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Influence of Red Blood Cells and Their Components on Protein Adsorption

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Earlier observations from this laboratory showed that red blood cells have an inhibitory effect on adsorption of albumin and fibrinogen to polyethylene surfaces. The present work extends observations of this "red cell effect" to the glass–fibrinogen system. Adsorption in the presence of red cells was inhibited up to 50%, the inhibition increasing with increasing hematocrit at constant protein concentration in the free fluid volume. Since some hemolysis occurs in these experiments, the effect of deliberately added hemolysate was investigated, but found to be negligible. Adsorption in the presence of red cell ghosts was inhibited strongly. These results suggest that the red cell effect is not attributable to leakage of cell contents, but rather is a membrane-related effect.

Adsorption of proteins is the primary event upon contact between blood and foreign surfaces (*1*), and subsequent cellular interactions leading to thrombus formation are determined by these adsorbed proteins (*2*). Much of the early work on the study of adsorption was done in buffered solutions of single proteins or relatively simple mixtures (*3–7*). More recently, studies have been conducted using more "realistic" media, particularly plasma (*8, 9*). These studies have shown that the plasma interacts subsequently with the initially adsorbed proteins, causing some unexpected effects. For example, initially adsorbed fibrinogen is desorbed rapidly from several surfaces in the presence of plasma (*8*).

The question might well be asked whether the red cells, another major component of blood, have any effect on protein adsorption. The effect of red cells on platelet sticking has been noted widely, causing an augmentation of the rate of adhesion, probably by a combination of physical and biochemical mechanisms (*10*). However, studies of protein adsorption in the presence of

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red cells are, surprisingly, almost entirely lacking. In a previous study from this laboratory (*11*), the addition of red blood cells to buffered solutions of plasma proteins caused a decrease in the quantity of protein adsorbed from these solutions to a polyethylene surface. The present work extends these observations to the glass–fibrinogen system, and provides new information relevant to the mechanism of adsorption inhibition. In particular, we wanted to establish whether the red cell effect has intracellular or membrane origins. Therefore, separate experiments were carried out in the presence of hemolysate from red cells and red cell ghosts.

Experimental

Materials. Fibrinogen (human, Grade L) was obtained from Kabi (Stockholm, Sweden). The lyophilized product was dissolved in distilled water (concentration: 1 g/100 mL) and dialyzed against an appropriate buffer, usually isotonic phosphate-buffered saline (PBS), pH 7.35 (*see* below). The solution was frozen in 5-mL portions until required. Hemoglobin (human, Type IV, twice crystallized) was purchased from Sigma Chemical Co. and was used as received. The $Na^{125}I$ was from New England Nuclear. Glass tubing (0.25 cm in diameter) was Corning code 7740, Pyrex glass, and was washed for 1 h with chromic acid cleaning mixture (Chromerge), then rinsed with copious amounts of distilled water. The surface of this glass previously has been shown to be essentially smooth and featureless from a topographical standpoint by electron microscopy (*12*).

The "normal" adsorption medium was isotonic PBS (0.15*M*, pH 7.35) containing labeled fibrinogen at a specified concentration. To this medium were added, variously, washed whole red cells, red cell ghosts, or red cell hemolysate in sufficient quantity to give a specified final concentration of hemoglobin. Fibrinogen concentrations for the media containing red cells or ghost cells were based on free fluid volume rather than total volume.

Fibrinogen Labeling. Labeling with ^{125}I was carried out as described previously (*12*), using a twofold molar excess of iodine monochloride. This procedure gives labeled fibrinogen identical in its adsorption on glass to unlabeled protein (*13*). Other properties of fibrinogen, including biological properties, also are unaffected by this labeling method (*14*, *15*). In general, the fibrinogen solutions used in the present work contained 30% labeled and 70% unlabeled material.

Preparation of Washed Red Cells. Freshly drawn human blood, collected into acid citrate dextrose (ACD) anticoagulant, was centrifuged for 15 min at 630 × *g*, and the plasma and buffy coat were removed. The red cells were washed three times using 3–4 volumes of PBS, centrifuging between each wash at 2000 × *g* for 10 min. Packed red cells [hematocrit (HCT) about 90%] were added to appropriate volumes of fibrinogen solution (in PBS) to give the required hematocrit.

Preparation of Ghost Cells. The method of Steck (*16*) was modified for use in the present work. Washed, packed red cells prepared as indicated above were lysed in 40 volumes of 5m*M* sodium phosphate (pH 8.0), then washed three times using 40 volumes of 5m*M* sodium phosphate. This procedure gives so-called "unsealed" ghost cells (*16*) from which essentially all the hemoglobin has been removed. At the same time, normal red cell morphology (biconcave disc) and size are maintained as shown by the results of scanning electron microscopy (SEM) (Figure 1). Cells were prepared for SEM by allowing them to settle on Nuclepore polycarbonate membranes. They were then fixed sequentially with glutaraldehyde and osmium tetroxide, and dehydrated with ethanol.

