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The Structure of Myelin Figures and Microemulsions as Observed with the Electron Microscope

By Walther Stoeckenius, Jack H. Schulman and Leon M. Prince

With 13 figures and 5 diagrams

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Part I - Myelin Figures[1])

Myelin figures develop if certain lipids or mixtures of lipids are brought into contact with water. The conditions of their formation have been thoroughly studied by *Dervichian* (6, 11). They appear in the form of cylinders or spheres of light microscopical dimensions, and have been studied by polarized light, *mechanical* and X-ray diffraction techniques (28, 6). The accepted interpretation of the results is that they consist of a concentric arrangement of bimolecular leaflets of lipid intercalated layers of water. In each leaflet the hydrocarbon chains of the lipid molecules face inward, whereas the polar groups face outward forming the hydrophilic surfaces of the bimolecular layers. The interior of the layers in tangential direction can be considered fluid, whereas in the direction normal to the plane of the layers there is a regular arrangement with a precise pattern repetition which gives rise to sharp X-ray reflections.

The longest known material which forms myelin figures is a mixture of lipids, mainly phospholipids, found in extracts of animal and plant tissues. Myelinic forms of these lipids can be rendered insoluble by treatment with OsO_4 or $KMnO_4$. This makes it possible to embed them in methacrylate and cut thin sections suitable for observation in the electron microscope according to techniques which have been developed for animal and plant tissues. A lamellar structure has been seen in such sections (20, 29, 33, 35).

This observation is interesting not only as a confirmation of the information gained by the indirect methods mentioned above, but it should also be useful in the interpretation of electron micrographs of cells and tissues, since bimolecular lipid layers are assumed to be an integral part of cellular membranes. It was mainly with this point in view that the following investigations have been carried out.

Material and Techniques

As lipid material we used a phospholipid extract of brain tissue containing mainly cephalins, lecithins and some phosphoinositols. A small amount of the substance brought into contact with water readily developed myelin figures which were subsequently exposed to osmium tetroxide vapor. The excess water was drained off, the material further dehydrated in a series of graded acetone-water mixtures and finally transferred over dry acetone to a mixture of butyl- and methylmethacrylate monomer. By polymerization of the methacrylate firm blocks were obtained which could be sectioned thin enough for high-resolution electron microscopy. A more detailed account of the techniques used has been given elsewhere (35).

The X-ray diffraction work was done with a *Hilger* microfocus unit and a phinole camera. Nickel-filtered copper Kα radiation was used[2]). The material to be investigated was either pressed between two thin glass coverslips or sucked into a thin walled glass capillary. From the material embedded in methacrylate, sections about 100 μ thick were mounted on the tip of a needle for examination. The diffraction pattern was recorded on photographic film.

[1]) Most of the material presented in this paper, especially as far as myelin forms are concerned, has been published in a previous article (35). The discussion given here includes however, an alternative interpretation of the findings which had been thought too remote to be included in the first version but which, in the light of new observations, has become more probable and as such can no longer be neglected.

[2]) Our thanks are due to Dr. *B. Magdoff* from the *Boyce-Thompson* Institute for Plant Research, Yonkers, N. Y., for constant advice and permission to use her equipment.

Results

X-ray diffraction: The dry lipid mixture gives an intense and sharp diffraction band at 42.5 Å, which probably corresponds to twice the length of the lipid molecules (2). Addition of small amounts of water will increase that spacing. But if a large surplus of water is added (1 ml. to 50 mg. of lipid) the whole material is transformed into myelin figures and a diffraction pattern can no longer be obtained. Addition of a divalent cation to such a preparation is known to restrict the swelling of the myelin figures. We used 1,3 mM $BaCl_2$ and got a sharp diffraction pattern with a long spacing of 53.6 Å. Exposure to OsO_4 vapor reduced this spacing to 45.0 Å, and small further shrinkage to 44.0 Å may or may not occur during dehydration in acetone. The following monomer methacrylate causes an increase in the long spacing to 47.0 Å. Polymerisation finally brings about a reduction of the spacing to a value of 41.5 Å which is close to that of the original dry material i. e. 42.6 Å. During the whole procedure the lamellar structure was preserved (diagram 1). In addition to this long spacing a diffuse ring between 4.5 Å and 4.9 Å was frequently found. If the material was slightly oriented by sucking it into a capillary, an accentuation of this ring was always found at right angles to the accentuation of the long-spacing ring. This diffuse ring probably corresponds to the side spacing between the fatty acid chains within each layer (2).

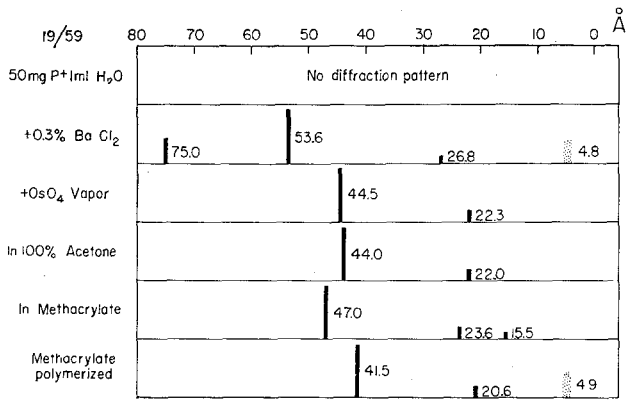

Diagram 1. Changes in the X-ray diffraction pattern of phospholipids during fixation, dehydration and embedding. The spacings for each step are illustrated in the horizontal columns, and the sequence of changes is seen by comparing the columns, commencing with the uppermost

Electron microscopy: Thin sections of the same material used for the X-ray examination were observed in the microscope. They showed broad bands, which could be identified as sections of various incidence through cylinders or spheres. At higher magnifications these bands could be resolved in many places into a pattern of alternating light and dense lines (fig. 1 and 2). The center to center distance between two neighboring dense or light lines was about 40 Å. The width of the dense lines can not be measured accurately because they do not show sharp edges, but they usually appear a bit narrower than the light lines. An average width of about 17 Å should come close to the real value. That leaves about 23 Å for the width of a light line.

