Paramagnetic Isoprenoid Carrier Lipids. 2. Dispersion and Dynamics in Lipid Membranes†

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ABSTRACT: The transbilayer diffusion rates and self-association of spin-labeled glycosyl carrier lipids and other isoprenoids have been studied in model phospholipid membranes. Transposition rates of phosphorylated species in small (300 Å) or large (≥1000 Å) diameter unilamellar phosphatidylcholine (PC) vesicles are slow ($t_{1/2}$ > 5 h at 25 °C); this argues against their proposed role in the transbilayer portage of sugar units during polysaccharide and glycoprotein assembly. The same probes mix well with several host lipids, remaining monomolecularly dispersed even at high ionic strength (0.5 M NaCl) or in the presence of various polyvalent cations. This behavior persists for several degrees beneath the transition temperature (T_c) of saturated lecithins. In contrast, neutral carboxylate analogues undergo pronounced reversible self-association. Aggregation is quite dependent on the probe concentration and nature of the host but only weakly temperature dependent above the T_c. Even at low probe concentrations (<0.5 mol %) in fluid membranes, aggregates persist above 75 °C. Unsaturation in the lecithin fatty acyl chains dramatically increases isoprenoid monomer solubility. Segregation appears to involve relocation of the entire molecule in the membrane interior.

The transmembrane migration rates of isoprenyl carrier lipids are of immediate interest in light of their suggested function in transmembrane carbohydrate transport. The notion that undecaprenyl pyrophosphate physically transports covalently attached sugars from the cytoplasmic to the external face of bacterial plasma membranes is longstanding and widespread (Anderson et al., 1965; Kanegasaki & Wright, 1970; Lehninger, 1975; Stryer, 1975; Robyt, 1979). A similar facilitating role has been proposed for the dolichyl chain in glycoprotein assembly (Depierre & Dallner, 1975; Lennarz, 1975; Parodi & Leloir, 1979). Supporting rationale has been twofold. First, the final polymerized saccharide units reside in an aqueous space separated from the site of synthesis of the original nucleotide–sugar substrates by a membrane which is presumed to be impermeable to those substrates. Second, is is asserted that the extremely hydrophobic nature of lipid intermediates should permit polar carbohydrate units to surmount the permeability barrier of the lipid bilayer.

Lipid-mediated oligo- and polysaccharide elaboration must often be rapid, e.g., in an exponentially growing bacterial culture or in plasma cells actively producing antibodies. The transbilayer exchange rates of carriers must accordingly be fast to account for this with the above mechanism. The permeability agrument would predict rapid transposition even in model lipid systems. Therefore, as a test of this hypothesis we have estimated the transverse diffusion rates of spin-labeled carriers in unilamellar phosphatidylcholine vesicles.

The spatial distribution of lipid carriers within the membrane plane has also been a matter of some concern in the literature. Although two reports have argued for sequestration of certain peptidoglycan and techoic acid intermediates within transferase complexes, not all the phosphorylated species may necessarily act the same (Anderson et al., 1972; Weppner & Neuhaus, 1978). Kanegasaki & Wright (1970) have interpreted their data to mean that undecaprenyl intermediates of *Salmonella* O-antigen synthesis are freely mobile within the hydrophobic membrane domain. Moreover, in both prokaryotes and eukaryotes a major portion of poly-cis-isoprenoid is often present as "inactive" free alcohol or fatty acyl ester (Bohnenberger & Sandermann, 1976 and references cited therein). Nothing is known about the function or mode of intergration of this material in the membrane. In order to ascertain limits on the in vivo possibilites for organization of these "superlipids" within the bilayer, we have studied the distribution of spin-labeled analogues in model systems of variable lipid composition. We report here on the results of this work as well as on the transbilayer movement of these probes.

Materials and Methods

Much of the general protocol, including the preparation and structures of the spin-labeled polyprenyl phosphodiesters of dolichol (IIa), ficaprenol (IIb), solanesol (IIc), farnesol (IId), and phytol (IIe) and the spin-labeled polyprenyl carboxylate esters of dolichol (IIIa), ficaprenol (IIIb), solanesol (IIIc), and farnesol (IIId) (Figure 1, accompanying manuscript), is described in McCloskey & Troy (1980). HGSL-I[1] was synthesized according to Kornberg & McConnell (1971).

Sample Preparation and Characterization. Stock lipid solutions were stored in chloroform or ethanol; their concentrations were determined by phosphate assay or weighing. Appropriate aliquots were mixed in a small flask to give the desired molar ratios, and the bulk of solvent was removed under a stream of N_2 at 40–50 °C. The samples were then placed on a lyophilizer overnight (10–20 h). Buffer was added and the sample was heated to 50–65 °C and then vortexed or stirred magnetically for 10–20 min to achieve complete suspension. If the vacuum treatment was too short, residual $CHCl_3$ had profound effects on β values (see below).