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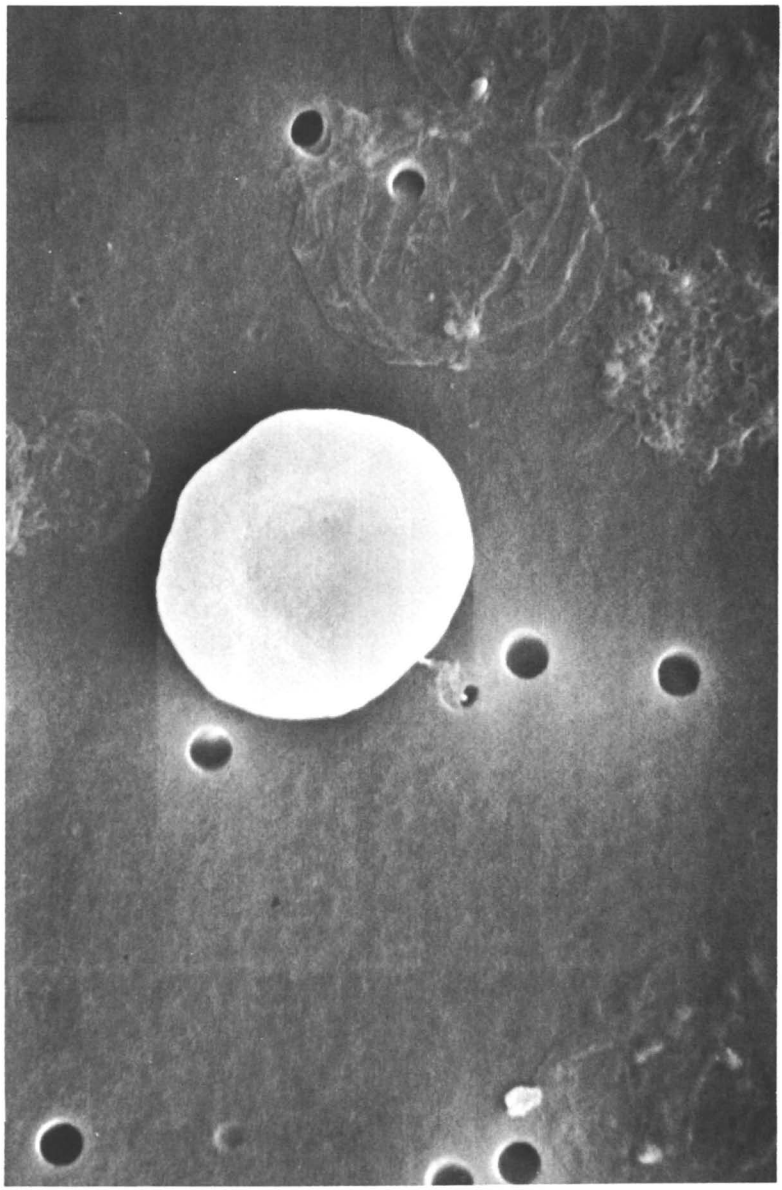

Figure 1. SEM of a typical ghost cell. Cells were deposited on a polycarbonate membrane (Nuclepore) whose pores are visible. The small white marker lines represent 1 μm.

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In addition to SEM, size analysis of the ghost cells was performed with a Coulter counter, and as shown in Figure 2, the size distribution is similar to that of the washed red cell preparation.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of ghost membrane proteins was performed by a modification of the method of Laemmli (*17*). Samples were reduced with β-mercaptoethanol and run in 11% gels. Molecular weight (MW) standards were obtained from Pharmacia Canada Ltd.

Media containing ghost cells for adsorption studies were prepared by mixing appropriate volumes of packed ghost cells and labeled fibrinogen in PBS. Ghost hematocrits were determined by cell counting (Coulter counter), assuming an average ghost cell volume of 75 μm^3.

Preparation of Hemolysate. Washed red cells were lysed in distilled water (20% HCT) for 15 min. The stroma was extracted in carbon tetrachloride, and the aqueous layer containing the hemolysate was recovered. This solution was diluted appropriately with PBS to a specified hemoglobin concentration, and labeled fibrinogen was added. The final solution was adjusted to isotonicity and pH 7.35. Hemoglobin concentrations were determined by the cyanmethemoglobin method (*18*).

Adsorption Experiments. Adsorptions were carried out as described previously (*11*). A circuit was set up consisting of several glass tubing segments and a roller pump connected in series. The tubes were positioned vertically, and were connected together using Silastic medical-grade tubing (Dow–Corning) and three-way valves. The total length of the Silastic connectors was about one-third that of the glass test segments. The circuit was primed with PBS, which was then displaced by the test medium (a suspension of red blood cells, ghosts, etc.) in a manner (using the three-way valves to eliminate air bubbles) such that no air–solution–solid interface was created. Experiments were run at a flow rate of 50 mL/min (540 s^{-1} surface shear rate) at room temperature. To determine the time course of adsorption, tubing segments were disconnected at various times up to 4 h and were rinsed three times with PBS (20 volumes), and the associated radioactivity was determined (Beckman Biogamma system). An aliquot of the labeled fibrinogen solution (of known concentration) prepared for a given experiment also was counted, and surface concentration Γ ($\mu g/cm^2$) was calculated from the relation:

$$\Gamma = \frac{C_p R_f}{A R_s}$$

where C_p is the solution concentration of fibrinogen ($\mu g/mL$); R_f, the count rate of surface; R_s, the count rate of solution (per mL); and A, the area of surface (cm^2).

