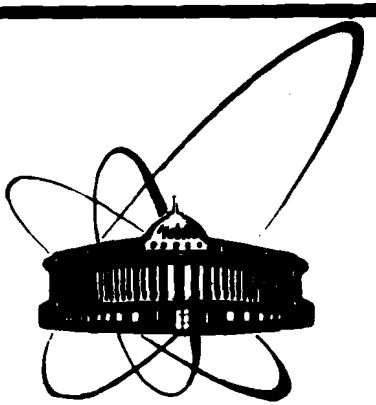

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DETECTION OF STRUCTURAL DEFECTS IN LECITHIN MEMBRANES BY SMALL-ANGLE NEUTRON SCATTERING. THE CLUSTER MODEL OF A LIPID BILAYER

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1. Introduction

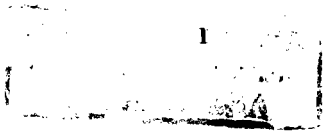
The "fluid-mosaic" model of a biological membrane has been postulated due to the efforts of many researchers [93]. It is based on the Gorter and Grendel idea [38] of a "liquid" bimolecular lipid layer (lipid bilayer) which surrounds any cell. Extensive regions of a "liquid" bilayer are also the important components of any membrane structures described by more perfect models [51, 93] including those taking into consideration the protein "frameworks" (cytoskeleton)[60].

The lipid bilayer parameters, the "average" conformations of lipid molecules in bilayers and their dynamics are well characterized by numerous physical-chemical methods [1,14,15, 19-22, 26, 35, 45-47, 50, 51, 54, 63, 86-89, 26, 109-112] but the arrangements of molecules and their interactions are still the object of the discussion due to high conformation flexibility of lipid molecules in "liquid" bilayers. The model of a homogeneous hydrocarbon matrix similar to a liquid paraffine layer has widely been used up till now [29, 31, 45, 47, 63, 79], although this approximation is very rough and the behaviour of even a simple molecule as water or n-alkanes in bilayers differs from that in isotropic hydrocarbone liquid essentially [57, 79, 91, 92, 103, 107, 108].

In 1974 Lee et al. [61] suggested the hypothesis of "liquid" clusters in bilayers to explain the discontinuity in Arrhenius plots of the ESR spin-probe distribution between water and dioleoylphosphatidylcholine (DOPC0). Clusters in a physical-chemical sense were pictured as "short-living more density pasked arrangements of molecules within the environment of freely dispersed molecules" [61]. They are found in bilayers of both homogeneous and heterogeneous compositions in spite of the fact whether there is or there is not some molecular segregation into homogeneous pools for the latter.

Recently Bashford et al. [5] have used the analogous approach for explanation of the fluorescent probe behaviour in membranes. In principle the term "cluster" is widely applied to numerous fields of the science especially in chemistry [43].We use it in the same sense as in [61] unlike other authors using it, for example, in the meaning of the new phase nuclei in the phase transition region [32] or simply for pools of homogeneous lipids in mixtures [19, 33]. Obviously the term "composition cluster" must be used for the latters[33].

It is obvious that clusters, in our comprehension, are similar to "cybotaxic groups" (regions of a partical order) in liquids suggested by Stewart in 1928 to explain X-ray diffraction pictures for liquid hydrocarbons [34, 94]. Segerman [90] was an apparently one of



the first who postulated clusters in biological membranes and had roughly estimated their dimensions from the X-ray peak sharpness. For the erythrocite "ghosts" he estimated the average cluster diameter 24Å along a bilayer plane /90/ which coincides surprisingly well with our results described below.

The existence of clusters follows inevitably from the collective tilting of chain "segments" adjacent to polar head groups of lipids with respect to the bilayer normal if we take into consideration the fact that the partial specific volume a "liquid" is only 2-4% more than that for a solid one /27,73,75/ where the fully extended alkyl chains are packed closely /45, 47, 50, 54, 63, 88/.

The collective tilting of chains has been postulated by McFarland and McConnell /71/ on the basis of the ESR spin-label behaviour in the egg lecithin (EPC) oriented multilayers and it has been confirmed by more recent researches /7, 39/. This idea has been used in /76/ to describe molecular arrangement in lipid monolayers at oil-water interfaces. The collective tilt of chains exists only on the time scale less than 10^{-6}s as it follows from the axial symmetry of the chain motion respectively to the normal to the bilayer surface observed for multilayers of lecithins ^2H - labelled in the C-5 position of the acyl chains by the ^2H-NMR method /87, 89/.

In principle the NMR data may be explained without any collective chain tilt /89/ but the characteristic "plateau" for order profile and relaxation time /2, 10, 13, 19-23, 26, 45, 62-64, 69, 71, 72, 78, 80, 82, 86-89, 110/ does not contradict this model by no means. On the contrary the pure "pink" model /99/ which proposes the motion of gauche-trans-gauche(g^- - t - g^+) conformers along chains preferentially oriented along normal is not in accordance with the properties of a fluid lipid bilayer /87, 89/.

The hetorogenity of a hydrocarbon bilayer interior along the normal direction must also be a result of the collective tilting in the first half of the acyl chains (C3-C9). It has been suggested in /1/ exclusively on the basis of the structural features of lipid molecules. The similar hypothesis has been suggested from the analysis of the solubility of different substances in membranes /57/ and of the black film conductivity /103/.

The cluster model predicts essentially the existence of defects in the molecular arrangement between adjacent clusters. Defects can be sharp or "melted" but they exist without fail /53/. Two kinds of defects - static and dynamic were postulated by D.L.Rubinshtein in 1947 /83/: "... some analogy of pores can exist in a fluid membrane,

having the thickness of the molecular size, as a result of the dynamic equilibrium between more ordered and partially disordered regions of a monomolecular layer... Thus the pores are static structures in the solid molecular layers and dynamic sructures in the fluid ones". Up to the recent time most of the researchers took into consideration only one kind of defects - the "kinks" /99/ which would explain only the motion of small molecules and ions across a bilayer. To explain these processes for large molecules "the solution" was postulated without any detailed analysis of its mechanism. The first attempt to describe qualitatively the nature of the bilayer permeability through the transient defects which appears as a result of the thermal fluctuations has been made in /25/. Two kinds of dynamic defects in a bilayer have been suggested: "i-defects" at a membrane-water interfase and "m-defects" in a hydrocarbon interior. The latter denotes all inner defects capable to accomodate water or other single small molecules and ions /25/.

