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Erythrocyte Membrane Proteins: A Modified Gorter-Grendel Experiment

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Summary. The pressure-area isotherm and shear resistance of spectrin-actin monolayers indicate a close-packed structure at about 1.0 m^2/mg protein. This surface area is equivalent to a thickness of about two monolayers at the erythrocyte membrane inner face. The maximum elasticity (lowest compressibility) occurs at 0.7 m^2/mg protein, indicating the limit of reversible compression. The mechanical properties of the monolayers approximate those of the intact membrane, suggesting that the structures are similar and that these monolayers may account for many of the *in vivo* properties.

The Gorter-Grendel (1925) experiment, originally published over 50 years ago, set out to answer a simple quantitative question about erythrocyte lipids and thereby helped to shape our ideas about the organization of the erythrocyte membrane. By extracting the lipids from a known amount of membrane material and subsequently determining the area that these molecules occupied in a surface film, they were able to determine that there was sufficient lipid to cover about twice the area of the membrane. Bar, Deamer and Cornwell (1966) recently repeated the experiment and pointed out some of the difficulties in the interpretation of the original results. They nevertheless confirmed the basic observations and the conclusion about the presence of sufficient membrane lipid to form a bilayer structure.

The outline of this experiment is so simple that one might ask why it has never been repeated with proteins, the other major class of membrane components. The simple answer, of course, is that, in sharp contrast to lipids, membrane proteins present greater experimental problems in extraction, spreading, etc., and until recently they were not even very well characterized. In the last few years much has been learned about the erythrocyte membrane proteins—amounts, molecular weights, location in the membrane structure (Steck, 1974; Kirkpatrick, 1976), and it is now possible to ask the same quantitative question posed by Gorter and Grendel with regard to proteins.

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Several years ago we (Blank & Britten, 1975; Blank, 1976) speculated about the outcome of such an experiment based on the known amounts of protein in the membrane and the measured properties of protein films. Since many proteins form "close-packed" monolayers at areas of about 1 m^2/mg when spread on a surface, we calculated that the known amount of membrane protein in a red cell is equivalent to about 7 monolayers of protein. It was hard to interpret this approximate figure in terms of a structure because of the inclusion of both integral and peripheral proteins, but there did appear to be a lot more protein present than was generally depicted in models.

In this paper we have now measured the surface isotherm of proteins obtained from the erythrocyte membrane. Furthermore, we have used a protein fraction that constitutes a known percentage of the total membrane protein and whose location is solely at the inner face of the structure. Therefore, our modified Gorter and Grendel calculations, relating the surface packing of these proteins to the membrane organization, have greater relevance to the *in vivo* structure and enable us to draw some tentative conclusions about function.

Materials and Methods

The proteins of the red cell membrane are generally characterized in terms of the bands they form on SDS polyacrylamide gel during electrophoresis, and we have used a 1:1 mixture of the proteins spectrin (bands 1 and 2) and actin (band 5) in our study. These proteins (S+A) are known to be present on the inner face of the membrane and account for one third of the total protein (Steck, 1974; Kirkpatrick, 1976; Nicholson, Marchesi & Singer, 1971).

Solutions of S+A were prepared by standard methods and without the aid of detergents. Human erythrocytes were obtained from freshly outdated blood bank blood. Following centrifugation and removal of the plasma and buffy coat, the erythrocytes were washed with cold isotonic buffer (30 mM sodium phosphate, pH 7.4, 117 mM NaCl, and 2.8 mM KCl). The washed packed cells were hemolyzed in 12 volumes of cold 8 mM sodium phosphate, pH 7.4, for 30 min at 4 °C, and ghost membranes isolated (Dodge, Mitchell & Hanahan, 1963). The washed ghosts were stored frozen at −20 °C overnight, and then extracted with 10 volumes 0.1 mM sodium EDTA (pH 8.0) for 20 min at 37 °C (Fairbanks, Steck & Wallach, 1971). Centrifugation at 78×10^3 *g* (30 min; 4 °C) yielded a clear supernatant (typical protein concentration, about 0.2 mg/ml) which was a 1:1 mixture of spectrin (bands 1 and 2) and actin (band 5) as judged by the Coomassie Brilliant Blue bands seen after SDS-polyacrylamide gel electrophoresis.

