FREE ENERGY POTENTIAL FOR AGGREGATION OF ERYTHROCYTES AND PHOSPHATIDYLCHOLINE/PHOSPHATIDYLSERINE VESICLES IN DEXTRAN (36,500 MW) SOLUTIONS AND IN PLASMA

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ABSTRACT The free energy potential (affinity) for aggregation of human red blood cells and lipid vesicles in Dextran solutions and blood plasma has been quantitated by measuring to what extent a vesicle is encapsulated by the red cell surface. The free energy reduction per unit area of contact formation (affinity) was computed from the observation of the fractional extent of encapsulation at equilibrium with the use of a relation based on the elastic compliance of the red cell membrane as it is deformed to adhere to the vesicle surface. Micromanipulation methods were used to select and transfer single lipid vesicles (2–3 $\times$ 10^{-4} cm diameter) from a chamber that contained the vesicle suspension to a separate chamber on the microscope stage that contained red cells in an EDTA buffer with Dextran or whole plasma. The vesicle and a red cell were maneuvered into close proximity and contact allowed to take place without forcing the cells together. To evaluate the effects of surface charge density and steric interactions on aggregation, vesicles were made from mixtures of egg phosphatidylcholine (PC) and bovine phosphatidylserine (PS) over a range of mole ratios (PC/PS)from (1:0) to (1:1); the vesicles were formed by rehydration in buffer. The Dextran solutions were made with a sharp-cut fraction of 36,500 MW in a concentration range of 0–10% by weight in grams (wt/wt). It was found that the Dextran 36,500 MW fraction produced aggregation behavior for red cells and vesicles similar to red cell–red cell aggregation in Dextran 70,000–150,000 MW fractions, when the vesicle surface charge density was comparable with that of normal red cells (i.e., PC/PS ratio of ~3:1). This result indicated that Dextran molecules penetrate between the carbohydrate groups on the red cell surface and that either steric interactions between cell surface carbohydrates are important or many of the charge groups on red cells are superficial. Electrostatic repulsion effects were apparent, as no aggregation in Dextran 36,500 MW occurred for PC/PS ratios <2.6:1; the level of affinity increased with the PC content at a specific Dextran concentration. Affinities were measured in the range of 0–2 $\times$ 10^{-2} ergs/cm^2. When adherent red cell–vesicle pairs were transferred into a Dextran-free buffer, the pair did not spontaneously separate. They maintained adhesive contact until forcibly parted, after which they would not readhere. This demonstrates that Dextran forms a "cross-bridge" between the membrane surfaces. Red cell–vesicle aggregation was also tested in whole plasma, which normally yields affinity values in the range of 2–4 $\times$ 10^{-3} ergs/cm^2 for red cell–red cell aggregation. However, no red cell–vesicle aggregation occurred in plasma, even for pure PC vesicles. This result indicates that either the aggregating plasma proteins (primarily fibrinogen) do not bind sufficiently to the lecithin surface, or they are shielded from binding to the surface by the presence of other nonaggregating components (perhaps albumin).

INTRODUCTION

Plasma proteins and Dextran, a glucose polymer, promote aggregation of red blood cells in solution. The aggregates exhibit close, uniformly spread cell-cell contact over large areas of the cell surfaces. Important features of the aggregation have been well described by Brooks (1973 *b*), and Chien and associates (Jan and Chien, 1973): (*a*) normal red cells are not aggregated by Dextrans with molecular weights less than ~50,000, (*b*) aggregation commences at low concentrations of <1% by weight in grams (wt/wt), (*c*) aggregation of normal red cells does not occur for Dextran concentrations above 5–10% by weight in grams, and (*d*) aggregation depends on cell surface charge. Thus, there is a window of Dextran concentrations within which normal red cells are aggregated; outside this window, aggregation does not occur even though the adsorption of Dextran is not saturated (Brooks, 1973 *a*; Brooks et al., 1980; and Chien, 1980). These features have been verified in controlled aggregation tests of single red blood cells; in addition, the