Large diameter vesicles were prepared using the ether injection technique of Deamer & Bangham (1976). Probe

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[1] Abbreviations used: HGSL-I, head-group spin-labeled phosphatidylcholine; PC, phosphatidylcholine; EPC, egg PC; DML, dimyristoyl-PC; DPL, dipalmitoyl-PC; Chol, cholesterol; NaAsc, sodium ascorbate; M_i, z component of ^{14}N nuclear spin (0, ±1); EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; t_0, zero time; T_c, transition temperature.

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concentration was 1–3 mol % with 6–8% phosphatidic acid included to prevent aggregation. The dispersion was filtered through a Millipore filter (0.22 μm) to remove large liposomes and passed through Sephadex G-50 to eliminate most of the residual ether. Approximately 50–55% of the spin was protected from $NiSO_4$ at 25 °C (see below) and NaAsc at 2 °C, indicating ≤5% contamination with multilamellar or concentric liposomes.

Small diameter vesicles were formed either by sonication or by cholate removal (Brunner et al., 1976). Sonication was for 30 min in an ice bath using a power setting of 40 W. A Heat Systems Ultrasonics device with microtip was employed. Typically, 3 mL of suspension was sonicated in a tube 1 cm in diameter. "Sonic" vesicles were purified by ultracentrifugation as described by Barenholz et al. (1977). Only region III vesicles were used. "Cholate" vesicles were formed as described previously; retention of cholate was monitored with tritiated sodium cholate (New England Nuclear, 14 mCi/mmol) and amounted to <0.5 mol % relative to the PC. For all samples the buffer was either 50 mM phosphate–0.1 M NaCl (pH 7.2) or 50 mM Tris–0.1 M NaCl (pH 7.3).

Negative-stain electron microscopy was performed with 1.5% uranyl acetate on Formvar-coated copper grids.

^1H NMR spectra (360 MHz) were obtained on a Nicolet NPC/FT-1180 instrument. Vesicles were dialyzed four times vs. D_2O prior to acquisition of spectra. Inner and outer choline methyl resonances were separated with europium(III) as described elsewhere (Bystrov et al., 1971).

Transverse Diffusion. Transbilayer motion of spin-labeled polyprenyl phosphodiester probes IIa–d was followed by using a modification of a previously reported technique (Kornberg & McConnell, 1971). Nitroxide head groups in the outer monolayer of single bilayer vesicles were reduced by treatment with a 100-fold excess of NaAsc at 0 °C for 5–10 min. NaAsc was removed (>99.99%, ^{14}C) by a rapid gel filtration step (Sephadex G-25, 1 × 25 cm) at 4 °C, and the vesicles were brought back to ambient temperature. At regular intervals after elution, aliquots were removed and diluted 1:1 with either buffer or 2.0 M nickel sulfate. Nickel(II) acts as a nonpenetrating paramagnetic broadening agent to quench signals from accessible spin-labels (Keith et al., 1977; Polnaszek et al., 1978). The amplitude of the low field (+1) EPR line was measured and used as an estimate of total ($+H_2O$) or internal (+Ni) paramagnetism. The amplitude of this signal decreased linearly with the logarithm of Ni^{II} concentration (25 °C) up to 1.0 M, after which it leveled off. For all nonreduced samples the signal remained constant for over 2 h in the presence of nickel, and the percent of signal protected agreed within ±2% with the value obtained using NaAsc at 0 °C in the spectrometer cavity (Kornberg & McConnell, 1971). The inside–outside ratios using nickel were therefore determined.

EPR Spectra. EPR spectra were obtained as described before or on a Varian E-12 instrument equipped with an automatic field-frequency lock and interfaced with a Digital PDP8 computer. The two-component spectra of compound IIIc were decomposed by subtracting variable amounts of exchange-collapsed spectrum (obtained at high levels concentration) until a nearly exchange free three-line spectrum emerged (Jost & Griffith, 1976). Alternatively, the fractional area of the exchange-free component, β, was estimated with

$$\beta = \frac{h(\mathrm{s})W(\mathrm{s})^2}{h(\mathrm{s})W(\mathrm{s})^2 + (3/2)[h(+1)W(+1)^2 + h(-1)W(-1)^2]}$$

with $h(\mathrm{s})$, $h(\pm1)$ being the heights of collapsed or exchange-free (±1) lines and $W(\mathrm{s})$, $W(\pm1)$ their respective widths. This formula assumes a Lorentzian line shape for both spectral components and is based upon the proportionality between the area under an absorption curve and the height × (width)^2 of its first derivative. Widths are measured from peak to trough and $h(\pm1)$ and are corrected for the immediately underlying contribution from exchange-collapsed spectrum. Results of these methods differed by as much as 10% for low values of β. The latter method was used for analyzing spectra of dolichyl and ficaprenyl carboxylates IIIa,b.

