ELECTRON MICROSCOPE AND X-RAY DIFFRACTION STUDIES ON A HOMOLOGOUS SERIES OF SATURATED PHOSPHATIDYLCHOLINES

P. F. ELBERS, Ph.D., and P. H. J. T. VERVERGAERT

From the Centrum voor Submicroscopisch Onderzoek van Biologische Objecten, Utrecht, The Netherlands

ABSTRACT

Three homologous saturated phosphatidylcholines were studied by electron microscopy after tricomplex fixation. The results are compared with those obtained by x-ray diffraction analysis of the same and some other homologous compounds, in the dry crystalline state and after tricomplex fixation. By electron microscopy alternating dark and light bands are observed which are likely to correspond to phosphatide double layers. X-Ray diffraction reveals the presence of lamellar structures of regular spacing. The layer spacings obtained by both methods are in good agreement. From the electron micrographs the width of the polar parts of the double layers can be derived directly. The width of the carboxylglyceryl-phosphorylcholine moiety of the layers is found by extrapolating the x-ray diffraction data to zero chain length of the fatty acids. When from this width the contribution of the carboxyl-glyceryl part of the molecules is subtracted, again we find good agreement with the electron microscope measurements. An attempt has been made to account for the different layer spacings measured in terms of orientation of the molecules within the double layers.

INTRODUCTION

The study of lipid model structures is obviously of great importance for the interpretation of data from biological membrane studies. This is especially the case when electron microscope and x-ray diffraction analysis can be applied to the same material. The electron microscope results can then be checked by a method that has no disturbing influence on the structure studied.

X-Ray diffraction studies on isolated lipids from natural sources have been carried out by Palmer and Schmitt (1941). They explained the layer spacings found in 33 per cent lipid emulsions by assuming that lipid double layers were separated by water layers. The thickness of the water layers proved to depend on the salt content of the medium.

Such a layering was also found by Luzzati and Husson (1962) in their x-ray studies on brain phospholipids. These authors investigated the structure of lipid-water systems as a function of concentration and temperature. Besides a lamellar structure, in certain concentration and temperature ranges, they proved the existence of a hexagonal liquid crystalline structure in which the lipid molecules occur in cylindrical arrangement with the polar parts facing the interior.

Stoeckenius, Schulman, and Prince (1960) studied brain phospholipids by electron microscopy and x-ray diffraction analysis. The dry lipid showed an x-ray diffraction spacing of 42.5 A. In a 5 per cent lipid emulsion the diffraction pattern was lost. A spacing of 53.6 A was found, however, after the addition of 1.3 M $BaCl_2$. Fixation by OsO_4 vapor reduced the 53.6 A spacing to

45 A. An increase of the spacing to 47 A was observed in methacrylate monomer and a reduction again, to 41.5 A, occurred on polymerization of the methacrylate. The spacing observed in the electron microscope was about 40 A, the dark bands measuring about 17 A in width, the light bands 23 A.

In a brain lipid system containing 30 per cent water, Stoeckenius (1962) found, by means of x-ray diffraction analysis, a fundamental spacing of 58 A. After OsO_4 fixation, a repeating period of 38 A was revealed by electron microscopy. The thickness of the lipid leaflets proper in lipid-water systems can be calculated according to Luzzati and Husson (1962). In the system containing 30 per cent water, used by Stoeckenius, this gives a thickness of 40.6 A for the bimolecular lipid leaflets.

The investigations mentioned so far have been concerned with mixed lipids from natural sources that contain large amounts of unsaturated fatty acids. Some results of observations on pure, saturated lipids are also available.

Trurnit and Schidlovsky (1961) studied multilayer stacks of barium soaps of fully saturated fatty acids. With the Langmuir-Blodgett technique monomolecular films were deposited as bimolecular layers, the polar groups facing each other, on a supporting slide. These experiments were made with fatty acids containing 16, 22, and 36 carbon atoms. After exposure to OsO_4 the multilayered stacks revealed a periodic structure of alternating dark and light bands in the electron microscope. The respective periodicities showed a ratio of approximately 16 to 22 to 36, but only when the sections were sufficiently protected by a carbon coating on both sides and low beam current was used. In the absence of these precautions smaller periodicities were observed. Multilayers containing the fatty acid with 22 carbon atoms showed, by electron microscopy, a layer spacing of 60 A. This was identical, within a few Angstroms, with the layer spacing derived from x-ray diffraction analysis.

Finean (1959) studied the layer spacing of a synthetic L-α-(distearoyl)phosphatidyl-L-serine sample by x-ray diffraction and electron microscopy. By x-ray diffraction the spacing of the crystalline material at room temperature was 63 A. This remained the same after exposure to a buffered OsO_4 solution, dehydration, and embedding in Araldite. In the electron microscope, the layer spacing was about 50 A. In the micrographs the dense lines were noticeably narrower than the light interspaces. By x-ray diffraction the layer spacing measured at a temperature of 70 to 80°C was 55 A to 50 A, and when measured at 90 to 100°C only 38 A. It seems likely, therefore, that heating of the sections by the electron beam caused a shortening of the layer spacing. In his x-ray diffraction analysis of some isolated and synthetic phospholipids Finean (1953) observed the general feature that the layer spacings tend to decrease with rise in temperature, the decrease being associated with a tilting of the long axes of the molecules with respect to the plane of the bimolecular leaflet.

