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INTERFACIAL FREE ENERGIES OF INTACT AND RECONSTITUTED ERYTHROCYTE SURFACES

IMPLICATIONS FOR BIOLOGICAL ADHESION

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Model cell surfaces consisting of phospholipids or phospholipids and the erythrocyte membrane glycoprotein glycophorin have been formed at an oil/water interface. Interfacial free energies have been estimated from surface wetting by both hydrophobic and hydrophilic test droplets on both the model surfaces and on intact erythrocytes. The use of a dense fluorocarbon oil to form the oil/water interface facilitates analysis by minimising surface deformation by the test drop. Hydrophobic test droplets (polar hydrocarbon oils) show increasing contact angles (decreasing wetting) with increasing hydrophilicity (decreasing interfacial free energy) of the model interface. Hydrophilic test droplets (phase separated aqueous polymer systems) show the opposite behaviour, spreading more as the interfacial free energy is decreased. Both systems give similar estimates of the interfacial free energy. Glycophorin reproduces the wetting properties of intact cell surfaces by reducing the lipid-water interfacial free energy from $5 \cdot 10^{-3}$ J·m^{-2} to $1 \cdot 10^{-6}$ J·m^{-2}. From molecular considerations it is concluded that 'cell surface free energy' is an ambiguous term; its magnitude depends on the location of the interface in question. Thus, in a thermodynamic analysis of interactions at biosurfaces (such as cellular adhesion, chemotaxis or membrane fusion), the interfacial free energies may vary by more than three orders of magnitude depending on the location of the particular interface.

Introduction

The formation and reversal of adhesive contacts between biological surfaces plays a key role in a wide variety of physiological and pathological processes. Adhesion is an important event in cell fertilization, motility, growth, differentiation and repair [1], as well as in stimulus-secretion coupling and stimulus-contraction coupling [2]. Pathological processes involving alterations in the adhesiveness of cell surface include inflammation, thrombosis, infection and metastasis [3]. The driving force for adhesive interactions between all surfaces is the change in interfacial free energy [4], and it has been recognized since the 1920's that living cells are no exception to this general rule [5]. The early workers were well aware of the importance of the free energy at the cell surface, but were limited by the methods of the day in their ability to estimate its magnitude. With the doubtful assumption that cells behaved as isotropic fluids, estimates of cell surface energies were made from deformation measurements, and were found to lie in the range 0.6–0.3 mJ · m^{-2} [6–8].

More recently, a number of attempts have been made to avoid the problems of cell deformation,

and to estimate cell surface energies from measurements of cell wetting by fluids of known surface free energies [9–11]. As a result of these studies it has become clear that wetting and deformation measurements do not reflect the same properties of the cell surface, since the interfacial free energies estimated from wetting studies are about 3 orders of magnitude lower than those estimated from cell deformation (about 1 $\mu J \cdot m^{-2}$ compared with 1 $mJ \cdot m^{-2}$ [11]).

Which of these estimates (if either) reflects the 'real' free energy of the cell surface? The energies inferred from wetting studies are typical of interfaces between polymer solutions, and indeed it has been suggested that a particle covered by terminally anchored polymers (such as cell membrane proteins) would have similar interfacial properties to those of the corresponding polymers in free solution [12]. On the other hand, the energies inferred from deformation experiments are typical of those between lipids and water [13].

The current view of the structure of cell surfaces is that they are composed of quasi-fluid hydrated phospholipids, predominantly arranged in a bilayer configuration in which embedded macromolecules have varying degrees of mobility [14].

If wetting measurements respond to low-energy (polymer) components and deformation measurements respond to high-energy (lipid) components of composite biological interfaces this discrepancy in interfacial energy measurements would be resolved *. Testing this hypothesis requires separate measurements of the interfacial energies of components of a biological interface and comparison with similar measurements on an intact interface.

In the present study we have used dipalmitoylphosphatidylcholine as a representative of membrane phospholipids. Membrane polymers are generally glycoproteins and carbohydrate-containing cell surface macromolecules have emerged as prime candidates for mediating the stickiness involved in adhesive interactions such as cell recognition [15], platelet aggregation [16], bacterial toxin ingestion [17], virus mediated membrane fusion [18] and as the pre-synaptic calcium receptors involved in neurotransmitter release [19]. We have used the purified erythrocyte sialoglycoprotein, glycophorin, to form composite lipid/protein interfaces and we have compared interfacial energy measurements on these surfaces with similar measurements on intact erythrocytes.