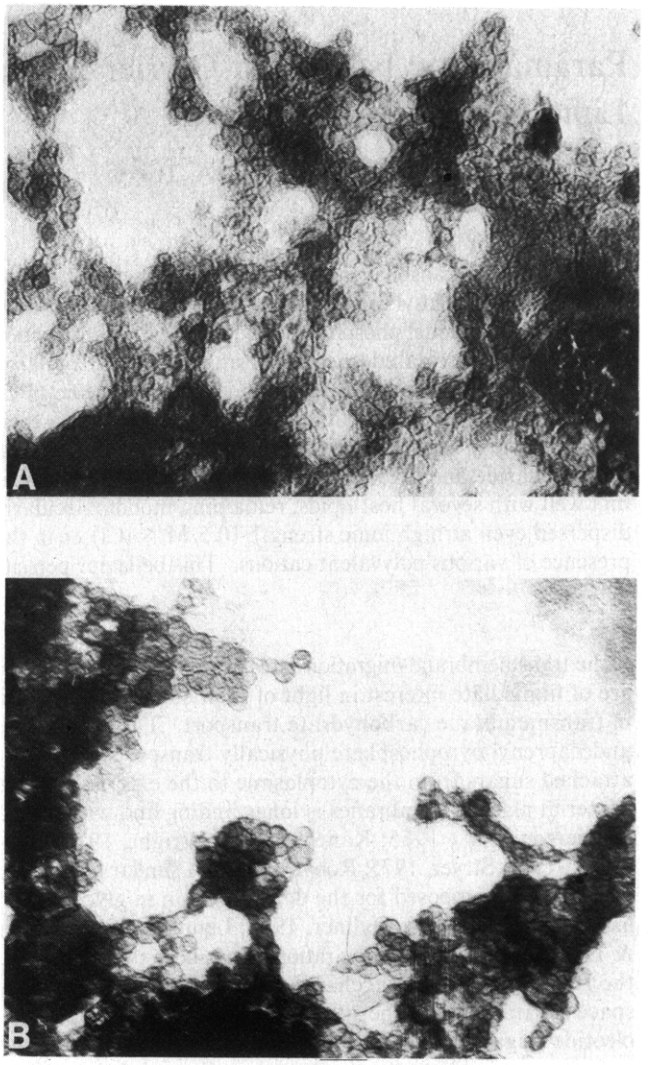

FIGURE 1: Negative-stain electron microscopy of egg PC vesicles (A) with and (B) without 3 mol % of the spin-labeled phosphodiester of ficaprenol (IIb). Magnification is 117 000 diameters.

Results

Vesicle Structure. Figure 1 shows electron microscope pictures of EPC vesicles containing 3% IIb. A relatively homogeneous population with an apparent average diameter of ~270 Å is present.

In the presence of Eu^{III}, ^1H NMR on the same samples revealed two *N*-methyl peaks with an upfield (outer) to downfield (inner) area ratio of ~1.9. The two peaks remained distinct for several hours at room temperature. These results are consistent with the existence of stable single bilayer vesicles containing the probe.

The inside–outside partitioning of HGSL-I, as measured with $NiSO_4$ at 25 °C or NaAsc at 0 °C, was approximately 35% inside and 65% outside (cf. Kornberg & McConnell, 1971). In contrast, the polyisoprenoid lipids II and III had a greater preference for the inner compartment, the distri-

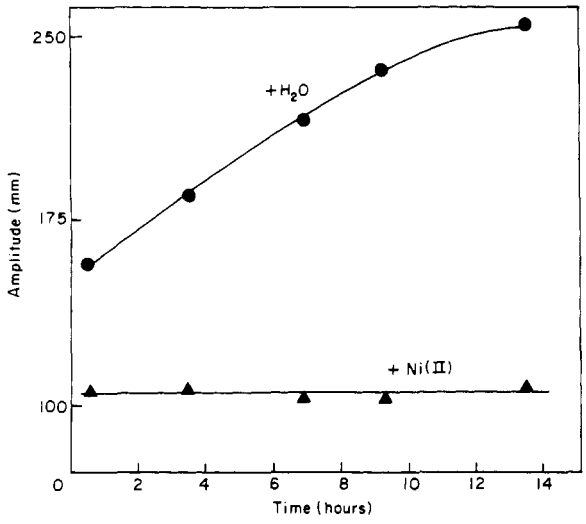

FIGURE 2: A plot of the total ($+H_2O$) and internal (+Ni) paramagnetism of "cholate" vesicles containing 1.5 mol % phosphatidyltempo in egg PC as a function of time after initial ascorbate reduction. Vesicles (1 mL, ~0.67 mM label) were mixed with 50 μL of NaAsc (335 mg/mL) and kept on ice for 7 min. The entire sample was then passed through a 1 × 25 cm column of Sephadex G-25 at 4 °C and the void volume collected and returned to 27 °C. The constant amplitude of internal signal was only repeated once for this label, but HGSL-I consistently gave a flat line for internal signal. The reoxidation was faster for HGSL-I, being complete within 7 h.

bution for IIb approaching 55% inside and 45% outside.

