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RECOMBINATION OF HUMAN ERYTHROCYTE APOPROTEIN AND LIPID

I. Interaction of Apoprotein and Lipid at the Air-Water Interface

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The formation and stabilization of a complex between total erythrocyte apoprotein and monolayers of total erythrocyte lipid as measured by changes of surface pressure ($\Delta\pi$) and rate of change of surface pressure ($d\pi/dt$) was studied as a function of pH, ionic strength, and lipid surface pressure. Penetration of apoprotein into lipid monolayers was favored by conditions in which lipid and apoprotein were oppositely charged. Once the interaction was completed, the resultant surface complex was resistant to large changes in subphase pH and ionic strength as shown by the insensitivity of $\Delta\pi$ to these parameters. The $d\pi/dt$, however, showed strong dependence on pH and ionic strength, but not on lipid surface pressure. A sharp decrease in $d\pi/dt$ around pH 3.5–4.5 is associated with the change in apoprotein charge from (+) to (–). Comparison of complex formation between apoprotein and bovine serum albumin, cytochrome c, and human hemoglobin suggests that erythrocyte apoprotein was specialized in its interaction with erythrocyte lipids. The data show that formation of an apoprotein-lipid complex at the air-water interface has both electrostatic and hydrophobic components. This contradicts results from other laboratories studying erythrocyte membrane recombination by bulk methods.

INTRODUCTION

Bulk recombination of proteins and lipids extracted from biological membranes has provided some insight into the forces stabilizing membrane structure. In particular, erythrocyte membrane components have been extensively probed by this technique. Zwaal and van Deenen (1) used 2-butanol extracted erythrocyte proteins and suggested that both electrostatic and hydrophobic forces may be involved in recombination with lipids. Zahler and Weibel (2) used 2-chloroethanol extracted erythrocyte proteins and found that recombination with lipid was insensitive to ionic strength. This suggested that hydrophobic forces were primarily involved. Schubert et al. (3) and Schubert (4) found

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that such results depend strongly on the manner in which the proteins were isolated. Thus, considerable disagreement exists on the relative importance of electrostatic and hydrophobic forces in formation of protein-lipid recombinates.

A requirement of bulk recombination techniques is that the protein and lipid must be kept in contact for long periods of time. Little is known of the time course of recombination and the relative amounts of lipid and protein in the complex are difficult to control. These difficulties may be overcome by use of the surface chemistry tehcniques in which protein is permitted to interact with lipid monlayers. In this system, controlled amounts of protein are introduced beneath lipid monolayers and changes in surface pressure provide an estimate of both the rate and extent of protein-lipid interaction. The total change in surface pressure ($\Delta\pi$) is related to penetration of protein into the lipid monolayer (5), and the degree of electrostatic faciliation or recombination is reflected in measurements on the rate of surface pressure change, $d\pi/dt$ (6). Furthermore, introduction of protein beneath a lipid monolayer is a model for membrane synthesis and later incorporation of protein into pre-existing membranes.

This paper reports a study of the interaction between total erythrocyte membrane protein (apoprotein) and total erythrocyte lipids spread as monolayers. By comparative measurements of $\Delta\pi$ and $d\pi/dt$, I have attempted to determine the relative contributions of electrostatic and hydrophobic interactions during recombination of erythrocyte lipid and apoprotein in monolayers. In particular, the effect of ionic strength, pH, and the surface pressure of the lipids prior to introduction of apoprotein (π_i) on these parameters was investigated. The results are compared to previous studies of bulk-phase recombinates. Since several erythrocyte proteins should be large enough to visualize by electron microscopy, it was important to determine whether the penetration reactions of apoprotein into the lipid could be directly demonstrated. In the accompanying paper (7) we report the first direct correlation of an electron microscopic image with protein-lipid penetration reactions in monolayers.

MATERIALS AND METHODS

All chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany), except glycine, bovine serum albumin (BSA), and cytochrome c, which were obtained from Sigma (St. Louis). Human hemoglobin was obtained from lysed erythrocytes. Water was deionized and redistilled from quartz, and 2-chloroethanol was also redistilled.

