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SPREADING OF LIPOSOMES AT THE AIR/WATER INTERFACE

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Summary

Two types of film structure are formed when liposomes are spread at the air/water interface. At zero surface pressure, there is a slow transformation of the closed bilayered structure into a lipid monolayer. The internal content of the liposomes is released into the aqueous subphase. In contrast, when multilamellar liposomes are spread against a surface pressure, they retain their internal content at the air/water interface by forming multilayered structures. Among the liposomes which dipped through the interface an important fraction loses its internal content. During the spreading process at zero surface pressure, it seems that the outer layer of the liposome spreads with a better yield as compared with the inner layer. It is possible to use this spreading technique to determine the asymmetrical distribution of lipids across bilayers.

Introduction

The classical models of biological membranes arising from the observations of Gorter and Grendel [1] and the proposals of Danielli and Davson [2] led to the view that biomembranes could be regarded as the association of two monomolecular layers. If this conception is correct, it would be possible to transform a lipid bilayer into a monolayer and vice versa. The formation of black lipid membranes from lipid monolayers was investigated and represent a promising approach in reconstitution studies [3,4,5].

On the other hand, a method for spreading biomembranes at the air/water interface was described recently [6]. The changes in architecture of membranes, during spreading at different surface pressures, can provide a simple method to study the influence of lipid packing on the catalytic activity of membrane bound enzymes.

Liposomes can be considered as the simplest closed membrane model. Their

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structure and osmotic behaviour have been extensively studied. This paper is an attempt to study the mechanism of formation and the structure of the films obtained by spreading liposomes at the air/water interface. The compression isotherm obtained from spread liposomes was compared to the usual isotherm given by the same phospholipids spread from a chloroform solution. Liposomes with glucose trapped inside were spread in order to follow the release of the internal content into the aqueous subphase. Finally, the behaviour of the inner and outer liposome layer was investigated by spreading liposomes containing two different radioactive isotopes located respectively on the two liposome layers.

Materials and Methods

Lipids

^{14}C-labelled and ^{3}H-labelled phosphatidylcholines were isolated from the liver microsomes of two fasted rats after intraperitoneal injection of 0.5 μCi of ^{14}C-labelled or 1 μCi of ^{3}H-labelled choline respectively. The livers were excised 1 h after the injection. The phosphatidylcholines prepared from the microsomes as described by Wirtz et al. [7] were found pure by thin layer chromatography. The specific radioactivities were, respectively, $1.2 \cdot 10^6$ cpm/μmol and $3 \cdot 10^5$ cpm/μmol for the ^{14}C and ^{3}H-labelled phosphatidylcholines. The egg phosphatidylcholines were purified by silicic acid (Mallinckrodt, 60—140 mesh) column chromatography [8] and checked for homogeneity by thin-layer chromatography on microscope slides (26 × 75 mm) covered with silica G (Merck, Germany).

Dicetyl phosphate was purchased from Sigma and phosphatidic acid from Serdary Research Inc. (Canada). Lipid phosphorus was estimated as orthophosphate by the modified method of Fisk and Subba Row [9] after mineralization in $HClO_4$.

Radioactive chemicals

[*Me*-^{14}C]choline chloride (52 mCi/mmol) and [*Me*-^{3}H]choline chloride (10.1 Ci/mmol) were purchased from the Radiochemical Center (Amersham). [G-^{3}H]Cholesterol (4 Ci/mmol), [$^{3}H_1$]glucose (3.75 Ci/mmol) and [^{3}H]oleic acid (40 Ci/mmol) were obtained from the Commissariat a l'Energie Atomique ^{14}C-labelled phosphatidylcholines (1.76 Ci/mmol) from algae were purchased from New England Nuclear.

Liposome preparations

Two types of liposomes were used: the unilamellar liposomes prepared by ethanolic flash according to Batzri and Korn [10] and the multilamellar liposomes obtained by vortexing a lipid suspension as described by Bangham [11]. In each case, trace amounts of ^{14}C-labelled phosphatidylcholines from algae were added in order to facilitate the phospholipid detection.

