Phillips, D. R., Jennings, L. K., & Edwards, H. H. (1980) *J. Biol. Chem. 86*, 77-86.
Rao, A. (1979) *J. Biol. Chem. 254*, 3503-3511.
Robey, F. A., Jamieson, G. A., & Hunt, J. B. (1979) *J. Biol. Chem. 254*, 1010-1012.
Rose, J. K., Adams, G. A., & Gallione, C. J. (1984) *Proc. Natl. Acad Sci. U.S.A. 81*, 2050-2054.
Rotman, A., Heldman, J., & Linder, S. (1982) *Biochemistry 21*, 1713-1719.
Scouten, W. H., Lubcher, R., & Baughman, W. (1973) *Biochim. Biophys. Acta 336*, 421-426.
Silk, S. T., Kenneth, T. H., & Marcus, A. J. (1981) *Biochemistry 20*, 391-397.
Smith, D. E., Mosher, D. F., Johnson, R. B., & Furcht, L. T. (1982) *J. Biol. Chem. 257*, 5831-5838.
Smith, D. K., & Palek, J. (1982) *Nature (London) 297*, 424-425.
Smith, D. K., & Palek, J. (1983) *Blood* 62, 1190-1196.
Solum, N. O., Olsen, T. M., Gogstad, G. O., Hagen, I., & Brosstad, F. (1983) *Biochim. Biophys. Acta 729*, 53-61.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237-248.
Wagner, D. D., & Hynes, R. O. (1980) *J. Biol. Chem. 255*, 4304-4312.
Weiss, H. J., Tschopp, T. B., Baumgartner, H. R., Sussman, I. I., Johnson, M. M., & Egan, J. J. (1974) *Am. J. Med. 57*, 920-925.
Zucker, M. B., Kim, S.-J., McPherson, J., & Grant, R. A. (1977) *Br. J. Haematol. 35*, 535-549.
Zucker, M. B., Varon, D., Masiello, N. C., & Karpatkin, S. (1983) *Thromb. Haemostasis 50*, 848-851.

Fusogenic Capacities of Divalent Cations and Effect of Liposome Size†

Joe Bentz*,‡ and Nejat Düzgüneş§

Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, and Cancer Research Institute, School of Medicine, University of California, San Francisco, California 94143

Received January 4, 1985

ABSTRACT: The initial kinetics of divalent cation (Ca^{2+}, Ba^{2+}, Sr^{2+}) induced fusion of phosphatidylserine (PS) liposomes, LUV, is examined to obtain the fusion rate constant, f_{11}, for two apposed liposomes as a function of bound divalent cation. The aggregation of dimers is rendered very rapid by having Mg^{2+} in the electrolyte, so that their subsequent fusion is rate limiting to the overall reaction. In this way the fusion kinetics are observed directly. The bound Mg^{2+}, which by itself is unable to induce the PS LUV to fuse, is shown to affect only the aggregation kinetics when the other divalent cations are present. There is a threshold amount of bound divalent cation below which the fusion rate constant f_{11} is small and above which it rapidly increases with bound divalent cation. These threshold amounts increase in the sequence $Ca^{2+} < Ba^{2+} < Sr^{2+}$, which is the same as found previously for sonicated PS liposomes, SUV. While Mg^{2+} cannot induce fusion of the LUV and much more bound Sr^{2+} is required to reach the fusion threshold, for Ca^{2+} and Ba^{2+} the threshold is the same for PS SUV and LUV. The fusion rate constant for PS liposomes clearly depends upon the amount and identity of bound divalent cation and the size of the liposomes. However, for Ca^{2+} and Ba^{2+}, this size dependence manifests itself only in the rate of increase of f_{11} with bound divalent cation, rather than in any greater intrinsic instability of the PS SUV. The destabilization of PS LUV by Mn^{2+} and Ni^{2+} is shown to be qualitatively distinct from that induced by the alkaline earth metals.

Recent studies on the fusion of phosphatidylserine (PS)[1] liposomes induced by divalent cations have elucidated the basic criteria that any proposed molecular mechanism must satisfy [for reviews, see Nir et al. (1983a) and Düzgüneş & Papahadjopoulos (1983)]. Basic to any study of liposome fusion is recognizing that the first step of the overall fusion process is given by the mass action reaction:

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \xrightarrow{f_{11}} F_2 \qquad (1)$$

where V_1 denotes the liposome, V_2 denotes the dimer aggregate, and F_2 denotes the fused doublet, i.e., when the bilayers have merged and the encapsulated contents of the liposomes have mixed.

Thus, to evaluate the effect of environmental parameters on the fusion rate constant, f_{11}, it is necessary to monitor fusion at early times so that higher order aggregates do not interfere and, of equal importance, to know that the rate-limiting step for the overall reaction is the fusion reaction itself. Recently, we have established the theoretical and experimental methodology necessary to obtain these rate constants (Wilschut et al., 1980, 1981; Nir et al., 1980b, 1982, 1983b; Bentz et al., 1983a,b, 1985). However, we have also developed a simple method for accelerating the aggregation rate and slowing down the fusion rate, so that we can observe the value of f_{11} directly. In Bentz et al. (1983b), we used PS SUV with high Na^+ and Li^+ concentrations (300-500 mM) to accelerate aggregation (Bentz & Nir, 1981a,b; Nir et al., 1981) and to keep the amounts of bound divalent cation low by direct competition of the divalent and monovalent cations for binding to the PS

† This investigation was supported by Research Grants GM-31506 (J.B.) and GM-28117 (N.D.) from the National Institutes of Health and a Grant-in-Aid from the American Heart Association with funds contributed in part by the California Affiliate (N.D.).

‡ Departments of Pharmacy and Pharmaceutical Chemistry.
§ Department of Pharmaceutical Chemistry and Cancer Research Institute.

[1] Abbreviations: DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetate; CF, carboxyfluorescein; LUV, large unilamellar vesicle, diameter ~150 nm; MLV, multilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SUV, small unilamellar vesicle, diameter ~30 nm.

0006-2960/85/0424-5436$01.50/0

head groups (Nir et al., 1978, 1983b; Newton et al., 1978; Portis et al. 1979; Düzgüneş et al., 1981a; McLaughlin et al., 1981; Ohki & Kurland, 1981). We found that f_{11} increases sharply with bound divalent cation once the amount exceeds a threshold level specific for each cation. For a given amount of bound divalent cation, the value of f_{11} decreases in the sequence $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$, which is defined as the fusogenic capacity of the divalent cations.

These results established that any molecular mechanism for fusion of SUV (PS) must account for the intrinsic differences in the capacities of the divalent cations to induce fusion, i.e., the amounts of bound divalent cation necessary for fusion to occur. This value was found to be ~0.15 Ca(bound) per PS when the fusion step was rate limiting, e.g., in the presence of 500 mM Na^+. With 20–100 mM Na^+, however, 0.3–0.4 Ca(bound) per PS was necessary to observe fusion, since the liposomes could not aggregate at lower Ca(bound) per PS values; i.e., C_{11} was too small (Bentz et al., 1983a,b).