Erythrocyte ghosts were prepared from freshly collected human blood by the method of Hoogeveen et al. (8) or by the method of Dodge et al. (9). No measurable differences in apoprotein were found between these procedures. Apoprotein was prepared by the method of Zahler and Weibel (2) in the laboratory of Zahler. Ghosts were solubilized in 90% 2-chloroethanol, and apoprotein was separated from the lipids by LH-20 Sephadex and stored in 2-chloroethanol at $-10^{\circ}C$ at a concentration of 5 mg/ml. The apoprotein was heterogeneous and gave several reproducible bands by disc-gel electrophoresis (2). Total erythrocyte lipids were prepared from erythrocyte ghosts by the method of Folch et al. (10) and stored at $-10^{\circ}C$ at 2 mg/ml in chloroform. Protein was measured by the method of Lowry et al. (11).

Surface pressure was measured by a platinum Wilhelmy plate (12) of perimeter 39.6 mm coated with platinum grey. The plate was suspended from a Statham Universal Transducing Cell, Model UC-2. The transducer was fitted with a micro-scale accessory. A

Hewlett-Packard transducer/amplifier indicator, Model 311A, amplified the signal. The output was recorded by a Philips 12-channel recorder, Model PR 3500. The Teflon trough was divided into three compartments, each with a volume of 100 ml and a surface area of 100 cm^2. A frame was constructed to shift the protein-lipid film from one compartment to another while continuously monitoring surface pressure. In this way, the film could be exposed to different subphases. The subphase was stirred magnetically; the apparatus was kept in a constant temperature room at 20°C.

The subphase usually consisted of 10 mM buffer (glycine-NaOH at pH 2.5-3.5, citrate-NaOH at pH 4.5, Tricine-NaOH at pH 5.5-6.5, or Tris-HCl at pH 7.5-8.5) and 1 mM Na_2EDTA. The lipids were spread onto the subphase surface with a 10 μl Terumo microsyringe. Apoprotein dissolved in 2-chloroethanol or other proteins dissolved in water were slowly injected into the subphase through a hole in the frame to prevent protein from contacting the lipid film prematurely. Protein concentration in the subphase was always 10 μg/ml and apoprotein was completely soluble at this concentration. The surface pressure was not affected by 2-chloroethanol alone. Each point on the graphs to follow represents a separate experiment.

RESULTS

To compare the effect of lipid on surface pressure changes generated by apoprotein interaction at the interface, it was first necessary to measure $\Delta\pi$ and $d\pi/dt$ in the absence of lipid. These results are summarized in Table I. It is apparent that the apoprotein is surface active, and that subphase conditions affected the rate and extent of protein entry into the interface. The $\Delta\pi$ values were not greatly affected by pH, but $d\pi/dt$ values were increased approximately 15-fold at pH 3.5 compared to pH 8.5.

In the results to follow, it will be seen that the presence of lipid monolayers further increased both $\Delta\pi$ and $d\pi/dt$, the latter by nearly 10-fold. These results are best explained in terms of formation of protein-lipid complexes at the interface. I will assume that relatively high $\Delta\pi$ values and insensitivity to subphase pH and ionic strength conditions suggest greater stability of hydrophobic interactions in the complex (13, 14). Relatively high $d\pi/dt$ values indicate electrostatic facilitation of complex formation (6).

Effect of Salts on Apoprotein-Lipid Interaction

When apoprotein was injected beneath a monolayer of erythrocyte lipid (2 dynes/cm) at pH 3.5, $\Delta\pi$ rose rapidly to a value of 22 dynes/cm (Fig. 1). This demonstrated the presence of a complex between apoprotein and lipid at the surface. Once the interaction was completed, as indicated by no further change in surface pressure, the apoprotein-lipid film was washed free of excess apoprotein by moving it onto compartments on the trough containing only water. When the washed film was exposed to a subphase of pH 1-12 or subphase containing 4.5 M NaCl or ~~1 M~~ $LaCl_3$, no change in $\Delta\pi$ occurred. This indicates that the forces stabilizing the complex are strongly hydrophobic in nature. Both $\Delta\pi$ and $d\pi/dt$ decreased when 1 M NaCl was present in the subphase during formation of the complex. This indicates that formation of the complex was influenced by electrostatic charge.

Table I. Surface parameters for Spreading of Eryhtrocyte Apoprotein in the Absence of Lipid[a]

		$\Delta\pi$ dynes/cm	$d\pi/dt$ dynes/cm/min
pH	3.5	16.5	1.3
	8.3	11.2	0.2

[a]The subphase contained 10 mM buffer (glycine-NaOH at pH 3.5, Tris-HCl at pH 8.5) and 1 mM Na_2 EDTA. Protein was injected to a final subphase concentration of 10 μg/ml. In the presence of lipids, values greater than these signify formation of an apoprotein-lipid complex. Each value is an average of two experiments.