Transmembrane Movement. Figure 2 is a plot of the flip-flop results for phosphatidyltempo in EPC vesicles. The control amplitude rises with time because the initially reduced label undergoes reoxidation. This was particularly fast when vesiculation was by sonication in a Tris buffer, in some cases being completed within 4 h. The problems was less severe when vesicles were formed by cholate removal in a phosphate buffer. Contrary to previous reports (Sharom & Grant, 1978; Castle & Hubbell, 1976), it could not be eliminated. Similarly, while the disagreement between extrapolated zero time amplitudes of the control and nickel-treated samples could be held at 10–15% by extensive degassing of the gel and thorough N_2 purging of the buffer, it could not be entirely eliminated. It probably represents a rapid, column-mediated reoxidation of external label.[2] Since reoxidation destroys the transmembrane gradient of nitroxide concentration it limits the experimental time scale. However, the constant amplitude of protected signal indicates that over a period of 14 h little or no transbilayer exchange of internal and external label has occurred.

When the experiment was repeated with any of the spin-labeled polyprenyl phosphodiesters (compounds IIa–d), results typified by Figure 3A were obtained. The signal height of internal as well as external label rises with time; thus, some of the internal probe must have been reduced prior to the first measurements. This was shown by using the radioactive dolichyl probe [^{3}H]IIa and comparing the signal heights (at constant instrument gain) per mole of protected label both before and after the ascorbate–Sephadex G-25 procedure. As much as 30% of the protected label was sometimes reduced at t_0.[3] This was not due to either rapid flip-flop at 0–4 °C or leaky vesicles, as small-scale control reductions performed

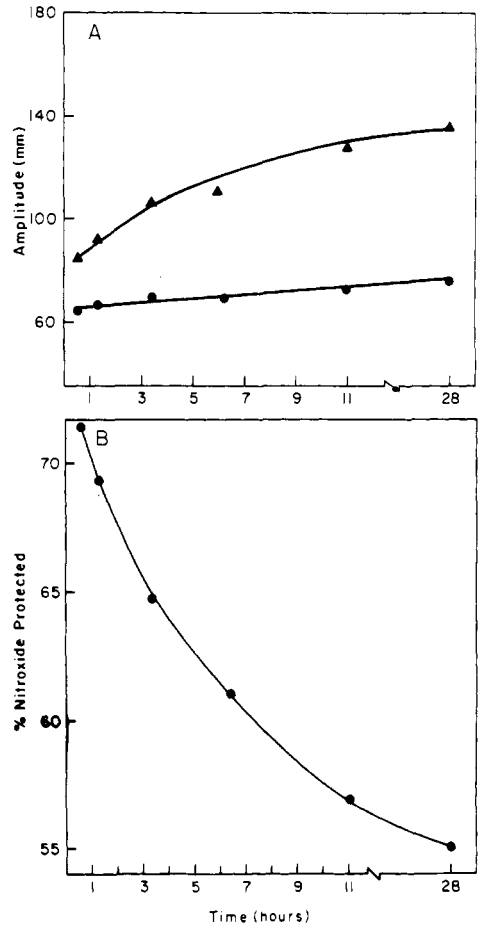

FIGURE 3: (A) Plot of total (▲—▲—▲) and internal (●—●—●) paramagnetism for the spin-labeled phosphodiester of dolichol (IIa, 1.5 mol %) in "cholate" vesicles as a function of time after the initial reduction. Experimental procedure the same as described under the legend of Figure 2. (B) The percent of total signal from IIa which is internal as a function of time.

in the EPR cavity gave a constant magnitude of protected signal for more than 20 min at 0–4 °C. Access of ascorbate to internal label must have occurred on the column. Numerous attempts to circumvent this problem were carried out but all to no avail.[4]

While the internal reduction/reoxidation could not be circumvented, it does not preclude an estimation of transbilayer diffusion rates. As shown in Figure 3B, the transmembrane gradient of nitroxide decays by 50% within 5 h. This decay is a sum of terms due to inside–outside transitions and reoxidation. Whatever the rate law for reoxidation is, with the observed levels of internal and external reduction at t_0 reoxidation could only act to decrease the transmembrane concentration gradient of oxidized labels. From the plot one can therefore assign a minimum half-time for head-group transposition of about 5 h to dolichyl compound IIa in this system. The same result is obtained for the shorter chain diesters IIb–d in small or large diameter vesicles.

Probe Dispersion. The EPR spectra of phosphodiesters IIa–d in fluid membranes give no evidence of strong spin exchange at concentrations of 3 mol % or less. Even at 10 °C below the T_c of dimyristoyl-PC or dipalmitoyl-PC in the presence of 0.5 M NaCl, the line broadening for 2% dolichol (IIa) or ficaprenol (IIb) corresponds only to the "weak exchange" limit (increased widths, but no inward shifts of M_I

[2] Since the signal amplitude of protected label was constant for HGSL-I after elution, the zero time discrepancy is not caused by partial reduction of internal label.

[3] The same result is obtained with phosphatidyltempo and cholesterylphosphoryltempo, indicating that the nature of the hydrophobic anchor is not important in causing this problem and implicating the phosphoryltempo head group.

[4] The details of these experiments are described fully in McCloskey (1979).

