Transbilayer Redistribution of Phosphatidylethanolamine during Fusion of Phospholipid Vesicles. Dependence on Fusion Rate, Lipid Phase Separation, and Formation of Nonbilayer Structures†

Dick Hoekstra* and Ona C. Martin

ABSTRACT: The effect of membrane fusion on the transbilayer distribution of dioleoyl- and dipalmitoylphosphatidylethanolamine (DOPE and DPPE, respectively) in phosphatidylserine (PS) vesicles was investigated. A 7-fold increase in the external pool of DOPE, as determined by labeling of the vesicle surface with 2,4,6-trinitrobenzenesulfonic acid, was observed when multilamellar vesicles (MLV) consisting of PS and DOPE were incubated with small unilamellar vesicles (SUV) of PS in the presence of Ca^{2+}. In contrast, no significant redistribution of DPPE was seen when similar experiments were performed by using PS bilayers that contained DPPE instead of DOPE. Redistribution of neither DOPE nor DPPE could be detected during SUV–SUV fusion. By using the resonance energy transfer fusion assay for mixing of membrane lipids [Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099; Hoekstra, D. (1982) *Biochemistry 21*, 2833–2840], it was demonstrated that fusion between SUV and MLV had actually occurred. The results also suggested that (partial) fusion of internal bilayers within the multilamellar system must have occurred. Although Mg^{2+}-induced fusion between SUV and MLV was also observed, no redistribution of DOPE was seen in this case. It is concluded that the observed translocation of DOPE during fusion was probably mediated via inverted micellar structures, which were formed when the lipid was converted to the hexagonal (H_{II}) phase resulting from lipid phase separation between PS and DOPE. However, induction of the hexagonal phase per se, i.e., in the absence of fusion, did not cause substantial transbilayer redistribution of DOPE, suggesting that fusion was intimately involved in this phenomenon. It is suggested that fusion represents the "driving force" for transbilayer DOPE redistribution, requiring a (partial) overlap between the kinetics of phase separation and fusion.

Studies of Ca^{2+}-induced fusion between phosphatidylserine (PS)[1]-containing lipid vesicles have attracted widespread attention in recent years as a potential model for fusion of biological membranes [for reviews, see Poste & Nicolson (1978) and Papahadjopoulos et al. (1979)]. Using a variety of techniques, it has been demonstrated that fusion between such vesicles may indeed occur (Papahadjopoulos et al., 1975; Maeda & Ohnishi, 1974; Liao & Prestegard, 1979a; Vanderwerf & Ullman, 1980; Hoekstra et al., 1979; Wilschut et al., 1981; Hark & Ho, 1980; Struck et al., 1981). Although the details of the mechanism(s) involved have yet to be explained, increasing evidence suggests that lipid bilayers of apposing membranes may come into close proximity through local dehydrated areas (Newton et al., 1978; Portis et al., 1979; Wilschut et al., 1981; Hoekstra, 1982b) and subsequent local transient destabilization of the bilayer may facilitate the fusion process (Papahadjopoulos et al., 1979; Liao & Prestegard, 1979b; Cullis & de Kruijff, 1979; Wilschut et al., 1981; Hoekstra, 1982b). Although the *overall* bilayer structure seems to be retained (Hauser et al., 1977), it is conceivable that at some stage during the fusion event, a portion of the lipid molecules may deviate from the bilayer structure, resulting in a (partial) redistribution of lipid molecules.

In this study, the lipid topography of two species of PE was investigated during fusion of artificial lipid vesicles to gain further insight into the molecular details of membrane fusion processes. If membrane topography is conserved during fusion, lipids initially present in the external leaflet of the vesicle bilayer should reside in the external leaflet following fusion, while lipids initially present in the inner leaflet of the vesicle bilayer should remain in the inner leaflet. For examination of this possibility, DOPE or DPPE was incorporated into PS bilayers, and the transbilayer distribution of the PE species was determined before and after Ca^{2+}- or Mg^{2+}-induced fusion of the PS/PE vesicles. The orientation of PE was determined by using the amino-reactive reagent TNBS, which, under appropriate conditions, reacts only with externally disposed amino groups at the vesicle surface (Litman, 1974). Experiments are presented which demonstrate that transbilayer redistribution of DOPE, *but not DPPE*, could be induced only during fusion between membranes with different bilayer curvatures. The results suggest that redistribution appeared to be determined by the difference in physical properties of the PE species in isolation, caused by elimination of the bilayer stabilizing effect of PS on PE (Cullis & Verkleij, 1979) when PS is complexed by Ca^{2+}. The results suggest that the observed lipid redistribution was determined by (i) the rate of fusion, (ii) the rate of lipid phase separation, and (iii) the ability of the particular PE species to form hexagonal (H_{II}) phases in isolation, which occurs in the case of DOPE (van

† From the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210. *Received March 15, 1982.* This investigation was supported by a fellowship grant to D.H. from The Netherlands Organization for the Advancement of Pure Research (ZWO) and by National Institutes of Health Grant GM 22942 to R. E. Pagano.

* Correspondence should be addressed to this author at the Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands.

[1] Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; PS, phosphatidylserine; NBD, 4-nitro-2,1,3-benzoxadiazole; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; SUV, small unilamellar vesicle(s); MLV, multilamellar vesicle(s); LUV, large unilamellar vesicle(s); HCMF, calcium- and magnesium-free Puck's saline, buffered with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; TNBS, 2,4,6-trinitrobenzenesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tnp, trinitrophenyl.

0006-2960/82/0421-6097$01.25/0

Dijck et al., 1976) but not in the case of DPPE (Seelig & Gally, 1976; Cullis & de Kruijff, 1979).

Materials and Methods

Lipids and Lipid Vesicles. Phosphatidylserine (PS) from bovine brain, dioleoylphosphatidylethanolamine (DOPE), dipalmitoylphosphatidylethanolamine (DPPE), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) were purchased from Avanti Biochemicals (Birmingham, AL). [^{3}H]DOPE was prepared from [^{3}H]DOPC [synthesized as described in Boss et al. (1975)] by using phospholipase D and ethanolamine (Comfurius & Zwaal, 1977). [^{14}C]DPPE was obtained from New England Nuclear. *N*-(Lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) was synthesized and purified as described elsewhere (Struck et al., 1981). All lipids were periodically monitored for purity and exhibited a single spot by thin-layer chromatography.