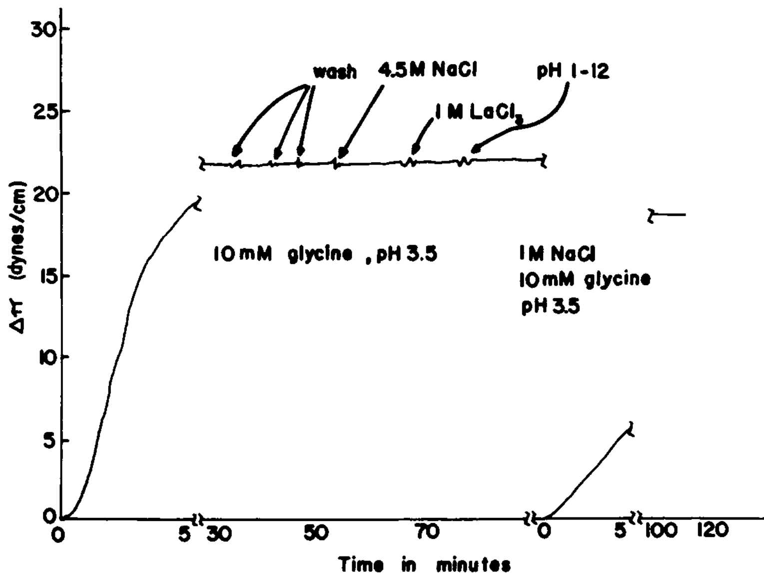

Fig. 1. Interaction of erythrocyte apoprotein with erythrocyte lipid. The lipid was first spread to a π_i of 2 dynes/cm. Apoprotein was then injected to a final subphase concentration of 10 μg/ml (time 0). Composition of the subphase is given above. In the first experiment, $\Delta\pi$ is 22 dynes/cm and $d\pi/dt$ is 13 dynes/cm/min. Once π ceased to change, the complex was washed free of uncomplexed apoprotein and subjected to a number of treatments. In the second experiment, $\Delta\pi$ is 17 dynes/cm and $d\pi/dt$ is 4 dynes/cm/min. This figure is a composite of several experiments.

Effect of pH and Initial Lipid Surface Pressure (π_i) on Apoprotein-Lipid Interaction

To measure the extent of apoprotein conformation changes at different pH values (13, 14), $\Delta\pi$ was studied at pH 3.5 and 8.5 (Fig. 2a). In addition, π_i was varied to determine the limiting value of $\Delta\pi$ at which apoprotein could penetrate. This provides information about the relative thermodynamic work necessary for penetration (13, 14).

At all values of π_i, $\Delta\pi$ at pH 3.5 was somewhat greater than at pH 8.5. This suggests that the protein conformation at the surface is affected by pH. Increasing π_i caused linear decreases in $\Delta\pi$. This is characteristic of most proteins injected beneath lipid monolayers. The slopes of these curves are the same at both pH 3.5 and 8.5, but at pH 3.5, apoprotein can penetrate erythrocyte lipid at a higher π_i. Thus, the work required for apoprotein penetration at pH 3.5 is less than at pH 8.5.

The $d\pi/dt$ was strongly influenced by pH and much less by π_i (Fig. 2b). Apoprotein penetration proceeded much faster at pH 3.5 and remained high as π_i increased up to 20 dynes/cm. A peak in $d\pi/dt$ was observed at 2 dynes/cm as was present in some simpler systems (6).

The effect of aproprotein charge on complex formation and stabilization was observed by interacting aproprotein and lipid at various subphase pH values. When $\Delta\pi$ and $d\pi/dt$ were studied between pH 2.5 and 8.5 (Fig. 3) and π_i held constant at 2 dynes/cm, $d\pi/dt$ showed a steep decrease between pH 3.5 and 4.5. This corresponds to the isoprecipitation range of n-butanol extracted erythrocyte apoprotein (1). The $\Delta\pi$ showed, as expected, a slight decrease with increasing pH. Since erythrocyte lipids are negatively charged over the pH range studied, one explanation of the results is that the change in apoprotein charge from (+) to (–) in the isoprecipitation range was responsible for the change in $d\pi/dt$.

