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PHASE DIAGRAMS AND THE KINETICS OF PHOSPHOLIPID EXCHANGE FOR VESICLES OF DIFFERENT COMPOSITION AND RADIUS

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Summary

Interactions between dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles have been studied by light scattering of dilute dispersions (concn $\leqslant$ 1 mg/ml). It was found that at 50°C, the two types of vesicles exchange lipid molecules, irrespective of the initial radii of the pure vesicles. When the initial radii are different, the process leads eventually to vesicles of intermediate size. The reaction follows second order kinetics. At room temperature no reaction was observed. The phase diagrams for several lipid systems are discussed.

Introduction

Vesicles constitute a much used model system for the study of chemical and physical properties of biological membranes. One of the most important properties of the membrane is its fluidity, since many biological processes, such as permeability, aggregation and fusion [1,2] depend on the fluidity of the membrane. The fluidity of the membrane can be influenced by changing the temperature [3,4], or adding other components [5].

A good review of experimental work on the phase transition temperatures (at which the fluidity of the membrane changes) for pure lipids has been published [6]. Many experimental studies have also been devoted to phase diagrams [7—10] of mixtures of biologically important lipids. Also, some papers have appeared in which the observed chain melting temperatures are related to the chain length by means of a statistical mechanical description of the chain dynamics [6,11]. Since a reaction involving the exchange of lipids leads to a shift of the phase transition temperature, measurements of this shift can be used to study the interactions between vesicles of different lipids.

Whereas there is good agreement between the experimentally determined phase transition temperatures, there appears to be a large discrepancy between

the experimental results on the lipid exchange between phospholipid vesicles as a function of the fluidity of the membrane. Also, the molecular mechanism of the exchange of lipid molecules is at present not well understood. Lentz et al. [9] did not find any interaction between vesicles of neutral lipids. Papahadjopoulos et al. [12] supported these findings, observing exchange of lipid only in the case of charged lipids that are in the fluid state. As a mechanism for the observed process they proposed an exchange of lipids via diffusion of molecules. Only in the presence of some agents (hydrophobic protein or dimethylsulfoxide), did they observe fusion between entire vesicles. Martin and McDonald [2] on the other hand, did find an interaction between neutral phospholipid vesicles above the phase transition temperatures of the lipids used. They explained the observed transfer of lipid by assuming that the higher solubility of the smallest phospholipid caused diffusion of this lipid to the phospholipid vesicles with the largest alkyl chain. Haran and Sphorer [13] reported transfer of cholesterol from cholesterol-rich to pure egg lecithin vesicles without the occurrence of fusion. The accuracy of their kinetic measurements, however, was not sufficient to decide on the order of the reaction with respect to vesicle concentration.

In order to understand the mechanism underlying the exchange of lipid, we used a light scattering technique to study the interaction between dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles above and below the phase transition temperatures. Both phase transitions (light scattering intensity versus temperature) and kinetics (intensity versus time) can be determined with this technique. Also, light scattering allows the study of samples of very low concentration (concn. $\leqslant$ 1 mg/ml), so that the occurring process can be followed in an early stage.

Materials and Methods

Dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine were obtained from Koch and Light Laboratories, Colnbrook, Bucks, U.K. (Art. 2203t, batch nr. 65508 and Art. 3402t, batch nr. 67082, respectively). Since the purchased dimyristoyl phosphatidylcholine was contaminated with myristic acid, a fusogenic component, the sample was purified by column chromatography on a silica column (Merck, Kieselgel 60 reinst. 70—230 mesh) using a gradient of chloroform and methanol (up to 70% methanol) as eluant. Fractions were analyzed by thin layer chromatography with a chloroform/methanol/water mixture (65 : 35 : 4, by vol.) as developer. The first and last fractions showing the presence of dimyristoyl phosphatidylcholine were discarded to avoid contamination with small amounts of impurities, that would not be visible with this low sensitivity analysis. The dimyristoyl phosphatidylcholine thus obtained was kept at $-20°$ C. Dipalmitoyl phosphatidylcholine was used as received.