* N.B. The terminology used here of 'high energy' and 'low energy' referring to solid/liquid interfaces is the opposite to the convention developed for solid/vapour interfaces by Zisman and co-workers [21].

Materials and Methods

Human red blood cells were separated by centrifugation from freshly drawn citrated whole blood.

Monolayers of isolated cells were prepared by gentle filtration of cells from suspensions onto hydrated cellulose acetate membranes with a pore diameter of 0.45 μm (Millipore Corporation). Cells were counted in a haemocytometer, and the final cell density on the filter was adjusted to $2.5 \cdot 10^3$ cells/μm^2.

Human erythrocyte glycophorin was kindly provided by Dr. C.W.M. Grant, Department of Biochemistry, University of Western Ontario. The glycoprotein was isolated from out-dated bank blood by the method of Marchesi and Andrews [20], and was extensively washed with ethanol.

Phospholipids were obtained from Serdary Research, London, Ontario, and were judged pure by thin-layer chromatography.

Hydrocarbon test fluids, dibutyl phthalate and dioctyl phthalate, mixed to yield a final density of 1.02 g/ml, slightly above that of 0.9% saline (1.01 g/ml) were obtained from Eastman Kodak.

Fluorocarbon liquid FC 40 (density 1.85 g/ml) was purchased from the 3M Company, St. Paul, MN.

Aqueous two-phase systems composed of dextran M_r 2000000 (Pharmacia) and poly(ethylene glycol) M_r 20000 (Polysciences) were made in Hepes-buffered saline as previously described [11].

Measurement of interfacial free energies

A variety of methods is available for the estimation of interfacial free energies at liquid-vapour or liquid-liquid interfaces (see, for example, Adamson [21]). However, when one of the phases is a solid, virtually all estimates of solid surface free energies have been based on wetting of the solid by liquids. In a three phase system, when a non-deformable solid is wetted by two immiscible

liquids, the equilibrium contact angle between the liquids and the solid is a function of the interfacial free energies, γ, given by Young's equation (Fig. 1)

$$\gamma_{AC} = \gamma_{BC} + \gamma_{AB} \cos\theta \tag{1}$$

γ_{AC} is a function of the intermolecular forces across the interface ac, and is in aqueous systems largely determined by the surface-water interaction. Only γ_{AB} and the contact angle θ are experimentally accessible; solving Young's equation requires knowledge of an additional relationship between the interfacial free energies. Such a relationship may be derived either empirically or theoretically.

Estimation of interfacial free energies from surface wetting by hydrophobic test fluids. The difficulty in estimating γ_{AC} or γ_{BC} limited the practical application of Young's equation until Neumann and his colleagues succeeded in calculating the γ_{BC} term by employing Good's interaction parameter φ and an empirical formulation of an equation-of-state relationship among the solid/vapor, and solid/liquid interfacial tensions [22]. The equation

$$\gamma_{BC} = \frac{\left((\gamma_{AC})^{1/2} - (\gamma_{AB})^{1/2}\right)^2}{1 - 0.015(\gamma_{AC}\gamma_{AB})^{1/2}} \tag{2}$$

was substituted in Young's equation to give the relation

$$\cos\theta = \frac{(0.015\gamma_{AC} - 2.00)(\gamma_{AC}\gamma_{AB})^{1/2} + \gamma_{AB}}{\gamma_{AB}\left(0.015(\gamma_{AC}\gamma_{AB})^{1/2} - 1\right)} \tag{3}$$

This equation allows interfacial tensions of solid surfaces to be calculated with relative ease once values of θ and γ_{AB} are determined experimentally.

However, both the derivation and the application of the equation of state have so far been limited to solid/liquid/vapor interfaces in two-component, three-phase systems.

We have recently shown that the equation-of-state relationship also holds in oil-water test systems, and the method has been described in detail elsewhere [23]. Briefly, the approach involves measuring wetting of a liquid fluorocarbon/aqueous solution interface, by a water- and fluorocarbon-immiscible test droplet. By spreading different substances at the fluorocarbon/water interface different interfaces are created whose interfacial free energies can be estimated from measurements of the contact angle and γ_{AB}, using the equation-of-state relationship between the variables.

