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Lipid mixing in dimyristoyl phosphatidylcholine-dimyristoyl glycerol dispersions: spin label ESR studies

Karl Schorn, Derek Marsh *

Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, Am Faßberg, D-37077 Göttingen, Germany

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Abstract

The lipid transfer and mixing properties in hydrated dispersions of dimyristoyl phosphatidylcholine (DMPC)/dimyristoyl glycerol (DMG) binary mixtures have been investigated by using electron spin resonance spectroscopy of the spin-labelled lipid components. The assay system is based on the reduction in spectral broadening from spin–spin interactions that takes place by dilution of the spin-labelled lipid on transfer to a 9-fold excess of dispersions that contain no spin label. Lipid dispersions with DMPC:DMG compositions of 70:30, 40:60 and 20:80 mol/mol, for which the fluid phases have lamellar, inverted hexagonal and isotropic structures, respectively, have been studied. Essentially no transfer of spin-labelled lipid takes place for any of the lipid mixtures in the lamellar gel phase, or in dispersions of DMPC alone at all temperatures studied. The greatest degrees of transfer are found in the fluid phase of the DMPC/DMG mixtures. In general, the extent of lipid transfer is greater for the diacylglycerol component than for the phosphatidylcholine component. The extent of transfer of phosphatidylcholine is very low in the fluid lamellar phase of the 70:30 mol/mol DMPC/DMG, as compared with that of diacylglycerol. Only in the case of the 40:60 mol/mol DMPC/DMG mixture, in the inverted hexagonal phase, are the extents of transfer comparable for both phosphatidylcholine and diacylglycerol components, indicating a bulk transfer of lipid within the dispersions. The largest extent of transfer is found for diacylglyerol in the 20:80 mol/mol DMPC/DMG mixture in the isotropic phase.

Keywords: Diacylglycerol; Lipid mixing; Lipid exchange; Non-lamellar phase; Spin label; Electron spin resonance

1. Introduction

There is considerable interest in the role of diacylglycerol in the fusion of lipid vesicles [1–3] and biological membranes [4–6]. Diacylglycerol, produced transiently by the action of a G-protein coupled phospholipase C, is involved in the exocytotic signalling pathway [7–9], and may, therefore, be directly implicated in this membrane fusion process. In addition, diacylglycerol has been found to potenti-

Abbreviations: DMG, 1,2-dimyristoyl-*sn*-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 14-DGSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycerol; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulphonic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; L_α, fluid lamellar phase; H_{II}, inverted hexagonal phase.

* Corresponding author. Fax: +49 551 2011501.

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ate the enzymatic action of various phospholipases, including phospholipase C [10–12]. The latter has been found to promote the aggregation and fusion of phospholipid vesicles by the mediation of the enzymatically produced diacylglycerol [13,14]. Interestingly, it was found that high concentrations of the enzymatically generated product could also exert a controlling inhibitory effect on the fusion process.

The studies on the fusion of phospholipid vesicles have suggested that the action of diacylglycerols arises from their ability to promote the formation of non-bilayer lipid phases [1,2]. Such structures are likely to be formed at high local concentrations of the diacylglycerol component. Binary phase diagrams have been established for mixtures of saturated chain diacylglycerols with phosphatidylcholines [15,16]. These serve to define the composition regions for which non-lamellar fluid phases exist. The gel phases have a lamellar structure for all compositions. For binary mixtures of dimyristoyl glycerol (DMG) with dimyristoyl phosphatidylcholine (DMPC), or the corresponding systems with palmitoyl chains, the fluid phase is found to be of lamellar structure for compositions up to approximately equimolar. For higher DMG contents, up to a DMG:DMPC stoichiometry of approx. 2:1 mol/mol, the fluid phase is no longer lamellar but has an inverted hexagonal structure. At DMG contents above the 2:1 mol/mol stoichiometry, the fluid phase is of the isotropic type, with the phosphatidylcholine component being present as inverted micelles [17].