Small unilamellar vesicles of various compositions were prepared by sonication in HCMF at 10 °C under an inert atmosphere of argon gas. Multilamellar vesicles were prepared by suspending the dried lipids in HCMF, followed by vortexing and repeated centrifugation (20 min, 15000*g*). The final pellet of MLV was suspended in HCMF. Large unilamellar vesicles (LUV) were prepared by fusion of SUV (Szoka & Papahadjopoulos, 1980) in the presence of 1.5 mM Ca^{2+} for 15 s. The reaction was stopped by addition of a 2-fold molar excess of EDTA. The preparation was then centrifuged twice (20 min at 30000*g*), and the pellet was resuspended in HCMF.

Vesicle Fusion. Fusion between vesicles was initiated by addition of 4 mM Ca^{2+} (final concentration), and the reaction was stopped by addition of 8 mM EDTA. The fusion kinetics were monitored by the resonance energy transfer fusion assay (Struck et al., 1981) as described elsewhere (Hoekstra, 1982b). Briefly, two nonexchangeable fluorescent lipid markers, *N*-NBD-PE and *N*-Rh-PE, are incorporated into separate bilayers. Upon Ca^{2+}- or Mg^{2+}-induced fusion of the vesicles, mixing of the probes within the same bilayer occurs, permitting resonance energy transfer between the fluorescent donor (*N*-NBD-PE) and acceptor (*N*-Rh-PE) molecules, resulting in quenching of NBD fluorescence. The relative fluorescent signal of the starting NBD-containing vesicles is taken as 100% fluorescence, representing the absence of fusion. More than 95% quenching of NBD fluorescence is obtained when vesicles are prepared, containing both *N*-NBD-PE and *N*-Rh-PE ("mock" fusion), in a ratio identical with their ratio in the (separate) starting vesicles, which is considered to represent 100% fusion. The amount of resonance energy transfer is approximately proportional to the extent of probe intermixing, and the extent of fusion can then be determined by comparing the relative fluorescence with a mock fused control [for further details, see Struck et al. (1981) and Hoekstra (1982b)]. The quenching process, as a measure of fusion, was monitored continuously in an Aminco-Bowman spectrophotofluorometer (American Instrument Co.) equipped with a chart recorder. Samples were excited at 475 nm and monitored at 530 nm (excitation and emission wavelengths, respectively, of NBD) by using narrow band-pass slits (1 mm) and crossed polarizers to minimize light scattering.

Determination of Lipid Phase Separation. The kinetics of phase separation were measured as described previously (Hoekstra, 1982a). Briefly, PS/*N*-NBD-PE vesicles (95:5) were incubated in the presence of Ca^{2+}. Upon Ca^{2+}-induced phase separation, the *local* concentration of NBD lipid in the bilayer increases, resulting in fluorescence quenching due to self-quenching of the NBD fluorophore. Thus, the kinetics of phase separation can be determined continuously by monitoring NBD fluorescence quenching as described above.

Phase separation and fusion can be distinguished from one another by addition of EDTA [see Hoekstra (1982b)]. NBD quenching resulting from Ca^{2+}-induced phase separation is governed solely by the presence of the cation and is therefore reversible upon displacement of Ca^{2+} from the membrane. In contrast, after fusion of NBD-containing vesicles with Rh-containing vesicles, NBD fluorescence quenching will persist following addition of EDTA, since fusion leads to intermixing of fluorescent donor and acceptor molecules in the *same* bilayer.

Determination of PE Distribution. The outer vesicle surface PE content was determined by chemical labeling with 2,4,6-trinitrobenzenesulfonic acid (TNBS) as described by Litman (1974). Aliquots of the vesicle suspension were chilled on ice, and the pH was adjusted to pH 8.5. TNBS (10 mM final concentration) in 0.1 M $NaHCO_3$, pH 8.5, was added, and the samples were incubated in the dark for 2 h on ice. The reaction was stopped by acidification of the incubation mixture. The lipids were then extracted and separated by thin-layer chromatography, using chloroform/methanol/acetone/acetic acid/water (3:1:4:1:0.5) as the running solvent. Areas corresponding to isotopically labeled PE and its trinitrophenyl derivative were scraped, and the radioactivity in each area was determined.

Results

Transbilayer Redistribution of DOPE in Multilamellar and Small Unilamellar PS/DOPE Vesicles. When small unilamellar vesicles consisting of PS and various amounts of DOPE (5–20 mol %) were fused in the presence of Ca^{2+} (see below), the ratio of DOPE in the outer vesicle surface to the total DOPE content (as determined by TNBS labeling) did not significantly change when the TNBS-accessible DOPE pools were compared before and after fusion (unpublished observations). Consistent with these results was the observation that 12–15% of the total DOPE pool in multilamellar PS/DOPE vesicles (95:5) could be converted to Tnp-DOPE before addition of Ca^{2+}, but no substantial changes were detected in the Tnp-DOPE pool size upon incubation of these vesicles in the presence of Ca^{2+} (Figure 1). These results suggest that the transbilayer distribution of DOPE in the initial vesicle preparation was essentially conserved during fusion of DOPE-containing unilamellar or multilamellar PS vesicles.

However, when similar experiments were performed by using vesicles with different degrees of curvature, the pool of DOPE in the surface monolayer of multilamellar PS/DOPE vesicles (95:5) rapidly increased (from ~11% to ~74% Tnp-DOPE) when incubated with small unilamellar PS vesicles in the presence of Ca^{2+} (Figure 1, □). The results were similar when unilamellar vesicles consisting of PS and DOPE (95:5) were incubated with multilamellar PS vesicles in the presence of Ca^{2+} (○), and the Tnp-DOPE pool increased from ~34% to ~64%.

