PROTEIN PENETRATION AS A TOOL FOR THE INVESTIGATION OF LANGMUIR FILMS DERIVED FROM ERYTHROCYTES

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1. Introduction

Study of lipid bilayers by such techniques as the formation of black lipid membranes shows that these structures have a low mechanical strength and stability as compared with naturally occurring biomembranes. Nevertheless it is generally believed that the major structural components of membranes are the phospholipids and that the proteins which reside in them largely consist of enzymes. This experiment was designed to test the hypothesis that there exists a non-lipid component in some membranes which makes a considerable contribution to their mechanical coherence and stability. It was decided to study erythrocyte membranes as there exist well-known techniques for their isolation and for the removal of extrinsic proteins, such as spectrin, which are attached to them. We used such purified membranes as a starting point for the preparation of Langmuir films. We then injected water-soluble proteins beneath these layers and studied the effects of protein penetration on their isotherms. The behaviour of these monolayers was quite different from that of monolayers prepared from various mixtures of lipids alone. Experiments described below show that the additional component which brings about this difference is a protein.

2. Materials and methods

The materials used were of the highest purity commercially available and were used without further purification except in the case of NaCl which was roasted for 8 h at 700°C before use to remove extraneous organic contamination.

Glass-distilled water was used and was passed through a 0.22 μm filter prior to addition of salts and buffer. Periodical checks of the surface tension of water thus prepared indicated that this quantity always lay well within 2% of the expected value. Erythrocyte ghosts were prepared as in [1]. The extrinsic water-soluble proteins were removed from the erythrocyte ghosts using a standard technique [2,3]. The erythrocyte ghosts were stirred at 0–4°C in 0.1 mM disodium EDTA (adjusted to pH 8.0) for 12 h and subsequently centrifuged at 100 000 × g (MSE Prepspin 50) for 90 min at 4°C. The pellet was examined by light microscopy and was seen to consist of small vesicles of ghost membrane. It was deduced from these observations that very little extrinsic protein remained after this treatment. In this preparation, the lipid:protein ratio is 2:1 (w/w) [2,3].

The pellet obtained from the final stage of the removal of extrinsic proteins was dispersed with distilled water and freeze dried in small amounts. The material obtained was suspended at 0.2 mg/ml in n-hexane and sonicated at 4°C for 5 min using a 180 G disintegrator (Ultrasonics Ltd) with a 3 mm tip on power setting 5. The solution was maintained at 4°C by a jacketed vessel connected to an external water circulator. The resultant suspension had a slightly turbid appearance. Examination using an optical microscope indicated the presence of some liposome-like structures large enough to be resolved. This result would indeed be expected. It was found that the appearance of the suspension was sensitive to the sonication time for $\leqslant$ 5 min but longer times had no significant influence on the appearance. The resulting suspension was used either directly or was filtered through a PTFE (polytetrafluoroethylene) FH Millipore membrane (500 nm pore size) and used

to form a layer at the surface of the Langmuir trough.

The Langmuir trough was machined from a single block of polytetrafluoroethylene and the general experimental arrangements were very similar to those in [4]. A PTFE barrier was driven mechanically and the surface pressure was measured by a piece of filter paper acting as a Wilhelmy plate suspended from a CI Electronics Microforce balance. The subphase used was 100 mM NaCl buffered to pH 7.2 (Tris/acetate) at 22°C.

To check that the layers formed from the erythrocyte extract were indeed one molecule thick, calculations were made to relate the mass (and hence volume) of non-volatile material/unit volume erythrocyte extract to the area of spread film produced from such a volume. These calculations indicated the presence of a monolayer. As a further check as separate trough was used in order to deposit a monolayer on a clean glass slide. This trough was deep enough to allow travel of the slide through a monolayer formed at the surface. An automatic barrier device maintained the surface pressure at 30 mN/m whilst the slide of known area was withdrawn vertically through the pre-formed monolayer. By this means it was checked that the area of the slide covered by the monolayer corresponded to the decrease in area of the Langmuir film. Small drops of water were placed on the surface and the angle of contact indicated that the surface was hydrophobic as expected on the assumption that one monolayer only was present.

Penetration studies were done by spreading a measured amount of solution using a micropipette

