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IMAGES OF DIVALENT CATIONS IN UNSTAINED SYMMETRIC AND ASYMMETRIC LIPID BILAYERS

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SUMMARY

Divalent cations have been microscopically visualized in association with simple lipid bilayers. Symmetric and asymmetric oriented bilayers were constructed from fatty acid monolayers and were cut in thin transverse sections for examination by bright field electron microscopy in the absence of stains, fixatives or embedding materials. It has been found that bilayers formed of lipid molecules having alkaline earth head groups exhibit natural electron contrast. The intrinsic image has been linked to local variations in the bilayer absolute electron density profile determined by X-ray diffraction analysis of the same specimens (McIntosh, T. J., Waldbillig, R. C. and Robertson, J. D. (1976) Biochim. Biophys. Acta 448, 15–33). By combining the microscopic, chemical and X-ray evidence it has been estimated that local increments of about 1 g/cm^3 can produce detectable electron contrast in 500 Å transverse sections of bilayers.

INTRODUCTION

Biological membranes are generally considered to be lipid bilayer structures in specific association with both hydrophilic and hydrophobic proteins. It is now clear that the bilayer is penetrated in some way by protein. The location and distribution of these transmembrane structures has often been related to the occurrence of intramembrane particles seen in freeze fracture replicas of membranes [1, 2]. One of the major paradoxes of modern electron microscopy is the virtual absence of intramembraneous detail in specimens prepared by thin sectioning methods. This conflict has created uncertainty as to the true structure of biological membranes. In an effort to clarify this situation we have undertaken a study of model systems for the purpose of establishing the electron contrast characteristics of molecules arranged as bilayers.

In our efforts to relate the chemical composition of a bilayer to its electron microscopic image we have examined unstained, unembedded and unfixed bilayers formed from lipid monolayers spread at the air/water interface. Schidlovsky's techniques [3] of preparing thin sections of Langmuir-Blodgett [4, 5] multilayers have been modified to permit ultrastructural analysis of the same specimen by both

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electron microscopic and X-ray diffraction methods. As has been noted in preliminary reports [6, 7] a convergence of surface chemical, electron microscopic and X-ray information indicates that the atomic substructure of a bilayer can be related to its electron imaging characteristics.

METHODS

Lipid monolayers of behenic acid (i.e. docosanoic acid = $C_{22}H_{44}O_2$) were spread at the air/water interface by conventional techniques [8]. The crystalline fatty acid (+99 % Applied Sci. Inc.) was dissolved in warm *n*-hexane (99 % Fisher Chem. Inc.) for spreading at the interface. Unsaponified monolayers were formed over subsolutions of glass distilled water. Other monolayers were saponified at the interface by spreading the lipid over unbuffered subsolutions of 10 mM alkaline earth chlorides freshly titrated to pH 9 with NaOH/HCl. Exceptions to these saponification conditions are noted in the text.

All monolayers were formed in a surfactometer device equipped with a tetrafluoroethylene trough, a motor driven tetrafluorethylene surface piston and a platinum foil Wilhelmy plate (Kim-Ray Inc. Oklahoma City, Okla.). The interface of the subsolution was swept and suction polished immediately prior to depositing the spreading solution. The expanded solvent free film was partially covered to exclude dust and was slowly [9, 10] compressed to a surface pressure of 53 dyne/cm (22 °C) which is about 8 dyne/cm below the collapse pressure. This condensed state (20 Å^2/

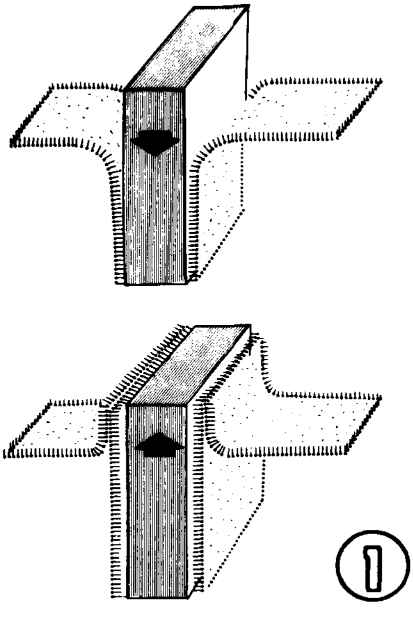

Fig. 1. Schematic of the initial dipping cycle in the formation of oriented bilayers from lipid monolayers by the Langmuir-Blodgett dipping method. Each subsequent down stroke of the hydrophobic wafer yields a lipid bilayer by joining together the terminal CH_3 ends of molecules from two monolayers.

molecule) was automatically maintained by a surface pressure sensitive servo control of the surface piston.