An x-ray diffraction study by Finean and Millington (1955) of three homologous series of phospholipids has revealed the existence of three polymorphic forms in these compounds. The homologous series consisted of pure crystalline phosphatidylethanolamines and phosphatidylcholines containing saturated fatty acids with 12, 14, 16, and 18 carbon atoms, respectively. With the phosphatidylethanolamines the layer spacings decreased markedly, in well defined steps, with rise in temperature. The changes observed indicated the existence of distinct polymorphic forms of these lipids. Three types, the A, B, and C polymorphs, could be distinguished, corresponding to increasing temperatures. The layer spacings in the homologous series showed a linear relationship with the number of carbon atoms in the hydrocarbon chain of the fatty acids. The slope of this curve is characteristic of the arrangement of the hydrocarbon chains, and extrapolation to zero chain length gives the contribution of the end group to the layer spacing. Finean and Millington used these data as a basis for their diagrams which indicate the mode of packing of phospholipid molecules into bimolecular leaflets in the A, B, and C polymorphs. Their data will be compared with the data of the present study in the Discussion section of this paper.

For reasons set forth in a previous paper (Elbers, Ververgaert, and Demel, 1965) it was also thought necessary to investigate fully saturated phospholipids by electron microscopy. Such a study would permit a critical evaluation of the observed structural details by comparison with the corresponding x-ray data. Pure substances of known molecular composition would make the interpretation of the data more straightforward

than was possible with the relatively crude materials studied formerly. The tricomplex fixation method offered the means to start this study, on the well founded theoretical basis provided by the work of Bungenberg de Jong on complex colloid systems.

MATERIAL AND METHODS

Electron Microscopy

Chromatographically pure samples of three saturated phosphatidylcholines were studied, *viz* L-α-(diheptanoyl)-, L-α-(didecanoyl)-, and L-α-(dipentadecanoyl)lecithin. They had been synthesized according to the methods described by Kögl, de Haas, and van Deenen (1960) and de Haas (1963). Dispersions of these substances in water were made with a lipid content of 0.5 per cent. The L-α-(diheptanoyl)lecithin yielded a water-clear sol, the L-α-(didecanoyl)lecithin a slightly opalescent sol, and the L-α-(dipentadecanoyl)lecithin a turbid sol. Fixation was done according to the tricomplex method described by Elbers, Ververgaert, and Demel (1965). Cobalt nitrate and ammonium molybdate in 0.1 N solutions provided the tricomplex microions. The tricomplex floccules were centrifuged down, dehydrated with acetone, and embedded in a butyl-methylmethacrylate—divinylbenzene mixture (Kushida, 1961). Thin sections were mounted on carbon-coated, fenestrated (Jaffe, 1948) collodion films. In one group of experiments they were covered by a carbon coating on both sides.

Electron micrographs at an initial magnification of about 57,000 were made with a Siemens Elmiskop I at 60 kv. The magnification of each micrograph could be determined with an accuracy higher than 1 per cent with a method devised by Elbers and Pieters (1964). Through-focus series of micrographs were made with an objective current increment corresponding to one step of the fine-focus control. Only plates in focus were selected for measuring purposes. Density variations in selected areas of the photographic plates could be recorded by means of a Kipp recording microdensitometer. The densitometer track was carefully oriented perpendicular to the lamellar structures found on the photographic plates. All dimensions reported were taken from these densitometer recordings. For each figure the standard deviation (SD) and the number of measurements (n) are given.

X-Ray Diffraction

The x-ray diffraction experiments were carried out at room temperature, using a Kratky camera operated *in vacuo*. In all exposures unfiltered copper radiation was used. The samples to be investigated were held in slits milled in brass specimen carriers. These slits had a length of 5 mm, a height of 0.5 mm and a depth of 0.3 mm. They were covered on both sides by adhesive tape, which provided a vacuum-tight enclosure of the samples. In addition to the three lecithins mentioned above, also L-α-(diacetyl)-, L-α-(dibutyryl)-, DL-α-(ditetradecanoyl)-, and L-α-(ditetracosanoyl)lecithin were studied by x-ray diffraction. In all samples the Bragg spacings of the sharp lines proved to be the integral orders of one fundamental spacing. This is characteristic of a lamellar structure (Luzzati and Husson, 1962).

In some cases a few faint extra lines were observed. These were not taken into consideration in the interpretation of the diagrams. The accuracy of the x-ray diffraction measurements in this investigation was about 1 per cent.

RESULTS

L-α-(DIHEPTANOYL)LECITHIN: This lecithin is slightly soluble in water, readily soluble in ethanol, and very soluble in acetone (de Haas, 1963). On the electron micrographs configurations of parallel dark and light bands are observed. Their mean layer spacing is 27 A (SD = 2.6; n = 48) in uncoated sections. With carbon-coated sections a mean layer spacing of 35 A (SD = 2.1; n = 89) is found (Fig. 1 *a* and 1 *b*).