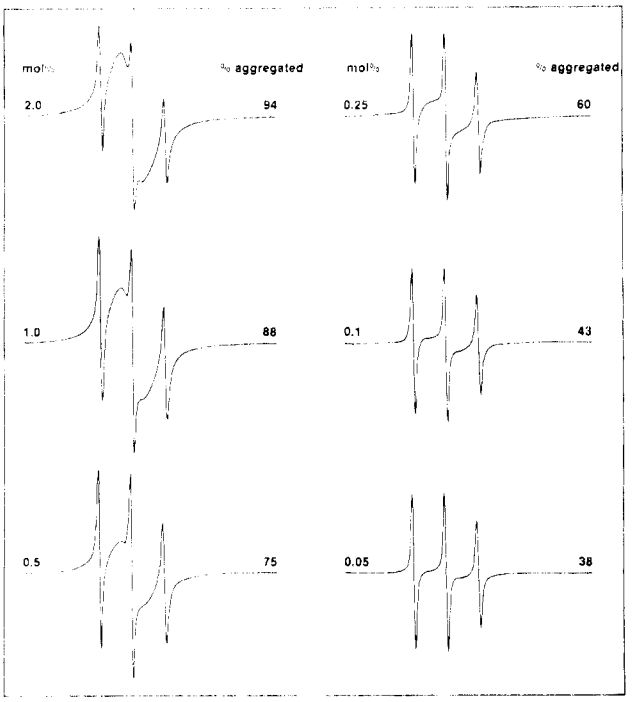

FIGURE 4: Series of EPR spectra of the neutral carboxylate ester of solanesol (IIIc), at increasing concentrations in dipalmitoyl-PC liposomes. The percent aggregated, as determined by computer subtraction, is given on the right of each spectrum and the corresponding concentration of IIIc on the left. Temperature is 52 °C.

Table I: β Values for IIIa–c at 45.5 °C and 1.3 mol %

host	probe			
	C_{15}	C_{45} trans	C_{55} cis	C_{95} cis
EPC	0[a]	0[a]	0[a]	0.48[a]
DML	0	ND[b]	0.71, 0.73[c]	0.89, 0.88[c]
DPL	0	0.92	0.93, 0.95[c]	0.93
EPC–Chol (3:2)	0	0.62	0.77	0.93, 0.97[c]
SM–Chol (3:2)	0	0.89	0.97	0.99

[a] 2 mol %, 24 °C. [b] Not determined. [c] 77 °C.

$= \pm 1$ lines). In sharp contrast is the behavior of the neutral species, IIIa–c. Figure 4 gives a series of resonance spectra of the carboxylate ester of solanesol (IIIc) in dipalmitoyl-PC liposomes above the T_c. The broad single-line spectrum is due to strong intermolecular spin exchange and emanates from probes in very close proximity to one another. At a total probe concentration of 2 mol % it overwhelms a minor three-line component due to monomeric probes. Most of the radicals are therefore in an aggregated state in this membrane. As previously pointed out, if this aggregation process is a unique equilibrium between monomers and aggregates with a well-defined size, then a plot of the data according to Figure 5 should be linear (Rey & McConnell, 1977). This follows from a simple rearrangement and logarithmic transformation of the balanced equilibrium equation. Apparently there is not a unique domain size for the solanesyl compound IIIc.

The temperature dependence of the clustering equilibrium appears minimal above the host T_c (Table I). On the other hand, as shown in Figure 6, the line width of aggregated labels markedly decreases with temperature elevation above the T_c. This line narrowing reflects a more efficient exchange process at the higher temperatures.

The resonance lines for monomeric carboxylates IIIa–c change dramatically near the host T_c, with the steepest change occurring at 2–4 °C below the T_c for pure host lipid. This

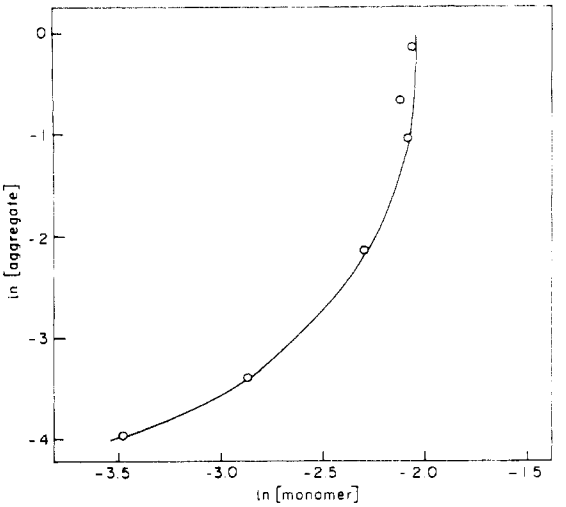

FIGURE 5: A log–log plot of the aggregate concentration vs. monomer concentration for the spin-labeled carboxylate ester of solanesol (IIIc) in DPL. Concentration is in units of mol %. The nonlinearity of this plot demonstrates that a unique equilibrium, e.g., dimerization, trimerization, etc., does not exist.