The semiquantitative cluster model of a "liquid" lecithin bilayer was suggested in 1982 /51/. In the frames of this model the average interchain distance in clusters /50, 51/, the average cluster dimensions along and across the bilayer surface /51, 52/, the probable geometry, sizes and apparent free energy of the defects /49, 51/, the own area of hydrated polar head groups at a bilayer surface /49/ have been estimated. The statistical-mechanical calculations show that the cluster model could explain the experimental parameters of gel-liquid crystal phase transitions /48/.

The purpose of the present paper is to make an attempt to describe quantitatively the structural organisation of a clustered lipid bilayer on the basis of all known experimental data including a recent study of the cluster parameters by the small - angle neutron scattering /6/.

2. Elements of Theory

The small-angle scattering of X-rays and neutrons is a method for the observation of some inhomogeneities of atomic composition or packing /63, 77, 95, 97/. The scattered intensity I depends on the scattering vector $\vec{q} = \vec{K} - \vec{K}_0$, where $\vec{K}$ and $\vec{K}_0$ are the wave vectors before and after the scattering. Extrapolation of the scattered intensity to the zero scattering vector length $q = 4\pi \sin(\theta/2)/\lambda$ (where θ is the scattering angle and λ is the wavelength) gives the forward scattered intensity $I(0)$ per unit volume of the sample:

$$I(0) = I_e \; n \; (\rho - \rho_s)^2 \; V^2 \quad (cm^{-1}\ sterad^{-1}) \qquad (1)$$

where I is the incoming beam intensity, n is the number of inhomogeneities per unit volume of the sample, V is the volume of a single inhomogeneity and $\rho - \rho_s$ is the difference of the scattering length densities of the inhomogeneity under consideration and the one averaged over the volume of the whole sampe. This difference is often called a contrast. Eq. (1) is a reasonable approximation at $nV \ll 1$ and it allows one to estimate any part of the right-hand side quantity of the intensity I(0) is adequately compared with I (the scattering crossection is put on to absolute scale).

The scattered intensity depends stringly on the kind of the radiation used (X-rays, neutrons, electrons) and in the case of neutrons - on the isotope composition of a sample. The important case is the ^1H to ^2H substitution which increases greatly the contrast for neutrons. Supposing the defects in a multilayer system are empty or filled with heavy water ^2H_2O, the ratio of the squared contrast for neutrons and X-rays can be easily calculated /6/; the results are given in Table 1. From this one concludes that neutrons would be preferred for observation of heavy water filled defects embedded in hydrocarbon medium as it is expected in wetted multilayers.

Another observable quantity which characterises the inhomogeneity follows from the slope of $\ln I(q)$ vs. q^2 plot (the so-called Guinier plot) which is called the radius of gyration. In the case under discussion (oriented multilayers and scattering vector directed along the bilayer surface) the intensity is given by

$$I(q) = I(0) \cdot \exp\ (-\ q^2\ R_g^2/2), \qquad (2)$$

where

$$R_g^2 = \int (\rho(r) - \rho_s) \cdot r^2\, dV \Big/ \int_V (\rho(r) - \rho_s)\, dV \qquad (3)$$

and the origin is taken in the centre of mass of the density $(\rho - \rho_s)$. Some simple cases will be used hereafter. Assuming the scattering vector perpendicular to the cylinder axis we will get:
for the continuous cylinder of the radius R

$$R_g^2 = R^2/2; \qquad (4)$$

for the annular cylinder with the outer radius R_2 and the inner radius R_1:

$$R_g^2 = (R_1^2 + R_2^2)/2 = \bar{R}^2 + \Delta^2, \qquad (5)$$

Table 1

The caculated ratio of the scattering intensities by defects in the lipid bilayers for neutrons and X-rays /6/

Medium	Defect filling	$(\rho - \rho_s)^2 \times 10^{-20}(cm^{-4})$ neutrons	X-rays	Ratio of intensities neutrons/X-rays
Hydrocarbon	D_2O	45.2	1.44	31.4
chains	Empty	0.116	67.0	$1.7 \cdot 10^{-3}$
Polar head	D_2O	20.8	12.9	1.62
groups	Empty	3.24	169.0	$1.9 \cdot 10^{-2}$

where $R = (R_1 + R_2)/2$ is the average radius and $2\Delta = R_2 - R_1$ is the annulus thickness; for the annulus of the triangular cross section:

$$R_g^2 = R^2 + \Delta^2/2, \qquad (6)$$

where Δ is the half of the triangle base 2Δ.

On the other hand the size of the cluster can be estimated from the width of the diffuse neutron or X-ray "packing" reflex. If a scattering bulk is an ensemble of microcrystals (domains) of the size about L, the full "width" of some diffraction peak at the half of its height $\Delta S_{1/2} \sim 1/L$, where $S = q/2\pi$. It is necessary to emphasize that the "packing" reflex at $q = (4.6\ \text{Å})^{-1}$ is not necessary the Bragg reflection for the crystalline lattice of the hexagonal symmetry as some authors suggested /12, 45, 69, 84, 88/. Its position rather reflects the average distance between the adjacent chains ($\bar{d}$) and it is determined by the chain cross section /50, 52/ analogous l_y to liquid paraffins /94/. The width of the peak depends on the short range order of the molecular arrangement characterised by the correlation radius /102/. If the instant chain distribution is described respectively to their average position by the Gauss function with dispersion δ, the full width of the packing peak is given by /102/:

$$\Delta S_{1/2} = \frac{1}{\bar{d}}\, \pi^2 h^2 \left(\frac{\delta}{\bar{d}}\right)^2, \qquad (7)$$

where h is the order of the Bragg reflex and $\bar{d}$ is the average interchain separation.

