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FURTHER STUDIES ON THE SPREADING OF BIOMEMBRANES AT THE AIR/WATER INTERFACE

STRUCTURE, COMPOSITION, ENZYMATIC ACTIVITIES OF HUMAN ERYTHROCYTE AND SARCOPLASMIC RETICULUM MEMBRANE FILMS

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Air/water interface films were obtained from human erythrocytes and rabbit sarcoplasmic reticulum membranes at 'zero surface pressure. according to Verger, R. and Pattus, F. (Chem. Phys. Lipids (1976) 16, 285–291). The lipid and protein distribution of these membrane films suggest that the film composition is determined by the composition of the membrane and the mode of integration of its components. When kept at low surface pressure, slow film expansion occurred due to unfolding of proteins at the interface. This process can be stopped by compressing the films at a higher surface pressure than 15 dyn/cm. Acetylcholinesterase activity from human erythrocyte films is highly dependent on the condensation state of the film. Ca^{2+}-ATPase from sarcoplasmic reticulum films was still activable by Ca^{2+}. Freeze-fracture studies on erythrocyte membrane films suggest that such films are monolayers in which proteins are randomly distributed.

Introduction

In the past years the monolayer technique has been shown to be a useful tool in studying the interfacial properties of lipids in relation to their organization in biological membranes [1].

The monolayer system is the only one which allows a continuous and controlled variation of the 'quality of the interface' [2] and the latter was shown to be of prime importance for the recognition of lipid interfaces by lipolytic enzymes [3,4].

Although monomolecular films seem very attractive to study lipid-protein interactions, this technique was restricted to a few membrane proteins. This is mainly because membrane proteins are insoluble in water making aggregates which display no affinity for lipid interfaces. Since the development of a technique of formation of asymmetrical bilayers from two monolayers [5,6] it was of interest to be able to form lipoprotein films at the air/water interface: these films containing biologically active proteins.

In order to tackle this problem, we developed a new technique for spreading membranes or reconstituted lipoproteins at the air/water interface [7]. Using phospholipid vesicles [8], we showed that spreading at low surface pressure ($\pi < 2$ dyn/cm) was required to obtain true monomolecular films at the air/water interface. When spreading was performed at a higher surface pressure, the vesicles retained part of their internal content, the surface pressure-area isotherms deviating from a monomolecular film.

Similar results were found independently by Schindler by a different film formation [10]. In this case, the lipid film was built by self assembly of the lipid phase at the interface of a homogeneous vesicle

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Abbreviation: DTNB, dithiobis(2-nitrobenzoic acid).

solution. Quite similar behaviour was found with natural membrane vesicles [9]. Furthermore, membrane bound enzymatic activities were retained in vesicle spread films.

The unilamellar structure of films from intestinal brush-border was indicated by the complete accessibility of aminopeptidase to its substrate and to proteolytic enzymes from the subphase. However, we were not able to establish that such films were true monolayers.

The spreading technique was also used to demonstrate the anchoring capacity of the hydrophobic peptide from the detergent form of pig intestinal aminopeptidase [11].

Recently, successful formation of planar bilayers from two vesicle spread monolayers have been described (see H. Schindler (1980) FEBS Lett. 122, 77). This technique was employed to study membrane channels in bilayers formed from native membrane vesicles rich in acetylcholine receptor [29] and in bilayers formed from reassembled vesicles containing matrix protein from *Escherichia coli* outer membrane [12].

We used human erythrocytes and rabbit white muscle sarcoplasmic reticulum to further characterize membrane films. The interfacial behaviour of a membrane enzyme and of a spanning protein was investigated as a function of the spreading time and surface pressure. Finally the structure of membrane films was studied by electron microscopy after deposition on a solid support.

Materials and Methods

Erythrocyte and sarcoplasmic reticulum membranes. Erythrocyte ghosts were prepared according to Dodge et al. [13]. Protein concentration, measured by the method of Lowry et al. [14] was adjusted to 10–12 mg protein/ml. Ghosts were discarded after 3 days when stored at 0–4°C. Acetylcholinesterase was assayed with acetylthiocholine (Merck) (30 mg/100 ml) as substrate in presence of dithiobis(2-nitrobenzoic acid) (DTNB) (5 mg/100 ml) 412 nm = 13 600. All experiments with erythrocyte membranes were carried out in 10 mM Tris-HCl buffer (pH 7.4), 0.1 M NaCl, 10 mM $CaCl_2$.

Rabbit white muscle sarcoplasmic reticulum membranes (9 mg protein/ml) prepared according to Martonosi [15] were kindly supplied by H. Sigrist (Bern). Ca^{2+}-ATPase activity was measured according to Mac Lennon [16] with 10 mM ATP (Merck) and 0.05 mM $CaCl_2$ or 5 mM EGTA in 10 mM Tris-HCl buffer (pH 7.4), 0.2 M KCl, 5 mM $MgCl_2$ [17].