level of chemical affinity between red cell membranes has been determined in these tests as a function of Dextran concentration and molecular weight (Buxbaum et al., 1982). The affinity between membrane surfaces is defined by the reduction in free energy per unit area that is associated with the initial formation of adhesive contact. As such, the potential for cells to aggregate is represented by surface affinity. Values of affinity for normal red cells range from low values characteristic of aggregation in plasma (10^{-3} ergs/cm^2) to values above 10^{-2} ergs/cm^2 in solutions of 150,000 MW Dextran (Buxbaum et al., 1982). The peak affinityfor aggregation of normal cells goes up with Dextran molecular weight. When cells have been treated with neuraminidase to partially remove cell surface charge (Haydon and Seaman, 1967), the levels of affinity are much higher for a given molecular weight and concentration of Dextran. Also, molecular weights as low as 28,000 give measurable levels of affinity between neuraminidase-treated cells. This demonstrates that electrostatic repulsion is a prominent mechanism in red cell aggregation. In addition, it has been proposed (Brooks, 1973 *b*) that the adsorption of Dextran may act to expand the electric double layer and thus be responsible for quenching the aggregation process at high Dextran concentrations. This thesis was consistent with the observation that removal of large amounts of cell surface charge by neuraminidase treatment appeared to abolish the quenching affect and with the observed effect of ionic strength on the quenching concentration.

The cell-surface charge is distributed in a thick layer of carbohydrate groups on the red cell membrane (Steck and Dawson, 1974; Steck, 1974); thus,it is difficult to separate electrostatic repulsion from steric interactions that occur between red cell surfaces. Enzymatic removal of these groups clearly affects both interactions. To evaluate the effects of cell-surface charge distribution and density on cell aggregation, we have performed controlled aggregation tests of single red blood cells with large lipid vesicles made from mixtures of egg phosphatidylcholine (PC) and bovine phosphatidylserine (PS). From these tests, we have derived the affinity (defined by the free energy reduction per unit area of contact) between the red cell and vesicle membrane as a function of the vesicle surface charge density and Dextran concentration. Similarly, we have examined the potential for aggregation of red cells and lipid vesicles in native plasma.

EXPERIMENTAL PROCEDURES

Experiments were performed with red blood cells (RBC) from a single healthy donor (type O, Rh$^+$) obtained by finger prick. The cells were suspended at very low hematocrit in either filtered plasma or phosphate buffered saline (PBS)–Dextran solution that contained human serum albumin (0.5% by weight in grams). The presence of albumin had no affect on the aggregation; however, it did preserve the discoidal shape of the red cells and eliminated adhesion to the glass micropipettes.

Plasma was obtained as the supernate from blood-type compatible samples centrifuged at 8,000 *g* and room temperature for 2 min to remove cells. The buffy coat and red cells were removed, then the plasma was filtered. Concentrated stock solutions of Dextran (36,500 MW) were made in distilled water with 0.2% by weight in grams sodium azide to retard bacterial growth. Concentrations of stock solutions were accurately measured by polarimetry. Subsequent dilutions of the Dextran stock solution into concentrated PBS were made before experimentation to obtain the desired concentration and ionic strength. The PBS contained 25–30 mM phosphate to maintain PH at 7.4, 0.1 mM EDTA, with the final osmotic strength of 250 mOsM. The hypoosmotic buffer was used to slightly swell the red cells and vesicles for better observation of the vesicle encapsulation. The Dextran was a sharp-cut fraction with a weight-averaged molecular weight of 36,500 and a ratio of weight average to number average molecular weights of 1.34 (kindly supplied by Dr. Kirsti Granath, Pharmacia designation FDR 7314, Pharmacia Fine Chemicals, Div. Pharmacia Inc., Piscataway, NJ).

Vesicles were prepared from stock solutions of PC/PS mixtures (PC, Sigma Chemical Co., St. Louis, MO; PS, Avanti Polar Lipids, Inc., Birmingham, AL; both components 99% pure) in chloroform methanol (9:1 solution). The stock solutions were stored in a freezer at −10°C. Aliquots of the stock solutions were dried under argon and placed in vacuo for 4–5 h. The samples were then suspended in 270 mOsM sucrose buffer containing 0.1 mM EDTA and allowed to sit for 1h at room temperature. Finally, the vesicle suspension in sucrose was dialyzed overnight against 270 mOsM PBS to provide the test suspension.