The experimental arrangement is shown schematically in Fig. 2. In each experiment a large fluorocarbon drop (FC 40, 3M Company, Saint Paul, MN) was formed on the agar block inside the saline bathing solution. After cleaning the FC 40/saline interface by aspiration with a micropipet, we spread a monolayer of dipalmitoyl- or distearoylphosphatidylcholine (DPPC or DSPC) from a solution of 2 mg/ml of phospholipid in ethanol. By removal of FC 40 fluid from the interior of the sessile drop we were able to reduce the interfacial area and to compress the monolayer in a manner similar to compressing monolayers in a Langmuir surface balance. By adding or removing FC 40 fluid from the drop a range of phospholipid/water interfacial tensions could be generated, and the simultaneous changes in contact angle of the upper drop could be followed.

Under these circumstances, the absence of contact angle hysteresis (a result of the smooth and homogeneous microtopography of the interface) means that Young's equation may be applied. All of the experiments reported in the present paper

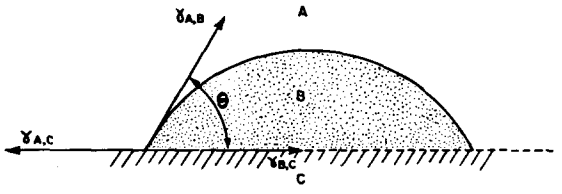

Fig. 1. Relationship between interfacial free energies (γ) and contact angle (θ) at a three-phase boundary.

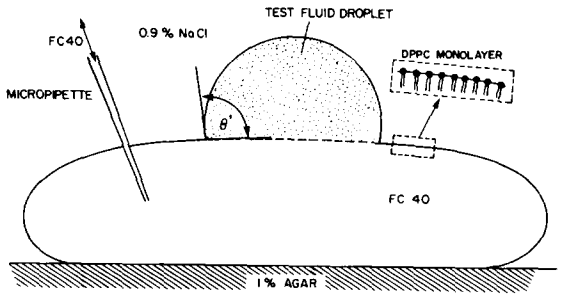

Fig. 2. Schematic diagram of the model interface.

were performed using uncompressed interfacial monolayers.

The contact angles were measured either directly by using a Nikon SMZ microscope equipped with a protractor eyepiece, or the angles were determined from slide projection of photographs.

Estimation of interfacial free energies from surface wetting by hydrophilic phase separated polymer systems. The use of hydrophobic test fluids and the equation of state indicates that the biosurface/water interfacial free energy is very low (approx. $1 \cdot 10^{-6}$ J $\cdot$ m^{-2} [10]) but this procedure is not without problems: the high interfacial free energy of the test fluid and the low interfacial free energy of the test surface gives rise to an imbalance which severely limits the sensitivity of the method. Contact angles of less than 150° are not obtained on healthy cells. Improvements in sensitivity (lower contact angles) can only be achieved by lowering the free energy of the test fluid towards that of the biological surface.

Phase separated aqueous polymer systems such as dextran and poly(ethylene glycol) provide a partial solution to this problem. By varying the polymer concentration γ_{AB} may be set at any value between approx. $1.0 \cdot 10^{-3}$ and $1 \cdot 10^{-7}$ J $\cdot$ m^{-2}, and a corresponding range of contact angles between 120° and 0° is obtained when drops of one polymer phase are allowed to spread on biological surfaces immersed in the other phase.

The use of phase separated polymer systems to estimate biosurface free energies has been described elsewhere [11,24]. Cell/medium interfacial free energies derived from this approach range between $10 \cdot 10^{-6}$ and $0.6 \cdot 10^{-6}$ J $\cdot$ m^{-2}, they vary in a reproducible fashion between different cell types, and between the same cell types under different conditions [11]. Briefly, solutions of dextran molecular weight two million (Pharmacia) and poly(ethylene glycol) molecular weight 20000 (Fisher) were mixed and allowed to separate [25]. Droplets of the denser, dextran-rich phase were spread on the test surface immersed in the lighter, poly(ethylene glycol)-rich phase. The viscosity of the polymer solutions varies with the polymer concentration, so that the kinetics of droplet spreading vary between the different test solutions. With the lowest polymer concentrations constant contact angles were achieved within 1 min; with the highest concentrations 10–15 min were required for equilibration. After this initial period no further changes in contact angle were observed for 2 h or more. The right and the left contact angle of each drop was measured either directly with a protractor eyepiece or determined from photographs. The precision for these measurements was $\pm 1°$. The angle was taken as the final advancing contact angle. However, the polymer/polymer test system despite its sensitivity to low interfacial free energies, has an additional problem not encountered with the earlier method: the equation-of-state relationship as formulated by Neumann et al. [22] (Eqn. 2) between the three phases does not hold for phase-separated aqueous polymers. This is probably because Eqn. 2 is based on an empirical linear relationship between Good's interaction parameter φ and γ_{BC} [22]; in phase-separated aqueous polymer systems this relationship appears to be highly non-linear (Schürch, S. and McIver, D.J.L., unpublished data). Thus, until sufficient experimental data are available to make an empirical formulation of an equation-of-state relationship for polymer systems (if indeed, such a relationship exists), alternative methods must be used to estimate γ_{AC} under these conditions.