In this study we have discovered how the ability of divalent cations (Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mg^{2+}) to induce the fusion of PS liposomes depends upon the size of the liposomes. Specifically, we have determined that the value of the fusion rate constant, f_{11}, (see eq 1) depends upon the amount and type of divalent cation bound to the PS head groups. These data have been compared with similar studies using PS SUV (Bentz et al., 1983b), thereby providing the liposome radial dependence of f_{11}. For both PS LUV and SUV, the fusogenic capacities of the divalent cations, i.e., the value of f_{11} for a given amount of bound divalent cation, decrease in the sequence $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$. In fact, Mg^{2+} cannot fuse the LUV, and Sr^{2+} has lost much of its fusogenic capacity relative to the SUV. On the other hand, at the threshold for fusion, the fusogenic capacities for Ca^{2+} and Ba^{2+} are the same for SUV and LUV. For these cations, the radial dependence of f_{11} is manifested as the more rapid rate of increase of f_{11} vs. bound divalent cation for the SUV. Finally, the transition metal divalent cations Mn^{2+} and Ni^{2+} are shown to exhibit different interactions with the PS LUV relative to the alkaline earth metals. A preliminary report of our results has been published (Düzgüneş et al., 1983a).

Materials and Methods

Phosphatidylserine from bovine brain was purchased from Avanti Polar Lipids (Birmingham, AL) and stored as a chloroform solution under argon at −40 °C. $TbCl_3$ was obtained from Alfa (Danvers, MA); dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid (TES), L-histidine, and LiCl were from Sigma; NaCl was from Mallinckrodt (Paris, KY); $CaCl_2$, $BaCl_2$, $SrCl_2$, $NiCl_2$, $MnCl_2$, and $MgCl_2$ were from Fisher. Cholate (Calbiochem) was recrystallized twice. Carboxyfluorescein (CF; Eastman Kodak, Rochester, NY) was purified by chromatography on Sephadex LH-20 (Ralston et al., 1981). Water was distilled twice, the second time in an all-glass apparatus, and further purified in a Barnstead Nanopure filtration system.

Large unilamellar liposomes were prepared by reverse-phase evaporation as described previously (Wilschut et al., 1980; Düzgüneş et al., 1983b) and contained either (i) 2.5 mM $TbCl_3$ and 50 mM sodium citrate, (ii) 50 mM DPA (sodium salt) and 20 mM NaCl, or (iii) 1.25 mM $TbCl_3$, 25 mM sodium citrate, 25 mM DPA, and 10 mM NaCl. All solutions were buffered with 2 mM TES and 2 mM L-histidine, pH 7.4. Unencapsulated material was eliminated by gel filtration on Sephadex G-75 (Pharmacia) with 100 mM NaCl, 2 mM TES, 2 mM L-histidine, pH 7.4 (NaCl buffer), and 1 mM ethylenediaminetetraacetate (EDTA) as elution buffer. For calibration of the fusion assay, a portion of the Tb–liposomes (type i above) was passed through another Sephadex G-75 column equilibrated with NaCl buffer (containing no EDTA). Liposome concentrations were determined by phosphate analysis (Bartlett, 1959).

The Tb fluorescence scale was calibrated by lysing 25 μM of the Tb–liposomes (freed of EDTA) with 0.5% (w/v) sodium cholate in the presence of 20 μM free DPA and sonicating for 5 min under argon in a bath-type sonicator. The fluorescence value obtained was set to 100%. Fluorescence and 90° light scattering measurements were made in an SLM-4000 fluorometer, which allows simultaneous monitoring of fluorescence (excitation at 276 nm and emission at 545 nm, with a Corning 3-68 cut-off filter to eliminate contribution to the signal from light scattering, which was always less than 1.5% of the maximal Tb fluorescence intensity) and light scattering (using a Corning 7-54 band-pass filter). The output of the fluorometer was recorded on an Omniscribe chart recorder, at fast chart speeds when necessary. Additional details of the fusion assay have been described elsewhere (Wilschut et al., 1980, 1983; Düzgüneş et al., 1981b; Bentz et al., 1983b, 1985).

The chelation of Tb by DPA is prevented outside the liposomes by the presence of the divalent cations and EDTA. Ba^{2+} and Sr^{2+} are very effective in quenching Tb fluorescence in the presence of 0.1 mM EDTA (Bentz et al., 1983b). The Tb/DPA complex that forms during the fusion of Tb– and DPA–liposomes will then be completely dissociated if it is released into the medium containing divalent cations and EDTA or if the medium enters the liposome interior. The dissociation was measured directly by encapsulating the Tb/DPA complex and following the decrease in fluorescence (initially set at 100%) when fusion was induced by divalent cations.

The *fusion experiment* starts with 25 μM each of the Tb–liposomes (type i above) and DPA–liposomes (type ii above) and measures the fluorescence kinetics of mixing of the vesicles' contents during fusion. The *dissociation experiment* starts with 50 μM Tb/DPA–liposomes (type iii above) and measures the kinetics of the dissociation of the Tb/DPA complex due to its leakage and the influx of divalent cations and EDTA into the liposomes during fusion. In fact, the fluorescence intensity measured at *any* time (either in the fusion experiment or the dissociation experiment) equals the percentage of the total amount of Tb that is complexed with DPA at that time. With both types of experiments, 0.1 mL of the liposome stock (0.5 μmol of lipid/mL) was suspended into 0.9 mL of the NaCl buffer (0.1 mM final EDTA concentration) in a quartz cuvette of 1-cm path length and stirred continuously at 25 °C. Divalent cations were introduced by adding an aliquot of a concentrated solution with a Hamilton syringe.

Release of liposome contents into the medium was measured by the relief of self-quenching of CF (Weinstein et al., 1977; Portis et al., 1979) encapsulated at a concentration of 50 mM (with 2 mM TES and 2 mM L-histidine). CF fluorescence was detected at wavelengths above 530 nm by means of the 3-68 filter, with an excitation wavelength of 430 nm.

Results

It is known that the alkaline earth metals Ba^{2+}, Ca^{2+}, and Sr^{2+} will induce the fusion of PS LUV (Düzgüneş et al., 1984; Bentz et al., 1985), while Mg^{2+} will induce only aggregation, at least below 30 °C (Wilschut et al., 1981, 1985). In this study we have used Mg^{2+} to accelerate the rate of aggregation so as to observe the effect of the other divalent cations on the fusion reaction per se. In Figure 1 we show the 90° light

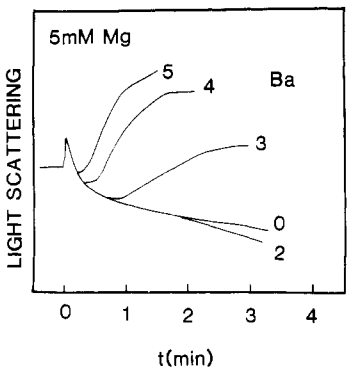

FIGURE 1: Aggregation over time of the PS LUV in 100 mM Na^+, 5 mM Mg^{2+}, and the indicated concentration of Ba^{2+} (mM) is shown by 90° light scattering (276 nm). Before addition of Ba^{2+} and Mg^{2+} (at $t = 0$), the vesicles are dispersed, and the light scattering intensity is normalized to 1. The light scattered or absorbed by the salt solutions alone is negligible on the scale shown.

scattering signal for PS LUV in 100 mM Na^+ buffer, 5 mM Mg^{2+}, and various Ba^{2+} concentrations, noted as millimolar values at the side of each curve. It is clear that 5 mM Mg^{2+} alone, i.e., 0 mM Ba^{2+}, will induce substantial aggregation and even including 2 mM Ba^{2+} does not change the light scattering signal, up to 2 min. Higher Ba^{2+} concentrations cause the light scattering signal to increase after shorter periods of time. This is indicative of the collapse of the fusing liposomes (Wilschut et al., 1980; Düzgüneş et al., 1981b). For all experiments described here, the divalent cations are injected into the stirred cuvette containing the liposomes in 100 mM Na^+ buffer. The cause of the initial increase in scattered light intensity is not known, although changes in lipid refractive index following cation binding are the most likely source.

