BBA 75489

THE STRUCTURE OF LYSOLECITHIN–WATER PHASES

NEGATIVE STAINING AND OPTICAL DIFFRACTION ANALYSIS OF THE ELECTRON MICROGRAPHS

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(Received May 11th, 1970)

SUMMARY

Lysolecithin–water mixtures have been investigated by electron microscopy using the negative-staining technique. The negatively stained micrographs were analyzed by optical diffraction. At high lysolecithin concentrations a rod-like phase has been observed. The features and the dimensions of this phase agree with the hexagonal phase Type I, described by X-ray studies[2]. At low lysolecithin concentrations a globular phase was found which usually occurs in a hexagonal close-packed form, seldom in a square arrangement.

INTRODUCTION

In electron microscopy, the negative-staining technique has become a convenient method for studying membrane or membrane-bound structures. The method certainly gives a sufficient resolution at the macromolecular level, but results should be critically accepted only. The structures of the lipid components, for example, which are extremely dependent on the water content, may be affected by the experimental procedures. In order to get some better information about the validity of the negative-staining technique, it seems necessary to work with model systems and to compare the results with those of other appropriate techniques. In addition, the electron microscopical examination of lipid model systems may provide some complementary aspects in membrane studies.

In a previous communication various liquid–paraffin phases of hydrated phosphatidyl ethanolamine have been investigated using the negative-staining technique[1]. The objective of this paper is to investigate lysolecithin–water mixtures under similar conditions. Pure lysolecithin has been chosen as the second model system because it represents a phospholipid with a single fatty acid chain, which shows phase characteristics similar to monoglycerides and soaps. From that morphological point of view, a comparison with phosphatidylethanolamine seems interesting, with respect to the opposite structural features of these two phospholipids. While for phosphatidyl

Abbreviations: FCC, face-centered cubic; HCP, hexagonal close-packed.

ethanolamine water-in-paraffin structures Type II have been observed by X-ray diffraction studies[2, 3] and by electron microscopy[1], lysolecithin is known to form paraffin-in-water structures Type I. The negative staining of these structures, which are dependent on temperature and concentration, implies uncertainties because it is impossible to maintain the conditions of the lipid–water equilibrium during the staining procedure. There is, however, a chance to preserve particles of the definite lipid–water mixtures, as shown by the different structures, which could be obtained at high and low lipid concentrations, respectively.

MATERIALS AND METHODS

According to the method of HANAHAN *et al.*[4] lysolecithin was prepared by enzymic treatment of egg lecithin with snake venom phospholipase A (*Crotalus terr. terr.*, Fa. Boehringer, Mannheim). After precipitation with diethyl ether from ethanol, lysolecithin was purified by column chromatography on silicic acid[5].

Commercial products from Sigma Chem. Co. and Nutritional Biochem. Corp. were also used.

The purity of the samples was controlled by one- and two-dimensional thin-layer chromatography on silica gel. The solvent systems were chloroform–methanol–water (65:25:4, by vol.) and chloroform–methanol–7 M NH_4OH (100:100:23, by vol.), respectively. The lysolecithin samples were chromatographically pure. No difference in the behaviour of the three products was found.

Two lysolecithin–water mixtures were prepared: (a) An approx. 0.1 % lysolecithin–water mixture was dehydrated in a desiccator at 40° over 6–8 h until a concentration of 50–80 % lysolecithin was reached. For negative staining the lysolecithin–water mixture was mechanically suspended in 2 % sodium phosphotungstate solution at pH 7–4 and 40°. A drop of this suspension was mounted immediately on formvar carbon-coated grids and dried. All manipulations were carried out as quickly as possible. (b) A lysolecithin–water dispersion containing lipid concentrations of 0.5–1 % was mixed with a 2 % sodium phosphotungstate solution. After 15–30 min, the grids were dipped into the mixture and dried. Experiments were carried out at 40, 25 and 0°. The pH of the stain solution has been varied between 7 and 4. Electron optical magnifications were 40000:1 and 100000:1, using a 50-μ objective aperture and an accelerating voltage of 80 and 100 kV, respectively.

