PLASMA PROTEIN ADSORPTION: IN VITRO AND EX VIVO OBSERVATIONS

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Abstract: The *in vitro* and *ex vivo* adsorption of blood proteins is studied in order to elucidate the protein-surface interactions which determine the thrombogenicity and thus the applicability of various polymers in blood contacting devices. The *in vitro* adsorption of albumin and fibrinogen to four polymers shows that at low solution concentrations, more fibrinogen is adsorbed than albumin. At higher solution concentrations, albumin adsorbs in multilayers while fibrinogen adsorbs, and then partially desorbs spontaneously from the surface. Sequential adsorption studies show that fibrinogen and albumin can partially replace each other. Fibrinogen is preferentially adsorbed over albumin in competitive adsorption studies. In *ex vivo* experiments, more albumin than fibrinogen is adsorbed from blood during the first 120 minutes of whole blood contact. When exposed to flowing whole blood, pre-adsorbed fibrinogen desorbs more rapidly than albumin.

INTRODUCTION

When blood contacts a polymer surface, adsorption of plasma protein to that surface occurs immediately. Several investigators have shown that the composition of the adsorbed protein appears to be dependent upon the chemical nature of the polymer surface [1–9]. The composition of the protein layer also appears to be dynamic with protein turnover and replacement a commonplace occurrence [3,5,10–13]. Over the past few years we have investigated several aspects of plasma protein adsorption, desorption, and replacement upon polymeric biomaterials. The premise of this research is that by understanding and controlling the protein adsorption process, one can understand and control the subsequent thrombogenic events such as platelet adhesion and activation, mural thrombus formation, embolization, and fibrin polymerization, which often occur when blood contacts a foreign surface.

Previous studies have investigated how adsorbed proteins affected platelet and fibrin deposition. This was accomplished by pre-adsorbing various plasma and platelet granule proteins onto polymer tubing, and then exposing the protein coated tubing to non-anticoagulated whole blood in a canine *ex vivo* shunt [1,8,14–16]. Exposure of bare, uncoated tubing to whole blood produced a characteristic platelet and fibrin deposition which was presumably influenced by the types of proteins adsorbing from the blood. The protein precoated tubings usually produced very different platelet and fibrin deposition profiles. Thus it appears that pre-adsorbed protein, while subjected to whole blood exchange, turnover, and desorption, does impart a characteristic thrombogenic

response when exposed to flowing whole blood. For example,it was observed that fibrinogen, fibronectin, γ-globulins, thrombospondin, von Willebrand factor, and vitronectin enhanced the number of platelets deposited and increased their rate of shape change and activation. Albumin and transferrin reduced the amount of platelet deposition and activation. Pre-adsorbed α_2-macroglobulin increased platelet deposition on plasticized polyvinyl chloride, but decreased it on silicone rubber.

This paper examines *in vitro* fibrinogen and albumin adsorption and exchange, and discusses and their correlation with *ex vivo* protein adsorption and desorption. These two proteins produce opposite results when adsorbed *ex vivo*, and can be considered model proteins which exacerbate and passivate the thrombogenic response of a polymer surface. These two proteins also exhibit very different *in vitro* adsorption characteristics: the amount of adsorbed albumin (like transferrin, fibronectin, α_2-macroglobulin and γ-globulin) appears to increase as the polymer substrate becomes more hydrophobic; however, the amount of adsorbed fibrinogen appears to increase as the polymer becomes more hydrophilic [17].

EXPERIMENTAL

Protein Preparation. The protein purification and characterization procedures have been reported previously in detail [1,8,18]. Protein purity was verified with polyacrylamide gel electrophoresis using sodium dodecyl sulfate. Each protein showed only a single band of the appropriate molecular weight. Portions of the purified proteins were labeled with ^{125}I using the lactoperoxidase method (Enzymobead, Bio Rad Laboratory).