To prevent aggregation of small vesicles and lipid debris to the red cells, the vesicle and red cell suspensions were placed in separate chambers on the microscope stage. The experimental procedure was to select a vesicle (2–3 μm diam) with one suction micropipette, insert the vesicle inside a much larger pipette (~15 μm i.d.), then translate the microscope stage to transfer the micropipette assembly into the second chamber. The vesicle was then removed from the large, transfer pipette and held with sufficient suction pressure to leave a rigid spherical portion outside the micropipette. Next, a red cell was aspirated with a third pipette at such a low suction pressure that little deformation of the red cell occurred. Fig. 1 *A* shows a vesicle and red cell held by micropipettes before the aggregation test. Finally, the red cell was maneuvered into position proximal to the vesicle surface (but not forced to spread over the vesicle), contact was allowed to take place, and the red cell suction pipette was removed. The equilibrium contact was observed as shown in Fig. 1 *B*. All experiments were performed at room temperature. When no spontaneous aggregation occurred, the red cell was released from the pipette in a position slightly above the vesicle and allowed to settle on the vesicle surface by gravity. If there was no potential for aggregation, then the red cell simply fell off the vesicle and floated away. The aggregation was quantitated by measurement of the fractional extent of encapsulation of the spherical vesicle surface at equilibrium. This was determined from measurements of the cap height, Z_c, at the contact zone and the vesicle diameter, 2 R_s, as illustrated in Fig. 1 *C*. The fractional extent of encapsulation is defined by $x = Z_c/2R_s$. As will be discussed in the next section, it has been shown that the fractional extent of encapsulation, x, of a spherical surface by a red blood cell is a progressive function of the affinity between the red cell and sphere surfaces (Evans and Buxbaum, 1981). This relationship is derived from the free energy for elastic deformation of the red cell membrane as it conforms to the sphere. Hence, the observation of encapsulation provides a direct method to quantitate the potential for red cell-vesicle aggregation. The practical limit for measurement of the encapsulation is $x = 0.7$. Values above 0.7 are often accompanied by folding of the red cell membrane and are intrinsically difficult to measure because the cell obscures the view of the vesicle. Because vesicles are transparent, it was necessary to use interference contrast optics to observe the vesicle geometry.

RESULTS AND ANALYSIS

Because the RBC has ~3 × 10^{13} charges/cm^2 (Seaman et al., 1977; Leviné et al., 1983), we investigated a range of PC/PS ratios from 1:0 to 1:1 which approximately repre-