Aqueous dispersions of dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine and of mixtures of these lipids were prepared according to a modification of the method of Batzri and Korn [14], as described in detail elsewhere [15]. In this method, 0.5 ml of an ethanolic solution containing different quantities of the lipids (up to 50 μmol per ml ethanol) is injected through

a Hamilton syringe into 10 ml of vigorously stirred water or of a buffer solution. The buffer solution used throughout this work was 0.1 M NaCl in 0.01 M Tris · HCl, adjusted to pH 7. All aqueous solutions were prepared with doubly distilled water. The injection velocity was 0.05 ml per min. During injection the temperature of the water or buffer solution was kept at about 50° C (above the phase transition temperatures of both lipids). The alcohol and the buffer solutions used for the preparation of vesicles were filtered through Solvinert filters (pore diameter, 200 nm) and Millipore filters (25 nm), respectively. All glassware used was made dust-free by cleaning with condensing acetone or ethanol.

The light scattering experiments were done with a FICA light scattering photometer equipped with a thermostat and an optically perfect, cylindrical cell (sample volume 2 ml), in which a thermistor was placed to measure the temperature of the vesicle samples. All light scattering experiments were done at a wavelength of 546 nm. The radii of the vesicles prepared at 50° C were determined from the angular dependence of the scattered intensity, using Equation 10 given by Pecora and Aragon [16] (this procedure is described in detail elsewhere [15]).

The phase transition temperatures of all types of vesicles were obtained by recording the intensity of the light scattered at an angle of 90° as a function of the temperature (temperature change, 1° C/min). When the particle size does not change during the temperature scan, this procedure monitors essentially the refractive index increment dn/dc, which is known to change sharply at the phase transition temperature [17] as a result of the change in lipid density.

The time dependence of the phase transitions of the mixtures of pure dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles was determined as follows. First, the mixtures were incubated for a certain time at the desired temperature (20 or 50° C). Then the solutions were diluted four times in order to diminish the rate of the lipid exchange that takes place during the temperature scan. Finally the intensity of the light scattered at an angle of 90° was measured as a function of temperature as described above.

The time dependence of the intensity of the light scattered at an angle of 90° directly after mixing two solutions of unequal vesicles was also studied. This time dependence was determined for different initial concentrations and different initial radii of the mixed vesicles.

Dilutions of the vesicle solutions were made with buffer solution.

Results

The change of the intensity of the scattered light as function of the temperature for the two types of pure vesicles and for vesicles consisting of an equimolar mixture of the two lipids, is given in Fig. 1. This figure also shows, how the phase transition temperature range ΔT_m and the phase transition temperature T_m are defined by an extrapolation procedure.

Fig. 2, part A, shows the transition temperature ranges (denoted by bars) for mixed dimyristoyl/dipalmitoyl phosphatidylcholine dispersions as functions of the mol fraction of the latter lipid present in the injected ethanolic solution. Also included in this figure are the phase diagrams, as reported in the literature, for dipalmitoyl/distearoyl phosphatidylcholine liposomes (part B and ref. 6)

