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Negative Staining of Phospholipids and their Structural Modification by Surface-active Agents as observed in the Electron Microscope

A. D. Bangham and R. W. Horne

Agricultural Research Council Institute of Animal Physiology
Babraham, Cambridge, England

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The negative-staining technique was applied to the study of lipid phase structures in the electron microscope. Lecithin dispersions were seen to contain spherulites composed of concentric lamellae. The width of the lipid layer was estimated to be 44 Å. Rod-shaped elements were also observed and interpreted as being groups of lipid molecules arranged at right angles to the long axis of the lamellae. Preparations of lecithin–cholesterol of equal molar proportions were described as being basically the same as lecithin alone.

Saponin modified the lamellar structure of lecithin–cholesterol in such a way as to produce an array of perforations with a centre-to-centre spacing of 120 Å and with a diameter of 85 Å. Separated rings together with helical structures estimated to be 220 Å in diameter with an axial hole of 68 Å were described. The helical structures showed a well-defined periodicity of 58 to 59 Å along the axis.

Lecithin dispersions were shown to be progressively disrupted after mixing with lysolecithin in water. Beading of the lamellae was a pronounced feature. Lecithin progesterone dispersions in water produced a line structure of about 20 to 22 Å across.

1. Introduction

A bimolecular leaflet arrangement of lipids depends as much on the presence of water in the region of the hydrophilic head-groups as it does on the precise composition and structure of the participating lipid molecules. It is therefore a structure which is extremely vulnerable to modification by procedures that remove water or involve even trace amounts of surface-active (amphipathic) compounds (reviewed by Bangham, 1964*a*,*b*). For these reasons, the validity of electron micrographs of fixed, embedded, dehydrated and sectioned materials must be questioned. Nevertheless, the degree of correlation between X-ray diffraction data on wet, unfixed specialized material, e.g. nerve myelin, and its appearance in the electron microscope after such treatments is more than sufficient to identify the presence of such a leaflet in myelin as well as in other cell membranes (Schmitt, Bear & Palmer, 1941; Fernández-Morán & Finean, 1957; Robertson, 1957,1960; Stoeckenius, 1962).

A bimolecular leaflet of lipids is a liquid crystalline structure capable of reversible structural modifications (Lawrence, 1961; Luzzati & Husson, 1962) and it is possible that the permeability properties of the biological cell depend upon such reversible changes. Certainly, the toxic effects of many compounds known to be surface active might be expected to produce their effects by altering the structure of the lipid leaflet (Bangham, 1964*b*). Recognition of these altered configurations has not been

forthcoming in fixed and embedded material although their presence might be expected. An early lead suggesting that structural modifications of a bimolecular lipid leaflet might be visualized in the electron microscope came when Bangham & Horne (1962) and Glauert, Dingle & Lucy (1962) simultaneously showed that a regular hexagonal structure was produced when saponin was allowed to react with mixtures of lecithin and cholesterol. This work was initiated by the negatively-stained micrographs obtained by Dourmashkin, Dougherty & Harris (1962), who showed that a similar structure was obtained when saponin reacted with certain mammalian cell and virus envelopes.

The use of potassium phosphotungstate as a negative stain offers several important advantages in the direct visualization of lipid phase structures. First, the rigid setting of the salt in the form of an electron-dense "glass" is believed to preserve structures as though they were in the presence of water. This is possibly due to the magnitude of the space charge surrounding the polyvalent phosphotungstate ion, no fixation in the histological sense being required. Secondly, a dilute solution of phosphotungstate is not itself surface active, and, thirdly, a very high degree of resolution can be obtained (cf. Horne, 1961). On the other hand, potassium phosphotungstate may be expected to exert an electrostatic stress in the region of the electrical double layer due to the inequality of the ionic valencies between the adsorbed phosphotungstate and potassium ions.

The purpose of this paper is to establish whether a negative-staining technique can visualize such bimolecular leaflet structure of lipids as might occur *in vivo*, and to see whether known lytic and other agents can modify such a structure and in what way.

2. Experimental

Ovolecithin, prepared by alumina and silicic acid column chromatography, was obtained through the generosity of Dr. Dawson, R.A.M.C. Lysolecithin, obtained from the same source, was recrystallized and largely freed of phospholipase A (*Naja naja*). Cholesterol (Roche) and white saponin (B.D.H.) were obtained commercially.

Dispersions for microscopy were prepared by taking 5 mg of the lipids dissolved in chloroform down to dryness *in vacuo* and resuspending in 1·0 ml. water either by gentle hand shaking or by sonication in a Dison Sontigrator, 80 kc/s at 80 w. Finally, an equal volume of a 2% solution of potassium phosphotungstate in water was added and a drop of the mixture placed on a cellulose-carbon coated grid. The drop was drained and, when dry, examined in an Elmiskop I (Siemens) microscope at instrumental magnifications of 40,000 and 80,000.

3. Results

Lecithin dispersion

Plate I shows a selected area of a grid in which the ratio of potassium phosphotungstate solution to the amount of lipid ensured the preservation of the structure of the lamellae. In other areas, containing large spherulites composed of many hundreds of concentric lamellae, insufficient potassium phosphotungstate was entrained to preserve the liquid crystalline structure, and the lipid appeared amorphous. There was no significant difference in the appearance of the small spherulites of lecithin whether they were prepared by hand shaking or sonication; the latter preparation merely contained fewer large spherulites. Stereo electron micrographs, obtained from negatively-stained air-dried and freeze-dried preparations, strongly suggested both large and small spherulites, rather than annuli of small depth.

The apparent progressive loss of the lamellae towards the centres of the spherulite may be interpreted as being due to the contrast effects illustrated in Fig. 1. The aqueous compartments containing the electron-dense material are always most clearly defined between the peripheral lamellae of lipid.