The x-ray studies give the following picture. The dry crystalline lecithin shows a repeating period of 32.4 A, the tricomplex of this material in water a period of 35.5 A, and the methacrylate-embedded tricomplex a repeating period of 33.5 A.

L-α-(DIDECANOYL)LECITHIN: This lecithin is also slightly soluble in water and readily soluble in ethanol, but less soluble than the first in acetone (de Haas, 1963). In the electron microscope it shows dark and light bands. Their mean layer spacing is 33 A (SD = 2.6; n = 77) in uncoated sections. With carbon-coated sections, a mean layer spacing of 39 A (SD = 2.9; n = 80) is found (Fig. 2 *a* and 2 *b*).

X-Ray studies show that the dry crystalline material has a repeating period of 40.5 A. The tricomplex of this lecithin in water has a period of 42.4 A. After cooling to −60°C for 1 hour this period is 41.5 A, and after heating to 100°C for 15 minutes the period is 42.3 A. The methacrylate-embedded tricomplex shows a repeating period of 42.0 A.

L-α-(DIPENTADECANOYL)LECITHIN: This lecithin is insoluble in water and soluble in

ethanol, but practically insoluble in acetone. It is very soluble in methacrylate monomer at 50°C. In the electron microscope parallel dark and light bands are again observed. Their mean layer spacing is 47 A (SD = 4.4; n = 26) in uncoated sections and 47 A (SD = 4.2; n = 142) in carbon-coated sections (Fig. 3).

X-Ray diffraction reveals that the dry crystalline material shows a repeating period of 59.5 A. After heating to 100°C for 15 minutes the same repeating period is found. Previous cooling to −25°C for 80 hours again gives the same repeating period, whereas after cooling to −60°C for one hour a period of 59.1 A is observed. The tricomplex of this lecithin in water shows a repeating period of 61.5 A. Heating to 100°C for 15 minutes results in a period of 47 A and after cooling to −60°C for 1 hour a repeating period of 62.1 A is observed. The methacrylate-embedded tricomplex has a repeating period of 63.5 A.

The other lecithins mentioned were investigated in the dry crystalline state by x-ray diffraction only. The L-α-(diacetyl)lecithin gave no x-ray reflections. The repeating period of L-α-(dibutyryl) lecithin was 25.4 A. The period was 25.1 A after heating to 100°C for 15 minutes and remained so after 80 hours at −25°C. The DL-α-(ditetradecanoyl)lecithin showed a repeating period of 55.2 A. This period was 54.5 A after heating to 100°C for 15 minutes and remained so after 80 hours at −25°C. The L-α-(ditetracosanoyl)-lecithin was characterized by a repeating period of 82 A. This period was 81.4 A after heating to 100°C for 15 minutes and remained so after 80 hours at −25°C. Fig. 6 shows a graphic representation of the major part of the data obtained.

For analysis by electron microscopy, layer spacing of the lecithin was determined by measuring peak distances in the densitometer recordings. The polar layer thickness will be derived from the half-width of these peaks. Their half-width, however, depends on the orientation of the micellar layer planes with respect to the optical axis of the microscope. Only an orientation of the layers perfectly parallel to the optical axis will allow the measuring of the true width of the electron-dense band. All other orientations will increase the measured thickness until, eventually, the structure becomes invisible. In contradistinction to the results obtained by Trurnit and Schidlovsky (1961) with heavy-metal soaps of saturated fatty acids, our preparations showed this change of the width of the electron-opaque bands with tilting of the specimen. We carefully selected a specimen site in which the dark bands ran perpendicular to the plane of tilting of the stereogrid holder used. This site was photographed before and after a tilt of 10° (Fig. 4 *a* and 4 *b*). The layers in the left part of this figure run perpendicular to the plane of tilting. They are, moreover, oriented in the section parallel to the electron beam except for a small twist in their central region. This region gives a wide projection of the electron-opaque bands. Apparently, after a specimen tilt of 10° the twisted region has become parallel to the electron beam. This results in a narrow projection of the electron-opaque bands in this region, together with a shift of the wide projection to the outer regions of this layer group. This phenomenon is not seen in specimen sites where the dark bands do not run perpendicular to the tilting plane as in the right part of Fig. 4 *a* and 4 *b*.