In experiments where the extent of hemolysis was needed, samples of test fluid were withdrawn from the circuit at various times, and the hemoglobin concentration in the supernatant was measured (*18*).

Results

Effect of Whole Red Cells. As indicated in Figure 3, whole red cells cause a diminution in the quantity of fibrinogen adsorbed to a glass surface. The extent of this "inhibition" increases with increasing hematocrit, and at 40% HCT, the surface concentration after 4 h was reduced to about 50% of its value in the absence of cells. It was shown previously (*11*), that such an effect was not due to the depletion of fibrinogen from the aqueous phase by adsorption to the cell surfaces. Although this may occur to some extent, it is insufficient to cause any detectable alteration in the concentration of fibrino-

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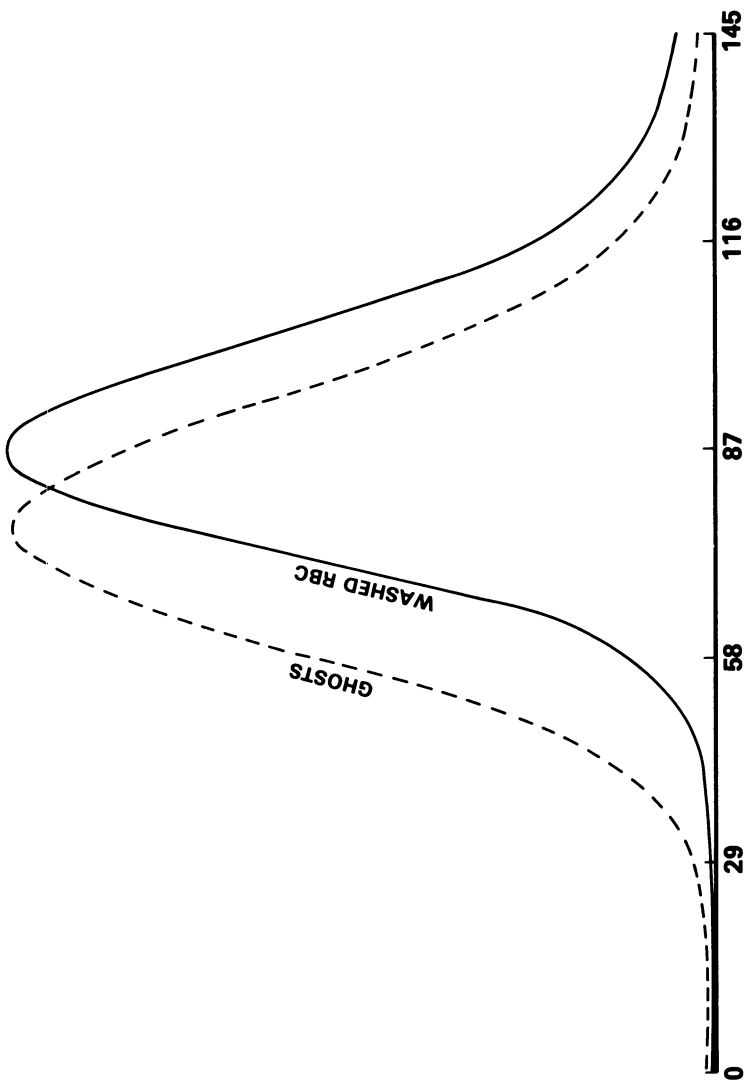

Figure 2. Distributions of cell volumes of typical red cell and ghost cell preparations, obtained by Coulter counter. Key to mean cell volumes: red cells, 87 μm³; and ghost cells, 76 μm³.