Figure 2 shows the fusion (denoted F) and dissociation (denoted D) signals for various Ca^{2+}, Ba^{2+}, and Sr^{2+} concentrations in the presence of 5 (upper set) and 10 mM Mg^{2+} (lower set). The fusion signal equals the percent of encapsulated Tb^{3+} that has been chelated by DPA within the fused liposomes. The dissociation signal equals the fraction of preencapsulated Tb/DPA complex that has been dissociated due to EDTA and divalent cation interaction, which arises either because the Tb/DPA complex leaks into the medium or because the medium enters the Tb/DPA-containing liposomes during fusion. The curves denoted F_c are the sum of the fusion curve plus half the dissociation curve[2] ($F_c = F + 0.5D$). As explained before, this corrected curve initially equals the total amount of aqueous contents mixing that would be observed if there were no dissociation of Tb/DPA complex due to either leakage or influx of medium (Bentz et al., 1983a,b, 1985).

The fusion curve for 5 mM Mg^{2+} plus 4 mM Ba^{2+} (Figure 2) reaches a maximum at about 30 s. This is the time point after which the dissociation of Tb/DPA complex formed is greater than the additional formation of Tb/DPA complex due to the continued fusion of the liposomes. Roughly speaking, one can take the maximum of the fusion curve to demark the end of the initial phase of liposome fusion, after which the

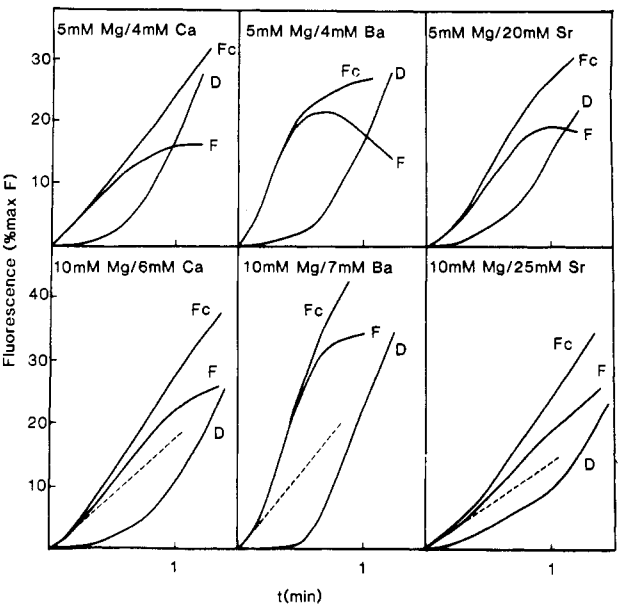

FIGURE 2: Fusion of PS LUV induced by each of the divalent cations in the presence of 100 mM Na^+ and the indicated Mg^{2+} concentration, either 5 or 10 mM. The fusion curve F is obtained from the mixing of Tb and DPA from separate PS LUV populations (25 μM each) and shows the percentage of encapsulated Tb that is complexed to DPA. The dissociation curve D is obtained from 50 μM of PS LUV containing the Tb/DPA complex and shows the percentage of Tb/DPA complex that has dissociated due to the leakage from and entry of medium into the liposomes. The contribution to the observed fluorescence levels from scattered light is always less than 1.5%. See Materials and Methods for further details. The line denoted F_c is the fusion corrected for the loss of fluorescence due to dissociation as estimated by the sum of $F + 0.5D$ (see footnote 2). The dashed line (--) gives the initial slope of the fusion curves.

formation of higher order fusion products and their collapse (Bentz et al., 1985) dominate the observed fluorescence curves (see Figure 1 for the correlation with the increase in scattered light intensity).

The most compelling evidence that the overall kinetics are rate limited by the fusion step under these ionic conditions is obtained by comparing the curves for each divalent cation in 5 vs. 10 mM Mg^{2+}. For example, the curves for 4 mM Ca^{2+} and 5 mM Mg^{2+} initially overlay the curves for 6 mM Ca^{2+} and 10 Mg^{2+}. Below, we will see that the amount of Ca bound per PS head group, calculated from binding constants, is essentially the same in both cases, although there is more Mg^{2+} bound in the latter case. If aggregation were involved in the observed kinetics, then substantially faster fusion should be seen for the larger Ca^{2+} and Mg^{2+} concentrations, since the rate of aggregation increases with divalent cation concentration (Nir & Bentz, 1978; Nir et al., 1980a; Bentz & Nir, 1981a,b; Ohki et al., 1982). Likewise, if Mg^{2+} were contributing directly to the fusion reaction, then again the curves with higher Mg^{2+} concentrations should be much faster. Hence, initially the bound Mg^{2+}, like the bound Na^+, affects the fusion rate constant only via competing with the other divalent cations for binding. On the other hand, over time it is clear that the upper and lower curves diverge. The importance of focusing on the initial events is evident. The later differences mostly arise from large aggregates, and their relevance to the mechanism of fusion between two liposomes is questionable.

As described in the introduction, we are concerned with the initial events of the dimerization and fusion of two liposomes. Figure 2 clearly shows that the initial slope of the fusion curve gives the initial kinetics of this event, since the dissociation curve is insignificant initially. The dashed lines on the indi-

[2] More refined corrections are made when the fusion kinetics are analyzed (Nir et al., 1980b; Bentz et al., 1983a, 1985). When we denote the fusion signal corrected for complex dissociation by F_c, then the observed fusion signal, F, and the observed dissociation symbol, D, rigorously yield $F_c = F + \delta D$, where the value δ depends upon the primary rate constants. Initially, $\delta = 0.5$ as explained in Bentz et al. (1983b) and in time $0.5 < \delta < 1.0$. However, for all practical purposes, since we only deal with the initial kinetics, taking $\delta = 0.5$ does not entail any serious error.