The correlation radius[102] is determined as:

$$r_M = \frac{d}{(2.5 \cdot \delta/d)^2} \cdot \qquad (8)$$

Combining (7) with (8) we get

$$r_M \simeq \frac{\pi^2 h^2}{6.25} \cdot \frac{1}{\Delta S_{1/2}} \simeq \frac{1.6 h^2}{\Delta S_{1/2}} \cdot \qquad (9)$$

3. Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC, SERVA) multibilayers were prepared analogously to [4]. 10% ethanol solution of the lipid was on the quarts glass (24 x 24 mm^2) and dried at 70°C which is higher than the temperature of the gel-liquid crystal phase transition. The slow annealing procedure [81] gives highly ordered samples (the FWHM of the rocking curve was about 2°). The dry weight of the lipids in each of the three independently studied and prepared samples was (50 ± 3) mg.

The samples were placed into the hermetic quarts glass container with a suitable salt or solution on its bottom. The relative humidity was RH = 97% (the aqueous solution of K_2SO_4) or RH = 0% (P_2O_5). Measurements were carried out at two temperatures: 23°C (the solid $L_{\beta'}$-phase) and 55°C (the "liquid" $L_\perp$-phase). A test of the state of lipid was carried out from position of the diffraction maxima: S_{max} = $(4{,}2\ Å)^{-1}$ for $L_{\beta'}$-phase and $S_{max} = (4{,}6\ Å)^{-1}$ for $L_\perp$-phase [50, 63]. The equilibration of the samples was examined for more than two hours.

Neutron scattering measurements were done on the time - of - flight small-angle scattering spectrometer at the IBR-2 pulsed reactor [101]. The incoming neutron beam perpendicular to the plane of the sample was collimated to the 22 mm diameter. The mean thermal neutron flux in the sample position was $3.2 \cdot 10^7\ cm^{-2}s^{-1}$. The sample-detector distance was 10.63 m and the range of the observed scattering vector lengths was $0.02 < q < 0.4\ Å^{-1}$. The measurements for any samples were bound with those for the standard scatterer (metallic vanadium) every five minutes. The whole experiment for one sample was being continued for about ten hours. The excess scattering was found by subtraction of the intensity values for "dry" lipid (RH 0%) from the ones for the hydrated lipid (RH = 97%). This allowed one to correct the results not only for the instrument background but for the scattering by the cuvette and by "dry lipid" also. The excess intensity in this case is determined by water introduced into the multilamellar structures.

4. Results and Discussion

The dependence of the excess scattering intensity I(q) on the square of the scattering vector q^2 is shown in Fig.1. The excess intensity is zero for the solid phase, consequently multilayers are homogeneous along the lateral direction. So the excess intensity becomes well detectable for the "liquid" phase.

The excess intensity in the Guinier coordinates (see section 2) is shown in Fig.2. It is well described by the Guinier approximation (eq.2) allowing one to determine the two parameters of the scatterer: I(0) and Rg. These values calculated be the least square method are shown in Table 2 for three samples. The Rg values correlate with each other sufficiently well (in the limit of errors) while the intensity data are more dispersed. The latter may be a result of the errors in the determination of the bilayer weight.

Table 2

Sample	I(0) (cm^{-1})	Rg (Å)	χ^2 per degree of freedom
No 1	0.48 ± 0.06	29.8 ± 2.6	1.1
No 2	0.34 ± 0.01	29.7 ± 6.9	0.5
No 3	0.36 ± 0.04	26.7 ± 3.8	0.8
The weighted average values for three samples	0.36 ± 0.04	29 ± 2	

The following discussion of the results demands the model analysis. We will discuss the two most probable models: the porous membrane and the clustered one. To calculate their structural parameters, the averaged values I(0) and Rg from Table 2 will be used.

To calculate the average number of inhomogeneities per unit of the bilayer area it is necessary to determine the repeat distance value (lamellar spacing), d, which depends on the temperature and relative humidity values. At 55°C and RH = 97% d = 54 Å, which is close enough to 54,1 Å in [112] for DPPC at 50°C and 25 wt % H_2O. The latter corresponds to 13.5 H_2O molecule per DPPC (molecular weight

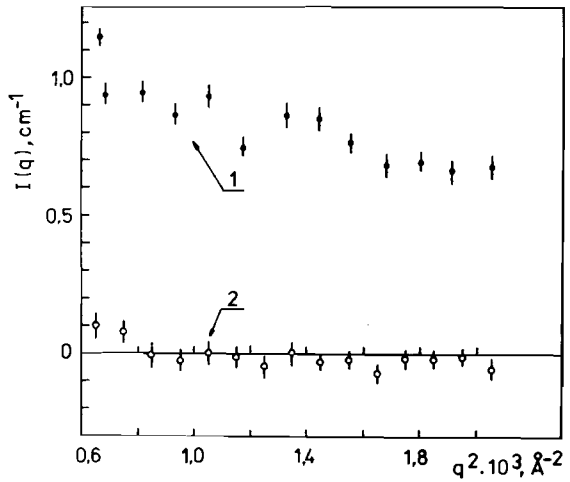


Fig.1. The excess intensity of the small-angle neutron scattering (at multilayer hydration by D_2O) in dependence on q^2($q = \frac{4\pi}{\lambda} \sin\frac{\theta}{2}$). The sample N2 - 8.7 mg.cm^{-2} of multilamellar DPPC structure: 1 - L_α -phase, 2 - L_β -phase.

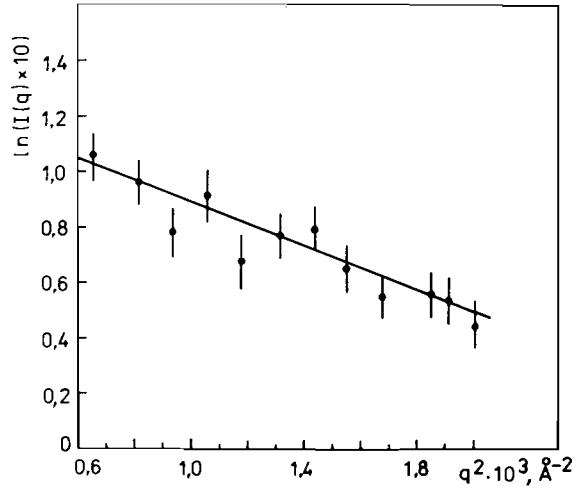


Fig.2. The excess intensity of the small-angle neutron scattering in Guinier approximation. The sample N2, RH = 97%, 55°C, L -phase.

is 733). The "liquid" EPC bilayers absorb 14 H_2O molecules per lipid molecule at RH = 97% /55/, and it can be assumed that the absorption by DPPC bilayers at 55°C does not significantly differ. The similar conditions in the present work and in /15,112/ make it possible to describe the DPPC bilayer more correctly.

