Biochimica et Biophysica Acta, 507 (1978) 71—82

BBA 77911

SPREADING OF BIOMEMBRANES AT THE AIR/WATER INTERFACE *

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Summary

This paper presents the compression isotherms obtained by spreading membranes of intestinal brush border, human erythrocyte and *Escherichia coli* (cytoplasmic) at the air/water interface. Unilamellar membrane films were formed, with a good yield, at zero surface pressure, whereas multilamellar structures were formed at high surface pressure. Once formed, the films were particularly stable and could be manipulated without any detectable loss. With doubly-labelled *E. coli* cytoplasmic membrane, we could show that phospholipids and proteins spread, with the same yield, as a single unit. Moreover, we studied the influence of hydrolytic enzymes, chemical agents and cations on the compression isotherm of biomembranes. The resultant changes in architecture of membrane films can provide a very simple method of studying the influence of membrane packing on catalytic activity and protein conformation of membrane-bound proteins.

Introduction

In recent years, many studies had been devoted to the reconstitution of membrane complexes by reincorporation of membranous proteins into organized lipid structures. These investigations were mainly based on the use of the liposomes as membrane model. This is due to the experimental simplicity and the possibilities of transport studies offered by the liposomes. Generally speaking, membranous proteins are amphipatic molecules, insoluble in water, which are 'solubilized' by organic solvents or detergent solutions. These solubility properties probably explain why relatively few studies of membranous proteins have been performed with the monolayer technique. Nevertheless, the monolayer system is the only one which allows a continuous

* This work is part of the "thèse de Docteur-Ingénieur" of F. Pattus, obtained at the Provence University, October 1976.

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and controlled variation of the 'quality of the interface' [1], and the latter was shown to be of prime importance for the recognition of lipid interfaces by lipolytic enzymes [2].

It is also probable that the 'quality' of the lipid matrix of a membrane plays a role in the regulation of the activity of membrane-bound enzymes. In order to tackle this problem we developed a new technique for spreading membranes at the air/water interface [3]. This technique was also used for a quantitative investigation of the interactions of protein with lipid [4]. After investigating, in the preceding paper, the mechanism of formation and the structure of films obtained by spreading liposomes [5], we studied the compression isotherms of films obtained by spreading membranes of intestinal brush border, *Escherichia coli* and erythrocytes at the air/water interface. An additional advantage, in the complicated case of natural membranes, is that the spreading technique does not use denaturating agents and very small amounts of material give unique information on the compression isotherms of a biomembrane. Furthermore, the changes that occur in architecture of membranes during spreading can provide a very simple method to study the influence of lipid packing on catalytic activity of membrane-bound enzymes. With doubly-labelled *E. coli* cytoplasmic membrane, we could show that phospholipids and membrane proteins spread as a single unit. Moreover, we studied the influence of hydrolytic enzymes, chemical agents and cations on the compression isotherms of biomembranes.

Materials and Methods

Intestinal brush border membrane

Previously published techniques were employed for the preparation of vesicles from pig jejunal brush border [6] and for the preparation of papain-treated vesicles [7]. After papain treatment, 90% of the disaccharidases, 86% of the aminopeptidase and 52% of the alkaline phosphatase activities of the brush border membrane were released in solution. Aminopeptidase and alkaline phosphatase activities were measured spectrophotometrically using, respectively, L-alanine-*p*-nitroanilide and *p*-nitrophenyl phosphate as substrate [6]. For very low aminopeptidase activities, the estimation of the *p*-nitroaniline released is based on diazotation of the reaction product [8] and absorbance measurements at 550 nm (ϵ_M = 24 700). Proteins were estimated according to Lowry et al. [9] or by their intrinsic fluorescence. The brush border vesicles used had the following characteristics: protein, 13 mg/ml; specific activity of aminopeptidase, 1360 U/mg; intravesicular volume, 1.5 μl/mg protein; lipid to protein ratio, 0.21; surface density of the aminopeptidase on the vesicles, 1.6 units/cm^2.

