TRICOMPLEX FIXATION OF PHOSPHOLIPIDS

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ABSTRACT

A basis for the interpretation of the structure of the cell membrane is often looked for in electron microscope investigations on the structure of lipid models. A difficulty in these investigations is our lack of knowledge of the effect of the preparative treatment on the structure studied. This applies especially to the strongly oxidizing fixatives: osmium tetroxide and potassium permanganate. These agents have, moreover, the drawback that they cannot be used to fix fully saturated lipids. On the basis of existing theories concerning complex colloid systems, a fixation method was developed that allows the electron microscope study of the structure of phospholipid models, irrespective of whether they consist of saturated or unsaturated compounds. With this fixation, namely tricomplex flocculation by means of suitable ions, the structure of the lipid molecules is not altered. Moreover, the site and mode of action of this fixation are well known. The first application of this method to the study of isolated beef brain phospholipids is described.

INTRODUCTION

The presence of layered structures at the borderline of cell territories has been demonstrated in electron micrographs of a wide variety of cells. Furthermore, strong evidence from chemical and physical investigations indicates that the cell membrane contains a bimolecular lipid leaflet. The layered structures revealed by electron microscopy are, therefore, often interpreted as representing parts of this leaflet with associated structures. They are, however, the result of an interaction of fixing and staining agents with the molecules of the membrane. The uncertainty of the interpretation now lies in the fact that our knowledge of the chemical nature of this interaction is rather scanty, not only as regards the reaction kinetics but also the extent of reorientation of the constituent molecules that may occur.

A possible way out of these difficulties is to study membrane structure by means that are independent of electron microscopy, such as low-angle x-ray analysis, and to study model structures of known composition by as many different techniques as possible.

To the first type of investigation belong the studies on nerve myelin structure by Schmidt (1936), Schmitt (1936), Schmitt, Bear, and Palmer (1941), Finean (1954), and Fernández-Morán and Finean (1957); to the second, the studies on model systems by Stoeckenius, Schulman, and Prince (1960), Finean and Millington (1955), Luzzati and Husson (1962). For a more extensive treatment of this subject we may refer to the review by Elbers (1964).

Even with lipid models, the interpretation, in terms of molecules or parts of them, of micrographs obtained by electron microscopy is hampered by our lack of knowledge of the action of the commonly used fixatives osmium tetroxide and potassium permanganate on the structure and position of the molecules. It is well known that lipids from biological sources contain large and varying amounts of mono- and polyunsaturated

fatty acids (Kögl *et al.*, 1960; de Gier *et al.*, 1961). Osmium tetroxide and potassium permanganate are strong oxidants and react, in the first place, with the double bonds of unsaturated fatty acids. Aziz Khan *et al.* (1961) and Riemersma and Booij (1962) have demonstrated that there is a stoichiometric relation between the amount of osmium tetroxide used up by and the number of double bonds present in a particular lipid sample. One molecule of OsO_4 reacts with one double bond.

According to the reaction scheme of Criegee (1936), the double bond is converted to a diol-configuration. This hydrophilization of part of the fatty acid hydrocarbon chain, especially in the case of polyunsaturated acids, must have a profound influence on the orientation of these chains. Experiments by Riemersma and Booij (1962) and Riemersma (1963) have shown that, as a result of the reaction with osmium tetroxide, the polar group of phosphatides will change properties too. Judging from the behaviour of osmium tetroxide–fixed phosphatides in the tricomplex staining reaction devised by Bungenberg de Jong and van Someren (1959), these authors suggested that after fixation the positive charge of the phospholipids has been partially removed by adsorption of hydrated osmium dioxide. It seems feasible that potassium permanganate reacts in much the same way, but it should be kept in mind that the non-ionized osmium tetroxide, because of its high lipid solubility, quickly permeates lipid layers, whereas potassium permanganate is used in the ionized state and thus its action depends upon the permeability to water of the structures to be fixed. Both fixatives are similar in that they act on lipids that contain unsaturated fatty acids. With lipids containing saturated fatty acids, normally no fixation is observed. A possible exception is the result obtained by Finean (1959) with L-α-(distearoyl) phosphatidylserine which, after prolonged reaction with osmium tetroxide, showed a system of parallel dark bands in the electron microscope.