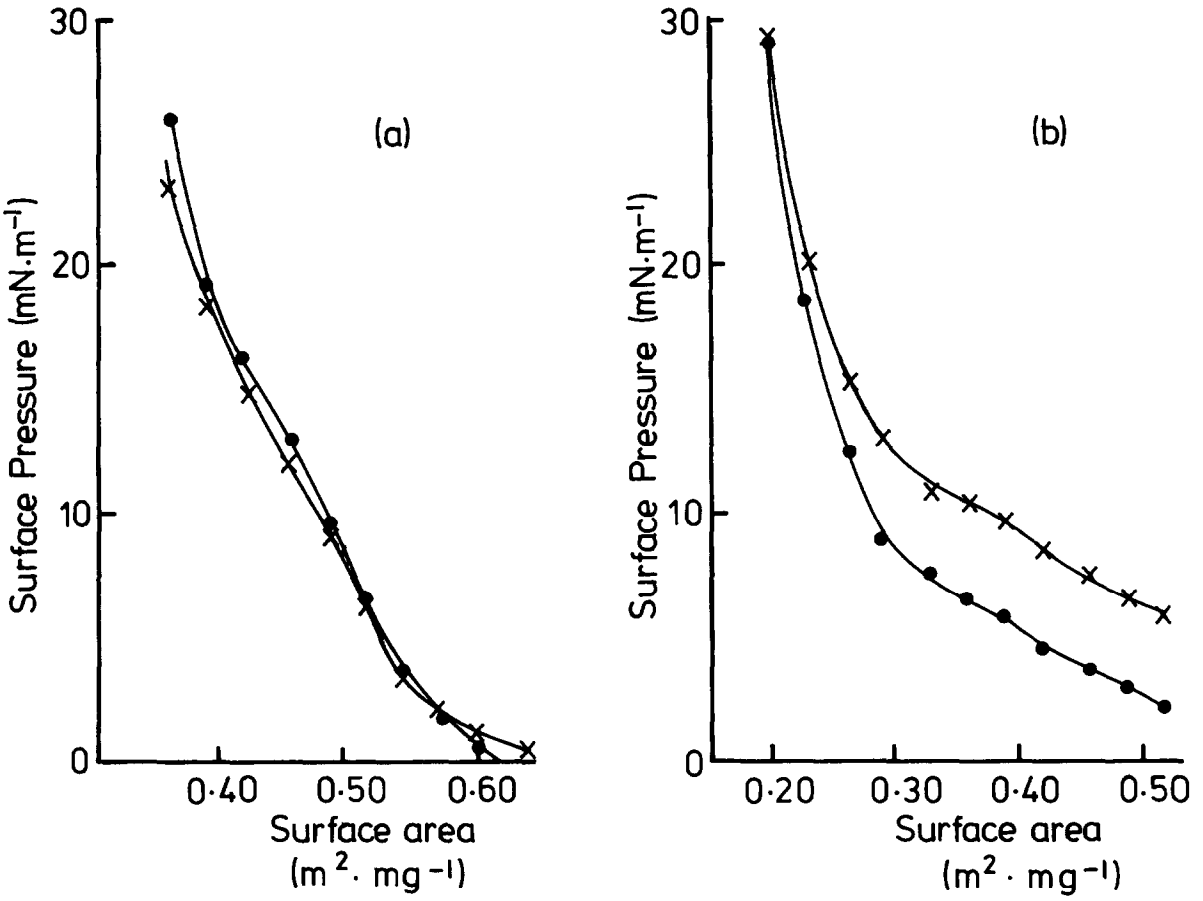

Fig.1. Monolayers formed from erythrocyte ghosts. (a) Unfiltered extract: the layer was compressed to 30 mN/m and bovine serum albumin added to 10 μg/ml; the monolayer was held at constant area for 90 min. (b) Filtered extract: the conditions were as for (a) except that the monolayer was compressed to produce a starting pressure of 35 mN/m. (•) Initial expansion; (×) expansion in the presence of bovine serum albumin.

when the barrier was at maximum opening. The layer was allowed to stand for ⩾ 5 min prior to compression. The barrier was then moved until ~30 mN/m was obtained. The layer was then expanded and recompressed, the pressure being meanwhile recorded. Serum albumin or spectrin was now injected beneath the monolayer and the subphase stirred using a magnetic stirrer. The layer was now re-expanded and a further isotherm recorded.

In some experiments monolayers prepared from whole ghost membrane and filtered ghost membrane extracts, were incubated with α-chymotrypsin at 1 μg/ml for 30–90 min. Enzyme pre-inactivated by addition of TPCK (L-1-tosylamide-2-phenylethyl-chloromethylketone) [5] in the molar proportion 1:2 for 8 h at pH 7.0 was also investigated for its effect on these monolayers.

3. Results

Fig.1a illustrates isotherms obtained using unfiltered ghost extract with and without injected serum albumin. Negligible penetration takes place. Fig.1b illustrates the result of an experiment using filtered ghost extract where now penetration is obvious.