Inorganic phosphate was determined according to Fiske and SubbaRow [17].

Spreading technique, film recovery. Membranes were spread at 'zero surface pressure' as described previously [7]. The membrane vesicle suspension (10 mg protein/ml) was allowed to flow down along a wet glass rod crossing the air/water interface. The spreading area was such that no rise of surface pressure could be detected as the membrane material spread over the surface ('zero surface pressure' spreading). The film is then transferred, washed and compressed to a given surface pressure. The monolayer trough and film recovery technique were described previously [18].

When needed, the recovered films were centrifuged for 30 min at 50 000 rev./min in a 60 Ti rotor (Beckman).

We checked that after centrifugation no lipids could be extracted from the supernatant and no protein could be detected. Identical enzymatic activities were found in the pellet as in the recovered film before centrifugation.

Acetylcholine esterase activity in the film 'in situ' was measured by injection of DTNB and acetylthiocholine into the subphase below the film at 25°C. These compounds did not affect the surface pressure of the films. Acetylthiocholine hydrolysis was measured on 1-ml aliquots of the subphase. Ca^{2+}-ATPase activity was measured by injection of Ca^{2+} (or EGTA) and ATP below the film at 37°C. Inorganic phosphate was determined on 3-ml aliquots from the subphase. Blanks of soluble enzymatic activities were determined by incubating samples from the subphase separately.

Lipid and protein analysis. Lipids from film pellets or membranes were extracted according to Renkonen et al. [19]. Lipid species were separated by two-dimensional thin-layer chromatography [20]. Phospholipid composition was determined by the content of inorganic phosphate of phospholipid spots after perchloric digestion [17]. Protein compositions were determined by polyacrylamide gel electrophoresis in SDS (5.8% and 7% acrylamide for erythrocyte membrane and sarcoplasmic membrane, respectively).

Preparation of the lipoprotein bilayer on a solid support. Films were deposited on a freshly split mica [23] as done first by Blodgett [21] with lipid monolayers (Fig. 1). Mica is hydrophilic. When it is first dipped into the subphase no change of surface pressure occurred. The mica plate was then raised up through the film. The film that clings to the plate is oriented with the hydrocarbon layer outward. The surface of the film-coated plate is hydrophobic. The plate was then dipped into the film covered surface, depositing a second layer 'back to back'. Success of the deposition was monitored by measuring the decrease of surface pressure. The mica plates were transferred, in water, to a large contained where they are attached to a U-shaped brass holder.

The holder with the mica was rinsed with 30% glycerol, rapidly frozen in Freon 22 and then placed on the pre-cooled (−150°C) stage of a Balzers BA 360 freeze-etch apparatus. Under vacuum (10^{-6} torr) the mica plate was broken away from the ice with the knife assembly (Fig. 1b, see also Ref. 23). The exposed surface was etched briefly (60 s at −100°C) and shadowed with platinium and carbon.

Electron microscopy. The carbon replica corresponding to the hydrophobic fracture face of the second monolayer deposited on the mica was placed on a 100 mesh copper net and used for electron microscopy in a Philips EM 200 at 60 kV. All micrographs were obtained at a scale 26 900X with subsequent photographic enlargement of 2.4X giving a final magnification of 64 560X.

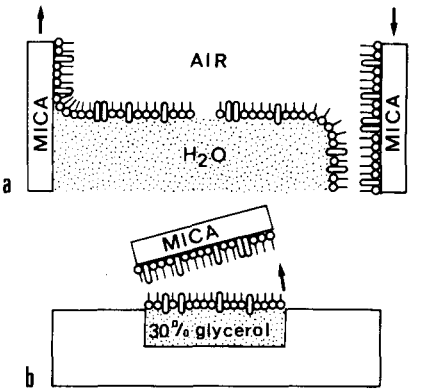


Fig. 1. (a) Dipping technique with the hydrophilic mica using a micromanipulator. (b) Fracturing of the bilayer by tearing off the mica from the U-shaped brass holder. The second monolayer remains on the frozen 30% glycerol.

Results

Lipid and protein composition of films obtained by spreading human erythrocyte ghosts and rabbit sarcoplasmic reticulum membranes at zero surface pressure