Hitherto, the difference in the fixing reaction of these two types of lipids was no serious drawback, for the lipid models studied so far were made from relatively crude extracts of brain or red blood cells which contain a large amount of unsaturated fatty acids. To get more insight into the structure of lipid systems as studied by electron microscopy, it was deemed necessary, however, that synthesized, chemically pure, lipids with fully saturated fatty acid chains should be more frequently the object of investigations. This would also lead to a more fruitful correlation with the numerous physicochemical investigations carried out on lipid-water systems; *e.g.* on their stability and properties in aqueous solutions (Haydon and Taylor, 1963), on the role of long range forces in the cohesion of lipoproteins (Salem, 1962), or on the relationship of properties of monolayers, at the air-water interface, to the fatty acid composition of phosphatides (van Deenen *et al.*, 1962). The first problem to be solved is the electron microscopic demonstration of phospholipids—saturated and unsaturated—in some ordered array. As a model, one may take the arrangement of phospholipid molecules, above the critical micelle concentration, in a watery environment. Because of

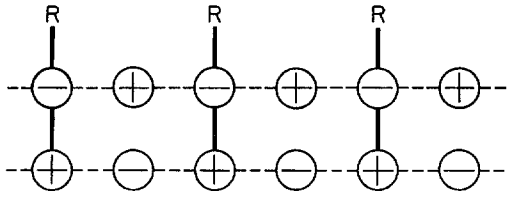

FIGURE 1 Model of a tricomplex system of phosphatide amphoions, microcations, and microanions. *R* represents the apolar part of the lipid molecules.

This figure was originally published in Biochemische Zeitschrift, 1936, volume 288, in an article by Dr. H. G. Bungenberg de Jong and Dr. G. G. P. Saubert (see Bibliography).

charge compensation in the polar groups, phospholipid molecules tend to form large, flat micelles, essentially bimolecular lipid layers. The distance between the individual micelles depends on the water and salt content of the system (Palmer and Schmitt, 1941; Luzzati and Husson, 1962).

Provisionally, we set aside a true electron microscopic representation of the intermicellar distances. First, a fixation of the micellar structure, as such, was aimed at. We had, therefore, to look for a fixation method that (1) would be effective regardless of whether the phospholipids are unsaturated or saturated; (2) would leave the chemical structure of the lipid molecules unchanged; (3) would allow the identification of electron-scattering regions of the molecules with certainty; and (4) would be accessible to theoretical interpretation, preferably within the frame of an existing theory on the stability of colloid systems.

A method that would satisfy the above condi-

FIGURE 2 Brain phospholipids fixed by the tricomplex method with cobalt nitrate and ammonium molybdate. Magnification, 450,000.

tions was found by applying a theory on the stability of biological membranes that was developed several years ago by Bungenberg de Jong and his co-workers. In this theory, for which Booij (1949) proposed the name "complex theory," the cell membrane is regarded as a tricomplex colloid system consisting of lipids, proteins, and cations tied together by complex relations (electrostatic interaction) and van der Waals dispersion forces. The theory was developed from considerations on a more general type of complex system containing colloid amphoions, cations, and anions. For a detailed account of the implications of this theory, the reader is referred to the outlines given by Bungenberg de Jong (1949), Booij and Bungenberg de Jong (1956), and also to a review by Elbers (1964).

A phenomenon that contributed to the abovementioned theory is of importance to the fixation of micellar structures from isolated phospholipids. This phenomenon is the so called tricomplex flocculation of phospholipid sols and emulsions, discovered by Bungenberg de Jong and Saubert (1936) in systems containing water, egg-lecithin, and suitable cations and anions. A hydrosol of purified egg-lecithin will not be flocculated by the addition of $CaCl_2$ or other chlorides and nitrates of mono-, di-, and trivalent cations. Nor will this be the case after the addition of ammonium molybdate. Flocculation occurs, however, when both salts are added simultaneously.

