spray described by Dunphy et al. (1966). High-pressure liquid chromatography (high-pressure LC) of dolichyl and ficaprenyl *p*-nitrobenzoates was performed exactly as described by Keenan et al. (1977b).

Elemental analyses were performed at the microanalytical lab of the University of California at Berkeley (Chemistry Department). To drive off the last traces of solvent it was necessary to heat the samples in vacuo at 60 °C for 1–2 h. Otherwise the carbon values were erroneously high. Quantitative phosphorus analyses were performed as described by McClare (1971).

IR spectra were recorded on a Perkin-Elmer Model 337 spectrometer. Samples (~2 mg) were held as thin films between sodium chloride plates. ^1H NMR spectra (100 MHz) were recorded on a Jeolco PS-100 P/EC-100 Fourier-transform instrument. Internal Me_4Si was the reference. $CDCl_3$, pyridine-d_5, and D_2O were purchased from Bio-Rad. Acquisition of ^1H spectra on paramagnetic samples initially posed a problem, in that previously successful solvent–reductant combinations (Lee & Keana, 1975; Rozantsev, 1970) failed to consume all the radicals and generated spurious upfield peaks. The combination of pyridine-d_5 and phenylhydrazine (1–2 equiv relative to label) gave unambiguous results.

X-Band electron paramagnetic resonance (EPR) spectra were recorded on a Varian E-4 instrument equipped with a variable temperature controller (Varian No. V-4540). Liposomal samples were contained in 50-μL micropipets sealed at one end and held in place with a cylindrical Teflon gasket (Gaffney & McNamee, 1974). Oriented multibilayers were held between quartz plates in a rotatable Kel-F holder designed by Dr. Wayne Hubbell. Hyperfine constants (A_N) were measured relative to tempol (17.05 G) and [^{15}N]tempone (22.8 G) in water.

Polyprenols. Farnesol and phytol were from Aldrich. Solanesol was isolated from flue-cured tobacco leaves (3 days, 16% moisture) generously donated by Dibrell Bros., Inc., Danville, VA. Purification was basically as described for ficaprenol below, except that the final step was crystallization from acetone (4 volumes) at 4 °C. The isolated material gave a single spot upon TLC in system D and comigrated with an authentic sample of solanesol supplied by Dr. R. L. Rowland of R. J. Reynolds Tobacco Co. (Rowland et al., 1956; Erickson et al., 1959). ^1H NMR in $CDCl_3$ was as expected for an *all-trans*-nonaprenol, with the main CH_3 peak at δ 1.59 and a minor one at δ 1.67 (Feeney & Hemming, 1967).

Ficaprenol was isolated from leaves of *Ficus elastica*, kindly supplied by Dennis Kucera of the botanical garden at the University of California, Riverside, CA. The procedure was essentially that described previously (Stone et al., 1967), although complete purification required an additional step described elsewhere.[1] Anal. Calcd for $C_{55}H_{90}O$: C, 86.09; H, 11.82. Found: C, 86.15; H, 11.86. Analysis of the isoprenologue composition by reversed-phase high-pressure LC revealed only three components, C_{50}, C_{55}, and C_{60}, with the 55-carbon species dominant.

Dolichol was isolated from two human livers which had been obtained at autopsy. The procedure was a modification[1] of that employed by Burgos et al. (1963). Anal. Calcd for $C_{95}H_{156}O$: C, 86.82; H, 11.96. Found: C, 86.89; H, 11.83. Six prenologues were detected by high-pressure LC. The relative amounts of two major components were more nearly equal than those reported for pig liver dolichols (35 and 39% vs. 46 and 28%). This could reflect an intra- or an interspecies variability.

Spin-Labels. 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy and tempol were made according to published procedures (Rozantsev, 1970). Radioactive tempol was prepared by reduction of triacetonylamine with [^3H]$NaBH_4$ (ICN, 250 mCi/mol) and oxidation to the nitroxide as above. Tempo phosphate (PT) was synthesized as described by Keith et al. (1977). The final material migrated as a single component (^3H, phosphate, nitroxide) upon paper chromatography and cochromatographed (R_f 0.44) with an authentic sample of PT (Syva, Palo Alto, CA). Stock solutions (0.04–0.6 M) in anhydrous pyridine were stored at −20 °C until use.