The surface isotherms of these solutions (surface pressure, π, *vs.* area, A, curves) were measured by the standard technique on a Langmuir trough made of Teflon. The original area for spreading the film was 300 cm^2 and the compression rate was 10 cm^2/sec. The surface pressure was measured with a Sanborn (Model 311A) transducer and an attached sandblasted platinum plate. The Teflon apparatus and a glass rod were cleaned

with detergent, rinsed several minutes in running cold water and finally with doubly-distilled water. The surface tension was recorded after filling the trough with subphase solution, sweeping the surface, and setting up the dipping plate. Precise amounts of S+A spreading solution were delivered from Lang-Levy pipets and spread down the glass rod to the surface. The rod was then rinsed several times with the subphase solution. After waiting 5 min, the movable Teflon barrier was started and the surface tension readings were automatically recorded. The π *vs.* A curves and the Gibbsian elasticity

$$E = -A(\mathrm{d}\pi/\mathrm{dA})$$

were calculated from these data.

The surface viscosity of protein monolayers is generally very high and we used this property to investigate intermolecular interactions on a surface. We have measured the shear resistance of a protein film at a decane/aqueous interface (Blank & Soo, 1976) using an adaptation of the model LVT Brookfield viscometer. A specially designed stainless steel spindle (4.75 cm OD, 4.4 cm ID) was used for making contact with the aqueous surface in a crystallizing dish (14.9 cm ID). The spindle was centered above the (thermostatted) crystallizing dish and lowered slowly with a racking device until there was contact with the surface. The S+A film was spread with a thin layer of decane (5 ml) on top, as in previous experiments. The speed of rotation of the spindle was 3 rpm, and the readings (which are related to the surface viscosity) were made directly on the scale of the Brookfield viscometer.

The decane/water interface has been used in the past as a suitable medium for studying the properties of protein surface films, so it is possible to compare the S+A films to known systems (e.g., Blank & Soo, 1976). This interface is also a reasonable model for the lipid bilayer/cytoplasm interface at the inner surface of the red cell membrane and therefore appropriate for the study of S+A films.

Results and Discussion

The area occupied by S+A molecules at an interface has been estimated by spreading known amounts of the proteins as monolayers and studying the changes in the surface properties with two dimensional compression. Depositing the S+A from a 0.1 mM EDTA solution on to a 1.5 M saline surface and compressing the film, we obtain the surface pressure data shown in Fig. 1. Upon extrapolation to zero surface pressure, the intercept is about 1.0 m^2/mg. (If the subphase concentration is 0.1 M NaCl, the entire surface pressure-area curve is displaced, with an extrapolated area of 0.7 m^2/mg.) The Gibbsian elasticity, plotted in Fig. 2, shows a maximum at an area of about 0.7 m^2/mg.

The above properties of S+A films can be related to the properties of the S+A molecules on a surface. The point where the spread protein molecules begin to make contact with each other occurs at the extrapolated area at zero pressure. When the molecules are compressed beyond this point up to the point of maximum elasticity, they occupy smaller areas per molecule without irreversible distortion. Further compression leads to irreversible changes in the conformation of the molecules. It