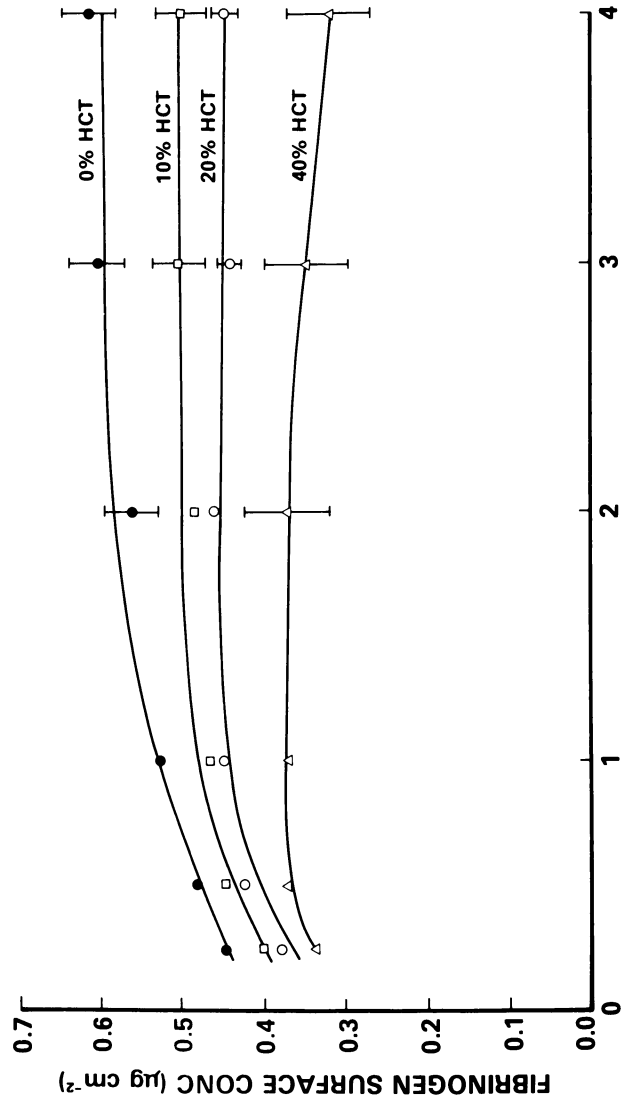

Figure 3. Adsorption of fibrinogen on glass as a function of time at various hematocrits. Conditions: buffer—PBS, pH 7.35; fibrinogen concentration—1.0 mg/mL in free volume; and shear rate at the surface—540 s^{-1}. Values are the average of at least three experiments; error limits are standard deviations. Key: ●, 0% HCT; □, 10% HCT; ○, 20% HCT; and △, 40% HCT.

gen in the liquid phase. In addition, for all suspensions used, the concentration in the cell-free volume was kept constant at 1.0 mg/mL. Unlike the results for polyethylene, published previously (*11*), the surface concentration does not pass through an early maximum. Instead, it shows "typical" kinetic behavior, approaching a steady-state value in times on the order of 1–2 h. In the case of the 40% HCT suspension, the surface concentration began to decrease from its steady-state value after 2–3 h.

Hemolysis During Experiments with Whole Red Cells. Although it was previously concluded (*11*) that hemolysis in these experiments is less than 1% and probably of no account, we wanted to ascertain the exact concentration of free hemoglobin and to determine whether this concentration increases with time. Horbett et al. (*19*) suggested that hemoglobin is a protein of high surface activity, and is preferentially adsorbed from plasma relative to fibrinogen and albumin. Thus, at least a part of the red cell–related inhibition of adsorption possibly could be due to preferential adsorption of hemoglobin. Figure 4 shows that the free hemoglobin concentration does indeed increase with time in typical adsorption experiments, suggesting that some hemolytic damage to cells is occurring. The effect is minimal at the lower hematocrit, but at 40% HCT, the concentration of free hemoglobin reaches a value on the order of 1.0 mg/mL, comparable to that of fibrinogen. The initial concentration of hemoglobin is finite and increases with increasing hematocrit. With the manipulations involved in preparing the red cells, this "residual" free hemoglobin could not be eliminated. Even at 20% HCT, the initial hemoglobin concentration was about 0.04 mg/mL.

Effects of Hemoglobin and Hemolysate. Because substantial concentrations of hemoglobin exist in the adsorption media, we wanted to know whether hemoglobin per se would exert an inhibitory effect on fibrinogen adsorption. Experiments using hemoglobin from a commercial source were thus carried out, using concentrations at the low and high ends of the range actually encountered in the red cell suspensions. Figure 5 shows the kinetics of adsorption at hemoglobin concentrations of 0.09 and 0.8 mg/mL, and indicates a strongly inhibitory effect such that adsorption of fibrinogen is all but eliminated at the higher concentration. The shape of the curves suggests that a certain amount of the fibrinogen adsorbed at short times is removed later. The spectacular effect of hemoglobin shown in Figure 5 is, in fact, greater than the red cell effect itself, suggesting that if the latter is due entirely to leakage of cell contents, then hemolysate is relatively less inhibitory than pure hemoblogin. To investigate further the role of cell contents, adsorption experiments were performed in the presence of controlled amounts of hemolysate, such that free hemoglobin concentrations were again in the range encountered in the red cell experiments. The results of these experiments are shown in Figure 6; the hemolysate effect was very small, and effectively negligible up to a hemoglobin concentration of 1.0 mg/mL. A slight decrease in the steady-state level of adsorption occurred at the higher

