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Translational diffusion of lipid monomers determined by spin label electron spin resonance

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The collision rates between spin-labelled valeric acid in water, and between the corresponding mixed-chain, spin-labelled phosphatidylcholine in water-methanol mixtures, and also between spin-labelled phosphatidylcholine monomers and micelles in water have been determined from the spin-spin broadening of the electron spin resonance spectrum. In each case the second order rate constants are consistent with a diffusion-controlled process. For spin-labelled valeric acid in water the translational diffusion coefficient at 20°C is $3.4 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$, and for spin-labelled phosphatidylcholine varies between $2.3 \cdot 10^{-6}$ and $3.8 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$ within the range 44 to 88 wt% methanol. The spin-labelled phosphatidylcholine monomer diffusion coefficient in water at 20°C is $2.4 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$, deduced from the monomer-micelle association rate, with an activation energy of 4.0 kcal $\cdot$ mol^{-1}. The much slower on-rates for association of lipid monomers with phospholipid bilayer vesicles reported in the literature, therefore indicate that incorporation into bilayers is not a diffusion-controlled process.

The transfer of lipid molecules between phospholipid vesicles and between lipoproteins and lipid vesicles has been shown in many cases to occur via monomer diffusion through the aqueous phase [1–4]. Although in most cases the release of the lipid molecule from the donor vesicle is likely to be the rate-limiting step, it is of considerable interest to know the rate of transfer in the aqueous phase. This is normally assumed to be diffusion controlled, even though measurements of the transfer kinetics in several systems have yielded on-rate constants which are considerably below the normally accepted diffusion controlled limit [5,6].

In the present study we have measured the monomer diffusion coefficient of a short-chain spin-labelled fatty acid in water, and of the corresponding phosphatidylcholine derivative in various methanol-water mixtures. The diffusion coefficient was obtained from the bimolecular collision rate, determined from the spin-spin exchange broadening of the electron spin resonance (ESR) spectrum, as a function of spin label concentration. The method was also applied to the measurement of the monomer-micelle association rate for the spin-labelled phosphatidylcholine in water. The latter is the relevant parameter for lipid exchange between vesicles.

The Heisenberg spin exchange frequency, τ_{ex}^{-1}, is related to the dependence of the Lorentzian

Abbreviations: ESR, electron spin resonance; VASL, 4-(4,4-dimethyloxazolidine-*N*-oxyl) valeric acid; VPCSL, 1-lauroyl-2-(4-(4,4-dimethyloxazolidine-*N*-oxyl)valeryl)-*sn*-glycero-3-phosphocholine.

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peak-to-peak linewidth, ΔH_{pp}^{L}, in the ESR spectrum on concentration, c, by (see, for example, Ref. 7)

$$\tau_{ex}^{-1}/c = (\sqrt{3}\,\gamma/2)\cdot d(\Delta H_{pp}^{L})/dc \quad (1)$$

where γ is the gyromagnetic ratio of the electron. Because of the rapid rates of translational diffusion in low-viscosity media, dipole-dipole interactions between the spin labels will be averaged to zero and therefore will not contribute to the spin-spin broadening. The exchange frequency is related to the spin label collision frequency, τ_{coll}^{-1}, by (see, for example, Ref. 7)

$$\tau_{ex}^{-1} = \left[\frac{2I}{2I+1}\right]\cdot p_{ex}\cdot \tau_{coll}^{-1} \quad (2)$$

where $I = 1$ is the ^{14}N nuclear spin quantum number and $p_{ex} = 1/2$ is the probability of spin exchange on collision. The second-order rate constant for diffusion-controlled collision, $k_{coll} = \tau_{coll}^{-1}/c$, is then related to the diffusion coefficients, D_A and D_B, of the colliding species by the Smoluchowski equation [8]

$$k_{coll} = 4\pi N_A 10^{-3}(r_A + r_B)(D_A + D_B) \quad (3)$$

where r_A and r_B are the collision radii of the two species, and N_A is Avogadro's number. The diffusion coefficients are related to the viscosity of the medium, η, by the Stokes-Einstein relation

$$D_A = kT/(6\pi\eta r_A) \quad (4)$$

where k is Boltzmann's constant and an effectively spherical molecule is assumed, with the Stokes radius equal to the collision radius, r_A.

Spin-labelled valeric acid, VASL, and spin-labelled phosphatidylcholine, VPCSL, with lauric acid on the *sn*-1 chain and VASL on the *sn*-2 chain, were synthesized essentially according to the methods of Ref. 9. ESR spectra were recorded on a Varian Century Line 9 GHz spectrometer, equipped with nitrogen gas flow temperature regulation. Spectra were stored and processed on a PDP 11 minicomputer, interfaced directly to the spectrometer. Lorentzian linewidths were extracted from the inhomogeneously broadened $m_I = +1$ hyperfine line, by measuring the relative intensity in the wings of the spectrum, as described in Ref. 11 (see Fig. 1B). Phospholipid concentration was determined by inorganic phosphate estimation, according to Ref. 10. VASL concentration was determined gravimetrically.