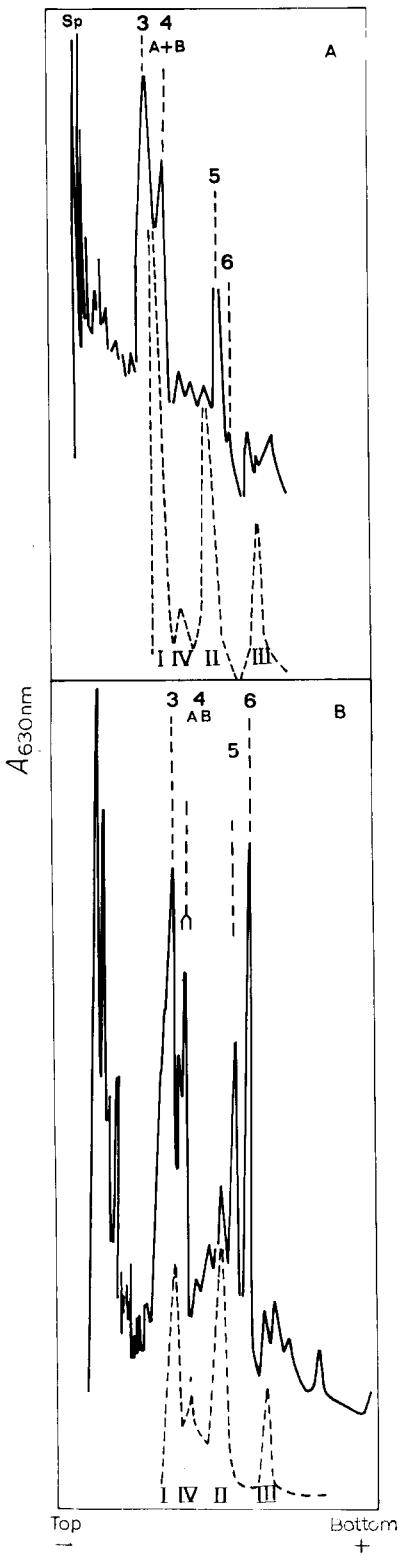
Erythrocyte membrane films are slightly enriched in phospholipids when comparing to the membrane (Table I), while with sarcoplasmic reticulum membranes, the lipid to protein ratio was not affected as found previously with *Escherichia coli* inner membrane [9].

However, for both membranes, the proportion of the different phospholipids is the same in the films as

TABLE I

LIPID COMPOSITION OF HUMAN ERYTHROCYTES AND RABBIT SARCOPLASMIC RETICULUM MEMBRANES AND CORRESPONDING FILMS

Lipid/protein ratio (μmol phosporus/mg of protein)	Lipid (%)			
	Native membrane 0.85 ± 0.05	Membrane film 1.00 ± 0.05	Native membrane 0.30 ± 0.05	Membrane film 0.35 ± 0.05
Sphingomyelin	20 ± 3	20 ± 1	2	1
Phosphatidylcholine	24 ± 3	29 ± 3	65 ± 4	60 ± 3
Phosphatidylserine + phosphatidylinositol	21 ± 1	21 ± 2	12 ± 2	15 ± 2
Phosphatidylethanolamine	31 ± 3	29 ± 2	20 ± 3	25 ± 3



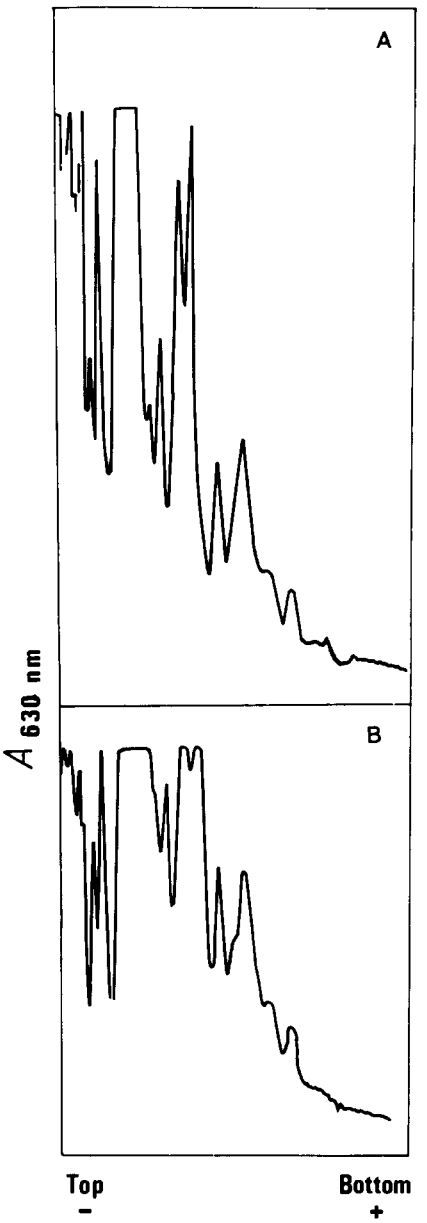


Fig. 3. Gel electrophoretic pattern of sarcoplasmic reticulum membrane films (A) and sarcoplasmic reticulum membranes (B). Coomassie blue staining. The gels were slightly overloaded in order to see minor bands. 7% acrylamide gels in SDS.

Fig. 2. Gel electrophoretic pattern of erythrocyte membrane films (A) and erythrocyte membranes (B). Film spreading time 1 min. Identical results were obtained after 25 min spreading time. ———, Coomassie blue-stained protein pattern; ------, Schiff reagent-stained glycoprotein pattern. 5.8% acrylamide gels in SDS.

in the membranes (Table I). Furthermore from the spots on the TLC places one can conclude qualitatively that cholesterol and minor lipids composition is also not affected by spreading.