In the present work, the ability of the spin-labelled diacylglycerol and phosphatidylcholine components to redistribute into unlabelled lipid dispersions has been investigated for DMPC/DMG mixtures with a wide range of DMG contents. Electron spin resonance spectroscopy was used to assay the reduction in concentration-dependent spin-spin interactions of the spin-labelled lipid component, on dilution into an unlabelled lipid dispersion of similar composition. The temperature dependence of the degree of lipid redistribution has been determined with DMPC/DMG mixtures for which the fluid lipid phase is of the lamellar (L_α), inverted hexagonal (H_{II}), or isotropic type. This covers the full range of non-lamellar structural polymorphism observed for these fully hydrated diacylglycerol/phosphatidylcholine mixtures. The results are relevant to the lipid transfer processes that may take place on close apposition in lipid systems with high local concentrations of diacylglycerol. The latter may be generated transiently by the action of phospholipase C and also may be found in the interlamellar attachments that are suggested to exist as intermediates in lipid membrane fusion and in the formation of non-lamellar structures in general [18]. This work concentrates on the effects specifically induced by the diacylglycerol component because it refers to a saturated phosphatidylcholine system that displays no tendency to fusion or the formation of non-lamellar phases, and for which rates of lipid transfer are rather slow (see e.g., Ref. [19]).

2. Materials and methods

2.1. Materials

DMPC was obtained from Fluka (Buchs, Switzerland). DMG was produced from DMPC by enzymatic cleavage with phospholipase C from *Bacillus cereus* (Boehringer-Mannheim, Mannheim, Germany), in ether/water (1:1, v/v) at 0°C. DMG was extracted from the ether phase after the reaction had gone to completion and purity was checked by thin-layer chromatography. Spin-labelled phosphatidylcholine bearing the nitroxide group on the C-14 atom of the *sn*-2 chain (14-PCSL) was synthesized as described in Ref. [20]. The corresponding spin-labelled diacylglycerol (14-DGSL) was synthesized from 14-PCSL by using the same enzymatic method as for DMG.

2.2. Sample preparation

The required amounts of DMPC and DMG (ca. 0.2–2 mg in total) were dissolved in dichloromethane, together with the desired spin label (14-PCSL or 14-DGSL). Each sample contained the same total amount of spin label. The spin label concentration relative to the host lipid in which it was incorporated was 10 mol%, or 1 mol% for the control. The organic solvent was evaporated with a stream of dry nitrogen and the sample then dried under vacuum for at least 3 h. The dry lipid was dispersed in 100 μl of buffer (10 mM Hepes, 1 mM EDTA, pH 7.4) with vortexing. The dispersion was warmed above the chain-

melting transition temperature to ensure complete hydration. The sample was then transferred to a 100-μl glass capillary (1 mm ID) that was sealed at one end and pelleted at room temperature by centrifugation in a bench-top centrifuge at 8000–10 000 rpm. The excess supernatant was removed to yield a fully hydrated pellet of the lipid mixture and the capillary was flame-sealed.

2.3. ESR spectroscopy

ESR spectra were recorded on a Varian E-Line 9 GHz spectrometer with nitrogen gas flow temperature regulation. The glass capillary was accommodated within a standard 4 mm-diameter quartz tube that contained light silicone oil for thermal stability. The temperature was regulated to ± 0.1°C by using a thermocouple that was placed in the silicone oil. Spectra were recorded with a field modulation frequency of 100 kHz and modulation amplitude of 1.25 gauss. ESR spectra were recorded as a function of increasing temperature over the range 0° to 70°C or 80°C in 10° steps. A 9-fold excess of unlabelled lipid dispersion was then added to the sample with 10 mol% spin label with mixing by centrifugation. Addition of the unlabelled dispersion required approx. 10 min and was performed at room temperature. ESR spectra were again recorded from 0°C to 70°C or 80°C in 10° steps. The time at each temperature was 15–20 min, including that for collection of the spectra. The spin label concentration in this mixed sample would be 1 mol% if a uniform distribution were achieved. The time involved in these mixing experiments is much longer than that required to establish phase equilibrium at a given temperature, as established by measurements on single samples with low spin label concentration.

