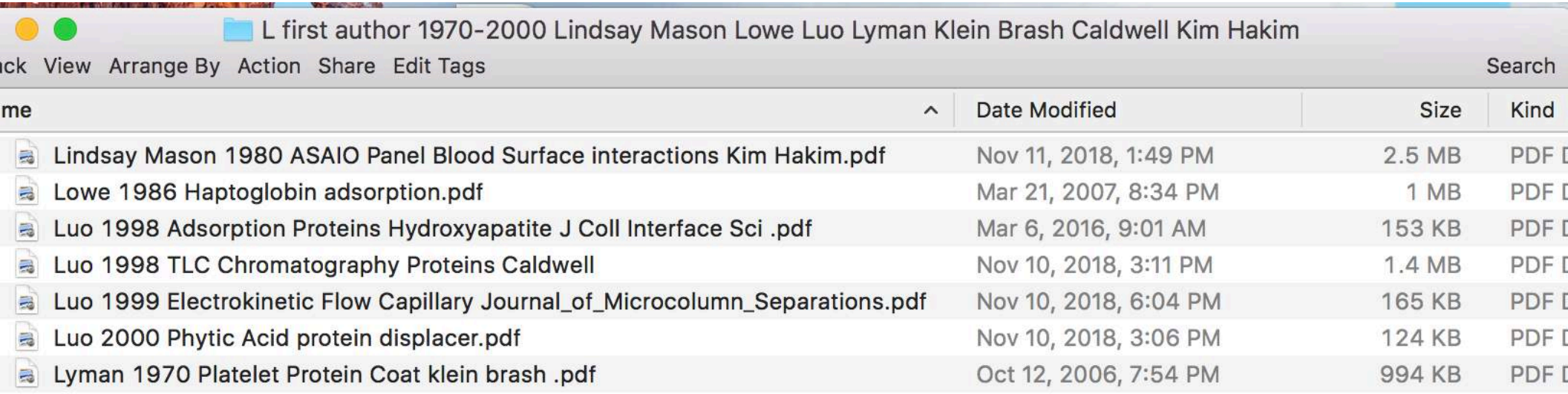
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PANEL CONFERENCE

BLOOD SURFACE INTERACTIONS

R. M. Lindsay[1], Chairman

Panelists:
R. G. Mason[2], S. W. Kim[2], J. D. Andrade[2], and R. M. Hakim[3]

The objective of this particular panel discussion on blood surface interactions was to highlight recent developments that appear to have clinical impact and to indicate how the clinical scientist could direct the bioengineer for future research development. A detailed discussion of the following topics was made: 1) The real problems of importance in the interaction between the formed elements of blood and coagulation parameters with the surface. 2) That the whole interrelationship between device and patient had to be examined rather than just the blood-surface interface. 3) How surfaces might be modified by pharmacological means and what seems to show promise for the future in this direction. 4) How the bioengineer should monitor surface modifications with regard to likely influence of these changes on thrombogenicity. 5) The complement story: is this a clinically significant phenomenon?

DISCUSSION

Interaction Between Blood and Coagulation Parameters with the Surface. The exposure of blood to artificial surfaces leads in many cases to adsorption of plasma proteins upon the material with subsequent deposition of platelets and leukocytes. Adsorption of plasma proteins can lead to activation of the blood coagulation, complement, and fibrinolytic systems. This activation may be of slight degree or, depending upon the degree of activation and blood flow conditions, fibrin may be deposited on the surface. In general, the sequence of events appears to be the following: adsorption of plasma proteins, adhesion of platelets and leukocytes, activation of platelets followed by the release reaction, recruitment of nearby platelets by released products including ADP and thromboxane A_2, aggregation of recruited platelets upon the layer of adherent platelets with eventual formation of a mural thrombus. This mural thrombus frequently is associated with fibrin formation, and the presence of fibrin stabilizes the thrombus. Leukocytes contribute to activation of the blood coagulation sequence as well as to removal of fibrin and markedly altered platelets. Erythrocytes play a small but significant role in mural thrombus formation also.