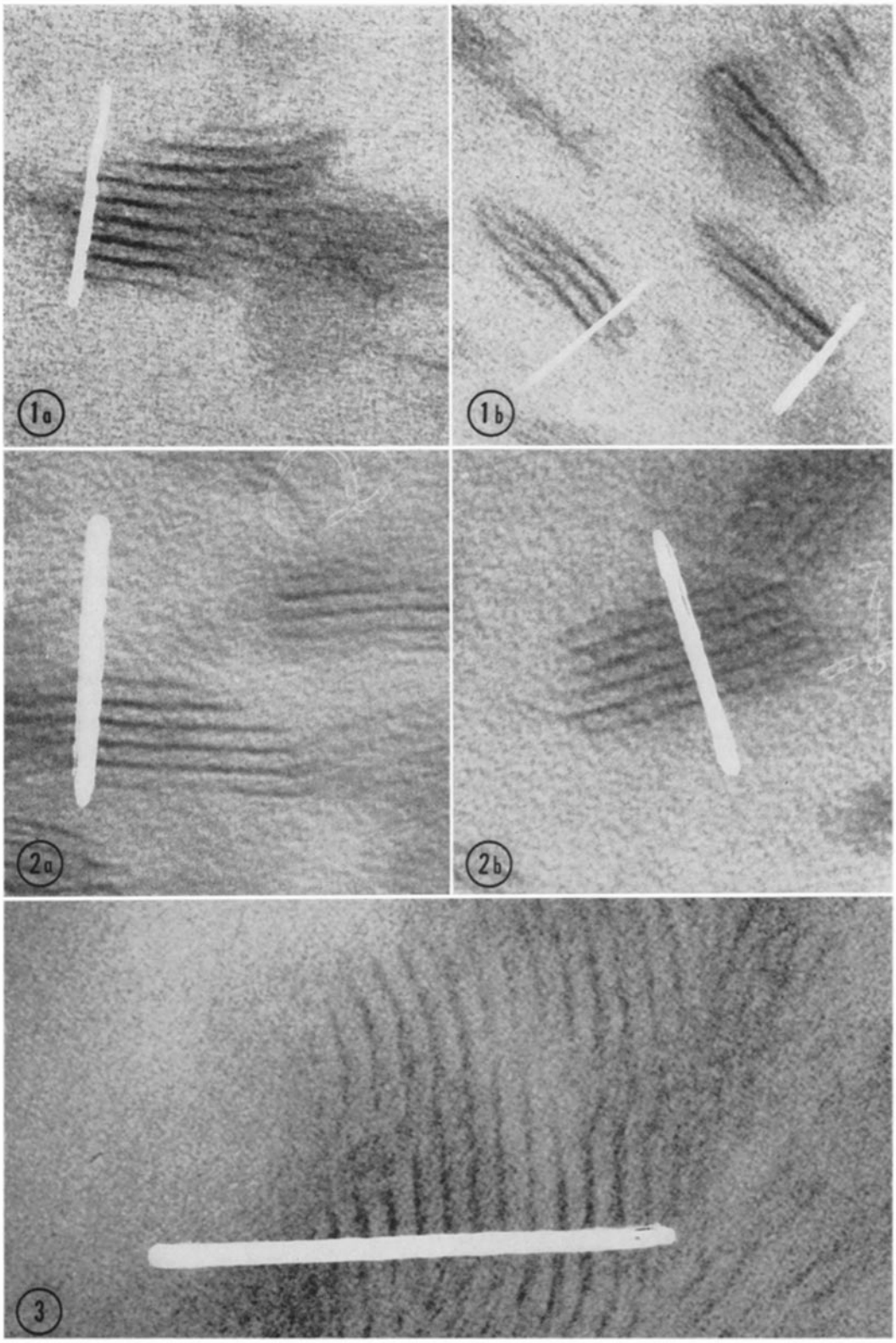
For the determination of the polar layer thickness, therefore, the smallest half-width of the peaks in the densitometer recordings was selected. This proved to be about 12 A and the same in the three lecithins investigated.

Fig. 5 gives an idea of the three-dimensional extension of the tricomplex floccules studied. A specimen grid was dipped in the suspension of the floccules, dried, and briefly washed with distilled

FIGURE 1 *a* and 1 *b* L-α-(diheptanoyl)lecithin tricomplex with cobalt and molybdate ions. (The white bands on this and the following figures served as guide lines for the densitometer tracings on the negative plates). × 827,000.

FIGURE 2 *a* and 2 *b* L-α-(didecanoyl)lecithin tricomplex with cobalt and molybdate ions. × 827,000.

FIGURE 3 L-α-(dipentadecanoyl)lecithin tricomplex with cobalt and molybdate ions. × 839,000.