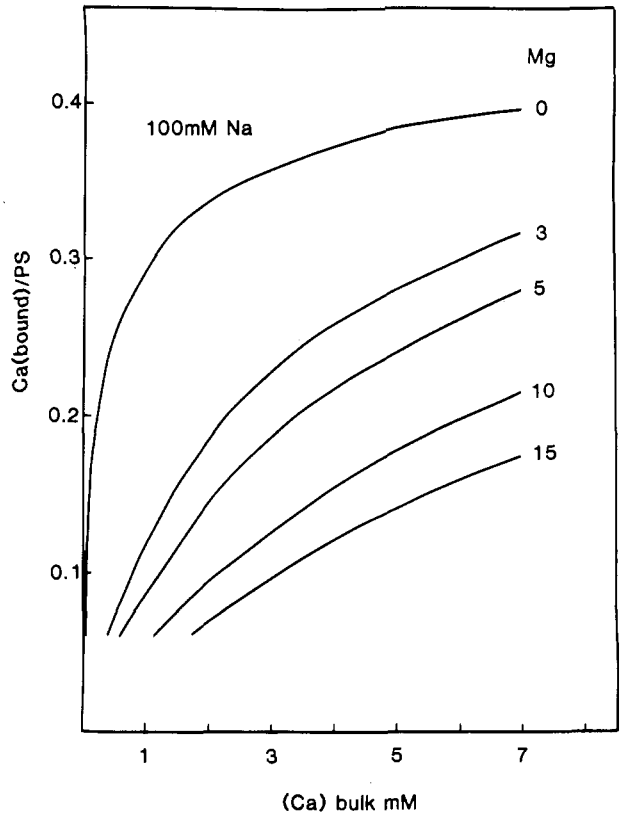

FIGURE 3: Calculated amount of Ca bound per PS is shown as a function of the bulk Ca^{2+} concentration with 100 mM Na^+ and the indicated bulk concentrations of Mg^{2+}. The calculations pertain to isolated PS LUV, i.e., before aggregation and fusion have begun. The binding constants of McLaughlin et al. (1981) were used: $K(Na) = 0.6\ M^{-1}$, $K(Mg) = 8\ M^{-1}$, and $K(Ca) = 12\ M^{-1}$ with Ca binding to a single PS head group. The other details of the calculations are described in footnote 3. The reduced Ca binding with increasing Mg^{2+} concentrations is due to the direct binding competition between Mg^{2+} and Ca^{2+} and to the reduction of the Ca^{2+} concentration near the liposomes' surface due to the increased ionic strength that reduces the surface potential of the liposome (Bentz, 1981).

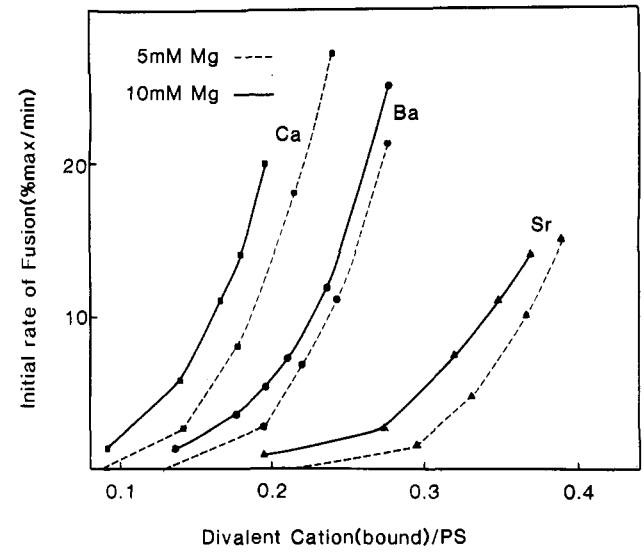

FIGURE 4: Initial rates of fusion (% max F/min, from the initial slope of the F curves as in Figure 2) are plotted against the bound divalent cation per PS head group for Ca^{2+} (■), Ba^{2+} (●), and Sr^{2+} (▲) with 100 mM Na^+ and either 5 mM (--) or 10 mM Mg^{2+} (—) in the medium, as indicated. In all cases, at the concentrations where the curves intersect the ordinate (zero initial fusion), there is substantial aggregation and no measurable fusion or dissociation (<1% max F or D) for at least 2 min. The calculated amounts of divalent cation bound per PS for LUV are calculated as described in Figure 3. The binding constants of McLaughlin et al. (1981) are used: $K(Ba) = 20\ M^{-1}$, $K(Sr) = 14\ M^{-1}$, $K(Ca) = 12\ M^{-1}$, and $K(Mg) = 8\ M^{-1}$. These binding constants were obtained by assuming that each of the cations can bind only to a single PS head group. Likewise, these calculations pertain only to the liposomes before aggregation and fusion have begun (see footnote 3).

vidual figures show the initial slopes of these fusion curves. As explained in Bentz et al. (1983b), under conditions of rapid aggregation, where the fusion reaction per se is rate limiting to the overall process, the initial slope of the fusion curve is directly related to the fusion rate constant f_{11} (see eq 1). For the PS LUV, initial slopes are always somewhat less than the maximum slopes of each curve, which is not the case with PS SUV (Bentz et al., 1983b) and may be related to the size distribution of the LUV. That is, the larger of the LUV may have significantly smaller fusion rate constants than the smaller of the LUV (Bentz et al., 1985). This is likely not the case with the SUV. Using the initial rates eliminates the contribution of the dissociation of the complex and is consistent with our stress on the importance of the initial events in studying fusion. However, the conclusions we reach below would also follow if maximum rates were used.

We now relate these initial fusion rates to the amount of bound divalent cation. Using cation binding constants to PS in liposomes, obtained with a variety of methods,[3] we solve the Poisson–Boltzmann equation for spherical surfaces and calculate the amounts of bound cations (Bentz, 1981, 1982). Once the liposomes begin to aggregate and fuse, the amounts of bound cations will change due to close apposition of charged surfaces (Bentz, 1982) and, more important, the emergence of new binding complexes (Portis et al., 1979; Rehfeld et al., 1981; Ekerdt & Papahadjopoulos, 1982; Bentz et al., 1983b; Nir, 1984).

In Figure 3 we show the calculated amounts of Ca^{2+} bound per PS head group on the exterior surface (since the calculation assumes no aggregation of fusion) vs. the bulk Ca^{2+} concentration. The remaining electrolyte contains 100 mM Na^+ and varying concentrations of Mg^{2+}, as denoted to the right of each curve. For our calculations, we use the binding constants obtained by McLaughlin et al. (1981), which assumes a 1:1 binding complex between the PS head group and the divalent cation. In Bentz et al. (1983b), we showed that other studies (Portis et al., 1979; Nir et al., 1978; Ohki & Kurland, 1981; Düzgüneş et al., 1981a) that assume a 2:1 binding complex (i.e., the divalent cation binding to two adjacent head groups) give essentially identical predictions for the amounts of bound divalent cation. Our calculations also assume that all of the cations compete for the same binding site on the PS head group. If Na^+ and Ca^+ bind to different sites, the calculated amounts of bound Ca^{2+} for the experimentally relevant electrolyte concentrations are not affected appreciably (Bentz et al., 1983b).