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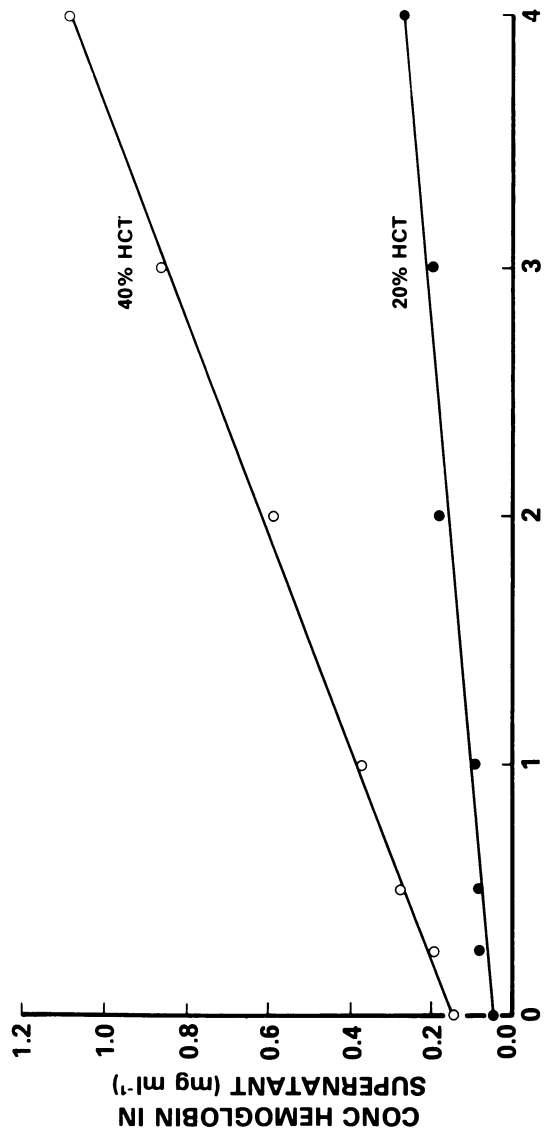

Figure 4. Development of hemoglobin in supernatant as a function of time during red cell experiments at two hematocrits. Values are the average of at least three experiments. Key: ○, 40% HCT and ●, 20% HCT.

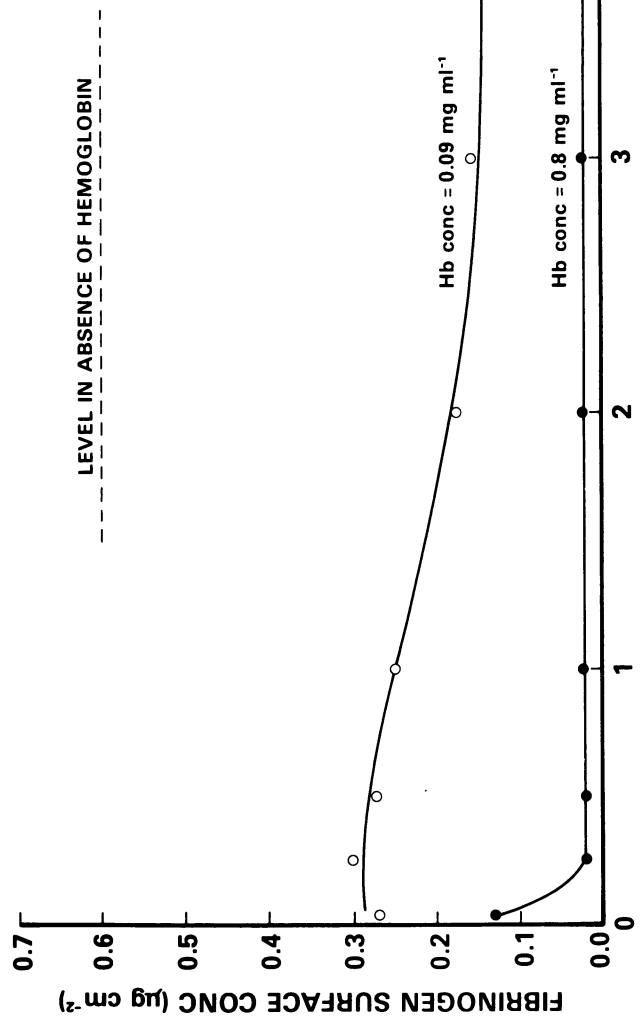

Figure 5. Adsorption of fibrinogen on glass in the presence of Sigma hemoglobin. Conditions: buffer—PBS, pH 7.35; fibrinogen concentration—1.0 mg/mL, and shear rate at the surface—540 s^{-1}. Values are the average of three experiments. Key to hemoglobin concentration: ○, 0.09 mg/mL and ●, 0.8 mg/mL; ---, level in absence of hemoglobin.

hemoglobin concentrations. The difference between the effects of the commercial hemoglobin and hemolysate is very pronounced. Among possible explanations is that other constituents of hemolysate act in opposition to hemoglobin. However, Figure 7 suggests another, perhaps more plausible, explanation. From this figure, showing visible spectra of the hemolysate and Sigma hemoglobin, the hemolysate hemoglobin is in the form of oxyhemoglobin, whereas the Sigma hemoglobin is in the form of methemoglobin (*20*). Consequently, methemoglobin appears to be adsorbed strongly in preference to fibrinogen, whereas oxyhemoglobin is not.