Surface Preparation and Characterization. Surface preparation techniques and contact angle characterization of the tubings have been reported previously [8,18]. The four tubings investigated in this study were a polyether-polyurethaneurea (PEUU, extruded Biomer, Ethicon, Sumerville, NJ, 0.125 inch ID), polyvinyl chloride (PVC, Tygon, R3603, Norton Plastics, Akron, OH, 0.125 inch ID), silicone rubber (SR, Medical Grade Silastic, Dow Corning, Midland, MI, 0.132 inch ID), and polyethylene (PE, Intramedic, Clay Adams, Parsippany, NJ, 0.125 inch ID). The results of contact angle experiments reported previously indicate that the tubing surfaces increase in non-polar nature in the order PEUU, PVC, SR and PE.

In Vitro Protein Adsorption. The details of the *in vitro* protein adsorption experiments have been previously reported [8,18,19]. Briefly, 3-5 cm lengths of the tubings were connected in a repeating series and filled with phosphate buffered saline (PBS, pH 7.4) and incubated at 4°C overnight. They were exposed to radiolabeled protein solutions at room temperature under a range of solution concentrations and exposure times. Adsorption was terminated by gently flushing the tubing with PBS to remove non-adsorbed protein.

Single, sequential, and competitive adsorption of canine albumin and fibrinogen from 0.3 mg/ml solutions at room temperature was studied on PVC, SR and PE tubings. Sequential protein adsorption was accomplished by exposing the polymer tubing surfaces to a single protein solution, displacing the solution with PBS, exposing the surface to a second protein solution, and again displacing the solution with PBS. The total time of exposure to both protein solutions was 2 hours, with the time of the first protein

exposure varying from 1 to 60 minutes. Competitive adsorption of canine albumin and fibrinogen involved a 2 hour exposure of the tubings to a 50-50 mixture of the 0.3 mg/ml albumin solution and the 0.3 mg/ml fibrinogen solution. Displacement with PBS after 2 hours terminated the competitive adsorption experiment.

In all *in vitro* experiments, the amount of adsorbed protein on each tubing segment was measured by counting its radioactivity in a gamma counter.

Ex Vivo Protein Adsorption and Desorption. The procedures for measuring protein adsorption during the first 90 seconds of contact in an *ex vivo* canine model were identical to those for platelet and fibrin deposition studies [1,8,20]. For adsorption times up to 120 minutes, the same procedure was employed with the exception that: (1) the canine subject was given an initial dose of 125 units/kg of sodium heparin (Porcine Mucosal, Lypho-Med., Inc.) followed by 40 units/kg given once every hour to prevent thrombus formation; and (2) hematological tests were omitted during the experiment. The details of quantitating *ex vivo* adsorption of ^{125}I-labeled proteins are given in reference [1].

For the desorption measurements, ^{125}I-labeled canine fibrinogen (0.15 mg/ml), canine albumin (0.15 mg/ml), canine fibronectin (0.10 mg/ml), and human transferrin (2.0 mg/ml) solutions were incubated in the tubing for 2 hours before implantation. The experimental protocol was identical to the *ex vivo* experiment described for platelet and fibrin deposition studies except for the following: (1) systemic platelets and fibrinogen were not radiolabeled; (2) surface-bound radiolabeled protein was counted (and averaged) over 80 second intervals every 2 minutes during the 2 hours of exposure; (3) the tubing was not flushed with saline during the 2 hours of exposure. See reference [8] for details.

RESULTS

In Vitro Adsorption. Figures 1 and 2 show the *in vitro* human serum albumin (HSA) adsorption on SR and PEUU. Adsorption on PVC and PE showed very similar kinetics [8,17]. The adsorption reached a plateau within 400 minutes when the solution concentration was below 5 mg/ml, but at higher concentrations, the adsorption continued without reaching a plateau. The amount adsorbed from higher solution concentrations is well in excess of what is expected for a monolayer of adsorbed protein (0.15 to 0.8 $\mu g/cm^2$), indicating that extensive multilayer adsorption occurs.