Double-labelled inner (cytoplasmic) and outer membranes from E. coli

E. coli CR 34 cultures (500 ml) were grown at 37°C in minimum salt medium 63 [10] supplemented with 0.5% casamino acids, thymine (50 μg/ml) and vitamin B1 (5 μg/ml). Glucose, 0.4%, was used as a carbon and energy source. An overnight culture was diluted 50 times and the cells were grown for 5—6 generations up to an absorbance of 0.1 at 600 nm. Then, carrier-free

[^{32}P]-orthophosphate (CEA France; final radioactivity, 4 μCi/ml) and ^{14}C-labelled casamino acids (final radioactivity, 0.3 μCi/ml) were added to the exponentially growing bacteria suspension. The cells were harvested when the absorbance at 600 nm reached 0.7.

Both types of membrane were prepared by first washing the cells twice in a 10 mM phosphate buffer (pH 7.4) 0.15 M NaCl and resuspending them in 50 ml of deionized iced water. Then, 5 ml of 100 mM Tris buffer (pH 7), 18 ml of 2 M sucrose and 2.5 ml of 1% EDTA were successively added. After addition of 1.25 mg of egg-white lysozyme (Sigma), the spheroplast formation was followed by the turbidity decrease. At the plateau value (after 10 min), $MgCl_2$ was added (final concentration, 1 mM). This suspension was centrifuged at 19 000 $\times g$. The pellet was resuspended in 50 ml of cold water in the presence of 1 μg/ml of DNAase and 1 mM $MgCl_2$. This procedure promoted the lysis of spheroplasts; after centrifugation, the enriched cytoplasmic membrane fraction was obtained. This fraction was further purified on sucrose gradient [11]. Two fractions were obtained; a major one of cytoplasmic membrane and a minor one of outer membrane. The supernatant of the first 19 000 $\times g$ centrifugation, containing the external membrane, was acidified to pH 5 to cause aggregation according to Wolf-Wartz et al. [12]. After recovery by centrifugation, the external membranes were washed in phosphate buffered saline and purified on discontinuous sucrose gradient as described by Osborn et al. [11]. The densities of the cytoplasmic and outer membrane were respectively 1.14 and 1.21. The NADH oxydase activity measured according to Osborn et al. [11] was found to be exclusively associated to the cytoplasmic membrane. Some characteristics of the *E. coli* membranes thus obtained are listed in Table I.

Human erythrocyte membrane

The human erythrocytes were labelled with [^{125}I]iodine according to Phillips and Morrisson [13]. Erythrocyte ghosts were prepared and their hemoglobin content was determined according to Dodge et al. [14]. More than 90% of the radioactive iodine was washed away during ghost preparation. The ghost suspension contained 6.3 mg of protein/ml with a specific radioactivity of 1920 cpm/mg protein.

TABLE I

SOME CHARACTERISTICS OF THE *E. COLI* MEMBRANES USED

Membrane type	Protein concentration (mg/ml)	NADH oxydase, specific activity (U/mg protein)	Protein specific radioactivity $\times 10^{-5}$ (^{14}C cpm/mg protein)	Lipid specific radioactivity $\times 10^{-5}$ (^{32}P cpm/mg lipids)
Cytoplasmic (inner) membrane	7.7	0.9	1.9	3.2
Outer membrane prepared on sucrose gradient	0.8	0.1	0.9	1.0
Outer membrane aggregated at pH 5	0.2	0.0	1.4	1.4

Spreading technique

The spreading technique used was the same as previously described [3], except for the fact that surface pressure was measured with a Wilhelmy plate instead of the Langmuir method.