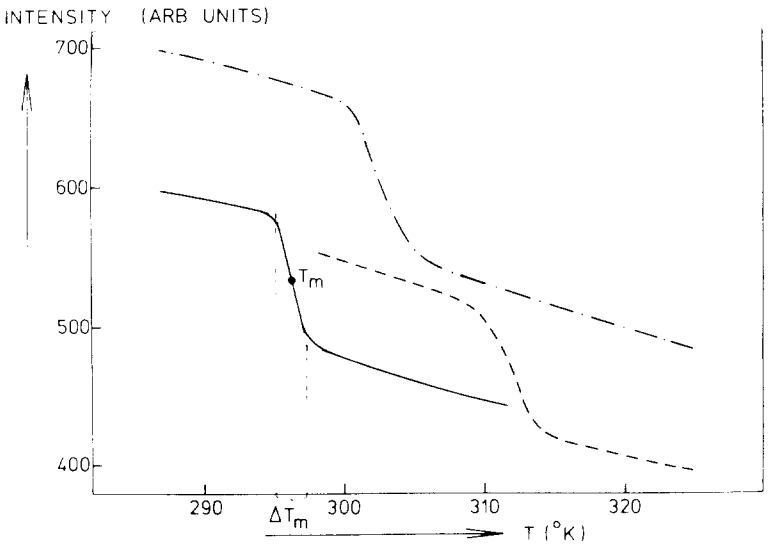

Fig. 1. Phase transitions of pure dimyristoyl phosphatidylcholine (———), pure dipalmitoyl phosphatidylcholine (- - - - - -) and of vesicles consisting of an equimolar mixture of these lipids (— · — ·), respectively. The transition temperature, T_m is defined by taking the intersection between the experimental curve and a straight line drawn so that the areas between the measured curve and the extrapolated lines are equal to each other. The values of T_m on the centigrade scale are 23°C for dimyristoyl phosphatidylcholine, 39°C for dipalmitoyl phosphatidylcholine and 31°C for the equimolar mixture.

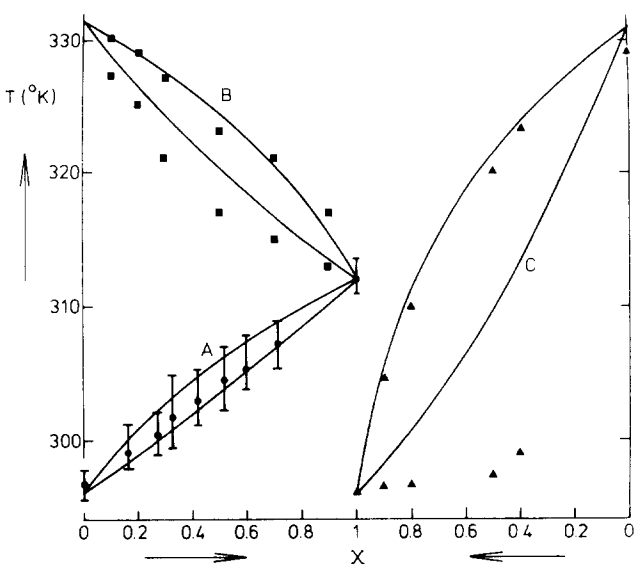

Fig. 2. Phase diagrams for mixtures of phospholipids. Part A: Dimyristoyl/dipalmitoyl phosphatidylcholine. Part B: Distearoyl/dipalmitoyl phosphatidylcholine. In A and B, X is the mol fraction of dipalmitoyl phosphatidylcholine. Part C: Dimyristoyl/distearoyl phosphatidylcholine. Here, X is the mol fraction of dimyristoyl phosphatidylcholine. In part A, the bars represent ΔT_m and the points give T_m as defined in Fig. 1. The experimental values derived from cooling and heating curves in differential scanning calorimetry, are shown by squares (ref. 6) and by triangles (ref. 7), respectively. The curves in Parts A, B and C have been calculated from Eqns. 1—5 (see text).

and dimyristoyl/distearyl phosphatidylcholine liposomes (part C and ref. 7).

Fig. 3 shows the time dependence of the phase transition for an equimolar mixture of the two lipids after incubation at 50°C. It is seen that the phase transitions shift to each other after incubation at 50°C and merge into one single phase transition. This final phase transition is identical with the phase transition (cf. Fig. 1) of the vesicles prepared by injecting an ethanolic solution containing an equimolar mixture of the two lipids. The same observation holds for all other ratios of the two lipids. The phase transitions shift towards each other when the radii of the two kinds of vesicles are equal as well as when they differ from each other. During incubation at 20°C, however, the phase transition never changed with time, which proves that the two kinds of vesicles did not exchange lipid molecules.

