PHASE SEPARATION AND DOMAIN INTERACTION IN PHEOPHYTIN CONTAINING MONOLAYERS

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Résumé. — On a mesuré simultanément la pression de surface et l'intensité de fluorescence des couches minces monomoléculaires qui se composent du lipide contenant de la phéophytine à la surface de séparation de l'eau et de l'air.

Nous avons trouvé une séparation de deux phases différentes. La phase 1 en domaines de phéophytine, par contre la phase 2 est composée d'un seul domaine de lipide contenant 2 à 15 % de phéophytine dépendant de l'état du lipide.

La transition des domaines phéophytiniques est décrite par un modèle qui explique correctement la dépendance observée de la pression de transition π_k de la concentration. L'analyse des résultats prouve que les domaines phéophytiniques sont étendus à deux dimensions et qu'ils sont de grandeur limitée (plus petites que 1 000 molécules).

Les domaines sont stabilisés par des forces stériques provenant des environs. Nous avons calculé le changement des interactions pendant la transition de phase et nous avons obtenu le résultat suivant : $0{,}6 \times 10^{-13}$ erg par molécule au milieu et $1{,}2 \times 10^{-13}$ erg par molécule au bord du domaine.

Abstract. — Surface pressure and fluorescence intensity of pheophytin containing lipid-monolayers are measured simultaneously at the air-water interface. A phase separation into two distinct phases is reported. Phase 1 exists of pheophytin domains whereas phase 2 is a lipid domain containing pheophytin in molar content between 2 % and 15 %, depending on the state of the lipid.

The transition of the pheophytin domains is described within a model explaining correctly the observed concentration dependence of the transition pressure π_k. The analysis shows that the pheophytin domains are two-dimensional and of limited size (smaller than 1 000 molecules). The domains are stabilized by their environment by steric forces. The change of the interactions during the phase transition is calculated. It amounts to 0.6×10^{-13} erg per molecule in the bulk of the domain and to 1.2×10^{-13} erg per molecule at the domain wall.

1. **Introduction.** — There is increasing evidence that the chloroplast thylakoid membrane contains the chlorophyll molecules in a non-random distribution [1, 2]. This is quite important since the functioning of the photosynthetic unit critically depends on the kind of molecular arrangement within the membrane [3]. In order to get structural information it is therefore of interest to study interactions between chlorophyll molecules and lipids, proteins, water or other chlorophylls.

Mixed monolayers containing chlorophylls have proved one of the most well-defined model systems to study these interactions [4, 5, 6]. We investigated these systems containing the lipid α-dimyristoyllecithin (DML) and pheophytin in a different relative amount [7]. The pheophytin fluorescence and the surface pressure were measured simultaneously as a function of molecular area at the air-water interface. This enabled us to detect and to characterize pressure induced phase changes of the monolayer [8]. It also enabled us to establish phase separations on the monolayer surface that will be briefly reviewed. The purpose of this work is the presentation of a model to describe domain interactions and will draw conclusions with respect to the type of interactions and the shape of the domains.

2. **Results and discussion.** — 2.1 PHASE SEPARATION BETWEEN PHEOPHYTIN AND DIMYRISTOYLLECITHIN. — Very recently we could establish a phase separation of the mixed monolayer into two distinct types of domains [8]. Domain (1) exists almost exclusively of pheophytin molecules whereas domain (2) exists of DML molecules solubilizing pheophytin in a concentration X_0, its solubility limit. X_0 was determined as 15 ± 5 mole % in the fluid DML phase and was supposed to decrease to only a few mole % on transition from the fluid to the solid phase. The phase separation was proved from the fluorescence as well as from the pressure data.