4.1. The Model of the Porous Membrane

The defects of the aqueous pore type have been suggested in "liquid" membranes to explain their permeabilities for water /56,58/. If the scatterers are the cylindrical pores inside the hydrocarbon bilayer interior of the thickness ℓ_h their radius and concentration (volume) can be calculated from (1) and (5). The value $\ell_h \simeq 30$ Å according to /15, 112/ and the other parameters are shown in Table 3. The total area fraction occupied by pores, α_s , allows one to calculate the membrane permeability, P , for molecules and ions if their selfdiffusion coefficient D is known. Let us assume that the pore diameter is large enough with respect to the size of water molecule and D can be taken similar to that in water. Then the membrane permeability for water /3,66,79/:

$$P = \frac{D}{\ell_h} \cdot \alpha_s . \qquad (10)$$

The experimentally found permeability of the egg-yolk lecithin bilayers is ~ $8\cdot10^{-3}$ cm·s^{-1} at 37°C and $4.5\cdot10^{-3}$ cm·s^{-1} at 25°C (large vesicles) and $1.7\cdot10^{-3}$cm·s^{-1} at 44°C /29/.

Applying the observed radius of gyration and intensity data to the cylindrical model we are able to calculate all necessary parameters of porous membrane (see Table 3). Combining them with respective values of D ($5.02\cdot10^{-5}$cm^2·s^{-1} at 50°C and $2.5\cdot10^{-5}$cm^2·s^{-1} at 25°C/16/) we can estimate the permeabilities of $160\cdot10^{-3}$ and $80\cdot10^{-3}$cm s^{-1} respectively which surpass the observed values more than for an order of magnitude. The discrepancies up to three orders of magnitude occur if we use the diffusion coefficients (10^{-5} - 10^{-6} cm^2·s^{-1}) and the observed permeabilities (10^{-8}cm s^{-1}, electrical measurement, or 10^{-6}cm s^{-1}, isotopic data /3/) for small ions. Thus we conclude that the detected inhomogeneities cannot be the simplest cylindrical pores piercing a membrane.

Table 3

The parameters of the porous membrane

Pore parameters	Formula	Value
LENGTH	ℓ_h	30 (Å)
radius	$R_p = \sqrt{2}\ Rg$	
volume	$V_p = \pi R_p^2 \cdot \ell_h$	$1.58 \cdot 10^5 (Å^3)$
area	$S = \pi \cdot R_p^2$	$5.3 \cdot 10^3 (Å^2)$
concentration	$n_p = I(0)/(\rho - \rho_s)^2 \cdot V_p^2$	$2.88 \cdot 10^{15} (cm^{-3})$
total volume fraction occupied by pores	$\alpha_v = n_p \cdot V_p$	$4.6 \cdot 10^{-4}$
number of pores per 1 cm^2 of bilayer	$n_s = n \cdot d$	$1.55 \cdot 10^9 (cm^{-2})$
total area fraction occupied by pores	$\alpha_s = n_s \cdot S_p$	$8.2 \cdot 10^{-4}$

4.2. The cluster model

The alternative assumption is that neutrons are scattered by D_2O-"contrasted" defects between the adjacent clusters which pool the hydrocarbon all-trans "segments" adjacent to the polar head groups (Fig.3). These chain segments tilt against the direction perpendicular to the plane of the bilayer as a result of the excess head group area with respect to the cross-section area of two hydrocarbon chains. The flat effective form of a two-chain lipid molecule allows one to think that defects are mainly of the linear from but in the first approximation they can be suggested as annular ones with the triangle cross-section form. To calculate the defect concentration from expression (1) it is necessary to estimate the average value of the defect on the basis of the cluster geometry, i.e. on the length of the all-trans "segment" and its tilting angle.

The effective length of an all-trans "segment" of a lipid chain can be estimated from nuclear magnetic resonance data /20-23,80,85-89/ where the characteristic "plateau" is observed for C3-C9 methylene groups in both order parameter and spin-lattice relaxation time profiles. Independently this value has been determined by Vogel and Jahnig /104/ on the basis of the analysis of peak positions and intensities in Raman spectra of various lipids. This length coincides very

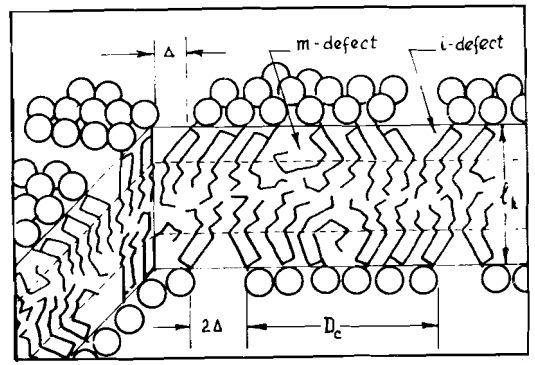


Fig.3. Schematic picture of lipid molecule arrangements in a liquid lipid bilayer.

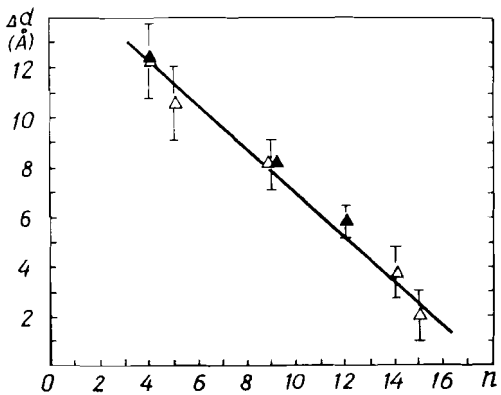


Fig.4. The distance between the bilayer center and the projections of labelled CH_2 groups on the bilayer normal direction in dependence on the label positions in acyl chains /112/:
1 - DPPC, 25 wt % H_2O, 50°C,
2 - DPPC, 10 wt % H_2O, 70°C.

well with NMR data and is equal to these of about 8 CH_2 groups (10Å) for the series of lecithins with the fully saturated acyl chains ($N_{all-t} \simeq 7.6$ methylene groups for "liquid" DMPC bilayers). The theoretical calculations /41, 42, 59, 67, 85/ also show preferentially transconformations of the methylene groups adjacent to the polar regions although do not assume initially the collective chain tilting.