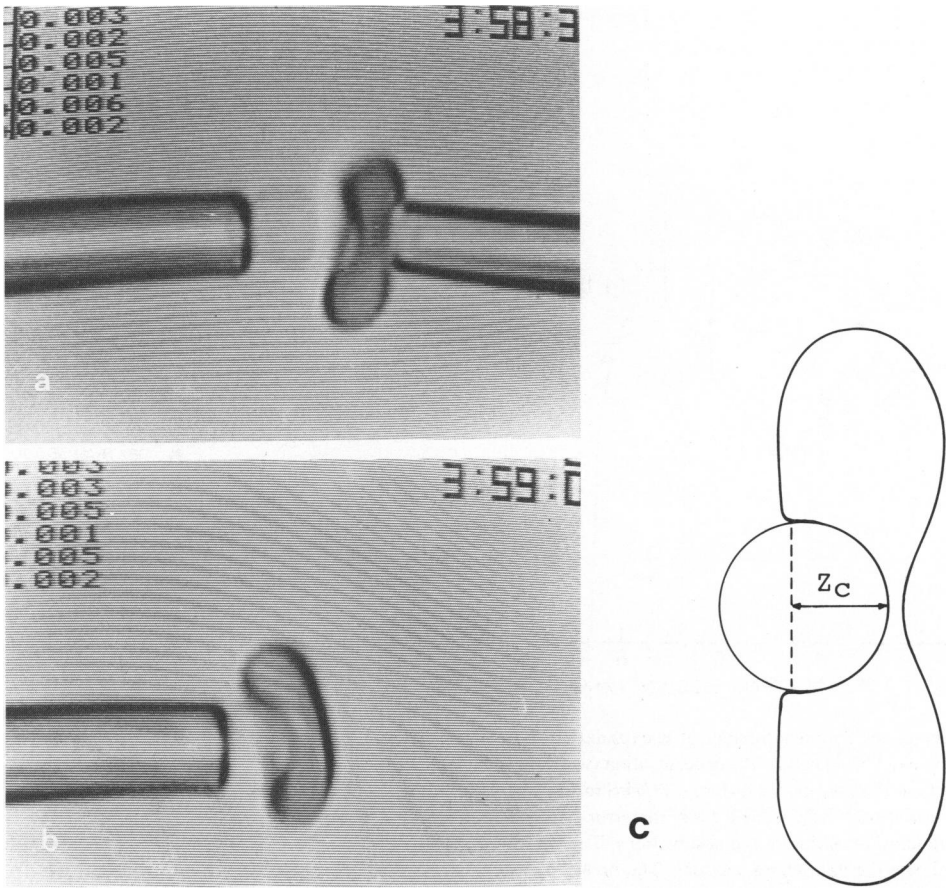

FIGURE 1 (*a*) Videomicrograph of a human red blood cell and lecithin vesicle held by micropipettes before aggregation in PBS-Dextran 36,500 MW solution. The major diameter of the red cell is $\sim 8 \times 10^{-4}$ cm; the inner diameters of the pipettes are $\sim 1 \times 10^{-4}$ cm. The image is enhanced by differential interference contrast optics. (*b*) Video micrograph of the equilibrium configuration of the red cell adhered to the vesicle after the cell was maneuvered into proximity of the vesicle and contact was allowed to take place. The red cell was observed to spread spontaneously over the vesicle surface without forcing the two capsules together. The extent of encapsulation is directly related to the affinity between the red cell and vesicle surfaces. (*c*) Minimum-energy contour, computed for a red cell encapsulation of a spherical substrate, that illustrates the parameters measured in each experiment, i.e., the cap height, Z_c, and the vesicle diameter, $2R_s$. The fractional extent of encapsulation is given by $x = Z_c/2R_s$.

sents a range of charge densities from 0–7 $\times$ 10^{13}/cm^2 (i.e., twice the RBC level). The estimates of charge densities for mixed lipids are calculated with the assumptions that the area per lipid head group is the same for either component and equal to 70 Å^2 per molecule. The assumptions are based on evidence from x-ray diffraction that the area per lipid head group in salt solutions is not greatly affected by the charge on the head group (Luzzati, 1968; Cowley et al., 1978). In our experiments, it was found that Dextran 36,500 MW produced RBC-vesicle aggregation behavior similar to RBC-RBC aggregation in Dextran 70,000 and 150,000 MW (Baxbaum et al., 1982) when the vesicle charge densities were comparable to RBC values. Thus, the aggregation tests were carried out with the sharp-cut Dextran 36,500 MW fraction. Data on the extent of encapsulation, x, as a function of Dextran concentration are cumulated in Fig. 2 for PC/PS ratios of 1:0, 4:1, 3:1, and 2.7:1. No RBC-vesicle aggregation was observed for PC/PS ratios <2.6:1 in Dextran 36,500 MW. For PC/PS compositions in the range of 4:1 to 2.7:1, the RBC-vesicle aggregation occurred in the same window of concentrations as RBC-RBC aggregation in Dextran 70,000 and 150,000 MW solutions. The data points are averages for 10–15 RBC-vesicle aggregation tests; the average values for the extent of encapsulation are shown bracketed by typical standard deviations of the measurements. The hand-drawn curves simply indicate the data characteristics and are not numerical correlations. As with RBC-RBC aggregation in Dextran, there existed an upper concentration of Dextran above which the aggregation was quenched. The concentration, where quenching commenced, was observed to depend on the vesicle surface charge density. Similarly, the onset of aggregation was observed to depend on Dextran concentration and vesicle surface density as with RBC-RBC aggregation in Dextran before and after neuraminidase treatment (Buxbaum et al., 1982). Fig. 3 is a plot of the threshold concentrations that bracket the RBC-vesicle aggregation vs. the mole fraction of PS. The solid line represents the behavior for the onset of aggregation and the dotted line characterizes