The Langmuir-Blodgett dipping method [4] was used to form oriented planar bilayers from the monolayers. Rectangular wafers which served as the solid support for the multilayers were made by curing Epon 812 epoxy resin (Shell Oil Co.) between two tetrafluoroethylene coated (MS-122 Miller/Stephenson Co. Danbury, Conn.) glass microscope slides separated by a glass cover slip spacer. The cured wafers were removed from the glass sandwich immediately prior to use and were thin (0.2 mm), flat, very smooth and hydrophobic.

Multilayers were deposited upon the surface of the wafer by dipping it vertically through a condensed monolayer as illustrated in Fig. 1. The transport was accomplished by an automatic control system having both cycle counting and variable speed capability.

The multilayer coated wafers were not fixed, stained or embedded in the ordinary manner but rather were placed directly in an ultramicrotome vice chuck for thin sectioning. The wafer was oriented in such a way that both of its lipid coated faces were perpendicular to the cutting edge of the diamond knife. The sections, which were judged to be less than 500 Å in thickness by their interference color, were cut directly from the trimmed wafer onto distilled water and the ribbons were transferred onto carbon film backed electron microscope specimen grids. The transverse sections were examined in a Philips EM301 electron microscope equipped with a high resolution stage. The microscope was operated in the bright field transmission mode (80 kV) with a 50 μm objective aperture.

RESULTS

Lipid monolayers were used to form oriented bilayers for the purpose of establishing the absolute limits of electron contrast in model systems having a molecular orientation analogous to that of biological membranes. The present study was confined to the simplest available case since the details of fatty acid molecular substructure are not in themselves a matter of controversy [8, 11, 12]. It has been known for many years [4] that monolayers of this type can be formed into well oriented bilayers having the same molecular organization as the parent monolayer [13–16]. Langmuir-Blodgett multilayers of saponified fatty acids contain little or no water and X-ray diffraction studies have shown [17–19] that their ultrastructural organization is very similar to that of crystals of the same material [20–22].

The manner in which monolayers are formed into stacks of well oriented bilayers has been evaluated in detail by others [4, 23, 24]. In the present study it was observed that the monolayers transferred onto the surface of the solid support according to the scheme illustrated in Fig. 1. The multilayers were of the Y-type [4] with a molecular deposition ratio [23] near unity.

It was noted that the wafer exhibited contact angles [25] at the monolayer interface as illustrated in Fig. 1. When the support first moved downward a monolayer of lipid adhered to the surface of the solid and when the wafer was withdrawn and second sheet of lipid was transferred. The fact that the support wafer emerged from the subsolution with a dry surface is in agreement with earlier observations [4] that the CH_3 ends of those lipids transferred during the upstroke of the cycle were directed into the air. During each of the subsequent downstrokes of the wafer a lipid bilayer was formed by the apposition of the CH_3 ends of lipids from two monolayers.

Oriented multilayers of the Langmuir-Blodgett type are thought to consist of stacks of planar bilayers having a molecular packing density near the 20 A^2/molecule observed in monolayers. Schidlovsky [3] examined thin sections of saponified multilayers which had been fixed with OsO_4 and embedded in methacrylate and observed the expected lamellar structure. We have confirmed his results with the important exception that pretreatment of the multilayer with fixatives, stains and embedding materials has been found to be completely unnecessary for the visualization of the multilayer. Our exclusion of foreign molecules from the specimen plus the exceptional physicochemical definition of the bilayers has made it possible to relate directly the microscopically observed structures to discrete atomic groups of the bilayers.