We have adapted the method of critical surface tension for spreading for estimating γ_{AC} in aqueous two-phase liquid/liquid/solid system. The concept of the critical surface tension for spreading was originally introduced by Zisman [26] to characterize the surface free energies of solids, and was subsequently modified by Good [27]. The critical surface tension, γ_c, determined by measuring contact angles with the substrate in question for each of a series of liquid droplets of known surface tension and plotting the cosine of these angles against the surface tension of the droplet. The critical tension of the substrate is then defined as the intercept of the line of best fit through the data points with the axis for $\cos \theta = 1$. Physically, γ_c separates liquids which do not form finite contact angles with the substrate (liquids that spread spontaneously) from those which do form finite contact angles and do not spread.

The change in droplet-cell contact angle with changing polymer concentration provides the basis for the determination of γ_c.

Preparation of surfaces

Glycophorin/DPPC surfaces were prepared by dissolving varying concentration of the glycoprotein in the aqueous phase and allowing it to absorb at the oil/water interface. The rate and extent of adsorption was estimated by measuring the contact angle as a function of time and glycophorin concentration (Figs. 5 and 6). The contact angles reported in Tables I and II were determined when the contact angle had reached its plateau with respect to time (2 h) and protein concentration (1 mg/ml).

Results

Surface wetting by hydrophobic test fluids

In Fig. 3 the fluorocarbon/saline interface is progressively altered by the adsorption of defined membrane components; the wetting of the three model interfaces (A–C) by a hydrocarbon test drop is compared with the wetting of intact erythrocytes (D) by the same test fluid. In Fig. 3A the test drop spreads nearly completely at the extremely hydrophobic FC 40/saline interface. When a surface active phospholipid (dipalmitoylphosphatidylcholine, DPPC) is spread on the FC 40 the interfacial energy is substantially lowered, and the fluorocarbon drop flattens under the influence of gravity; at the same time, the test fluid droplet rounds up as the interface becomes more hydrophilic, and the test drop forms a contact angle of 90° with the lipid/saline interface. However, only when glycophorin is adsorbed to the lipid/water interface (C) does the contact angle approach that observed on intact cells (D), demonstrating that the extremely low interfacial free energies at the cell-medium interface are due to membrane-bound polymers.

These results, and the estimated values of the interfacial free energies are summarized in Table I. Glycophorin-lipid interactions are clearly necessary for the expression of the surface activity of the glycoprotein, since in the absence of lipid glycophorin does not lower the FC 40/saline interfacial energy. Other experiments (not shown) indicated that non-membrane glycoproteins such as serum albumin are unable to produce this profound lowering of the interfacial free energy, even in the presence of a phospholipid monolayer.

Surface wetting by hydrophilic test fluids

Phase separated hydrophilic polymer test systems show exactly the opposite wetting behaviour to the hydrophobic test fluids: as the interfacial energy decreases (i.e., as the interface becomes more hydrophilic), the contact angle decreases. Thus (Table II), the hydrophobic FC 40/saline interface is completely non-wetted by the polymer test system; with reduction of the interfacial free energy contact angles of 150° on DPPC and 90° on glycophorin are obtained. Once again, the similarity of the contact angles on intact cells and the glycophorin model interface indicates that the wetting behaviour of the cell surface is dominated by the presence of high molecular weight hydrophilic polymers.