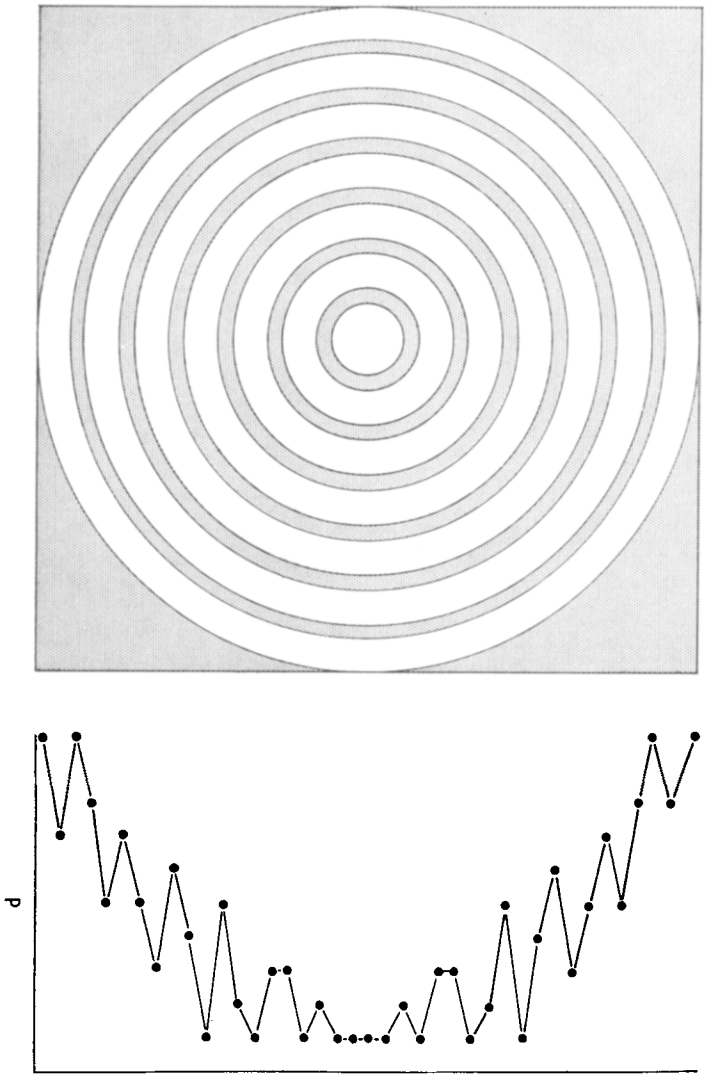

FIG. 1. Diagram illustrating the variations in density of an electron beam illuminating a structure consisting of concentric lamellae of high and low electron-dense material. The graph was obtained by summing the contributions of all the high-density lamellae in a vertical plane.

Plate II is an enlargement of a favourable area and shows, clearly, the structure of the lamellae. The mean width (50 measurements) of the lipid layer was estimated to be 44·2 Å and of the water compartment 25·6 Å. A number of micrographs have revealed rod-shaped elements (A) having a thickness of 10·2 Å lying at right angles to the long axis of the lamellae.

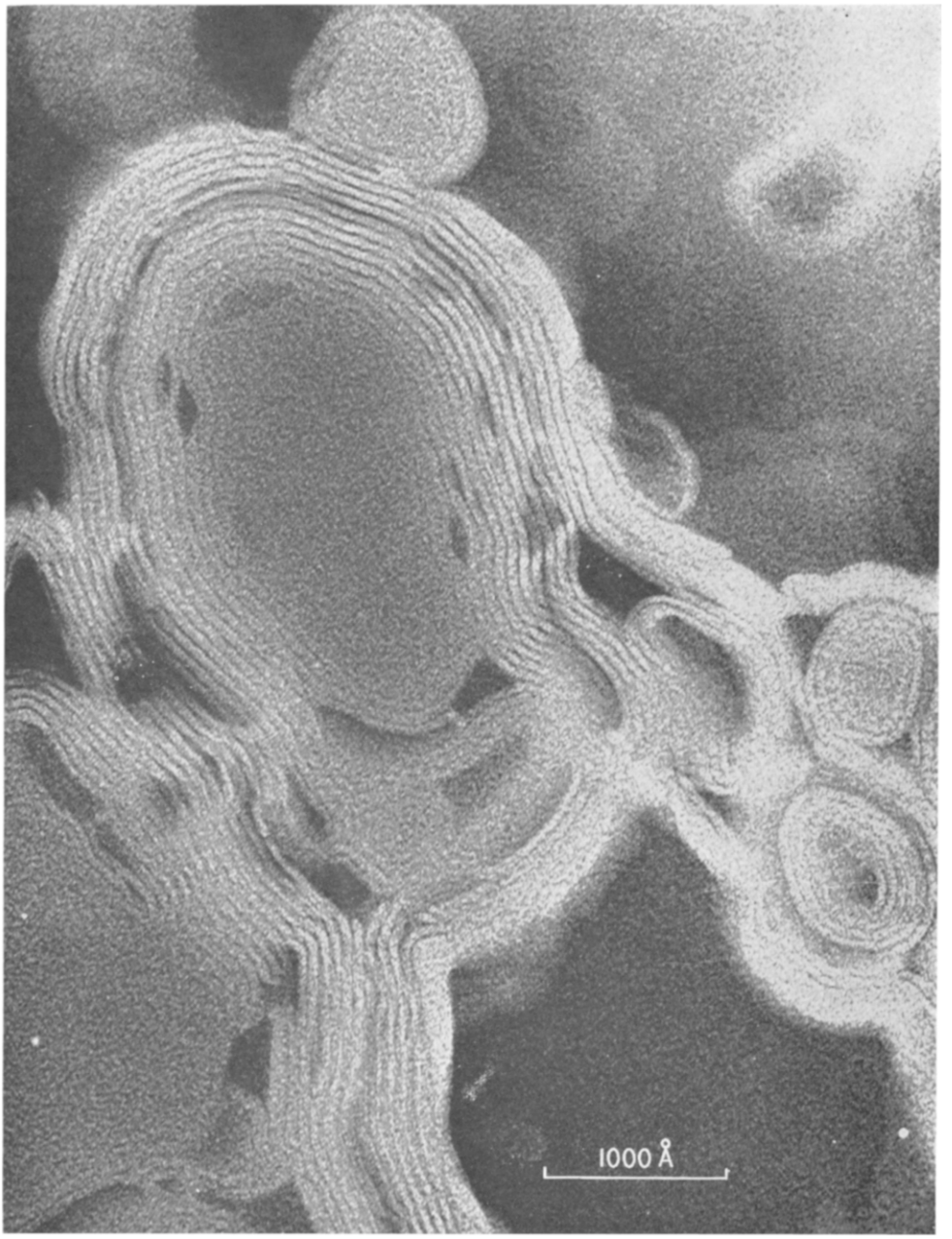

PLATE I. Electron micrograph of ovolecithin treated by ultrasound and mixed with an equal volume of 2% potassium phosphotungstate. Droplet deposited on to substrate.

Unless otherwise stated, all electron micrographs were obtained from negatively-stained preparations.