Figures 3 and 4 show the *in vitro* adsorption kinetics of human fibrinogen (HFGN) on PE and PEUU which are similar to the adsorption kinetics of HFGN on SR and PVC. Adsorption of HFGN on all of these surfaces is very different than that of any other protein studied. At low solution concentrations, the amount adsorbed reached a plateau concentration. However, adsorption from solution concentrations above 0.3 mg/ml increased to a maximum at about 150 minutes on PEUU, PVC and PE. On SR the maximum occurred at about 25 minutes. This adsorption maximum is reminiscent of the 'Vroman Effect' observed by several investigators [3,5,10,13,21] who claim that fibrinogen adsorbs and then is desorbed or displaced by adsorption of other plasma proteins. However, sequential adsorption and replacement cannot explain the *in vitro* adsorption of the single protein observed here, since electrophoretic data showed that the HFGN solutions were pure. These results suggest that fibrinogen may have a tendency

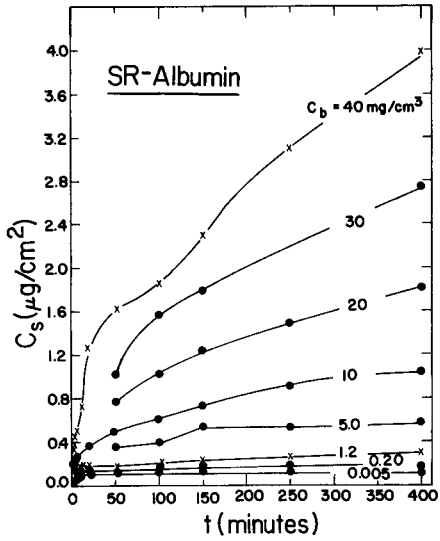

Figure 1: Human albumin adsorption kinetics on SR

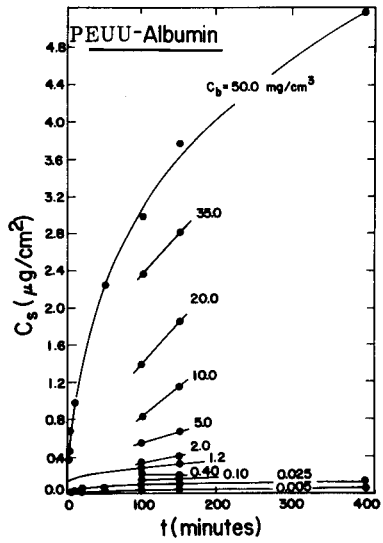

Figure 2: Human albumin adsorption kinetics on PEUU

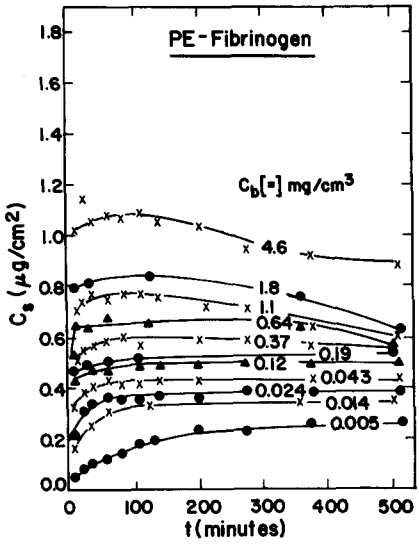

Figure 3: Human fibrinogen adsorption kinetics on PE

to spontaneously adsorb, then desorb, perhaps in response to a time dependent change in conformation of the protein, or to a change in orientation and packing on the surface. Sevastianov has observed and modeled a local maximum in the adsorption kinetics of HSA on PE, polyurethanes, and a ceramic surface [22,23], but this maximum occurred within one minute, and in his experiments, further protein adsorption continued beyond this local maximum. Soderquist and Walton [24] observed a local maximum in albumin adsorption kinetics, but not for fibrinogen adsorption, and not at the higher surface concentrations examined in this study.