The floccules are dispersed again by the addition of NaCl. This phenomenon and also the results of electrophoresis measurements point to the concept that the flocculation represents the result of complex relations between three components: colloid amphoion—crystalloid cation—crystalloid anion. With a $CaCl_2$ concentration of sufficient strength and at electroneutrality of the floccules, one gram-molecule of lecithin has been found to bind one gram-equivalent of molybdate ions to the ionized choline group, and one gram-equivalent of calcium ions to the ionized phosphate group. Ion pairs of Ca^{2+} and $Mo_7O_{24}^{6-}$ then form links between the phosphatide amphoions, as is shown in a two-dimensional model (Bungenberg de Jong and Saubert, 1936) in which a + represents ½ Ca ion and a − represents ⅙ molybdate ion (Fig. 1). The dotted lines represent Coulomb interactions which are responsible for the cohesion of the lipid layers in the plane of the polar group. It is quite conceivable that the cohesion between two adjacent phospholipid layers in the floccules is also due to Coulomb forces between the positive charges of the respective polar group layers and the six-valent molybdate ions. The fact that the flocculation can be reversed by means of NaCl shows that electrostatic interactions are mainly responsible for the observed binding between phospholipid molecules, the van der Waals dispersion forces between the hydrocarbon chains playing a minor part.

This same concept was taken as a basis for a fixation method destined for use in the electron microscopy of thin sections of phospholipid-containing material. One of the features of preparation of material for electron microscope investigations on thin sections is the treatment of the material with lipid solvents during the dehydration and embedding procedure. This treatment will, in any case, weaken the van der Waals cohesion of lipid molecules. On the other hand, it may enhance the electrostatic attraction between opposite charges, because of the lowering of the value of the dielectric constant of the medium—from that for water ($\epsilon = 81$) to that for (*e.g.*) acetone ($\epsilon = 21$). The latter reasoning applies only when we are dealing with the macroscopic dielectric constant. Salem (1962), however, points out that, in the calculation of the Coulomb attraction energy in the situation of the ion pairs mentioned here, the effective or microscopic dielectric constant has to be used. The last one might be nearer to 15 or even smaller, without regard to the dielectric constant of the medium. With $\epsilon = 15$ he calculated an attraction energy of 4.1 kilocalories per mole for two unit charges of opposite sign at a distance of 5 A.

Tricomplex flocculation was now regarded as a sign of enhanced coherence of the lipid molecules to such an extent that the lipid micelles would resist, without dissolution the preparative treatment necessary for electron microscopy. The action of a fixative, on this basis, is localized with certainty at the polar part of the lipid molecules, while these polar parts, and of course the apolar parts too, are left unchanged chemically. When, in the tricomplex reaction, cations and anions are used that contain heavy metal atoms, then places of strong electron-scattering in the electron microscope image will correspond to the places of electrostatic interaction and thus to the polar parts of the lipids. A bimolecular lipid layer, after suitable section cutting, will be represented as a system of two parallel dark bands, the distance of which most likely corresponds to twice the length of the

FIGURE 3 Negative staining of the floccules by the salts employed in the tricomplex reaction. Magnification, 280,000.

apolar part of the molecules, that is, chiefly the fatty acid hydrocarbon chains.

PROCEDURE

Bungenberg de Jong and Saubert (1936) give a list of two series of salts which produce tricomplex flocculation with egg lecithin. The salts of the first series contain suitable cations, the salts of the second series suitable anions. The salts are selected in such a way that neither lecithin sol + salt 1, nor lecithin sol + salt 2, nor salt 1 + salt 2 produce a precipitate. In our preliminary experiments beef brain phospholipids were used instead of egg lecithin.