Effect of Ghost Cells. The results of the experiments in the presence of hemolysate suggest that cell contents, per se, do not influence fibrinogen adsorption, thus implicating the cell membranes directly, or alternatively, the particulate character of the cells, as being responsible for the effect. To investigate this possibility, experiments with ghost cells were conducted. The preparative technique resulted in ghost cells of normal shape and size. Thus, SEM results such as those presented in Figure 1 show retention of bioconcave shape and a cell diameter between 6 and 7 μm. Coulter counter analysis (Figure 2) showed the distribution of cell volumes to be substantially maintained, although the mean cell volume was somewhat less than for the normal cells. Two concentrations of ghost cells, namely, 15% and 45% by volume, were used in these experiments. The results, presented in Figure 8, show a marked diminution in quantity adsorbed (on the order of 50% and 80% at 15% and 45% by volume of ghost cells, respectively). Again, as with

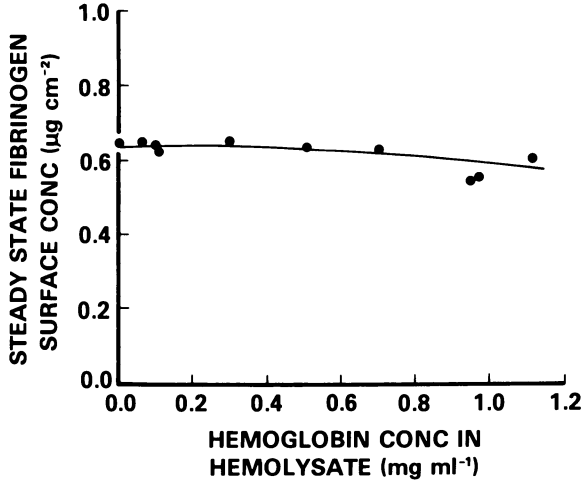

Figure 6. Adsorption of fibrinogen on glass in the presence of red cell hemolysate. Conditions: buffer—PBS, pH 7.35; fibrinogen concentration—1.0 mg/mL; and surface shear rate—540 s^{-1}. Values are the average of three experiments.

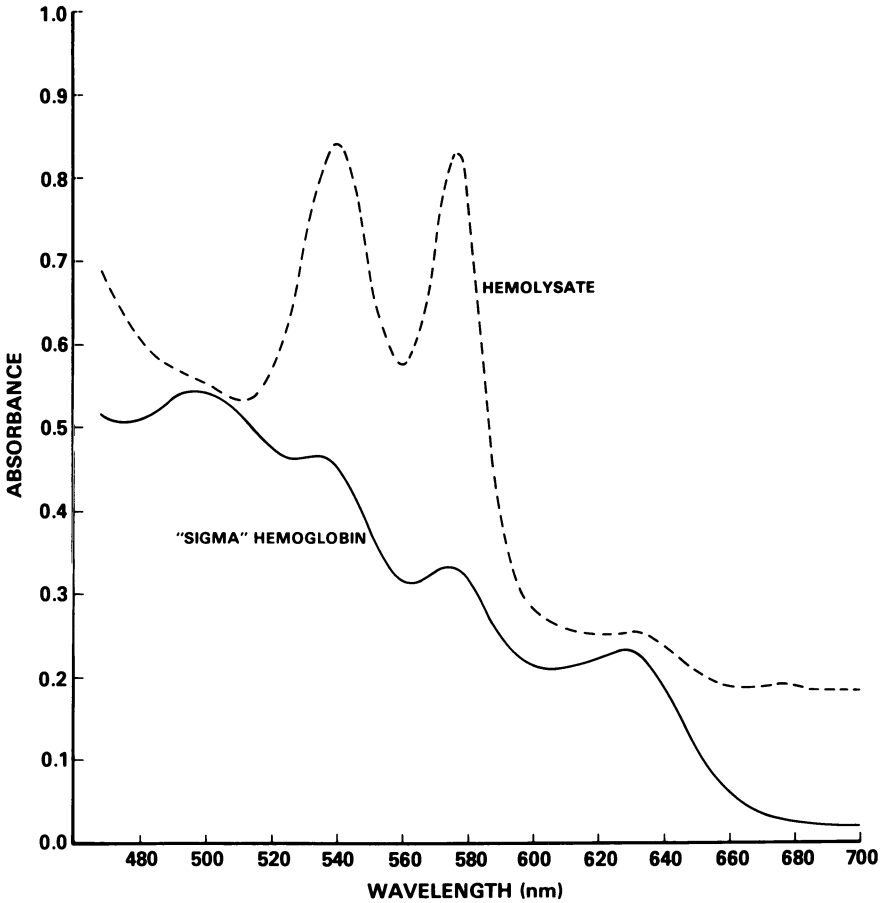

Figure 7. Visible spectra of hemolysate preparation and Sigma hemoglobin in the wavelength range 460–700 nm. Key: ---, hemolysate and —, Sigma hemoglobin.

the kinetics in the presence of methemoglobin, the ghost cells appear to be able to remove initially adsorbed fibrinogen.

SDS–PAGE of Adsorption Media. SDS–PAGE of various media used in this study was undertaken to obtain additional information on their protein compositions. Typical gels are presented in Figure 9. Gel 3 corresponds to the proteins from ghost cell membranes and shows a complex pattern of bands that agrees well with previously published results, for example, those of Fairbanks et al. (*21*).