The intensity of the scattered light as function of the time, after mixing dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles with different initial radii, is shown in Fig. 4 for different total lipid concentrations. The slope of the intensity versus time curves extrapolated to $t = 0$ as a function of the total lipid concentration is given in Fig. 5. This dependence can be described by second order kinetics as will be shown in the discussion.

The experiments represented by Figs. 4 and 5 were carried out for a number of combinations of vesicles of different radii and different mixing ratios. These data are given in the first three columns of Table I. The decrease of the scattering intensity (cf. Fig. 4) and the relation between dI/dt and c_t shown in

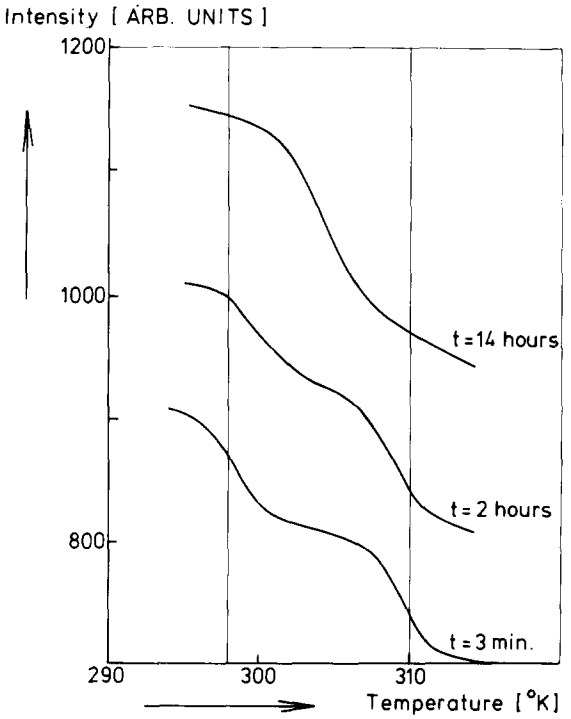

Fig. 3. The phase transition of a 1 : 1 mixture of dimyristoyl- and dipalmitoyl phosphatidylcholine molecules during incubation at 50°C. Total lipid concentration, 0.2 mg/ml.

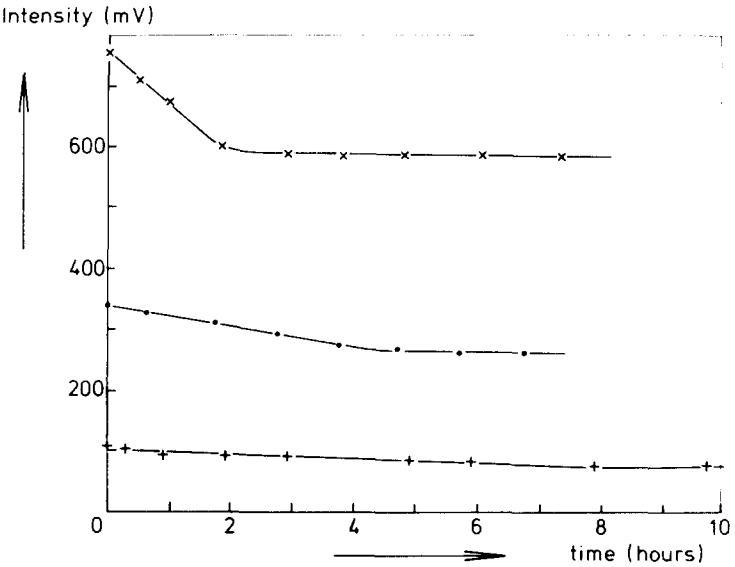

Fig. 4. The intensity (expressed as the voltage of the photomultiplier output) as a function of time for the mixture of dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles at total lipid concentrations of 1 mg/ml (×), 0.5 mg/ml (•) and 0.125 mg/ml (+) and at 50°C.