The formation of layers of adsorbed protein and adherent platelets and leukocytes upon artificial surfaces exposed to blood depends upon a number of factors. Among these are: the nature of the surface of the biomaterial, the specific types of plasma proteins adsorbed and the effect of this on activation of the blood coagulation and complement and fibrinolytic systems, the type of anticoagulant that may be present in the blood, the flow properties of blood near the interface with the biomaterial, and possible low grade activation of platelets and the blood coagulation system at points in the vascular system remote from the biomaterial. Some artificial surfaces appear to attract predominantly platelets, while others attract populations of neutrophils and macrophages. In either case, adherent platelets or leukocytes appear to contribute to activation of nearby platelets as well as the activation of the blood coagulation systems. These activated platelets and leukocytes appear to be capable of triggering both the intrinsic and extrinsic blood clotting systems; activation of these 2 systems reinforce each other.

Our knowledge of the mechanisms by which the intrinsic blood coagulation system is activated by contact of blood with an artificial surface is incomplete. A number of studies indicate that fibrinogen is a major component of the adsorbed protein layer that forms upon initial exposure of blood to biomaterials. Some of this fibrinogen appears to be replaced on the biomaterial surface fairly quickly by high molecular weight kininogen. This latter substance is necessary for the attachment and activation of factor XII. Once factor XII is activated, the intrinsic blood coagulation system will proceed to produce fibrin. Recent work indicates that certain of the components of the intrinsic blood coagulation system activated early in the cascade sequence can activate factor VII thus leading to activation of the extrinsic blood coagulation system also.

Important in the formation of mural thrombi are flow phenomena. Areas of stagnation of blood or areas of vortical flow tend to enhance thrombus formation in part because activated coagulation components are not diluted out by mixing with the main blood flow.

Antithrombin III is the most potent controlling factor for the blood coagulation systems, since it is a strong inactivator of most of the activated enzymes that comprise the cascade sequence. Antithrombin III reacts with heparin, and the reaction of antithrombin III with heparin immobilized on a surface may explain, in part, the antithrombotic effect of such surfaces.

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From a clinical standpoint, so long as artificial surfaces activate platelets, leukocytes, and the blood coagulation system it appears necessary to use anticoagulants and antithrombotic agents. Heparin is a potent anticoagulant and is best for short-term use, even though it does not inhibit activation of platelets. Dicumarol compounds are better suited for long-term use, since they can be given by the oral route. Studies are accumulating to indicate that in certain instances agents, such as aspirin and dipyridamole or sulfinpyrazone, can inhibit effectively platelet activation and consumption. More studies are needed in order to define the proper use of antithrombotic agents either alone or in combination with anticoagulants.

The Device-Patient Interrelationship: Observations Based Upon Heparin, Platelet Foreign Surface Interactions. It is very easy for the bioengineer to consider surface induced thrombus formation by examining the interface between blood and a given surface. It is our opinion, however, that this may not be enough and that the investigator must consider problems beyond that inferface; it may be that the device can modify the patient to such an extent that it changes the blood surface interaction. Furthermore, the situation may become complicated by the interrelation between anticoagulants, blood and surface and then, further, by an interaction between the patient and the anticoagulant. Examination of heparin-platelet-foreign surface interactions in patients with uremia undergoing regular hemodialysis exemplifies this problem.

It is well known that dialysis unit personnel often complain that the heparin they use for hemodialysis anticoagulation appears to be of variable potency. This assumption is derived from the observation that heparinization cannot be uniformly judged on a body weight basis and that a given patient may require different heparin dosages from time to time. It is well known that commercially available heparin preparations represent a variable polydisperse mixture of polysaccharides in terms of molecular weights and other properties. Furthermore, it has been shown that the material referred to as heparin can be separated into active and inactive fractions as far as its anticoagulant properties are concerned. Thus, it is very easy to incriminate the heparin preparation when faced with the clinical problem of a dialysis patient who either clots his dialyzer or experiences excessive bleeding from his cannulation sites. To this date, however, no one appears to have considered the possibility that the variability in heparin requirements might be related to changes occurring in the interrelationship between the patient and his extracorporeal circuit. It is our hypothesis that such changes are more likely to be the cause of variable heparin requirements than are any variations in the biological potency of present day, commercially available heparin. The reasons for this assertion are presented below.