The estimation of γ_{AC} by critical spreading is shown in Fig. 4. A series of droplets of differing polymer concentrations (and hence differing γ_{AB} values) form a series of contact angles on the same surface. Extrapolation to zero contact angle allows estimation of γ_{AC}; in the case illustrated for intact erythrocytes the estimate of γ_{AC} ($1.2 \cdot 10^{-6}$ J · m^{-2}) is very similar to the estimate derived from analysis of contact angles at the glycophorin/saline interface ($1 \cdot 10^{-6}$ J · m^{-2}).

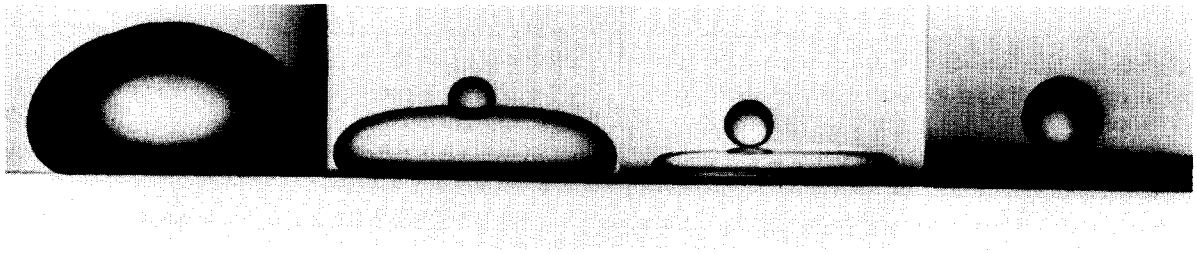

Fig. 3. Surface wetting by hydrophobic test drops on model surfaces (A–C) and intact erythrocytes (D).

TABLE I

SURFACE WETTING WITH HYDROPHOBIC TEST FLUIDS (1 : 1 DIBUTYL PHTHALATE: DIOCTYL PHTHALATE)

Abbreviations: FC 40, Fluorocarbon oil 'FC 40', 3M Company; DPPC, dipalmitoylphosphatidylcholine; GLYCO, human erythrocyte glycophorin. Nomenclature as in Fig. 1. Bathing fluid (phase A) is 0.9% NaCl. Methods: (a) pendant or sessile drop shape analysis [21]; (b) Critical spreading [11]; (c) DuNoüy ring or Wilhelmy plate method [21]; (d) equation-of-state [23].

Subphase	Contact angle ($\theta°$)	γ_{AB} ($J \cdot m^{-2}$)	γ_{AC} ($J \cdot m^{-2}$)
FC 40	~5 *	$2.25 \cdot 10^{-2}$ (a)	$5.4 \cdot 10^{-2}$ (c)
FC 40/DPPC	90	$2.25 \cdot 10^{-2}$ (a)	$5.6 \cdot 10^{-3}$ (d)
FC 40/DPPC/GLYCO	180	$2.25 \cdot 10^{-2}$ (a)	$1 \cdot 10^{-6}$ (d)
FC 40/GLYCO (no lipid)	5 *	$2.25 \cdot 10^{-2}$ (a)	$5 \cdot 10^{-2}$ * (d)
Erythrocytes	175	$2.25 \cdot 10^{-2}$ (a)	$1 \cdot 10^{-6}$ * (d)

* Approximate estimates for comparison purposes only: in this range of interfacial energies accurate results are not obtainable with this method (see Introduction).

TABLE II

SURFACE WETTING WITH HYDROPHILIC TEST FLUIDS (4% DEXTRAN M_r 2000000/4% POLY(ETHYLENE GLYCOL) M_r 20000)

Abbreviations: FC 40, Fluorocarbon oil 'FC 40', 3M Company; DPPC, dipalmitoylphosphatidylcholine; GLYCO, Human erythrocyte glycophorin. Nomenclature as in Fig. 1. Bathing fluid (phase A) is the poly(ethylene glycol)-rich phase of the two phase system. Methods: (a) pendant or sessile drop shape analysis [21]; (b) critical spreading [11]; (c) DuNoüy ring or Wilhelmy plate method [21]; (d) equation-of-state [23].