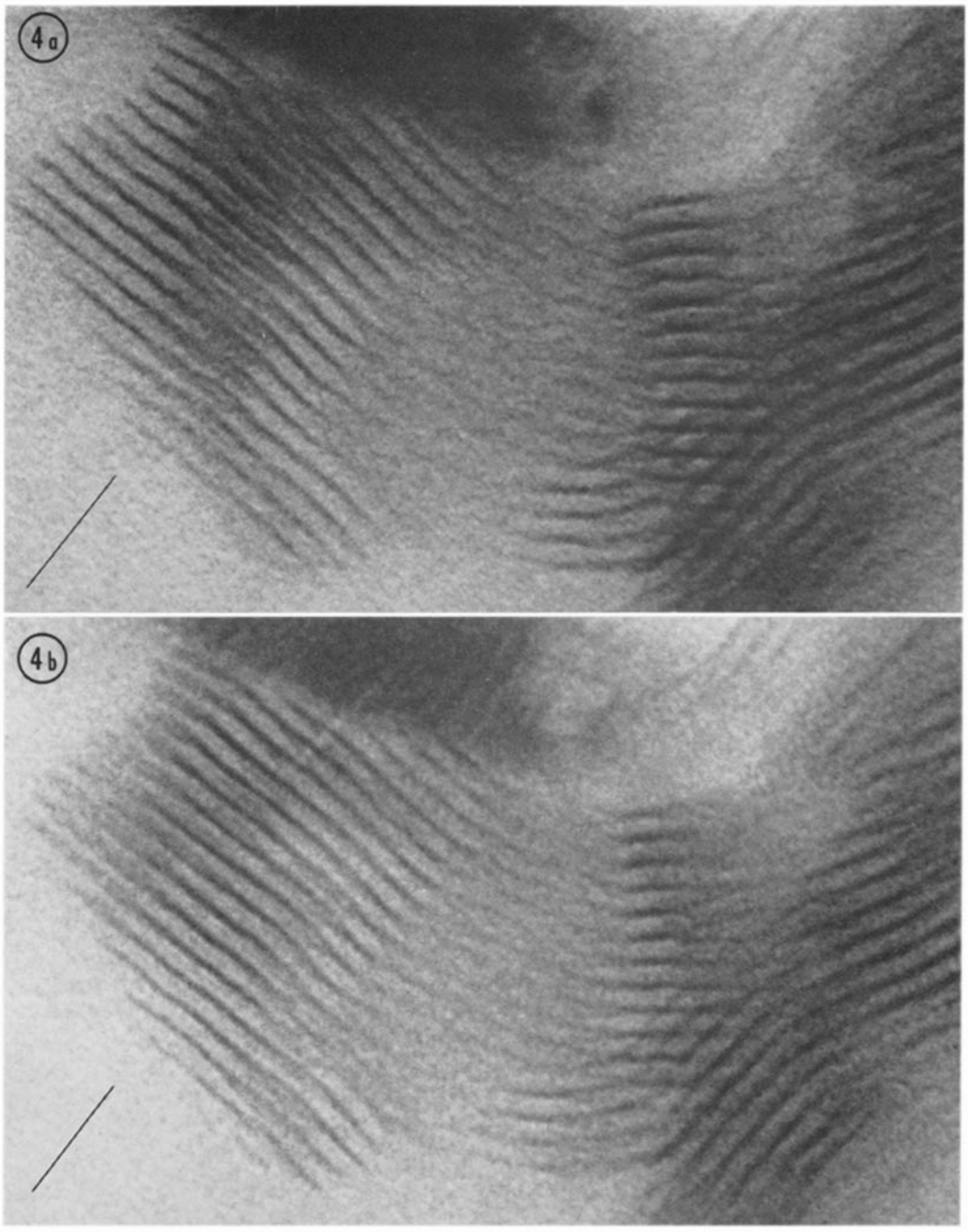


FIGURE 4 *a* L-α-(dipentadecanoyl)lecithin tricomplex with cobalt and molybdate ions. × 787,000.

FIGURE 4 *b* The same section as Fig. 4 *a* photographed after a tilt of 10°. The layers in the left part of the figures run perpendicularly to the plane of tilting of the specimen-grid holder. The secant of this plane with the plane of the section is indicated by a black line. Further explanation in the text.