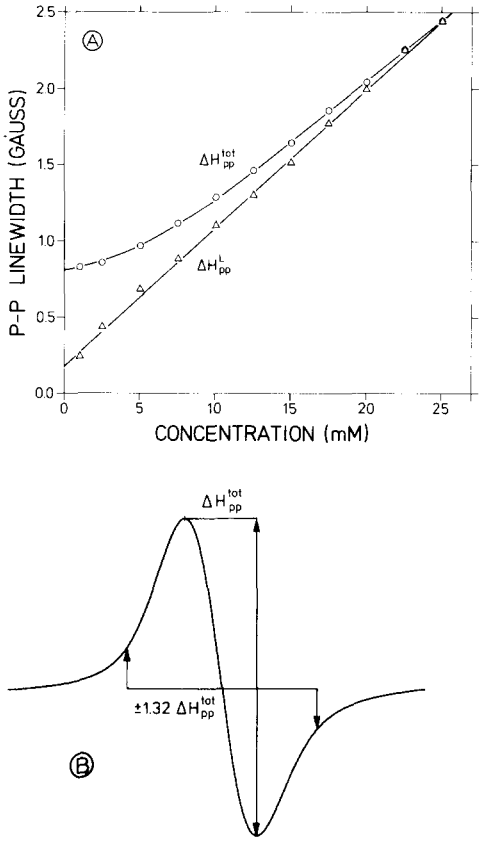

Fig. 1. (A) Concentration dependence of the total, ΔH_{pp}^{tot}, and Lorentzian, ΔH_{pp}^{L}, peak-to-peak linewidths of the low-field hyperfine line in the spectra of the VASL valeric acid spin label in water at 20°C. The circles are the experimental total linewidths and the triangles are the Lorentzian linewidths derived from intensity measurements in the wings of the ESR line, at the positions indicated in (B), using the calibrations given in Ref. 11.

The concentration dependence of the low-field peak-to-peak linewidth in the ESR spectrum of the VASL valeric acid spin label in water is given in Fig. 1. A non-linear dependence is observed because of the inhomogeneous broadening arising from unresolved proton hyperfine structure. The true Lorentzian linewidth has been obtained using

TABLE I

SECOND-ORDER COLLISION RATE CONSTANTS AND TRANSLATIONAL DIFFUSION COEFFICIENTS OF THE VPCSL PHOSPHATIDYLCHOLINE SPIN LABEL IN METHANOL-WATER MIXTURES AT 20°C

η and η_0 are the viscosities of the water-methanol mixtures and of water, respectively, at 20°C [12].

MeOH (wt %)	k_{coll} ($M^{-1} \cdot s^{-1}$)	k_{coll} (η/η_0) ($M^{-1} \cdot s^{-1}$)	D_T ($cm^2 \cdot s^{-1}$)
88	$4.3 \cdot 10^9$	$3.9 \cdot 10^9$	$3.5 \cdot 10^{-6}$
76	$4.2 \cdot 10^9$	$5.1 \cdot 10^9$	$3.5 \cdot 10^{-6}$
65	$4.2 \cdot 10^9$	$6.2 \cdot 10^9$	$3.5 \cdot 10^{-6}$
54	$4.6 \cdot 10^9$	$7.8 \cdot 10^9$	$3.8 \cdot 10^{-6}$
44	$2.7 \cdot 10^9$	$5.0 \cdot 10^9$	$2.3 \cdot 10^{-6}$

the deconvolution method described by Bales [11]. This is found to have an accurately linear dependence on spin label concentration (see Fig. 1). The second order rate constant for collision, obtained from Eqns. 1 and 2, is then $k_{coll} = 4.1 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, in reasonable agreement with the predicted rate constant for a diffusion-controlled collision process of $k_2^{diff} = 8RT/(3000\eta) = 6.5 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ at 20°C. Using a value of $r_A = r_B = 4$ Å, Eqn. 3, together with the rate constant deduced from Fig. 1, yields a value of $D_T = 3.4 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$ for the diffusion coefficient of VASL in water at 20°C *.

Similar measurements have also been made for the VPCSL spin-labelled phosphatidylcholine in water-methanol mixtures of various compositions, up to the monomer solubility limit. At lower methanol contents, the VPCSL spin label forms micelles, hence precluding measurement of the monomer collision rate in a water alone. For this reason the collision rates in the various mixtures have been normalized to the viscosity of pure water, according to the Stokes-Einstein relation. The monomer bimolecular collision rate constants, k_{coll}, in the water/methanol mixtures are given in Table I. The values all lie within the range $(4–8) \cdot 10^9$ $M^{-1} \cdot s^{-1}$ at 20°C, after correction for the different viscosities of the solvent mixtures. This is again close to the predictions for a diffusion-controlled collision process. The corresponding translational diffusion coefficients, D_T, calculated from Eqn. 3, with $r_A = r_B = 4$ Å are also listed in Table I.