To prevent thrombosis taking place during hemodialysis, the anticoagulant heparin is used. This agent acts on the intrinsic coagulation pathway by dramatically accelerating the rate at which antithrombin-heparin cofactor neutralizes the serial proteolytic enzymes in the hemostatic cascade mechanism. It has no such inhibitory effect on the platelet-foreign surface interaction. Indeed, some authors have actually shown that heparin in concentrations used during hemodialysis may actually enhance the retention of platelets on a foreign surface and, thus, heparin cannot be considered as an ideal anticoagulant for extracorporeal use. It has been clearly shown that thrombus formation can take place upon the artificial membranes of a hemodialyzer in spite of adequate heparinization and that this occurrence is due to platelet retention by the artificial surface. This has been proven by the finding that antiplatelet agents will reduce the platelet retention, and the amount of thrombus formed, during hemodialysis. Why then may variable amounts of heparin be required for dialysis with a given patient? One obvious situation would be if that patient changed his dialyzer to one with a greater thrombogenic potential. This invariably implies that some factor causing enhanced platelet surface retention is operative and, in this situation, the nature of the dialysis membrane and the geometry that the membrane conforms to, as induced by its support system, are important. Of even more relevance, however, is the fact that the patient's intrinsic platelet function (which, of course, will reflect its ability to adhere to foreign surfaces and to release its contents) will vary according to the adequacy of dialysis. Furthermore, we have to consider the fact that one of the constituents released by platelets has a direct antiheparin effect. These aspects should now be considered in some more detail.

It is accepted that platelet abnormalities form the major coagulation defect in uremic patients and these may be both quantitative and qualitative. Improvement in the qualitative defect by dialysis was first noted as far back as 1976 and several groups since then have confirmed this, indicating that the thrombocytopathy of uremia is due, at least in part, to a dialyzable factor or factors. It is now accepted that platelet function is relatively normal in chronic renal failure until the serum creatinine exceeds 6 mg% and thereafter progressively deteriorates. After the commencement of dialysis, however, platelet function can be normalized providing efficient techniques are used by either the hemo or peritoneal route and that this can occur in spite of the extracorporeal trauma associated with hemodialysis. Thus, it can be anticipated that platelet function in a given patient will be quite different at the commencement of renal replacement therapy from that found after 6 mos of adequate dialysis. Accordingly, his propensity to have platelet retention by relatively nonbiocompatible materials will change with improvement in the uremic status as brought about by his dialysis treatment.

As mentioned above, the platelet dialysis membrane interaction is implicated in thrombus formation during hemodialysis. Platelet retention by foreign surfaces in this situation is followed by release of platelet constituents including platelet factor 4 (PF_4) which is released in parallel with serotonin. PF_4 appears to be a substance in the molecular weight range of 7-9000 Daltons and which has heparin neutralizing activity (HNA).

Interestingly enough, HNA levels have been found to be increased in the plasma of patients with end-stage renal disease treated by maintenance hemodialysis. HNA levels, however, are not raised in nondialyzed patients with severe chronic renal failure, nor in patients with normal renal function who have been exposed to the extracorporeal circulation of the heart-lung bypass 72 hrs before testing and, furthermore, plasma HNA is normal in patients with end-stage renal disease treated by intermittent and chronic ambulatory peritoneal dialysis. It is postulated, therefore, that the trauma of the extracorporeal circulation causes platelets to release PF_4 which is not cleared by the dialysis membrane of the artificial kidney, but is by the human kidney. The elevation in plasma HNA so caused must have therapeutic implications for heparin dosage schedules during hemodialysis and, indeed, it has been confirmed that there is a correlation between an elevated plasma HNA level and the necessity to give greater than usual heparin dosages during hemodialysis.