The liposomes were made from egg or rat liver microsomes phosphatidylcholine and contained 2—9% of dicetylphosphate. The liposomes were prepared and the spreading was performed on a Tris · HCl buffer 10 mM, pH 7.5, NaCl 0.1 M.

Estimation of the glucose present outside and inside the liposomes

A multilamellar liposome suspension was percolated through a DEAE cellulose column (4 × 1 cm) equilibrated with Tris 10 mM (pH 8) according to Hellings et al. [12]. The external glucose was eluted with the void volume whereas the internal glucose was released only after elution of the column with 2 × 4 ml of a 1% Triton X 100, NaCl 0.5 M solution.

Preparation of the double labelled liposomes on the outer and inner layers

The pure bovine brain phosphatidylcholine exchange protein was used to label specifically the outer layer of unilamellar acceptor liposomes according to Wirtz et al. [12,13]. The donor liposomes (0.25 μmol of ^{3}H-labelled phosphatidylcholines containing 9 mol% phosphatidic acid) were incubated for 30 min at 25°C with the acceptor liposomes (0.50 μmol of ^{14}C-labelled phosphatidylcholines containing 2 mol% phosphatidic acid) in the presence of 2 μg of phosphatidylcholine exchange protein in a 10 mM Tris · HCl buffer, pH 7.5, NaCl 0.1 M. The mixture was then percolated through a DEAE cellulose column. Due to their higher anionic character, the donor liposomes were retained on the column; 40—60% of the acceptor liposomes were recovered with the void volume and concentrated 10-fold under vacuum in collodion bags (Sartorius). We have checked that donor and acceptor liposomes were totally separated during the filtration on DEAE-cellulose column. All liposomes were assumed to remain unilamellar during the concentration step.

Calculation of the spreading yield of the outer and inner liposome layer

The doubly labelled acceptor liposomes on the outer ^{3}H-labelled isotope and inner ^{14}C-labelled isotope layers, prepared as described above, were spread at the air/water interface. After film recovery, the amounts of the two radioactively labelled phosphatidylcholines were estimated and the spreading yield of each radioactive phosphatidylcholine was calculated. A is the spreading yield of the ^{3}H-labelled phosphatidylcholines coming from the donor liposomes, B is the spreading yield of the ^{14}C-labelled phosphatidylcholines present in the acceptor liposomes, C is the percentage of phosphatidylcholines exchanged by the phosphatidylcholine exchange protein and reflecting the molar percentage of ^{3}H-labelled phosphatidylcholines coming from the donor liposomes and transferred to the acceptor liposomes.

The spreading yield of the outer layer is given by the yield of the ^{3}H-labelled phosphatidylcholines coming from the donor liposomes (A) and exclusively localized on the outer layer. It is generally admitted that in unilamellar liposomes there are 65 and 35% of the phospholipid molecules on the outer and inner monolayers respectively [14,15]: thus the spreading yield of the inner layer is given by the formula: Spreading yield of the inner layer = $B(100 - C) - A(65 - C)/35$

Spreading technique

The spreading technique used was the same as previously described [6] except for the fact that the surface pressure was measured with a Wilhelmy plate, instead of the Langmuir method. Generally, higher spreading yields were obtained by using more concentrated liposomes suspensions.

Results

A. Spreading of liposomes at zero surface pressure

In order to study the mechanism of formation and the structure of the films obtained by spreading liposomes at the air/water interface, two series of experiments were performed using unilamellar and multilamellar liposomes.

In a first series, an aqueous suspension of well defined ^{14}C-labelled unilamellar liposomes made from egg phosphatidylcholine were spread at zero surface pressure. 20 min after spreading, approximately 74 ± 5% of the phospholipid material could be recovered from the air/water interface. After compression to 20 dynes/cm, the area occupied by the phospholipids was 81 ± 10 $Å^2$ per molecule. The compression isotherm yielded by spread unilamellar liposomes was observed to be very similar to the usual isotherm given by the same amount of phospholipid spread from a chloroform solution. In this latter case, the phospholipid covers at 20 dynes/cm an area of 90 ± 10 $Å^2$ per molecule and 87 ± 5% of the phospholipid could be recovered from the surface. Therefore, it is likely that the liposomes spread as a monomolecular film at the air/water interface, implying a drastic alteration of the phospholipid bilayer structure. The closed and spherical liposomes should be expected to open and to release their internal content into the aqueous subphase.