A crude ethanol-ether extract of fresh beef brain was purified by chromatography on a silica-gel column, using chloroform-methanol mixtures as eluents (Boldingh, 1963). The composition of the resultant phospholipid fraction was assessed from chromatography on silica-impregnated paper (Marinetti *et al.*, 1957). Elution was done with a mixture of diisobutylketone, acetic acid, and water (10:15:1), and the staining of the spots by means of the tricomplex staining reaction proposed by Bungenberg de Jong and van Someren (1959). The brain phospholipid fraction in these experiments contained about 40 per cent phosphatidylcholine and plasmalogencholine, 20 per cent sphingomyelin, 20 per cent phosphatidylserine, and 20 per cent phosphatidylethanolamine.

Emulsions of about 0.5 per cent lipid content were mixed with a suitable amount of the two salt solutions in such a way that the end concentration of the salts was about 0.05 N. After 17 hours the floccules were centrifuged down and dehydrated. The successive steps of the dehydration and embedding procedure were checked by polarisation microscopy. The floccules are birefringent, and the persistence of birefringence was taken as a sign of the continued presence of lipid lamellae in the precipitate. Dehydration was carried out by means of acetone, and embedding was done in either Araldite or butylmethylmethacrylate to which divinylbenzene was added according to Kushida (1961). Sections were studied with the Siemens Elmiskop I at 60 kv and a primary magnification of × 40,000.

Successful preparations were obtained with the following salt combinations: $Pb(NO_3)_2 + K_3Fe(CN)_6$; $PdCl_2 + Na_3PW_{12}O_{40}$; $Co(NO_3)_2 + (NH_4)_6Mo_7O_{24}$. Fig. 2 gives an example of the images obtained.

It proved possible to use the last-mentioned salt combination as a positive and negative staining agent at the same time. When the suspension of floccules containing the salts is sprayed onto carbon-coated specimen grids, droplets of sufficient transparency are found to permit electron microscopic investigation.

In the droplet area, structures are seen which seem to consist of flat (Fig. 3) and rolled-up sheets. From these micrographs no information is obtained about the thickness of the sheets. They are tentatively interpreted as representing large flat micelles of the phospholipid tricomplex.

In order to demonstrate that requirement No. 1, as mentioned above, is fulfilled, a synthetic, fully saturated phospholipid was also studied with the tricomplex method. With L-α-(ditetradecanoyl) lecithin, configurations of parallel bands were found, just as with the brain phospholipids, but with a more regular array (Fig. 4).

DISCUSSION

Phosphatides belong to the association colloids which tend to the formation of micelles in a milieu of sufficient water content. The micelles have different shapes, depending on the number of molecules that take part in their formation. Large micelles are essentially double films with a hydrophobic interior.

As the charged groups in the phosphatide molecule are fixed by covalent bonds at a short distance from each other, the conditions for intramicellar complex relations are fulfilled. Because of the charge compensation, large flat micelles are formed, the so called sandwich micelles (Booij and Bungenberg de Jong, 1956).

In our experiments, the original water content of the system was 99.5 per cent. The fixation process will, therefore, start with the above-mentioned sandwich micelles. These micelles are transformed into tricomplex systems which come closer together and thus flocculate. It is these condensed micelles that are revealed by electron microscopy. The results of the experiments led to the conclusion that the requirements for a new fixation method, as specified in the Introduction, are fulfilled. This new method promises to be a helpful tool in the study of artificial lipid systems of varied origin and, by comparison with x-ray data, of the influence of techniques of preparation and observation upon the final electron microscopic image. It also offers some means of checking the results obtained by fixation with osmium tetroxide or potassium permanganate.