From the argument presented above, therefore, it should be clear that heparin is not an ideal anticoagulant for hemodialysis, yet it is the only one available. Thrombus formation may take place during hemodialysis in spite of apparently adequate heparinization and the platelet foreign surface interaction is of major importance in this occurrence. The ability of platelets to release their constituents is likely to be enhanced by the relief of the uremic syndrome, as afforded by adequate dialysis, and also by materials used in the extracorporeal circuit which are of poor biocompatibility. It should be perfectly clear, therefore, that variations in heparin dosage for hemodialysis are likely in the dialysis population and cannot necessarily be predicted on a body weight basis. Thus, it seems clear to us that when questions of biocompatibility are being considered, it is no longer just possible to merely consider the blood foreign surface interaction, but one should rather consider the whole interrelationship between the patient and the device which is treating the patient.

Pharmacological Modifications of Polymers. Surface thrombosis is a major concern with the use of artificial organs and blood contacting biomedical devices. Numerous investigators have attempted to develop thromboresistant materials in the past, and research in this area remains equally active today; however, due to the complexity of the mechanisms involved in thrombogenesis at foreign surface interfaces, the development of thromboresistant materials has met only with limited success.

The rationale in the development of thromboresistant materials, as reported in the literature, is split into 2 philosophies. The first approach is to prevent activation of the thrombogenic pathways by tailoring the physiochemical properties of the polymer surface to either minimize all blood interactions, or to selectively adsorb a nonreactive albumin layer. Although this approach may perhaps be the more desirable and aesthetically pleasing of the 2, little real progress has been achieved and the final realization of a thromboresistant material is far from fruition.

The second approach is to incorporate pharmacologically active, anticoagulating agents with existing polymers either via bulk dispersion or by surface immobilization of the anticoagulants.

The anticoagulant most widely used today for prevention of thrombosis with the use of artificial organs is heparin. Although the pharmacological functions of heparin are not totally understood, it is generally believed that heparin prevents fibrin formation by first binding to antithrombin III (AT III). This heparin-AT III complex then irreversibly binds to thrombin, thereby preventing conversion of fibrinogen to fibrin. Although heparin therapy is relatively successful, it is not a panacea and many untoward side effects, including thrombocytopenia, are associated with acute and repeated administration. This is especially critical to patients with hemostatic disorders.

In view of heparin's wide use and untoward side effects when systematically administered, it is not surprising that a plethora of research in the development of thromboresistant materials has involved incorporation of heparin with existing polymers. The pioneering work in this area was initiated by Gott et al and further pursued by the Battelle group. In both cases a quaternary ammonium surfactant was first affixed to the polymer surface, heparin was then ionically bound to the surfactant. When this material was exposed to blood, heparin was released via an ion exchange mechanism, leaving an undesirable final surface. Tanzawa later introduced quaternary amine groups in hydrogel copolymers, to which heparin could be ionically bound. This material would then slowly release heparin as above. It was found that increasing the copolymer water content increased the heparin release rate and that only high water content copolymers significantly reduced fibrin formation, indicating that heparin directly affixed to the surface was biochemically inactive and that only released heparin was effective in prevention of fibrin formation.

This conclusion is further substantiated by the work of Saltzman et al who covalently bound heparin to surfaces of cellophane and sepharose beads. In these studies it was found that the final materials were less blood compatible than the starting materials. Olson et al have also attempted to permanently surface immobilize heparin. Their procedure is to first ionically bind heparin to surfactant molecules previously adsorbed onto polyethylene surfaces. The heparinized surface is then lightly crosslinked with glutaraldehyde. The above treatment only modifies the physiochemical properties of the surface to selectively adsorb a nonreactive protein layer from blood.

Our current studies would establish criteria for the design of long-term, biologically active immobilized heparin surfaces as follows:

1. Modify heparin by ligand substitution such that clotting activity is not affected. Intermolecular cross-linking is avoided by prior acetylation of free amines on heparin.

2. Immobilize substituted, N-acetylated heparin to existing polymer surfaces using the ligand substituion groups as a linkage between the surface and the heparin molecules.