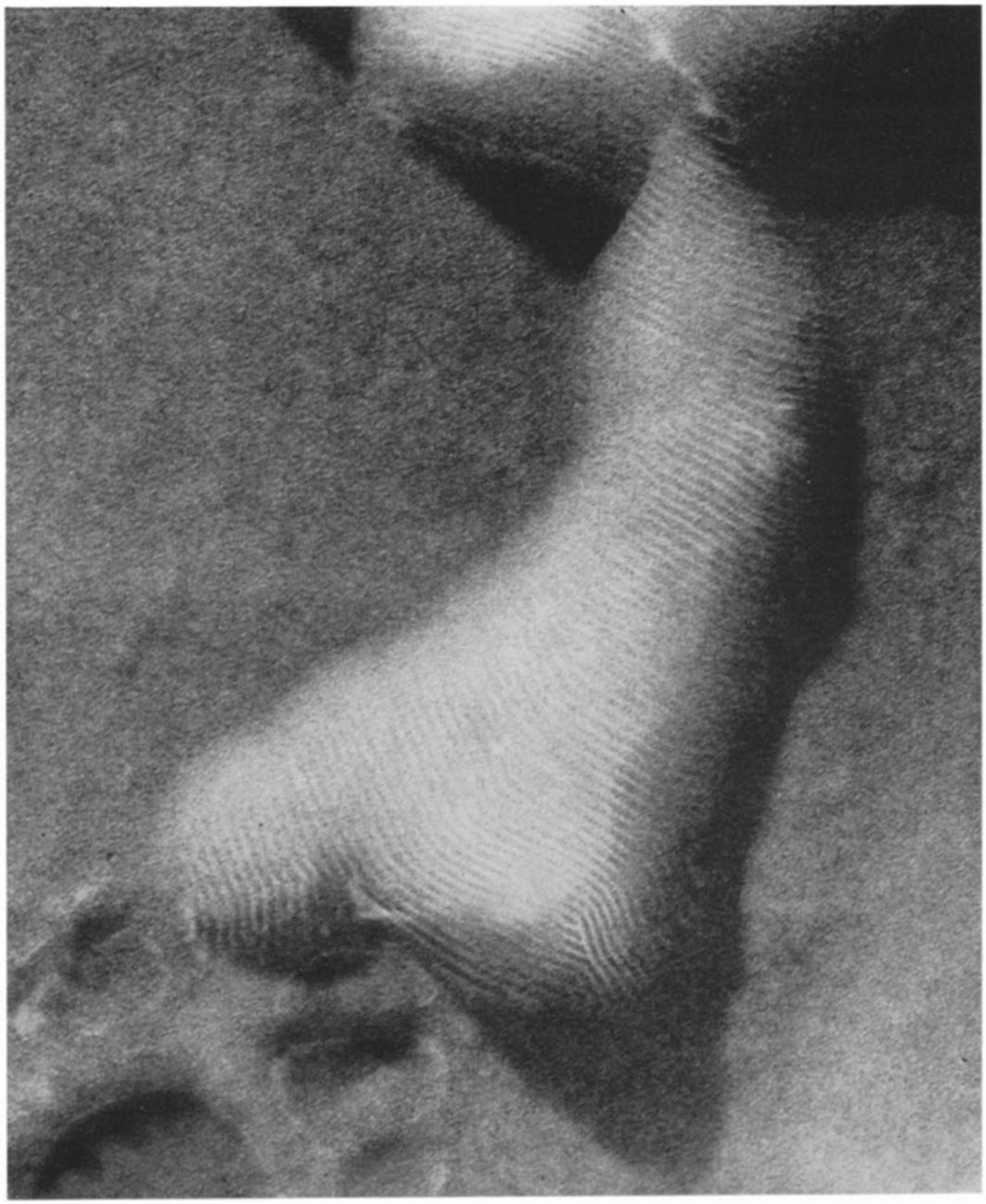


FIGURE 5 L-α-(dipentadecanoyl)lecithin tricomplex floccule shadow-casted with platinum-carbon. × 288,000.

water. Shadow-casting was done with platinum-carbon. In the floccules the polar layers are visible by their own contrast.

DISCUSSION

Before analyzing the meaning of the lipid layer dimensions as found in the experiments, it is necessary to consider what is represented by the dark and light bands revealed by electron microscopy. The contrast in the electron micrographs is undoubtedly caused by heavy metal atoms. To test this the tricomplex fixation was performed with ions of lighter elements. The electron microscope reveals the same parallel band configurations but only with low contrast. The dark layers in the electron micrographs are therefore the layers in which the heavy metal ions occur.

Now the question arises as to the nature of the atomic configuration in these layers. According to the theory on complex relations proposed by Bungenberg de Jong (1949) this configuration will consist of the phosphatide Zwitter ion, that is, the phosphorylcholine group, the cobalt cation and the molybdate anion, while the presence of some water of hydration has to be discussed. With regard to the last question, it is argued by Bungenberg de Jong that the phosphate group is considerably more polarizable than water. The cobalt ion, which is small and in addition divalent, in its turn shows a strongly polarizing action. This means that in the complex relation the cobalt ion will get rid of its water of hydration because of the large polarization energy, which is set free on the attachment of the anhydrous ion to the phosphate group. The positive group of the Zwitter ion has the properties of a large cation, which means that it exerts no polarizing action. The fixation of an anion to this group will be the easier the greater its size, that is, the less it is hydrated.

According to Sasaki, Lindquist, and Sillen, (1959) a watery solution of ammonium molybdate near pH 7 contains the six-valent Mo_7O_{24} ion as the main component. The molybdate ion thus belongs to the large anions. According to the theory of Bungenberg de Jong, we have to do here with favourable conditions for strong tricomplex relations. This points to a direct contact of the oppositely charged ionised groups and ions. On the other hand, in the tricomplex floccules charge compensation of the positive and negative groups and ions is found. This means that every cobalt ion is connected to two phosphate groups and every molybdate ion to six choline groups (Bungenberg de Jong and Saubert, 1936).

From the above conditions it is inferred that the polar layers of the tricomplex floccules will contain little or no water of hydration. In any case the lipid double layers will not alternate with water layers of different thickness as in the phospholipid-water mixtures of high lipid content which were studied by Luzzati and Husson (1962) in x-ray diffraction experiments. It is therefore thought likely that the small differences in layer spacing observed in our x-ray analysis of the pure crystalline lecithins, the tricomplex floccules in water, and those embedded in methacrylate reflect the addition of ions to the polar layers or changes in the orientation of the polar and/or apolar groups of the lipid double layers rather than differences in water content.

When the spacings of the crystalline dibutyryl-, diheptanoyl- and didecanoyllecithin, analyzed by x-ray diffraction, are plotted against the number of C atoms in the hydrocarbon chains (Fig. 6) a linear relationship is found. The slope of this curve is 2.5, the same as observed by Finean and Millington (1955) in the A polymorph of their homologous series of lecithins. The ordinate intercept, which gives the contribution of the end-groups to the layer spacing, is 17.5 A. The corresponding curve for the lecithin tricomplex in water has the same slope, but an ordinate intercept of 20 A. Embedding in methacrylate in the case of diheptanoyl lecithin reduces the layer spacing almost to that of the crystalline lecithin, whereas in the case of didecanoyllecithin the tri-complex layer spacing is left unchanged. With the A polymorph Finean and Millington assumed a configuration of the double layers in which the molecules lie perpendicular to the layer planes with the end-groups fully extended and in line with the hydrocarbon chains.