The amount of bound Mg^{2+} per PS head group, denoted RB(Mg), can be easily obtained from the amount of bound Ca, RB(Ca), from (Bentz & Nir, 1980)

$$RB(Mg) = \frac{K(Mg)[Mg^{2+}]}{K(Ca)[Ca^{2+}]}RB(Ca) \qquad (2)$$

[3] The values of bound cation per PS are obtained from the binding constants and equations described in Bentz (1981, 1982) and Bentz et al. (1983b). The most important point to recognize for these calculations is that they are valid only for isolated (unaggregated) liposomes. When the liposomes aggregate, the amount of bound divalent cation will increase by 20–30% simply due to the close approach of the charged surfaces (Bentz, 1982). In addition, there is substantial evidence that during and/or after fusion there is a new, stronger binding complex between (at least) Ca^{2+} and PS (Ekerdt & Papahadjopoulos, 1982; Nir, 1984).

Table I: Divalent Cation Concentrations and Divalent Cation Bound per PS at an Initial Fusion Rate of 10% Maximum Fluorescence per Minute for PS LUV and SUV[a]

	(A) PS LUV Mg^{2+} concn (mM)			
	3	5	10	15
bulk concn (mM)				
Ca	3.0	3.5	4.3	5.0
Ba	2.25	2.75	4.5	
Sr	13.0	15.0	17.3	
bound per PS				
Ca	0.23	0.20	0.16	0.15
Ba	0.26	0.23	0.23	
Sr	0.38	0.37	0.34	

	(B) PS SUV 300 mM		500 mM	
	Na^+	Li^+	Na^+	Li^+
bound per PS				
Ca	0.22	0.20	0.16	0.16
Ba	0.25	0.23	0.21	0.20
Sr	0.28	0.27	0.24	0.22
Mg	0.31	0.29	0.26	0.24

[a] The bulk concentration of divalent cation at the fusion threshold (defined as an initial rate of fusion of 10% max F/min) is obtained directly from Figure 3. The data for the PS SUV are taken from Bentz et al. (1983b). The calculated amounts of divalent cation bound per PS are obtained from the binding constants of Eisenberg et al. (1979) and McLaughlin et al. (1981) as described in Bentz et al. (1983b). The experimental uncertainties in the values of these binding constants produce an uncertainty of about ±0.01 in these calculated values. These values pertain only to the vesicles before the fusion process begins; see footnote 3.

where $[Mg^{2+}]$ and $[Ca^{2+}]$ are the bulk electrolyte concentrations. $K(Mg) = 8\ M^{-1}$ and $K(Ca) = 12\ M^{-1}$ are the 1:1 binding constants (McLaughlin et al., 1981; see the legend to Figure 4 for the other divalent cation binding constants). For the previously cited case of 5 mM Mg^{2+} and 4 mM Ca^{2+}, RB(Ca) = 0.22, and by eq (2) RB(Mg) = 0.18.

In Figure 4, we combine these data to show the initial rate of fusion, taken from curves like those shown in Figure 2, as a function of the amount of bound divalent cation, calculated as described in Figure 3. There are curves for Ca^{2+}, Ba^{2+}, and Sr^{2+} in the presence of 5 and 10 mM Mg^{2+}. Just as we found for PS SUV (Bentz et al., 1983b), the order in fusogenic capacities of the cations is $Ca^{2+} > Ba^{2+} > Sr^{2+}$; i.e., for a fixed amount of bound divalent cation, the fusion rate constant is greatest for Ca^{2+}, followed by Ba^{2+}, and then Sr^{2+}. For the PS SUV, Mg^{2+} fell behind Sr^{2+}. The error bars on the initial rates are ~1%/min; hence, for Ca^{2+} and Sr^{2+} there is somewhat faster fusion with 10 mM Mg^{2+} for the same amount of bound divalent cation, whereas for Ba^{2+} the curves are essentially identical.

In all cases it is clear how the fusion rate constant increases rapidly with bound divalent cation once a threshold value is exceeded. As a matter of convenience, we previously defined the value of 10% max F/min to demark this kinetic threshold (Bentz et al., 1983b). This value is a compromise between having enough fusion to reliably measure and still being able to examine the threshold for fusion. In Table I, we have assembled these data and that previously reported for PS SUV (Bentz et al., 1983b) to show how the fusion threshold depends on cations and liposome size. Part A of the table contains the data for the PS LUV described here, and part B gives the bound amounts of divalent cations at the fusion threshold for PS SUV in 300 and 500 mM Na^+ and Li^+, i.e., the monovalent cation concentrations used to effect rapid aggregation of the PS SUV.

For the PS LUV, as the Mg^{2+} concentrations are increased, the threshold value of bound divalent cation decreases to some minimum value, e.g., 0.23 Ba(bound) per PS head group. With enough Mg^{2+} present, the fusion reaction is completely rate limiting to the overall process. The same behavior is noted for PS SUV with increasing Na^+ or Li^+ concentrations. It is worth noting here that high Na^+ or Li^+ concentrations were not used to aggregate the PS LUV because of unknown contributions from osmotic stress (Cohen et al., 1982; Ohki, 1984). Likewise, Mg^{2+} could not be used with PS SUV since it fuses these liposomes by itself.

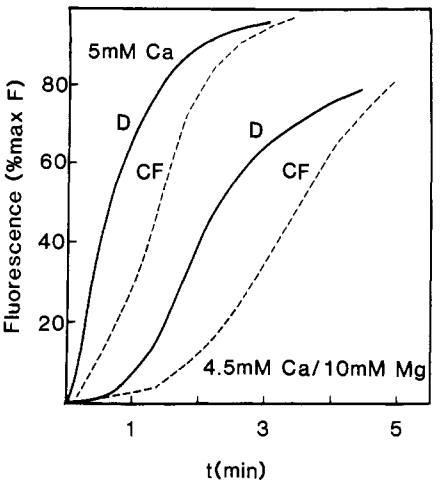

FIGURE 5: Destabilization of PS LUV by 5 mM Ca^{2+} (left pair of curves) and by 4.5 mM Ca^{2+} plus 10 mM Mg^{2+} (right pair of curves) is monitored by dissociation of preencapsulated Tb/DPA complex (D, —) or the relief of self-quenching of carboxyfluorescein (CF, --) due to leakage. The difference between dissociation and carboxyfluorescein leakage, i.e., D − CF, gives a measure of the influx of medium into the fusing liposomes.

When we compare the threshold values of bound divalent cation for PS LUV in 10 mM Mg^{2+} and for PS SUV in 500 mM Na^+ or Li^+, which are the minimal values, we find a startling result. For Ca^{2+}, the threshold is the same, ~0.16 Ca(bound) per PS, and for Ba^{2+}, the threshold for the LUV is only slightly higher. For Ca^{2+} and Ba^{2+}, the fusion *threshold* is not sensitive to liposome size. For Sr^{2+}, there has been a substantial loss of fusogenic capacity with the LUV, and Mg^{2+} has lost all of its fusogenic capacity. We will return to this issue under Discussion.

Since the relationship between f_{11} and bound divalent cation is the issue of this and previous works, we wanted to expand the divalent cations studied to include some transition metals, notably Ni^{2+} and Mn^{2+}, which are known to bind to PS liposomes (McLaughlin et al., 1981). The result of this study was that there is a substantial difference between the way in which the alkaline earth metals (Ca^{2+}, Ba^{2+}, Mg^{2+}, and Sr^{2+}) interact with PS and the interactions of these transition metals.