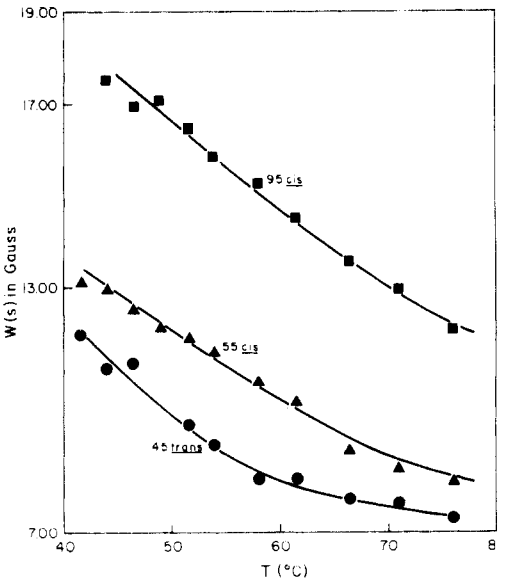

FIGURE 6: Plot of the line width from clustered carboxylates for IIIa–c in dipalmitoyl-PC as a function of temperature. The width was measured peak to trough, and a 100-G scan width was used.

corresponds to "partial immobilization" of isolated probe molecules and reflects a 2–3-fold increase in rotational correlation time through the transition ($\tau_c \sim 10^{-9}$ s at 26 °C in dipalmitoyl-PC). Phosphodiesters IIa–c are much less sensitive indicators of the transition, there being only a gradual and less marked change in the spectra near the T_c.

Discussion

Our rationale for investigating transbilayer diffusion was that the intrinsic rates of carrier lipids should be orders of magnitude faster than those measured for other phospholipids in model or biological systems in order to support their involvement as direct porters of sugars across the bilayer. Such a circumstance was at least conceivable, given the unique and perhaps perturbing structures of undecaprenol and dolichol. In practice, the experimental method was restricted for an absolute measurement, and we were only able to estimate an upper bound for the rate ($t_{1/2} > 5$ h, 25 °C).

In comparison, one may calculate that in an exponentially growing culture of *Micrococcus lysodeikticus*, the undeca-

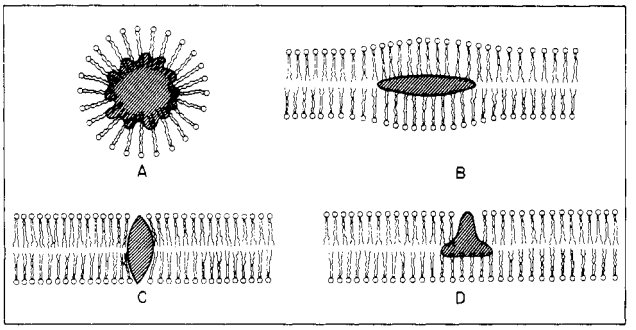

FIGURE 7: Alternative pictures for host–aggregate structure. No implications regarding the extent of pure III regions are intended. C and D emphasize proximity of head group to water phase. C indicates transbilayer extension of individual molecules or coordinate grouping of molecules anchored on opposing halves of bilayer.

prenyl carriers must be oscillating back and forth across the membrane with a frequency of ca. 1–3 Hz, which corresponds to a first-order decay half-time of 230–690 ms (Appendix A). This is an approximate but conservative estimate of the rate predicted by the "shuttle" mechanism for peptidoglycan export. Similar rates would theoretically be required for dolichyl phosphate in immunoglobulin glycosylation (Appendix B). The disparity between observed and calculated rates leads us to suggest that incipient polymer units are not transported across the bilayer while covalently bound to lipid; if they are, the process is probably mediated by a specific protein. Neither the unusual length nor the poly-cis unsaturation of isoprenoid carriers affords them a uniquely rapid rate of unassisted flip-flop. On this basis, the permeability argument per se seems inadequate to explain their hypothesized function in sugar translocation. It is true that in certain protein–lipid models and biological membranes the transposition rates of glycerophospholipids are significantly faster then in lipid model systems (De Kruijff et al., 1978; Zilversmit & Hughes, 1977; McNamee & McConnell, 1973). Still, the shortest half-times yet reported are in the order of several minutes. Nevertheless, it would perhaps be of interest to incorporate these compounds into bacterial or microsomal membranes and monitor transposition here.[5]

The pronounced self-association of neutral isoprenoids reported here is perhaps not surprising in view of their dissimilarity with the host PC's. Although a detailed quantitative study was not performed, certain characteristic features have emerged. First, aggregation is not an artifact of the spin-label, as the short-chain compound IIId does not cluster except at high concentrations and below the T_c. Recalling that β is proportional to the percent of self-associated label, Table I demonstrates that unsaturation in the host fatty acyl chains dramatically enhances solubility of the probes in the liquid-crystalline phase. For membranes in the fluid phase the extent of clustering is relatively temperature insensitive (Table I). Assuming monomer–cluster equilibria exist, this suggests that either there is a minimal ΔG of aggregation or the driving force is an entropic and not an enthalpic one. An increase in the translational and/or rotational entropy of the host molecules upon segregation of the isoprenoids would be in accord with this and might be expected if polymethylene chains adjacent to the isoprenyl tails were partially entangled in them and/or rotationally constrained. An increase correlation time for temp tumbling has been observed for different PC's upon addition of 20 mol % ficaprenol (Gennis et al., 1976).