Phosphodiesters.[3] Several small-scale (40 mg of ficaprenol) reactions were carried out to optimize conditions. Independent of the amount of prenol, variation in the TPS:PT ratio controlled the level of prenol sulfonylation. For ratios ≤1 no sulfonate was formed. The apparent yield of phosphodiester increased gradually with increase in the PT:prenol ratio between 0.85 and 3.0. Within 60 h progress had ended, and the putative sulfonate became evident. Heating at 37 °C did not enhance the yield. These observations led to the following general scheme. Prenol (1.0 equiv), PT (2.15 equiv), and TPS (2.05 equiv) are dried separately as above. The PT and TPS are dissolved in a minimum volume of pyridine and allowed to react at room temperature for 15 min or until the precipitation of pyridinium chloride is complete. Prenol is added

[2] Abbreviations used: IR, infrared; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; A_N, nitrogen hyperfine coupling constant; T_c, transition temperature; high-pressure LC, high-pressure liquid chromatography; TLC, thin-layer chromatography; PLC, preparative thick-layer chromatography; PT, tempo phosphate (2,2,6,6-tetramethyl-4-phosphopiperidinyl-1-oxy); TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy; PC, phosphatidylcholine; $R_{\parallel}$, rotational diffusion constant about unique axis of axially symmetric tensor.

[3] The presumed diesters of cholesteryl phosphate and dipalmitoylphosphatidic acid with tempol were made using TPS and purified to yield a single spot upon TLC.

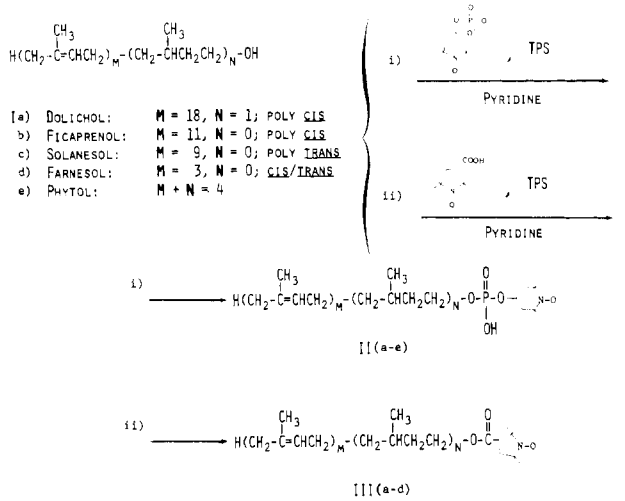

FIGURE 1: Summary of the synthetic schemes which were employed in preparation of spin-labeled polyisoprenoids.

under exclusion of moisture and the sealed reaction flask stored under dry N_2 in a desiccator for 2–3 days. Water is added just to the point of incipient emulsification and the mixture stirred for 5–15 h at room temperature, the time depending on the chain structure of the prenol. Most of the solvent is then evaporated and the orange residue partitioned between equal volumes of $CHCl_3$ and 5% $NaHCO_3$. The chloroform is rinsed with water, concentrated, and passed through a small column of sodium sulfate in a Pasteur pipet. The crude product in chloroform is purified by PLC in solvent B. If a uniform salt form is desired, a very dilute solution is slowly percolated through a large excess of MP50 resin (Bio-Rad Laboratories) in $CHCl_3$–CH_3OH (1:1). The isolated yields, based upon mass and phosphorus content, are 36% for ficaprenol (IIb) and ≥66% for dolichol (IIa), based on percent of theoretical.

Spin-labeled polyprenyl phosphodiester of ficaprenol (compound IIb): Anal. Calcd for $C_{64}H_{108}NO_6PNa{\cdot}H_2O$: C, 73.81; H, 10.45; N, 1.34; P, 2.97. Found: C, 73.95; H, 10.59; N, 1.33; P, 3.00. IR 3500 (br, H_2O), 2950, 2920, 2850 (CH stretch, aliphatic), 1660 (C=C), 1450 (CH_2, CH_3 bending), 1220 (P=O), 1045 (POC), 835 cm^{-1} (R_2C=CH bend); ^1H NMR δ 1.38, 1.42 (ax, eq ring CH_3), 1.71 (chain *trans*-CH_3), 1.82 (chain *cis*-CH_3), (CH_2), 5.28 (C=CH), 5.67 ppm (t, α-C=CH); TLC (0.25-mm SG 60), R_f 0.51 (A), 0.64 (B). The ratio of spin to phosphate was ca. 1.0 initially, but declined with time upon storage at −20 °C in $CHCl_3$.