In order to check directly this point, a second series of experiments were performed using multilamellar liposomes. This type of liposome, notwithstanding its size inhomogeneity, has the advantage of entrapping significant amounts of glucose. This provides the means of measuring the release of the internal content of the liposomes into the aqueous subphase. We first checked that when the glucose was present only at the outside of the liposomes, no surface excess of glucose could be detected after spreading. Therefore, no detectable glucose adsorption occurs at the phospholipid film and, consequently, the interfacial glucose excess in all subsequent assays will only be attributed to trapped glucose in closed lipid structures. An aqueous suspension of ^{14}C-labelled multilamellar liposomes made from egg phosphatidylcholine and containing ^{3}H-labelled glucose was prepared and spread at zero surface pressure. The amount of glucose present in excess at the surface was always very low (4 ± 4%). This result strongly favors the idea that at zero surface pressure, the closed bimolecular leaflets of the multilamellar liposomes are mostly converted into an open structure at the air/water interface.

After spreading multilamellar liposomes at zero surface pressure, the amount of phospholipid recovered from the air/water interface was lower and less reproducible (55 ± 25%) than in the case of unilamellar liposomes (74 ± 5%). Nevertheless, in the former case, if the time interval between spreading and film recovery was longer than 20 min, the lipid packing was observed to be very similar to the usual packing given by the same amount of phospholipid spread from a chloroform solution (see Table I). This result is in perfect agreement with those obtained with unilamellar liposomes and shows that the spreading of liposomes at the air/water interface is a slow process. Furthermore, Table I shows that glucose remained trapped in the surface film when not enough time was allowed for liposomes to spread.

TABLE I

TIME DEPENDENCE OF THE LIPID SURFACE DENSITY AND THE GLUCOSE RETENTION IN THE FILM, AFTER SPREADING MULTILAMELLAR LIPOSOMES AT ZERO SURFACE PRESSURE

The liposomes suspension (1 mg/ml) entrapping [^{3}H]glucose (10^7 cpm/mg lipid) was prepared from egg phosphatidylcholines containing, 5% weight, dicetyl phosphate. The specific radioactivity of the liposomes varied from 5 to $20 \cdot 10^5$ cpm ^{14}C/mg of lipid. We spread 9 μg of lipid on a surface of 700 cm^2.

Spreading conditions	Lipid surface density at 15 dynes/cm (molecules/cm^2) $\times 10^{-12}$		Glucose retention in the film (% of total spread)
Time interval between spreading and film recovery	5 min	200	10.1
	10 min	143	5.1
	20 min	116	3.1
Chloroform solution		110	—

B. Influence on the film structure of the amount of liposomes spread per unit surface

All previously described experiments were performed under conditions where surface pressure remained zero. However, when large quantities of liposomes were spread on a given area, the surface pressure raised progressively and then stabilized after 15 to 20 min. As shown in Fig. 1, the relative amount of glucose trapped at the surface and the amount of phospholipid recovered from the surface increased when increasing amounts of multilamellar liposomes containing glucose were spread at the air/water interface.

However, in the results presented in Fig. 1, the surface pressure varied with time and from one experiment to another. In order to study more precisely the