The angle of the chain tilting against the normal can be estimated in principle from ESR data for spin-labelled lipid probes. Near the polar region it is ~30-47° for EPC bilayers depending on the probe structure and the experimental conditions /7, 39, 71/. But labels distort obviously molecule arrangements in their environment, that is why these results can be only regarded as qualitative characteristics. The correct quantitative estimations have been received from the neutron diffraction data of Zaccai et al. /15, 112/. Fig.4 shows the dependence of the average distances of deuterium CH_2-groups from the bilayer centre on their positions in acyl chains for DPPC multilamellar structures at various water concentrations and temperatures. The linear plot allows us to calculate the distance increment on the CH_2-group and consequently the average angle of the chain tilting. The increment $\Delta d_{CH_2} \simeq 0.9$ Å and the angle of the tilt about the normal to the bilayer surface: φ = arc cos (0.91/1.25) = arc cos 0.72 ≃ 44°. The value 1.25 Å is the effective length of the CH_2 - link, i. e. the length of the C-C bond projection on the long axis of the hydrocarbon chain in the all-trans conformation.

The collective tilt existence for the chain segment adjacent to the polar group is also confirmed by the small width of the peaks (~1.5 Å) which characterize the deuterium label location in a bilayer /112/.

Our experimental conditions are similar enough to those of /15, 112/ at 25 wt % of water and it is resonable to suppose that the angle of the chain tilting is the same, i.e. φ = 44°.

4.3. Annular defects. Concentration and Size

The geometry of defects is schematically shown in Fig.3. Similarly to /25/ we mark them as "interfacial" (i-defects) and "inner" (m-defects). The latter are probably "filled" in the main due to the trans-gauche isomerisation of the chains near the cluster boundaries. It is reasonable to suppose that the defects of 2Δ and Δ width are equally probable (they depend on the mutual orientation of the planes in which the chain bending takes place). If $N_{all-t} \simeq 10$ Å (8 CH_2 groups) and φ = 44° the defect parameters are: $\Delta \simeq 7$ Å, $2\Delta \simeq 14$ Å, the depth $\ell_d \simeq 7.2$ Å and the respective cross-section areas, S_c , are 25 or 50 Å^2.

For the sake of simplicity we suppose that a cluster is surrounded by the "average" annular defect with the triangle base of the width $\frac{3}{2}\Delta$ and $S_c \sim 37$ Å^2. The average diameter of D_2 O-contrasted annular defects (i-defects) according to (7): 2Rg = 58 Å because $Rg \gg \Delta^2/2$. The effective diameter of the liquid core (a cluster diameter):

$$D_c \simeq 2Rg - (3/2)\Delta \simeq 48 \text{ Å}$$

Thus the size of a cluster along the bilayer surface $r_c = D_c/2 \simeq 24$Å , correlating with the estimations of Segerman /90/. It is obvious that a part of the i-defect area is screened by lipid polar groups against water. The volume of the defect can be calculated from the above geometric notions and the defect concentration can be determined from the expression (1). The parameters of i-defects in clustered lecithin bilayers are shown in Table 4 taking into consideration that the multibilayer repeat distance is d = 54 Å.

Permeation of water through such a clustered bilayer can be roughly estimated if only the central "disordered" hydrocarbon layer (see Fig.3) acts as a diffusion barrier and the defects but not the clusters are fully permeable for water and other molecules and ions. At maximum hydration the DPPC and EPC bilayers have the hydrocarbon interior thickness $\ell_h \simeq 26$ Å /65/ and the thickness of two clustered layers is ~ 14 Å.

Table 4

Parameters of i-defects in the clustered liquid-crystalline DPPC bilayers at 97% relative humidity and 55°C

Parameters of defects	Formula	Value
1	2	3
ground width	$(3/2)\Delta$	10.5 (Å)
depth	ℓ_d	7.0 (Å)
cross-section area	$S_c = (3/4)\ell_d \cdot \Delta$	37 (Å)^2
radius of annular defects	$R \simeq Rg$	29 (Å)
area at the bilayer surface	$S_d = 3\pi \cdot Rg \cdot \Delta$	$1.9 \cdot 10^3$(Å)^2
volume	$V_d = 2\pi RS_c$	$6.7 \cdot 10^3$(Å)^3
concentration	$n = I(o)/(\rho - \rho_s)^2 \cdot V_d^2$	$1.7 \cdot 10^{18}$(cm^{-3})

Table 4 (continue)

1	2	3
fraction of the total sample volume occupied by defects	$\alpha_v = n \cdot V_d$	$1.14 \cdot 10^{-2}$
the number of defects per 1 cm^2 of a bilayer	$n_s = nd$	$9.2 \cdot 10^{11}$ (cm^{-2})
the number of defects per 1 cm^2 of a monolayer	$n_s / 2$	$4.6 \cdot 10^{11}$ (cm^{-2})
fraction of a monolayer area occupied by defects	$\alpha_s = n \cdot S_{d/2}$	0.088

Thus the thickness of a central "disordered" layer $\ell \simeq 12$ Å and its properties are supposed to be similar to those of liquid paraffins. For hexadecane the diffusion and partition coefficients are /31/: $D \sim 2 \cdot 10^{-5} cm^2 \cdot s^{-1}$ and $K = 4.2 \cdot 10^{-5}$. The permeability coefficient

$$P = \alpha_s \cdot \frac{K \cdot D}{\ell} \simeq 0{,}1 \times \frac{4.2 \times 10^{-5} \times 5 \cdot 10^{-5}}{12 \cdot 10^{-8}} \simeq 1.7 \times 10^{3} cm \cdot s^{-1}.$$

This value is of the same order as the experimental one /79/, however this fact is not yet a direct evidence for the cluster model validity because the values K and D really unknown for the bilayer interior.