Results

1. Compression isotherms of various biomembranes

A. Spreading at zero surface pressure. Erythrocytes and brush border membranes spread with comparable yields (33—37% and 25—30%, respectively). *E. coli* cytoplasmic membranes spread with a higher yield than the other membranes. When *E. coli* cytoplasmic membranes, containing ^{32}P-labelled phospholipids and ^{14}C-labelled proteins, were spread, similar proportions of the two radioactive labels (56—60%) were recovered from the film. This result indicates that the protein/lipid ratio in the surface film is the same as that in the native membrane and, consequently, that there is no preferential spreading of proteins or lipids. The protein to lipid ratio is higher in the *E. coli* outer membrane than in the inner membrane. After spreading the outer membrane prepared on a sucrose gradient, we obtained a stable film (spreading yield, 35—45%) having the same lipid to protein ratio as the native membranes. If the same membrane type was prepared by aggregation at pH 5, the film obtained was now more expanded. This difference may be due to the denaturation of some proteins during the acidic treatment.

Fig. 1 shows the compression isotherms of membranes of intestinal brush border, *E. coli* (cytoplasmic) and human erythrocytes spread at zero surface

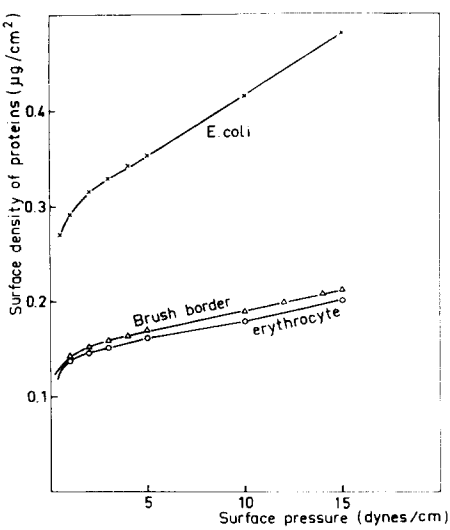

Fig. 1. Compression isotherms of intestinal brush border, human erythrocyte and *E. coli* cytoplasmic membranes. The membranes were spread at zero surface pressure and 15 min later compressed stepwise up to 15 dynes/cm and then collected. △———△, Intestinal brush border membrane spread over Tris · HCl buffer 10 mM (pH 7.5) NaCl 0.1 M, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM; X———X, cytoplasmic membrane from *E. coli* spread over Tris · HCl buffer 10 mM (pH 7.5) NaCl 0.1 M, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM; ○———○, human erythrocyte membrane spread over Tris · HCl buffer 10 mM (pH 7.5) NaCl 0.15 M. 20—50 μg of membrane proteins were spread over 700 cm^2.

pressure and compressed stepwise up to 15 dynes/cm. The films obtained are stable on a stirred subphase at pH 7.5 containing NaCl, 0.1 M. Erythrocytes and brush border membranes give similar isotherms, whereas *E. coli* cytoplasmic membrane spread as a more condensed and more compressible film. In contrast with the simpler case of liposomes [5], when biomembranes are spread at the air/water interface at zero surface pressure it is difficult to demonstrate that monomolecular films are formed. This important point will be discussed later on.

The enzyme activities of the spread membranes were either measured by collection of the film followed by a standard assay or by injecting the substrate under the film compressed at 15 dynes/cm. The specific activities of brush border aminopeptidase and alkaline phosphatase measured by both techniques were 80% of the values found with native vesicles [3]. In contrast, NADH oxydase activity, measured by both techniques, was no more than 44—60% of the activity measured with native *E. coli* cytoplasmic membranes. Linear kinetics were obtained during 1 h when NADH oxidase activity was measured by injecting NADH under a film of *E. coli* cytoplasmic membranes spread at zero surface pressure and further compressed to 15 dynes/cm. No dissolution of the enzyme in the subphase occurred during the time of the experiment.

It was of interest to determine if intact erythrocytes could spread at the air/water interface and to compare the compression isotherm with the one obtained from erythrocyte ghosts. Nicolson and Singer reported the lysis of erythrocytes when a cell suspension was dropped on a surface of distilled water [15]. We spread directly intact ^{125}I-labelled human erythrocyte cells at the air/water interface on an isotonic buffer (NaCl, 0.15 M) and obtained stable films containing undetectable amounts of hemoglobin. This indicates a complete hemolysis of the cells at the air/water interface under isotonic conditions. The surface radioactivity measured after spreading ^{125}I-labelled erythrocytes was half the value found by spreading the corresponding ghosts. This difference suggests that unlabelled proteins, which were eliminated during the ghost preparation, were still bound to the film obtained by spreading erythrocyte cells.