When the reaction proceeded beyond 10 min, the fraction of the total DOPE pool that was accessible to TNBS labeling decreased slightly, as evidenced by a decrease in the pool of Tnp-DOPE. As mentioned above, no substantial increase in the ratio of outer vesicle surface DOPE to the total DOPE content was observed when only multilamellar vesicles were incubated in the presence of Ca^{2+} (■), excluding the possibilities either that a massive Ca^{2+}-induced flip-flop of DOPE was the cause of the transbilayer redistribution (Figure 1) or that leakage of TNBS across the bilayers during the chemical labeling procedure had occurred (also see below).

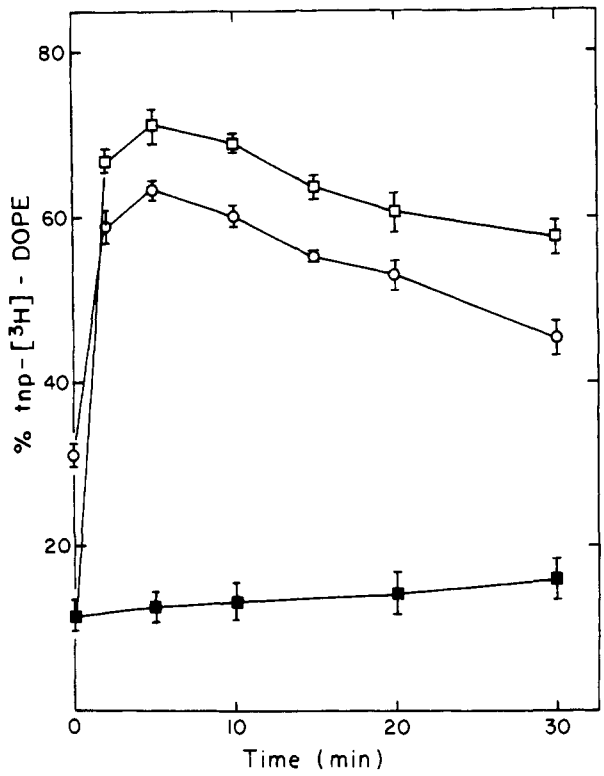

FIGURE 1: Transbilayer redistribution of DOPE in small unilamellar and multilamellar PS/DOPE vesicles. Vesicle mixtures consisting of multilamellar PS/DOPE vesicles (95:5, 0.63 μmol of lipid) and small unilamellar PS vesicles (0.80 μmol of lipid), multilamellar PS vesicles (1 μmol of lipid) and small unilamellar PS/DOPE vesicles (95:5, 0.63 μmol of lipid), or multilamellar PS/DOPE vesicles (95:5, 0.63 μmol of lipid) alone were prepared. All DOPE-containing vesicle preparations contained in addition a trace amount of [^{3}H]DOPE. 4 mM Ca^{2+} (final concentration) was added, and after different time intervals, 8 mM EDTA (final concentration) was added. The pH of the incubation mixtures was increased to 8.5, and the pool of vesicle surface DOPE was determined by TNBS labeling as described under Materials and Methods. The percentage of [^{3}H]DOPE that could be converted to its Tnp derivative was plotted vs. the incubation time in the presence of Ca^{2+}: (□) MLV (PS/DOPE) and SUV (PS); (○) MLV (PS) and SUV (PS/DOPE); (■) MLV (PS/DOPE).

Inner Bilayer Fusion in Multilamellar PS/DOPE Vesicles. Since only 10–15% of the total (DOPE) lipid is located in the external leaflet of a multilamellar vesicle (Bangham et al., 1967; DiCorleto & Zilversmit, 1977), it appears that during the interaction of multilamellar PS/DOPE vesicles and small unilamellar PS vesicles, inner bilayer DOPE must also have become accessible to TNBS. One possibility could be that DOPE which was initially located in inner bilayers may have been transferred to the vesicle surface via interconnecting bilayers established by fusion of inner MLV bilayers. For investigation of this possibility, a previously described assay for monitoring membrane fusion based on lipid intermixing was used (Struck et al., 1981; Hoekstra, 1982b). In Figure 2, the results of such an experiment are shown in which multilamellar PS/*N*-NBD-PE (95:5) vesicles were mixed with small unilamellar PS/*N*-Rh-PE (95:5) vesicles. Upon addition of Ca^{2+}, a rapid decrease in NBD fluorescence was observed (×), indicating that fusion between both populations had actually occurred. Identical kinetics of fusion were observed when Ca^{2+} was added to a mixture of multilamellar PS/DOPE/*N*-NBD-PE vesicles (95:4:1) and small unilamellar PS/*N*-Rh-PE vesicles (99:1) (not shown, cf. Figure 2).

However, fusion under these experimental conditions is probably a rather complicated process, since, in principle, at least three different types of interaction may occur. First, a