In erythrocyte, negatively charged lipids are mostly located in the inner monolayer of the bilayer. The results of Table I indicate that lipids from the two halves of the bilayer spread at the air/water interface with the same yield.

The gel electrophoresis pattern from sarcoplasmic reticulum films and membranes (Fig. 3A and B, respectively) suggests that also the protein composition is not affected by spreading. This is in agreement with the conservation of the lipid to protein ratio. In contrast, erythrocyte membrane films have a slight different protein composition than the membrane (Fig. 2). While Band 3 and the glycoproteins are present in the same proportions, Band 6, a loosely bound protein is nearly missing. Some spectrin is also released during spreading. This explains the slight phospholipid enrichment in these films (Table I).

Erythrocyte membrane films were also formed by the method described by Schindler [10]. We found the same lipid and protein components in 'desintegrated films' at low surface pressure as in spread films. Band 6 was also released in the subphase during membrane desintegration at the air/water interface (data not shown).

Influence of the spreading time at zero surface pressure on the isotherm of compression of the films

At low surface pressure, there is a high interfacial energy which is known to unfold proteins at the air/water interface [22]. However such a low pressure was needed to disrupt the vesicles and form unilamellar films [8,9]. As shown in Table II, if the time interval between spreading at zero surface pressure and film compression increases, the surface density of protein decreases indicating a film expansion with time at low surface pressure. A parallel loss of acetylcholinesterase activity is observed. After 50 min spreading time, the film is 1.8-times more expanded than after 1 min. Acetylcholinesterase activity is, in this case, almost completely and irreversibly destroyed. The spreading time has no influence on the spreading yield of membrane components. The lipid and protein composition of the film is unaffected.

Unfolding and denaturation of the proteins at the air/water interface does not occur only at zero surface pressure. In order to follow the denaturation, acetylcholinesterase activity was measured at different surface pressures (Fig. 4). In these experiments, spreading time at zero surface pressure was 1 min. The film was then compressed and surface pressure adjusted to a given value. At time zero, acetylthiocholine was injected in the subphase. Surface pressure

TABLE II

INFLUENCE OF THE TIME ELLAPSED AFTER SPREADING AT ZERO SURFACE PRESSURE ON THE FILM STRUCTURE AND ACETYLCHOLINESTERASE ACTIVITY

Human erythrocyte membranes were spread at zero surface pressure on 10 mM Tris-HCl buffer (pH 7.4), 0.1 M NaCl, 10 mM $CaCl_2$. At different times the film was compressed until a surface pressure of 20 dyn/cm was reached. Films were recovered and analysed for protein, phosphorus or acetylcholinesterase activity as described in Material and Methods.

Time ellapsed after spreading	Spreading yield of proteins (%)	μg protein/cm² at 20 dyn/cm	Specific activity acetylcholinesterase	μmol phosphorus/mg protein
0 [a]	–	–	1.8 [a]	0.85 ± 0.05 [a]
1	37.5 ± 2	0.145	1.4	1.0 ± 0.1
10	38 ± 2	0.125	0.74	
25	37 ± 2	0.100	0.54	0.95 ± 0.1
50	–	0.090	0.20	

[a] Values for native membranes.

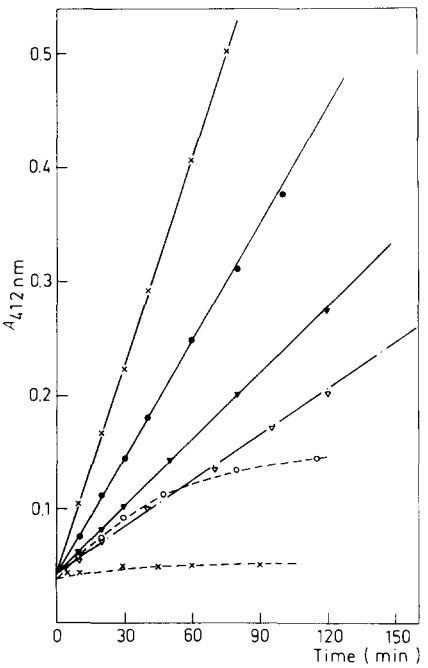


Fig. 4. Kinetics of hydrolysis of acetylthiocholine injected below erythrocyte membrane films. Membranes were spread at zero surface pressure. After 1 min, the films were compressed at different surface pressures. Acetylthiocholine was injected in the subphase. Hydrolysis was measured at 25°C in 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 10 mM $CaCl_2$. X-----X, 10 dyn/cm; ○———○, 13 dyn/cm; ▼———▼, 17 dyn/cm; ▽———▽, 20 dyn/cm; ●———●, 25 dyn/cm; X———X, 32 dyn/cm.

was recorded and acetylthiocholine hydrolysis measured.