Comparison with Bovine Serum Albumin-Erythrocyte Lipid Interaction

To test this hypothesis, the experiments shown in Fig. 3 were repeated using a single, purified protein (Fig. 4). BSA was chosen because it has a pK_a of 4.6. The $d\pi/dt$, as expected, showed a sharp decrease between pH 4 and 5.6. However, $\Delta\pi$ decreased drastically at pH values above 6. This corresponds to large changes in BSA conformation at pH values above its isoelectric point (15).

Comparison of Protein-Lipid Interaction with Cytochrome c, Bovine Serum Albumin, and Human Hemoglobin

By far the most prevalent protein in the erythrocyte is hemoglobin, but little, if any, is present in the membrane (9). To determine if the formation and stabilization of the apoprotein-erythrocyte lipid complex was favored over a complex between erythrocyte lipids and hemoglobin or other water-soluble proteins, the $\Delta\pi$ and $d\pi/dt$ of apoprotein, BSA, cytochrome c, and human hemoglobin were studied as a function of π_i. This was done at pH 8.5.

Apoprotein was first injected into one compartment of the trough and stirred for about 30 minutes. Since apoprotein precipitates rapidly when injected at high concentrations into water (unpublished observation) this period was considered adequate for apoprotein to make any conformational changes related to the change from an organic to an aqueous environment. Erythrocyte lipid was then spread on another compartment