Comparison of the adsorption of HSA and HFGN from low solution concentrations reveals that fibrinogen adsorbs much faster than albumin. This indicates that there is greater adhesion between the surface and fibrinogen than between the surface and albumin. Comparison of the adsorption from higher solution concentrations shows that albumin has the ability to adsorb in multilayers, while fibrinogen shows little or no tendency toward multilayer formation. This suggests that protein-protein attractive interactions are greater for albumin than for fibrinogen.

The adsorption data for canine albumin (CSA) and fibrinogen (CFGN) to PE, SR, and PVC *in vitro* from 0.3 mg/ml solutions for 120 minutes were similar to the human protein kinetics (compare refs. [19,25] with refs. [8,17]). Both the shape of the kinetic adsorption profiles and the total amount adsorbed were similar. This indicates that species variation has less influence in determining the *in vitro* adsorption behavior than does the biological function or the physical chemistry of the proteins.

The surface concentrations of sequentially and competitively adsorbed canine albumin and fibrinogen are presented in Table 1. The adsorption times for the first and second sequentially adsorbed protein are given on the left, and the surface concentrations are listed on the right. The adsorption data from single protein solutions onto PVC

ed	Second Protein Adsorbed		Surface Concentration (μg/cm²)					
			PVC		PE		SR	
n)	Type	Adsorption Time (min)	Albumin	Fibrinogen	Albumin	Fibrinogen	Albumin	Fibrinogen
	Albumin (0.3 mg/ml)	119	0.25±0.04	0.32±0.07	0.15±0.05	0.21±0.07	0.14±0.08	0.24±0.07
		117	0.15±0.04	0.31±0.10	0.05±0.02	0.24±0.07	0.12±0.06	0.27±0.06
		115	0.16±0.06	0.32±0.10	0.06±0.05	0.27±0.07	0.12±0.04	0.31±0.08
		110	0.12±0.07	0.40±0.08	0.03±0.02	0.28±0.07	0.12±0.08	0.36±0.11
		90	0.10±0.01	0.60±0.02	0.06±0.05	0.37±0.08	0.06±0.01	0.43±0.03
		60	0.06±0.01	0.62±0.05	0.02±0.01	0.37±0.04	0.04±0.01	0.44±0.02
		0	-	0.79±0.09	-	0.43±0.04	-	0.50±0.06
	Fibrinogen (0.3 mg/ml)	119	0.20±0.01	0.61±0.06	0.13±0.04	0.33±0.08	0.18±0.05	0.39±0.16
		117	0.26±0.04	0.53±0.40	0.17±0.01	0.28±0.07	0.21±0.01	0.34±0.11
		115	0.28±0.05	0.79±0.20	0.18±0.02	0.25±0.06	0.19±0.03	0.38±0.08
		110	0.32±0.05	0.41±0.20	0.20±0.04	0.23±0.15	0.23±0.02	0.07±0.06
		90	0.41±0.01	0.34±0.20	0.25±0.01	0.07±0.01	0.32±0.04	0.05±0.01
		60	0.45±0.03	0.20±0.04	0.25±0.02	0.03±0.01	0.30±0.03	0.05±0.02
		0	0.57±0.07	-	0.32±0.05	-	0.46±0.07	-
	lon for 120 Minutes and 0.3 mg/ml Fibrinogen)		0.30±0.07	0.73±0.08	0.17±0.03	0.26±0.03	0.28±0.03	0.33±0.03

oncentrations for sequential and competitive adsorption of canine albumin and fibrinogen
), n = 4)