The exact meaning of "fixation" on the molecular level will have to be settled, however, by the study of well defined molecular species. In this paper only the general background and the four

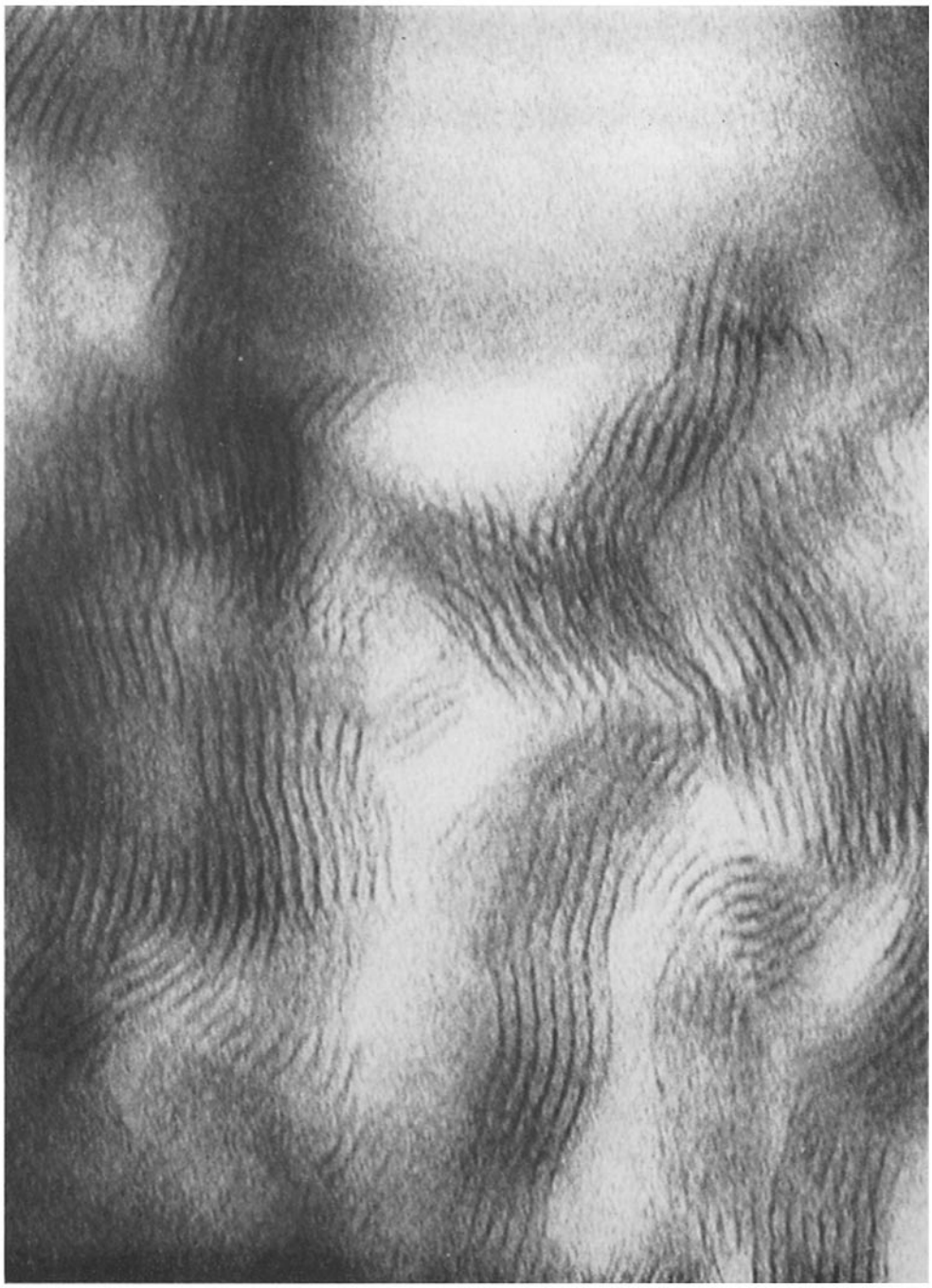

FIGURE 4 L-α-(ditetradecanoyl)lecithin fixed by the tricomplex method with cobalt nitrate and ammonium molybdate. The width of the dark bands, the polar layers, depends upon their orientation with respect to the electron beam. Magnification, 540,000.

special points of the tricomplex fixation are treated. The application of this method to the study of a homologous series of synthetic, fully saturated, phospholipids by electron microscopy, as well as low-angle x-ray diffraction, will be the subject of a forthcoming publication.

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