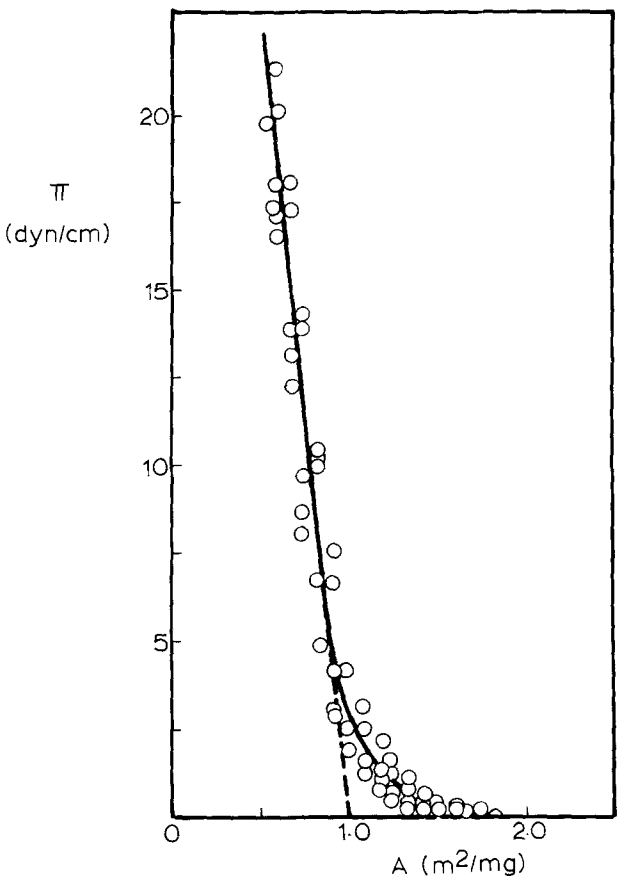

Fig. 1. The surface pressure, π, in dyn/cm as a function of the surface area per weight of S+A in m^2/mg. The temperature is 25 °C and the subphase is 1.5 M KCl

is therefore reasonable to assume that the surface density range of 0.7–1.0 m^2/mg represents the probable surface concentration of the extracted membrane proteins on a surface.

Another measurement we have made with spread films of S+A is a determination of the shear resistance, R, shown in Fig. 3. Here again when we extrapolate to zero retarding force on the spindle due to the S+A film, we obtain an intercept at about 1.0 mg/m^2. At this point the S+A molecules presumably start to exert a resistance to shear because of intermolecular interactions in the surface.

The three physical properties shown in Figs. 1–3 help to identify the range in which the protein molecules on the surface are in contact and can be reversibly compressed. We can compare this range of area to the expected surface concentration of S+A based on the 10^{-12} g

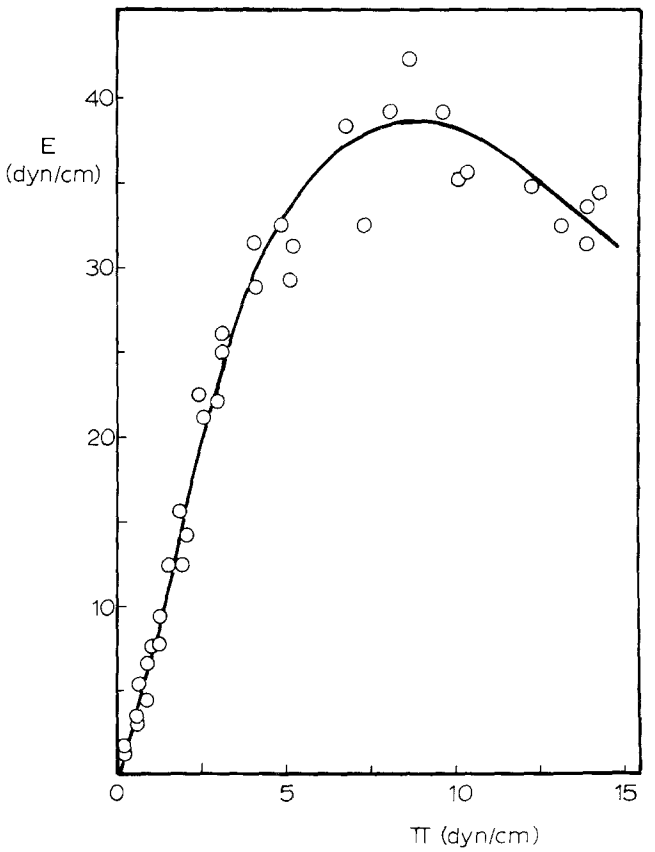

Fig. 2. The Gibbsian elasticity, E, in dyn/cm as a function of the surface pressure, π, in dyn/cm. The temperature is 25 °C, and the subphase is 1.5 M KCl