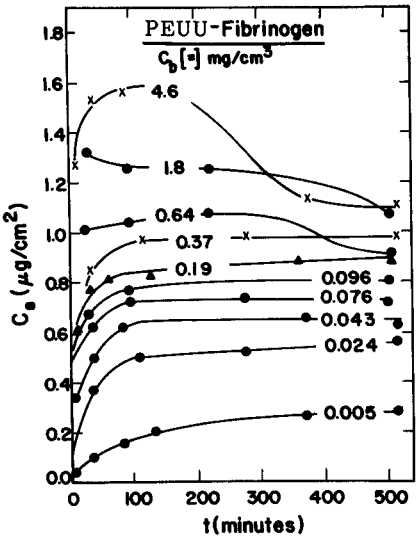

Figure 4: Human fibrinogen adsorption kinetics on PEUU

and PE are listed in Table 2. This table also lists the amount of the first adsorbed protein which was displaced or desorbed from the surface during adsorption of the second protein in the sequential adsorption experiments. This protein desorption was determined by subtracting the final surface concentrations of the sequential adsorptions from the surface concentrations of the single protein adsorption experiments. It is possible that some loosely bound protein was rapidly desorbed while the first or second protein solution was displaced by the PBS buffer, but this type of desorption cannot be independently distinguished by this kind of experiment.

The concentration of protein remaining bound to the surface at the completion of the sequential adsorption of albumin followed by fibrinogen is shown in Figure 5. This figure shows that when albumin adsorption preceded fibrinogen adsorption, the amount of adsorbed CFGN is linearly related to the CSA surface concentration on PVC. On SR and PE, this linearity is observed up to an albumin concentration of about 0.22 μg/cm^2, which is what one would expect for a monolayer of side-on adsorbed albumin. For higher albumin surface concentrations, the fibrinogen surface concentration appears to be small and independent of albumin concentration. When fibrinogen was the first adsobed protein, no linear regions in a similarly constructed cross plot were observed (see ref. [19]).

The competitive adsorption data in Table 1 indicate that the presence of the second competing protein decreases the amount adsorbed compared to single protein adsorption. More CFGN is adsorbed than CSA on each surface, indicating that CFGN competes better for the surface sites. Fibrinogen competes most effectively on PVC and least effectively on SR as shown by the ratios of adsorbed CFGN to CSA of 2.4, 1.5, and 1.2 for PVC, PE, and SR,respectively.

Ex Vivo Adsorption. The first 90 seconds of *ex vivo* adsorption data for radiolabeled

First Protein adsorbed	Adsorption time (min)	Single adsorption surface concentration ($\mu g/cm^2$)		Sequential displacement of first protein ($\mu g/cm^2$)	
		PVC	PE	PVC	PE
Albumin	1	0.24 ± 0.03	0.20 ± 0.05	0.04	0.07
0.3 mg/ml	3	0.31 ± 0.02	0.23 ± 0.02	0.05	0.06
	5	0.36 ± 0.03	0.25 ± 0.03	0.08	0.07
	10	0.40 ± 0.03	0.27 ± 0.02	0.08	0.07
	30	0.47 ± 0.03	0.30 ± 0.03	0.06	0.05
	60	0.52 ± 0.05	0.31 ± 0.02	0.07	0.06
	120	0.56 ± 0.07	0.32 ± 0.05	—	—
Fibrinogen	1	0.32 ± 0.02	0.29 ± 0.03	0.00	0.08
0.3 mg/ml	3	0.38 ± 0.02	0.32 ± 0.03	0.07	0.08
	5	0.40 ± 0.02	0.33 ± 0.04	0.08	0.06
	10	0.45 ± 0.03	0.35 ± 0.03	0.05	0.07
	30	0.55 ± 0.04	0.39 ± 0.05	—	0.02
	60	0.66 ± 0.05	0.40 ± 0.03	0.04	0.03
	120	0.78 ± 0.09	0.43 ± 0.04	—	—

Table 2: Single protein adsorption and displacement of the first adsorbed protein by the second protein in sequential adsorption (mean $\pm$ SD, n=4)