An example for these measurements gives figure 1 that shows the surface pressure *versus* area diagrams for monolayers containing DML and pheophytin in varying amount. For pheophytin concentrations below 10 mole % one clearly observes the DML main transition at a pressure between 14 and

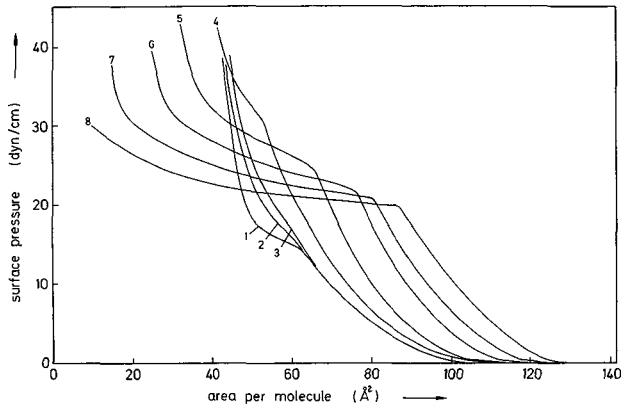

FIG. 1. — Surface pressure *versus* area diagram of DML monolayers containing pheophytin in different concentrations X. Temperature 8 °C. (1) : $X = 0$ mole %, (2) : $X = 4$ mole %, (3) : $X = 10$ mole %, (4) : $X = 20$ mole %, (5) : $X = 40$ mole %, (6) : $X = 60$ mole %, (7) : $X = 80$ mole %, (8) : $X = 100$ mole %.

20 dynes/cm, whereas for larger pheophytin concentrations the pheophytin main transition is clearly observed. It is important to note that the pressure corresponding to the latter transition strongly depends on concentration. The model that we will subsequently present describes this concentration dependence of the pheophytin transition more quantitatively.

2.2 THE PHEOPHYTIN MAIN TRANSITION. — Figure 2 gives data on a pheophytin monolayer that are relevant with respect to a physical interpretation of the phase transition. The pressure *versus* area diagram (curve 1) changes abruptly at an area of 87 Å²/molecule to become nearly horizontal at smaller areas. At an area below 45 Å²/molecule the slope increases again, but remains finite for even the smallest areas measurable with our film balance (10 Å²/molecule). The simultaneously recorded fluorescence intensity (at 680 nm) changes drastically at an area of 130 Å²/molecule which is probably due to an unwrapping of pheophytin from water molecules. The main transition is only slightly reflected as a change in the slope (arrow 4 in figure 2). A more pronounced change in the slope of the fluorescence *versus* area curve occurs at an area of about 45 Å²/molecule (arrow 5). It is remarkable that, although the molecular density is doubled in going from arrow 4 to arrow 5, the fluorescence intensity of the layer does hardly increase. This indicates that a change in the area between these two points does not involve a change in the density of fluorescing molecules. It can be understood if during the pheophytin transition the molecular density of the fluorescent surface layer remains unchanged. Hence on further increasing the pressure molecules are pushed into the subphase where they do not fluoresce.

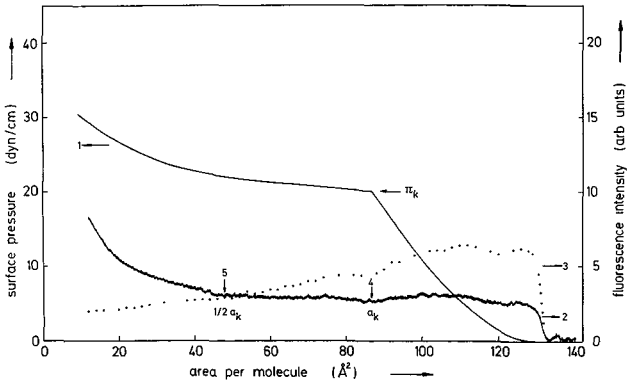

FIG. 2. — Surface pressure (1) and fluorescence intensity J (2) as a function of area for a pheophytin monolayer. $T = 8$ °C. Curve (3) represents the product Jx molecular density.

There are also other facts showing that during the transition pheophytin molecules are pushed into the subphase, still interacting with the surface layer :

1) Curves 1 and 2 in figure 2 are reversible, reproducible and do not show any discontinuities, as to be expected for an unstable layer.

2) The absorption spectrum measured by Bellamy *et al.* [4] for their so-called unstable region shows an additional band near 700 nm. This band has to be expected for pheophytin aggregates which are supposedly formed below the surface [9].

3) At a pressure of 30 dynes/cm the area per molecule in figure 2 is less than 10 Å². This is much less than the area needed by a pheophytin molecule.

4) Figure 3 gives the area per molecule as a function of concentration at a pressure of 40 dynes/cm. The measured points are rather close to line II. This shows that the area is essentially determined by the fraction of DML molecules within the layer. Hence only a small part of pheophytin molecules remains within the surface layer at this pressure.