B. Spreading at constant surface pressure. We previously reported that when increasing amounts of liposomes were spread at the air/water interface, the relative amount of lipid recovered from the surface decreased and that multilayered structures were formed [5]. Similarly, the results in Table II show that the spreading yield decreases and the surface density of proteins increases, when membranes are spread at higher surface pressures. The specific activities of brush border aminopeptidase and alkaline phosphatase in the recovered film were the same as in the native membrane, whereas the specific activity of NADH oxydase increased gradually with the spreading surface pressure. These results indicate that the NADH oxydase activity, but not the other enzymes, may be influenced by its lipid environment or that the oxydase is more susceptible to surface inactivation at low pressure as found for lipolytic enzymes [16].

2. Influence of extrinsic proteins on the compression isotherm of intestinal brush border membrane

A. Spreading at zero surface pressure. It has recently been shown in our

TABLE II

INFLUENCE OF THE SPREADING SURFACE PRESSURE ON THE SPREADING YIELDS, PROTEIN SURFACE DENSITIES AND SPECIFIC ENZYME ACTIVITIES OF INTESTINAL BRUSH BORDER, CYTOPLASMIC *E. COLI* AND HUMAN ERYTHROCYTE MEMBRANES

The membranes were spread at various surface pressures and 15 min later further compressed to the fixed value of 15 dynes/cm and then collected at this pressure. The other experimental conditions are the same as described in Fig. 1. The spreading yields were very low for intestinal brush border vesicles, as well as for liposomes when spread over a low ionic strength buffer (Tris · HCl 10 mM, pH 7.5, sucrose 0.2 M). Furthermore, the specific activity of the aminopeptidase measured after collecting the surface film was 2 times higher than that of intact membranes. This result suggests that during the spreading of intestinal brush border vesicles on a buffer at low ionic strength, the internal fibers could be depolymerized and solubilized in the water phase.

Spreading surface pressure (dynes/cm)	Intestinal brush border membrane			*E. coli* cytoplasmic membrane			Human erythrocyte membrane	
	Spreading yield (%)	Protein surface density ($\mu g/cm^2$)	Aminopeptidase or alkaline phosphatase, specific activities (% of native)	Spreading yield (%)	Protein surface density ($\mu g/cm^2$)	NADH oxydase specific activity (% of native)	Spreading yield (%)	Protein surface density ($\mu g/cm^2$)
0	27	—	80	58	—	50	35	—
5	17	0.23	100	32	0.42	66	—	—
10	13	0.31	100	—	—	—	19	0.86
15	11	0.38	100	19	0.80	89	—	—

laboratory that the intestinal brush border aminopeptidase is anchored to the lipid bilayer by a short hydrophobic domain [17]. Upon treatment of the membrane with papain, the catalytic unit of the enzyme is solubilized without significant specific activity decrease. This characteristic, common to other brush border hydrolases, strongly suggests that the catalytic activity of this class of membrane-immobilized enzymes is not dependent on the lipid environment of the membrane matrix. The membrane-bound hydrolases, released by proteolytic enzymes, represent 50% of the total proteins of the vesicles. This behaviour prompted us to choose brush border membrane vesicles to characterize enzymatically lipoprotein films spread at the air/water interface. Native vesicles and papain-treated vesicles prepared from brush border spread at the air/water interface with the same yield (23%). The compression isotherms (lipid density as function of surface pressure) of both types of membrane are identical. This result shows that the catalytic units of the brush border hydrolases do not affect the lipid surface density of the film.