of membrane protein per cell (Dodge *et al.*, 1963; Hoogeveen *et al.*, 1970). Of the 10^{-12} g of protein per cell membrane, one third is S+A (Steck, 1974). Dividing the area of a cell, 140 μm^2, by 3.3×10^{-13} g S+A per cell, we calculate that the total average surface area of S+A *in vitro* is 0.42 m^2/mg in an erythrocyte. Therefore, there appears to be enough protein to form about 2 monolayers of S+A on the inner face of the membrane. (This would correspond to an average thickness of about 35 Å if we assume that the protein has the same partial molar volume in a monolayer as in solution.) On the basis of the molecular properties of dissolved spectrin, Kirkpatrick (1976) has suggested that the spectrin covers only about 30% of the membrane area, although more recently, Ralston (1978) has suggested that S+A may form a complete monolayer. Regardless of the extrapolations from data on

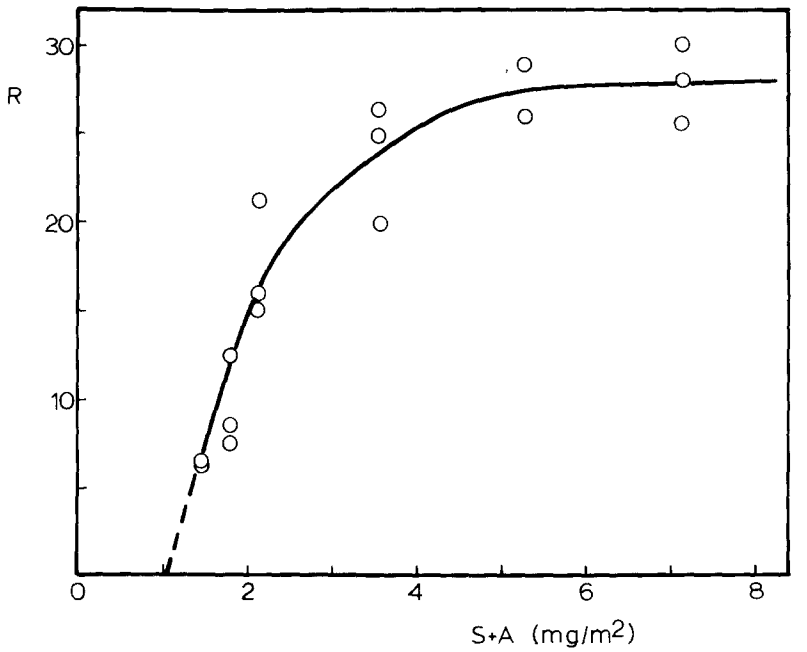

Fig. 3. The shear resistance, R (the reading on a Brookfield viscometer), when using the specially designed surface spindle described in the text, as a function of the surface concentration of S+A in mg/m^2 at a decane/water

dissolved proteins, the fuzzy layer due to S+A on the inner face of the erythrocyte membrane is well known from earlier measurements (Nicholson *et al.*, 1971), and Hainfeld and Steck (1977) have recently shown the presence of a continuous web-like reticulum on the inner face of the membrane, using scanning electron microscopy. Our measurements contribute an experimentally derived value for the area occupied by an extracted S+A mixture of membrane proteins to the discussion of membrane structure.

The surface film experiments and the calculations are straightforward and reproducible, but there are several obvious questions regarding the conclusions that can be drawn from a surface film experiment involving proteins, and soluble ones at that. The two questions we shall now consider are:

1) Does the solubility of the proteins impose limitations on the experiments and on the conclusions that can be drawn from them?

2) Does the film structure on the surface resemble the organization of the same molecules on the inner face of the membrane?