2.3 QUALITATIVE MANIFESTATION OF THE DOMAIN INTERACTION. — During the previous two sections we have established the existence of two types of domains within the monolayer surface and we gave some interpretation of the transition of the pure pheophytin monolayer. The latter is expected to hold also for the transition of the pheophytin domains (1) in the mixed monolayer. We now turn to the main topic of this work, the interaction between the domains. In our understanding a domain exists of a regular arrangement of molecules, in the environment the molecules are arranged differently.

There are essentially two experimental facts proving that there is an interaction between a pheophytin domain and its environment and that this interaction tends to stabilize the pheophytin surface layer :

1) The pheophytin solubility in the DML phase was estimated to about 2 mole % [8]. However, at a pressure as large as 40 dynes/cm the area per molecule increases with pheophytin concentration in the concentration range between 2 and 10 mole % (Fig. 3). This shows that pheophytin present in excess of 2 mole % is not completely segregated into the

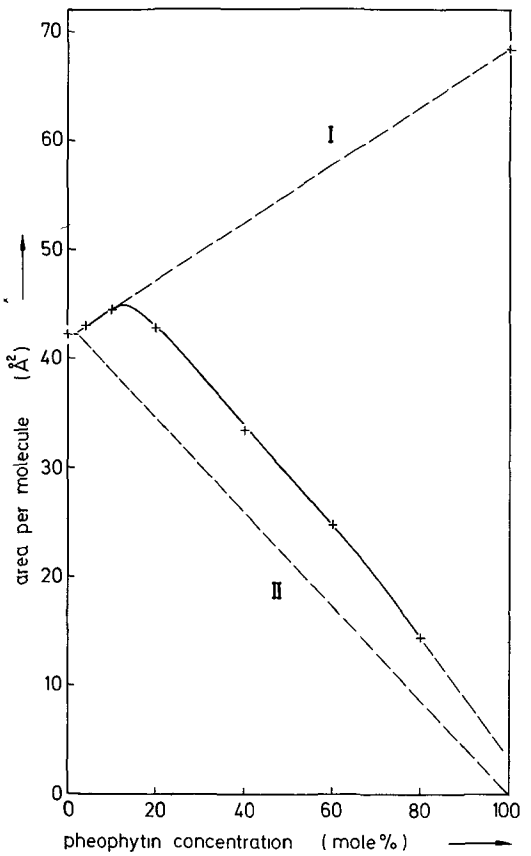

FIG. 3. — Area per molecule for a surface pressure of 40 dynes/cm. Line II gives the area of the solidified DML domains containing 2 mole % of pheophytin. The measurements would obeye line I, if the pheophytin domains would remain in the surface layer. The area of these domains was calculated from an extrapolation of curve 8 in figure 1 to higher pressures (see footnote in the text).

water phase. Thus there exist pheophytin domains within the surface layer and these domains are also stable at a pressure well above the transition pressure π_k of the pure pheophytin monolayer

$$(\pi_k = 20 \text{ dynes/cm}) .$$

The domains of the mixed monolayer are obviously stabilized by their environment.

2) The pressure π_k increases with increasing DML concentration. This can be understood if the domain interaction depends on concentration. In the next section we will describe a model that gives a physical picture of this concentration dependence.

2.4 MODEL FOR THE QUANTITATIVE EVALUATION OF THE DOMAIN INTERACTION. — We consider a pheophytin domain containing N molecules, of which N_w form the domain wall. The free energy G of the domain in two states (1) and (2) is then given by the equation

$$G(1) = f_i^{1)} . N + (f_w^{1)} - f_i^{1)}) N_w^{1)} + \pi^{1)} a^{1)} N \quad (1)$$

$$G(2) = f_i^{2)} . N + (f_w^{2)} - f_i^{2)}) N_w^{2)} + \pi^{2)} a^{2)} N \quad (2)$$

where $\pi^{i)}$ is the pressure, $a^{i)}$ the area per molecule, $f_i^{i)}$ the interaction energy per molecule in the interior, $f_w^{i)}$ the interaction energy per molecule at the wall in state i.