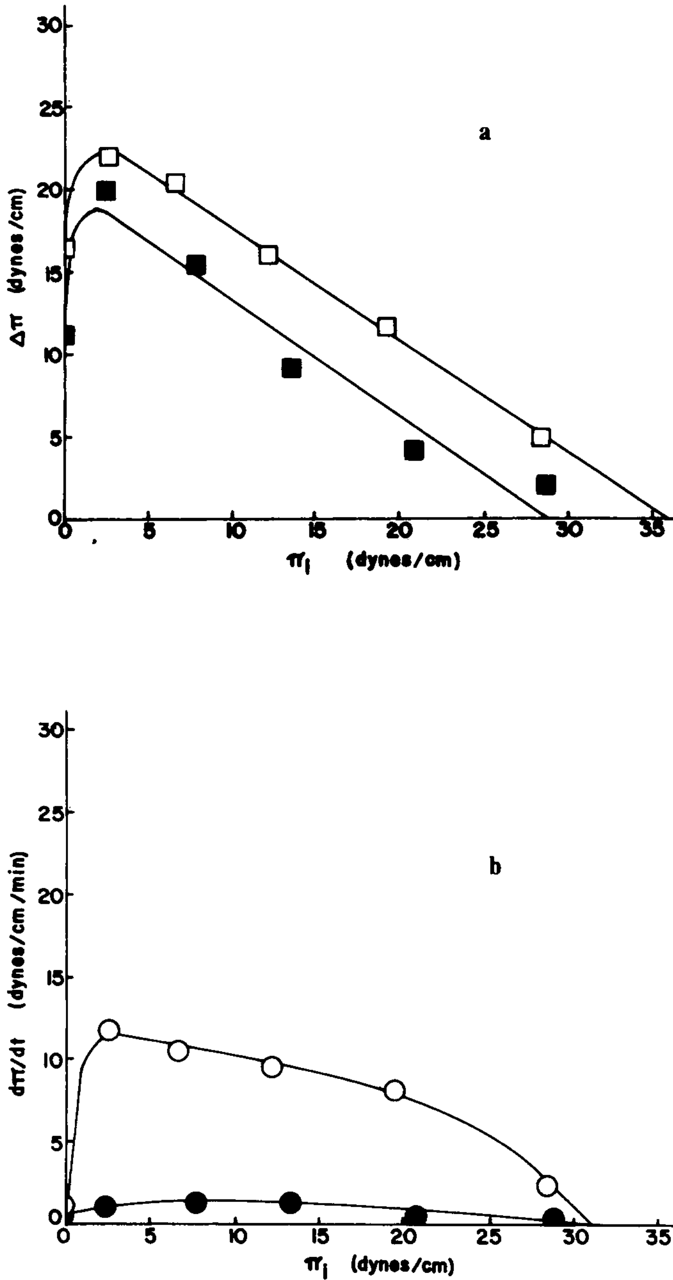

Fig. 2. (a) Dependence of $\Delta\pi$ on π_i and pH. The subphase contained 10 mM buffer (glycine-NaOH at pH 3.5 and Tris-HCl at pH 8.5) and 1 mM Na_2EDTA. The lipid was initially spread to the indicated surface pressure and apoprotein injected beneath to a subphase concentration of 10 μg/ml. The calculated regression lines give the values where apoprotein can no longer penetrate the lipid monolayer as 28 dynes/cm and 36 dynes/cm at pH 8.5 and 3.5, respectively. □, pH 3.5: ■,pH 8.5. (b) Dedendence of $d\pi/dt$ for apoprotein on π_i and pH. All conditions are the same as (a). ○ pH 3.5;
●, pH 8.5

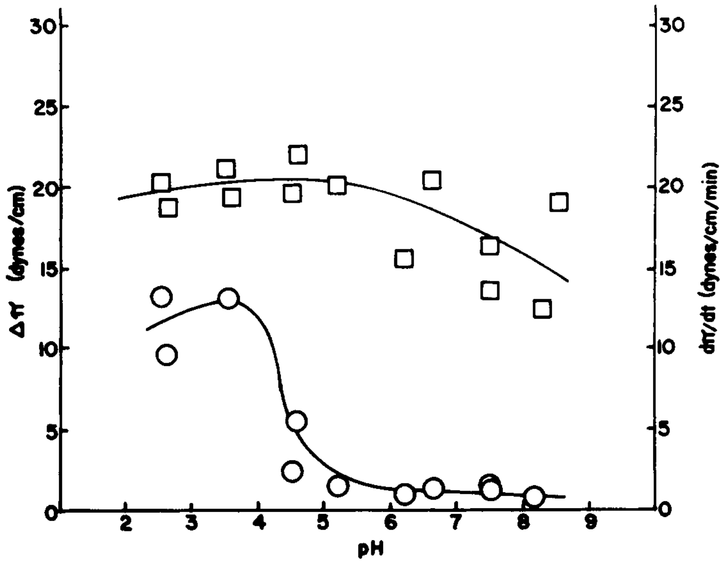

Fig. 3. Dependence of $\Delta\pi$ and $d\pi/dt$ on pH. Lipid surface pressure was 2 dynes/cm. The subphase contained 10 mM buffer (glycine-NaOH at pH 2.5-3.5, sodium citrate-NaOH at pH 4.5, Tricine-NaOH at pH 5.5-6.5, and Tris-HCl at pH 7.5–8.5) and 1 mM Na_2EDTA. Erythrocyte apoprotein was injected to a final subphase concentration of 10 μg/ml. □, $\Delta\pi$; ○ $d\pi/dt$.

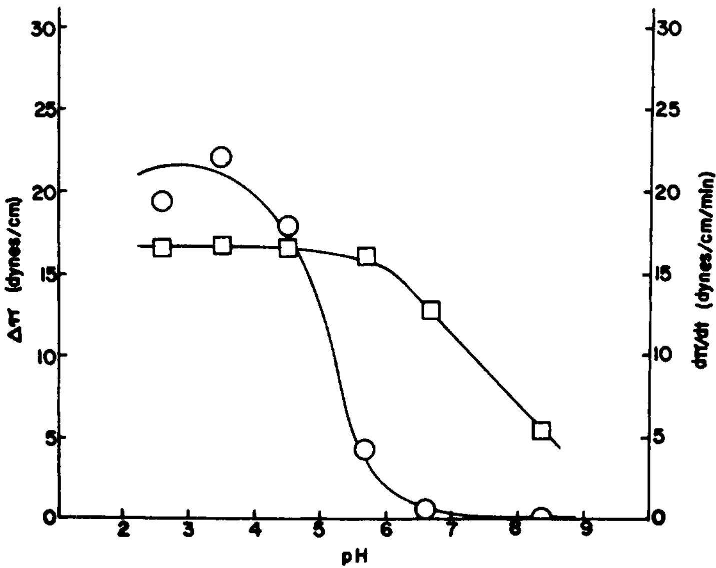

Fig. 4. Bovine serum albumin erythrocyte lipid complex formation. All conditions similar to Fig. 3. □, $\Delta\pi$; ○, $d\pi/dt$.

of the trough and moved over the compartment containing apoprotein. Results for both $d\pi/dt$ and $\Delta\pi$ obtained in this manner were comparable to $d\pi/dt$ and $\Delta\pi$ values when apoprotein in 2-chloroethanol was injected directly beneath the lipid monolayer.