Comparison of Gels 2 and 4, corresponding to Kabi fibrinogen and fibrinogen plus supernatant from a ghost cell suspension, respectively, shows the presence of an additional band in Gel 4 at a molecular weight of about 37,000. This protein presumably originated in the ghost membranes, and

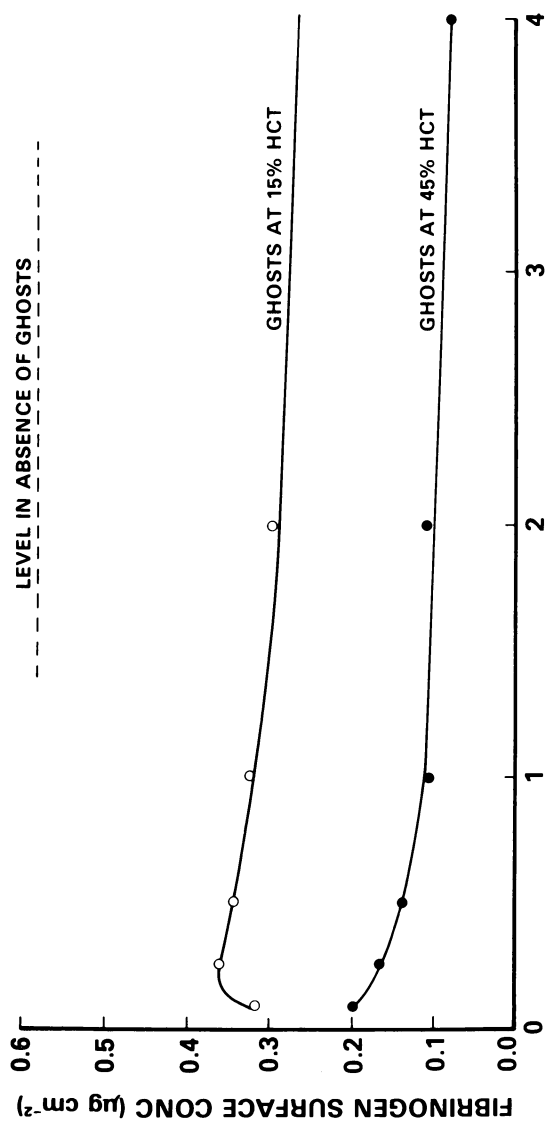

Figure 8. Adsorption of fibrinogen on glass in the presence of ghost cells. Conditions: buffer—PBS, pH 7.35; and fibrinogen concentration—1.0 mg/mL in free volume. Values are the average of three experiments. Key: ○, ghosts at 15% HCT; ●, ghosts at 45% HCT; and ---, level in absence of ghosts.

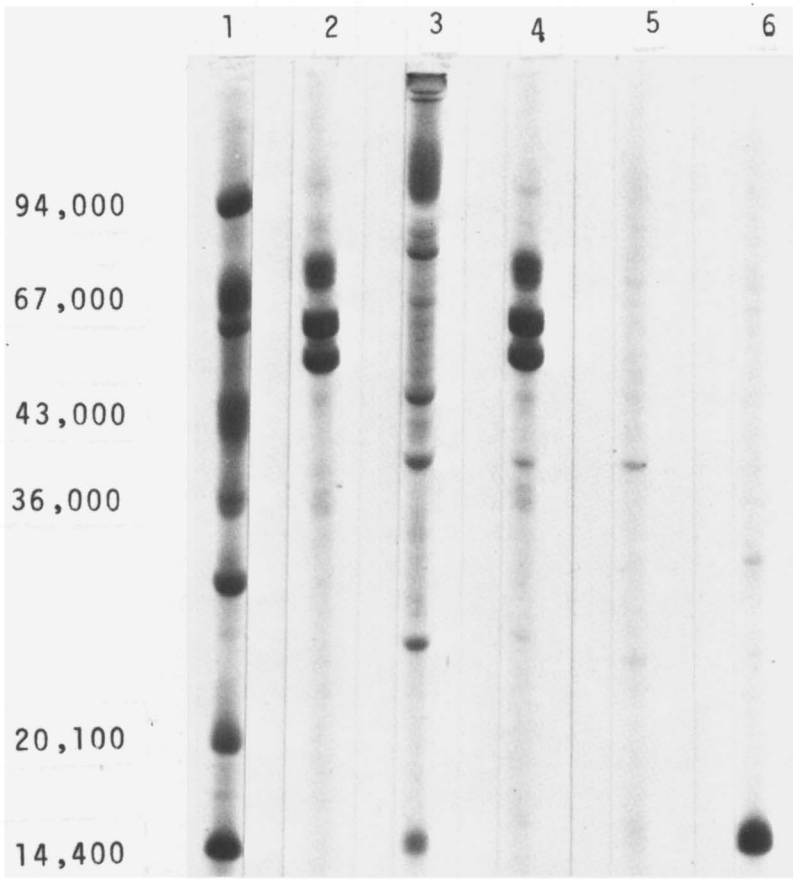

Figure 9. SDS–PAGE of various materials.