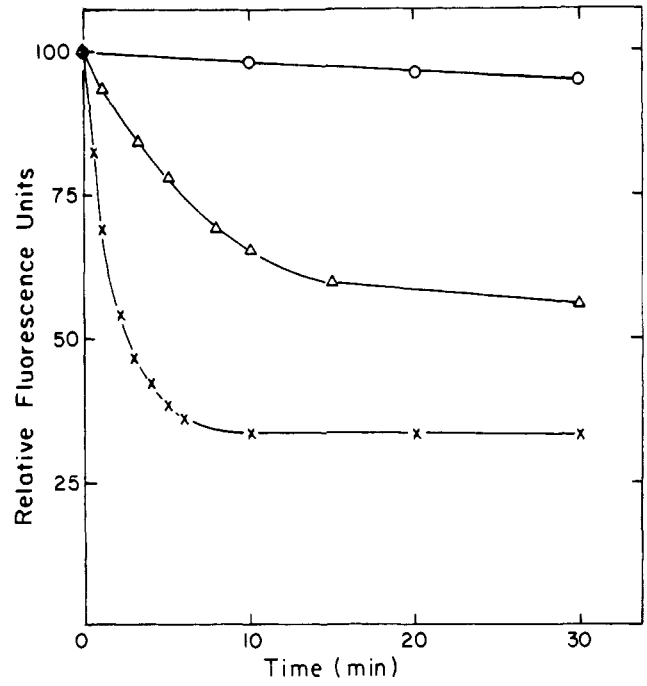

FIGURE 2: Kinetics of Ca^{2+}-induced fusion of vesicle mixtures consisting of multilamellar and unilamellar vesicles, and kinetics of fusion and phase separation of multilamellar vesicles. MLV (PS/*N*-NBD-PE, 95/5) were mixed with SUV (PS/*N*-Rh-PE, 95/5) in 2 mL of HCMF at room temperature. 4 mM Ca^{2+} (final concentration) was added, and the reaction was stopped after different incubation periods by addition of 8 mM EDTA (final concentration). NBD fluorescence was measured as indicated under Materials and Methods (×). Fusion between MLV was measured similarly except that instead of SUV, MLV consisting of PS and *N*-Rh-PE were added (○). Ca^{2+}-induced phase separation in MLV (△) was determined by incubating MLV (PS/*N*-NBD-PE, 95/5) in the presence of 4 mM Ca^{2+}. NBD fluorescence was measured as described under Materials and Methods. Lipid concentrations were as in Figure 1.

majority of the SUV probably fused with one another, although such a process would obviously not lead to changes in fluorescence, since mixing of *N*-Rh-PE in the SUV with *N*-NBD-PE does not occur if only SUV–SUV fusion is taking place. Second, MLV–MLV fusion may also occur, which was tested in a separate experiment in which two MLV PS populations, one containing 5 mol % *N*-NBD-PE and the other 5 mol % *N*-Rh-PE, were mixed and incubated in the presence of 4 mM Ca^{2+}. However, as shown in Figure 2 (○), only a very small decrease in NBD fluorescence was seen during a 30-min incubation period, excluding substantial MLV–MLV fusion. The results of this experiment also indicate that the MLV preparations were not significantly contaminated with smaller vesicles, which would fuse more rapidly and thus contribute substantially to the observed rate and extent of NBD quenching in the MLV–SUV fusion experiment. Third, nonfused (and possibly fused) SUV may fuse with MLV, and since the NBD fluorescence decreased (Figure 2), it appears that this process must have occurred.

Since only 10–15% of the total NBD lipid is localized in the external leaflet of a multilamellar vesicle (Pagano et al., 1981), a much larger pool of NBD lipid than only those molecules located in the external surface monolayer must have become intermixed with (SUV) *N*-Rh-PE in order to account for the observed decrease of approximately 70% in NBD fluorescence. Since exchange or anomalous flip-flop of these fluorescent derivatives can be excluded (Struck et al., 1981; Pagano et al., 1981; D. Hoekstra, unpublished experiments), these results would thus be consistent with interbilayer fusion and subsequent lateral diffusion-mediated mixing of the

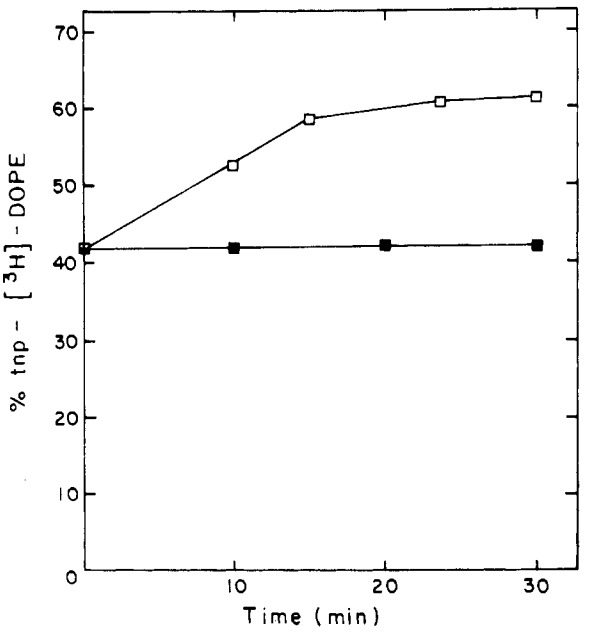

FIGURE 3: Transbilayer redistribution of DOPE in large unilamellar PS/DOPE vesicles. LUV consisting of PS, 5 mol % DOPE, and a trace amount of [^{3}H]DOPE were prepared as described under Materials and Methods. LUV alone (■) or LUV premixed with a 2-fold excess of SUV consisting of PS (□) were subsequently incubated in the presence of 4 mM Ca^{2+}. After different incubation periods, samples were treated as described in Figure 1 for determination of the vesicle surface DOPE pool.

fluorescent probes within the multilamellar system.

DOPE Redistribution upon Fusion of Differently Curved Bilayers. It is interesting to note that the kinetics of TNBS labeling of vesicle surface DOPE (Figure 1) and the kinetics of fusion (Figure 2) immediately after addition of Ca^{2+} proceed at similar rates. The maximal extents of both TNBS labeling and fusion-mediated lipid mixing were observed after 5–10 min, suggesting a possible relationship between fusion and lipid redistribution, although some "spontaneous" redistribution may occur after the fusion process has ceased (>10–15 min, Figure 1).

Since redistribution of DOPE was not observed during fusion of vesicles with similar curvature (see above), the results suggest that fusion per se did not suffice to cause redistribution but that, in addition, the difference in the degree of curvature of the interacting bilayers may be closely related to this phenomenon. This suggestion was confirmed by an experiment in which the extent of DOPE redistribution was examined during an incubation of large unilamellar PS/DOPE vesicles (95:5) with small unilamellar PS vesicles in the presence of Ca^{2+}. As shown in Figure 3, no significant redistribution of DOPE was seen during or after LUV–LUV fusion. However, when Ca^{2+} was added to a mixture of LUV and SUV, an increase in the pool of vesicle surface DOPE was observed, presumably as a result of LUV–SUV fusion, which continued up to 15 min after the addition of Ca^{2+}. The fraction of DOPE accessible to TNBS increased from 40% to approximately 58% during this time and leveled off in 30 min at approximately 63%. These results provide further support for the observation that DOPE redistribution occurred only when membranes with different degrees of curvature were fused. It should be noted, however, that when LUV and SUV were mixed, a complicated series of vesicle–vesicle interaction events, similar to those described above for MLV–SUV interactions, may have occurred. Since LUV–LUV and SUV–SUV fusion did not result in lipid redistribution, it is assumed that LUV–SUV fusion represented the primary reaction which caused DOPE lipid redistribution.