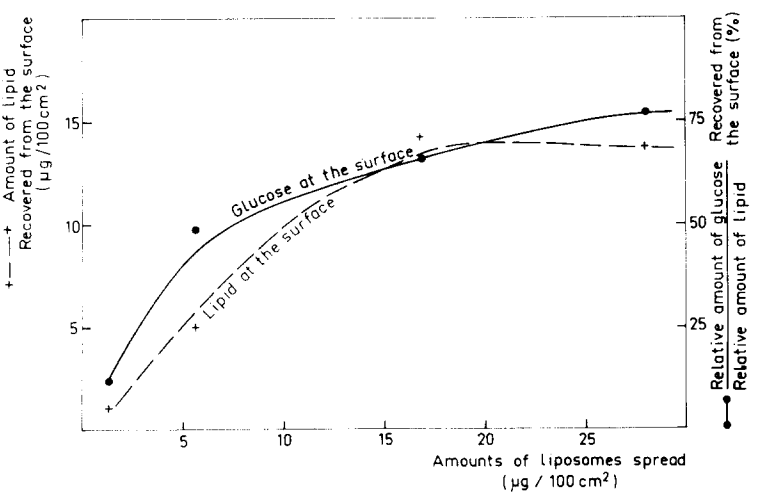

Fig. 1. Influence of the amount of liposomes spread per unit surface on the amounts of lipid and glucose recovered from the surface. The multilamellar liposomes suspension (10 mg/ml) entrapping [^{3}H]glucose (10^7 cpm/mg lipid) was made from egg phosphatidylcholines containing, 5% by weight, dicetyl phosphate. The specific radioactivity of the liposomes, labelled with trace amounts of ^{14}C, ——— algae phosphatidylcholines, varied from 5 to $20 \cdot 10^5$ ^{14}C cpm/mg of lipid. The observed rise in surface pressure stabilized 15 to 20 min after spreading to the value of: 0, 0.5, 7 and 5 dynes/cm for 1.3, 5.6, 16.9 and 28 μg of liposomes spread per 100 cm^2, respectively.

influence of surface pressure, the spreading of the same type of liposomes was performed at constant surface pressure (10 dynes/cm) by using the barostat [16]. Only 25% of the lipid material was recovered from the surface. 25% of the glucose initially present inside the liposomes was also associated with these recovered lipids. This experiment shows that when the liposomes are spread against a surface pressure, they retain their internal content at the air/water interface.

As mentioned previously, only a fraction of the total liposome material spread at the air/water interface. Among the liposomes which dipped through the interface, the glucose present outside and inside the liposomes was estimated by the technique described under Materials and Methods. After spreading multilamellar liposomes containing glucose at constant surface pressure (10 dynes/cm), about one third of the liposomes found in the aqueous subphase had lost their internal glucose. When very large quantities of liposomes were spread on a given area, in order to reach the collapse pressure of the phosphatidylcholine film, about half the liposomes collected in the subphase lost their internal content. This indicates an increased permeability of the osmotic barrier of the liposomes when they dipped into the subphase after crossing the interface.

C. Lipid segregation during liposome spreading

During all previously described experiments, we only measured an overall spreading yield (maximal value 79%) which reflected a mean behaviour of the inner and outer liposome layers. These experiments could not be used to discriminate the particular behaviour of each layer. It is generally admitted that in unilamellar liposomes there are 65% and 35% of the phospholipid molecules on the outer and inner monolayers respectively [14,15]. It is thus possible to imagine that the two layers spread differently at the air/water interface. In order to check this possibility we used the specific property of the phosphatidylcholine exchange protein to transfer and randomize the phosphatidylcholine molecules exclusively located on the outer layers of membranes [13,17]. We prepared double-labelled liposomes having an asymmetrical distribution of 2 isotopically labelled phosphatidylcholines. One isotope, ^{3}H, was exclusively located on the outer layer and the other isotope, ^{14}C, was present on both layers. After spreading these doubly-labelled liposomes, the percentage of each isotope recovered from the surface was determined and the spreading yields of each layer calculated (for details see Materials and Methods). Table II shows the spreading yields at zero surface pressure of the inner and outer layers. Independently from the fatty-acid pattern of the radioactively labelled phosphatidylcholines used, we observed a better spreading of the phosphatidylcholine molecules located on the outer layer as compared with the phosphatidylcholines present in the inner layer.

In control experiments, we have found identical spreading yields of the two radioactively labelled phosphatidylcholines after randomization of the labels by solvent extraction of the lipids and preparing new liposomes from this extract. These results strongly suggest that in the liposome structure the topological localization of a phosphatidylcholine molecule will influence its spreading properties at the air/water interface.