Subphase	Contact angle (θ)	γ_{AB} ($J \cdot m^{-2}$)	γ_{AC} ($J \cdot m^{-2}$)
FC 40	180	$3.47 \cdot 10^{-6}$ (a)	$5.4 \cdot 10^{-2}$ (c)
FC 40/DPPC	150	$3.47 \cdot 10^{-6}$ (a)	$1 \cdot 10^{-3}$ * (b)
FC 40/DPPC/GLYCO	95	$3.47 \cdot 10^{-6}$ (a)	$1 \cdot 10^{-6}$ (b)
FC 40/GLYCO (no lipid)	180	$3.47 \cdot 10^{-6}$ (a)	$1 \cdot 10^{-2}$ * (b)
Erythrocytes	100	$3.47 \cdot 10^{-6}$ (a)	$1.2 \cdot 10^{-6}$ (b)

* Approximate estimates for comparison purposes only: in this range of interfacial energies accurate results are not obtainable with this method (see Introduction).

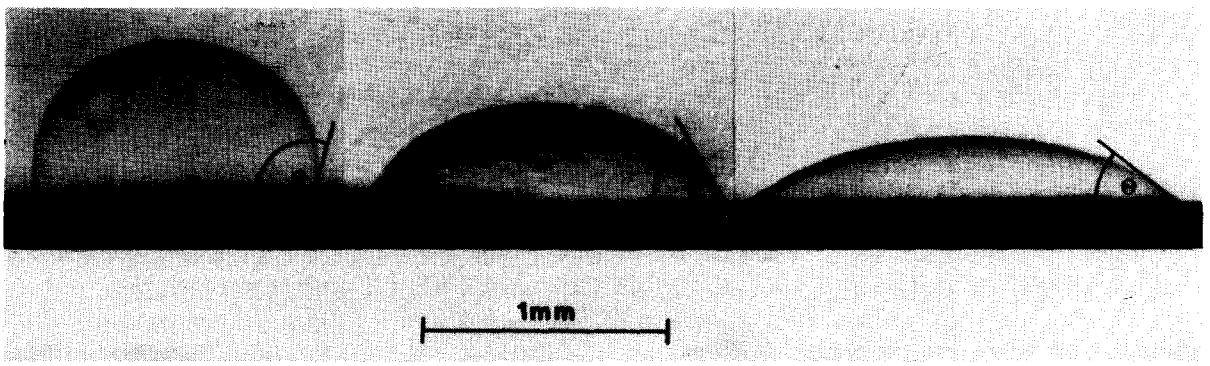

Fig. 4. The spreading of two-phase polymer droplets on erythrocyte layers.

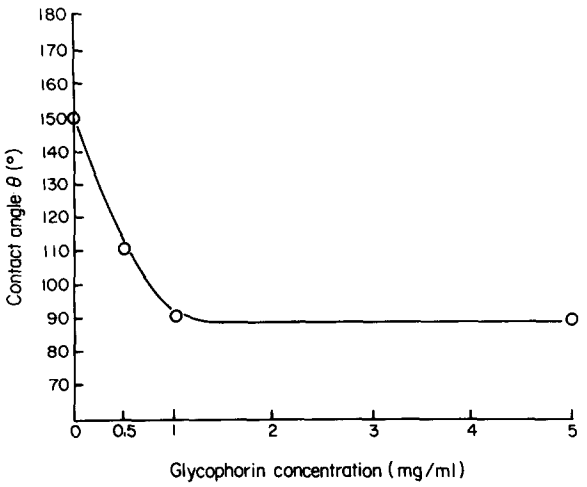

Fig. 5. Effect of glycophorin concentration on dextran/poly(ethylene glycol) contact angle.

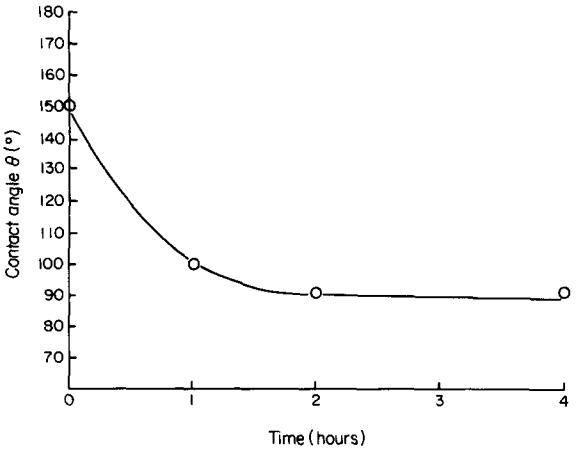

Fig. 6. Effect of time of exposure to glycophorin on dextran/poly(ethylene glycol) contact angle.