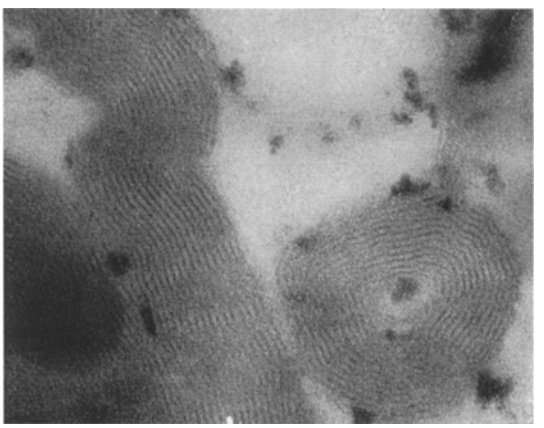

Fig. 1. Electron micrograph of a thin section through myelin figures from $BaCl_2$ solution fixed with OsO_4 and embedded in methacrylate. Magnification 430,000×

Highly hydrated specimens of the lipid are obtained when the material is allowed to swell for about 24 hours in distilled water or when purified samples of cephaline[3]) are used. They do not show the compact, broad bands described above but are made up of a loose concentric array of narrow bands about 40 Å wide separated by unequal distances of several hundred Å. At higher magnifications these bands can be resolved into two outer dense lines separated by a light line of the same width as those found in the broad bands of myelin figures formed in $BaCl_2$ solution i. e. 23 Å (fig. 3). In a few places in which such narrow bands are found to converge, their close packing gives rise to a pattern indistinguishable from the one

[3]) Pure cephalins were obtained from Dr. *H. Debuch*, Physiologisch-Chemisches Institut der Universität Köln

seen in less hydrated samples. Occasionally the narrow band splits along the center of the light line, giving rise to single dense lines (fig. 4).

The addition of a basic protein to the water in which myelin figures develop (we used globin prepared from beef hemoglobin

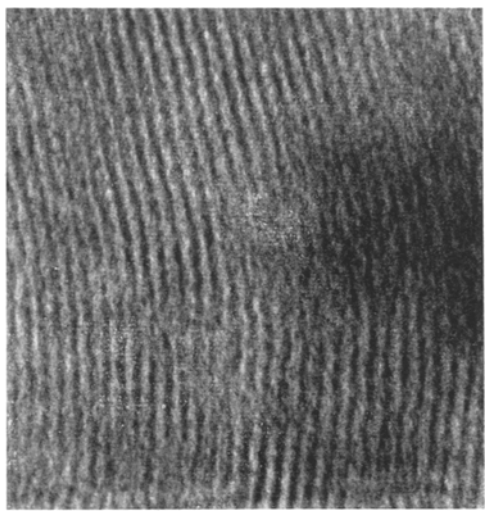

Fig. 2. Same as fig. 1 at higher magnification 1,100,000 ×

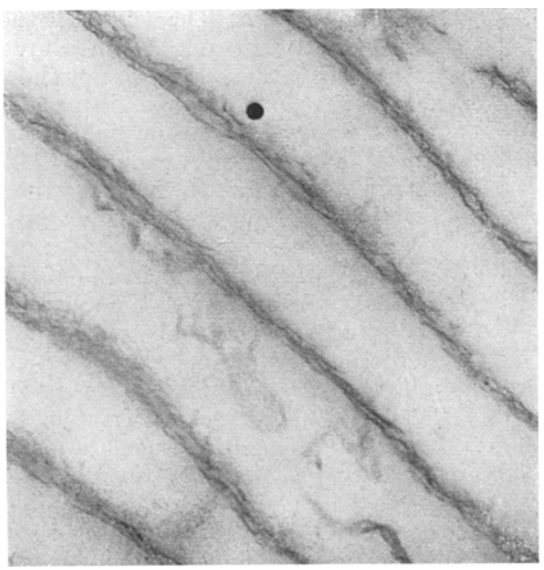

Fig. 3. Electron micrograph of highly hydrated phospholipids. Magnification 470,000 ×

at p_H 4.0) also limits their hydration and leads to the formation of figures whose walls appear in the electron microscope as compact, broad bands. The pattern of light and dense lines in the interior of these bands is exactly the same as in the myelin figures formed in water without the addition of protein. But on both surfaces of each broad band there is a thicker and denser line, the width of which may vary from 25 to 50 Å (fig. 5). Often the width is different on the two surfaces of the same band. One can find any number of narrow dense lines inside the band, from one (fig. 6) to thirty or forty and more,

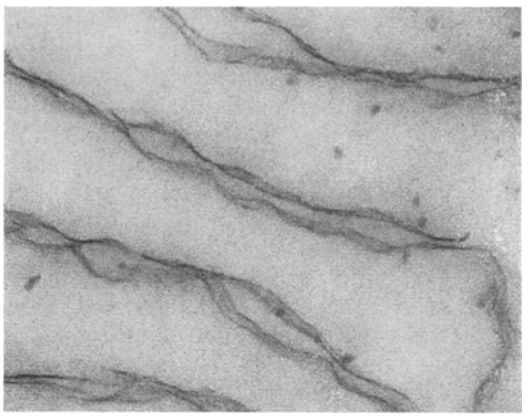

Fig. 4. Another area of the same specimen shown in fig. 3. Magnification 470,000 ×