3. Results and discussion

3.1. Lipid mixing assay

The temperature dependence of the central line height, h_o, of the 14-DGSL spin label at low concentration (1 mol%) in aqueous dispersions of DMPC and of a 38:62 mol/mol DMPC/DMG mixture is given in Fig. 1. The line heights are normalized to the number of spins in the sample by means of the second integral of the spectrum and are scaled to a value of 1.0 for 1 mol% 14-DGSL in DMPC at 0°C. All spectral line heights reported here are normalized with this convention. Although there are sharp discontinuities in the line height at the chain-melting

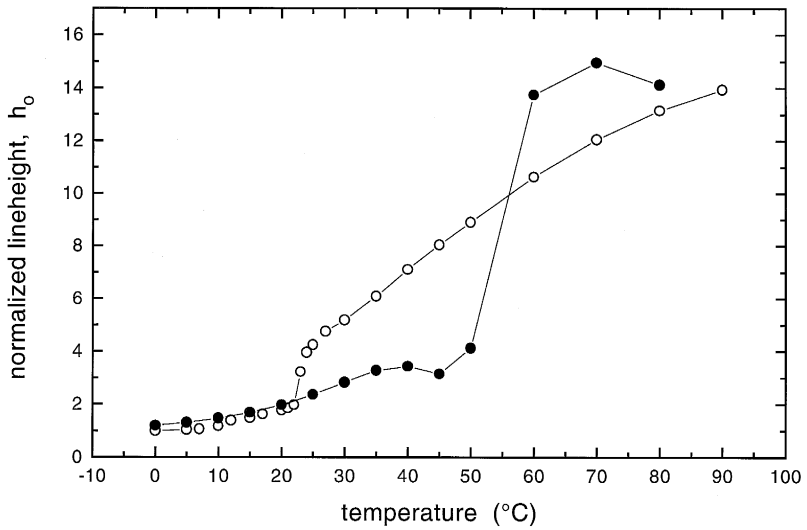

Fig. 1. Temperature dependence of the line height of the central maximum in the ESR spectra of 1 mol% 14-DGSL spin label in aqueous dispersions of DMPC (open circles) and of a DMPC/DMG 38:62 mol/mol mixture (filled circles). Line heights are normalized to the double-integral of the entire spectrum. All values are referred to a line height of unity for 1 mol% 14-DGSL in DMPC at 0°C.

phase transition (for DMPC) or in the region of lateral phase separation (for DMPC:DMG = 38:62 mol/mol), the normalized line heights achieved at the same temperature in the fluid phase are comparable. For various DMPC/DMG mixtures of different compositions, the normalized line heights at 80°C for samples containing uniformly 1 mol% spin label all lie in the range between 9 and 14, and all are very similar at 0°C. This makes the central line height a useful parameter for assaying lipid mixing when using over-labelled dispersions mixed with unlabelled dispersions. The assay is based on dilution of the concentrated spin label from a dispersion in which the spin-label concentration is 10 mol%, as is illustrated in Fig. 2. The ESR spectra displayed in Fig. 2 all correspond to the same amount of spin label (but at different concentrations relative to the unlabelled host lipid) and reflect their true line heights. The spectra from the sample containing 10 mol% spin label (dashed line) are broadened by spin-spin interactions, resulting in lower line heights than those from a sample containing the same amount of spin label but at a relative concentration of 1 mol% (dotted line). This is the case for samples both in the gel phase at 0°C and in the fluid phase at 70°C; the line height for the sample with 1 mol% spin label is ca. 3.5 times greater than that for the sample with 10 mol% spin label at 0°C and ca. 15 times larger at 70°C. The difference is greater at the higher temperature because the spin–spin broadening is greater relative to the smaller intrinsic line width in the fluid phase. The effect of mixing a 9-fold excess of unlabelled lipid dispersion with the dispersion that contains 10 mol% spin label is given by the spectra that are indicated by the arrows in Fig. 2. If the spin label were homogeneously distributed throughout the entire lipid, then the spectra should be identical to those of the sample containing 1 mol% spin label. In the gel phase at 0°C, the spectra are very similar to those of the original sample containing 10 mol% spin label. This indicates that very little of the spin-labelled lipid has mixed with the unlabelled lipid. In the fluid phase at 70°C, the line height of the spectrum for the sample containing 10 mol% spin label in the presence of unlabelled lipid is more than twice that in its absence. The spin-labelled lipid has partly, but not fully, mixed with the unlabelled lipid in the fluid phase. The effective degree of lipid mixing can be quantitated as:

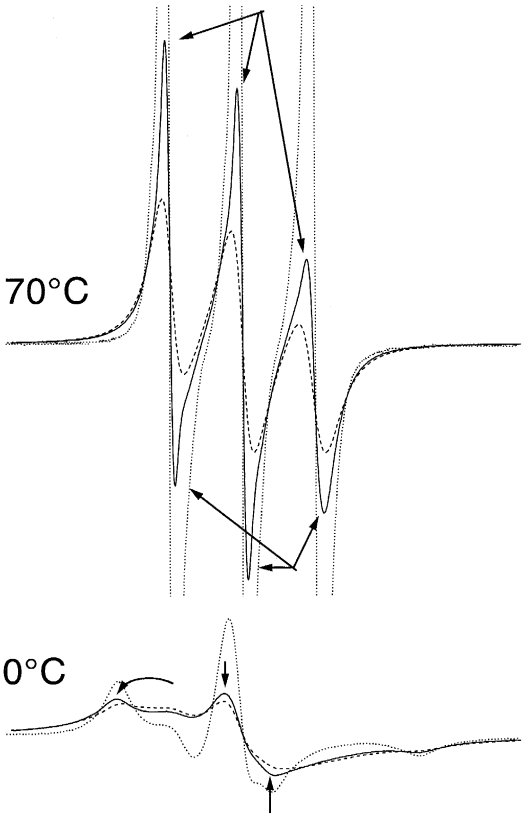

Fig. 2. ESR spectra of 10 mol% (dashed line) or 1 mol% (dotted line) 14-PCSL spin label in a hydrated DMPC/DMG 70:30 mol/mol dispersion at 70°C (upper) and 0°C (lower). Samples all contain the same amount of spin label and spectra are displayed with their true relative line heights. The spectra indicated by arrows are from a sample with 10 mol% spin label that is mixed with a 9-fold excess of unlabelled lipid dispersion. Total scan width = 100 G.

$$f_{\mathrm{eff}} = \left[h_{\mathrm{mix}}/h_{\mathrm{o}}(10\%) - 1\right] / \left[h_{\mathrm{o}}(1\%)/h_{\mathrm{o}}(10\%) - 1\right] \quad (1)$$

where h_{mix} is the normalized central line height in the spectra from the spin-labelled lipid dispersion mixed with unlabelled lipid dispersion and $h_{\mathrm{o}}(1\%)$, $h_{\mathrm{o}}(10\%)$ are the normalized central line heights in the spectra from control samples containing a uniform distribution of spin labels at relative concentrations of 1 mol% and 10 mol%, respectively. For complete mixing: $f_{\mathrm{eff}} = 1$ and for no mixing: $f_{\mathrm{eff}} = 0$.

3.2. Temperature and composition dependence of lipid mixing

The temperature dependence of the normalized line heights, h_{mix}, of the 14-PCSL phosphatidylcholine spin label in the lipid-mixing assay for DMPC/DMG dispersions of different compositions is given in Fig. 3. The lipid dispersions are all lamellar in the gel phase, at low temperature, and also in the fluid phase for DMPC alone and for the 70:30 mol/mol mixture with DMG. For the 40:60 mol/mol and 20:80 mol/mol dispersions, the fluid phases are of the inverted hexagonal and isotropic types, respectively. The structures of the phases at these compositions were established by X-ray diffraction and ^{31}P-NMR spectroscopy [15,17]. The fluid phases at these compositions also correspond to single-phase regions of the binary phase diagram [15].