TABLE II

SPREADING YIELDS, AT ZERO SURFACE PRESSURE, OF THE INNER AND OUTER LAYERS OF UNILAMELLAR LIPOSOMES CONTAINING TWO DIFFERENT ISOTOPICALLY LABELLED PHOSPHATIDYLCHOLINES

In the first 4 experiments (I–IV) liposomes were prepared from egg lecithins containing, 5% by weight, dicetyl phosphate and trace amounts of [^{14}C]labelled algae phosphatidylcholines. The phosphatidylcholine exchange protein was used to label specifically the outer layer of these liposomes with ^{3}H-labelled phosphatidylcholines prepared from rat liver microsomes. In experiment number V, the double-labelled liposomes were prepared from ^{3}H- and ^{14}C-labelled phosphatidylcholines obtained from liver microsomes of two rats (for details, see Materials and Methods). Control experiments were carried out by spreading liposomes with the two radioactively labelled phosphatidylcholines randomized between the inner and outer layers. This randomization was achieved by $CHCl_3$/MeOH (2/1) extraction of the liposomes used in experiments I—V and preparation of new liposomes. The values of the spreading yield of the inner layer were obtained by the use of the formula given under Materials and Methods.

Experiment number	Amount of phosphatidylcholines exchanged (% of total phosphatidylcholines from the acceptor) (C)	Spreading yield of the outer layer (%) (A)	Spreading yield of the inner layer (%)	Ratio of spreading yields (outer/inner)
I	4.3	23.5	11.1	2.1
II	5.8	50.2	34.6	1.5
III	11.9	31.2	13.6	2.3
IV	49.7	40.2	30.6	1.3
V	29.6	21.6	14.3	1.5
Control (mean of 3 experiments)				1.0

Because the outer layer spreads with a higher yield than the inner layer, it was of interest to investigate the asymmetrical distribution of different lipids across the phospholipid bilayer of mixed liposomes. Mixed liposomes (uni- or multilamellar) made from ^{14}C-labelled phosphatidylcholines and containing trace amounts of [^{3}H]cholesterol or [^{3}H]oleic acid were spread at zero surface pressure. 20 min after spreading the cholesterol-containing liposomes, we recovered from the surface 0.4-times less cholesterol than phosphatidylcholines, whereas in the other type of liposomes 1.9 times more oleic acid than phosphatidylcholines was recovered. It is rather unlikely that this behaviour reflects a difference in tensioactive properties of the lipids used, because fatty acid soaps are much more water soluble than cholesterol and phosphatidylcholines. Furthermore, in a control experiment, the same lipid mixtures were spread from a total chloroform extract of the above used liposomes and as much cholesterol and oleic acid as phosphatidylcholines was recovered from the surface. A possible explanation of this behaviour of the mixed liposomes during spreading is to assume an asymmetrical distribution of the cholesterol and the oleic acid among the phospholipid bilayer.

Discussion

From the results presented in this paper, it seems clear that two types of films are formed when liposomes are spread at the air/water interface. A drastic shuffling of the phospholipid bilayer must occur during spreading at zero surface pressure and with the internal content of the liposomes being released into

the aqueous subphase. The thin water-film wetting the glass rod probably favors the contact between the liposomes and the air/water interface. The compression isotherm obtained from spread liposomes is very similar to the usual isotherm given by the same phospholipids spread from a chloroform solution. Consequently, at zero surface pressure, there is a slow transformation of the closed bilayered structure into a lipid monolayer. This process can be seen as a fusion of a bilayer at the air/water interface. In contrast, when multilamellar liposomes were spread against a surface pressure, they retained a part of their internal content at the air/water interface. This can be explained by the formation of multilamellar structures as schematically illustrated by Fig. 2. Among the liposomes which dipped into the subphase after crossing the interface, a significant fraction had lost their internal content, whereas the remainder was apparently intact. The relative importance of the above fractions depends upon the value of the interfacial energy (surface tension). Several hypotheses can be formulated to explain this fact. Either a fraction of closed multilamellar liposomes is destroyed giving open 'myelin figures' with a simultaneous leakage of the glucose initially entrapped into the liposomes or the closed structure is preserved during the passage across the interface with a transient increase in permeability and a partial loss of the internal glucose.