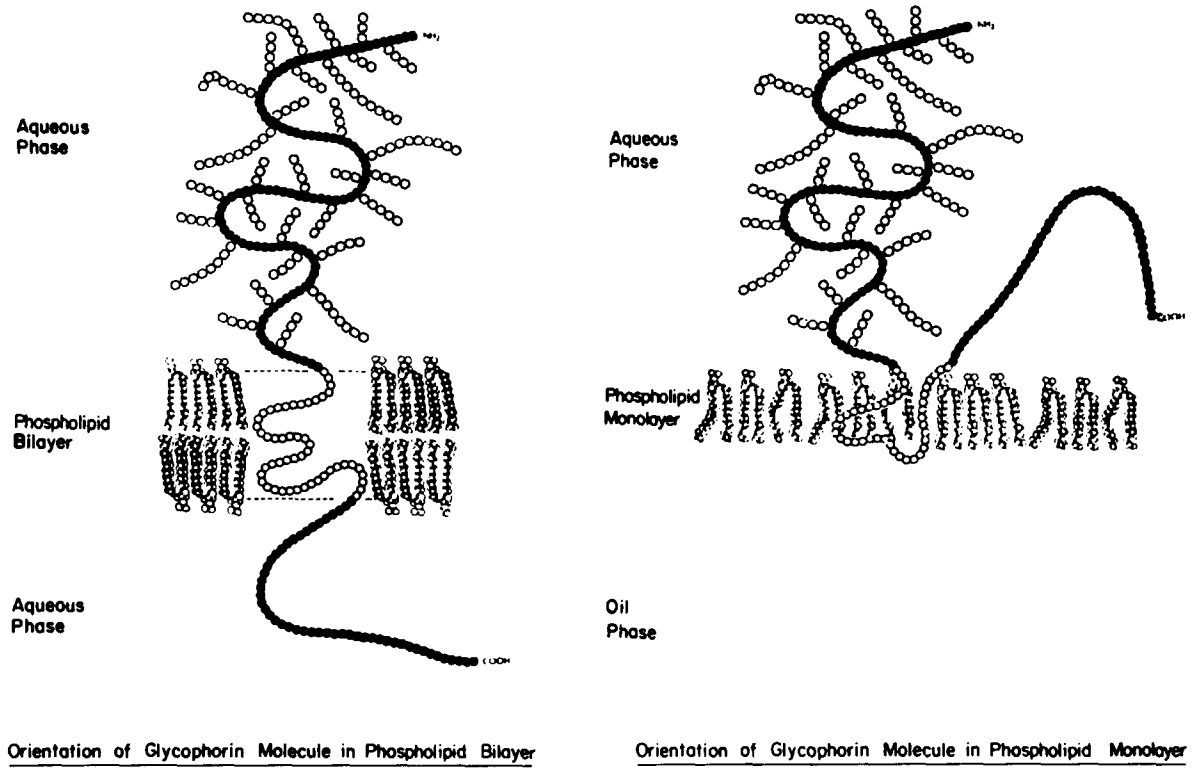

Fig. 7. The orientation of glycophorin molecules at a lipid bilayer/water interface (left) and a lipid monolayer/water interface (right).

Time- and concentration-dependence of glycophorin adsorption

The effect on the dextran/poly(ethylene glycol) contact angle of glycophorin adsorption at the oil/water interface is shown in Figs. 5 and 6. With the 4% dextran M_r 2000000)/4% poly(ethylene glycol) (M_r 20000) system the contact angle attains its minimum value of 90° at a bulk phase glycophorin concentration of 1 mg/ml. This protein concentration was used in the experiments reported in Table II and Fig. 3 C. The decline in contact angle with time (Fig. 6) presumably reflects the rate of transfer of glycoprotein molecules from the aqueous phase to the interface; the relatively show rate of adsorption may result from the time required for intercalation of the hydrophobic amino acids of the protein into the lipid monolayer (Fig. 7) and/or the time required for dissociation of aqueous aggregates of protein molecules. The experiments reported in Table II were conducted after at least 2 h had been allowed for adsorption.

Discussion

The present work demonstrates that the thermodynamic properties of intact cell surfaces can be reproduced using defined membrane components. The probable structural arrangement of glycoprotein in the phospholipid monolayer and bilayer is illustrated schematically in Fig. 7. Despite the differences in orientation of the 'cytoplasmic' polypeptide chain in the two configurations, the similarity in the interfacial energies indicates that the hydrophilic glycosylated portion of the molecule dominates the interfacial activity.