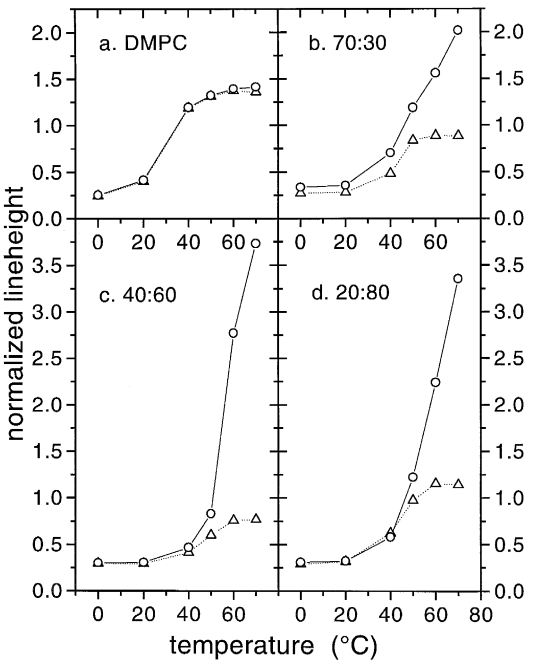

Fig. 3. Temperature dependence of the normalized spectral line height of 10 mol% spin-labelled phosphatidylcholine (14-PCSL) in hydrated DMPC/DMG mixtures in the presence (full lines) and absence (broken lines) of a 9-fold excess of the corresponding unlabelled lipid dispersion. (a) dispersion of DMPC alone, (b) 70:30 mol/mol DMPC/DMG dispersion, (c) 40:60 mol/mol DMPC/DMG dispersion, (d) 20:80 mol/mol DMPC/DMG dispersion. The error in measurement of the line height is no greater than the size of the symbols (as seen from comparison of the independent measurements where no transfer takes place).

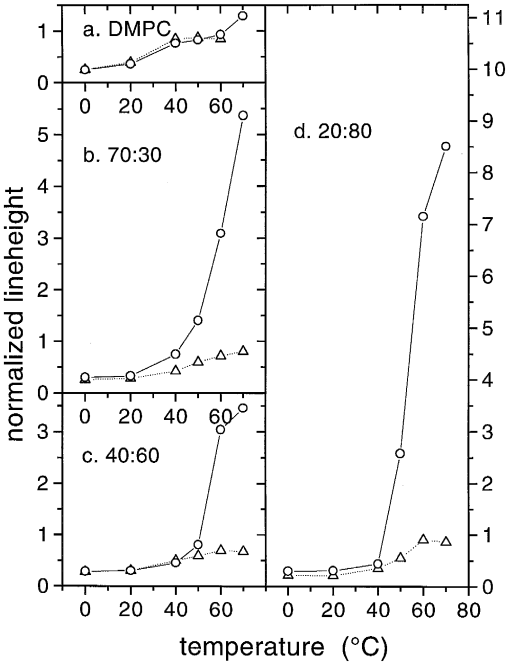

Fig. 4. Temperature dependence of the normalized spectral line height of 10 mol% spin-labelled diacylglycerol (14-DGSL) in hydrated DMPC/DMG mixtures in the presence (full lines) and absence (broken lines) of a 9-fold excess of the corresponding unlabelled lipid dispersion. (a) dispersion of DMPC alone, (b) 70:30 mol/mol DMPC/DMG dispersion, (c) 40:60 mol/mol DMPC/DMG dispersion, (d) 20:80 mol/mol DMPC/DMG dispersion. The error in measurement of the line height is no greater than the size of the symbols.

As seen from Fig. 3, little redistribution of the spin-labelled phosphatidylcholine takes place in the mixture of DMPC vesicles alone, nor in the gel phase of any of the dispersions containing diacylglycerol (cf. also Fig. 2). Only in the fluid phase is there evidence for a redistribution of the spin-labelled phosphatidylcholine into the unlabelled lipid dispersions and this only for the dispersions that contain diacylglycerol. Under the conditions used, the degree of mixing of the labelled phosphatidylcholine is limited and is comparable for the different compositions, being only somewhat less for the 70:30 mol/mol lamellar mixture than for the mixtures with higher DMG content.

The temperature dependence of the normalized line heights of the 14-DGSL diacylglycerol spin label in the same mixed lipid dispersions is given in Fig. 4. Again there is little redistribution of the spin-labelled lipid in vesicles of DMPC alone, nor in the other lipid dispersions in the gel phase. This is consistent

with the results obtained for the spin-labelled phosphatidylcholine component in Fig. 3. Mixing of the spin-labelled diacylglycerol with the unlabelled lipid dispersions takes place in the fluid phase for all dispersions containing diacylglycerol. The extent of mixing is considerably greater than that for the spin-labelled phosphatidylcholine component under comparable conditions. It is particularly extensive for the mixture of 20:80 mol/mol DMPC:DMG composition that is isotropic in the fluid phase. The normalized line heights of control samples and of the samples in the mixing assays are summarized for the various lipid compositions in Table 1.