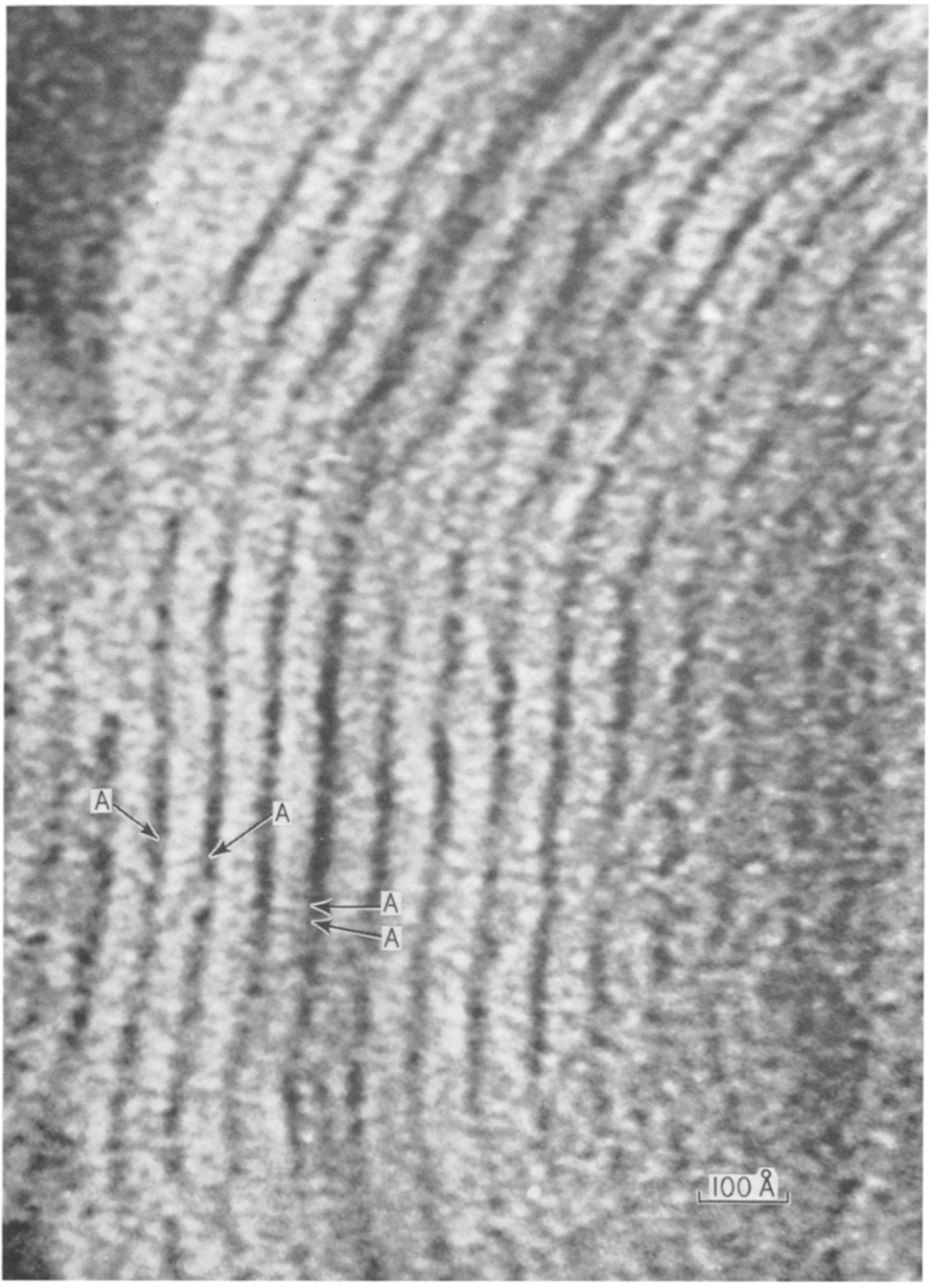

PLATE II. High-resolution micrograph of area from Plate I showing rod-shaped elements at "A" lying at right angles to the long axis of the lamellae.

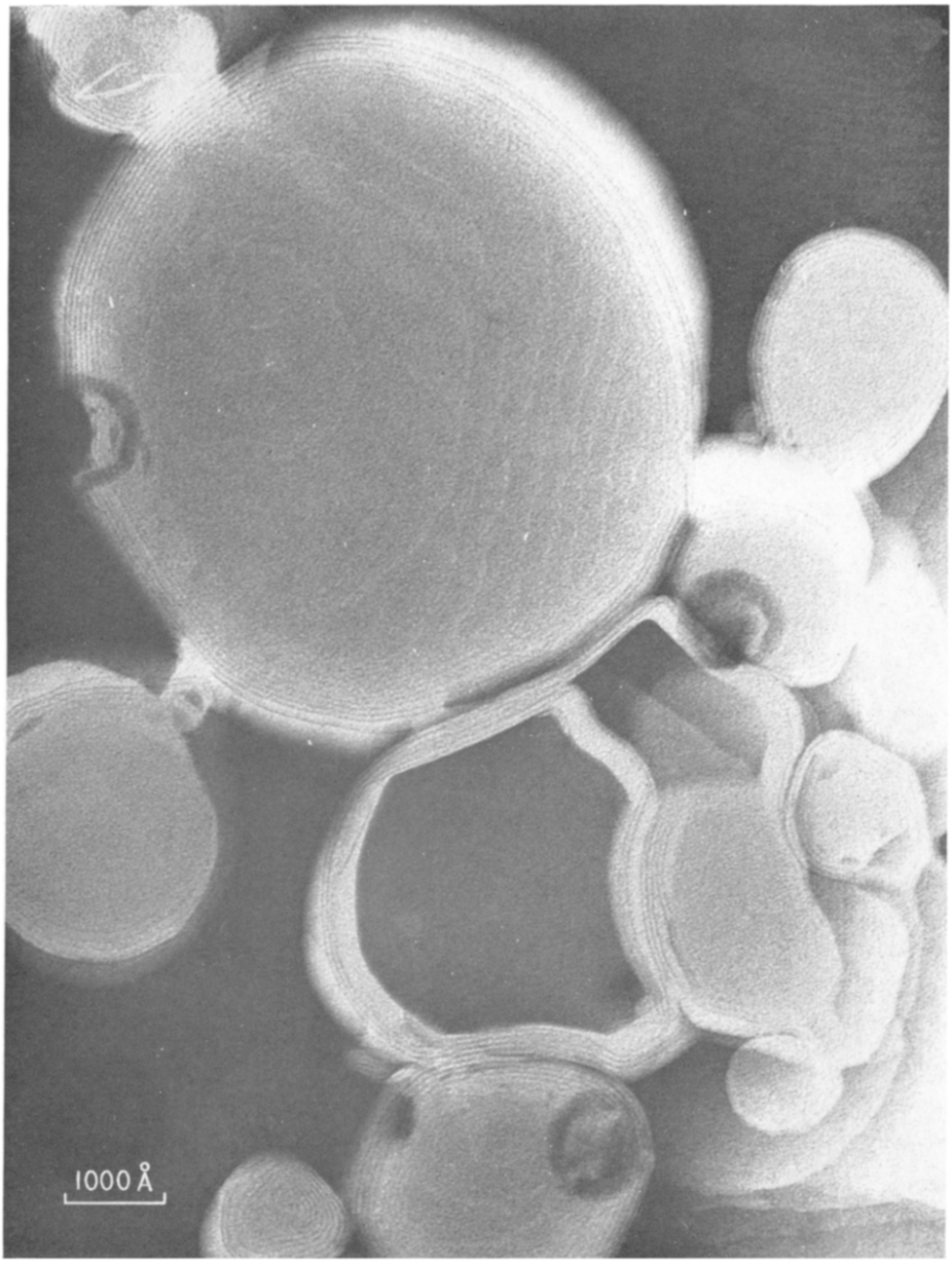

PLATE III. Electron micrograph of equal molar proportions of lecithin and cholesterol treated by ultrasound. The dimensions and appearance of the lamellae are similar to those shown in Plate I.

PLATE IV. The micrograph shows an area from a preparation of lecithin and cholesterol. Elongated structures possibly corresponding to the arrangement shown in Fig. 1 were frequently seen.