Following the earlier example [3] we constructed a sandwich-like multilayer in which bilayers of known chemical composition were deposited in a pre-determined sequence. The first zone of the multilayer was formed from monolayers of barium behenate (22 carbons), the second zone was from behenic acid and the third zone contained bilayers of barium palmitate (16 carbons). Electron microscopic examination of the untreated multilayer revealed (Fig. 2) that those zones containing lipids with barium head groups had a lamellar appearance whereas those zones without the

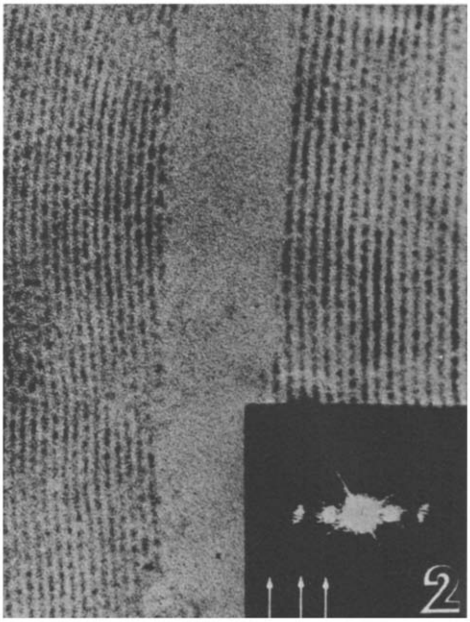

Fig. 2. The intrinsic image of lipid bilayers of known hydrocarbon chain length and head group composition. The lamellar zone to the left contained bilayers of barium palmitate (16 carbons) while the lamellar zone to the right contained bilayers of barium behenate (22 carbons). The non-lamellar central zone contained 10 bilayers of barium-free behenic acid. Note that the 54 Å thickness of the barium behenate bilayers is 22/16 that of the barium palmitate bilayers and that the head groups are visible when barium is attached to the lipid carboxyl moiety. Inset: three orders of optical diffraction from a barium behenate micrograph corresponding to a resolution of 18 Å. Occasionally four orders of diffraction are observed. All specimens unstained, unfixed and unembedded.

metal lacked lamellar electron contrast. It has been found that the width of the fine electronlucent lines in the barium behenate and barium palmitate zones differed by the same ratio (22 : 16) as the number of carbons in the fatty acid molecules. This result, which is in agreement with earlier observations [3], suggests that the fine electronlucent lines of the multilayer image correspond to the hydrocarbon regions of the bilayers seen in side view.

It has been found that the physicochemical integrity of the individual bilayer was not drastically altered by the preparation and imaging process. When multilayers were constructed to contain a specified number of barium behenate bilayers an equal number of lamellae appeared in the image. This relationship would not have been maintained if significant thin sectioning artifacts had been present. Thus, the individual repeating unit of the electron image corresponds to a side view of a barium behenate bilayer with an electronlucent hydrocarbon region and an electron opaque carboxylate head group.

Careful measurements of the image lamellar repeat period of barium behenate bilayers has shown it to be 54 Å. Consideration of the bond angles and lengths of the behenate indicates that two fully extended molecules oriented perpendicular to the plane of the bilayer would span a distance of about 60 Å. When the same multilayers which yielded the thin sections were analyzed by X-ray diffraction methods [22] it was found that the repeating units were 58 Å apart and that the lipid chains were slightly tilted. The shrinkage which the barium behenate bilayers suffered in the electron images can not be attributed to dehydration since it is well established [4, 33] that multilayers of this compound contain no water. It is likely that reduced spacing is due to disruption of the bilayer hydrocarbon organization by the electron beam since it has been shown by others [26], and confirmed by us, that bilayer wide angle electron diffraction patterns fade very rapidly and irreversibly upon electron illumination of the specimen. This disordering, together with other factors such as specimen orientation and thickness, may be a cause for the limited resolution of the multilayer electron micrographs (18–13.5 Å, Fig. 2 inset) as compared to the much better resolution (6 Å) obtained by X-ray diffraction analysis of the same multilayers [22].

It has been suggested [3] that the lack of lamellar detail in images of behenic acid zones of multilayers is not an accurate reflection of actual molecular structure. We have confirmed in the case of untreated multilayers that the overall width of behenic acid zones situated between barium behenate zones corresponds to the number of unsaponified bilayers deposited in the electronlucent areas (Fig. 2).

Furthermore, X-ray diffraction analysis of the behenic acid multilayers has shown them to be oriented lamellar structures [22]. Based upon these results it can be concluded that the lack of a lamellar image from behenic acid bilayers is attributable to the limited electron scattering power of the COOH head group.