Numbers on left are molecular weights of standards shown in Gel 1. Key to gels: 1, molecular weight standards; 2, human fibrinogen (Kabi); 3, ghost cell membrane polypeptides; 4, fibrinogen with supernatant from ghost cell preparation; 5, ghost cell supernatant alone; and 6, hemolysate from red cells.

appears to correspond to "Band 6" in the nomenclature of Fairbanks et al. (*21*). Band 6 has been identified with the monomeric form of glyceraldehyde-3-phosphate dehydrogenase (*22*), which is believed to be an "extrinsic" protein, loosely bound to the cytoplasmic surface of the membrane. Gel 5 represents a sample of hemolysate, and shows essentially a single band corresponding to the hemoglobin subunit, thus confirming that the hemolysate does not contain any membrane or plasma protein contaminants.

Discussion

The three principal conclusions emerging from this study are that: (1) the "red cell effect" can now be further generalized to include glass surface; (2) a considerable difference in competitive adsorption exists between oxyhemoglobin and methemoglobin; and (3) inhibition of adsorption by red cells appears to be a cell membrane–related effect.

With regard to the first point, initial work with polyethylene (*11*) postulated that the mechanism of adsorption inhibition involved collision of red cells with the surface, and transfer of some material from the cell surface to the tube wall. Such an effect might be considered to be more probable if it involved hydrophobic interactions, which would be more likely for polyethylene than for glass. However, as is shown by the present study, the extent of inhibition is about the same for both surfaces, so that whatever the mechanism or mechanisms, the inhibition appears equally likely for hydrophobic and hydrophilic surfaces. The effect may be a general one that would occur for any surface.

Vroman et al. (*9*) observed transient adsorption (i.e., adsorption followed by rapid desorption) of fibrinogen on glass in the presence of plasma, and attributed this result to replacement of fibrinogen with high molecular weight kininogen. The present results could possibly be explained by this plasma effect resulting from carry over of plasma with the red cells. Since the cells are washed very extensively, we do not believe such an effect would be important. In addition, we have observed a red cell effect for other proteins including albumin (*11*) and IgG (*23*).

The dramatic difference between the effects of hemolysate and Sigma hemoglobin is perhaps somewhat surprising, and must remain, for the moment, without explanation. Certainly, however, only the oxyhemoglobin situation is relevant to blood, since methemoglobin constitutes only 0.5–3% of total hemoglobin (*24*). The observations of Horbett et al. (*19*), suggesting that hemoglobin adsorption might be important in extracorporeal circulations where hemolysis is more likely to occur, were based on work with methemoglobin (*25*). Therefore, the question of the possible role of hemoglobin in blood–surface phenomena needs to be re-examined. From a more fundamental standpoint, how such differences in adsorption behavior might arise should be considered based on subtle structural differences between the various hemoglobin types. Apparently, the oxidation state of the iron affects adsorption properties, thus suggesting that the heme groups may be involved. Clearly, more systematic studies relevant to this question are required to resolve the various issues.

The results with ghost cells suggest that the membranes play a key role in the "red cell effect." This point of view is in accord with conclusions from

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our earlier work (*11*) that the red cell–surface collision results in transfer of material from the cell to the surface. Keller and Yum (*26*) also provided evidence that such a transfer of material may occur. Two possible mechanisms probably are operative in this connection: (1) the deposition of material from the cell as just noted, providing a new surface that is less adsorptive toward proteins than the original substrate, and (2) the ability of the red cell to "strip" the surface of previously adsorbed protein, as suggested by the shape of the curves in Figures 5 and 8. In the case of polyethylene (*11*), red cells circulated over a previously adsorbed protein layer did not remove the layer. Such experiments were not performed in the present work, but probably should be carried out to test the possibility that stripping can occur.

The relevance of these results to blood–biomaterial interactions has several facets. If red cell material is deposited on the surface, then this becomes an interaction of potential importance that has not been recognized previously. Previous observations of early events in blood–material interactions (*1*, *3*) have emphasized the rapid deposition of a protein layer, generally assuming it to consist of proteins originating from the plasma. However, red cell interactions also may possibly contribute to this layer. Identification of material deposited from the red cell and the acquisition of knowledge of how its presence on the surface might influence subsequent interactions such as platelet adhesion, are clearly tasks of some significance. In addition, whether the quantity and composition of the deposit depend on the specific biomaterial surface should be investigated.

Red cell–surface interactions may play a role in the dynamics of protein adsorption. We have been investigating the turnover of protein between solution and surface for several years (*27–29*), and have established that turnover occurs on a variety of surfaces. The rate and extent of turnover depend strongly on the surface character, with hydrophilic materials, for example, showing much more rapid turnover than hydrophobic materials. If red cells have the ability to strip protein off a biomaterial surface, then clearly this effect could influence the characteristics of the turnover process, particularly from a rate point of view. This process, in turn, could affect the development of the protein layer over a period of time.

Finally, based on results from the present work, if studies of protein adsorption are to be meaningful in terms of blood–biomaterial interactions, then they should be carried out in the presence of red blood cells.

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