3. Nonthrombogenicity would be due to the biochemical effect of heparin (i.e., via interaction with appropriate blood factors) rather than simply due to surface modification to selectively adsorb inert blood protein layer.

4. Determine optimal spacing distance (i.e., length of ligand substituent) for proper heparin interaction with blood factors.

We have found that N-acetylated heparin maintains biological activity following amidation of carboxylic groups with n-butylamine; however, nonacetylated heparin loses its activity significantly upon modification. Significant progress in the effective immobilization of heparin is currently being made in our laboratory.

Since Sutherland and Rall first showed evidence that cAMP functions as an intracellular messenger in biological systems, several laboratories have offered data suggesting a regulatory role for platelet cAMP. Platelet aggregation is known to be associated with a fall in platelet cAMP levels, while inhibition of aggregation is associated with a rise in cAMP levels. A reduction in cAMP may result either from inhibition of adenylate cyclase or by stimulation of phosphodiesterase. Prostaglandins such as PGE_1, PGD_2 and PGI_2 (prostacyclin) stimulate adenylate cyclase activity while caffeine, dipyridamole and papaverine inhibit phosphodiesterase. Prostaglandins, especially PGI_2, are known to be a potent inhibitor of ADP induced platelet aggregation. Our studies have shown that ADP induced platelet aggregation in platelet rich bovine plasma is inhibited 50% by PGI_2, PGE_1 and PGD_2 at plasma concentrations of 5.3×10^{-10} M, 1.3×10^{-7} M and 1.4×10^{-6} M, respectively.

Bunting et al documented improved hemocompatibility for extracorporeal, charcoal hemoperfusion systems evaluated with dogs receiving PGI_2 infusions at 50 ng/kg/hr. The prostacyclin infusion dramatically reduced platelet cytopenia without the formation of thromboemboli or changes in plasma fibrinogen levels, relative to control animals. Furthermore, Coppe et al have determined that platelet number and function are preserved during extracorporeal perfusion of membrane oxygenators with sheep receiving regional infusions of PGI_2.

Although animal studies and limited human elevations have yielded promising results, the infusion of PGI_2 into human patients will not likely be recommended in the near future due to the difficulties in the verification of adequate dosing and the possibility of side effects such as vasodilation. If the use of prostaglandins is limited to reduce local thrombus formation mainly at blood/polymer interfaces, constant release of small amounts of these agents at the interface can be effective anticoagulation strategy, with minimal systemic side effects. We have shown that prostaglandins combined with polyurethanes and polyvinylchloride were pharmacologically active when released from the polymer matrix, even after prolonged storage of the matrix systems. These prostaglandin releasing polymers (typically releasing microgram quantities per square centimeter per day) showed no platelet deposition on surfaces during in vitro experiments, and long-term in vivo experiments are in progress at the Division of Artificial Organs, University of Utah.

Two independent but overlapping mechanisms are involved in thrombus formation, namely, fibrin formation via activation of the intrinsic coagulation pathway and platelet adhesion and aggregation. Heparin and prostaglandins are effective for the prevention of fibrin formation and platelet interaction respectively. An interesting phenomena observed by Longmore et al is that routine heparinization with simultaneous PGI_2 infusion during extracorporeal circulation with dogs produced no change in plasma platelet levels or in platelet function. These results suggest that adequate anticoagulation may be achieved by simultaneous infusions of both heparin and PGI_2 at significantly reduced levels relative to normal, separate dosing regimens.