4.4. Cluster dimensions and the Interior Structure

A cluster diameter along the bilayer plane has been calculated in section 4.3 as the half of the lipid "core" diameter for the annular defect of the radius R. For liquid DPPC bilayers at 55°C and 97% of the relative humidity this value is $r_c \simeq 24$ Å. By the independent way it has been estimated in /51,52/ for various lipids from the $(4.6$ Å$)^{-1}$ diffuse X-ray reflex "packing".

These estimations (with the respectful corrections) are given in Table 5.

It is necessary to emphasize that in paper /52/ the estimations of the cluster size are probably not correct because the cluster latteral size was calculated as $2 r_c$. The size equal to r_c is apparently more reasonable.

The gyration radius of cluster is several liquid was determined in /37/ by the neutron scattering method. Supposing the cylindrical from of cluster transverse size (diameter) $r_c \simeq 2\sqrt{2} Rg$ and this value is 12.6 Å (C12), 13.5 Å (C 16), 14.3 Å (C 20) and 17.4 Å (C 32). We can not explain now the divergence of these data with those in Table 5.

Thus the hydrocarbon interior of a bilayer can be schematically imagined as a "sandwich" of two structural (clustered) and one central

Table 5

The correlation radius (minimum cluster dimensions) in liquid lipid bilayers and n-alkanes *

Parameter	DPPC		Azolectin		EPC		n-alkanes		
	I	II	I	II	I	II	C10	C16	C17
t, °C	70	45	20	20	20	20	20	25	25
d, Å	4.65	4.6	4.6	4.6	4.6	4.55	4.65	4.6	4.55
$S_{1/2}$, $Å^{-1}$	0.074	0.057	0.074	0.054	0.086	0.058	0.074	0.068	0.064
r_c, Å	22	28	22	29	19	27	22	24	25

* The corrected data from /52/; I - "dry" phospholipid (at relative humidity 85%), II - maximum hydration, t - temperature, d - interchain separation (±0.05 Å), $S_{1/2}$ - the "semiwidth" of the $(4.6$ Å$)^{-1}$-reflex, r_c - the correlation radius.

"liquid" (more disordered) layers (see Fig.3). For PC bilayers the thickness of the clustered layer is approximately 7-7.5 Å and it is constant enough at various chain lengths, temperatures and humidities judging by the NMR data /2, 20-22, 50, 51, 87-89/. This value considered as a distance between the centre C1-C2 and C9-C10 bonds can be calculated from the neutron diffraction data /112/ (see Fig.4). It is easy to see that it is 7 Å. This relative constancy is apparently explained by the fact that the chain tilting is determined by the area of the hydrated polar head group but not by the average area per lipid molecule.

The thickness of the "clustered" and "disordered" layers of the hydrocarbon interior have been independently estimated from the comparison of free energies and entropies of solvation for various substances in the fluid DMPC bilayers and in several solvents /57/. The authors /57/ proposed the existence of two structured layers ("domains") adjacent to the polar regions which dissolve ethers and of central "liquid" layer which dissolves n-alkanes. The dependence of the thermodynamic parameters of solvation on the molecule dimensions allows one to estimate the layer thickness. For example the structured layer thickness is commensurable with the decanol molecule length/57/.

The similar molecule arrangement is typical for any liquid-crystalline lipid bilayer independently of the number of fatty-asid resi-

dues in lipid molecule. Vodyanoy and Hall [103] have shown that the ion-motion through monoacylglyceride membranes is well described in the terms of the rectangular barrier of a variable height and width (in dependence on the acyl chain length) is situated in the bilayer centre. The distance from this barrier to the polar-hydrocarbon bilayer interface is estimated 9.2 ± 0.3 Å [103] for various lipids. This "structured" layer is permeable probably due to the defects. Water penetration into defects can probably explain the experimental values of apparent molar heat capacities for different phospholipids which Blume [9] calls "hydrophobic hydration".

Unlike the thickness clustered layers that of the central "disordered" layer depends immediately on the chain length.

It can be estimated as $\ell \simeq \ell_h - 14$ (Å) and for the series of lecithins [65] it is equal to 13 Å (DOPC), 15.5 Å (DSPC), 12 Å (DPPC), 9 Å (DMPC) and 5.5 Å (DLPC). If the methyl group size (2.7 - 2.9 Å) is taken into account, the high permeability and the little stability of the bilayers formed from the lipids with the chains containing the carbon atom number less than 14 becomes reasonable [8, 50]. It is unexpected, on the face of it, that the density of the chain packing is practically the same through the whole bilayer interior. Apparently this idea was at first suggested by Green [40] basing on his statistical-mechanical model of a billayer as the following improvement of the Marcelja model [67, 85]. Confirmation of it is both the linear plot in Fig.4 and the linear dependence of ℓ_h on the chain length for the series of synthetic lecithins with the increment per CH_2 group ~ 0.88Å which is practically equal to that in Fig.4. The some increase of the average area per molecule is possible with an increase of the chain length or when short-chain alkanes penetrate to the centre of the bilayer interior as a result of the central layer expansion due to the increase of the number of transgauche isomers. It partially conforms to the conception of the paper [18] (the resuts of [65] suggesting the area constancy or some decrease are based on the erroneous assumptions of the molecular volume constancy in the wide range of conditions).

The inner defect volume in the hydrocarbon interior (m-defects) can be estimated from the α_v value (see Table 4). In our case to a first approximation the volumes of i-defects and m-defects are the same. If d = 54 Å and $\alpha_v = 1.14 \cdot 10^{-2}$ the i-defect volume is $\alpha_v \frac{d}{2} \cdot \ell_d \simeq 4.4 \cdot 10^{-2}$ of the clustered layer volume because these defects are only localized in the clustered layers. The existence of m-defects explains apparently the strange fact of very large vertical fluctuations of chains in a liquid bilayer which were detected by the double electron-

electron resonance of the ^{14}N and ^{15}N labelled lipid probes [24,28], by the double nuclear magnetic resonance (proton-enhanced ^{13}C NMR) [110] and by the dynamic quenching of the fluorescent labels [106]. The time scale range of this process is 10^{-8}s judging by the life time of the label excited state [106].