The $\Delta\pi$ of apoprotein increased 9 dynes/cm over the $\Delta\pi$ of apoprotein alone. In the presence of an expanded lipid film (Fig. 5a), the $\Delta\pi$ for BSA and human hemoglobin only decreased as π_i increased. Cytochrome c was intermediate at 3 dynes/cm. The $d\pi/dt$ for cytochrome c and hemoglobin were greater than the $d\pi/dt$ of apoprotein (Fig. 5b). This suggests facilitation of binding of cytochrome c and hemoglobin, but the complex formed in both cases is much weaker than for apoprotein. In this sense, erythrocyte apoprotein seems specialized in its interaction with erythrocyte lipid.

DISCUSSION

Although extraction of erythrocyte apoprotein by 2-chloroethanol may cause denaturation, this study has shown that apoprotein can penetrate into monolayers of erythrocyte lipid in a strong and predictable manner. Formation of the complex is in part dependent upon favorable electrostatic interaction between lipid and protein, while stabilization of the complex is due largely to hydrophobic forces. The strong hydrophobic binding between apoprotein and lipid was expected from bulk recombination data of this preparation (2). Because of the hydrophobic nature of apoprotein, it was surprising that the apoprotein-lipid complex formed over a wide range of pH, ionic strength, and lipid surface pressure. This shows that the electrostatic component in complex formation was significant under all the above conditions. Apoprotein apparently behaves similarly to water-soluble proteins in this respect.

Nature of the Apoprotein and Lipid Fractions

The composition of human erythrocyte apoprotein is heterogeneous. Glycophorin represents about 10–30% of the total protein (16, 17), spectrin contributes about 20% (18), and "component a" contributes about 25% (17). The phospholipid fraction contains about 29% phosphatidyl choline, 27% phosphatidyl ethanolamine, 27% sphingomyelin, and 13% phosphatidyl serine; cholesterol represents about 30% of the total lipids (19). Both protein and lipid may be distributed asymmetrically between the two sides of the intact erythrocyte membrane (17, 20), but such asymmetry is absent from a protein-lipid complex formed from extracted components. A single protein could be selectively incorporated into the monolayer and be totally responsible for $\Delta\pi$, but this seems unlikely. Probably the final complex consists of a mixture of proteins penetrating the lipid monolayer, but not necessarily duplicating the protein distribution found in the intact erythrocyte membrane. Future work should be directed toward studies of specific erythrocyte membrane proteins interacting with lipid monolayers.

$\Delta\pi$ and $d\pi/dt$

Quinn and Dawson (13) show that large changes in $\Delta\pi$ with pH reflect conformational changes of protein at the interface. The $\Delta\pi$ of apoprotein, however, shows no wide variations over a considerable range of pH (Figs. 1 and 3). This supports the assumption that