Fig. 5, were observed in all cases reported in the table. The fourth column of Table I gives the radii of the vesicles that are present when the process is completed. These radii were calculated from the angular dependence of the scattering intensity that was observed when the intensity had become independent of time (cf. Fig. 4).

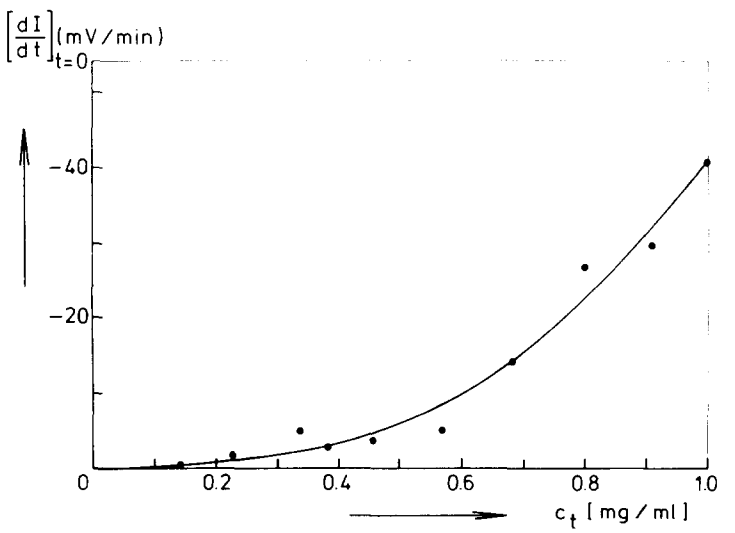

Fig. 5. The change of the intensity with time at $t = 0$ for an approximately equimolar mixture of dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles as a function of the total lipid concentration, c_t at 50°C. The points represent the experimental values for the system given in the 2nd line of Table I, and the solid line gives the quadratic function predicted by second order kinetics.

TABLE I

RESULTS FOR A NUMBER OF VESICLE SYSTEMS

R_{14} and R_{16} are the radii of the dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles before mixing; f is the ratio of the initial molar concentrations of these vesicles. R_{exp} is the final radius determined experimentally after completion of the reaction; R_{theor} is the final radius calculated assuming conservation of particle number and k is the rate constant of the process (6), calculated from Eqn. 7. Errors in R_{14}, R_{16} and R_{exp} are approx. 15%. For further explanation see text.

R_{14} (nm)	R_{16} (nm)	f	R_{exp} (nm)	R_{theor} (nm)	k(l/mol per s)
30	43	0.95	40	38	$2 \cdot 10^6$
33	46	0.83	44	41	$1 \cdot 10^6$
47	21	0.75	40	36	$1 \cdot 10^5$
30	42	1.07	37	38	$3 \cdot 10^6$
60	32	0.23	44	42	$2 \cdot 10^5$

When vesicles of dimyristoyl- and dipalmitoyl phosphatidylcholine with equal radii were mixed, the intensity of the scattered light did not change with time. Mixtures containing only vesicles of pure lipids, but with unequal radii, also showed no time dependence of the scattered intensity.

The described experimental results did not depend on whether water or a buffer solution was used to prepare the lipid dispersions. Also, the ethanol concentration in the medium (which varied from 5 to 0.5% because the solutions were diluted with buffer containing no ethanol) did not influence the decrease of the scattered intensity with time.

Although the solutions, after mixing, showed a decrease of the scattered intensity with time, as a result of the formation of particles with radii intermediate between the initial radii, the solutions often showed an increase in scattering intensity after incubation for about one day or longer at 50°C. This increase of the intensity with time was also observed with concentrated vesicle solutions (>1 mg/ml) directly after mixing. Analysis of the angular dependence of the light scattering shows that in these cases the increase of the intensity was caused by an increase in particle size.

For convenience we have summarized our main observations in Table II.