The large wobbling amplitude for membrane fluorescent probe motion in the nanosecond time scales [46,58] can be also explained by probe location in the defects, outer or inner in dependence on the probe molecular structure.

4.5. Chain packing density in a lipid bilayer

To describe transport processes through a membrane it is enough in some cases to approximate the whole bilayer interior by a liquid n-alkanes [31, 79]. Although the real bilayer interior behaviour differs from that an isotopic three-dimensional liquid [91, 92] such approach is widespread. The sandwiched spatial organisation of a hydrocarbon bilayer interior has been described in the previous section. Here we will examine the average chain packing density in clusters characterized by the value of the cross-section area per chain.

The main assertion is that the angle of the chain tilting φ is determined by the area of the hydrated polar head group of a lipid molecule. If the lipid chains in clusters are packed closely the single chain cross-section area:

$$a_o = \frac{1}{2} A_o \cdot \cos\varphi \qquad (11)$$

analogously to the procedure for $L_{\beta'}$-phase [63]. According to [49] A_0 = 59.2 Å for lecithins* and $\varphi \simeq 44°$ as it was shown in the previous sections. Then a_o = 21.3 Å^2 and the effective volume of the CH_2-group is $v_{CH_2} = 1.25 \cdot a_o$ = 26.6 Å^3 which is very close to 27 Å^3 found in [96].

Quite independently a_o can be estimated from the position of the X-ray and neutron diffraction maximum (4.6 Å)$^{-1}$. If the chains in clusters are closely packed and the "statistical rotation" of chains arround their long axes is present the scattering centre distribution can be suggested as the superposition of two distinct hexa-

*The average area of the polar head group at the bilayer surface can be rougly estimated from the X-ray diffraction and hydration data. The phosphocholine group volume is 360 Å^3 [96] and the volume of the bound water molecules (~12 H_2O per PC molecule [30, 55] is 360 Å^3 too. The thickness of the polar layer is ~12 Å [98] then the polar group area is ~60 Å^2.

gonal lattices for the closest and for the friable packing /102/. The peak maximum corresponds to the distance:

$$\overline{d}_{hc} = \frac{1}{2}(d_{min} + d_{max}) \simeq 4.65\ \text{Å},$$

where d_{min} = 4.1 Å, d_{max} = 5.2 Å are taken from the cross-section form of a chain /50-52, 102/. The average area per chain can be apparently calculated as a mean of that for two "quasilattices". The closest packing corresponds to the solid (gel) state and the cross-section area $a_g \simeq 4.1^2/\sin 60° \simeq 19.4$ Å^2. For free rotating chains (the "friable" lattice) the scattering maximum corresponds to the neighbouring chain separation analogously to liquids and $a_l \simeq 5.2^2 \sin 60° \simeq$ 23.4 Å^2. The mean for these lattices gives

$$a_o = \frac{1}{2}(a_g + a_l) \simeq 21.4\ \text{Å}^2.$$

Comparing this cross-section area with that one in a solid $L_{\beta'}$-phase ($\overline{d}_{hc}$ = 4.1/cos 60° = 4.75 Å) the interchain separation in a cluster

$$\overline{d}_{hc} = 4.75\sqrt{a_o/a_g} \simeq 5.0\ \text{Å}.$$

According to /11/ the critical separation for the gauche-conformation generating is $\geqslant$ 4.9 Å. Petersen and Chan /80/ estimated the probability of transconformation for the first half of a chain as $1.0 \geqslant p_t \geqslant 0.8$ in liquid bilayers. Thus the clustered chains have some quantity of the gauche-conformers (for example, one kink per chain).

The alternative estimations of the interchain separation are widespread in literature. They suppose that the transition from a solid to "liquid" bilayer results in the hexagonal lattice "swelling" with the long-range order preservation which is reflected as the Bragg reflex displacement from (4.2 Å)$^{-1}$ to (4.6 Å)$^{-1}$ /11, 45, 67/. It is assumed that chains keep their all-trans conformation and are parallel to the bilayer normal. Then the average separation between the adjacent chains is estimated from the reflex position 5.21 Å /84/, 5.3 Å /45/ or 5.56 Å /11/. The average area per lecithin molecule is respectively 47 + 50 Å^2 /84/ which is significantly less than 60 + 70 Å^2 received from the experimental data /45, 50, 51, 63/.

The disribution of the substance density along the normal direction to the bilayer plane can be calculated in the frames of the cluster model compare the bilayer hydrocarbon interior with liquid hydrocarbons. We propose that the bilayer parameters should be analogous to that in /112/ for DPPC at 25 wt % of water and the partial specific DPPC volume at 55°C $\bar{v}_l$ = 1.01 ml·g^{-1} /75/. The molecular mass of DPPC is 733, the thickness of the clustered layer is 7.2 Å. The clustered layer volume per DPPC molecule (8 CH_2) is 2 x 8 x 1.25 x a_o = 426 Å^3, where a_o = 21.3 Å^2. Under these conditions $l_h \simeq$ 29 Å

and the central layer thickness $l = l_h - 2 \times 7.2 \simeq 14.5$ Å, i.e. the thickness of the clustered and disordered regions are practically the same. The molecular weight of two clustered chain "segments" is 224. The value $\alpha_V = 1.14 \cdot 10^{-2}$ is in accordance with $4.4 \cdot 10^{-2}$ from the volume of the clustered layers. This value is also the contribution of m-defects to the layer volume (we suggest that m-defects should be vacant). Then 95.6% of the layer volume is occupied by chains and the total volume of these layers is 426/0.956 $\simeq$ 450 Å^3. The effective density

$$\rho_c = \frac{224}{N_A \cdot 450 \cdot 10^{-24}} \simeq 0.83\ g \cdot cm^{-3}$$

and the m-defect volume per DPPC molecule is ~ 24 Å^3. (N_A is the Avogadro's number).