We now assume a transition from state (1) to state (2) specified by the following conditions :

a) $\pi^{1)} = \pi^{2)} = \pi_k$. The transition occurs at a constant pressure. This corresponds to a horizontal surface pressure *versus* area diagram and is reasonably well fulfilled in figures 1 and 2. Under this condition the enthalpy is also conserved, i.e. $G(1) = G(2)$

$$a^1 = 2\, a^2 = a_k .$$

b) $a^1 = 2\, a^2 = a_k$. We define state (2) as the state where the molecular area of the domain amounts to half of its value in state (1). This definition was used since between a_k and $1/2\, a_k$ (arrow 4 and 5 in figure 2) the surface pressure in figures 1 and 2 does not change significantly and since near an area of $1/2\, a_k$ the slopes of surface pressure and fluorescence curves change significantly.

c) $N_w^{1)} = N_w^{2)}$. The assumption of identical numbers of barrier molecules is not necessarily valid in the system under investigation. It holds if the monolayer bends and forms ripples as is observed from electron micrographs of vesicles [10]. One may also imagine changes where an ordered structure in the subphase builds up [8]. In that case a relation $N_w^{1)} = cN_w^{2)}$ is expected, c being a constant of the order of unity. Using this more general relation would somewhat complicate the calculation and change the numbers finally presented, but it would not qualitatively alter our calculation.

With these assumptions we obtain from eqs. (1) and (2)

$$1/2\, \pi_k\, a_k = (f_i^{2)} - f_i^{1)}) + \\ + [(f_w^{2)} - f_w^{1)}) - (f_i^{2)} - f_i^{1)})] \frac{N_w}{N} . \quad (3)$$

If the domain size is rather small the number of molecules near the barrier is not negligible with respect to the total number of molecules within the domain. Therefore the second contribution in equation (3), due to changes in internal energy of barrier molecules, is also important. It is essentially this term that determines the observed concentration dependence of the transition pressure π_k.

d) For a twodimensional domain the relation

$$\frac{N_w}{N} \propto \frac{1}{\sqrt{N}}$$

generally holds. For simplicity we assume a circular domain for which we obtain the relation

$$\frac{N_w}{N} = \frac{2\sqrt{\pi}}{\sqrt{N}} . \quad (4)$$

e) Whereas equation (3) gives a relation between transition pressure π_k and domain size, the measurements yield π_k as function of concentration. Therefore we have to connect domain size and concentration. This is done by using a model that holds to describe phase separation on lipid bilayer vesicles : we assume a constant number of pheophytin domains in the concentration range 20 mole % $\leqslant x' \leqslant$ 80 mole %, hence the domain size depends on concentration according to :

$$N = x' . N^0 \tag{5}$$

$$x' = x - \frac{x_0}{1 - x_0}(1 - x)$$

is the concentration of pheophytin that is not dissolved within the DML domains ($x_0 \approx 2$ mole %).

N^0 represents a maximum domains size which for monolayers and vesicles is estimated to be some hundred [11-13].

From equations (3), (4) and (5) one finally obtains a relation between the volume work done during the transition and the pheophytin concentration x'

$$\pi_k a_k = 2(f_i^{2)} - f_i^{1)}) + \\ + [(f_w^{2)} - f_w^{1)}) - (f_i^{2)} - f_i^{1)})] \sqrt{\frac{\pi}{N^0}} \cdot \frac{4}{\sqrt{x'}}. \tag{6}$$

This is exactly the functional dependence that is observed in the experiment and is represented in figure 4 ([1]). This figure is the highlight of our work giving rise to the conclusion discussed in the next paragraph.

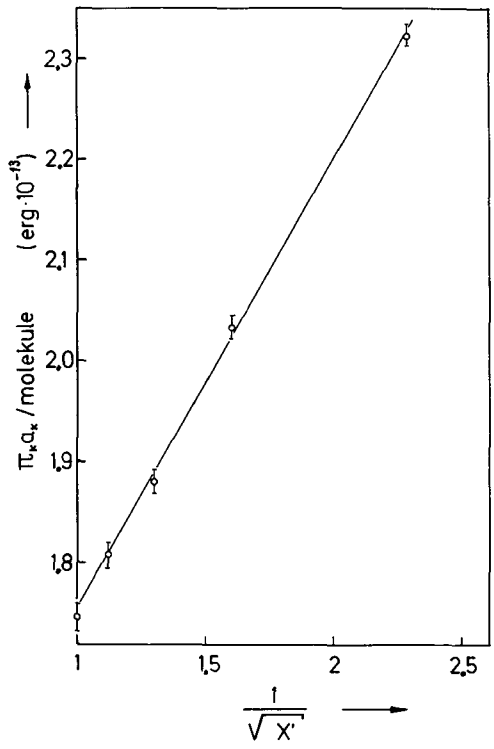

FIG. 4. — Volume work performed during the pheophytin transition as a function of pheophytin concentration x'.