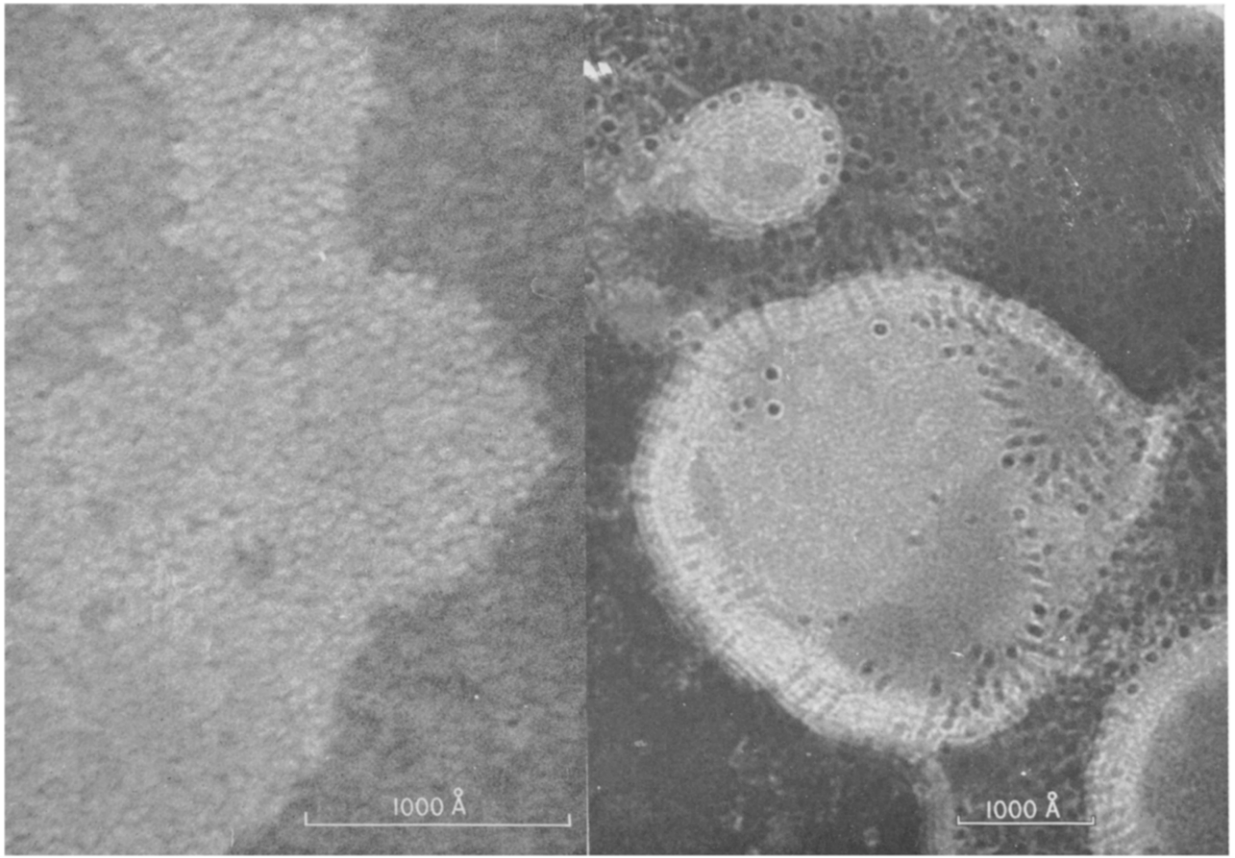

PLATE V

PLATE VI

PLATE V. Lysolecithin in water mixed with an equal volume of potassium phosphotungstate. Small, roughly spherical particles are seen in the micrograph as isolated components or in various stages of aggregation.

PLATE VI. The micrograph shows a dispersion of 2 moles of lecithin and 1 mole of cholesterol in water, treated with an equal volume of 0·2% (w/v) saponin, and finally mixed with an equal volume of 2% potassium phosphotungstate.

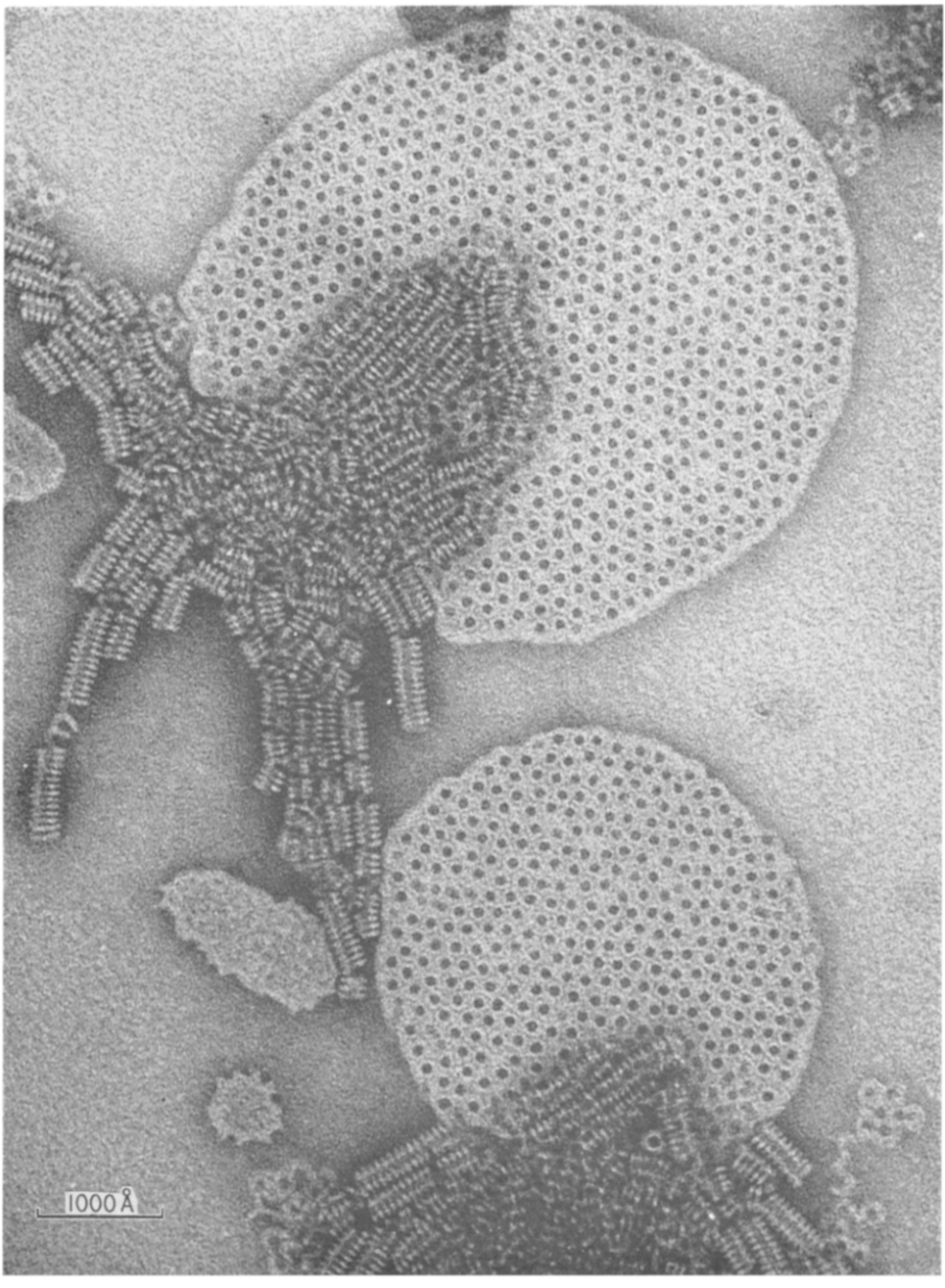

PLATE VII. A similar preparation to that shown in Plate VI with a molar ratio of 1:1.