Another factor which must be considered with respect to the absence of a lamellar image from the behenic acid is the known tendency of fatty acids to sublime in a high vacuum environment [27, 28]. We have found that the lack of substructural contrast in the images of these bilayers is not attributable to their sublimation in the electron microscope. Micrographs of the multilayers were placed in a scanning microdensitometer and the absorbances were recorded. It was found that the image of the non-lamellar zone upon the carbon film had an absorbance greater than that of the bare carbon film. Optical gravimetric analysis of the micrographs by the method of

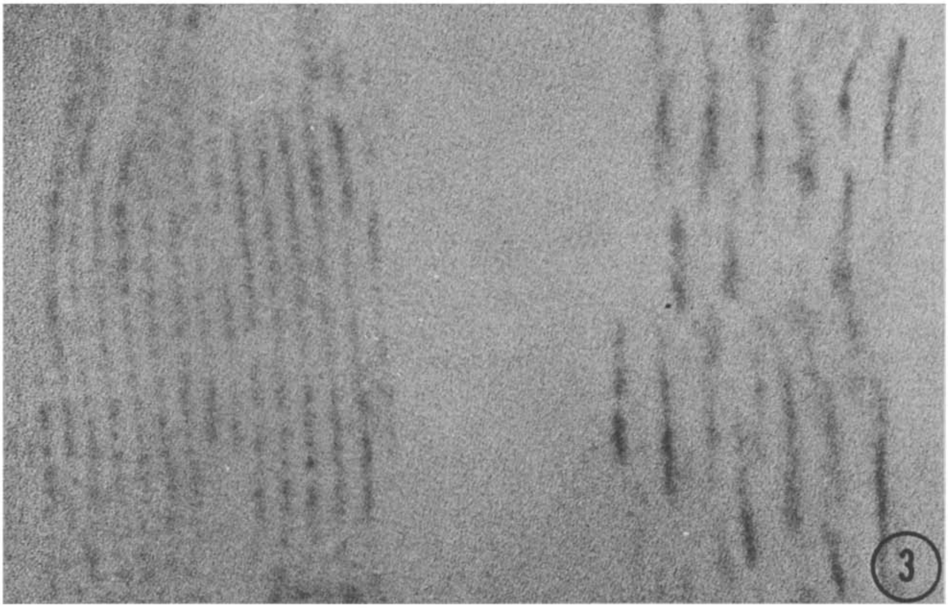

Fig. 3. Intrinsic image of bilayers of defined symmetry and asymmetry. Left, symmetric bilayers of barium behenate having 54 Å repeat period. Center, symmetric bilayers of behenic acid. Right, asymmetric bilayers with one face of barium behenate and one face of behenic acid. Note the doubling of the dense spacing in the asymmetric bilayers. Also note the occurrence of secondary fine densities between the major asymmetric lines and the step-like indication of ensemble bilayer flip-flop. All specimens: Unstained, unfixed and unembedded.

Bahr and Zeitler [29] has confirmed the presence of a measurable mass in these regions of the multilayer. Furthermore, this mass maintains its orientation during the imaging process since it has been shown by others [3, 6] and confirmed by us that a single bilayer of visible barium behenate remains oriented in an electronlucent zone of behenic acid bilayers.

The observed differences in the electron scattering power of the behenate and behenic acid head groups have been used to evaluate the electron imaging properties of chemically asymmetric bilayers. Consideration of the transfer process details (see Fig. 1) indicates that asymmetric bilayers could be created by alternately dipping the support wafer through two different kinds of monolayers causing the formation of centrosymmetric bilayers repeating in mirror fashion in the multilayer (i.e. Ba--COOH, COOH--Ba, Ba--COOH, etc.). Since chemical asymmetry is one of the fundamental characteristics of biological membranes it is important to consider the influence of such a molecular organization upon bilayer images.

Large multilayer arrays were made to contain zones of both symmetric and asymmetric bilayers. It can be seen by examining the multilayer electron micrograph in Fig. 3 that the asymmetric bilayer zone (right) exhibits lamellar image densities which repeat primarily at double the spacing of the symmetric barium behenate control bilayers (left). This result agrees with the surface chemical expectation since microscopically "invisible" COOH head group planes would alternate between the visible planes of the barium containing head groups.

The disorderliness of the asymmetric bilayers is not unexpected. It was demonstrated long ago that a type of molecular flip-flop known as an X-Y inversion [4, 23, 24, 30] occurs during the formation in Langmuir-Blodgett multilayers. We have used both microscopic and X-ray diffraction methods to evaluate further the molecular inversion in asymmetric bilayers.