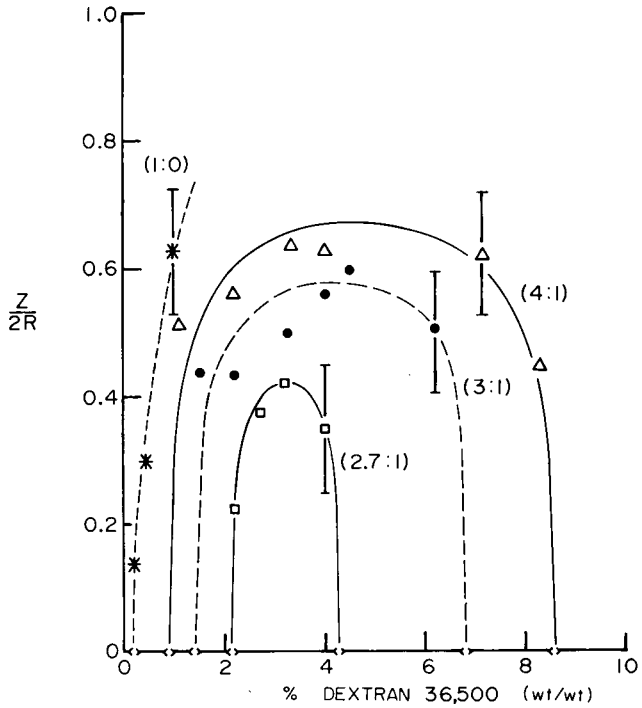

FIGURE 2 Observations of the fractional extent of encapsulation of a lecithin vesicle surface by a red blood cell vs. the concentration (wt/wt) of Dextran 36,500 MW polymer in solution for different PC/PS ratios. The data points are averages of 10–15 RBC-vesicle tests; the error bars are shown for the largest standard deviation in the observations. The curves were hand drawn to indicate the data characteristics. The breaks along the abscissa are the upper and lower concentration limits where aggregation was observed to either commence or cease for a given vesicle composition. Aggregation was observed for vesicle compositions in the range from pure PC and no PS (1:0) to 72% PC and 28% PS (2.6:1).

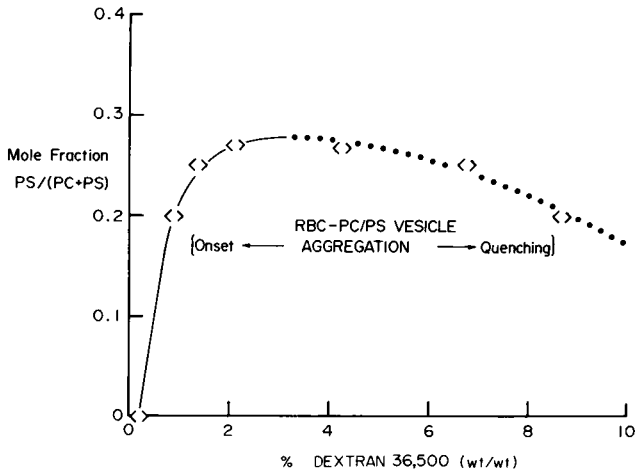

FIGURE 3 Plot of the threshold concentrations for the onset (—) and quenching (···) of the RBC-vesicle aggregation vs. the mole fraction of PS, i.e., PS/(PC + PS). The region under the curves represents the conditions where aggregation occurred in Dextran 36,500 MW solutions.

the upper concentration where the aggregation process was quenched. The curves converge at the PS fraction equivalent to a PC/PS ratio of 2.6:1, which was the maximum charge content where aggregation (very weak) could be observed. For pure vesicles (1:0), the aggregation appeared to increase without bound although no useful measurements could be made above 1% by weight in grams because the fractional encapsulation of the vesicle was >0.7. Even for Dextran concentrations as high as 25% by weight in grams, the encapsulation was greater than the observation limit. Higher concentrations were extremely difficult to use because of the high viscosity of the solutions. In support of the RBC-PC vesicle observations, our recent measurements of affinity for pure PC vesicle-vesicle aggregation show a linear increase with concentration over this range of Dextran concentrations (Evans and Metcalfe, submitted for publication).