Spin-labeled polyprenyl phosphodiester of dolichol (compound IIa): Anal. Calcd for $C_{104}H_{174}NO_6PNa{\cdot}H_2O$: C, 78.64; H, 11.04; N, 0.88; P, 1.95. Found: C, 78.77; H, 10.96; N, 0.81; P, 1.87. IR 3450 (H_2O), 3000, 2940, 2860, 1670, 1460, 1375, 1310, 1215 (P=O), 1060 (POC), 840, 760 cm^{-1}; ^1H NMR δ 1.41, 1.45 (ring CH_3), 1.72 (chain *trans*-CH_3), 1.83 (chain *cis*-CH_3), 2.25 (chain CH_2), 4.29 (m, α-CH_2), 5.34 ppm (C=CH); TLC, R_f 0.59 (A). A similar loss of paramagnetism occurred with this compound, being 50% within 10 months. Remaining diesters were purified to apparent homogeneity.

Carboxylate Esters. Prenols were esterified with 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy using TPS in pyridine (Sharom & Grant, 1978). A single major product was obtained in each case. It was purified either by PLC in toluene–ethyl acetate (95:5) or by silica column chromatography. Compound IIIc was purified by three recrystallizations from acetone at 5 °C. Each compound gave a strong carbonyl absorption in the IR (1740 cm^{-1}).

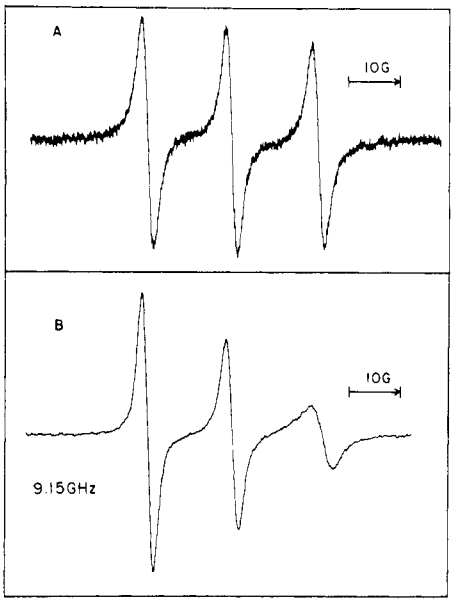

FIGURE 2: (A) EPR spectrum of the spin-labeled phosphodiester of ficaprenol (IIb, 10^{-5} M) in undegassed $CHCl_3$. Sweep width 100 G, magnetic field 3262 G, frequency 9.15 GHz, power 10 mW, modulation amplitude 0.5 G, and temperature 25 °C. (B) EPR spectrum of compound IIb (1.5 mol %) in egg PC liposomes at 25 °C. Instrument settings same as in (A).

Results and Discussion

Syntheses. Figure 1 outlines the general procedure that was used to construct spin-labeled polyprenyl phosphates and carboxylates. Several alternative means of forming the phosphodiester linkage were attempted before we concentrated on optimizing conditions of the TPS-mediated reaction.[1] Two major difficulties in obtaining phosphodiesters IIa–e via the TPS route were (1) formation of a considerable amount of prenylsulfonate biproduct and (2) complete failure of ion exchange using conventional resins.[1] To avoid sulfonylation of prenol it was necessary to keep the ratio of TPS:PT ≤ 1 and also to allow a short prior reaction of these components before adding prenol. Effective counterion exchange was only achieved with the use of a macroporous resin (AGMP50, Na^+).

Since treatment of prenols with TPS and 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy gave quick and apparently quantitative (TLC-D) conversion to a single paramagnetic product containing terpene, this procedure was adopted. After aqueous extraction and column or preparative thick-layer chromatography, the yields averaged 50%. Solanesyl compound IIIc was purified by recrystallization from acetone at 5 °C. None of the remaining compounds II (Na^+ salt) or III were obtained in crystalline form. Each is a pale yellow (III) or orange (II) syrup soluble in a variety of nonpolar solvents, including petroleum and ethyl ether. While the longer chain (≥45 C) members of both series are essentially water insoluble ($<10^{-5}$ M), phosphodiesters IId and IIe are appreciably soluble ($>10^{-2}$ M).