Close examination of the images of the asymmetric bilayers indicates that weak densities tend to appear in a stepwise manner between the stronger densities of the barium head group planes (Fig. 3). The distribution of the secondary densities at integral spacings of the bilayer indicates that some of the electron opaque barium relocates onto the "invisible" COOH groups of the opposite face of the bilayer. This redistribution is also evident in electron density profiles obtained by X-ray diffraction analysis (see ref. 22, Fig. 10). Inclusion of EDTA (0.1 mM) in the subsolution of the docosanoic acid monolayers has no effect upon the occurrence of the secondary densities in the asymmetric bilayers. It might be expected that this chelating agent would bind free barium if the cation dissociated from the behenate during the dipping. This result indicates that the inversion of mass is due to an ensemble transposition of blocks of the bilayer rather than simple cation dissociation during the formation process.

If behenic acid and barium behenate molecules segregated to one side of the bilayer can exchange places, then it is important to know if these two molecules can coexist as a mixed phase when on the same side of a bilayer. We have evaluated the

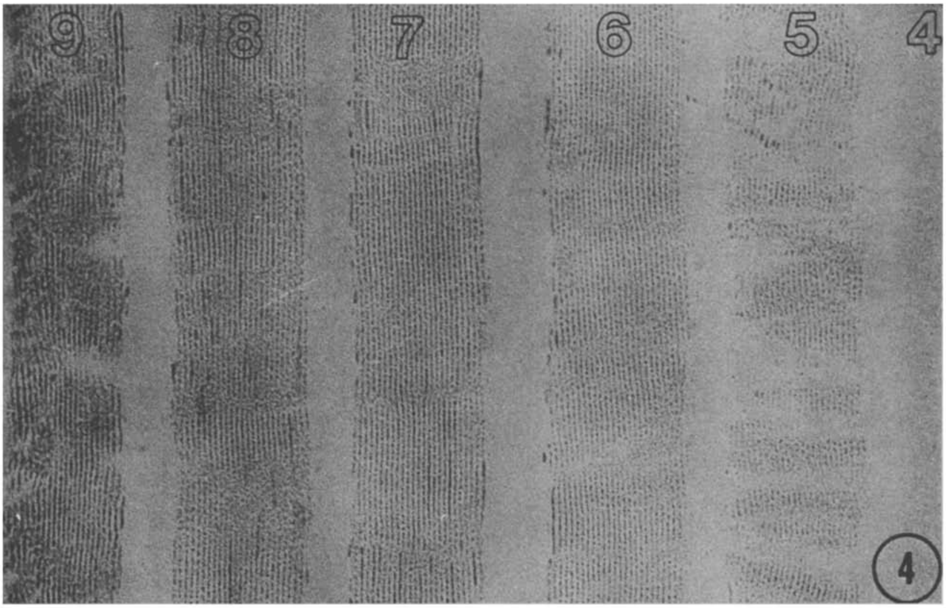

Fig. 4. Multilayer formed from barium behenate monolayers over subsolutions of different bulk pH. Left, most lamellar zone of 25 bilayers of barium behenate at pH 9 is next to the epoxy edge and electronlucent zones between lamellar stacks contain 10 bilayers of behenic acid. Notice the decrease in head group image density as the saponifying pH was lowered. Subsolution, 10 mM $BaCl_2$ for barium behenate and distilled water for behenic acid. Lamellar repeat 54 Å. All specimens, unstained, unfixed and unembedded.

possibility of a phase separation by forming symmetric bilayers which contained mixtures of the acid and salt form of the lipid.

It is known that the extent of divalent cation binding to fatty acid monolayers depends upon the pH of the saponification solution [13, 28, 30–34]. We have constructed multilayer arrays from behenic acid monolayers spread over 10 mM barium chloride subsolutions of different pH for the purpose of forming partially saponified bilayers. It is apparent upon examination of electron micrographs of such arrays (Fig. 4) that the headgroups image density decreases with the pH of the subsolution of the parent monolayer. Clustering of the barium head groups, which might be attributable to a phase separation of the behenate and behenic acid, can be seen only at extremely low pH values.