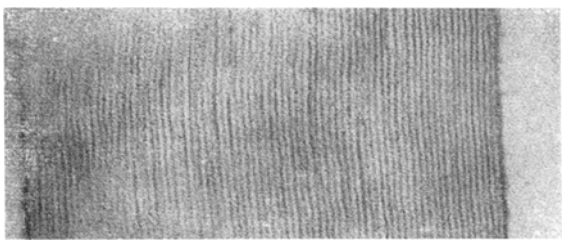

Fig. 5. Myelin figure formed in a globin solution, showing the broader and denser lines on both surfaces. The structure in the interior of the bands is indistinguishable from that of myelin figures formed in water or $BaCl_2$ solution. Magnification 430,000 ×

according to the width of the broad dense band. But always the outermost narrow dense line is separated from the broader and denser line on the surface by a light line, of usual width (about 23 Å). The thinnest structure which we found among protein treated myelin figures consisted of two dense lines separated by a single light line. Here also the width of the two dense lines might be different but they are always broader and denser than the narrow dense lines within the wide compact bands of myelin figures without protein (fig. 7).

Discussion

X-ray diffraction: The long spacing of 42 Å found in the dry lipid material probably corresponds to the length of two lipid mole-

cules packed end-on in the structural unit (2, 16, 17,). In the case of the myelin figures all evidence indicates that the structure is lamellar and the increased spacing of 53 Å is explained by an uptake of water between the hydrophilic surfaces of adjacent lipid layers. The shrinkage during fixation and dehydration should reflect then a removal of this water from the structure. Whether the slight swelling observed in monomer methacrylate is due to a change in

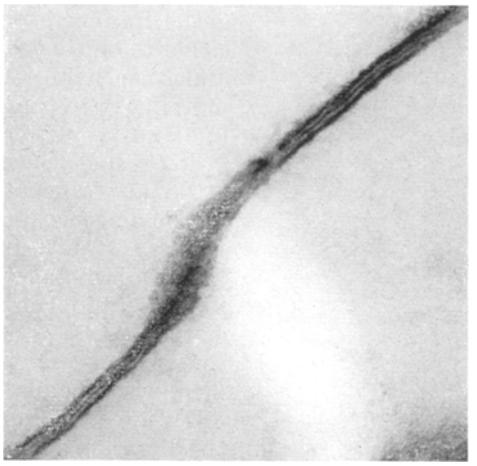

Fig. 6. Same specimen as in fig. 5 showing a smaller band containing only one narrow dense line in its interior. Magnification 430,000 ×

the shape of the lipid molecule or to an uptake of methacrylate into the structure cannot be decided yet. Similar changes have been observed in the myelin sheat of nerve fibers (15). After polymerization we find a value very close to that of the dry lipid and this makes any inclusion of extraneous material in between the lipid layers very improbable. Evidently the lamellar structure has been preserved during the whole procedure, which is also shown directly in the electron micrographs.

Like other investigators (2, 18), we occasionally observed an additional long spacing between 63 and 75 Å which cannot be accounted for at the moment. But 42 Å seems to be about the lower limit for the thickness of one bimolecular layer of lipid. *Finean* (16, 17) has found that in cephalins, which constitute the bulk of our material, the polar end-groups account for 18 Å of the layer thickness. This leaves about 24 Å for the fatty acid chains of two opposed molecules. If we assume that the average fatty acid chain in our material contains 18 C-atoms (22) a space only 24 Å wide appears very small as compared, for instance, to a double layer of stearic acid which has been found to be 43.8 Å thick (25). But this value is derived from a true three-dimensional crystal, whereas in our phospholipid the structure in the plane of the layer is fluid, as indicated by the diffuse 4.8 Å ring. Comparable conditions are encountered in soap-water systems at higher temperatures. For instance, the thickness of one bimolecular leaflet of sodium stearate including the COO^- groups and

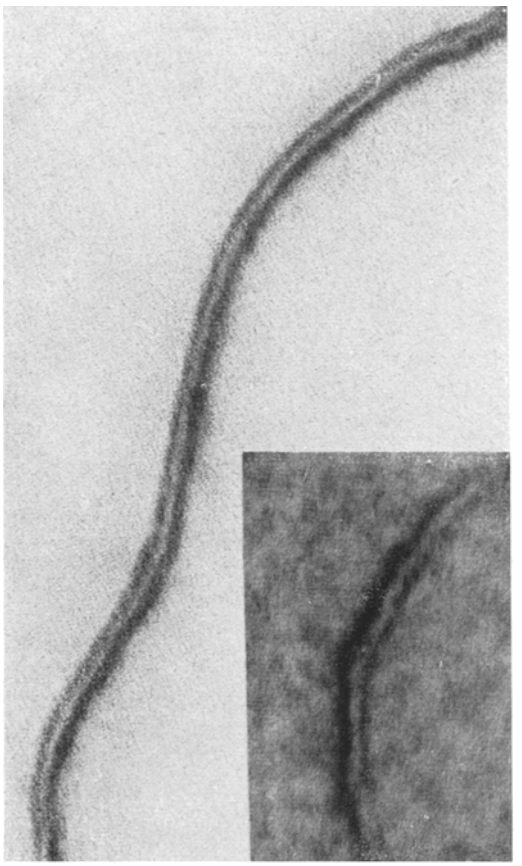

Fig. 7. Same specimen as in fig. 5 and 6 showing the smallest structure observed in this preparation. For comparison the electron microscope picture of the cell membrane of a muscle fiber is given in the inset. Magnification 530,000 ×

counter ions was found to be as low as 26.5 Å at 100° C (23). This figure fits in very well with our value of about 42 Å for the double layer thickness in phospholipid, assuming that the hydrophilic end groups occupy 18 Å, the value found by *Finean*.