We demonstrated that the Dextran molecules definitely form "cross-bridges" between the two membrane surfaces by the following test: after the red cell had formed adhesive contact with the vesicle, we transferred the red cell-vesicle pair to an adjacent chamber that contained only buffer with no Dextran in solution. The red cell remained adherent to the vesicle for as long as we chose (~1 h). When the red cell and vesicle were forced to separated (by micromanipulation), no reaggregation was possible.

Because RBC-RBC aggregation in plasma is comparable with aggregation in Dextran 70,000 MW and also with the RBC-vesicle aggregation in Dextran 36,500 MW, we set out to determine the PC/PS ratio that would give comparable RBC-vesicle aggregation in plasma. A very surprising result was obtained: no RBC-vesicle aggregation occurred in plasma even for pure PC vesicles without charge. This result indicates that either the aggregating plasma proteins (primarily fibrinogen) do not bind sufficiently to the lecithin surface or these aggregating plasma proteins are shielded from binding to the surface by the presence of other nonaggregating constituents (e.g., albumin). As stated in the last section, the fractional extent of encapsulation of the vesicle by the red cell surface at equilibrium provides a direct measure of the surface-to-surface affinity. This free energy reduction per unit area of contact formation is derived from the elastic compliance of the red cell membrane as it is deformed to cover the sphere. The equilibrium conformation is established when small (virtual) increases in elastic deformation energy of the red cell are just balanced by small (virtual) decreases in the free energy due to contact, i.e.,

$$\gamma = \frac{\partial W_D}{\partial A_C}, \tag{1}$$

where γ is the affinity (in ergs per square centimeter); W_D is the elastic work of deformation(in ergs); A_c is the contact area between the RBC and vesicle (in square centimeters). Because the red cell interior is liquid, the reversible work of cell deformation depends only on the membrane elastic properties and the membrane deformation. It has been shown that the compliance relation (Eq. 1) for red cell encapsulation of a spherical surface is well approximated

by

$$\gamma \simeq \mu[x^2/(1-x) - x \cdot \ln(1-x)] + B[2/R_s^2 + (2\mu/B \cdot R_s^2)^{1/2} \cdot x \cdot (1 + 3x/8 + x^2/4)], \quad (2)$$

where μ is the elastic shear (extensional) modulus and B is the bending elastic modulus of the RBC membrane (Evans and Buxbaum, 1981). This relation is not affected by the size of the sphere (other than through the bending term shown in Eq. 2) provided that the red cell has sufficient surface area in relation to its volume to allow for encapsulation of the sphere without increase in surface area. The practical size limit for spherical particles is ~3 μm when the RBC are in isotonic media. If the sphere is too large to be encapsulated, the membrane tension builds up rapidly in proportion to the affinity at essentially a fixed extent of encapsulation determined by the surface area and volume of the RBC (Evans and Parsegian, 1983).

The elastic properties of the RBC membrane have been measured and exhibit little variation between normal, healthy individuals. The membrane shear modulus is in the range of 6–7 $\times$ 10^{-3} erg/cm (Waugh and Evans, 1979). The membrane bending or curvature elastic modulus is not known as precisely because of its small value; but within a factor of 2, it is 1 $\times$ 10^{-12} erg (Evans, 1980; Evans, 1983). Hence, for vesicles on the order of 1 μm in radius, the bending elastic contribution to the compliance relation (Eq. 2) is small. Also, our measurements and the extensive work of Nash and Meiselman (1983) have shown that the elastic behavior of the RBC membrane is not affected by Dextran adsorption. Thus, with these elastic properties, the observations of the extent of encapsulation shown in Fig. 2, and the compliance relation from Eq. 2, we have derived the levels of membrane-membrane affinity for aggregation of RBC and vesicles in the Dextran solutions. The results are plotted in Fig. 4 for the data characteristics sketched in Fig. 2. Fig. 4 represents the free energy potential for aggregation of RBC and mixed PC/PS vesicles in solutions of Dextran 36,500 MW.