These experiments strongly suggest that transbilayer redistribution of DOPE occurred only upon fusion of differently curved bilayers, and, in addition, a lack of DOPE redistribution during fusion between vesicles with similar degrees of membrane curvature indicated that fusion itself was apparently not sufficient to induce DOPE redistribution.

Mechanism of DOPE Transbilayer Redistribution. So that the results described above could be explained, the possibility was considered that the transbilayer movement of DOPE was mediated by the formation of nonbilayer lipid structures, since DOPE can adopt the hexagonal (H_{II}) phase via inverted micellar structures (van Dijck et al., 1976). The involvement of such nonbilayer structures as a potential mechanism in transbilayer redistribution of lipids has been suggested by Cullis & de Kruijff (1979). A necessary condition for the formation of a hexagonal (H_{II}) phase in the PS/DOPE bilayer involves the occurrence of lipid phase separation between PS and DOPE, thus forcing DOPE to adopt nonbilayer structures. For determination of the kinetics of Ca^{2+}-induced phase separation, multilamellar vesicles were prepared consisting of PS and 5 mol % *N*-NBD-PE. Subsequently, 4 mM Ca^{2+} was added, and the quenching of NBD fluorescence (as a measure of phase separation) was monitored (Figure 2, △) as a function of time (Hoekstra, 1982a). [Similar kinetics of phase separation in MLV were observed in the presence of SUV. However, in this case, the extent of fluorescence quenching was less, presumably due to dilution of the fluorophore upon fusion between MLV and non-probe-containing SUV; see Hoekstra (1982a).] The time courses of surface appearance of DOPE and phase separation (Figure 1, □, vs. Figure 2, △, respectively) indicate that a significant amount of phase separation had occurred within the time interval in which the external pool of DOPE sharply increased. Presumably, substantial amounts of nonbilayer lipidic particles were formed during this time interval, which could have facilitated transbilayer redistribution of DOPE. However, MLV–SUV fusion is apparently an additional prerequisite, since solely the occurrence of phase separation in MLV (i.e., in the absence of SUV) was not sufficient to cause substantial redistribution of DOPE (Figure 1, ■).

Two additional types of experiments provided further evidence to support the suggestion that nonbilayer lipid structures were involved in the observed redistribution of DOPE. We examined (i) the potential redistribution of *DPPE*, a PE species which does not form the hexagonal phase (Cullis & de Kruijff, 1979), upon fusion of multilamellar PS/DPPE vesicles with unilamellar PS vesicles and (ii) the transbilayer distribution of *DOPE* before and after Mg^{2+}-induced fusion of multilamellar PS/DOPE vesicles with unilamellar PS vesicles, since Mg^{2+} does *not* induce phase separation of PS and DOPE, thus preventing DOPE from forming the hexagonal phase.

(i) Multilamellar vesicles consisting of PS and DPPE (instead of DOPE) were generated and incubated with small unilamellar PS vesicles in the presence of 4 mM Ca^{2+} by using conditions identical with those described in the experiments using DOPE-containing vesicles. Using the resonance energy transfer fusion assay, it was found that the kinetics of fusion in this case were identical with those observed during fusion of the DOPE-containing vesicle mixture (cf. Figure 2, ×). After different time intervals, the reaction was stopped by addition of excess EDTA, and the surface pool of DPPE was determined by TNBS labeling. In contrast to the results obtained for DOPE-containing PS vesicles, the external pool

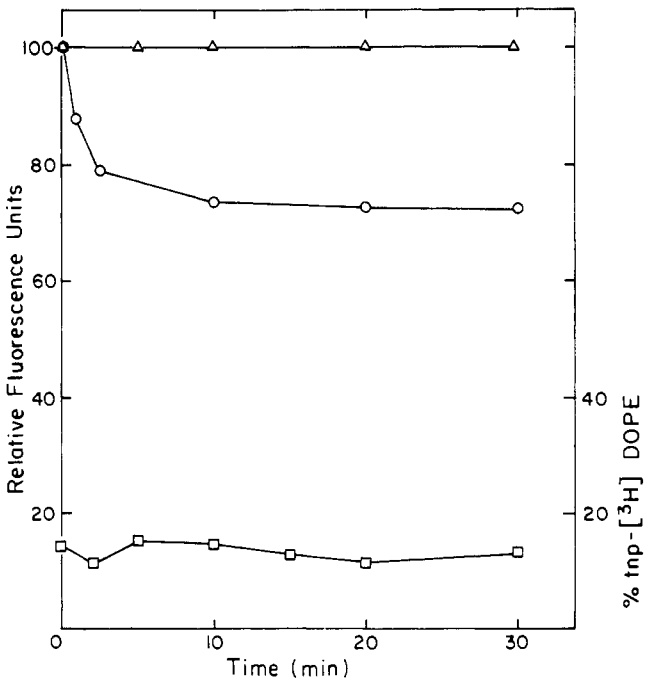

FIGURE 4: Effect of Mg^{2+}-induced fusion between MLV and SUV on transbilayer distribution of DOPE. MLV (0.6 μmol; PS/DOPE, 95/5) were mixed with SUV (0.9 μmol, PS) followed by addition of 25 mM Mg^{2+} (final concentration). At the indicated times, the reaction was stopped by addition of EDTA (50 mM final concentration), and the external pool of DOPE was determined as described in Figure 1 (□). Mg^{2+}-induced fusion between MLV and SUV (○), as monitored by resonance energy transfer and Mg^{2+}-induced phase separation in MLV (△), was performed as described for Ca^{2+} in Figure 2, except that the final concentration of Mg^{2+} was 25 mM.

of DPPE (relative to the pool size before addition of Ca^{2+}) remained virtually unaltered during a 30-min time interval, fluctuating between 9.5% and 13.9% of the total [^{3}H]DPPE pool, suggesting that only DPPE in the multilamellar external leaflet was accessible to TNBS labeling and, furthermore, that leakage of TNBS across the bilayer(s) did not occur. It is concluded that, in contrast to DOPE, transbilayer redistribution of *DPPE* did not occur during MLV–SUV fusion. Since DPPE does not undergo an H_{II} phase change and maintains a bilayer structure well above its phase transition temperature (Seelig & Gally, 1976; Cullis & de Kruijff, 1979), this result supports the involvement of nonbilayer structures as a mechanism for DOPE redistribution.