The topology of a bilayer assumes that the volume of the central layer per molecule in this base is equal to 450 Å^3 per DPPC too. The equality of the layer thickness change per CH_2 group (the increment ~0.9 Å from the CH_2-group position /112/ and 0.88 Å from the bilayer interior thickness /65/ allows one to suggest the similar average tilt of every methylene "link" respective to the normal and approximately similar density of the chain packing. 6 CH_2 and CH_3 groups from every chain occupy 26.6 x 6 + 54 = 213 Å^3 or 426 Å^3 per DPPC. Then the effective defect volume is 450 - 426 = 24 Å^3 i.e. it is equal to that for clustered layers. The molecular weigth of this part of molecule is 198 and the effective density

$$\rho = \frac{198}{N_A \cdot 450 \cdot 10^{-24}} = 0.735\ g \cdot cm^{-3}$$

the density of tetradecane is 0.755 g cm^{-3} at 20°C or taking into consideration the coefficient of the thermal expansion ~$1 \cdot 10^{-3}$ deg^{-1}

$$\rho_T \simeq 0.74\ g \cdot cm^{-3} \text{ at } 55°C.$$

The total hydrocarbon volume per DPPC molecule is ~900 Å^3, the molecular weigth of two chains is 422. Then the average density of the hydrocarbon interior is

$$\rho_h = \frac{422}{N_A \cdot 900 \cdot 10^{-24}} = 0.78\ g \cdot cm^{-3}.$$

The total volume of the molecule (the volume of the polar head group is 360 Å^3 /96/ V = 900 + 360 = 1260 Å^3, moreover 48 Å^3 from this value is the volume of m-defects. The molecular volume can be calculated from the partial specific volume of DPPC:

$$V = \frac{\bar{v}_e \cdot M}{N_A} = \frac{1.01 \times 733 \times 10^{24}}{6.02 \times 10^{23}} = 1230\ \text{Å}^3.$$

These values are close enough taking into account the simplifications used in our calculations.

Thus the DPPC hydrocarbon interior at 55°C and 25 wt % H_2O can be imagined as a "sandwich" of two clustered layers adjacent to the polar regions and the central liquid (disordered) layer with its density identical to tetradecane. The i-defects filled with water are wedged into the clustered layers (24 $Å^3$ per DPPC molecule). The respective volume of m-defects in the whole hydrocarbon interior is 48 $Å^3$ per DPPC molecule. The direct confirmation of such dynamic structural organization of a bilayer follows from the double nuclear magnetic resonance data /100/. The high efficient transfer of polarization from protones to carbon nuclei takes place in DMPC bilayers during 2 msec analogously to genuine solids unlike that for terminal methylene and methyl groups /100/.

There is extensive information on the lipid molecule dynamics in lipid bilayers /13, 17, 19-24, 28, 35, 36, 62, 64, 70, 74, 80, 82, 83, 87, 89, 105, 11 / but for the present it is impossible to describe unequivocally the whole chain motions. Nevertheless it is reasonable to suggest that the "plateau" in ^2H and ^{13}C spin-relaxation time and order parameter profiles should be determined by the cluster organization of a bilayer. The correlation time $\tau_{R_\perp}$ of the long axis reorientation relative to the local bilayer normal /22/ is then a result of the cluster reorientation and it is $6.1 \cdot 10^{-8}$ s (23°C, DMPC) and $1.3 \cdot 10^{-8}$ s (50°C, DMPC) according to the ESR data /22/. These values are close to those for ultrasonic relaxation: $2.1 \cdot 10^{-8}$ s at 44°C and $1.5 \cdot 10^{-8}$ s at 47°C for DPPC /74/. Such interpretation is confirmed by the absence of the significant dependence of the motion parameters on the ester (or either) binding of the acyl chain at the C2 position /2/. Petersen and Chan /80/ have estimated the characteristic time of chain reorientation about the normal direction 10^{-7} s. Apparently the more slow fluctuations found from the frequency dependence of the NMR relaxation times ($2.2 \cdot 10^5$ rad·s^{-1} /17/) characterize other motional modes. Indeed, the cluster lifetime τ_ℓ can't be larger than the time τ_D needed for a lipid molecule to diffuse from the centre to cluster boundary, i.e.

$$\tau_\ell \leq \tau_D \sim R^2/4D, \qquad (11)$$

where R - the cluster radius. D - lateral diffusion constant for lipid molecules. For R ~ 15 Å and D = $2 \cdot 10^{-8}$ cm^2/s (for DPPC membrane at T = 42°C in water excess)

$$\tau_\ell \leq \frac{(15 \cdot 10^{-8})^2}{4.2 \times 10^{-8}} \sim 2.8 \times 10^{-7} \text{ s}.$$

Conclusion

The structural inhomogeneities of the molecular arrangements in DPPC liquid-crystalline bilayers have been observed by the method of the small-angle neutron scattering. They have been interpreted as D_2O-contrasted annular defects between adjacent short-lived clusters pooling the all-trans chain segments of 8 CH_2-group length adjacent to the polar region. The average radius of gyration ~29 Å and the total volume of those inhomogeneities have been estimated in the Gunier approximation. The cluster size has been estimated ~24 Å, which agrees with the estimations of the size of partially order regions (19 ÷ 29 Å) in chain arrangements obtained from the width of the X-ray "packing" reflex $(4.6 Å)^{-1}$.

The liquid" bilayer hydrocarbon interior of DPPC can be imagined as a "sandwich" with the central disordered layer analogous to liquid tetradecane which is put between two clustered layers of the thickness ~7 Å. The interface i-defects (~24 $Å^3$ per DPPC molecule in our experimental conditions in the clustered layers are filled with water as a rule, the inner m-defects of the volume ~48 $Å^3$ per DPPC molecule can be partially filled with flexible chains. The chain packing density is practically constant through the whole hydrocarbon interior and it is characterized by the average cross-section area of a chain ~21.3 $Å^2$ in both the clusters and the "cybotaxic groups" of the central layer.

The angle of the collective chain tilting in clusters is determined by the hydrated polar head group area which is ~59.2 $Å^2$ for lecithin bilayers at the maximum water binding. The clustered chains rotate around the normal direction with the characteristic times 10^{-8} - 10^{-7} s. These motions can determine the transport processes for the molecules and ions included into the defects. The cluster model explains satisfactorily the numerous experimental data received by various physical-chemical methods (NMR, ESR, Raman-specrtoscopy, X-ray and neutron diffraction, calorimetry, etc.) and it can be useful for the explanation of the passive transport, bilayer confluence, molecule insertion into membranes and other membrane processes.

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