By way of identifying this difference, we show in Figure 5 both the dissociation of the preencapsulated Tb/DPA complex and the leakage of CF, encapsulated at a self-quenching concentration. The experiment was performed in 5 mM Ca^{2+}, the two curves on the left, and in 4.5 mM Ca^{2+} plus 10 mM Mg^{2+}, the two curves on the right. It is clear that dissociation is more rapid than CF release in both electrolytes. This makes sense in that the dissociation reaction depends upon leakage and the influx of medium. The biggest difference between the two assays is early on, indicating that initially dissociation is due to the influx of medium followed, somewhat later, by collapse of the fused structures (Bentz et al., 1985). If two fused liposomes relax back into a spherical shape, then the encapsulated volume is 50% greater than the sum of the en-

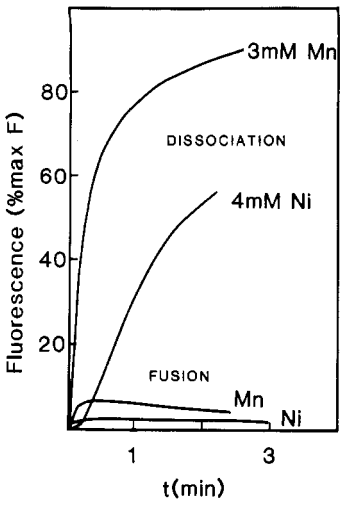

FIGURE 6: Effect of transition metals Mn^{2+} (3 mM) and Ni^{2+} (4 mM), with 100 mM Na^+ buffer, on the Tb/DPA fusion signal (lower pair of curves) and the dissociation signal (upper pair of curves) is shown. It is evident that both cations readily dissociate the Tb/DPA complex. The very small fusion signals imply either that there is very little mixing of contents or that a rapid influx of Ni^{2+} of Mn^{2+} into the fusing liposomes prevents the Tb/DPA chelation.

capsulated volumes of the two primary liposomes, assuming an initial exterior radius of 500 Å and a bilayer thickness of 50 Å. Thus, an initial phase of influx of divalent cations and EDTA in the medium causing Tb/DPA dissociation is easily understood.

In Figure 6, we show the fusion and dissociation curves for PS LUV in the presence of 3 mM Mn^{2+} and of 4 mM Ni^{2+}. The fusion signals are essentially nonexistent. With Mn^{2+} the dissociation signal is faster than that for 5 mM Ca^{2+}, whereas, with 4 mM Ni^{2+}, the dissociation is slower than that for 5 mM Ca^{2+} (see Figure 5). Having Mg^{2+} also in the medium gave qualitatively the same results. It is clear that these transition metal cations can destabilize the liposomes, but the events following destablization are distinct from those produced by the alkaline earth metals.

We can envisage two possible explanations for these data. First, Mn^{2+} and Ni^{2+} induce a very rapid collapse of the liposomes that prevents the formation of the Tb/DPA complex within the liposomes. However, with Ni^{2+} the dissociation signal is slower than that seen with 5 mM Ca^{2+}, which gives a substantial fusion signal. The second explanation is that these transition metal cations do not promote resealing of the destabilized bilayers, enhancing the influx of medium into the liposomes, thereby preventing the formation of the Tb/DPA complex. Establishing whether Ni^{2+} and Mn^{2+} promote fusion with the mixing of aqueous contents will require the use of another fusion assay that is not influenced by the influx of these ions.

Discussion

The basic conclusions to be drawn from this work are straightforward. Amongst the other factors that determine the fusion rate constant for PS liposomes, e.g., temperature (Bentz et al., 1985; Ohki, 1984; Wilschut et al., 1985), the amount of bound divalent cation and the radius of the liposomes are of prime importance. Previous studies have shown that when the amounts of bound divalent cation are large, i.e., ~0.35–0.40 for Ca^{2+}, which occur in 100 mM Na^+ and >2 mM Ca^{2+} (see Figure 3), then the fusion rate constant for the SUV is 10–100 times larger than that for the LUV (Nir et al., 1982; Bentz et al., 1983a, 1985). These results and other more qualitative studies (Wilschut et al., 1980, 1981, 1985; Ohki, 1984) revealed that the high curvature of the SUV made them more fusogenic. A conclusion similar to that was found for pure PC liposomes (Lichtenberg et al., 1981; Wong et al., 1982).

Now we find that the story is far more interesting for the PS liposomes, because at the threshold for fusion, where f_{11} begins its rapid increase as a function of bound divalent cation, each divalent cation provides a unique radial dependence (Table I). Somewhere between SUV and these LUV, i.e., average diameters of 30–150 nm, Mg^{2+} loses its fusogenic capacity completely (although a slight recovery is possible above 40 °C; J. Wilschut, N. Düzgüneş, and D. Papahadjopoulos, unpublished data), and Sr^{2+} has lost much of its fusogenic capacity. On the other hand, Ca^{2+} and Ba^{2+} are essentially insensitive to liposome size—at and near the threshold. Ohki (1984) has observed Ca^{2+}-induced fusion of very large PS liposomes, ~1000-nm average diameter. It would be interesting to extend these studies to liposomes of such large size to determine if Ca^{2+} and Ba^{2+} eventually lose some of their fusogenic capacity.

We have emphasized here and elsewhere (Bentz et al., 1983b, 1985) the importance of knowing whether the overall fusion kinetics are rate limited by the aggregation step or the fusion step. The parameter $K = f_{11}/(C_{11}X_0)$, where X_0 is the liposome concentration, prescribes which step is rate limiting. When $K > 100$, then the aggregation step is essentially completely rate limiting, and when $K < 1$, the fusion step is rate limiting (Bentz et al., 1983a). For intermediate values of K, both steps contribute to the overall kinetics. Under aggregation rate-limiting conditions (100 mM Na^+ buffer and 1–5 mM divalent cation), we have found that the fusion signal is greater for Ba^{2+} than for Ca^{2+}, but this is because Ba^{2+} has a greater binding constant to PS than Ca^{2+} and so Ba^{2+} could induce more rapid aggregation (Bentz et al. 1985). This, of course, says nothing about the mechanism of fusion.

Knowing the fusogenic sequence of the divalent cations can explain much about how the addition of other lipids to PS affects the aggregation and/or fusion rate constants. For example, we can deduce that adding PC to PS liposomes decreases the fusion rate constant f_{11}. When PS/PC (3:1) LUV are mixed with these divalent cations in 100 mM Na^+ buffer, the overall fusion kinetics decrease in the sequence $Ca^{2+} > Ba^{2+} \gg Sr^{2+}$ (Düzgüneş et al., 1983a), and this is in terms of bulk concentrations. For pure PS, the value of K is large, and the observed kinetics reflect only the values of the aggregation rate constants. Adding PC to the bilayers, under otherwise identical conditions, obviously *decreases* the value of K until the divalent cation fusogenic capacities emerge. A decrease in the value of K, for identical liposome concentrations, requires that f_{11} decreases and/or C_{11} increases. It is very unlikely that adding PC to the PS would increase C_{11} substantially; therefore, it must be that the addition of PC strongly reduces the fusion rate constant, f_{11}. This is quite consistent with the observation that PS/PC (1:1) LUV aggregate but do not fuse on a time scale of minutes in the presence of Ca^{2+} (Düzgüneş et al., 1981b) and that pure PC SUV require days to weeks to show significant membrane fusion (Wong et al., 1982).