(ii) As noted above, hexagonal phase formation depends on the induction of bilayer lipid phase separation, predicting that unless phase separation in the PS/DOPE bilayer occurs, DOPE transbilayer redistribution will most likely not occur. Experimental support for this prediction is provided by an experiment in which the interaction of multilamellar PS/DOPE vesicles (95:5) and small unilamellar PS vesicles was studied in the presence of Mg^{2+} (Figure 4). Addition of Mg^{2+} to the vesicle mixture did not induce phase separation (△), which is consistent with previous observations on the inability of Mg^{2+} to induce phase separations in PS-containing vesicles (Hoekstra, 1982a). Fusion did occur to a limited extent between the multilamellar and unilamellar vesicles, as assessed by the resonance energy transfer assay (○). However, significant redistribution of DOPE was not observed, as demonstrated by the essentially unaltered Tnp-[^{3}H]DOPE pool in the multilamellar PS/DOPE vesicle surface (□).

Discussion

Redistribution of DOPE and Hexagonal Lipid Phases. In this report, the potential redistribution of DOPE during fusion of PS/DOPE vesicles was investigated, and it was shown that the surface pool of [^{3}H]DOPE (as determined by TNBS labeling) did not substantially change when either small unilamellar, multilamellar, or large unilamellar PS/DOPE vesicles (95:5) were incubated in the presence of Ca^{2+}. However, a rather unexpected result was obtained when SUV were mixed with either MLV (Figure 1) or LUV (Figure 3) and subsequently incubated in the presence of Ca^{2+}, resulting in a considerable increase in the TNBS-accessible DOPE pool. It further appears that DOPE transbilayer redistribution in these cases was not due to some peculiarity of the fusion process between these vesicle mixtures per se, since Mg^{2+}-induced fusion between MLV and SUV did not result in DOPE redistribution (Figure 4). This was corroborated by an experiment in which Ca^{2+}-induced fusion of multilamellar PS/DPPE vesicles with small unilamellar PS vesicles was not accompanied by significant changes in the initial, surface-localized pool of DPPE, excluding random flip-flop due to the fusion process itself as a mechanism for DOPE transbilayer redistribution.

Rather, the results were best explained by a mechanism involving a translocation of DOPE via inverted micellar structures. In particular, the absence of lipid redistribution in the experiments in which Mg^{2+} was used to induce fusion (Figure 4) and the experiment showing a lack of DPPE redistribution upon Ca^{2+}-induced fusion of DPPE-containing PS vesicles argue strongly in favor of this mechanism as a major intermediate process in DOPE transbilayer redistribution. Cullis & de Kruijff (1979) have considered the potential significance of intrabilayer inverted lipid structures as a mechanism for transbilayer lipid transport. The formation of such nonbilayer structures is facilitated by a phase separation between PS and DOPE (Cullis & de Kruijff, 1979), a process that occurs in the presence of Ca^{2+} but not Mg^{2+} (cf. open triangles in Figures 2 and 4; Hoekstra, 1982a). However, not all molecular species of PE in isolation prefer the H_{II} phase following lateral segregation of the bilayer-stabilizing lipid species (in this case PS) and PE. In contrast to DOPE (van Dijck et al., 1976; Cullis & de Kruijff, 1976), DPPE maintains the bilayer structure in isolation (Seelig & Gally, 1976) and, hence, would be expected to maintain the bilayer structure after phase separation. Therefore, the absence of DOPE transbilayer redistribution in the case of Mg^{2+}-induced fusion between SUV and MLV and the absence of DPPE redistribution during Ca^{2+}-induced fusion of a similar vesicle mixture are consistent with the mechanism proposed above.

Dependence of DOPE Redistribution on the Rates of Fusion and Phase Separation. The formation of the H_{II} phase per se did not necessarily result in a significant redistribution of DOPE, as was seen when MLV alone were incubated in the presence of Ca^{2+} (Figure 1). Under these conditions, phase separation did occur, but fusion was almost undetectable. In addition, as shown previously (Hoekstra, 1982b), the rate of lipid phase separation is a much slower process than the rate of fusion during fusion of SUV (see below), and substantial lipid phase separation is detected only after the fusion process is virtually complete. Thus, although lipid phase separation is ultimately induced in such vesicles, significant lipid redistribution was not observed. This implies that fusion was a necessary, concomitant process in order to accomplish DOPE redistribution. A most intriguing question is, however, why redistribution of DOPE was observed only when *differently curved* membranes were fused and not during fusion of, for

instance, only SUV. Although the experiments do not provide a clear explanation, it could be suggested that the fusion *rate* in the distribution process might be important. In addition, the *rate* of phase separation must be considered, since phase separation is necessary to induce the formation of the hexagonal phase. Since both fusion and phase separation are apparently cooperative processes in causing DOPE redistribution (see above), it is tempting to suggest that a partial overlap of these processes is necessary. In the case of SUV–SUV fusion, any overlap is virtually nonexistent (Hoekstra, 1982a), since the half-times for fusion and phase separation are <5 s and approximately 1 min, respectively. However, for SUV–MLV fusion, the half-times were 1.5 and 5 min, respectively, and in this case, fusion may conceivably provide the "driving force" for the redistribution process, while during SUV–SUV fusion the fusion reaction is essentially complete before significant phase separation has occurred. [It should be noted that in the absence of fusion, significant DOPE redistribution was not observed in PS/DOPE bilayers (Figures 1 and 2) although the lipids were in a phase-separated state.]