The observed decrease in lamellar contrast with saponifying pH is in agreement with known effects of this treatment upon the ratio of salt to fatty acid in the multilayer. It has been shown by others [35] that when corrections are made for monolayer surface potential effects upon interfacial hydrogen ion concentration that the p*K* of fatty acid monolayers is near 5.5. We observed a sudden loss of lamellar image density below pH 5 and no discernable difference between lipids exposed to barium at pH 3 and lipids exposed to distilled water. The reduction in the lamellar contrast over this pH range is also consistent with the fact that the buffering capacity of atmospheric CO_2 at the interface is low near pH 5 [36].

We have also systematically altered the equilibrium of the interfacial saponification reaction by changing the concentration of barium chloride in the subsolution.

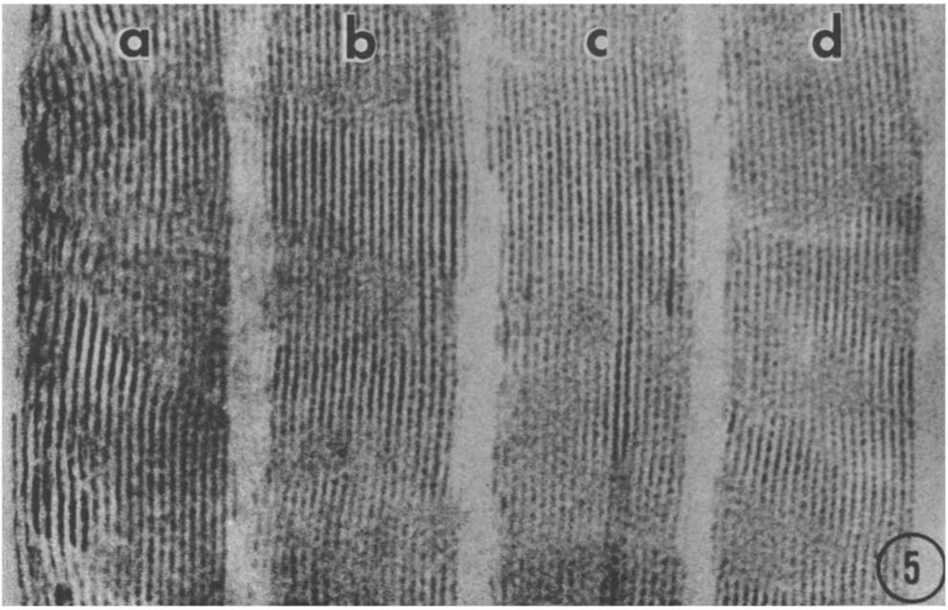

Fig. 5. Multilayer formed from monolayers over subsolutions of different $BaCl_2$ concentration. The 54 Å repeating lamellae correspond to the barium behenate bilayers formed from monolayer subsolutions containing a, 100 mM; b, 10 mM; c, 1 mM; d, 0.1 mM $BaCl_2$. Each lamellar zone was separated by 5 bilayers of behenic acid and the wafer surface is visible on the left. All specimens unstained, unfixed and unembedded.

Stacks of bilayers were assembled from monolayers spread over solutions of 100, 10, 1.0 and 0.1 mM $BaCl_2$ at pH 9. Electron micrographs of the resultant multilayer (Fig. 5) show that the electron contrast of lamellae decreases with the concentration of the saponifying cations. However, the zones of the multilayer formed from subsolutions containing 100 and 10 mM $BaCl_2$ exhibit nearly equal lamellar densities. It would appear that the standard saponification condition of 10 mM $BaCl_2$ at pH 9 was sufficient to fully react available sites. This saturation of sites by the barium atoms has been of value in the determination of the bilayer ultrastructure by X-ray diffraction methods [22]. Other workers have used radiotracer [16, 37], chemical [13] and optical [14, 15, 28, 33] methods to evaluate the binding of metals to fatty acid multilayer head groups.

Having established that the attachment of barium to the head group is sufficient to introduce local electron contrast into the bilayer polar region, it becomes important to delineate the imaging characteristics of other head groups. Large multilayer arrays were constructed from monolayers of behenic acid spread over pH 9 solutions having 10 mM concentrations of one of several different alkaline earth chlorides. The sequence in which the various zones of the multilayer were assembled was used to determine the intrinsic imaging properties of the different kinds of the cationic head groups. It can be seen upon examination of Fig. 6 that all of the physiologically important divalent cations are detectable in thin sections of the multilayers.