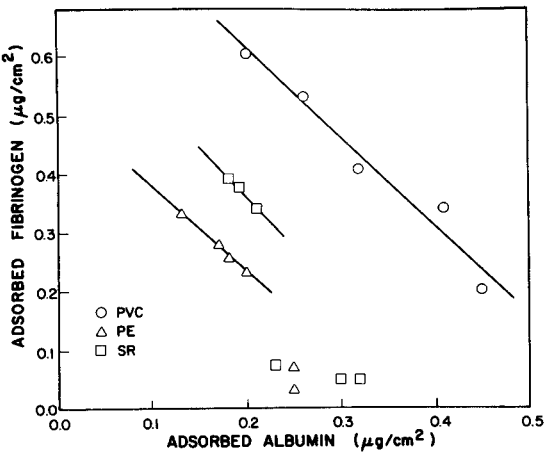

Figure 5: Cross plot of sequential adsorption of 0.3 mg/ml canine albumin followed by 0.3 mg/ml canine fibrinogen on PVC (○), PE (△), and SR (□)

Protein	Time (sec)	PEUU	PVC	SR	PE
Fibrinogen	30	27±8	23±13	85±49	89±12
	60	36±3	43±14	81±56	76±11
	90	42	69	30	89
Albumin	30	150	67	120	140
	60	180	61	85	85
	90	210	76	85	130

Table 3: *Ex vivo* protein adsorption (ng/cm^2). Data is from single experiments except for duplicate FGN adsorptions at 30 and 60 seconds

canine albumin and fibrinogen from non-heparinized blood is given in Table 3. Both albumin and fibrinogen adsorptions are lower on PVC than on the other surfaces. The amount of albumin adsorbed at these early adsorption times is greater than fibrinogen on each of the surfaces. Upon initial contact with blood, the transport of albumin to the surface occurs more quickly than for fibrinogen because albumin has both a greater concentration and a greater diffusion coefficient than fibrinogen. However, as the time of blood contact increases, albumin can be replaced by other blood proteins. Indeed Vroman has shown that *in vitro* on glass and metal oxide surfaces, albumin is replaced by immunoglobulins and fibrinogen at the surface, which in turn is replaced by other proteins at later times [11,13,21]. If fibrinogen replaces albumin on these polymer surfaces *ex vivo,* it does not appear to do so within the first 90 seconds in non-heparinized blood.

Figures 6 and 7 show that on PVC and SR, more albumin is adsorbed than fibrinogen from heparinized blood, even at much longer contact times. It appears that fibrinogen adsorption has a maximum at a very early time on both surfaces. Albumin adsorption also goes through a maximum, but at longer blood contact times. The amount of adsorbed CFGN is similar on both surfaces, but CSA adsorption is two to three times greater on SR than on PVC.

Ex Vivo Protein Desorption. Radiolabeled proteins which had been previously adsorbed on polymer tubings were exposed to whole blood in the *ex vivo* experiment, and their desorption rates were measured. Figure 8 shows the *ex vivo* surface concentration of these pre-adsorbed proteins as a function of blood exposure time on PVC, SR, PE, and Biomer (PEUU). An initial large decrease in the pre-adsorbed protein surface concentrations occurred on all surfaces within the first 10 minutes. Since the initial amount of pre-adsorbed protein was probably more than a monolayer [18], this initial rapid desorption is attributed to desorption of protein which is bound less tightly to the surface since it is not in direct contact with the surface. After 20 to 40 minutes of blood exposure, the desorption rate became constant with similar values on each surface. Albumin desorption at 0.125 ng/cm^2/min was an order of magnitude slower than fibrinogen desorption at 1.1 ng/cm^2/min. The increased fibrinogen desorption may be due in part to the removal of fibrinogen which binds to platelets and is pulled off the surface as the platelets embolize.