Fusion of Inner Bilayers and DOPE Redistribution in MLV. In previous studies from this laboratory (Struck et al., 1981; Hoekstra, 1982b), the resonance energy transfer assay has been conveniently used to monitor vesicle fusion. Since molecular exchange of the (head group labeled) fluorescent phospholipid analogues *N*-NBD-PE and *N*-Rh-PE does not occur (Struck et al., 1981; Pagano et al., 1981; Hoekstra, 1982a,b), the observation that NBD fluorescence in MLV became quenched after addition of SUV containing *N*-Rh-PE and Ca^{2+} suggests that fusion between both populations had actually occurred. However, as described under Results, membrane fusion in this vesicle mixture is most likely not a "clean" fusion process since several vesicle–vesicle fusion events may occur, most notably SUV–SUV fusion.

As indicated by the fusion assay and as may be inferred from a lack of redistribution during MLV–MLV (Figure 1) and SUV–SUV fusion, substantial SUV–MLV fusion must also have taken place. Since ~70% of the NBD fluorescence became quenched during SUV–MLV fusion, the results suggest that interbilayer fusion between bilayers within the multilamellar system had occurred, probably at attachment sites of adjacent lamellae in multilamellar vesicles, permitting equilibration of lipids via lateral diffusion. Furthermore, the distribution of DPPE before and after interaction of multilamellar PS/DPPE and unilamellar PS vesicles did not suggest that fusion between adjacent bilayers within the multilamellar system resulted in *complete* merging of the lipid molecules in both bilayers. In that case, an ~2-fold enhancement in the surface pool of DPPE would have been expected, which was not observed. This result suggests that only areas of adjacent bilayers had fused, rather than complete intermixing of adjacent bilayers, which is consistent with electron microscopic observations on other vesicle systems (Hui et al., 1981; Rand et al., 1981).

It has been proposed that membrane fusion may proceed not only via interbilayer, anhydrous Ca^{2+}–PS complexes (Newton et al., 1978; Portis et al., 1979) but also via formation of nonbilayer structures such as inverted micelles (de Kruijff et al., 1979; Verkleij et al., 1979; Cullis & Hope, 1978). It seems unlikely, however, that the formation of inverted micellar structures is required to induce fusion of small unilamellar PS/PE vesicles, since it has been shown previously (Hoekstra, 1982b) that extensive and virtually complete SUV fusion can take place before significant lipid phase separation occurs. The absence of DOPE redistribution during fusion of small unilamellar PS/DOPE vesicles, as described in the present study, is consistent with this observation. However, when fusion and phase separation proceed according to similar kinetics, as appears to be the case in MLV–SUV fusion, it is possible that interbilayer anhydrous Ca^{2+}–PS complexes as well as poorly hydrated nonbilayer DOPE structures may coexist in the plane of the bilayer and *both* conditions may further facilitate the fusion process. Since DOPE thus accumulates in areas where fusion between adjacent bilayers will occur (Cullis & Hope, 1978; de Kruijff et al., 1979; Hui et al., 1981; Rand, 1981), a direct, interbilayer, DOPE interaction site is established, and a rapid transfer between bilayers and intrabilayer flip-flop via the inverted DOPE micelles may occur. Through a series of such properly aligned structures in subsequent bilayers, DOPE could ultimately gain access to the external surface bilayer, inducing an asymmetric distribution of DOPE in the bilayer(s). It thus appears not only that these structures facilitate transbilayer movement of other, nonrelated lipids (Gerritsen et al., 1980) but also that apparently DOPE itself may become translocated between bilayers.

Finally, it is relevant to consider whether DOPE initially *accumulated* in the external leaflet of the surface MLV bilayer or if it was transferred to acceptor membranes [cf. Gerritsen et al. (1980)]. Acceptor membranes may have been formed by fusion of small unilamellar PS vesicles in the MLV–SUV mixture upon addition of Ca^{2+} (see above), assuming that substantial fusion of such structures with MLV did not occur. However, if these structures functioned as acceptor membranes for DOPE, an increase in the external pool of DOPE during LUV–LUV interaction would also have been expected, which was evidently not observed (Figure 3). Therefore, it is concluded that DOPE probably accumulated in the external leaflet of the MLV, increasing the DOPE/PS ratio from 1/20 to approximately 1/3 to 1/4 after short incubation times (<10 min), assuming that 10–15% of the total MLV lipid is located in the external leaflet (Bangham et al., 1967; DiCorleto & Zilversmit, 1977; Pagano et al., 1981).

In summary, it was shown that redistribution of DOPE, but not DPPE, could be induced upon fusion of small unilamellar PS vesicles with multilamellar or large unilamellar PS/PE vesicles. The results suggest that lipid redistribution was facilitated by (nonbilayer) inverted micellar DOPE structures, which were formed as a result of Ca^{2+}-induced lipid phase separation between PS and DOPE. Phase separation per se did not result in an asymmetric redistribution of DOPE across the bilayer, and membrane fusion appeared to be a necessary, accompanying process.

Finally, hexagonal phase formation does not occur (i) before the fusion of small unilamellar PS/DOPE vesicles is essentially complete, (ii) upon Mg^{2+}-induced fusion, or (iii) during fusion of PS vesicles containing DPPE instead of DOPE. Under these conditions, lipid redistribution was also not observed. Thus, these observations further indicated the dependence of lipid transbilayer movement on the rate of fusion and of phase separation, and on the ability of the particular lipid to form hexagonal phases.

Acknowledgments

Susan Satchell is gratefully acknowledged for her expertise in the preparation of the manuscript.

References

Bangham, A. D., de Gier, J., & Greville, G. D. (1967) *Chem. Phys. Lipids 1*, 225–246.