EPR Spectra. The EPR spectrum of ficaprenyl diester IIb in undegassed chloroform is shown in Figure 2A. Three sharp lines of approximately equal amplitudes indicate that the probe is tumbling rapidly and isotropically in this medium. In contrast, when the same molecule is incorporated into egg PC bilayers its motion becomes highly anisotropic (Figure 2B). At 9.15 GHz the serial decrease in amplitudes of low-, mid-, and high-field lines is characteristic of anisotropic tumbling (rotation or large amplitude oscillation) about an axis coincident, or nearly so, with the nitroxide N–O bond axis (Nordio,

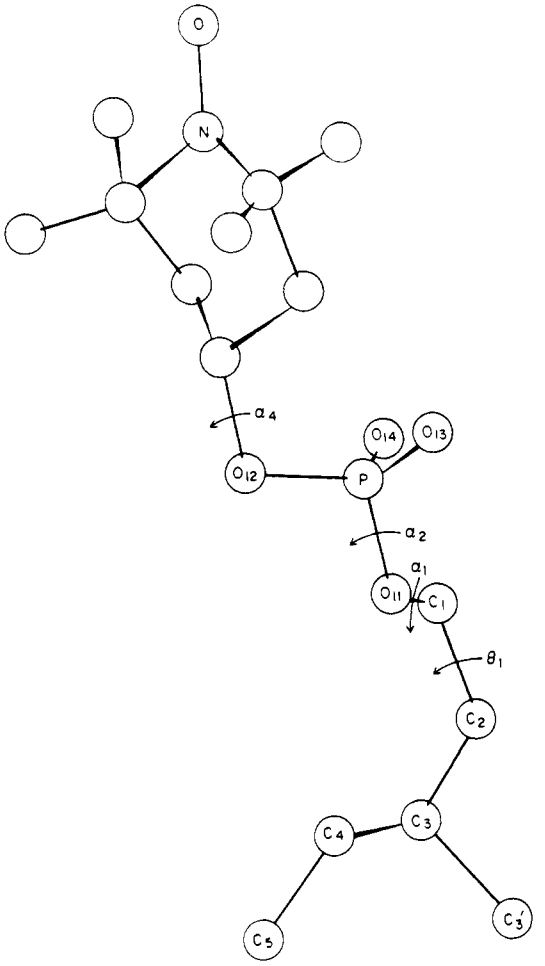

FIGURE 3: Perspective drawing of a reasonable conformation in the head group region of the spin-labeled polyprenyl phosphodiester of ficaprenol (IIb). Numbering and torsion angles follow from McAlister et al. (1973).

1970; Williams et al.,[4] 1971; Birrell et al., 1973; Van et al., 1974). As deduced from the orientation-dependent hyperfine splitting ($2A_{max}$ = 36.5 G, $2A_{min}$ = 27.5 G), this axis is aligned preferentially normal to the bilayer plane. The isotropic splitting of IIa–c in egg PC (A_N = 16.3 G) is intermediate between that of tempol in water and that of tempol in hexane and indicates that the nitroxyl group is incompletely exposed to bulk water. Comparatively slow reaction with ascorbate supports this (an 80–100-fold excess is required for complete reduction within 5 min at 0 °C; cf. Kornberg & McConnell, 1971). An alternative explanation of the reduced A_N is that the ring is rapidly ($\nu_{ex} \gg \sim 5 \times 10^7$ Hz) exchanging between fully exposed and hydrophobic positions. However, the observation of a net orientation in multilayer samples militates against this.