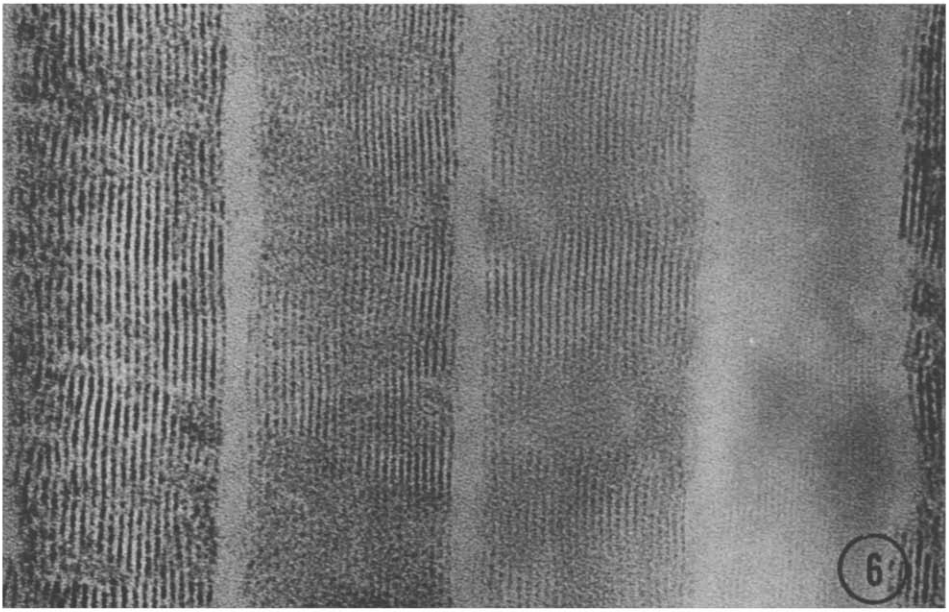

Fig. 6. Alkaline earth cation head groups on untreated behenate bilayers. The multilayer formation sequence (left to right) was Ba, Sr, Ca, Mg, Ba with each zone of 25 behenate bilayers separated by 5 bilayers of electronlucent behenic acid. The wafer edge is on the far left and a few control barium behenate bilayers can be seen on the far right of the micrograph. Note the faintly lamellar appearance of the magnesium behenate bilayers and the non-lamellar appearance of the behenic acid bilayers. All alkaline earth chloride subsolutions 10 mM at pH 9. Barium behenate repeat 54 Å. All specimens unstained, unfixed and unembedded.

The electron scattering power of the cations decreases with their atomic weight in the sequence Ba > Sr > Ca > Mg with the latter exhibiting very weak lamellar electron contrast.

It may seem remarkable from a biological perspective that bilayer atomic groups of such low atomic weight can be directly visualized in unstained thin sections. However, the observed differences in head group imaging characteristics are in accord with physical chemical characteristics of the polycrystalline specimen. The fact that the multilayers were all on the same thin section indicates that the observed difference in electron contrast cannot be attributed to section thickness variations. This unlikely possibility was excluded by examining multilayers of the same compounds arranged in different sequences and by directly examining unsectioned skimmed monolayers [4]. It was found that the contrast relationships of the divalent cations were independent of both the thin sectioning procedure and proximity to the epoxy wafer.

It is reasonable to expect that the electron imaging characteristics of the alkaline earth head groups would depend upon their difference in mass. It is known from condensed monolayer studies [8, 12, 33] that the distance between adjacent fatty acid chains is not significantly influenced by different alkaline earth head groups. Thus, it can be expected that each specimen bilayer contained the same number of cations as its saponified neighbors. Since all of the bilayers had the same width [4] and depth (section thickness), it follows that they occupied equal volumes and therefore that the electron contrast differences arise from the mass differences. It may be concluded that subtle variations in bilayer molecular organization can be detected by conventional methods of bright field electron microsocpy if care is taken to optimize the orientation and chemical composition of the specimen.

DISCUSSION

The central role which lipids play in the structural organization of biological membranes has prompted many studies of lipid bilayer morphology. Nearly all of the previous electron microscopic studies have used either extrinsic modifications of the lipid structure with treatments such as fixation, staining, freezing and metallic replication [38–48] or sophisticated physical techniques [19, 51–55]. Most notable in this regard are methods recently employed in the study of crystalline membranes [49] and protein crystals [50]. In the present study, we have been able to examine certain unstained lipid bilayers using conventional electron microscopic techniques by carefully controlling the molecular composition and orientation of the bilayers.