When the films were compressed above 15 dyn/cm, linear kinetics were obtained. This indicates that no denaturation of acetylcholine esterase occurred above 15 dyn/cm. Surface pressure remained constant all along the time course of the experiments (not shown). Below 15 dyn/cm, the hydrolysis rate, given by the slope of the kinetics, decreased with time. Surface pressure increased with time indicating a film expansion. Lipoprotein film expansion at low surface pressure is a general phenomenon. Similar behaviour was observed with sarcoplasmic reticulum films. In the experiments described in the other sections of this paper, the spreading time was kept as short as possible (1 min). Then, the films were further compressed to a higher surface pressure than 15 dyn/cm in order to minimize interfacial denaturation of proteins.

Compression isotherm and enzymatic activities

The compression isotherms of films obtained by spreading four different types of membranes are presented in Fig. 5. These compression isotherms depend on the composition of the membrane used. *E. coli* and sarcoplasmic reticulum membranes are rich in proteins as compared to intestinal brush border or erythrocyte membranes. Protein surface density at a fixed surface pressure is much higher for the former and films are more compressible.

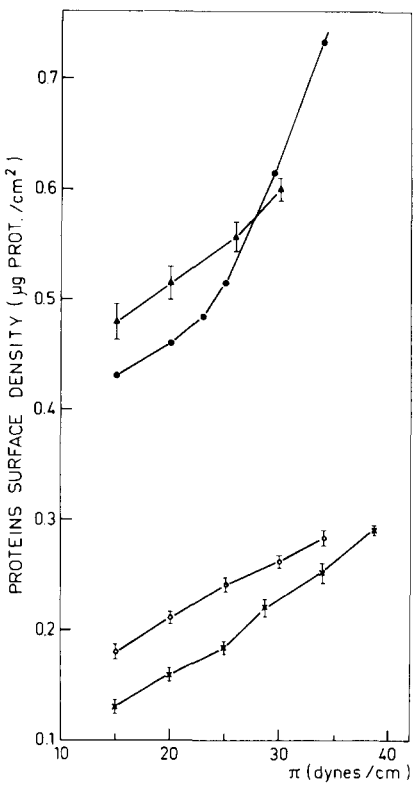


Fig. 5. Compression isotherms of pig intestinal brush border, human erythrocyte, *E. coli* cytoplasmic and rabbit sarcoplasmic reticulum membranes. The membranes were spread at zero surface pressure. After 1 min the films were compressed stepwise above 15 dyn/cm. ○———○, Intestinal brush border membranes; 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$. X———X, Erythrocytes membranes, 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 10 mM $CaCl_2$. △———△, *E. coli* cytoplasmic membranes, 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$. ●———●, Sarcoplasmic reticulum membranes, 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 5 mM $MgCl_2$.

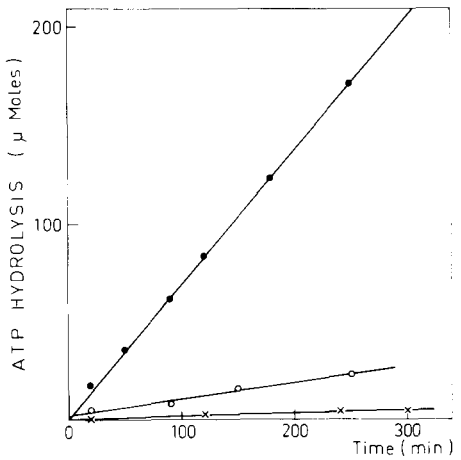


Fig. 6. Kinetics of hydrolysis of ATP injected below sarcoplasmic reticulum films at 35 dyn/cm, 37°C. •———•, 50 μM $CaCl_2$; ○———○, 1 mM EGTA. At time zero ATP (10 mM final concentration) was injected in the subphase. Inorganic phosphate was measured in 3 ml aliquots of the subphase. Blanks of soluble ATPase activity were measured on 3-ml aliquots kept at 37°C away from the film (X———X).