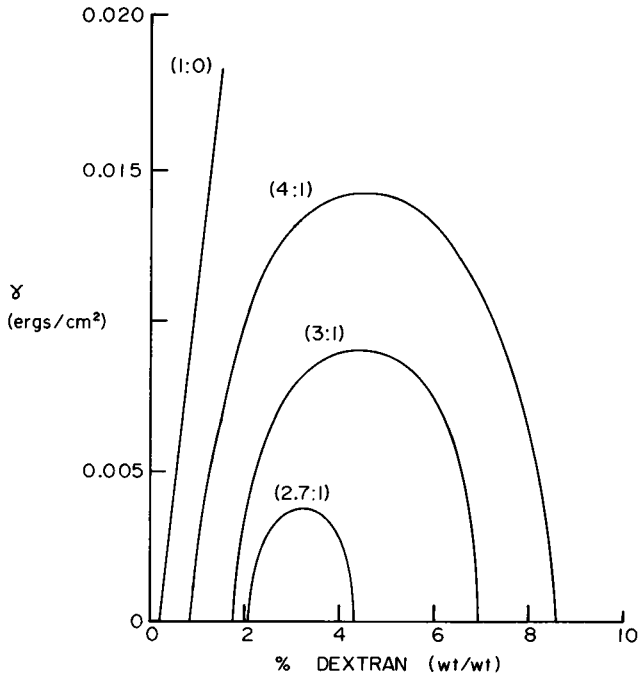

FIGURE 4 Free energy potential (affinity) for aggregation of RBC and mixed (PC/PS) vesicles in Dextran 36,500 MW solutions. The curves are based on the data characteristics shown in Fig. 2 and the elastic compliance relation (Eq. 2) for deformation of the red cell membrane as it encapsulates the spherical vesicle surface. The scale for affinity is established by measurements of the elastic shear and bending moduli of the red cell membrane.

DISCUSSION

The affinity levels for RBC-PC/PS vesicle aggregation in Dextran 36,500 MW solutions are comparable with the values determined previously for RBC-RBC aggregation in Dextran 70,000 and 150,000 MW solutions, i.e., on the order of 1–2 $\times$ 10^{-2} erg/cm^2 or less. Also, the window of concentrations for the aggregation is similar in both cases when the vesicle surface charge density is comparable with that of the normal RBC. It is obvious, however, that the size of the Dextran 36,500 MW is much smaller than either the 70,000 or 150,000 MW molecules (the size in solution varies approximately as the square root of the molecular weight. The peak level of affinity at a given Dextran concentration increases with the PC content. Also, both the onset and quenching of aggregation depended on the vesicle surface charge density, but no quenching affect was observed with neutral lipid vesicles. As was noted previously, RBC-RBC and RBC-lipid vesicle aggregation in Dextran solutions and in plasma are different in that there was no RBC-vesicle aggregation (even for uncharged lipid) in plasma, which indicates that either the aggregating proteins in plasma do not adsorb to the lecithin surface or are shielded from the surface by another adsorbed component.

One obvious explanation (there may be others as well) for the result that small Dextran molecules can produce red cell–charged vesicle adhesion as effectively as larger Dextrans do in red cell–red cell adhesion is the following: if Dextran molecules need to penetrate between the surface carbohydrates on the red cell to produce cross-bridges, then larger molecules would be required for red cell–red cell adhesion than for red cell–vesicle adhesion where the vesicle surface is much "smoother." In addition, penetration would increase the likelihood of steric interactions between carbohydrates on opposite red cell surfaces and would cause the interaction between superficial charge groups on these cell surfaces to be stronger. As it appears that Dextran molecules have to penetrate the surface carbohydrate layer to form cross-bridges, it is possible that the exposed regions (between carbohydrates) become saturated with Dextran and that further adsorption interferes with (quenches) the aggregation process. This speculation is consistent with our observation tht vesicle-vesicle aggre-

gation in Dextran is not quenched at high concentrations (Evans and Metcalfe, submitted for publication); quenching is definitely associated with the presence of surface carbohydrate groups.