When snake venom phospholipase A_2 (final concentration, 7.7 μg/ml) was injected under a brush border film, we observed no change in surface pressure and no solubilization of the brush border hydrolases. In contrast, when papain or trypsin (final concentration, 7.7 μg/ml) was injected under the film, a rapid release of the aminopeptidase activity into the aqueous subphase could be observed without any change in surface pressure. This fact confirms the previous observation that the catalytic part of the brush border hydrolases do not affect the surface packing of the film. A point of great interest is that trypsin, in contrast to papain, does not solubilize the brush border hydrolases from the native membrane vesicles [7] but does so after spreading. This situation is illustrated by Fig. 2. Even after compression up to 35 dynes/cm, we could not find a critical surface pressure above which trypsin was no longer able to solubilize the aminopeptidase from the film.

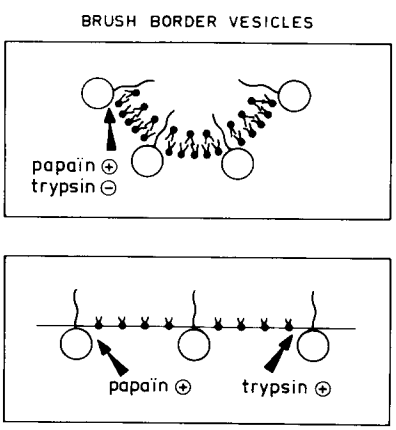

Fig. 2. Schematic representation indicating the asymmetrical distribution and the accessibility to papain and trypsin of the intestinal brush border hydrolases. The upper drawing illustrates the conclusions reached by Louvard and Maroux using the brush border vesicles [17]. The lower drawing is a hypothetical view of the film obtained by spreading the brush border membrane at the air/water interface.

B. Spreading at constant surface pressure. Fig. 3 shows the surface density of the aminopeptidase activity at different spreading pressures. The enzyme activity was measured in 3 different ways: (a) by collecting the film followed by a standard assay on the substrate alanine *p*-nitroanilide [6], (b) by injecting the substrate under the film compressed at a fixed surface pressure (15 dynes/cm) [3], (c) by measuring by the same technique as above [6] the aminopeptidase activity solubilized after papain injection. When the aminopeptidase activity was measured after film recovery, the enzyme surface density increased continuously with the spreading surface pressure. In contrast, the measurable aminopeptidase activity after substrate or trypsin injection was optimal around 2 dynes/cm, decreased with the spreading pressure and became negligible at 10 dynes/cm and above. This behaviour can be interpreted, as in the case of model membranes [5], in terms of multilayer formation, at higher pressure, inducing a decrease in enzyme accessibility. Nevertheless, this aminopeptidase accessibility was found higher for its substrate (aniline *p*-nitroanilide) than for papain.

3. Influence of the protein conformation on the compression isotherms of biomembranes

A. Effect of cysteine on intestinal brush border films. Under bulk conditions at pH 7.4 the soluble or membrane-bound aminopeptidase was readily inhibited by 1 mM cysteine. The inhibition was fully reversed by lowering the pH to 6.5. Cysteine could not be substituted by other reducing agents such as