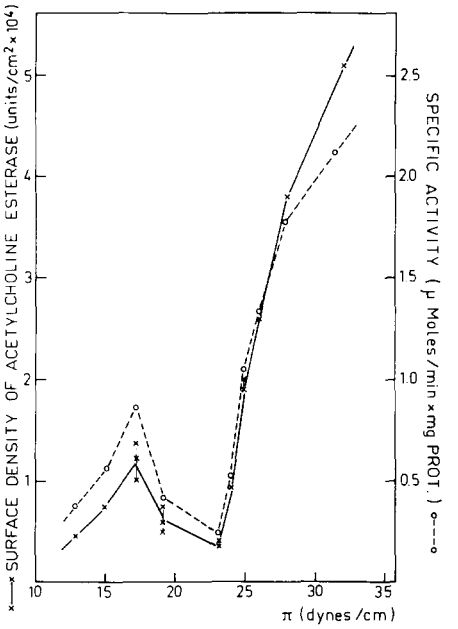


Fig. 7. Influence of surface pressure on the activity and specific activity of acetylcholinesterase in erythrocyte membrane films. Enzymatic activities were taken from the slopes of the kinetics shown in Fig. 4. The specific activity was calculated by dividing the surface density of acetylcholinesterase activity (units/cm^2) by the surface density of proteins at the same surface pressure (Fig. 5). 1 unit = 1 μmol of acetylthiocholine hydrolyzed per min. DTNB and acetylthiocholine did not affect surface pressure during the time course of the experiment. X———X, Surface density of acetylcholinesterase activity; ○- - - - -○, specific activity of acetylcholinesterase.

Fig. 6 shows the kinetics of ATP hydrolysis catalyzed by the Ca^{2+}-ATPase in films of sarcoplasmic reticulum. There is a linear release of mineral phosphate during the first 3 h of the reaction. As shown by blank of soluble activity there is no detectable release of the Ca^{2+}-ATPase during the time course of the experiment. The specific activity of the Ca^{2+}-ATPase, in the film, estimated from the kinetics of Fig. 6 and from the surface density of proteins (Fig. 5) (4.6 μmol ATP hydrolyzed per min per mg of protein) corresponds to about 30% of the specific activity of the Ca^{2+}-ATPase in the native membrane (12.5 μmol ATP hydrolyzed per min per mg protein). If however the film is recovered, the specific Ca^{2+}-ATPase activity is fully retained.

Fig. 7 shows the influence of surface pressure on the acetylcholinesterase activity of erythrocyte membrane films. The enzymatic activity was measured by injection of acetylthiocholine below films at different surface pressures. As show in Fig. 4, below 15 dyn/cm denaturation of proteins at the interface occurred. Enzymatic activities are not reliable. Above 15 dyn/cm, linear kinetics were obtained. There is no denaturation of the protein with time. There is a first optimum of activity at 17.5 dyn/cm. Then the activity decreases and is minimal at 23 dyn/cm. Above 23 dyn/cm the acetylcholinesterase activity is highly enhanced when surface pressure is increased. These minima and maxima are significant as compared to the experimental uncertainty. Identical behaviour was observed with different membrane preparations (freshly prepared or frozen ghost).

Film deposition on mica and freeze-fracture of bilayers formed from two membrane films

From previous studies [9,10] it was shown that liposomes, spread at zero surface pressure, give rise to monomolecular films. When intestinal brush-border membranes were spread at zero surface pressure, aminopeptidase was completely accessible towards its substrate or papain action.

This latter result suggests that membrane films obtained under these conditions were also true mono-