Although globular proteins are soluble in aqueous solution, they form stable films on aqueous subphases when properly spread. The stabil-

ity is probably achieved as a result of changes in molecular conformation upon spreading that concentrate the polar groups in the aqueous phase and the hydrophobic residues outside. The solubility of proteins in the aqueous phase can also be reduced by increasing the concentration of dissolved salt, as we have done. The π-A curves on 0.1 M NaCl show an intercept at .7 m^2/mg while on 1–3 M NaCl the intercept is 1.0 m^2/mg. (At the higher salt concentration, electrostatic effects would tend to make the area smaller so this factor cannot be the cause of the observed expansion.) Another factor that is apt to contribute to the stability of an S+A surface film is the interaction between the S and A components. In any case it appears that S+A films can be formed, and that they are slightly soluble on 0.1-M subphases. On 1-M subphases the solubility is descreased considerably and may even be insignificant. But on both subphases our experiments indicate that the observed areas per mg protein are in line with other protein surface films. Also, if some protein were lost from the film we would have to conclude that the *in vivo* S+A layer was even thicker.

Let us now consider the relation between the S+A film structure *in vitro* and the organization of the same molecules on the inner face of the membrane. There are undoubtedly conformational changes in the molecules as a result of extraction and spreading. (Some of the structural changes on extraction are probably reversed when the molecules are spread at an interface that is similar in asymmetry to the inner face of the membrane.) However, the major point in support of the similarity of structure and the applicability of this experiment to the conditions in the membrane is the parallelism between some of the properties of the S+A film and of the intact membrane.

If the S+A on the inner face of the erythrocyte membrane does form a close packed monolayer, the mechanical properties of the membrane should reflect those of the protein monolayer (Blank, 1976). We have measured some of these properties of S+A films (Blank et al., 1979), and can compare our observations to those made on intact erythrocyte membranes (Chien, 1977). For example, the dilational elastic modulus of the membrane is about 10^2 dyn/cm, while the S+A film values, shown in Fig. 2 are 10–40 dyn/cm. The extensional elastic modulus of the membrane is given as about 10^{-2} dyn/cm, while the S+A film is on the order of 10^{-1} dyn/cm and a function of frequency. The membrane viscosity, which has been measured under special conditions that involve a range of different frequencies, mostly low frequencies, is on the order of 10^{-3} dyn sec/cm. The viscosity of the S+A films vary with frequency

and at low frequency ($\sim 1\ sec^{-1}$) the values are on the order of 10^{-2}–10^{-3} dyn sec/cm. From these comparisons there appears to be good agreement regarding orders of magnitude, especially when one considers that the properties of lipid bilayers are very different. Other protein film properties such as the effects of cations on the two dimensional yield (Blank, 1976) and the effects of lipids on the surface viscosity (Blank & Soo, 1976) also suggest that the properties of an S+A film at the inner face of the erythrocyte membrane can account for some of the observed properties of the intact cell.

Another set of measurements that we have made on S+A films is the permeability to ions (Blank, Soo & Abbott, 1979). These observations have indicated that the S+A film is relatively impermeable to anions at normal body pH, and that the S+A layer is apt to be a significant barrier to ion movement. (In this connection we note that Lepke and Passow (1976) have shown that internal trypsin can cause a slight increase in anion equilibrium exchange.) These properties indicate that an intact S+A film at the inner face of the erythrocyte membrane may necessitate the extension of the anion transporting component (band 3 protein) through the S+A layer. This structure would achieve a greater mechanical stability as well as a greater permeability to ions.

Conclusion

Classic film balance techniques have been used for a long time, and so the deficiencies and problems of the approach are well known. Despite these limitations it is possible to formulate interesting questions about two dimensional organization and to obtain data in answer to these questions. The results of these balance experiments indicate that simple Gorter-Grendel type experiments appear to be useful in trying to understand the properties of complicated membrane structures. In particular, the surface isotherms of S+A films suggest the presence of a relatively thick (average ~ 35Å) layer of protein on the endofacial surface of the membrane. The rheological properties and the permeabilities of the S+A films appear to be related to the analogous properties of the intact erythrocyte membrane, suggesting that the endofacial protein layer plays a significant role in membrane function.

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