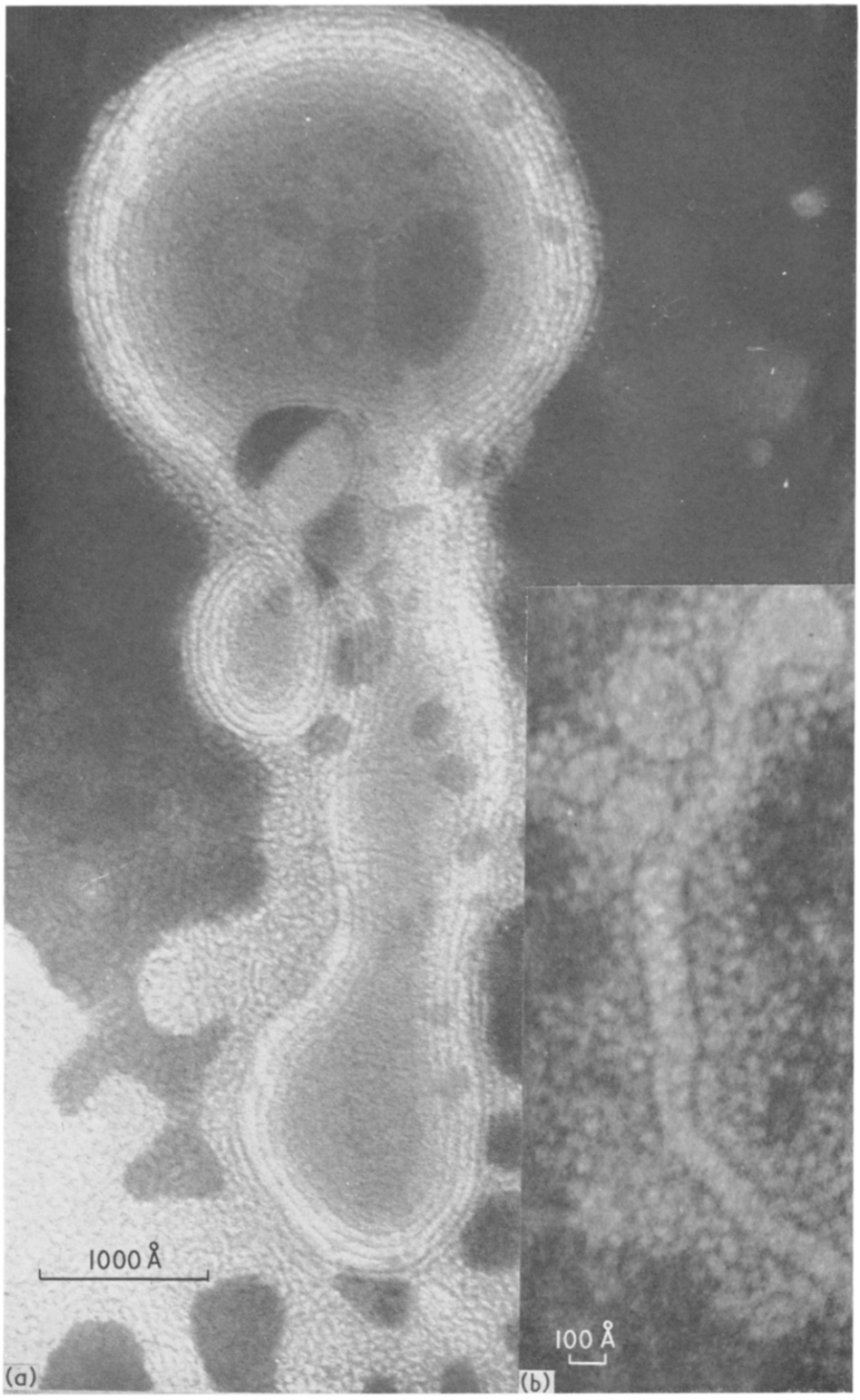

PLATE VIII. (a) Lecithin dispersion treated with lysolecithin in water, negatively stained with an equal volume of potassium phosphotungstate. (b) Inset shows a high-resolution micrograph of a similar preparation to Plate VIII(a). Particles estimated to be about 70 to 80 Å across are visible.