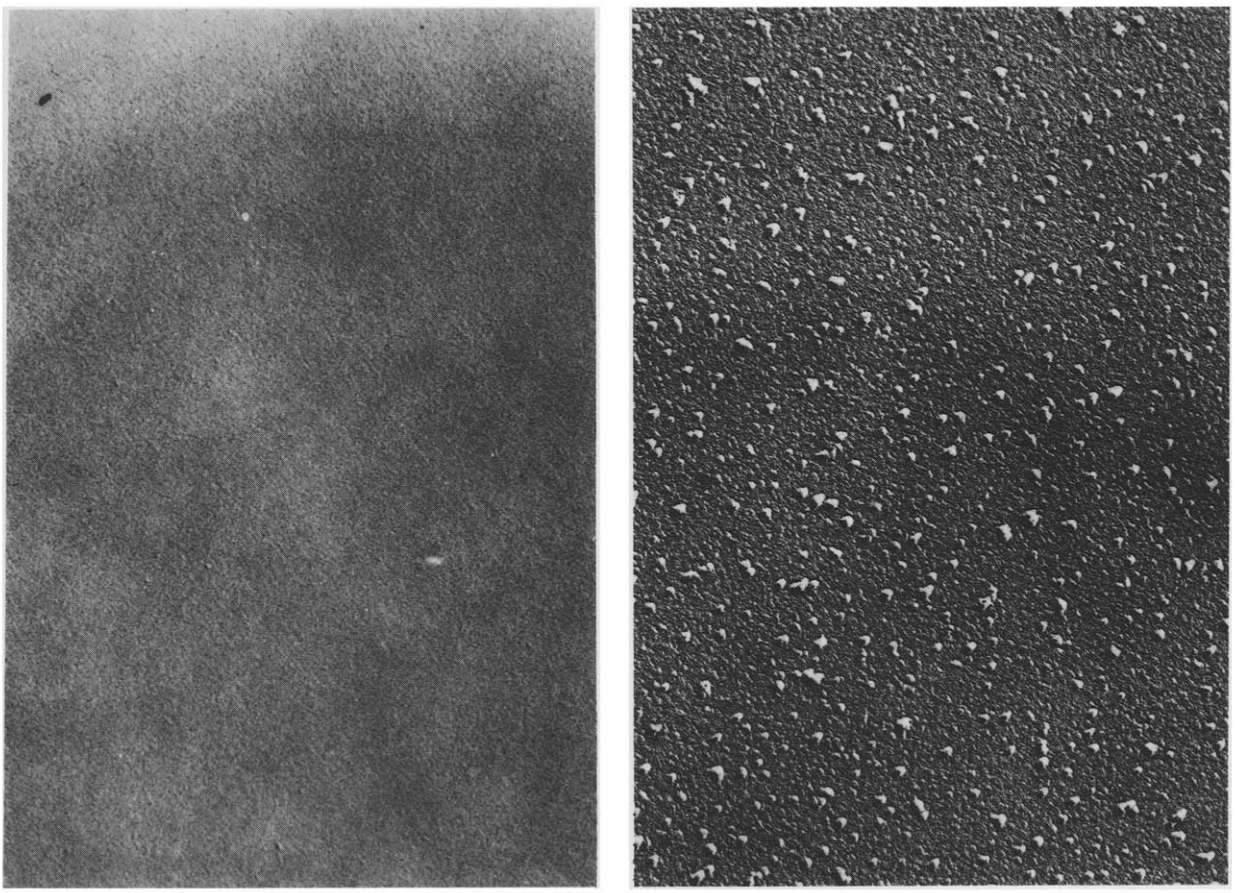


Fig. 8. Electron micrographs of freeze-fractured face of films deposited on mica. (A) Hydrophobic fractured face of a bilayer formed by deposition on mica of two monolayers from total lipid extract from erythrocyte. (B) Hydrophobic fractured face of a 'bilayer' formed by deposition of erythrocyte membrane films on mica. Erythrocyte were spread at zero surface pressure after 1 min the film was compressed untill 36 dyn/cm.

layers. However, the presence of bilayer patches at the air/water interface could not be excluded. To confirm the organization of membrane films we used the Blodgett technique [21] to prepare bilayers on freshly split mica (see Fig. 1 in Material and Methods). With lipoprotein films bilayers could be formed on mica only at a surface pressure higher than 35 dyn/cm. Successful deposition of the layers on mica was checked by measuring the surface pressure decrease during the deposition. This decrease was identical (within 10%) for both depositions. This surface pressure decrease was proportional to the surface of mica and close to the theoretical value expected from the compression isotherm of the films. The bilayer on the mica was then fractured and freeze etched and a carbon replica made as described in Material and Methods. Control experiments with mica alone, water, 30% glycerol gave identical results as found previously [23].

Fig. 8a shows the highly smooth and even surface of a phospholipid monolayer. Fig. 8b shows the hydrophobic face of a film obtained by spreading human erythrocyte ghosts at zero surface pressure. One can see only one fracture plane, no additional planes are visible indicating no bi or multi-layer patches are present. Particles are visible randomly distributed. The diameter is 8 to 10 nm comparable to the particle size found with the native red cell membrane.

Discussion

In previous studies [7–9], conditions for formation of lipoprotein films at the air/water interface, which preserve membrane bound enzymatic activities, were described. In the present report further description of the structure, composition of these films is presented.

The results from Table I and Figs. 2 and 3 indicate that the film composition is determined by the composition of the membrane. A slight difference in protein composition was observed. Loosely bound proteins are partially released into the subphase during spreading. The lipid composition remained unchanged. This indicates that both sides of the membrane spread with the same yield. If there was a preferential spreading of one of the membrane layers, one should expect a change in the lipid composition after spreading since lipids are asymmetrically distributed.

As a consequence, compression isotherms of membrane films are highly dependent on the lipid protein ratio of the membrane used (Fig. 5). Membrane rich in proteins give films with high protein surface densities and with a high compressibility.

In contrast with liposomes [8] the spreading process itself is fast. After 1 min the film composition is already established. A low spreading surface pressure is required to form unilamellar films [8,9]. However at this low pressure a second but slow process occurs. The film slowly expands with time followed by an irreversible loss of enzymatic activities (Table II). The high interfacial energy may unfold proteins at the interface producing film expansion. This is not too surprising. Unfolding of proteins in absence or presence of lipids at the air/water interface is well documented [22,18]. Completely unfolded proteins generally cover a surface of 1 mg/m^2 at 10 dyn/cm. From the compression isotherms of membrane films (Fig. 5), one can expect a film expansion factor as high as 5 between native and completely unfolded films. By compressing the films, surface pressure increases and surface energy decreases. Experimentally we found that a surface pressure of 15 dyn/cm or higher prevents further inactivation. 15 dyn/cm is also the surface pressure where denaturation of lipolytic enzymes became negligible [18].

The intestinal aminopeptidase, *E. coli* NADH oxidase activities measured in a previous study [9] and acetylcholinesterase activity (Figs. 4, 6) belong to non-spanning proteins. It is expected that these enzymes remain active at the air/water interface. More interesting is the activation by Ca^{2+} of the ATPase from sarcoplasmic reticulum films since the protein spans the membrane.