TABLE II

OBSERVATIONS FOR FOUR DIFFERENT TYPES OF VESICLE SYSTEMS AT A TEMPERATURE ABOVE THE PHASE TRANSITIONS OF THE VESICLES INITIALLY PRESENT AND AT LIPID CONCENTRATIONS BELOW 1 mg/ml

For further explanation see text.

Initial vesicle system			Observations	
	Lipid composition	Radii	Phase transition	Radii
1	Equal	Equal	No change	No change
2	Equal	Unequal	No change	No change
3	Unequal	Equal	Change to intermediate temperature	No change
4	Unequal	Unequal	Change to intermediate temperature	Change to intermediate radius

Discussion

A. Phase diagrams

The phase transition temperatures for pure dimyristoyl- and dipalmitoyl phosphatidylcholine (Fig. 1) are found to be 23°C and 39°C, respectively. They agree well with those obtained from other sources, e.g. from differential scanning calorimetry, viz. 23.78°C and 41.75°C (see ref. 4 for a comprehensive list). The slight discrepancy may be caused by differences in the purity of the samples, or by the different model systems used (vesicles versus liposomes [9]), or to different criteria used in defining T_m. The temperature-composition diagram (Fig. 2) for dimyristoyl/dipalmitoyl phosphatidylcholine vesicles, obtained from light scattering experiments, is in good agreement with that for liposomes of the same composition, obtained with the aid of a fluorescence technique [8].

The process occurring at the phase transition temperature involves the change from a gel crystalline (s) to a fluid (l) state of the alkyl chains of the lipid molecules in the bilayer. Thus, the chain melting process is analogous to the normal melting of solids and can in fact be described as a first order phase transition [5]. Therefore, one can apply classical thermodynamic arguments to the phase diagrams in Fig. 2. From thermodynamics it can be shown [18] that the following equations describe the phase diagram of an ideal two-component system, where T is the temperature, X_1^l the mol fraction in the liquid state and X_1^s the mol fraction in the gel crystalline state:

$$X_1^l = \frac{1-B}{A-B} \tag{1}$$

$$X_1^s = \frac{1-B}{A-B}A \tag{2}$$

$$A = \exp[\beta\Delta S_1(T_1 - T)] \tag{3}$$

$$B = \exp[\beta\Delta S_2(T_2 - T)] \tag{4}$$

$$\beta = 1/RT \tag{5}$$

The subscripts 1 and 2 refer to the two components. ΔS is the melting entropy defined as $S_l - S_s$ and R is the gas constant. The melting temperatures of the two components are given by T_1 and T_2. From these equations we have constructed the phase diagrams for the lipid mixtures, using the data given by Phillips et al. [19]. These theoretical curves have been drawn in Fig. 2.

As can be seen from Fig. 2 the liquidus curve in all three cases is described very well by the theory, but the solidus curves deviate, especially for dimyristoyl/distearoyl phosphatidylcholine (curve C). This indicates that the liquid mixture, down to the "freezing" points, behaves ideally, but that the solid phase formed at T_m does not consist of the ideal composition. In fact, as follows from Fig. 2, the solid phase is always richer in the component with the largest alkyl chain than predicted by theory. This may be explained on a molecular level as follows. For a mixed sample in the liquid phase the bilayer thick-

ness is expected to be in between that of the pure components. Since the alkyl chains are free to move and bend, the motions of the largest chains are not seriously affected by the opposite half of the bilayer. Upon lowering the temperature, the largest chains will first notice the influence of the opposite half. This will restrict their motion more than that of the short chains present. Thus the gel crystalline phase formed will be richer in the larger chain component than can be expected on the basis of ideal behaviour. This effect is more pronounced for dimyristoyl/distearoyl phosphatidylcholine, which differ more in chain length than dimyristoyl/dipalmitoyl phosphatidylcholine and distearoyl/dipalmitoyl phosphatidylcholine, as can be expected from the above arguments. It thus seems that the phase diagrams shown in Fig. 2 can be adequately described by ideal thermodynamics in the liquid state and that the deviation of the solidus line may be understood from a molecular point of view.