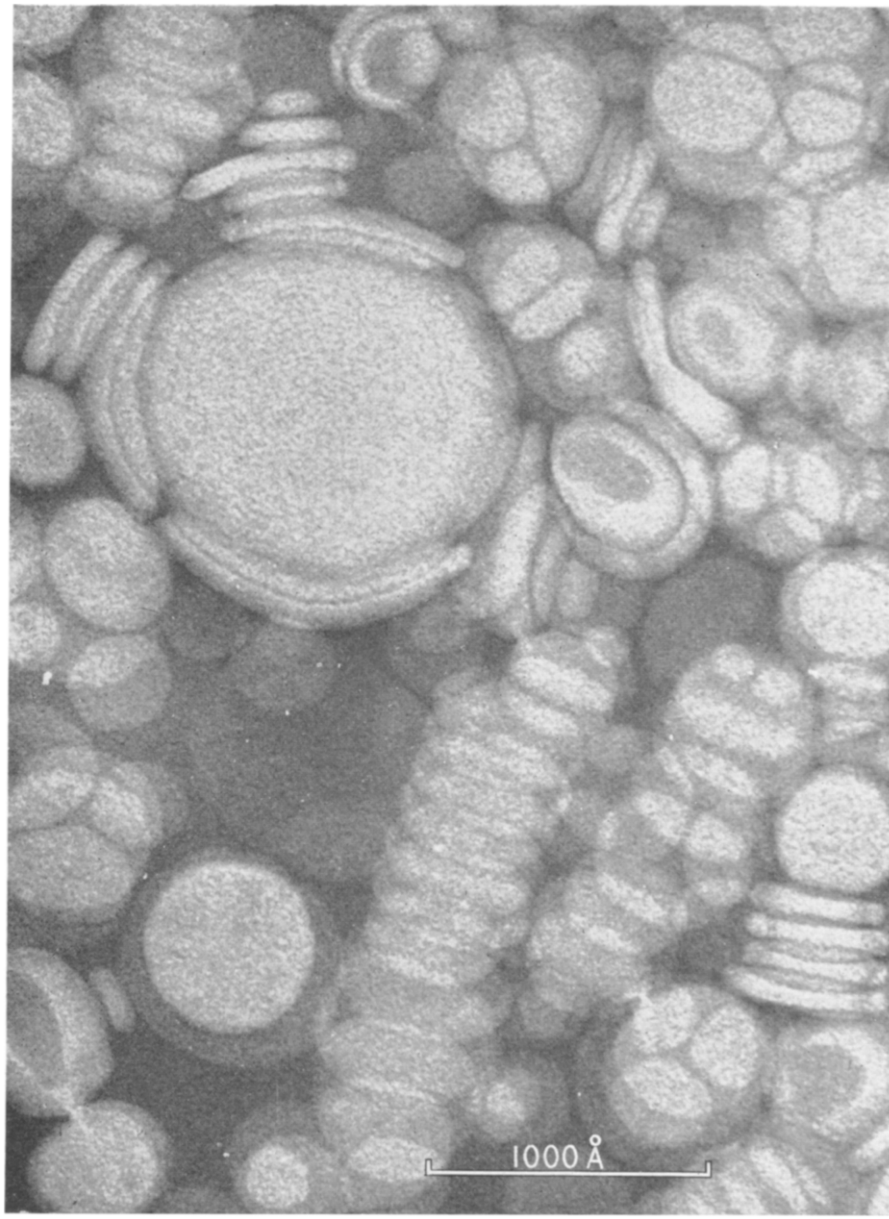

PLATE IX. Equal molar quantities of lecithin and cholesterol dispersed in water, treated wit lysolecithin in water. Preparation mixed with 2% potassium phosphotungstate.

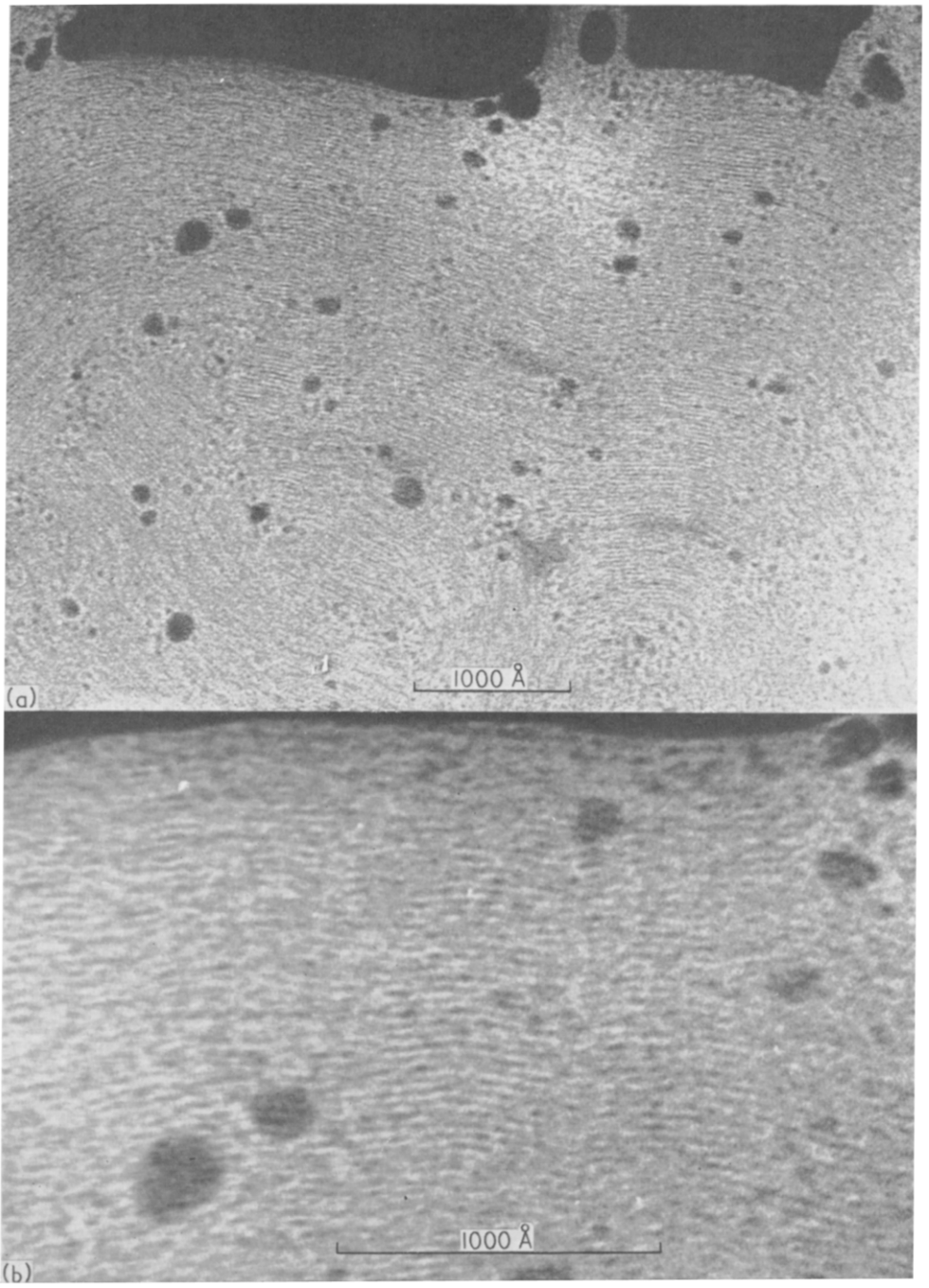

PLATE X. (a) Dispersion of equal molar quantities of lecithin and progesterone in water and mixed with equal volume of 2% potassium phosphotungstate. (b) High magnification from region in Plate X(a) showing a line structure (light regions) estimated to be 20 to 22 Å across.

Lecithin–cholesterol dispersions

Equal molar proportions of lecithin and cholesterol gave essentially similar lamellar structures to those already described for pure lecithin.

Plate III is a relatively low-power electron micrograph showing a number of spherulites and a toroidal myelin form. The dimensions of the lamellae were the same as those for lecithin. However, aberrant forms were occasionally seen. Plate IV shows an area which might be interpreted as consisting of bimolecular fragments of spherulites or, alternatively, as elongated rod-like micelles (Fig. 2). The width of the lipid structure was estimated to be 39 to 40 Å.

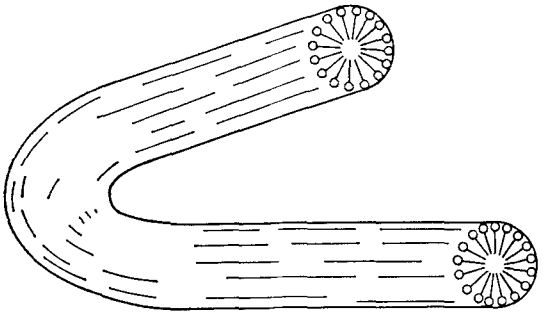

FIG. 2. Elongated structures could result from an arrangement of the lipid molecules radially arranged about a central axis as illustrated in the diagram.