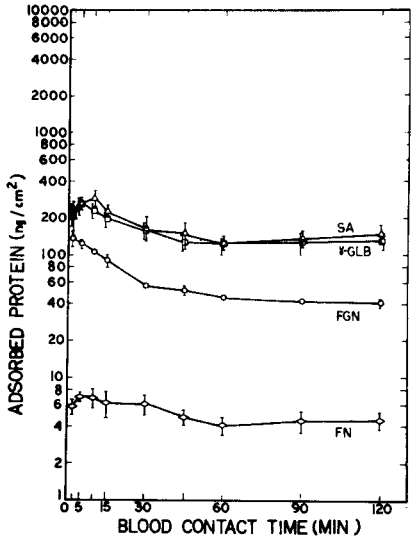

Figure 6: *Ex vivo* adsorption kinetics of canine albumin (SA), canine fibrinogen (FGN), canine γ-globulin (γ-GLB), and human fibronectin (FN) on PVC

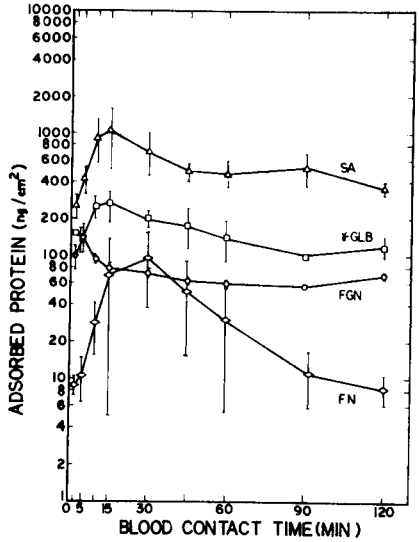

Figure 7: *Ex vivo* adsorption kinetics of canine albumin (SA), canine fibrinogen (FGN), canine γ-globulin (γ-GLB), and human fibronectin (FN) on SR

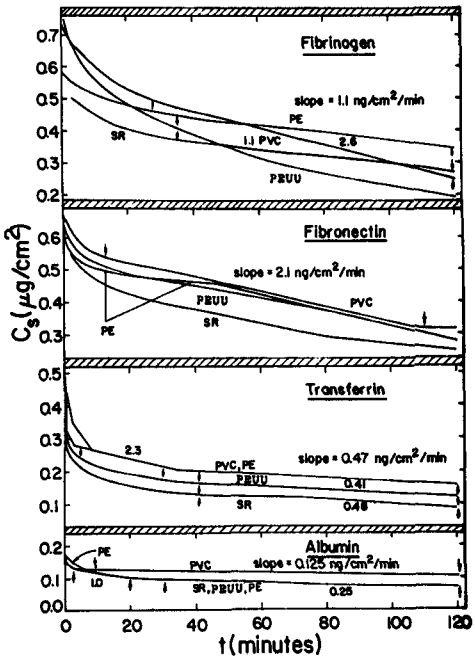

Figure 8: *Ex vivo* desorption kinetics of pre-adsorbed canine fibrinogen, human fibronectin, human transferrin ,and canine albumin on PVC

DISCUSSION

The adsorption kinetics of albumin and fibrinogen from single component solutions are different than the *ex vivo* adsorption kinetics. In the *ex vivo* experiments with blood containing about 40 mg/ml and 3 mg/ml albumin and fibrinogen,respectively, the surface concentrations are reduced by 50–90% of that observed *in vitro* for these same concentrations. The *in vitro* competitive adsorption data indicate that the qualitative result of having more than one type of protein present is to reduce the amount of a given protein from that observed in single component solutions. Not only do proteins compete for sites during adsorption, but proteins arriving later can displace previously adsorbed proteins. In blood which contains hundreds of proteins, the competitive and sequential adsorption of proteins is very complicated.

Although single protein adsorption studies do not reveal all of the interactions influencing protein adsorption, they are still essential in revealing the primary adhesive interaction between the protein and surface. But research should also be directed toward understanding sequential and competitive adsorption studies from multicomponent solutions. Another productive research direction would be to use model surfaces and model proteins systems in which the molecular structures are known well enough that the interactions can be more easily studied [26,27]. Further research is very much called for since despite intensive recent work on protein adsorption, a comprehensive understanding of how adsorbed blood proteins behave at biomaterial surfaces has not yet been achieved.

ACKNOWLEDGEMENTS

The authors wish to acknowledge partial support of this work through the National Institutes of Health grants HL-21001 and HL-24046, and fellowship support for WGP from the Standard Oil company.

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