The authors greatly appreciate helpful discussions with their colleague Dr. D. E. Brooks.

This work was supported by National Institutes of Health grant HL 26965.

Received for publication 2 March 1983 and in final form 1 July 1983.

REFERENCES

Brooks, D. E. 1973 *a*. The effect of neutral polymers on the electrokinetic potential of cells and other charged particles. II. A model for the effect of adsorbed polymer on the diffuse double layer. *J. Colloid Interface Sci.* 43:687–699.

Brooks, D. E. 1973 *b*. The effect of neutral polymers on the electrokinetic potential of cells and other charged particles. IV. Electrostatic effects in Dextran-mediated cellular interactions. *J. Colloid Interface Sci.* 43:714–726.

Brooks, D. E., R. G. Greig, and J. Janzen. 1980. Mechanisms of erythrocyte aggregation. *In* Erythrocyte Mechanics and Blood Flow. G. R. Cokelet, H. J. Meiselman, and D. E. Brooks, editors. Alan R. Liss, Inc., New York. 120–140.

Buxbaum, K., E. Evans, and D. E. Brooks. 1982. Quantitation of surface affinities of red blood cells in Dextran solutions and plasma. *Biochemistry.* 21:3235–3239.

Chien, S. 1980. Aggregation of red blood cells: an electrochemical and colloid chemical problem. *Adv. Chem. Ser.* 188:1.

Cowley, A. C., N. L. Fuller, R. P. Rand, and V. A. Parsegian. 1978. Measurements of repulsive forces between charged phospholipid bilayers. *Biochemistry.* 17:3163–3168.

Evans, E. A. 1980. Minimum energy analysis of membrane deformation applied to pipette aspiration and surface adhesion of red blood cells. *Biophys. J.* 30:265–284.

Evans, E. 1983. Bending elastic modulus of red blood cell membrane derived from buckling instability in micropipette aspiration tests. *Biophys. J.* 43:27–30.

Evans, E., and K. Buxbaum. 1981. Affinity of red blood cell membrane for particle surfaces measured by the extent of particle encapsulation. *Biophys. J.* 34:1–12.

Evans, E., and V. A. Parsegian. 1983. Energetics of membrane deformation and adhesion in cell and vesicle aggregation. *Ann. NY Acad. Sci.* In press.

Haydon, D. A., and G. V. F. Seaman. 1967. Electrokinetic studies on the ultrastructure of the human erythrocyte. I. Electrophoresis at high ionic strengths—the cell as a polyanion. *Arch. Biochem. Biophys.* 122:126–136.

Jan, K.-M., and S. Chien. 1973. Role of surface charge in red blood cell interaction. *J. Gen. Physiol.* 61:638–654.

Levine, S., M. Levine, K. A. Sharp, and D. E. Brooks. 1983. Theory of the electrokinetic behavior of human erythrocytes. *Biophys. J.* 42:127–144.

Luzzati, V. 1968. X-ray diffraction studies of lipid-water systems. *In* Biological Membranes: Physical Fact and Function. D. Chapman, editor. Academic Press, Inc., New York. 71–123.

Nash, G. B., and H. J. Meiselman. 1983. Effects of Dextran and polyvinylpyrrolidone on red cell geometry and membrane elasticity. *Ann. NY Acad. Sci.* In press.

Seaman, G. V. F., R. J. Knox, F. J. Nordt, and D. H. Regan. 1977. Red cell aging. I. Surface charge density and sialic acid content of density-fractionated human erythrocytes. *Blood.* 50:1001.

Steck, T. L. 1974. The organization of proteins in the human red blood cell membrane. *J. Cell Biol.* 62:1–19.

Steck, T. L., and G. Dawson. 1974. Topographical distribution of complex carbohydrates in the erythrocyte membrane. *J. Biol. Chem.* 249:2135.

Waugh, R., and E. A. Evans. 1979. Thermoelasticity of red blood cell membrane. *Biophys. J.* 26:115–132.