A more detailed discussion on the structure of lipid-water systems as derived from X-ray diffraction data has to await further work. The arrangement does not seem to be

lamellar in every case and the average dimensions of the lipid molecules probably change somewhat according to the amount of water and ions present and also with temperature, as shown by the results of investigations on soap-water systems by *Luzzati* and coworkers (23, 21). But is seems justified to assume that in our fixed and embedded myelin figures we are dealing with closely packed bimolecular leaflets of lipid.

Electron microscopy: Comparing the thickness of 42 Å for one bimolecular leaflet of the lipid, as derived from X-ray diffraction with 40 Å for the center to center–distance of the dense lines in the electron micrographs of thin sections, there can be little doubt that one bimolecular leaflet of the lipid corresponds either to one dense line and two halves of a light line or to one light line and two halves of a dense line. Since in our material contrast in the micrographs is almost certainly due to the osmium taken up by the lipid (for discussion see 27, 35), this change in density means an unequal distribution of osmium in the direction normal to the plane of the layers.

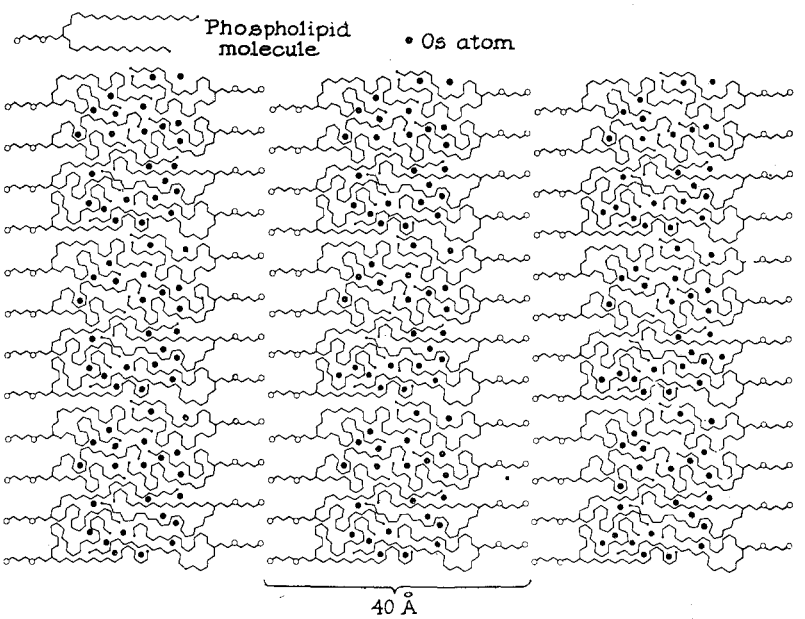

Diagram 2. Schema showing the arrangement of phospholipid molecules in the myelin figures and the distribution of osmium, assuming that this is only present at the site of the double bonds

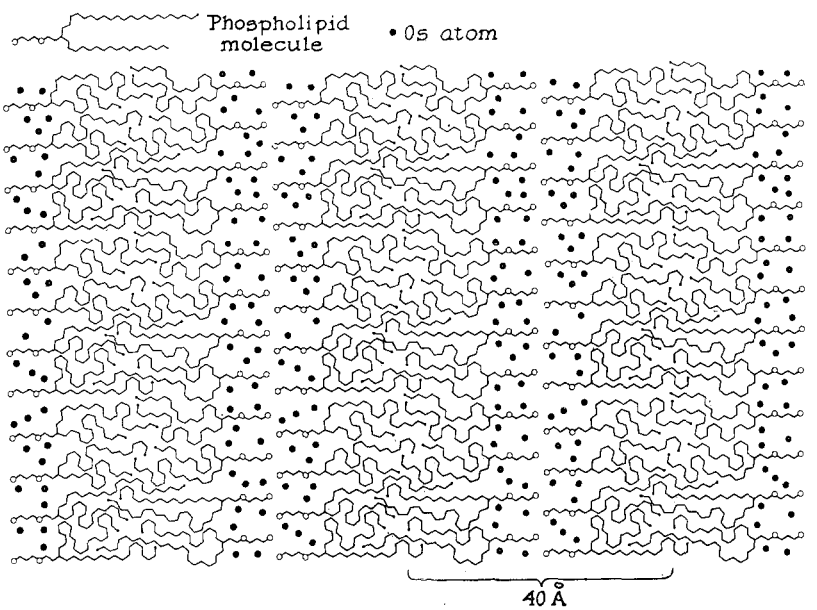

Diagram 3. Scheme showing the distribution of osmium in a fixed myelin figure under the assumption that it reacts only on the hydrophilic groups of the molecule. The electron microscope image of this model would be the same as that given by the model in diagram 2

Osmium tetroxide is known to react with double bonds in unsaturated fatty acids and at least one of the compounds – a diester forming cross linkages between olefines–could explain its "fixing" action very well (33, 34, 35, 36). Assuming that this is the only reaction taking place, the dark lines in the micrographs would correspond to the location of the fatty acid chains in the bimolecular leaflet, as shown schematically in diagram 2. On the other hand *Finean* (16, 17) has shown that synthetic serin cephalin with saturated fatty acid chains reacts with osmium tetroxide and is rendered insoluble in the process. As a re-

sult the lamellar structure of the material is preserved and can be shown in the electron microscope. Since saturated fatty acids do not react with osmium tetroxide, it follows that in the case of serin cephalin the reaction takes place only at the hydrophilic groups of the molecules and since serin cephalin is present in our material, we also have to consider the possibility that osmium might be located at the hydrophilic surface of the bimolecular layers. This would lead to the scheme shown in diagram 3.

Probably both the double bonds and at least some of the hydrophilic groups do react with the osmium tetroxide, but the contrast distribution in the pictures shows that there must be a much higher concentration in one zone as compared to the other. We are mainly concerned with the question of where this high concentration of osmium is located and shall therefore disregard for the moment the fact that there probably is some osmium also present in the remaining part of the leaflet, that is the part showing up as the light line.