The end-groups consist of the glycerylphosphorylcholine moiety of the molecules together with the two fatty acid carboxyl groups. From the dimensions of a lecithin molecular model it can be inferred that the part of the layer spacing occupied by the glycerylcarboxyl group of the lipid molecules will be about 8 A. Subtraction of this part from the end-group width gives about 12 A for the width of the phosphorylcholine moiety in the lecithin tricomplex, as determined by x-ray diffraction.

When it is assumed that the electron-scattering

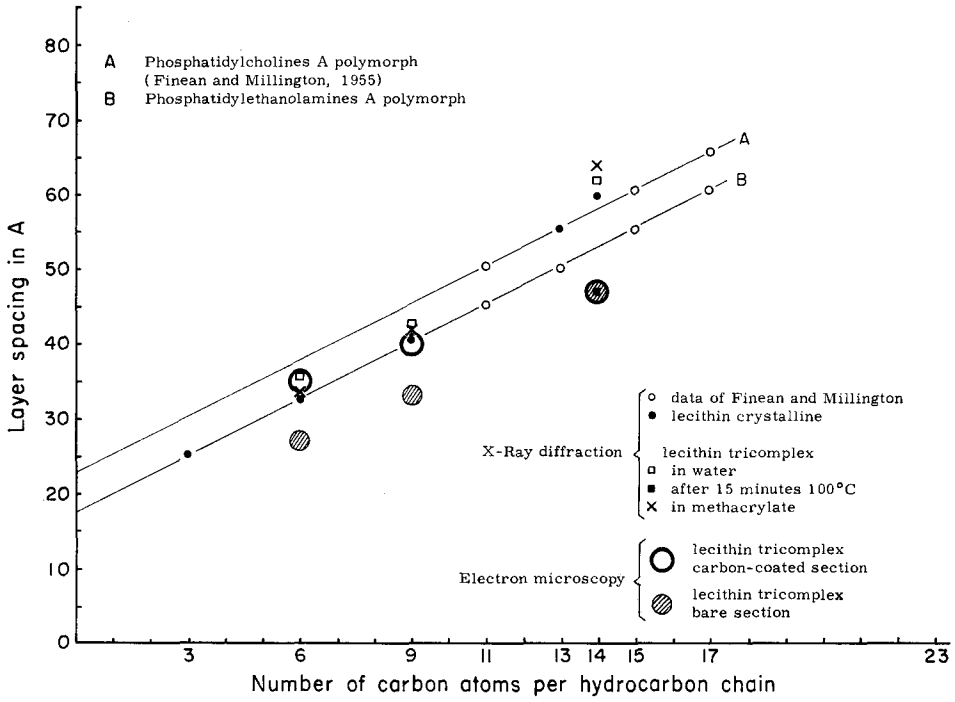


FIGURE 6 Graphic representation of the electron microscope and x-ray diffraction data together with some of the data given by Finean and Millington (1955). Explanation in the text.

heavy metal atoms are confined to the phosphorylcholine parts of the layers, then the width of the dark bands in the electron micrographs also corresponds to the width of the phosphorylcholine layers. This width, estimated from densitometer recordings, was about 12 A. With regard to the polar layer thickness of the bimolecular lipid lamellae, good agreement is therefore obtained between the x-ray and the electron microscope data. A similarly good agreement is found for the layer spacings themselves with diheptanoyl- and didecanoyllecithin, when carbon-coated sections are used in the electron microscope observations. In dipentadecanoyllecithin, even with carbon-coated sections, a large difference between electron microscope and x-ray data at first was obtained. When, however, in the x-ray experiments with the dipentadecanoyllecithin tricomplex in water the specimen was first heated to 100°C for 15 minutes and then studied at room temperature, the spacing was reduced exactly to the value found in the electron microscope observations. With the two lower lecithin homologues the two carbon layers on the sections are sufficiently heat conducting to prevent the molecules from transformation into a higher temperature polymorph. It seems that with the dipentadecanoyllecithin the carbon coating is insufficient to this end. The supposed heating effect in the electron microscope closely follows the effect of heating observed by x-ray diffraction. Fortunately the effect of heating on this substance persists at room temperature.

It can be precluded that the reduction in layer spacing occurred during the polymerization of the embedding material, because in the embedded lecithin tricomplex the layer spacing by x-ray diffraction was found to be even larger than that of the crystalline material. From these results it can be inferred that also, with regard to layer spacings, complete agreement between x-ray diffraction and electron microscope observation is obtained. The differences that were found can be explained by the heating effect of the electron beam.

Some differences between our x-ray diffraction data and those of Finean and Millington remain to be mentioned. These differences concern the end-group contribution to layer spacing as determined by extrapolation of the x-ray data to zero chain length. The slopes of our curves are identical

with that of the A polymorph of the lecithins investigated by these authors. Their curve shows an ordinate intercept of 23 A. Our curve for the dibutyryl-, diheptanoyl- and didecanoyllecithins in the crystalline state, however, has an intercept of 17.5 A, whereas the curve for the dipentadecanoyl- and ditetracosanoyllecithin has an ordinate intercept of 25 A. The layer spacing of the ditetradecanoyllecithin in the crystalline state coincides with that found by Finean and Millington for the same compound.