By spreading, at zero surface pressure, doubly-labelled unilamellar liposomes having an asymmetrical distribution of 2 isotopically labelled phosphatidyl-

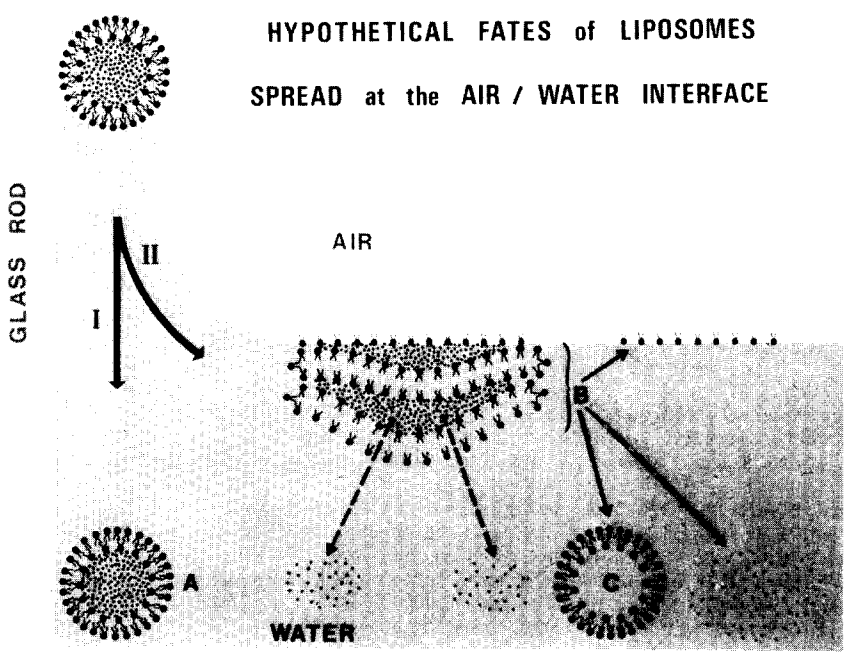

Fig. 2. Hypothetical fates of liposomes spread at the air/water interface. The arrow (I) indicates the flow of liposomes (A) which dipped through the interface. The arrow (II) indicates the flow of liposomes which form a multilamellar structure (B). Among the liposomes which dipped through the interface, a faction (C) loses its internal content, as shown by the dashed arrows. At zero surface pressure, there is a slow transformation of the closed multilamellar structure into a lipid monolayer.

cholines, it is clear that the outer layer spreads with a better yield than the inner layer. We can imagine this process occurring at the interface as a preferential peeling of the outer surface of an onion-like structure. If, in mixed liposomes made from phosphatidylcholines and other lipids, there is an asymmetrical distribution of these lipids across the bilayers, we must expect to recover from the interface relatively more lipid initially located in the outer layer and relatively less lipid from the inner layer. Accordingly, it seems probable that the cholesterol is mainly located on the inner liposome layer and the oleic acid on the outer layer. The same conclusion was reached by Huang et al. [18] and De Kruyff et al. [15]. These authors found that above 30 mol% cholesterol the distribution of lipid across the bilayer of all vesicles becomes asymmetrical with a disproportionately larger amount of cholesterol present in the inner monolayer. NMR has also been successfully used for the determination of the location of phospholipids in model membranes. However, this method is unsuitable or inaccurate when no signals of the polar head group of the lipid can be observed in mixed liposomes. The chemical and enzymatic methods, which have been used for the measurements of the distribution of phospholipids across the membrane, are less suitable for the determination of the location of lipids with the polar head group difficult to attack chemically or enzymatically. The spreading technique, which requires only double radioactive labelling, may be used to determine the asymmetrical distribution of such lipids across bilayers. In the following paper, we will see that part of the results of the present study can be generalized to the more complicated case of the biological membranes.

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