B. Kinetic measurements

The kinetic measurements are more difficult to interpret. The observation of a time dependent phase transition (Fig. 3) is evidence for a reaction between the two types of vesicles, involving the mixing of the two lipids. From the observation of a decrease in scattered light with time after mixing pure dimyristoyl- and dipalmitoyl phosphatidylcholine vesicles with different radii (Fig. 4) we conclude that a process of aggregation of fusion does not occur, since this would always lead to an increase in the scattered light intensity [20]. Also, the values of the particle size determined after completion of the process show that the average particle size has not increased, but has become intermediate between the initial radii (Table I). Fig. 5 shows that the decrease of scattering intensity with time follows second order kinetics. From this we conclude that the observed changes of the vesicle radii are caused by collisions between vesicles of unequal radii. Obviously, this involves the exchange of lipid molecules between vesicles. From the fact that the light scattering intensity does not change after mixing vesicles with different radii but equal composition, we conclude that the mixing of molecules of different kinds is an essential factor in the process.

In line with the above arguments we represent the overall process by

$$V_{14} + V_{16} \rightarrow 2V_{14/16} \tag{6}$$

where V_{14} and V_{16} stand for vesicles of pure dimyristoyl- and pure dipalmitoyl phosphatidylcholine, respectively, and $V_{14/16}$ represents a vesicle whose composition is determined by the initial molar concentrations of the pure lipids. We assume that the overall process consists of a series of "bimolecular" steps and characterize this process by a single second order rate constant k, which is of course an average of all specific rate constants of the separate steps.

The relation between the observed rate of change of the scattering intensity, the constant k, the particle size and initial concentrations of the reacting vesicles is derived in ref. 20. The result of the derivation is:

$$\left[\frac{1}{I_\theta}\frac{dI_\theta}{dt}\right]_{t=0} = \frac{2M_{14/16}^2 P_{14/16}(\theta) - M_{14}^2 P_{14}(\theta) - M_{16}^2 P_{16}(\theta)}{c_{14}(0)M_{14}^2 P_{14}(\theta) + c_{16}(0)M_{16}^2 P_{16}(\theta)} k c_{14}(0) c_{16}(0) \tag{7}$$

Here, I_θ is the intensity of the light scattered at angle θ, dI_θ/dt its rate of

change and $t = 0$ indicates initial time. M_i is the molar mass of a vesicle of kind i, $P_i(\theta)$ its form factor accounting for internal interference, and $c_{14}(0)$ and $c_{16}(0)$ are the molar concentrations at $t = 0$ of the two types of vesicles. To compare Eqn. 7 with experiment we use the equality:

$$\frac{c_{14}(0)c_{16}(0)}{c_{14}(0)M_{14}^2P_{14}(\theta) + c_{16}(0)M_{16}^2P_{16}(\theta)} = \frac{f}{[fM_{14}^2P_{14}(\theta) + M_{16}^2P_{16}(\theta)]\,(1+f)}\,c_t \qquad (8)$$

where $c_t = c_{14}(0) + c_{16}(0)$ and $f = c_{14}(0)/c_{16}(0)$. Hence, Eqn. 7 predicts that $[(1/I_\theta)\, dI_\theta/dt]_{t=0}$ is proportional to c_t when c_t is varied at constant ratio (f) of initial concentrations. This prediction holds for any values of M_i and $P_i(\theta)$. In Fig. 6, the experimental values of the left hand side of Eqn. 7 are plotted against c_t for one mixture of vesicles. The plots for the other vesicle systems (Table I) show the same correlation. We wish to point out that the model proposed by Martin and McDonald [2] (exchange of lipids through diffusion of free molecules) predicts that $[(1/I)\, dI/dt]_{t=0}$ is independent of c_t, as shown in ref. 20. We conclude that our light-scattering experiments are consistent with exchange of lipids through collision between vesicles.