It should be kept in mind that the x-ray diffraction data of the phosphatidylcholines were obtained by these authors with specimens at −20°C, whereas our specimens were studied at room temperature. Neither previous cooling of our crystalline specimens to −25°C or −60°C nor previous heating to 100°C changed the layer spacing found at room temperature.

Curiously enough the layer spacings observed by us for dibutyryl-, diheptanoyl-, and didecanoyllecithin in the crystalline state are found on the same curve as those obtained by Finean and Millington for the A polymorph of the higher homologous α-phosphatidylethanolamines at room temperature.

In general, it would seem that our x-ray diffraction studies were concerned with the A polymorph of the homologous lecithins. According to Finean and Millington the configuration of the lecithin molecules in the A polymorph is that of fully extended zig-zag hydrocarbon chains with the end-groups in line and perpendicularly oriented to the layer planes. This same configuration applies to the electron micrographs of the diheptanoyl and didecanoyl tricomplexes.

The molecular configuration in the electron micrographs of the dipentadecanoyllecithin tricomplex corresponds to that obtained by x-ray diffraction analysis after preheating to 100°C. With respect to the layer spacing before heating, a thickness reduction of about 13 A occurs. From the electron microscope observations it may be deduced that the tricomplex polar group width does not change by heat treatment. It seems likely therefore that the 20 per cent reduction in layer spacing has to be found in the hydrocarbon chain moiety of the lipid layers. This means that the hydrocarbon chain layer thickness is reduced to about 60 per cent of that of the fully extended zig-zag configuration. According to Finean and Millington this reduction could be obtained by the formation at higher temperatures of a tightly coiled spiral having four to five carbon atoms per turn. On the other hand, a tilt of 53° of the hydrocarbon chains would produce the same layer spacing reduction. In fact this last configuration was considered by Finean and Millington to occur in the C polymorph of their phosphatidylethanolamines at temperatures above 100°C. This problem of layer spacing reduction probably could be settled by the use of a suitable device for cooling specimens in the electron microscope.

It is not the object of the present study to go into more detail regarding the configuration of the lipid molecules in the tricomplex system. As a general conclusion it may be stated that with the tricomplex fixation method close agreement is obtained between electron microscope and x-ray diffraction data on a molecular association of synthetic, fully saturated phospholipids of known composition.

The authors are greatly indebted to Professor L. L. M. van Deenen, who supplied the synthetic lecithins used in this study.

Special thanks are due to Professor P. M. de Wolff and Mr. W. Broer at Delft for the first series of x-ray diffraction experiments. The main part of the x-ray diffraction work was done by the Technical Physics Department of T.N.O. and T.H. at Delft under the supervision of Drs. J. W. Visser, for which we express our sincerest thanks.

Received for publication, May 5, 1964.

REFERENCES

BUNGENBERG DE JONG, H. G., 1949, *in* Colloid Science (H. R. Kruyt, editor), Amsterdam, Elsevier Publishing Company **2**.

BUNGENBERG DE JONG, H. G., and SAUBERT, G. G. P., 1936, *Biochem. Z.*, **288**, 13.

DE HAAS, G. H., 1963, Glycerophosphatides, their chemical syntheses and application in biochemistry, Doctoral thesis, University of Utrecht.

ELBERS, P. F., and PIETERS, J., 1964, *J. Ultrastruct. Research*, **11**, 25.

ELBERS, P. F., VERVERGAERT, P. H. J. T., and DEMEL, R., 1965, *J. Cell Biol.*, **24**, 23.

FINEAN, J. B., 1953, *Biochim. et Biophysica Acta*, **10**, 371.

FINEAN, J. B., *J. Biophysic. and Biochem. Cytol.* 1959, **6**, 123.

FINEAN, J. B., and MILLINGTON, P. F., 1955, *Tr. Faraday Soc.*, **51**, 1008.

JAFFE, M. S., 1948, *J. Appl. Physics.*, **19**, 1191.

KÖGL, F., DE HAAS, G. H., and VAN DEENEN, L. L. M., 1960, *Rec. Trav. Chim.*, **79**, 661.

KUSHIDA, H., 1961, *J. Electronmicrosopy*, (*Tokyo*), **10**, 194.

LUZZATI, V., and HUSSON, F., 1962, *J. Cell Biol.*, **12**, 207.

PALMER, K. J., and SCHMITT, F. O., 1941, *J. Cell. and Comp. Physiol.*, **17**, 385.

SASAKI, Y., LINDQVIST, I., and SILLEN, L. G., 1959, *J. Inorg. Nucl. Chem.*, **9**, 93.

STOECKENIUS, W., 1962, *J. Cell Biol.*, **12**, 221.

STOECKENIUS, W., SCHULMAN, J. H., and PRINCE, L. M., 1960, *Kolloid Z.*, **169**, 170.

TRURNIT, H. J. and SCHIDLOVSKY, G., 1962. *Proc. European Regional Conf. Electron Microscopy, Delft, 1961*, **2**, 721.