While we have demonstrated that it is possible to identify certain regions of bilayers viewed in thin section, we have not achieved atomic resolution. It is clear from both the surface chemical and X-ray diffraction [22] evidence the specimen material was poly-crystalline in character and that imaged objects correspond to planes of superimposed atoms. The crystallinity of the multilayers makes it possible to estimate the physicochemical limits of electron contrast in oriented bilayers cut in thin section.

It is known from chemical studies of multilayers [37] that each divalent cation is shared by two fatty acid molecules. Both the area/molecule and X-ray diffraction evidence [22] indicates that the distance between neighboring hydrocarbon chains is

TABLE I

Estimated mass densities and projected mass densities (i.e. mass thickness) for different regions of a 500 Å transverse section of oriented Langmuir-Blodgett bilayer. Carboxylate values for 0.5 cation/lipid.

Moiety	Ba-COO	Sr-COO	Ca-COO	Mg-COO	COOH	$-CH_2-$	$-CH_3$	Units
Mass density	3.72	2.98	2.29	1.99	1.49	1.00	0.42	g/cm^3
Projected mass density	18.6	14.9	11.5	10.0	7.5	5.0	2.1	$\mu g/cm^2$

about 4 Å and therefore that the distance between neighboring cations is 8–9 Å. It follows that a 500 Å transverse section of a single monolayer would have about 60 cations superimposed to project as a point in an image. In the case of a multilayer the electron opaque line would be expected to have twice as many cations because the head group planes of two adjacent bilayers are abutted. Thus, while the observed structures do not correspond to single atoms it is interesting to note that remarkably few groups can produce the contrast.

What are the limits for the detection of substructural details in thin sections of the bilayers? Because the chemical composition of the bilayer is known it is a straightforward matter to use a given moiety's atomic weight and atomic number in combination with the volume information obtained by the X-ray analysis [22] to calculate the group's mass density. The values derived for different regions of the bilayer are shown in Table I and are extended to projected mass densities (i.e. mass thickness) for the same regions in 500 Å transverse sections [29].

It is clear that local increments in bilayer mass density correspond to differences in bilayer electron contrast. In the case of barium behenate it is estimated that there is density gradient of some 3.3 g/cm^3 within the width of a single monolayer (27 Å) with about 80 % of the increment occurring near the polar region. The fact that such a steep mass density gradient remained intact during the specimen preparation and imaging procedure suggests the thin sectioning method is compatible with detailed ultrastructural analysis.

The smallest increment in bilayer mass density which can give rise to detectable electron contrast in 500 Å sections can also be estimated. Because we were able to observe a small contrast difference between zones of the multilayer composed of unsaponified fatty acid bilayers and their supporting carbon film we have a clue as to the contrast limit. Since the total mass of the fatty acid molecule is dominated by the $-CH_2-$ groups it can be anticipated that a density of 1 g/cm^3 is near the limit of detectable bright field contrast in sections of this thickness. This value is in accord with the predictions of others [29].

The relationship between the bilayer substructure and electron contrast in thin sections can also be estimated by considering the structural variations as steps in mass density. Since there was no detectable difference between images of the hydrocarbon region of the bilayer and the COOH head group it would appear that a step of 0.5 g/cm^3 was inadequate to produce contrast. However, a larger step of about 1.0 g/cm^3 from the level of the $-CH_2-$ groups to the level of the magnesium carboxylate did yield weak electron contrast. An even larger step of about 1.3 g/cm^3 from the hydrocarbon chains to the calcium carboxylate head group resulted in good electron contrast. It would therefore appear that local variations of bilayer density on the order of 1 g/cm^3 are required to detect bilayer structures in 500 Å transverse sections.

While it is difficult to generalize from simple model systems to the more complex case it is possible to speculate about the electron microscopic visibility of biological molecules. The average mass density of hydrophillic proteins such as catalase [56] indicates that such compounds might be detectable when isolated in thin sections. However, the much lower density of hydrophobic proteins indicates that they would not be detectable in the unstained state when compared to lipid hydrocarbon. Such estimates must be approached with caution since they are based upon the average mass densities of the molecules without regard to local variations in the structure.

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