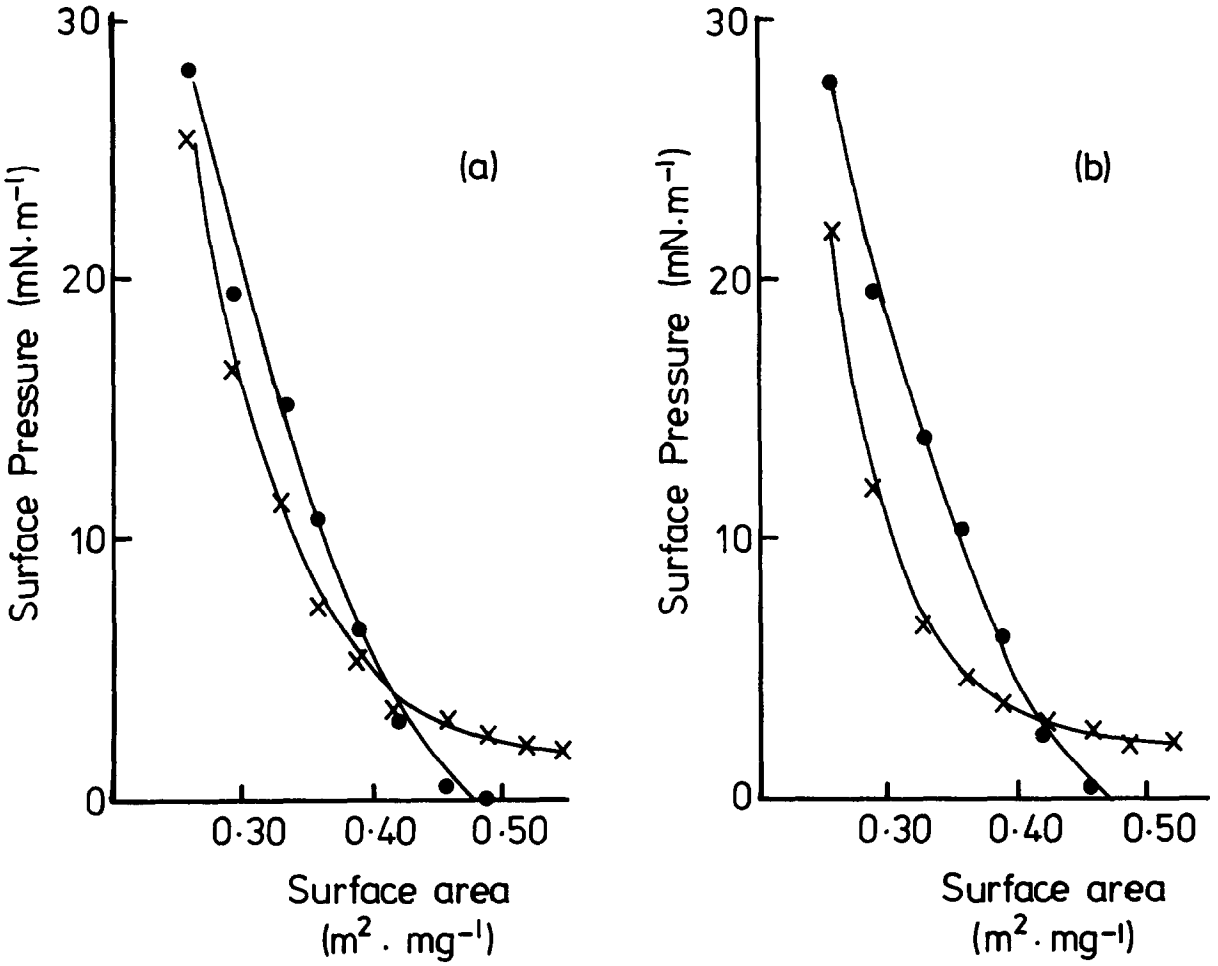

Fig.2. The effect of α-chymotrypsin on whole ghost extracts. Whole ghost extracts were spread as monolayers and compressed to a starting pressure of 30 mN/m. The enzyme was added beneath the layer (1 μg/ml) and the subphase stirred. Prior to expansion, bovine serum albumin was added to 10 μg/ml in the subphase. (a) Monolayer held at constant area for 30 min. (b) Monolayer held at constant area for 90 min. (•) Initial expansion; (X) expansion after treatment with the enzyme and addition of bovine serum albumin.

Fig.2 illustrates the effect of incubation with α- chymotrypsin on ghost extract for two different periods. It is clear that this proteolytic enzyme has had two effects:

(1) The shifting of the nearly vertical part of the curve to the left indicates that protein has been removed from the monolayer;
(2) The coherence of the layer has been reduced so that penetration becomes possible.

A repetition of this experiment using inactivated enzyme led to negative results as anticipated. All these experiments were repeated several times.

In order to test the effect of various lipid mixtures control experiments were also carried out using:

(1) A mixture of soyabean lipid with 25% cholesterol by weight.
(2) Lipids extracted from erythrocyte ghosts using a chloroform–ethanol mixture.

In both cases the results were similar to those obtained using filtered ghost extract.

Control experiments were carried out in which α-chymotrypsin was injected beneath ghost extract monolayers but in which no serum albumin was employed. The resultant isotherms showed no appreciable penetration of protein unlike the results in fig.2. Similar results were obtained using spectrin instead of bovine serum albumin.

4. Discussion and conclusions

The results above show clearly that, even when extrinsic proteins have been largely removed, the monolayers derived from erythrocyte ghosts have a far higher degree of coherence than do pure lipid layers. This coherence can be removed either by filtration (500 nm pore size) or by the action of α-chymotrypsin. It thus seems likely that there exists a protein network consisting of intrinsic proteins in addition to the extrinsic network of which spectrin forms a part.

We believe that, in the ghost extracts studied, very little extrinsic protein can survive the extraction procedures and the subsequent sonication in hexane. The coherence which we have observed must thus be due to the existence of an intrinsic protein network.

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