Some possibilities for host–aggregate structure are shown in Figure 7. The downfield shift in resonance position of grouped nitroxides ($\Delta H_0 \sim 0.6$ G) suggests that they are in a nonpolar environment. Although some form of mixed micelle containing PC and probe could explain this, it would have to be uncharacteristically dense, since essentially all the paramagnetic material in liposomal samples containing 1.3% neutral polyisoprenoids sediments rapidly (10 min) at low speed. Consistent with model B is the fact that spacing of radicals within the groups is not directly correlated with the lipid lattice spacing. One might have suspected a priori that clusters of types C and D would exhibit a decrease in ν_{ex} with thermal expansion of the matrix. The negative charge on type II labels may prevent them from migrating to mid-bilayer and hence prevent clustering. The electrostatic repulsion between these labels does not prevent their very close approach in water (IId) or $CHCl_3$ (IIa–d), as a strongly exchange-narrowed resonance can be observed at higher label concentration. It seems reasonable that close juxtaposition of type II or III probes when intercalated in a bilayer is precluded because of their lateral expansivity. A location at mid-bilayer would alleviate this constraint by permitting "end-to-end" as well as "side-by-side" approach of nitroxide groups. A mid-bilayer location for aggregates is also consistent with minimum steric perturbation of the membrane, since the greatest disorder and free volume is known to be near the fatty acyl methyl termini. Although inconclusive, current evidence favors model B.

The reason for stronger exchange at higher temperatures is not known but could be an increased lateral mobility of head groups within the aggregate or closer spacing of the isoprenyl chains. Over the temperature range studied, the exchange narrowing is greatest for solanesyl ester IIIc, somewhat less for ficaprenyl ester IIIb, and quite a bit less for dolichyl ester IIIa. This trend is probably due to the relative volumes of the three isoprenoid chains within the membrane.

The biological significance of this phenomenon is not obvious at this point. Data in the literature suggest that neutral undecaprenyl species could constitute between 0.1 and 0.4 mol % of the total cellular lipids in various bacteria (Bohnenberger & Sanderman, 1976; Sutherland, 1977 and references cited therein). At these concentrations aggregation would be significant in several fluid membranes. The autoradiographic work of Thorne et al. (1974) indicates a homogeneous dispersion of total undecaprenyl species around the cell membrane of *Lactobacillus casei*. It follows that if clustering does occur in vivo, the resulting lateral separations are of insufficient magnitude to explain the localized peptidoglycan assembly reported in certain bacteria (Pooley et al., 1978; Briles & Tomasz, 1970; Higgins & Shockman, 1970).

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Appendix A

Approximately 20 mg of undecaprenyl pyrophosphate accumulates in 660 g (wet weight) of *Micrococcus lysodeikticus*

[5] Following submission of this manuscript, Hanover & Lennarz (1979) reported no significant transverse diffusion of *N,N'*-diacetylchitobiosyl-pyrophosphoryldolichol (chitobiosyl-lipid) either in unilamellar liposomes made from phosphatidylcholine or in microsomes isolated from hen oviduct. These studies, utilizing a soluble galactosyltransferase to galactosylate chitobiosyl-lipid molecules on the external leaflet, showed no change in the transbilayer distribution of chitobiosyl-lipid during a 3-h incubation. These results provide compelling confirmation of the conclusions reached here.

which have been treated with bacitracin during exponential growth (Stone & Strominger, 1972). This value is within a factor of two of the concentrations of free and combined undecaprenol observed for several bacterial species. It corresponds to ca. 125 nmol of carrier per g cell dry weight. For *M. lysodeikticus* the wall (mostly peptidoglycan) constitutes ca. 28% of the dry cell weight (Salton, 1964). The average residue weight of a disaccharide tetrapeptide unit with a pentaglycine bridge is ca. 1130. This gives ca. 1980 mol of peptidoglycan monomer per mol of carrier per cell. Assuming a cell doubling time of 20 min to 1 h this requires 1980–5940 monomer units to be exported per h per molecule of carrier lipid; the implied transbilayer passage frequency for the carriers is ~ 1–$3\ s^{-1}$. For a first-order decay to equilibrium the half-time would be ca. 230–690 ms. This should be a conservative estimate, since the actual steady-state concentration of phosphorylated carriers is significantly lower than that which accumulates in the presence of bacitracin. Further, some of the active carriers may be committed to mannan synthesis.