The electron micrographs do not allow one to decide between these two possibilities, because their images would be the same in the microscope. One point in favor of scheme 3 is the frequent occurrence of "double lines" in highly hydrated specimens (fig. 3). If they formed in water they are most probably bimolecular leaflets with a width of about 40 Å and with the both surfaces marked by osmium located at the hydrophilic groups. This would correspond to the osmium distribution shown in scheme 3. If, on the other hand, the osmium distribution of scheme 2 be correct, each "double lines" would represent two bimolecular leaflets in close apposition, i. e. a much less probable occurrence in a highly hydrated specimen. A single bimolecular layer according to scheme 2 should show up as a single dense line. This can actually be found, in rare instances, but is more easily explained as a splitting of one bimolecular layer formed in water into two single layers after transfer of the specimen into methacrylate.

It may be argued that the width of one dense line in the double line structure could decide the question. If the "double line" is one bimolecular leaflet formed in water with the hydrophilic groups marked by osmium the dense lines should only be half as wide as the narrow dense lines in the compact bands where the leaflets are closely packed. But we are working close to the limit of resolution of the microscope and small differences in focus or a very slight tilt of the leaflets with respect to the plane of the section would alter appreciably the apparent width of the lines. If on the other hand the "double line", should be formed by two closely packed bimolecular leaflets and the osmium marks the interior of the layers as shown in scheme 2, then the total width of the structure should be 55–60 Å. But whereas in the dark broad bands the mean center to center distance of the narrow dense lines can be fairly accurately measured by counting the number of lines over a given distance, this is not possible in the "double line" structure. Therefore, no valid conclusions can be drawn at present from the measured width of the dense lines or the whole "double line" structure in the micrographs.

In the myelin figures formed in globin solution the thicker dense line on the outside of the broad bands obviously is bound or adsorbed protein. Cephalin which makes up more than 50% of the mixed lipids used in these experiments, is known to form salt-like compounds with globin (7, 8, 9). The

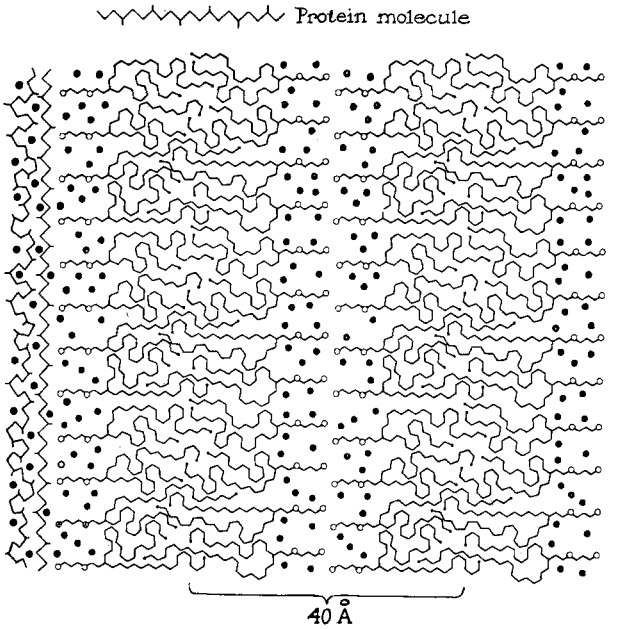

Diagram 4. Same as diagram 3 but showing two adsorbed layers of protein on the surface of the structure

varying thickness of the line is probably due to the adsorption of additional protein onto the first layer which is chemically bound (19, 24).

Apparently there is no penetration of protein into the interior of the band since the line pattern here is exactly the same as in pure lipid preparations. Scheme 3 offers the easiest explanation for the fact that each of the two most peripheral light lines is equal in width with any central light line. At the periphery the osmium marks the hydrophilic portions of the lipid molecules which form the surface of the myelin figure, i. e. the boundary of the broad dark bands in our micrographs. One would expect protein coating this surface to show up only as a broadening of this line (diagram 4). If, on the other hand, scheme 2 in which the osmium marks the fatty acid chains be correct, one would expect a narrower light line between a dark line given by the protein and the outermost narrow dark line formed

by the lipid. Unfortunately this most peripheral light line might be too narrow to be resolved in the micrographs, especially if penetration of the outermost lipid layer by the hydrophobic side chains of the protein occurs as it does in the reaction of surface films of lipid with protein (12, 24). (For a more detailed discussion of this possibility see 35). Therefore the evidence from the lipid-protein preparations, favors scheme 3 but cannot be considered conclusive.

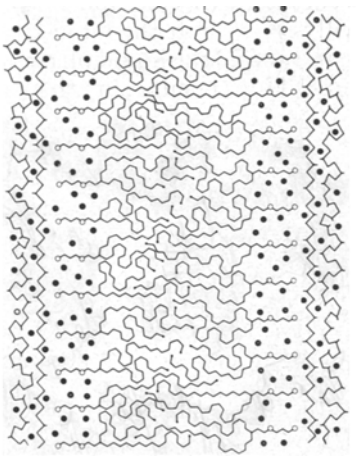

Diagram 5. Hypothetical scheme of the molecular arrangement of lipids and protein and the osmium distribution in the structure shown in fig. 7

Of special interest is the thinnest structure found in these preparations, which consists of only two thicker dark lines separated by one light line. Its appearance is the same as that of cell membranes in high-power electron micrographs of tissue sections (for ref. see 35, 37) an example of which is shown in the inset of fig 7. Assuming the osmium distribution of scheme 3, the molecular structure of the cell membrane would be that of one bimolecular leaflet of lipid covered on both sides by a layer of protein (diagram 5). This is essentially the structure postulated by *Danielli* 20 years ago, to explain the results of permeability studies (10). The present work can therefore be considered as independent morphological evidence compatible with this hypothesis.