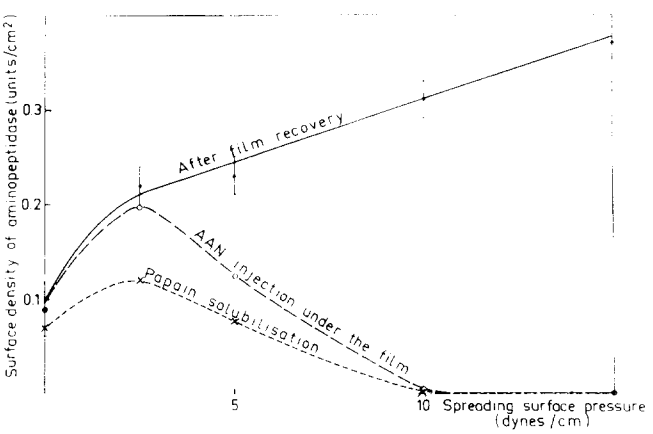

Fig. 3. Compression isotherms, obtained after spreading at various surface pressures and 15 min later further compression to 15 dynes/cm, of intestinal brush border vesicles. Subphase, phosphate buffer 10 mM (pH 6.2) NaCl 0.1 M, cysteine 1 mM. •———•, Aminopeptidase units measured after recovering the surface film by suction with a simple device described previously [16]. The aminopeptidase assay used is identical with the one described previously [6], except for the concentration of alanine *p*-nitroanilide (AAN) which here is 10 times lower (0.3 mM). The estimation of the *p*-nitroaniline released is based on diazoique coupling [8] and absorbance measurements at 550 nm (ϵ_M = 24 700). ○------○, After spreading and further compression to 15 dynes/cm, the film was rinsed and transferred [3]. At zero time, the substrate (AAN) was injected into the stirred subphase (final concentration, 0.3 mM) and the aminopeptidase activity of the membrane film measured as described [3]. X------X, Aminopeptidase activity released in the subphase 45 min after injection of a papain solution (final concentration, 7.7 μg/ml) under a membrane film. The aminopeptidase activity released, measured by sampling (1 ml) the subphase each 10 min, reached a plateau 40 min after the papain injection.

dithiothreitol and mercaptoethanol, or by the *S*-methyl derivative of cysteine.

When brush border vesicles were spread at zero surface pressure over 1 mM cysteine, we observed a complete and irreversible loss of the aminopeptidase activity. As under bulk conditions, this inhibition was specific for the cysteine molecule. Fig. 4 shows the compression isotherms of the films obtained by spreading native or papain-treated brush border vesicles at zero surface pressure over a subphase at pH 7.4, in presence or absence of cysteine. As mentioned before, the compression isotherms of both types of vesicles were similar on a subphase devoid of cysteine. In contrast, when spread over 1 mM cysteine, the native vesicles yielded a film 1.4 times more expanded and 1.7 times less compressible than a subphase without cysteine. With papain-treated vesicles, the film obtained in the presence of cysteine had an intermediate behaviour (1.2 times more expanded and 1.4 times less compressible). Injection of cysteine at pH 7.2 increased immediately the surface pressure of an intestinal brush border film from 15 to 22 dynes/cm (Fig. 5). When the pH was dropped to 6.0, we observed a decrease in surface pressure. We checked, in a control experiment, that the injection of cysteine (1 mM final concentration) at pH 6.2 had no influence on the surface pressure of brush border lipids or egg phosphatidylcholine films.

B. Effect of divalent cations on the compression isotherm of E. coli cytoplasmic membrane. When doubly-labelled, *E. coli* cytoplasmic membranes were spread at zero surface pressure over a solution containing EDTA (1 mM) and no added divalent cations, we recovered from the surface 40% of the lipids and 40% of the proteins. The compression isotherm was 3.4 times more expanded and 3.5 times less compressible than that obtained in the presence of Ca^{2+} and

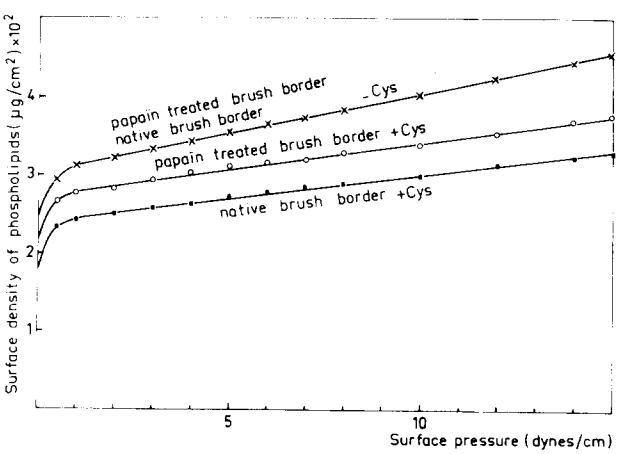

Fig. 4. Effect of cysteine on the compression isotherms of native or papain-treated intestinal brush border vesicles spread at zero surface pressure and compressed stepwise up to 15 dynes/cm. Subphase, Tris · HCl buffer 10 mM (pH 7.4) NaCl 0.1 M with or without cysteine, 1 mM. In order to estimate the amount of material present at the air/water interface, we recovered the surface film by suction with a simple device described previously [16]. Aliquots of the recovered film were tested for aminopeptidase, phospholipid and protein content. X———X, Compression isotherm of native or papain-treated vesicles in the absence of cysteine; ■———■, compression isotherm of native vesicles in the presence of cysteine, 1 mM; ○———○, compression isotherm of papain-treated vesicles in the presence of cysteine, 1 mM.