However Ca^{2+}-ATPase activity is three times lower at the air/water interface, at 35 dyn/cm, than in the membrane. After film recovery, bilayer structures are formed again (Pattus, F., unpublished data). The Ca^{2+}-ATPase activity is fully restored. One can explain these results by several ways. Either Ca^{2+}-ATPase needs the presence of a bilayer to express its full activity, or 35 dyn/cm is not the optimal surface pressure for the activity of the enzyme. One other possibility is that only part of the ATP binding sites are exposed towards the subphase.

Nevertheless, these results show that a spanning protein could keep at least part of its biological activity at the air/water interface. Acetylcholine receptor function has been reconstituted in planar bilayers from two membrane films at the air/water interface [29]. In this case too, the native conformation of a spanning protein was not irreversibly lost through the 'monolayer' state and could be reinduced when films were transformed into bilayers.

In 1959 Skou [24] studied the influence of surface pressure on the *Torpedo* acetylcholinesterase activity in monolayers. With acetylcholinesterase from red cells we found a first optimum in a similar range of surface pressure as Skou's report (Fig. 7). We also found that at high surface pressure acetylcholinesterase activity increases drastically. This behaviour is in contrast with the interfacial properties of aminopeptidase and alkaline phosphatase from films of intestinal brush border. These activities were found independent on surface pressure [8]. These first optimum and minimum of the activity profile (Fig. 7) are difficult to explain since many components are present in the film. A study with the pure enzyme reconstituted with lipids will be necessary. This is not the purpose of the present report. Recently, Ott and Brodbeck [25] showed that acetylcholinesterase solubilized in detergent is in equilibrium between monomers and dimers with different specific activities. The shape of the activity curve as a function of the surface pressure could be a consequence of this monomer-dimer equilibrium. The steep part of the

curve above 25 dyn/cm could be explained by a better interaction of lipids with acetylcholinesterase at higher lipid packing. One should stress that the specific activity in the film at a surface pressure of 30 dyn/cm is equivalent to the specific activity of the enzyme in the erythrocyte membrane. This surface pressure has been proposed as 'equivalent' surface pressure of the outer layer of human erythrocytes [26].

Recently Korenbrot and Pramik [27] used a similar spreading technique to form films containing cattle rhodopsin at the air/water interface. However these authors claimed that an osmotic shock was necessary to disrupt membrane vesicles and obtain surface films. The rhodopsin complex was spread on a subphase with no salt. Surface pressure was not measured during spreading and films were not transferred on a clean subphase.

In contrast to these authors, this osmotic shock was found unvaforable with each type of membrane we used. In our hand, at low ionic strength, very poor spreading yields and irreproducible results were obtained. 0.1 M salt or highest concentration were necessary to obtain good spreading yields. From the data of Korenbrot and Pramik [27], we can calculate that the spreading yield of egg phosphatidyl liposomes by the 'osmotic lysis' method was around 1% (75 ± 10% in our case).

From electron microscope studies of films containing rhodopsin after deposition on a solid support, Korenbrot and Pramik [27] concluded that the protein molecules are organized in membrane fragments (probably bilayers) coexisting with lipids in a monolayer state. However restructuration of the film after deposition on mica cannot be excluded [28] especially if the film was dried on the mica plate. In our study the hydrophobic face seen in Fig. 8B corresponds to a film which stays in the presence of water until freezing (see Material and Methods). This is in favour of the similarity of the structure visualized by electron microscopy with the structure of the films at the air/water interface. This freeze-fracture face of human erythrocytes membrane film suggest that during spreading at zero surface pressure, erythrocyte membranes, as lipid vesicles [9], form a true monolayer without membrane patches. If the particles seen in the electron micrograph can be attributed to the proteins, these are randomly distributed all over the surface.

The differences between the data of Korenbrot and Pramik [27] data and ours can be due to the large differences in the spreading conditions. An alternative explanation may be that the peculiar structure of rhodopsin (highly integrated in the bilayer) could also stabilize bilayer structures at the air/water interface. This is not the case with erythrocyte membranes containing many components with large hydrophilic segments (glycophorin for example).

The spreading at 'zero surface pressure' of membranes or reconstituted membrane complexes give well characterized films which reflect the composition of the membrane used. When a short period of time elapsed between spreading and compression of the films to a surface pressure higher than 15 dyn/cm asymmetrically distributed marker enzymes as well as spanning proteins keep their biological activities.

Although we cannot rule out completely the possible presence of bilayer patches in such membrane films, our previous results [8,9] and the ones presented in this report strongly suggest that membrane films formed at 'zero surface pressure' are indeed monolayers.

Bilayer formation from such membrane films as developped by Schindler's group [12,29] is a very attractive extension to study transport mechanisms.

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