Assuming the osmium distribution of scheme 2 the thinnest structure found in our preparations would correspond to two bimolecular leaflets of lipid with a layer of protein on both outer surfaces (for a more detailed description see 35). Although this possibility cannot be excluded at present, we shall briefly consider some preliminary results which at first appeared to favor scheme 2 (35), but now seem to lead to a different interpretation.

The bromination of our lipid which blocks the double bonds in the fatty acid chains shows that the double bonds are essential for fixation. For in contrast to unbrominated lipid, this material remained soluble in methacrylate after osmium tetroxide treatment and consequently could not be embedded for sectioning. But this finding does not necessarily mean that all the osmium taken up by the lipid is essential for fixation or that the osmium showing up as dark lines

Fig. 8. Potassium linolenate fixed with OsO_4 embedded and sectioned. The pattern is essentially the same as that of the phospholipids. Magnification 530,000×

in the micrographs is identical with the osmium which causes fixation. In fact, by measuring the amount of osmium uptake by the lipid, we found that it is much higher than can be accounted for by the number of double bonds in the fatty acids of the material and that brominated lipid will eventually take up as much as three quarters of the amount of osmium found in unbrominated material. In the light of these new findings the results of fixation experiments with brominated lipid lose their value as an argument in favor of scheme 2.

Since *Finean* has fixed saturated serin cephalin with osmium tetroxide (16, 17), experiments with lipids which have a hydrophilic group that does not react with osmium tetroxide became of interest. We chose potassium linolenate containing small amounts of water. Soaps are known to form bimolecular leaflets in the same way as phospholipids do (23). Treated with osmium tetroxide, embedded and sectioned, the

potassium linolenate shows a lamellar appearance in the electron microscope very similar to that of the phospholipids (fig. 8). This again, at first sight, seems to be a result in favor of scheme 2, for no reaction of the osmium tetroxide with the hydrophilic groups of the linolenate is to be expected and all the bound osmium should be at the sites of the double bonds. Therefore, from the similarity of the micrographs of linolenate and phospholipid layers one would be inclined to assume that the same holds true for the latter. But the potassium linolenate differs in important aspects from the phospholipids we used. It is a chemically uniform substance and the repeat pattern of 42 Å found by X-ray diffraction shows that the fatty acid chains in this case are not extensively bent, as is the case in the phospholipid layers. Moreover, since all three double bonds are close to the CH_3 group, we are dealing in linolenate layers with a very high concentration of double bonds in the central part of the bimolecular leaflet. Therefore enough osmium might be bound in this central zone to give rise to a dark line in the micrographs. This does not necessarily hold true for the phospholipids, where the absolute number of double bonds is lower – iodine number is approximately 110 as compared to 240 in the linolenate – and their distribution more even over the width of the fatty acid part of the layer. Even in the case of the linolenate the evidence for the interpretation given here is still insufficient, for one has also to consider the possibility that the osmium that gives contrast in the microscope is not necessarily that bound to the lipid. It could as well form a deposit of reduced osmium compounds between the hydrophilic surfaces of the layers.

As a tentative conclusion it could be said that as far as the phospholipids are concerned most of the findings agree better with the osmium distribution of scheme 3 rather than with that of scheme 2. For the linolenate the opposite seems to hold true. Although at present a definitive interpretation of the electron microscopical picture cannot be given, it is clear that the fixation of unsaturated lipids and lipid water systems used in this study opens up new possibilities for the direct visualization of colloidal systems. This possibility will be further discussed in Part II of this paper.

Part II - Micro Emulsions

Mechanism of Formation

The combination of the electron microscope and x-ray investigation of the myelinic structures of soaps above the *Krafft* point and phospholipids has given indication that the structures are liquid crystals in which the hydrocarbon chains are in the fluid state and the polar groups are arranged in a crystal order. This has been inferred from the diffuse nature of the side spacing and higher order spacings of the main layer structure unit from the x-ray pictures.

If further disorder is placed in these molecular liquid crystal arrays, the systems will become completely liquid and surface tension forces will take over at the interfaces between the monolayer and the liquid phases surrounding them. This results in the monolayer

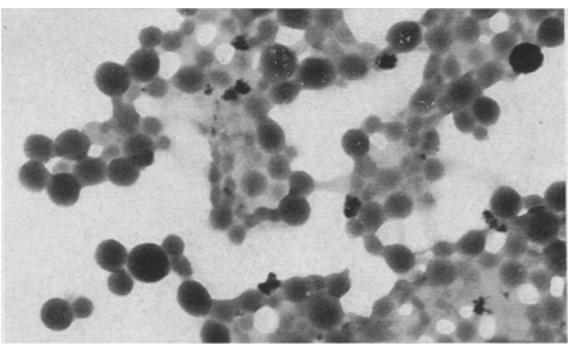

Fig. 9. Vide text

breaking up into spherical droplets. One phase or another is occluded inside the molecular array according to the tensions at each side of the monolayer non polar or polar surface (3).

It can be considered that if the surface tensions are positive the ultimate equilibrium condition will be for the systems to separate out into their separate phases, if, for example, the components are monolayer, aqueous and non aqueous immiscable phases.

This is the usual situation with emulsions made with surface active agents in which the components of the three above indicated phases do not inter-associate in the monolayer phase. If, for example, the interfacial tension becomes negative due to the surface pressure (π) of the interphase or monolayer phase being greater than the interfacial tension ($\gamma_{0/\omega}$) between the aqueous and non aqueous phases $\pi > \gamma_{0/\omega}$, then the equilibrium is the reverse, and the interfacial area will tend towards its greatest value. This necessitates that one phase breaks up into greatest dispersion of droplets according to the maximum interfacial area which can be produced by the available interphase molecules.