The divalent transition metals Mn^{2+} and Ni^{2+} interact with PS in a fundamentally different way than the alkaline earth metals. They have large binding constants to isolated PS liposomes (McLaughlin et al., 1981). But rather large concentrations of these metals are required to induce aggregation and destabilization of the PS liposomes. Even if Mn^{2+} and Ni^{2+} are promoting mixing of aqueous contents, but are de-

feating the Tb/DPA assay by a rapid influx of ions, it is clear that this influx is very rapid compared with that found for Ca^{2+}. It is possible that these transition metal ions are capable of forming an intramolecular chelation complex between the amine (with the release of a proton) and carboxyl groups of PS (Hendrickson & Fullington, 1965). It is unlikely that the alkaline earth metals could form complexes of this type (Sigel & Martin, 1982). Differences in the structure of the divalent cation–PS binding complex of this type are likely to substantially alter the subsequent destabilization of the liposomes.

The results of this study, together with previous work (Bentz et al., 1983a, 1985; Nir et al., 1983b), have established that the fusion rate constant, f_{11}, for PS liposomes is a smooth increasing function of the amount of bound divalent cation, temperature, and decreasing liposome size. This curve is shifted according to the fusogenic capacity of the various divalent cations. It is thought that membrane fusion proceeds via point defects in molecular packing (Papahadjopoulos et al., 1977; Hui et al., 1981; Düzgüneş et al., 1984). It now seems evident that the mechanism for fusion involves a critical density of bound divalent cation within the region of close apposition where the initial defect will form. Clearly, the defect area is within the area of close apposition; otherwise, no intermixing of aqueous contents would be observed. The value of this critical density may lie anywhere between the threshold values found here, e.g., 0.15 Ca^{2+} per PS or 0.2 Ba^{2+} per PS, up to the equilibrium (saturation) values found in *cochleates*, e.g., 0.5 Ca^{2+} per PS (Newton et al., 1978; Portis et al., 1979; Ekerdt & Papahadjopoulos, 1982). In any event, if an accumulation of bound divalent cation into the contact area is required to achieve the critical density, then the kinetics of this lateral movement or phase separation would become part of the fusion rate constant. Furthermore, the initial condition for this lateral phase separation is just the bound cation densities we calculate for the isolated liposomes.

The most important question that any molecular description of the fusion event must confront is explaining why the fusion rate constant increases with the amount of bound divalent cation. Our experiments have shown that by reducing the amount of bound divalent cation the fusion rate constant can be lowered to a level where the rate-limiting step of all the molecular events involved in membrane fusion is independent of liposome size. The data indicate that the effect of liposome size, and hence bilayer curvature, on divalent cation induced fusion is more subtle than previously suspected. It appears that point defects that lead to fusion occur at a threshold level of bound divalent cation regardless of the initial packing of the phospholipids in liposomes of different sizes.

Added in Proof

We have found that Mn^{2+} and Ni^{2+} induce mixing of aqueous contents using a recently developed assay (Ellens et al., 1985). Thus, it is evident that a rapid transport of these transition metals into the liposome interiors is responsible for the lack of the Tb/DPA fusion signal.

Acknowledgments

We thank Andy Phan for technical assistance and Vera Vaughn for preparing the manuscript.

Registry No. Ca, 7440-70-2; Ba, 7440-39-3; Sr, 7440-24-6; Mg, 7439-95-4; Mn, 7439-96-5; Ni, 7440-02-0.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.
Bentz, J. (1981) *J. Colloid Interface Sci 80*, 179–191.
Bentz, J. (1982) *J. Colloid Interface Sci. 90*, 164–182.
Bentz, J., & Nir, S. (1980) *Bull. Math. Biol. 42*, 191–220.
Bentz, J., & Nir, S. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1634–1637.
Bentz, J., & Nir, S. (1981b) *J. Chem. Soc., Faraday Trans. 1 77*, 1249–1275.
Bentz, J., Nir, S., & Wilschut, J. (1983a) *Colloids Surf. 6*, 33–66.
Bentz, J., Düzgüneş, N., & Nir, S. (1983b) *Biochemistry 22*, 3320–3330.
Bentz, J., Düzgüneş, N., & Nir, S. (1985) *Biochemistry 24*, 1064–1072.
Cohen, F. S., Akabas, M. H., & Finkelstein, A. (1982) *Science (Washington, D.C.) 217*, 458–460.
Düzgüneş, N., & Papahadjopoulos, D. (1983) in *Membrane Fluidity in Biology* (Aloia, R. C., Ed.) Vol 2, pp 187–216, Academic Press, New York.
Düzgüneş, N., Nir, S., Wilschut, J., Bentz, J., Newton, C., Portis, A., & Papahadjopoulos, D. (1981a) *J. Membr. Biol. 59*, 115–125.
Düzgüneş, N., Wilschut, J., Fraley, R., & Papahadjopoulos, D. (1981b) *Biochim. Biophys. Acta 642*, 182–195.
Düzgüneş, N., Bentz, J., Freeman, K. B., Nir, S., & Papahadjopoulos, D. (1983a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1771.
Düzgüneş, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D. S., James, T. L., & Papahadjopoulos, D. (1983b) *Biochim. Biophys. Acta 732*, 289–299.
Düzgüneş, N., Paiement, J., Freeman, K. B., Lopez, N. G., Wilschut, J., & Papahadjopoulos, D. (1984) *Biochemistry 23*, 3486–3494.
Ekerdt, R., & Papahadjopoulos, D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2273–2277.
Ellens, H., Bentz, J., & Szoka, F. C. (1985) *Biochemistry 24*, 3099–3106.
Hendrickson, H. S., & Fullington, J. G. (1965) *Biochemistry 4*, 1599–1605.
Hui, S. W., Stewart, T. P., Boni, L. T., & Yeagle, P. L. (1981) *Science (Washington, D.C.) 212*, 921–923.
Lichtenberg, D., Freire, E., Schmidt, C. F., Barenholz, Y., Felgner, P. L., & Thompson, T. E. (1981) *Biochemistry 20*, 3462–3467.
McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., & McLaughlin, A. (1981) *J. Gen. Physiol. 77*, 445–473.
Newton, C., Pangborn, W., Nir, S., & Papahadjopoulos, D. (1978) *Biochim. Biophys. Acta 506*, 281–287.
Nir, S. (1984) *J. Colloid Interface Sci. 102*, 313–321.
Nir, S., & Bentz, J. (1978) *J. Colloid Interface Sci. 65*, 399–414.
Nir, S., Newton, C., & Papahadjopoulos, D. (1978) *Bioelectrochem. Bioenerg. 5*, 116–133.
Nir, S., Bentz, J., & Portis, A. (1980a) *Adv. Chem. Ser. No. 188*, 75–106.
Nir, S., Bentz, J., & Wilschut, J. (1980b) *Biochemistry 19*, 6030–6036.
Nir, S., Bentz, J., & Düzgüneş, N. (1981) *J. Colloid Interface Sci. 84*, 266–269.
Nir, S., Wilschut, J., & Bentz, J. (1982) *Biochim. Biophys. Acta 688*, 275–278.
Nir, S., Bentz, J., Wilschut, J., & Düzgüneş, N. (1983a) *Prog. Surf. Sci. 13*, 1–124.
Nir, S., Düzgüneş, N., & Bentz, J. (1983b) *Biochim. Biophys. Acta 735*, 160–172.