The electron micrographs were evaluated by optical diffraction. A spatially filtered laser beam of 46-mm diameter between points of 13.5 % ($= 1/e^2$) of the maximum intensity passes through a 182-mm collimator lens and the micrograph mounted just behind it. After an optical path of 5.7 m the beam is focussed by the collimator on a camera back for viewing and registration. By this means, sufficiently parallel illumination, a convenient size of the diffraction pattern and the absence of further lenses, generating noise and unwanted reflexions are obtained. With respect to the instrumental arrangement and the resulting size of the diffraction pattern an electron microscopical magnification of 40000:1 was suitable. The marker in the diffraction diagrams corresponds to a 0.1-mm lattice period = 25 Å with regard to the electron optical magnification. The diffraction patterns of the electron micrographs were compared with those of model pictures. Therefore, models of a hexagonal close-packed (HCP) and a face-centered cubic (FCC) structure were built of 10-mm bearing balls.

An X-ray photograph was taken and the magnification was photographically matched to the dimensions of the structure of the electron micrographs.

RESULTS AND DISCUSSION

Figs. 1a and 1b show the phase which has been observed using the 50–80 % lysolecithin–water mixtures at 40°. Negative staining of this phase is difficult to reproduce because lysolecithin disperses in the stain solution. Fig. 1a represents the structures, which were frequently observed, when the staining procedure has been carried out as quickly as possible. The alternation between more or less compact regions refers to a beginning state of dissolution, but it is just this state which shows that this phase is formed by rods of indefinite length. The thickness of the rods, d_1, is about 70 Å (± 10 %). Only in a few close-packed areas can the cross sections of the rods and their hexagonal arrangement be seen (Fig. 1b). In these domains the original phase, which is formed at 80 % lysolecithin and 40°, seems to be preserved. The center to center distance, d, is 60 Å. The diameter of a rod appears slightly smaller, approx. 55 Å. The hexagonal areas partly change to linear patterns, which are caused by an inclination of the rods with respect to the beam direction. Depending on the angle of rotation, different periodical distances are observed, for example 50 Å corresponding to $d\sqrt{3}/2$ or 30 Å, corresponding to $d/2$ (Fig. 1b, arrows).

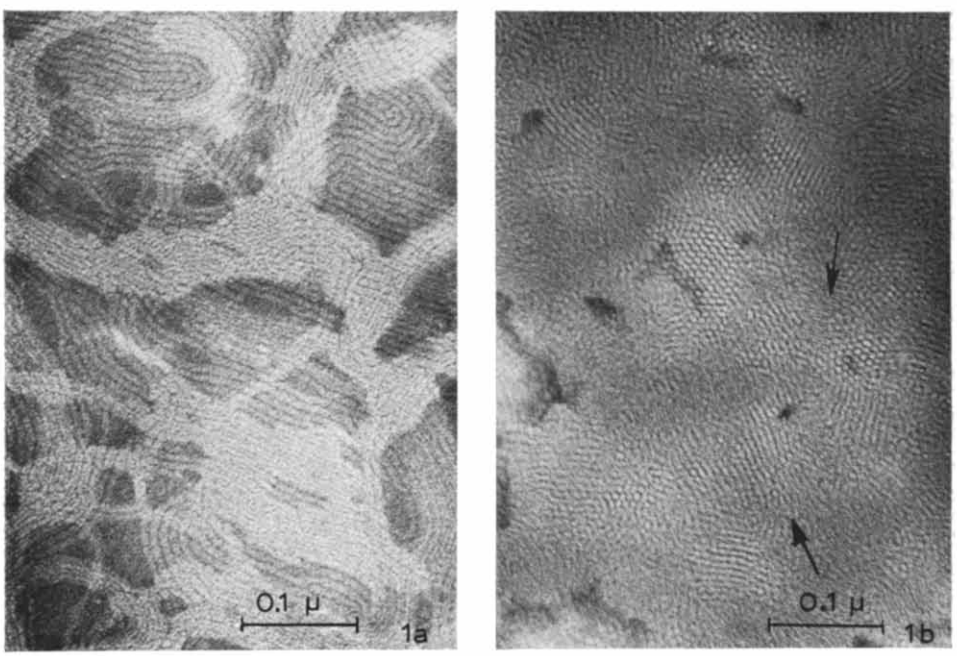


Fig. 1. Hexagonal phase, formed of indefinite rods.

These structural features and their dimensions agree with the hexagonal phase Type I, which has been described in an X-ray study by REISS-HUSSON[2].

Samples taken at 5-, 10- and 15-min intervals after the 80 % lysolecithin–water

TABLE I

COMPARISON OF OPTICAL DIFFRACTION DATA

R = radius of the diffraction maxima. s = spacing of the preparation.