The negative interfacial tension produced by the mixing of the components will, at equilibrium, become zero, and dispersion and

not separation will be the equilibrium condition. Possible ways of producing negative interfacial tensions and hence stable micro-emulsions have been indicated in a previous publication of the authors (30).

The essential feature of these systems is that the molecules of the oil phase must interpenetrate or associate with the molecules forming the interphase. Since the

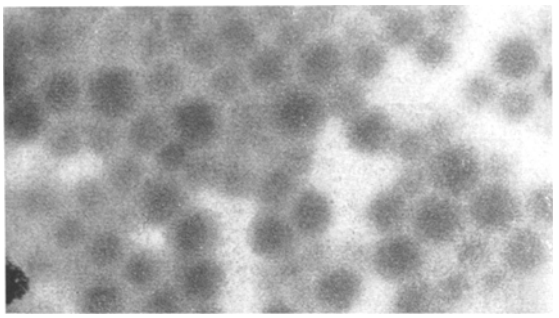

Fig. 10. Vide text

molecules forming the interphase are amphiphilic they are already associating with the water molecules from the aqueous phase. Negative interfacial tensions at the air/water interface would mean that the surface pressure of the adsorbed monolayer would have to be greater than 72 dynes/cm. Values approaching this figure have been obtained by monolayer penetration experiments. At oil/water interfaces with monolayer penetration, values of approximately zero interfacial tension have been measured. The interfacial tensions have not been measured in these cases with a horizontal boom of the *Langmuir* surface balance type, where surface pressures instead of surface tensions can be measured. Surface pressures of duplex films with monolayer penetration by a surface active component from the aqueous phase, could possibly measure directly negative surface tensions. This would be an extension of the experimental technique used by *Zisman* (30) for measuring the surface pressures of alkyl alcohol paraffin duplex films using the *Langmuir* surface balance. Experiments are in hand to try this out.

It has been shown experimentally (4) by plotting the interfacial tension against the concentration of the surface active agent in either the oil water phases that micro-emulsions form at concentrations definitely greater than those necessary to produce zero interfacial tension. This again indicates the feasibility of the negative interfacial tension concept.

Preparation of Micro Emulsions

In order to obtain high surface pressure liquid interphases necessary for the preparation of the stable micro-emulsions, which at the same time are compatible for viewing in the electron microscope, the following criteria can be followed.

It is essential in order to obtain a micro-emulsion for the molecules of the oil phase to penetrate the interfacial monolayer of additives, and produce sufficient disorder to obtain a liquid interfacial film. This can be obtained in a variety of ways.

If the additive is for example, K-oleate, which is normally in a liquid crystal structure, is charged by a large positive counter ion to replace the potassium, such as 2-amino 2-methyl 1 propanol (A.M.P.) disorder is brought into the structure, such that benzene molecules can now penetrate the interfacial monolayer. This permits the interfacial films to liquify and micro-emulsions to form. If benzene is replaced by kerosene in this system, order is brought back to the monolayer together with the fact that the interfacial tension for kerosene against water is

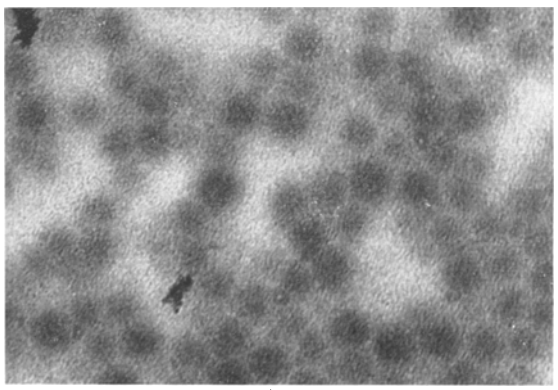

Fig. 11. Vide text

15 dynes/cm greater than benzene/water, which would make a negative interfacial tension more difficult to produce. For these systems the p_H must be greater than 10.5, since at lower p_H's the associated form of fatty acid is prevalent and specifically at p_H 8.8 the ratio is 1 : 1 for soap to fatty acid.

The free fatty acid can on its own be used as an amphiphile additive thus at p_H 8.8 kerosene can now penetrate the mixed acid soap (A.M.P. oleate) monolayer and produce micro-emulsions, but benzene cannot.

It has been shown that an amphiphile such as alkyl alcohols added to A.M.P. oleate (p_H 10.5) must have a hydrocarbon chain equal to or greater than the chain of the

hydrocarbon of the oil phase molecules, in order to produce liquid interfacial monolayers. Thus water, kerosene, cetyl alcohol and A.M.P. oleate will produce micro-emulsions. But replacement of kerosene with a C_{18} alkyl chain hydrocarbon will not. If the cetyl alcohol is replaced with myricyl alcohol, then micro-emulsions again immediately reform.

Disorder in the interfacial layer can therefore be brought about by penetration of the monolayer by an asymmetric alcohol or short chain hydrocarbon and by use of a surface active agent with a large ionic polar