3. **Conclusions.** — The model describing the domain interaction depends on the assumptions *a*)-*e*) just discussed. Some of these assumptions are necessary only for a quantitative evaluation of the data but some of them are crucial for the applicability of the model. On the other hand, we think that the validity of equation (6) also proves the validity of its basic assumptions and leads to the following conclusions :

1) An appreciable part of the number N of molecules within the domain are near the barrier. This indicates a domain size not exceeding 1 000 molecules. Otherwise the second term in equation (6) could be neglected, leading to a constant transition pressure π_k.

2) The domains are twodimensional. If they existed of single onedimensional arrays every molecule of the domain would also be one of the barrier.

($N = N_w$). Insertion of this identity into equation (3) would also result in a constant transition pressure π_k. Hence if the pheophytin molecules are stacked in arrays, as is expected from X-ray data of pheophorbides [9] and from optical measurements and exciton calculations [1, 2], these arrays are arranged in a twodimensional lattice within the domain. This result is also of interest with respect to the discussion of the chlorophyll arrangement on the photosynthetic membrane [1-3].

3) The assumption of a domain number independent of concentrations and a concentration dependent domain size seems to be valid on the monolayer surface too. An alternative picture regarding a concentration independent domain size does obviously not hold. It would lead to a concentration independent transition pressure.

4) An *a priori* not anticipated result is also that even the measurement of the pure pheophytin monolayer obeyes the straight line of figure 4. This indicates that with respect to the interaction between domain and environment it does not matter whether the latter is composed of pheophytin or of DML molecules. This indicates that the contributions of the steric forces dominate those of the chemical forces.

5) From slope and intercept of the line in figure 4 one can further calculate the energy changes per

([1]) In the derivation of figure 4 the area per pheophytin molecule a_k within a pheophytin domain had to be determined for different pressures π_k. This was done by extrapolating the pressure *versus* area curve 8 of the expanded state of figure 1 to higher pressures as if the phase transition at 20 dynes/cm would not occur. This is somewhat arbitrarily, but one estimates the error induced by this procedure to only about 2 %.

molecule involved in the transition. One obtains for the change in energy per molecule in the bulk of the domain :

$$f_i^{2)} - f_i^{1)} = 0.6 \times 10^{-13} \text{ erg} . \quad (7)$$

To calculate the changes in the interaction energy of molecules at the domain wall one has to know N^0, the maximum number of molecules per domain and the shape of the domain (see assumptions *c*) and *d*)). Therefore we can only give a rough estimate of this value. Assuming a realistic number $N^0 = 100$ [10, 12] one obtains from equation (6) and from the slope of figure 4 :

$$(f_w^{2)} - f_w^{1)}) - (f_i^{2)} - f_i^{1)}) = 0.6 \times 10^{-13} \text{ erg} . \quad (8)$$

Hence

$$f_w^{2)} - f_w^{1)} = 1.2 \times 10^{-13} . \quad (9)$$

The latter value is supposed to be accurate to only a factor of two. However, the positive slope of the line in figure 4 shows that according to equation (8) the value is larger than that given by equation (7). This again reflects the fact that it is the interaction at the domain walls that stabilizes the monolayer surface : more energy is needed to transfer a molecule at the wall from state (1) into state (2) than to transfer a bulk molecule into the area below the surface.

Secondly the values given by equations (7) and (9) differ by less than a factor of five. This is conceivable if the forces responsible for the interactions at the wall and in the interior are of the same type.

Acknowledgment. — Helpful discussions with Profs. E. Sackmann and H. Gruler are greatfully acknowledged. We also appreciate the technical assistance of Mr. U. Theilen and Mr. A. Kuttruff.

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