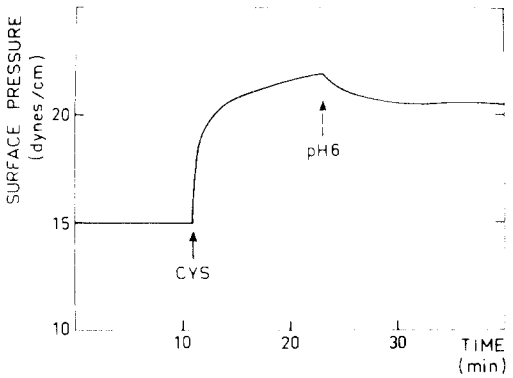

Fig. 5. Variation with time of the surface pressure of an intestinal brush border film spread at zero surface pressure and 15 min later further compressed up to 15 dynes/cm. Subphase, 130 ml of Tris · HCl buffer 10 mM (pH 7.2) NaCl 0.1 M. ←———, Injection of 0.5 ml of a 1 M cysteine solution; ←- - - - -, injection of 5 ml of a 1 M phosphate buffer (pH 6). After injection, the final pH of the subphase was 6.2.

Mg^{2+}. Furthermore, a total loss of NADH oxydase activity was observed in the absence of divalent cations.

Discussion

In a previous report [5], we observed that liposomes spread at zero surface pressure released their internal content and formed a monomolecular film at the air/water interface. In contrast, when spreading was performed against a given surface pressure, the liposomes retained a part of their internal content at the air/water interface, indicating the formation of multilamellar structures. In this respect, the biomembranes used during the present study behave similarly.

When we spread intestinal brush border vesicles at zero surface pressure, we obtained, with a good yield, well characterized films which retained their hydrolytic activities. The unilamellar structure of the films obtained is indicated by the complete accessibility of the aminopeptidase to its substrate and to proteolytic enzymes. Furthermore, no change in surface pressure of these films was observed during the solubilization of the membrane-bound hydrolases by the proteolytic treatment. This indicates that the catalytic units of the hydrolases, released by trypsin and papain treatment, had no effect on the surface packing of the membranous matrix. This conclusion is in agreement with the fact that native or papain-treated vesicles gave films having identical compression isotherms. The fact that trypsin could solubilize the aminopeptidase from the brush border films and not from the native vesicles could be explained in 2 ways: either the splitting bond in the aminopeptidase molecule is unmasked to the active site of trypsin during the spreading process due to a conformational change of the membrane bound aminopeptidase, or the surface packing of the membrane matrix in the native vesicles is higher than the packing obtained with a membrane film compressed at 35 dynes/cm. It could be possible, by analogy with the work of Demel et al. [18], to attribute a surface pressure higher than 35 dynes/cm to the brush border membrane. Furthermore, we observed that the surface density of the proteins was 4 times higher

in the native vesicles as compared with a membrane film spread at 0 dynes/cm and compressed up to 35 dynes/cm. This probably indicates that the bilayer of the membrane was transformed during the spreading process into an unilamellar structure with all the proteins of the native membrane facing the aqueous water subphase. It is also probable that during the spreading process part of the membrane proteins unfold and occupy a larger area at the interface.