Boss, W. F., Kelly, C. J., & Landsberger, F. R. (1975) *Anal. Biochem. 64*, 289–292.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.
Cullis, P. R., & de Kruijff, B. (1976) *Biochim. Biophys. Acta 436*, 523–540.
Cullis, P. R., & Hope, M. J. (1978) *Nature (London) 271*, 672–674.
Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.
Cullis, P. R., & Verkleij, A. J. (1979) *Biochim. Biophys. Acta 552*, 546–551.
de Kruijff, B., Verkleij, A. J., van Echteld, C. J. A., Gerritsen, W. J., Mombers, C., Noordam, P. C., & de Gier, J. (1979) *Biochim. Biophys. Acta 555*, 200–209.
DiCorleto, P. E., & Zilversmit, D. B. (1977) *Biochemistry 16*, 2145–2150.
Gerritsen, W. J., De Kruijff, B., Verkleij, A. J., de Gier, J., & Van Deenen, L. L. M. (1980) *Biochim. Biophys. Acta 598*, 554–560.
Hark, S. K., & Ho, J. T. (1980) *Biochim. Biophys. Acta 601*, 54–62.
Hauser, H., Finer, E. G., & Darke, A. (1977) *Biochem. Biophys. Res. Commun. 76*, 267–274.
Hoekstra, D. (1982a) *Biochemistry 21*, 1055–1061.
Hoekstra, D. (1982b) *Biochemistry 21*, 2833–2840.
Hoekstra, D., Yaron, A., Carmel, A., & Scherphof, G. (1979) *FEBS Lett. 106*, 176–180.
Hui, S. W., Stewart, T. P., Boni, L. T., & Yeagle, P. L. (1981) *Science (Washington, D.C.) 212*, 921–923.
Liao, M. J., & Prestegard, J. H. (1979a) *Biochim. Biophys. Acta 550*, 157–173.
Liao, M. J., & Prestegard, J. H. (1979b) *Biochem. Biophys. Res. Commun. 90*, 1274–1279.
Litman, B. J. (1974) *Biochemistry 13*, 2844–2848.
Maeda, T., & Ohnishi, S. I. (1974) *Biochem. Biophys. Res. Commun. 60*, 1509–1516.
Newton, C., Pangborn, W., Nir, S., & Papahadjopoulos, D. (1978) *Biochim. Biophys. Acta 506*, 281–287.
Pagano, R. E., Martin, O. C., Schroit, A. J., & Struck, D. K. (1981) *Biochemistry 20*, 4920–4927.
Papahadjopoulos, D., Vail, W. J., Jacobson, K., & Poste, G. (1975) *Biochim. Biophys. Acta 394*, 483–491.
Papahadjopoulos, D., Poste, G., & Vail, W. J. (1979) *Methods Membr. Biol. 10*, 1–121.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.
Poste, G., & Nicolson, G. L. (1978) *Cell Surf. Rev. 5*, 1–862.
Rand, R. P. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 227–314.
Rand, R. P., Reese, T. S., & Miller, R. G. (1981) *Nature (London) 293*, 237–238.
Seelig, J., & Gally, H. (1976) *Biochemistry 15*, 5199–5204.
Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.
Szoka, F., & Papahadjopoulos, D. (1980) *Annu. Rev. Biophys. Bioeng. 9*, 467–508.
Vanderwerf, P., & Ullman, E. F. (1980) *Biochim. Biophys. Acta 596*, 302–314.
van Dijck, P. W. M., de Kruijff, B., van Deenen, L. L. M., de Gier, J., & Demel, R. A. (1976) *Biochim. Biophys. Acta 455*, 576–587.
Verkleij, A. J., Mombers, C., Leunissen-Bijvelt, J., & Ververgaert, P. (1979) *Nature (London) 279*, 162–163.
Wilschut, J., Duzgunes, N., & Papahadjopoulos, D. (1981) *Biochemistry 20*, 3126–3133.

Effect of Lysine Modification on the Activity of the σ Subunit of *Escherichia coli* RNA Polymerase†

Chittampalli S. Narayanan and Joseph S. Krakow*

ABSTRACT: The function of lysyl residues of the σ subunit of the RNA polymerase from *Escherichia coli* was investigated by chemical modification with trinitrobenzenesulfonic acid (TNBS). Following reaction with TNBS, analysis of the modified σ indicated that trinitrophenylation was limited to the ϵ-amino groups of lysyl residues. Progressive loss in the activity of σ followed increasing trinitrophenylation as assayed by the ability to stimulate RNA polymerase core enzyme in a reaction directed by T7 DNA. Modification of five lysyl groups resulted in the complete loss of σ activity. Kinetic analysis indicated that one lysyl group is critical for the function of σ. TNP-σ was able to form a holoenzyme complex with a binding affinity comparable to that of σ. Promoter recognition studies were done by using *Hin*dIII fragments from T5 DNA. The TNP-σ core complex was unable to form a tight binary complex with the T5 promoters. Studies on RNA chain initiation were carried out by using $d(A\text{-}T)_n$ and T7 DNA templates. TNP-σ was unable to stimulate RNA chain initiation by core polymerase. Limited proteolytic digests of TNP-σ or σ using *Staphylococcus aureus* V8 protease were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The results suggested a change in the conformation of σ following trinitrophenylation.

The *Escherichia coli* DNA-dependent RNA polymerase consists of a catalytically competent core unit composed of three subunits (α_2, β, β') and a fourth dissociable subunit (σ). The presence of σ in the holoenzyme stimulates the overall rate of transcription by effecting promoter recognition and increasing the rate of RNA chain initiation. The effect of σ on DNA site selection could be due, in part, to a direct interaction of σ in the holoenzyme with the promoter (Simpson, 1979; Kudo & Doi, 1981). Alternatively, binding of σ may induce a specific conformation in the holoenzyme resulting in an increased affinity for promoter sites and a lowered affinity for DNA general sites (Wu et al., 1976). RNA chain initiation at nicks in double-stranded DNA seen with the core

† From the Department of Biological Sciences, Hunter College of the City University of New York, New York, New York 10021. *Received May 20, 1982.* This work was supported by a research grant from the National Institutes of Health (GM 18673).

0006-2960/82/0421-6103$01.25/0