On one occasion an area was observed in an electron micrograph of lecithin–cholesterol revealing structural features in the form of a "finger-print" pattern. The pattern of lines estimated to have an average thickness of about 10 to 12 Å were arranged in a similar configuration to those observed in lecithin–progesterone mixtures illustrated in Plate X(a) and (b). It is important to mention that the features were seen on one micrograph which was not part of a through-focal series, and interference effects cannot be excluded from the interpretation.

Lysolecithin

Lysolecithin forms an isotropic solution in water. Electron microscopic examination revealed the presence of small (70 to 72 Å) spherical, or possibly annular, micelles in large aggregates or as isolated particles (Plate V).

Lecithin–cholesterol–saponin structures in presence of potassium phosphotungstate

A modification of the lamellar structure of lecithin and cholesterol is clearly seen (Plate VI) when a ratio of 2 moles of lecithin and 1 mole of cholesterol were treated with 0·05 to 0·1% (w/v) saponin. The appearance in the electron micrographs was observed to be that of tubes penetrating into the spherulite at right angles to the planes of the lamellae. These preparations gave the best indication of the three-dimensional structure of the spherulites, particularly when viewed by stereoscopic methods. The diameter of the potassium phosphotungstate-filled hole was measured as being 85 Å, corresponding exactly to the holes seen end-on in Plate VII. When the mole ratio of cholesterol to lecithin is raised to 1:1, the complex presents two

strikingly different structures (Plate VII). The perforated disks were observed to be composed of rings made up of what appeared to be linked micelles. The potassium phosphotungstate-filled centres were measured as being 85 Å in diameter and the centre-to-centre spacing of the rings about 120 Å. The rings appeared to be separated from each other by randomly arranged discrete particles. The helical structures have an external width of about 220 Å, and an axial hole of about 68 Å. A well-defined periodicity of 58 to 59 Å was visible along the axis. There was some evidence that the polyvalent phosphotungstate anion contributes to the formation of these remarkable structures since a divalent salt, e.g. calcium, of phosphotungstate does not immediately produce the same structure† (Bangham & Horne, 1964). On the other hand, saponin does react with a mole ratio of 1:1 with lecithin–cholesterol mixtures in water alone and the intense birefringence seen between crossed Nicol prisms was modified.

Lecithin dispersions in water treated with lysolecithin in water

Plate VIII(a) illustrates the progressive breakdown of a lecithin spherulite allowed to form in water and then treated with a solution of lysolecithin in 2% potassium phosphotungstate. Beading of the lamellae, particularly of the peripheral layer, is well shown. The inset shows a high-power micrograph of the final appearance, in which all lamellar structure is lost; the average micellar size was about 70 to 80 Å (Plate VIII(b)).

Lecithin–cholesterol dispersion in water treated with lysolecithin in water

Adding lysolecithin to a dispersion composed of equimolar quantities of lecithin and cholesterol may be likened to the effect of thrusting a cork borer into an onion. A number of discs of approximately uniform diameter are released, each disk consisting of two lamellae annealed around their edges to enclose a water compartment. Plate IX illustrates a late stage of this process in which only the innermost skins of the spherulite remain intact.

In this preparation the lysolecithin was added to the lecithin–cholesterol dispersion before the potassium phosphotungstate. The centre-to-centre spacing of the disks was estimated to be approximately 100 Å and the diameter approximately 700 Å; each disk presumably contains a single water compartment unstained by potassium phosphotungstate. The disks, on the other hand, are separated from one another by a water layer containing potassium phosphotungstate. The water compartment within the disks can be revealed by adding the lysolecithin to a dispersion of lecithin–cholesterol in 1% potassium phosphotungstate.

Lecithin–progesterone dispersions in water

When equimolar quantities of lecithin and progesterone were dispersed in water and 2% potassium phosphotungstate, the most constant finding was a line structure having a maximum width of 20 to 22 Å (Plate X(a) and (b)). Quite unlike the lamellar structure of lecithin in water, it is at present difficult to interpret these pictures in terms of any three-dimensional structure.

† In a personal communication Glauert & Lucy have demonstrated that the structures seen in saponin-treated lecithin–cholesterol in potassium phosphotungstate can be produced in calcium phosphotungstate under certain conditions.

4. Discussion

Haydon & Taylor (1963) conclude that there are two important factors that predispose a single lipid, such as lecithin, to assume and retain a bimolecular leaflet structure. First, there is a very high free energy of transference of the head groups from the lipid phase to water due to their large size and ionic nature. It follows from this that the inter-facial tension—water/oil—on both sides of a lamella is zero and that incorporation of further molecules of lecithin results in a two-dimensional swelling of the structure. Secondly, the electrostatic balance of the ionic head group of lecithin (containing both a strong acid and a strong base) ensures that there is no repulsion between adjacent molecules even over a wide pH range.

The corollary of this argument is that the integrity of the bimolecular structure will be lost if the electrostatic, interfacial or intermolecular forces are disturbed. The appearance of an unbalanced, charged head group, for example, would cause instability and ultimately emulsification or micellization of the leaflet. This effect might be achieved either by introducing a long-chain anion or cation into the leaflet, or alternatively by selectively quenching either the phosphate or choline ion by altering the bulk pH or by the addition of divalent or polyvalent ions to the aqueous phase. From the physiological point of view, a local alteration of the pH might be the operative mechanism if the cell membrane contained a compound such as phosphatidyl ethanolamine, which differs from lecithin only by having a weak base with a pH in the region of 7·5. For obvious reasons it is not possible to demonstrate the end results of this type of instability by the negative-staining technique because one cannot control the final pH of the material on the substrate. It is even possible that the phosphotungstate ion, of unknown valency, may, as it becomes concentrated during the partial drying process, induce a sufficient net negative charge in the interface to produce local micellization (Bangham & Horne, work to be published).

The predominant structure of lecithin or lecithin and cholesterol in water is, however, lamellar under the conditions we have used here. The dimensions of the rod-shaped structure (44·2 × 13 Å) occasionally seen spanning the lipid lamella (Plate II) are compatible with those of a single dimer of lecithin molecules. The average area per molecule of a closely packed monolayer of lecithin at the air/water interface is 58 to 60 Å^2 (van Deenan, Houtsmuller, Haas & Mulder, 1962), and since there are two ionic head groups and two hydrocarbon chains per molecule, the cross-section of the molecule at the interface must be approximately rectangular. If the average ionic radius of a phosphate and choline ion is 4·0 to 4·5 Å, the long side of the head group must be about 15 Å.