The presence of cysteine at pH 7.4, but not at pH 6, induces two simultaneous effects: a total and irreversible loss of aminopeptidase activity and a large expansion of the membrane film. These effects could be attributed to the specific reduction of a disulfide bridge in the aminopeptidase molecule. The reduced enzyme may have simultaneously lost its catalytic activity and acquired a modified non-covalent structure inducing film expansion. Furthermore, proteins other than those released by papain could unfold on a cysteine solution (see Fig. 4). It is well known in surface biochemistry that protein unfolding at the air/water interface produces more expanded and less compressible films [19]. This surface denaturation induced by cysteine can only be observed at alkaline pH and, therefore, it cannot explain the papain effect on the native vesicles in the presence of cysteine at pH 6.

The multilamellar structure of the films obtained by spreading brush border membranes against increasing surface pressures was indicated by the decreasing accessibility of the membrane-bound aminopeptidase to its substrate and to the active site of papain (see Fig. 3). By spreading this membrane at 10 dynes/cm or a higher surface pressure, almost no enzyme activity could be detected by injecting substrate or papain below the membrane film, whereas after film recovery the entire enzyme activity could be measured. Furthermore, the membrane films obtained at high surface pressure were metastable. When these membrane films were expanded to a large area, in order to reach zero surface pressure value, the films obtained had all the characteristics of films spread at zero surface pressure. During this process, no release of aminopeptidase was observed.

Mg^{2+} deficiency in the gram-negative bacterium *E. coli* has been reported to cause the formation of filaments and changes in the permeability of the cell membrane [20]. During prolonged Mg^{2+} starvation, the plasma membrane proliferates and infolds near the ends of the cell [21—23]. The influence of Mg^{2+} and Ca^{2+} on the compression isotherms of films from *E. coli* cytoplasmic membranes is a good example of the effects of these cations on the physicochemical properties of a membrane. In the absence of cations, the isotherms were 3.4 times more expanded and 3.5 times less compressible than in presence of Mg^{2+} and Ca^{2+}. Part of this effect could be attributed to the well known condensing effect of the divalent cations on the packing of acidic phospholipid films [24]. However, a 1.2 expansion factor could be expected to result from the negatively charged phospholipid constituting the cytoplasmic *E. coli* membrane. It is very likely that the divalent cations stabilize the protein conformation in the membrane. The unfolding of these proteins in the absence of cations affects deeply the surface pressure and the enzyme activities bound to the membrane film. For instance, the NADH oxidase, which does not require cations for full catalytic activity, was totally and irreversibly inactivated after spreading the cytoplasmic *E. coli* membrane over a subphase free of Mg^{2+} and

Ca^{2+}. The effect of Mg^{2+} and Ca^{2+} on the packing of a membrane could explain the regulation exerted by these ions on the adsorption of periplasmic proteins on the cytoplasmic *E. coli* membrane. Preliminary experiments indicate that the ^{125}I-labelled aminopeptidase from *E. coli* adsorbs preferentially on cytoplasmic membrane films in the presence of Mg^{2+} and Ca^{2+}. Furthermore, in the presence of divalent cations, only 50% of the NADH oxydase activity was recovered from the surface after spreading cytoplasmic *E. coli* membrane at zero surface pressure, whereas 89% was recovered by spreading at 15 dynes/cm. This result shows that, in contrast with the intestinal brush border aminopeptidase, the NADH oxidase is influenced by the packing of its membranous environment.

The spreading of membranes, or cells, at the air/water interface could be a simple method to study the influence of the membrane packing on the biological functions of these membranes. On one hand, if there is a preferential peeling of the outer layer, or surface patches, during the spreading process, as found in the case of model membranes [5], it will be of interest to study the asymmetrical distribution of the membraneous components. On the other hand, it is very attractive to imagine the formation of a reconstituted bilayer from two membrane films previously spread at the air/water interface.

Acknowledgments

The authors thank Dr. C. Bovier-Lapierre (Centre National de Transfusion Sanguine, Marseille) for his generous gift of human erythrocytes. Thanks are due to Mrs. M.Th. Nicolas for typing the manuscript and Dr. R.O. Scow (N.I.H. Bethesda, U.S.A.) for correcting the manuscript.

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