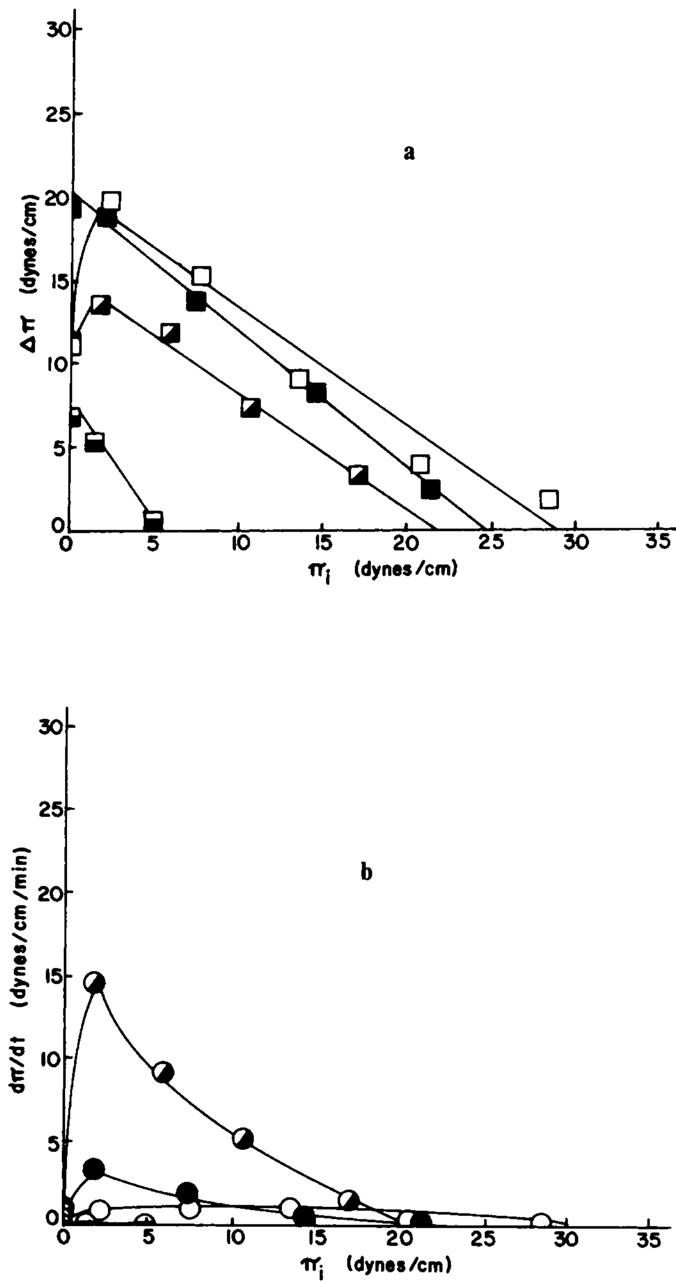

Fig. 5. (a) Dependence of $\Delta\pi$ on the complex formation of erythrocyte apoprotein (□), cytochrome c, (◪), human hemoglobin (■), and BSA (⊟) with erythrocyte lipids. The subphase contained 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.5. Protein was injected into the subphase to a final concentration of 10 μg/ml. The values for $\Delta\pi$ at π_i = 0 were taken with no lipid present on the surface. (b) Dependence of $d\pi/dt$ on the complex formation of erythrocyte apoprotein (○), cytochrome c (◐), human hemoglobin (●), and BSA (⊖) with erythrocyte lipids. Conditions were the same as in Fig. (a). In all cases, $d\pi/dt$ values in the absence of lipid were small.

no large conformational changes of the apoprotein occur under experimental conditions and that the $d\pi/dt$ results are most suitably explained by changes in electrostatic properties of apoprotein and lipid.

The strong dependence of $d\pi/dt$ on pH (Figs. 2a and 3) and ionic strength (Fig. 1) shows that the electrostatic components during complex formation are large. A π_i of 2 dynes/cm was chosen to maximize this effect, but Fig. 3 shows that the difference in $d\pi/dt$ between pH 3.5 and 8.5 extends over the entire range of π_i where protein will penetrate.

For a protein mixture, proteins for which penetration is electrostatically facilitated at a given pH presumably penetrate faster. The values of $d\pi/dt$ would reflect the penetration rate of the fastest component. The $d\pi/dt$ is not, however, diffusion limited. Assuming 19 sec are required to form a monolayer of protein beneath the lipid film (6), and that a monolayer of protein is required to produce the observed $\Delta\pi$ values (13, 14), the maximum $d\pi/dt$ would be

$$\frac{22 \text{ dynes/cm}}{10 \text{ sec}} \times 60 \text{ sec/min} = 70 \text{ dynes/cm/min} \quad (1)$$

Even under favorable charge conditions, $d\pi/dt$ is only 15 dynes/cm/min (Fig. 2b). Presumably, not every contact between apoprotein and lipid results in apoprotein penetration since the values of $d\pi/dt$ are correspondingly smaller.

Relation to Membrane Formation in the Erythrocyte

Bretscher (17) has suggested that erythrocyte membrane proteins may be synthesized inside the cell and incorporated into the membrane at a later time. Penetration of apoprotein into lipid monolayers is an analogous situation. Injection of apoprotein beneath the lipid would be a model for protein synthesis in vivo. Although the values of $d\pi/dt$ in the pH range 6-8 suggest that complex formation would be slow, it would be strongly stabilized by hydrophobic forces. Erythrocyte apoprotein would be selectively complexed with the membrane over water-soluble proteins.

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