Appendix B

Mouse MOPC 46 plasma cell tumors secrete a glycosylated κ-type light chain (LC) at the rate of $\sim 1.9 \times 10^4$ molecules $cell^{-1}\ s^{-1}$ (Melchers, 1970). Similar rates have been reported for human lymphoid cell lines (Fahey & Finegold, 1967). According to Sullivan & Salmon (1972) there are about a billion plasma cells per gram (in humans). Using this number one finds there is 3.16×10^{-12} mol of LC secreted g^{-1} of tissue in 1 s. The total level of dolichol (free and combined) in pig spleen cells is ca. 1 μg/g (Hemming, 1974). Mouse liver has a smaller concentration of dolichol than pig liver, and it is a reasonable assumption that mouse spleen cells (or plasma cells) do not contain more than 1 μg of dolichol g^{-1}. So even if all the dolichol were phosphorylated and active in transport, the minimum implied flip-flop rate is

$$(31.6 \times 10^{-12})(2)/[(1\ \mu g\ of\ C_{95})/(1312\ g\ mol^{-1}\ of\ C_{95})] = 4.8\ transits\ min^{-1}\ or\ half\text{-}time\ about\ 8\ s$$

Unfortunately, no hard data currently exist on endogenous molar concentrations of dolichyl phosphate in any mammalian species. However, the general consensus seems to be that it constitutes a small percentage (<10%) of the total dolichol. If this is true, it would imply a half-time of less than 1 s.

References

Anderson, J. S., Matsuhashi, M., Haskin, M. A., & Strominger, J. L. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 881–889.

Anderson, R. G., Hussey, H., & Baddiley, J. (1972) *Biochem. J. 127*, 11–25.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2810.

Bohenenberger, E., & Sandermann, H., Jr. (1976) *FEBS Lett. 67*, 85–89.

Briles, D. C., & Tomasz, A. (1970) *J. Cell Biol. 47*, 786–790.

Brunner, J., Skrabal, P., & Hauser, H. (1976) *Biochim. Biophys. Acta 455*, 322–331.

Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I., & Bergelson, L. D. (1971) *Chem. Phys. Lipids 6*, 343–350.

Castle, J. D., & Hubbell, W. L. (1976) *Biochemistry 15*, 4818–4831.

Deamer, D., & Bangham, A. D. (1976) *Biochim. Biophys. Acta 443*, 629–634.

De Kruijff, B., Van Zoelen, E. J. J., & Van Deenen, L. L. M. (1978) *Biochim. Biophys. Acta 509*, 537–542.

Depierre, J. W., & Dallner, G. (1975) *Biochim. Biophys. Acta 415*, 411–472.

Fahey, J. L., & Finegold, I. (1967) *Cold Spring Harbor Symp. Quant. Biol. 32*, 283.

Gennis, R. B., Sinensky, M., & Strominger, J. L. (1976) *J. Biol. Chem. 251*, 1270–1276.

Hanover, J. A., & Lennarz, W. J. (1979) *J. Biol. Chem. 254*, 9237–9246.

Higgins, M. L., & Shockman, G. D. (1970) *J. Bacteriol. 101*, 643–648.

Jost, P., & Griffith, O. H. (1976) in *Spin Labeling* (Berliner, L. J., Ed.) Vol. 1, pp 251–272, Academic Press, New York.

Kanegasaki, S., & Wright, A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 951–958.

Keith, A. D., Snipes, W., & Chapman D. (1977) *Biochemistry 16*, 634–641.

Kornberg, R. D., & McConnell, H. M. (1971) *Biochemistry 10*, 1111–1120.

Lehninger, A. L. (1975) *Biochemistry*, 2nd ed., pp 296–297, Worth Publishers, New York.

Lennarz, W. J. (1975) *Science 188*, 986–991.

McCloskey, M. A. (1979) Ph.D. Thesis, University of California, Davis, CA 95616.

McCloskey, M. A., & Troy, F. A. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 790.

McCloskey, M. A., & Troy, F. A. (1980) *Biochemistry* (preceding paper in this issue).

McNamee, M. G., & McConnell, H. M. (1973) *Biochemistry 12*, 2951–2958.

Melchers, F. (1970) *Biochem. J. 119*, 765–772.

Parodi, A. J., & Leloir, L. F. (1979) *Biochim. Biophys. Acta* 559, 1–37.

Polnaszek, C. F., Schreier, S., Butler, K. W., & Smith, I. C. P. (1978) *J. Am. Chem. Soc. 100*, 8223–8232.

Pooley, H. M., Schlaeppi, J. M., & Karamata, D. (1978) *Nature (London) 274*, 264–266.

Rey, P., & McConnell, H. M. (1977) *J. Am. Chem. Soc. 99*, 1637–1642.

Robyt, J. F. (1979) *Trends Biochem. Sci. 4*, 47–49.

Salton, M. R. J. (1964) *The Bacterial Cell Wall*, p 245, Elsevier, Amsterdam.

Sharom, F. J., & Grant, C. W. M. (1978) *Biochim. Biophys. Acta 507*, 280–293.

Stone, K. J., & Strominger, J. L. (1972) *J. Biol. Chem. 247*, 5107–5112.

Stryer, L. (1975) *Biochemistry*, pp 758, W. H. Freeman, San Francisco.

Sullivan, P. W., & Salmon, S. E. (1972) *J. Clin. Invest. 51*, 1697.

Sutherland, I. W. (1977) in *Surface Carbohydrates of the Prokaryotic Cell* (Sutherland, I., Ed.) p 62, Academic Press, New York.

Weppner, W. A., & Neuhaus, F. C. (1978) *J. Biol. Chem. 253*, 472–478.

Zilversmit, D. B., & Hughes, M. E. (1977) *Biochim. Biophys. Acta 469*, 99–110.