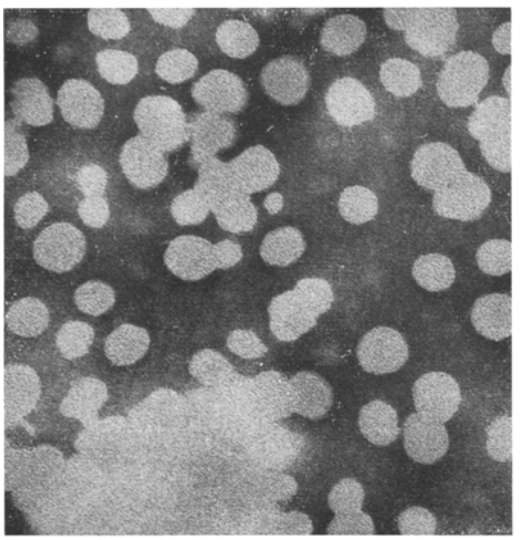

Fig. 12. Vide text

group or by the presence of large sized counter ions. This together with the appropriate stereochemistry of the oil phase molecules can produce high surface pressures with mixed interfacial films with a liquid structure and hence micro-emulsions. The phase continuity can be controlled readily by surface charge. If the concentration of the counter ions for the ionic surface active agent is high and the diffuse electrical double layer at the interface is suppressed water in oil droplets are produced. If the concentration of the counter ions is reduced sufficiently to produce a surface charge at the oil/water interface, the emulsion immediately inverts to an oil/water type. Each disperse system can be diluted with the appropriate continuous phase. This is important when an investigation of the size and shape of the dispersed droplets is undertaken, by low angle x-ray or light scattering and ultra centrifuge sedimentation techniques. References to previous work on these subjects have been given by *Schulman, Stoeckenius* and *Prince* (30). Previous work has also been given describing systems using non ionic emulsifying agents (31).

Electron Microscope Investigation

Since the diameter of the droplets in the micro-emulsions is usually well below that of the 1/20 of the wave length of visible light, the systems are transparent and if the droplets are spherical they are isotropic and Newtonian in flow with one diffuse x-ray band. If they are cylindrical, they are optically anisotropic and non Newtonian of

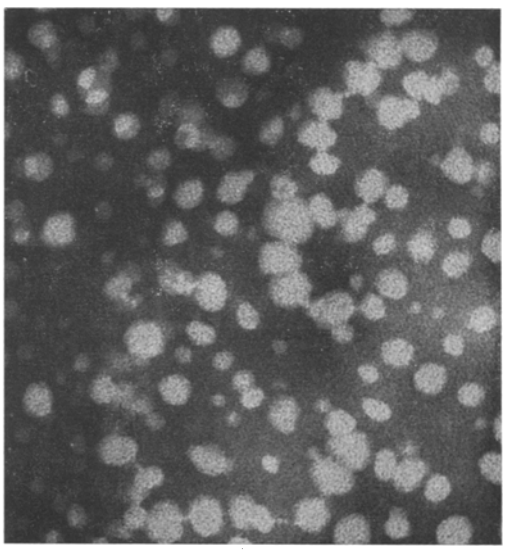

Fig. 13. Vide text

flow and show two diffuse x-ray bands giving dimensions related by a $\sqrt{3}$ factor (31).

The different physical methods of investigating the size and shape of the disperse droplets for the same system give very reasonable confirmation of the diameter of the droplets. Micro-emulsions were therefore prepared to give droplet sizes over the range of 75 Å to 1500 Å and the chemicals so chosen that they would readily react with gaseous osmium tetroxide. Oil phase molecules and surface active agents were used which contained a number of double bonds in non-conjugated form, such as salts of linoleic, linolenic acids and glyceride oils such as tung, linseed or soya-bean. Micro-emulsions with these systems of the oil-in-water type were exposed to osmium tetroxide vapor for several hours. The emulsion droplets blacken immediately if they are *o/w* type. These fixed and stained droplets can be washed and further diluted. Sedimentation in an ultra centrifuge gives a good indication of the uniformity of the dispersion.

Fig. 9 gives the picture at 10,000× magnification of a standard alkyl resin emulsion made with A.M.P. linolenate and non-ionic emulsifying agents. This preparation has been fixed and stained with osmic acid as described above and the stained droplets placed directly on the grid which has been given a nitrocellulose film coating. It can be seen that the droplets are not particulary uniform and melt into one another on irradiation by the electron beam. The diameter of droplets is approximately 1200 Å. Figs. 10 and 11 give pictures of micro-emulsions of much smaller dimensions, 450 Å and 350 Å diameter droplets respectively, and these dimensions agree directly with those determined by the physical methods already indicated. The electron optical magnification is 40,000× and is further enlarged to 140,000× on the plate. It can be seen that the droplets become much more uniform as the micro-emulsions are made with more agents to give greater interfacial area, and small droplet size.

Droplets of smaller diameters than 300 Å do not give enough contrast in the microscope to show them clearly. We therefore tried a technique which had proven to be very useful in the demonstration of small virus particles (5). The material is dried down on the grid from a suspension in 1% phosphotungstate. This forms a very dense film in which the much less dense virus particles, or in our case the fixed emulsion droplets, are embedded. In places where the thickness of the phosphotungstate film is of the same order of magnitude as the droplet diameter these can therefore be clearly seen standing out brightly against a dark background.

Figure 2 shows droplets of ca. 200 Å diameter at 280,000× magnification and fig. 13 droplets of 75 Å and 150 Å. In the ultra centrifuge the two sizes appear as sedimentation zones. The 75 Å diameter droplets approximate to the dimensions of a swollen micelle and agree with the low angle x-ray pictures (32) taken of these systems. They are also of the same dimensions as taken in the first section of myelinic figures both by the electron microscope and x-ray equipment.

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Diskussion

H. L. Booij (Leiden): Haben Sie auch andere Fixiermittel benutzt? Insbesondere möchte ich Sie nach dem Benehmen der Uranylsalze fragen.

A. Kleinschmidt (Frankfurt/M.): Welche Vorstellungen haben Sie, Herr *Stoeckenius*, über die Entfaltung und Adsorption des Globins an die hydrophile Außenseite der lamellar gebauten Phospholipide? Läßt sich über die vollständige oder partielle Entfaltung eine Abhängigkeit vom Salzgehalt und dem p_H-Wert annehmen oder nachweisen?

Antwort der Vortragenden lag bei Druckbeginn nicht vor. *(Die Herausgeber)*