Optical diffraction pattern of	R (*mm*)	s (*Å*)	*Factor*	
Monolayer (Fig. 2)	6.2	76	$d\sqrt{3}/2$	d(calc.)* = 89 Å d(meas.)** = 90 Å
Multilayer, HCP (Fig. 3)	7.25	70	$d\sqrt{3}/2$	d(calc.) = 80 Å
	12.25	40	$d/2$	d(meas.) = 80 Å
	14	35	$d\sqrt{3}/4$	
Multilayer, square and HCP areas (Fig. 5)	5.4	83	d	
	6.25	72	$d\sqrt{3}/2$	d(meas.) = 85 Å
	7.9	57	$d\sqrt{2}/2$	
	10.9	41	$d/2$	

* calc. = calculated from the optical diffraction pattern.
** meas. = measured in electron micrographs.

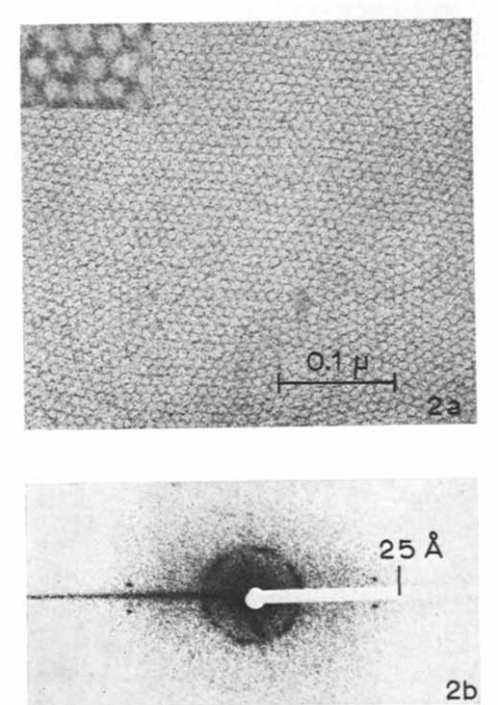
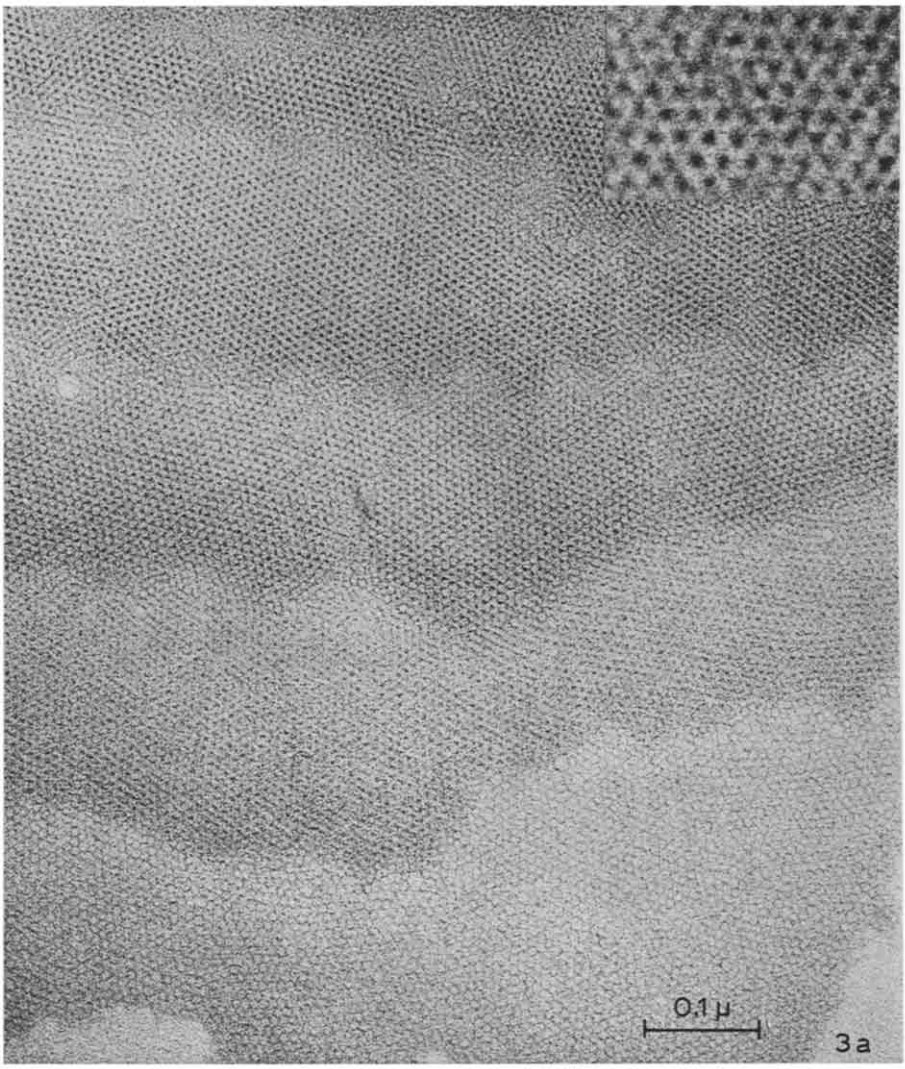