Monomer-micelle collision rates for the VPCSL phosphatidylcholine spin label in water have also been measured. In 0.15 NaCl the critical micelle concentration is sufficiently low ($1.0 \cdot 10^{-4}$ M at 20°C) that the spin–spin interactions between monomers are extremely small and also constant. Therefore the concentration dependence of the spin-spin broadening of the monomer ESR signal arises solely from monomer-micelle collisions. The temperature dependence of the on-rate constant, k^+, for the monomer-micelle interaction is given as an Arrhenius plot in Fig. 2. A value of $n = 63$ for the micelle size, obtained from light scattering [13], has been used to obtain the micelle concentrations required for calculating the rate con-

* The collision radii, r_A and r_B, in the Smoluchowski equation, Eqn. 3, refer to collisions between the corresponding spin label groups. Based on molecular models, the value of $r_A = 4$ Å provides a reasonably good estimate for both the VASL and VPCSL monomers (but for VPCSL micelles the micelle radius must be taken for r_B). For comparison, the classical diffusion radius of the somewhat smaller di-*tert*-butyl nitroxide is $r_{diff} = 3.2$ Å [14]. The elongated nature of the VPCSL molecule comes into consideration only in the theoretical estimate of the diffusion-controlled, second-order rate constant, k_2^{diff}, via the Stokes-Einstein equation, Eqn. 4. This value will be reduced by a factor determined by the frictional coefficient for the elongated molecule. Comparing with the experimentally determined collision rate constants, k_{coll}, yields a frictional ratio in the range $F \approx 1.3–1.6$, which corresponds to an axial ratio of the equivalent prolate ellipsoid of $a/b \approx 5–10$ [15].

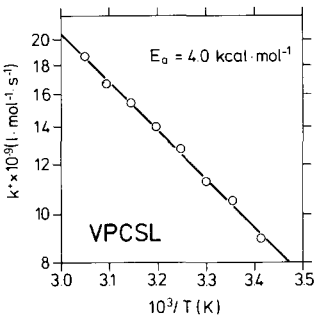

Fig. 2. Arrhenius plot of the temperature dependence of the monomer-micelle association rate constant, k^+, for the VPCSL phosphatidylcholine spin label in 0.15 M NaCl (pH 7). A micellar number of $n = 63$ [13] has been used in calculating the rate constant.

stants in Fig. 2. The logarithm of the rate constant decreases linearly with $1/T$, clearly demonstrating the lack of any contribution to the spin-spin broadening from dipole-dipole interactions, which would have the opposite temperature dependence. An activation energy of 4.0 kcal · mol^{-1} is obtained from the Arrhenius plot, which is very close to the effective activation energy of 3.7 kcal · mol^{-1} associated with the viscosity of water [12]. This again is suggestive of a diffusion-controlled process.

The on-rate constant for the monomer-micelle association at 20°C is $k^+ = 5.1 \cdot 10^9$ M$^{-1} \cdot$ s^{-1}, compared with a diffusion controlled rate of $k_2^{diff} = 2RT/(3000\eta) \cdot (r_B/r_A) = 6.5 \cdot 10^9$ M$^{-1} \cdot$ s^{-1} at 20°C, where the approximation $r_B/r_A = n^{1/3}$ has been used in determining the ratio of micelle to monomer radii. Again this strongly suggests that the monomer-micelle on-rate is a diffusion-controlled process. Since the spin-label group in VPCSL is located close to the polar-apolar interface of the micelle, it cannot be absolutely excluded that the on-rate represents monomer-micelle collision, rather than true incorporation of the monomer into the micelle. However, if the latter is the case, the phospholipid incorporation into micelles occurs at a much faster rate than that of diacylphospholipids into bilayers, since for dimyristoylphosphatidylcholine the association rate constant has been measured to be $k^+ = 2.1 \cdot 10^3$ M$^{-1} \cdot$ s^{-1} [6]. The VPCSL monomer diffusion coefficient at 20°C, calculated from Eqn. 3, using a micelle radius of $r_B = 28.5$ Å [13] and assuming that the micelle diffusion rate may be neglected ($D_B = 0$), is $D_T = 10^3 k^+/(4\pi N_A r_B) = 2.4 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}. This number is in good agreement with the translational diffusion coefficients measured for the monomer alone in water-methanol mixtures (see Table I), especially in view of the fact that the micelle radius rather than the monomer radius is used in the calculation.

In summary, the collision frequencies of a spin-labelled fatty acid in water, of a spin-labelled phosphatidylcholine in water-methanol mixtures, and between spin-labelled phosphatidylcholine monomers and micelles are consistent with a diffusion-controlled process. The translational diffusion coefficients at 20°C are in the region of $(2-4) \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1} for all three systems, with an activation energy for the monomer-micelle association of 4.0 kcal · mol^{-1} which is close to that for the viscosity of water. The measured diffusion coefficients are dependent on the values assumed for the collision radii, but the good agreement between the calculations involving the quite different radii of monomer and micelle indicate that these are essentially correct. The much slower rates of association of phosphatidylcholine monomers with phospholipid bilayer vesicles [5,6], therefore indicate that the on-rate for phospholipid incorporation into membranes is not diffusion-controlled, but must be in some way sterically hindered.

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