The values of the rate constant k for the different vesicle systems have been calculated from Eqn. 7, using our experimental values of the molar masses and the appropriate values of $P(\theta)$ obtained from the work of Pecora and Aragon [16]. For $M_{14/16}$ and $P_{14/16}(\theta)$ we have taken the values corresponding to the end product of reaction 6. This is an approximation, because we have measured the left hand side of Eqn. 7 at initial time. However, this approximation affects only the values of k, not the conclusion concerning the order of the process. The results are reported in the last column of Table I. The differences between the values of k can be attributed to differences in the area of contact between the two vesicles and to the approximation mentioned above.

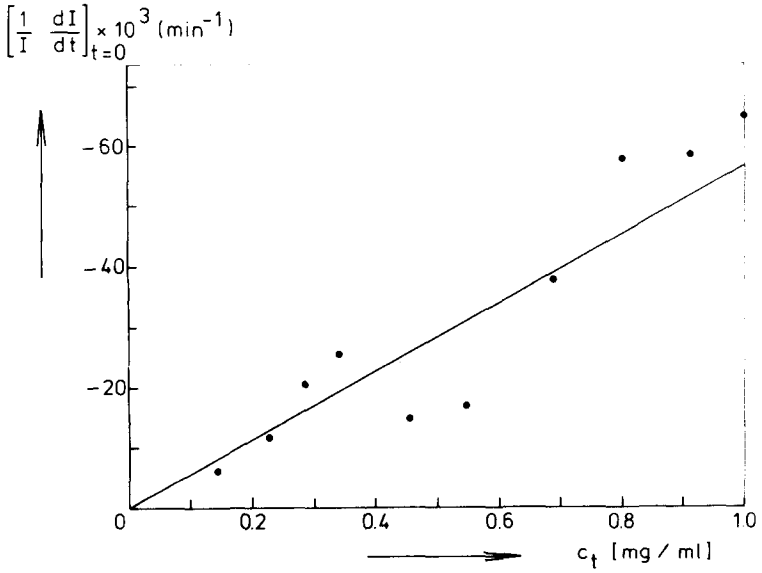

Fig. 6. Left hand side of Eqn. 7 as a function of total lipid concentration, c_t. Points represent the experimental values for the system given in the 2nd line of Table I.

We have carried out additional experiments with the vesicle system described in the last line of Table I. In these experiments, $c_{14}(0)$ was kept constant at $5 \cdot 10^{-9}$ mol/l and $c_{16}(0)$ was varied between $3 \cdot 10^{-9}$ and $20 \cdot 10^{-9}$ mol/l. The values of k, calculated from these experiments, varied between $1 \cdot 10^5$ and $3 \cdot 10^5$ l/mol per s.

The observed rate constant is always much smaller than that of a second order process which is controlled only by diffusion ($k_{diff} \simeq 10^{10}$ l/(mol/s)). This indicates that encounters between vesicles do not lead to total exchange of molecules, as is of course to be expected.

The observed increase in particle size after long incubation times (24 h) or at high vesicle concentrations is probably caused by fusion of vesicles.

In ref. 20 it is shown that the observed exchange process (cf. of Table II) is thermodynamically possible and why it is faster if mixing of different lipids is involved. Fusion of vesicles requires a higher activation energy than exchange [20] and will thus be a slower process.

Finally, we wish to point out that the light-scattering method is very well suited for this type of kinetic study because the processes can be followed continuously and especially because the high sensitivity of the method makes it possible to work with very low vesicle concentrations. Using dilute dispersions, one has time to follow processes with a high rate constant that take place initially. These processes may escape attention with methods such as turbidimetry, NMR, differential scanning calorimetry and electron microscopy, which require lipid concentrations that are at least one order of magnitude larger than those used in the present work. This advantage of the light scattering method applies especially to second order processes, whose rates are decreased by a factor of 100 by means of a 10-fold dilution.

Acknowledgements

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