Fig. 2. a. Monolayer, formed of close-packed globules; inset: 500000:1. b. Optical diffraction pattern from a.



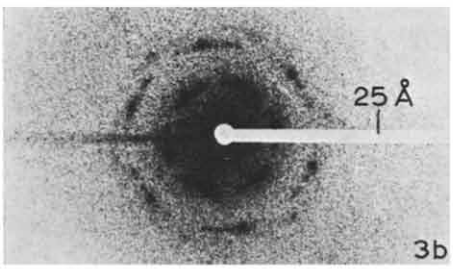


Fig. 3. a. Hexagonal close-packed (HCP) phase; inset: 500000:1. b. Optical diffraction pattern from a.

mixture was suspended in the stain solution show a progressive transformation to a globular phase.

Negative staining of a 0.1 % lysolecithin dispersion in water also reveals phases which are formed by globular subunits. In our experiments neither an influence of temperature within the range of 0–40° nor an influence of the pH of the stain solution on the formation of this phase could be noticed, but a dependence on time became apparent. The structures could only be observed 15–30 min after the phospholipid had been dispersed in water. The globules or spheres are packed together in a hexagonal array, as shown by the electron micrograph of a single layer (Fig. 2). The center to center distance, d, is about 80 Å. In the diffraction pattern a reflex appears which corresponds to $d\sqrt{3}/2$ (Table I).

Usually several layers form a close-packed crystal exhibiting characteristic superposition patterns.

As known through crystallographic studies, two different ways of closest layer stacking are possible. On the first layer A, the second layer B is stacked so that the spheres fit into the corresponding hollows. The third layer C, however, can be placed either in register with A or in the position which does not have its globules directly over A. Consequently the stacking sequence may be either ABABAB, due to a HCP lattice, or ABCABC, due to a FCC lattice.

Fig. 3 now gives a view normal to the stacking plane of the close-packed structure. The layers are normal to the [111] direction and lie in planes $\{111\}$. The contrast, caused by the phosphotungstate, obviously increases with the number of superimposed layers, suggesting that the gaps between the lipid spheres form vertical continuous channels. The heavy contrast of the gaps would indicate a HCP lattice, corresponding to a layer stacking ABABAB, but the increasing contrast cannot be used as a sufficient criterion to define the structure. To obtain a clearer picture of the structure, the electron micrographs were analyzed by optical diffraction.

According to the extinction rule, the $\{111\}$ planes of the HCP and the FCC structures are easy to distinguish. This is shown by the diffraction patterns of models of these two structures (Figs. 4a and 4b). If d is the center to center distance, the reflexions of the HCP structure correspond to $d\sqrt{3}/2$, $d/2$, $d\sqrt{3}/4$, ... giving a spacing ratio of $1:1/\sqrt{3}:1/\sqrt{4}$... In the diffraction pattern of the FCC structure, the reflexions corresponding to $d\sqrt{3}/2$ are extinguished. A spacing ratio of $1:\sqrt{3}:\sqrt{8}$ ... results. Comparing now the diffraction patterns of the models to that of Fig. 3, a HCP arrangement of the lipid globules can be deduced (see also Table I).

Besides this usually observed HCP structure, square areas were found. They appear very seldom, less than 1 %. Fig. 5a shows square regions alternating with HCP regions. The laser diagram (Fig. 5 b) of the total area contains reflexions corresponding to d, $d\sqrt{3}/2$, $d\sqrt{2}/2$ and $d/2$. If the hexagonal domains are masked, the $d\sqrt{3}/2$ reflexion disappears (Table I). The present material, however, the electron micrographs as well as the optical diffraction patterns, do not confirm the determination of the lattice type.