The effective degrees of mixing, f_{eff}, of the spin-labelled diacylglycerol and phosphatidylcholine components, calculated according to Eqn. 1, are given in Fig. 5 for the various DMPC/DMG mixtures. As expected from the data in Figs. 3 and 4, $f_{\mathrm{eff}} \approx 0$ for both spin-labelled lipids in DMPC vesicles. The multilamellar DMPC vesicles alone do not appear to fuse at an appreciable rate, throughout the temperature range studied. A similar conclusion has been reached for the interaction of small unilamellar vesicles interacting with multilamellar vesicles of a saturated-chain phosphatidylcholine [21]. Also, all values of f_{eff} are low in the gel phase, but increase with increasing temperature on entering the fluid phase for the mixtures containing diacylglycerol. Fluidization of the lipid chains is necessary for lipid mixing between aqueous dispersions of these particular compositions.

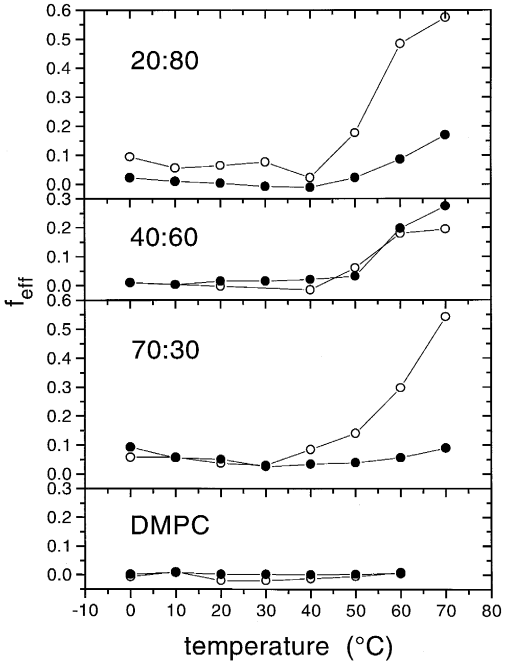

Fig. 5. Temperature dependence of the effective fractional redistribution, f_{eff}, of spin-labelled 14-PCSL (●) and 14-DGSL (○) into unlabelled lipid dispersions. From lower to upper: DMPC alone; DMPC/DMG 40/30 mol/mol; DMPC/DMG 40:60 mol/mol; DMPG/DMG 20:80 mol/mol. Deduced from the data of Fig. 3Fig. 4. The error in measurement of f_{eff} is determined primarily by the residual time dependence (see Fig. 6).

Table 1
Normalized line heights of 14-PCSL and 14-DGSL spin labels in DMPC/DMG mixtures at 70°C

Spin label	concentration (mol%)	DMPC/DMG (mol/mol)			
		1:0	70:30	40:60	20:80
14-PCSL	1.0	–	15.0	13.9	12.9
14-DGSL	1.0	14.0	12.0	12.0	11.5
14-PCSL	10	1.3	0.85	0.75	1.1
14-DGSL	10	0.9	0.9	0.7	0.9
14-PCSL	1 (mix) [a]	1.4	2.0	3.75	3.3
14-DGSL	1 (mix) [a]	1.3	5.5	3.5	8.5

The line heights are referred to 1 mol% 14-DGSL in DMPC alone at 0°C.

[a] Total spin label concentration is 1 mol% in the mixing assay which consists of a 1:9 mixture of labelled (10 mol%) with unlabelled dispersions.

The error in normalized lineheight measurement is no greater than ± 0.15.

An interesting pattern of behaviour is observed for the different lipid mixtures and for the two spin-labelled lipid components. The greatest degree of mixing is observed for the spin-labelled diacylglycerol component in the fluid phase of the 20:80 mol/mol DMPC/DMG mixture. In this case, the maximum degree of mixing obtained is $f_{\mathrm{eff}} \sim 0.6$ at 70°C. A complete mixing is probably hindered only by the fact that not all droplets of the fluid diacylglycerol are able to coalesce in the constrained volume of the sample capillary. The time dependence of the values of f_{eff} for a sample of this composition is given in Fig. 6. At 70°C, the redistribution has reached a limiting value on the time-scale of the experiments, whereas at 60°C the value of f_{eff} is still increasing towards the limiting value achieved at 70°C. (For all other compositions of the lipid mixtures, the values of f_{eff} were found to have achieved a constant level