The DPPC molecule is approx. 2.8 nm long, depending on the conformation of the headgroup. Molecular modelling indicates that the glycophorin molecule in a fully extended configuration will project 20–25 nm from the phospholipid headgroups. These dimensions, along with the assumption that the fluorocarbon/DPPC interface is located at the alkyl terminal of the DPPC molecule (based on the complete immiscibility of DPPC and FC 40) allows the structure of the reconstituted interface to be correlated with the thermodynamic measurements of the individual components.

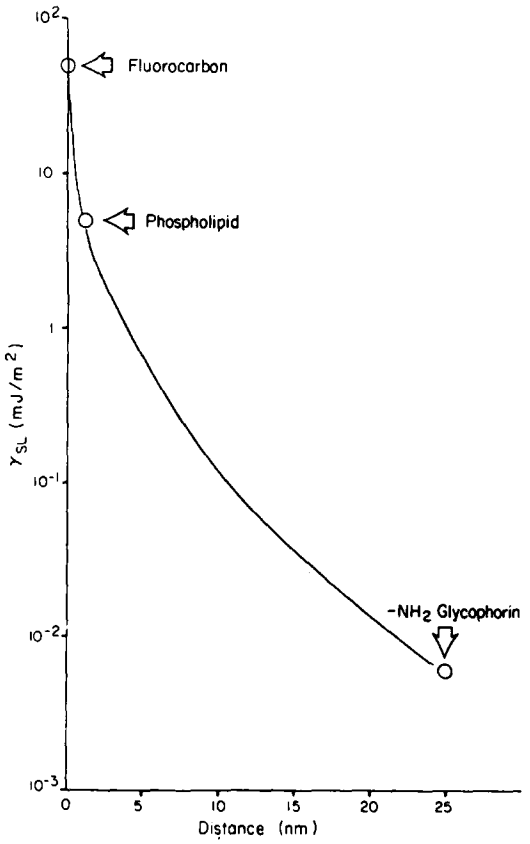

Fig. 8. Relationship between molecular dimensions and interfacial free energy in the oil/lipid/glycoprotein/water/system.

This point may be clarified by reference to Fig. 8 in which the interfacial free energies from Tables I and II are plotted against the molecular dimensions of the mixed monolayer. Taking the fluorocarbon/water interface as the origin, γ_{AC} falls by more than four orders of magnitude over a distance of about 25 nm.

Thus, the question 'what is the interfacial free energy at the cell surface?' does not have a unique answer; it is dependent on the precise location of the interfacial plane, and in a complex solid/liquid/gel system like the surface of a living cell there are many interfaces. Patchwise heterogeneity is well recognized as a source of variation in interfacial energy [28] and hysteresis of contact angles. In interfaces having appreciable thickness, such as those at biological surfaces, an additional

source of variation in interfacial energy arises from the thickness of the interfacial zone.

The biological relevance of these conclusions lies in the fact that the free energies of interactions occurring at different locations in biological surfaces may vary by more than three orders of magnitude. Thus, interactions occurring primarily at the glycocalyx/water interface (for example, platelet adhesion) will probably exhibit free energies of adhesion in the range of $\mu J \cdot m^{-2}$, while interactions taking place primarily at the phospholipid/water interface (for example, membrane fusion and phagocytosis) will probably have free energies of adhesion in the range of $mJ \cdot m^{-2}$.

The low magnitude of the glycocalyx/water interfacial energy indicates that interactions at this interface will be highly reversible and thus suited to the adhesion characteristics of cell motility and chemotactic phenomena [1]. By contrast, the much higher free energies of the lipid/water interface suggest that stable, relatively irreversible biosurface interactions probably involve the deeper parts of the interface [2].

The thermodynamic complexity resulting from structural heterogeneity of the biosurface is probably of considerable importance in attempts to selectively control adhesion to biological surfaces. Thus, the apparently diverse fields of drug carriers [29] and thromboresistant surfaces [30,31] involve attempts to maximize and minimize adhesion between biological surfaces and artificial macromolecular structures. In the case of drug carriers the object is to enhance the interaction between the carrier and the target cell; in the case of thromboresistant surfaces, the converse is true. In both cases, rational design of the artificial system requires knowledge of the relationship between thermodynamics and structure of the appropriate interfacial interactions.

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