The experiments reveal evidentially different phases formed by indefinite rods and globules, respectively. There remains the problem of determining to what extent the structures were affected by the staining procedure. Detailed information about the liquid paraffin water-containing phases obtained from X-ray studies facilitates

the detection of structural changes and an understanding of the influence of the staining procedure.

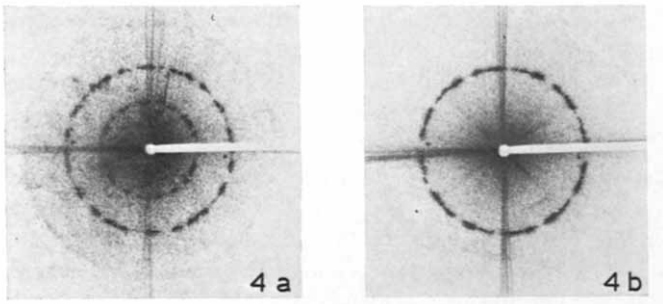


Fig. 4. a. Optical diffraction pattern from a hexagonal close-packed model. b. Optical diffraction pattern from a face-centered cubic model.

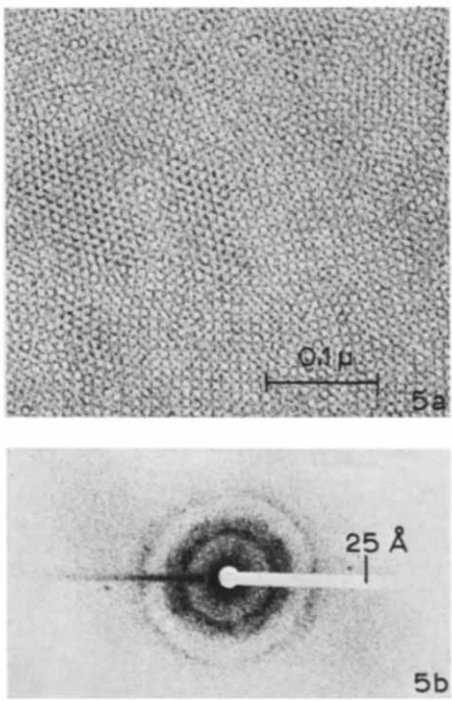


Fig. 5. a. Square areas alternating with hexagonal close-packed areas. b. Optical diffraction pattern from a.

The ionic constitution and the pH of the stain solution have recently been discussed[6] and doubtless have a considerable influence. Calculating the specific properties of lipids, these factors as well as temperature might be controlled and accommodated to any lipid system. The staining technique, however, requires a suitable dispersion of particles, implying thus a suspension of the lipid–water system in an excess of liquid. Since the range of existence of the lipid phases is a function of

concentration and temperature, the kinetics of water uptake into the lipid system and of phase formation must be considered a determining factor.

This may be shown by a comparison of the negatively stained phosphatidyl ethanolamine–water mixtures with lysolecithin–water mixtures. Compared to phosphatidyl ethanolamine the water uptake into the lysolecithin system takes place more rapidly. While the proportion of water in the hydrated phosphatidyl ethanolamine can be kept nearly constant by employing a short preparation time, for lysolecithin a considerable shift to higher water proportions cannot be avoided during the staining procedure. Therefore, the negatively stained samples of lysolecithin are not uniform at all and results are rather difficult to reproduce. In contrast to phosphatidyl ethanolamine, only a very small part of the hexagonal phase, which is known to be formed at high lysolecithin concentrations and at 40°, remains unaffected. The applied technique, however, permits one to demonstrate the existence of the hexagonal phase I within its range of existence.

The appearance of the close-packed structures is not explicable at this point of investigation. These globular phases are obtained from a dilute lysolecithin dispersion. According to optical and X-ray data an isotropic micellar solution is existent for this concentration range. It may be that more or less extended crystalline aggregates are already existent in the dispersion. The other possibility is that the structural elements are arranged in a close-packed lattice, when the specimen is dried. The phosphotungstate seems to have no influence on the formation of this phase.

ACKNOWLEDGEMENTS

The authors would like to thank Priv. Doz. Dr. W. Pitsch and Dr. P. L. Ryder for helpful discussions concerning the diffraction analysis. They are also most grateful to Dr. V. Luzzati for stimulating discussion.

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