Ohki, S. (1984) *J. Membr. Biol. 77*, 265–275.
Ohki, S., & Kurland, R. (1981) *Biochim. Biophys. Acta 645*, 170–176.
Ohki, S., Düzgüneş, N., & Leonards, K. (1982) *Biochemistry 21*, 2127–2133.
Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.
Ralston, E., Hjelmeland, L. M., Klausner, R. D., Weinstein, J. N., & Blumenthal, R. (1981) *Biochim. Biophys. Acta 649*, 133–137.
Rehfeld, S. J., Düzgüneş, N., Newton, C., Papahadjopoulos, D., & Eatough, D. J. (1981) *FEBS Lett. 123*, 249–251.
Sigel, H., & Martin, R. B. (1982) *Chem. Rev. 82*, 385–426.
Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R., & Hagins, W. A. (1977) *Science (Washington, D.C.) 195*, 489–492.
Wilschut, J., Düzgüneş, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.
Wilschut, J., Düzgüneş, N., & Papahadjopoulos, D. (1981) *Biochemistry 20*, 3126–3133.
Wilschut, J., Düzgüneş, N., Hong, K., Hoekstra, D., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 734*, 309–318.
Wilschut, J., Düzgüneş, N. Hoekstra, D., & Papahadjopoulos, D. (1985) *Biochemistry 24*, 8–14.
Wong, M., Anthony, F. H., Tillack, T. W., & Thompson, T. E. (1982) *Biochemistry 21*, 4126–4132.

Kinetics of Hemoprotein Reduction and Interprotein Heme Transfer†

Robert F. Pasternack,*,‡ Esther J. Gibbs,§ A. Grant Mauk,∥ Lorne S. Reid,∥ Ngai M. Wong,‡ Ko Kurokawa,‡ Mark Hashim,‡ and Ursula Muller-Eberhard⊥

Department of Chemistry, Swarthmore College, Swarthmore, Pennsylvania 19081, and Departments of Pediatrics, Pharmacology, and Biochemistry, New York Hospital/Cornell University Medical College, New York, New York 10021

Received December 5, 1984; Revised Manuscript Received April 10, 1985

ABSTRACT: The transfer of hemin from one protein to another is an event biologically important for the conservation of heme iron. Hemin entering the circulation (or added to serum) is mainly bound by albumin and then transferred to hemopexin [Morgan, W. T., Liem, H. H., Sutor, R. P., & Muller-Eberhard, U. (1976) *Biochim. Biophys. Acta 444*, 435–445], and we are now investigating which mechanisms may be operative in enhancing this process. The presence of imidazole has been demonstrated to accelerate hemin transfer from albumin to hemopexin [Pasternack, R. F., Gibbs, E. J., Hoeflin, E., Kosar, W. P., Kubera, G., Skowronek, C. A., Wong, N. M., & Muller-Eberhard, U. (1983) *Biochemistry 22*, 1753–1758]. The present work is an examination of the effect of the reduction of albumin-bound hemin on the rate of its transfer to hemopexin. Hemin (Hm^{III}; ferriprotoporphyrin IX) was reduced to Hm^{II} (ferroprotoporphyrin IX) by the addition of sodium dithionite under argon. The reduction kinetics of Hm^{III} to Hm^{II} were studied separately in the two complexes: with human serum albumin (HSA), which binds up to 20 mol of heme/mol (the first mole with $K \simeq 10^8$), and with hemopexin (HHx), which binds heme equimolarly ($K \simeq 10^{13}$). The rate of reduction of Hm^{III} to Hm^{II} on HSA was first order over several half-lives and linearly dependent on $[S_2O_4^{2-}]^{1/2}$. At $[HSA]_0/[Hm^{III}] = 3$, the k_{obsd} was $(5 \times 10^{-3}) + 0.75[S_2O_4^{2-}]^{1/2}$, and with $[HSA]/[Hm^{III}] \sim 25$, the k_{obsd} was $(2 \times 10^{-3}) + 0.25[S_2O_4^{2-}]^{1/2}$. The reduction of Hm^{III} to Hm^{II} on human hemopexin (HHx) is much more rapid with $k_{obsd} = (2.5 \times 10^3)[S_2O_4^{2-}]^{1/2}$. The transfer of Hm^{II} from HSA to HHx was studied by adding dithionite to Hm^{III}·HSA and mixing this with HHx. The transfer was biphasic, consisting of two first-order processes, k_f and k_s, independent of $[Hm^{III}\cdot HSA]_0$, $[HSA]_0$, and $[HHx]_0$, but with a slight dependence on pH and ionic strength. The transfer of Hm^{II} from HSA involves two steps that may be due to HSA existing as two noninterconverting conformers [Moehring, G. A., Chu, A. H., Kurlansik, L., & Williams, T. J. (1983) *Biochemistry 22*, 3381–3386]. Since the overall rate of the "redox" transfer pathway of Hm^{II} from HSA to HHx is as efficient as the pathway catalyzed by the presence of 50 mM imidazole, a catalyst much more effective than this nitrogen base would have to be present in vivo to enhance the transfer of heme from HSA to HHx.

The transfer of small substrate molecules between macromolecules such as proteins and nucleic acids is a step of considerable importance in many biological processes. An example is the conservation of the iron of circulating hemin during hemolytic events through a pathway that involves the passage of the metalloporphyrin moiety from human albumin (HSA)[1] to hemopexin (HHx) (Muller-Eberhard, 1978).

Hemopexin, which is present in the serum at 1.5–2% of the concentration of albumin, binds hemin (Hm^{III}; ferriproto-

† This research has been supported by grants from the National Institutes of Health to R.F.P. (GM-17574) and to U.M.-E. (AM-30203) and an MRC of Canada grant to A.G.M.

‡ Swarthmore College.
§ Department of Chemistry, Goucher College, Towson, MD 21204.
∥ Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5.
⊥ New York Hospital/Cornell University Medical College.

[1] Abbreviations: HSA, human serum albumin; HHx, human hemopexin; Hm^{III}, ferriprotoporphyrin IX; Hm^{II}, ferroprotoporphyrin IX; Me_2SO, dimethyl sulfoxide; ESR, electron spin resonance; NHE, normal hydrogen electrode; emf, electromotive force.

0006-2960/85/0424-5443$01.50/0