Motional Contributions. It is not surprising that probe motion in the interface region is restricted. However, one would like to assess the contributions of individual segments to the overall x-axis motion. Of the low-energy phosphodiester conformations, only the $t(\alpha 3),g^+(\alpha 2)$ state permits simultaneous outward orientation of the ring, approximately perpendicular alignment of the N–O bond axis, and reasonably parallel packing arrangement of the upper isoprenoid chain (Figure 3; also McAlister et al., 1973). In this geometry the O_{14}–O_{13} vector is nearly parallel to the membrane surface, and the dihedral angle between the O_{14}–P–O_{13} plane and the surface is within 10–15° of that observed for several phospholipids. Fluctuation of the torsion angles $\alpha 4$ and $\alpha 2$ could impart a strong component of x-axis motion to the label, while variation in $\alpha 1$ and $\alpha 3$ would not. Combined rotations and/or jumps of $\alpha 2$ and $\theta 1$ in glycerophospholipids have been postulated in order to explain the axial motion averaging of the static phosphorus-31 chemical shift tensor (Seelig & Gally, 1976; Kohler & Klein, 1976). The motion of tempo-labeled cholesteryl phosphate in EPC is even more anisotropic than that of the diesters IIa–c or of spin-labeled phosphatidic acid,[5] suggesting that rotation about the C_1–C_2 bond or any others within the chain does not contribute positively here. This is also apparent upon inspection of molecular models fixed in the above-mentioned conformation (Figure 3).

Rotation of an entire phospholipid molecule about its long axis has also been invoked to explain ^{31}P NMR results, and it has been directly observed and quantitated for spin-labeled steroids in bilayer systems (Schindler & Seelig, 1974; Seelig, 1970). If rotational constants for the entire isoprenoid chain could be extracted from EPR spectra, then potential insight into their molecular shape and size within the membrane would be at hand. One can place a lower limit on the frequency of this motion by assuming an axially symmetric diffusion tensor which is colinear with the nitroxide x principal axis (roughly the N–O bond axis). Assuming complete averaging of the y and z magnetic tensor components, the whole molecule rotational frequency must be 90 MHz or faster.[5] A different type of calculation yields values for the actual $R_\parallel$ of IIa,b in egg PC (25 °C) of 70–420 MHz.[6] This compares with a value of ca. 780 MHz for steroid spin probes in decanol–decanoic acid bilayers. Since rotation is slower in phospholipid membranes than in this system (because of their approximately tenfold greater viscosity), the rates are in order of magnitude agreement. While this suggests that whole molecule rotation could contribute, it does not replace the elaborate spectral simulations that would ultimately be required to handle the problem.

The EPR spectra of the carboxylates IIIa–d dissolved at low concentrations in egg PC are virtually identical. The sharp three-line signals reveal rapid ($\tau_c \sim 3$–8×10^{-10}) and apparently isotropic tumbling. As for the phosphates, the splitting of these compounds (A_N = 15.0 ± 0.2 G) is intermediate between that of IIId in H_2O (16 G) and that of IIId in heptane (14 G). This suggests that the polarity of combined nitroxide and ester functions is great enough to anchor the head

[4] Calculated with (Williams et al., 1971)

$$\tau_c = (6.5 \times 10^{-10}\ \text{s})\left[\left(\frac{h_0}{h-1}\right)^{1/2} - 1\right]W_0$$

[5] The following equation was used:

$$\nu_{min} = (\mu_\beta H_0/h)(g_{zz} - g_{yy}) + (\gamma_e/2\pi)(A_{zz} - A_{yy})$$

A and g tensor components are not available for tempo phosphate, so single-crystal values for DTBN were used (Libertini & Griffith, 1970). μ_β is the Bohr magneton, H_0 is the static field strength, h is Planck's constant, g_{zz} and g_{yy} are z and y principal components of the g tensor, γ_e is the electron's magnetogyric ratio, and A_{zz} and A_{yy} are y and z principal components of the hyperfine tensor.

[6] Under the assumption that rotational diffusion is continuous and described by an axially symmetric tensor, Vasserman et al. (1971) have calculated the dependence of a line-width parameter, ϵ, on the diffusional anisotropy ratio, $d = R_\parallel/R_\perp$, for various relative orientations of the nitroxide magnetic and diffusion tensors. For x-axis motion of ficaprenyl diester IIb in egg PC, we interpolate values of d = 4–18 using the average and extreme values of g and A components listed. Since the exact g and A values for IIa–e are not available, an unequivocal determination of d from ϵ is not possible.

group near the interface region. Even in a homogeneous, saturated host-like dipalmitoyl-PC, the large isoprenoid bulk is an insufficient perturbation to force the entire chain into the central, most disordered region of the bilayer.

These lipids were constructed primarily as probes for studying lateral and transverse diffusion rates as well as the state of aggregation of long chain isoprenoid lipids in various membranes. The following paper deals with measurements of the latter two types in model systems (McCloskey & Troy, 1980).

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