The penetration of the bimolecular leaflet by lysolecithin illustrates the instability produced by a wedge-shaped molecule in accordance with the theoretical reasons put forward by Haydon & Taylor (1963). Lysolecithin by itself exists in water in the form of small micelles (Saunders & Thomas, 1957); our results confirm this structure (Plate V). The interaction between lysolecithin and lecithin–cholesterol spherulites appears to release segments of bimolecular lipid, annealed to form discrete disks with a centre-to-centre spacing of approximately 100 Å, consisting of an aqueous compartment surrounded by a single, continuous bimolecular leaflet (Plate IX). Presumably the region of the leaflet with the smallest radius of curvature would contain the highest concentration of lysolecithin. As the mole fraction of lysolecithin to lecithin or lecithin–cholesterol increased, so the area of planar leaflet would decrease and the disks would become smaller, but have the same thickness. Ultimately,

a bimolecular structure would become energetically unfavourable and classical micellization would result (Plate VIII). It seems unlikely that the results obtained are due to the contamination of the lysolecithin with residual phospholipase A, since micrographs obtained from material containing inhibitory concentrations of Ca^{2+} (10^{-4} M) were identical with those to which no calcium had been added. This point is of some importance since the structure obtained by the direct treatment of lecithin dispersions by phospholipase A might well be different due to the simultaneous release *in situ* of a fatty acid as well as the wedge-shaped lysolecithin molecule. An interesting redistribution of lipid residues has been proposed by Bangham & Dawson (1962) following the enzymic digestion of lecithin by a phospholipase C (α toxic *Clostridium perfringens*)—the residues being diglyceride and water-soluble phosphoryl choline.

The effect of saponin is more difficult to interpret, partly because of the complexity of the structure formed and partly because the saponin used is of unknown composition. There is no doubt that the fully developed structure (Plate VII) resulting from the interaction of 0·05 to 0·1% saponin with an equimolecular mixture of lecithin and cholesterol is identical in all respects with that obtained by similar treatment of certain cell membranes. The inference to be drawn from this is that the reactions studied by Dourmashkin *et al.* (1962) involve only the lipids in the membrane and that the most likely lipids are lecithins and cholesterol. Our present results suggest that membranes that do not contain cholesterol will not show this type of reaction.

Lucy & Glauert (1964) have studied the differences in the structures produced by the action of saponin on cholesterol alone and those with lecithin and cholesterol. Their conclusions lead them to suggest that two types of micelle are formed. The network structure obtained with a saponin and cholesterol alone, they suggest, is formed by a joining together of saponin–cholesterol micelles, whereas the hexagonal and helical structures (Plate VII) found when lecithin is also present represent composite arrangements of two types of micelle—that of saponin–cholesterol and lecithin–cholesterol (Schulman & Rideal, 1937*a*,*b*). It is probable that as a result of the mobilization of the cholesterol from the bimolecular membrane, the lecithin molecules themselves form micelles—assisted to do so by the presence of the phosphotungstate ion (Bangham & Horne, work to be published).

The aberrant forms of lecithin and cholesterol shown in Plate IV are of particular interest. These structures could not be predictably produced on an electron microscope specimen substrate although the solid-looking hairpins were often seen. It is thought that the hairpin structure might represent a phase transition from liquid crystalline to an elongated micellar phase due to a drying artefact of the lipids before the drying of the sodium phosphotungstate. The extremely fine and fragmented leaflets mentioned earlier were seen only very occasionally in lecithin–cholesterol preparations; on the other hand, it was a consistent finding if lecithin and progesterone were allowed to swell together in water. This last observation, together with the dimension of the leaflet (20 to 25 Å), suggests that the lipid molecules are actually interdigitating with one another to form a symmetrical, unimolecular filament. That progesterone enhances this structure may be ascribed to the presence of hydrophilic groups at both ends of the steroid nucleus.

Finean & Rumsby (1963) have challenged the possibility that potassium or sodium phosphotungstate actually preserves lipid structure as though it were still in the

presence of water. They argued quite rightly that the precise structure of amphipathic substances such as soaps, lecithins and long-chain alcohols varies, their phase structure depending upon their concentration in water (Luzzati & Husson, 1962). It is true that in areas of the grid where large amounts of lipid are stranded, there is little or no recognizable structure. However, there remains certain circumstantial evidence that strongly suggests that potassium phosphotungstate can "freeze" the hydrated structure of lipids and that the morphological features seen are genuine (Horne, Whittaker & Bangham, 1963). Furthermore, preparations of lecithin–cholesterol identical to the ones used here for negative staining have been examined for us by Drs Finch and Klug by X-ray diffraction methods and found to contain lamellar structures of approximately the same spacings as those reported here. Certainly the structure of lecithin prepared in the presence of potassium phosphotungstate (Plate I) is entirely compatible with the birefringence of the same material in aqueous 1% potassium phosphotungstate although the birefringence disappears gradually in this salt solution.

However, the birefringence of lecithin–cholesterol myelin forms in water is rapidly modified when saponin is added, although Finean & Rumsby (1963) did not observe any alteration in the low-angle scattering of X-rays. If cholesterol is not present together with the lecithin, birefringence persists. Likewise, the birefringence of lecithin with or without cholesterol is rapidly lost if lysolecithin is added.

A further criticism of the use of potassium or sodium phosphotungstate rests upon the electrostatic stress which may develop at the lipid–water interface due to the extreme discrepancy in the valency of the phosphotungstate and univalent potassium ions. Lecithin dispersions have been shown to acquire a net positive charge in the presence of bi- or ter-valent salts of univalent acids and the ferricyanide ion has been shown to have the opposite effect (Bangham & Dawson, 1962). Although a 1% solution of potassium phosphotungstate might not impose an intolerable electrostatic force of repulsion between lipid head groups, it must be remembered that the concentration will rise considerably before effective "freezing" occurs. It is likely that bi- or ter-valent salts of phosphotungstic acid might give more valid pictures of lipid and other structures.

It is suggested, therefore, that the bimolecular lipid leaflet, although inherently a stable arrangement, is nevertheless vulnerable to modification in a variety of ways. Certainly the lesions produced in the model membrane of lecithin–cholesterol are commensurate with the damage obtained in biological cells when either saponin or lysolecithin is used as a lytic agent. Schulman & Rideal (1937*a*,*b*) and later Rideal & Taylor (1957,1958) proposed and showed quite clearly that the lytic action of surface-active agents like saponin was effective by virtue of their ability to penetrate and react with the lipids in the cell membrane. The results obtained with lysolecithin have an obvious relevance to the demyelinating diseases of the central nervous system and are compatible with its known macroscopic clearing action on crude lecithin dispersions (Saunders, Thomas & Robinson, 1958) and mixed brain phospholipids (Webster, 1957). Thompson (1964) has suggested that a vicious circle develops whereby damage to the myelin membrane is followed by damage to intracellular lysosomes and release of phospholipases. In turn, the degradation products of phospholipase activity, i.e. release of lysolecithin, involves further damage to myelin. If the original damage could be attributed to an undue accumulation of surface-active anions, e.g. fatty acids, causing discrete areas of micellization, reconstitution of a bimolecular

membrane is theoretically possible if surface-active cations could be incorporated at the site. Bangham, Rees & Schotlander (1962) have suggested that this might be the mechanism whereby liver necrosis in rats following carbon tetrachloride poisoning is prevented by intraperitoneal administration of cetyltrimethyl ammonium bromide. It is also possible that electrostatically-induced instabilities due to transient variation in local pH or bivalent ion concentration occur in excitable membranes and might in this way function as regulators for the passage of molecules through the membrane. This form of structural alteration might be too transient or too small to perceive in the electron microscope, but it is hoped to demonstrate its occurrence by other techniques.

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