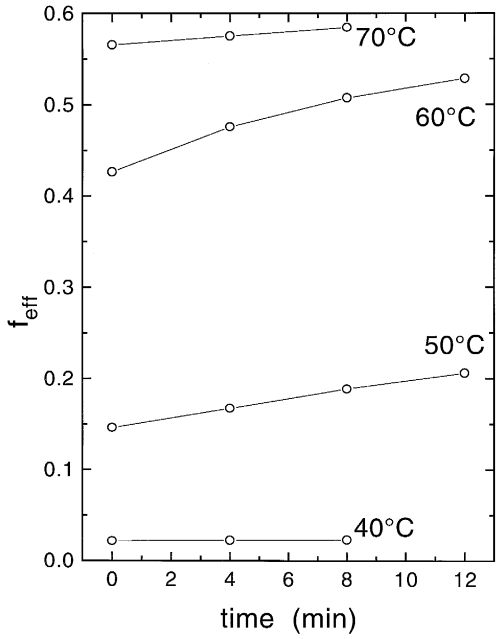

Fig. 6. Dependence of the effective fraction of homogeneously redistributed 14-DGSL into unlabelled 20:80 mol/mol DMPC/DMG mixture on time of incubation. Time zero corresponds to the first spectrum recorded immediately after mixing labelled and unlabelled dispersions at room temperature. The error in measurement of f_{eff} is of the order of the size of the symbols.

within the time-scale of the experiments.) Interestingly, the degree of lipid mixing is much smaller for the phosphatidylcholine component than for the diacylglycerol component in the 20:80 mol/mol DMPC/DMG mixture. It has been concluded earlier that the phosphatidylcholine component is present as inverted micelles in an isotropic melt of diacylglycerol, for samples of these compositions in the fluid phase [17]. These aggregated micellar structures, and the absence of monomeric phosphatidylcholine, could account for the slower rate of mixing of this component relative to diacylglycerol.

The lamellar structures formed by the 70:30 mol/mol DMPC/DMG mixtures are interesting because, whereas there is a high degree of mixing of the spin-labelled diaglycerol component in the fluid phase, the phosphatidylcholine component redistributes relatively little within the mixed lipid dispersion at all temperatures. The mixing of the diacylglycerol component cannot, therefore, be caused by fusion of the multilamellar vesicles, because then both components would redistribute equally. There is therefore an exchange of diacylglycerol with the unlabelled lipid dispersions. This transfer of diacylglycerol between vesicles must be mediated by close contacts between the respective bilayers. It is known that diacylglycerol can flip-flop rapidly between the monolayers of a given vesicle bilayer [22], whereas phosphatidylcholine cannot [23]. Possibly, the barriers to intervesicle transfer on close apposition may be similar to those for lipid flip-flop.

The 40:60 mol/mol DMPC/DMG dispersion is unique in that both the phosphatidylcholine and diacylglycerol components redistribute to comparable extents in the fluid phase. This suggests that a rearrangement involving bulk transfer of the lipid components takes place. To this extent, the process represents a true fusion of the lipid dispersions. This process takes place between inverted hexagonal (H_{II}) phase structures, however, rather than between lipid vesicles. This is consistent with the suggestion [18] that precursors of inverted non-lamellar phases may be involved as intermediates in the membrane fusion process.

The present results therefore serve to define the molecular transfer that takes place in lipid mixtures containing diacylglycerol under various conditions. Phosphatidylcholine serves as an inert host lipid; all transfer processes are induced by the presence of the diacylglycerol component. The results have relevance to the mechanisms by which diacylglycerol can induce vesicle fusion, initiate the formation of non-lamellar lipid phases and facilitate the action of lipolytic enzymes. In these processes, the transfer or exchange of the diacylglycerol molecules is likely to play a role, also in the case in which the diacylglycerol is generated in situ by the action of phospholipase C. Diacylglycerol has a controlling effect on exocytosis and it has been suggested that it may be directly involved in the fusion event that triggers exocytotic release [24]. Under these circumstances, the transfer of diacylglycerol may well also be important. Additionally, transfer processes may control the binding of diacylglycerol to protein kinase C during activation [25], or at least indirectly reflect the way in which diacylglycerol is presented to this regulatory enzyme at the membrane surface.

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