Recently we designed a simultaneously releasing prostaglandin-heparin polymer using poly-(hydroxyethylmethacrylate) (p-HEMA). In this design, heparin antagonism of the PGI_2 antiaggregatory effect on platelets, as shown by Mason's group, was a concern. We have determined the neutralization effects on prostaglandins caused by heparin, using a variety of heparin doses. The results indicated that PGI_2 function is sensitive to the heparin doses. Heparin at a concentration of 100 U/ml PRP significantly reduced the PGI_2 inhibitory activity of ADP induced platelet aggregation, while PGE_1 and PGD_2 showed no reduction of their antiaggregatory activities at identical heparin levels. According to our preliminary data, PGI_2 binding to heparin is drastic while PGE_1 and PGD_2 bindings are low, which may explain the heparin effect on PGI_2. In addition, PGE_1 is much more stable than PGI_2 in aqueous systems. Therefore, we have selected PGE_1 to design the heparin-prostaglandin combined release systems, even though PGE_1 is less potent than PGI_2. We have found that 1% PGE_1 substantially slows the release of heparin from p-HEMA. Heparin was found to increase the release of PGE_1 from the hydrogel matrix as compared to PGE_1 release without heparin. The released amount is always linearly proportional to the square root of time. In order for heparin to be released, special techniques for the fabrication of matrix systems are required to allow diffusion of macromolecules through the polymers. The extent of biological activity for the released PGE_1 was very close to the control data, which indicates the independence of PGE_1 on the existence of heparin in the polymer matrix. The released heparin also provided unchanged activity, as shown by APTT. Our data suggests that both heparin and PGE_1 released from polymer matrices proved to be biologically active according to APTT and platelet aggregation experiments respectively.

The use of controlled release bioactive agents or immobilized agents, by our experience, significantly reduces surface thrombosis and could be a promising system for application with blood contacting polymeric devices.

In Vitro Blood-Materials Interactions: A Multi-parameter Approach. The importance of surface and interface characteristics in blood materials interactions has been the subject of much controversy. This controversy is partially the result of a lack of characterization of materials and partly due to the lack of standard test methods for blood materials evaluation (NHLBI Task Force Reports on Blood-Material Interactions).

This study was designed to test several currently popular hypotheses of blood compatibility using well characterized model surfaces and simple in vitro blood evaluation methods. The hypotheses tested are the hydrophilic/hydrophobic ratio hypothesis, the interfacial free energy hypothesis, the negative surface charge hypothesis and the surface mobility hypothesis.

We chose a series of model polymers based on the methacrylate family to develop materials with the requisite properties for the testing of the various hypotheses. Methylmethacrylate (MMA) and hydroxyethylmethacrylate (HEMA) can be copolymerized to produce copolymers varying in bulk water contents from approximately one wt% for polyMMA to approximately 40 wt% for polyHEMA. This series of copolymers allows one to probe the interfacial free energy hypothesis and the hydrophilic/hydrophobic ratio hypothesis as the degree of hydrophilicity increases linearly with HEMA content of the copolymer. It has already been shown that for a 40 wt% water material the interfacial free energy is already as close to zero as can be measured using conventional contact angle methods.

The negative surface charge hypothesis can be probed by copolymerizing methacrylic acid with HEMA to provide negative charge in a hydrophilic environment or copolymerized with MMA to provide negative charge in an initially hydrophobic environment. The role of positive charge can also be assessed by using quaternized derivatives of the methacrylates, such as quaternized dimethyl aminoethyl methacrylate.

The surface mobility hypothesis is probed by using a series of hydrophobic methacrylates of different alkyl side chain lengths ranging from methyl to octadecyl. As the chain length increases, the polymers become less rigid and of a softer and more elastomeric nature. The surface mobility hypothesis can thus be probed in an essentially hydrophobic environment of relatively constant surface character.

In addition to these materials, a number of control materials have been used including glass, unfilled poly(dimethyl siloxane), and various common biomedical elastomers, particularly Biomer. The materials evaluation protocol consisted of fabrication in a dust-free environment using filtered polymer solutions spun cast onto ultra-clean glass coverslips and microscope slides. The materials are characterized by differential scanning calorimetry to determine their glass transition temperature.

Surface characterization includes light microscopy as a check on surface roughness and for particulates, and x-ray photoelectron spectroscopy (ESCA) as a measure of surface chemistry and surface elemental composition. Extensively water-extracted materials are equilibrated in 0.01 M KCl to measure streaming potentials from which zeta potential is calculated. Extensively water extracted samples are also equilibrated in phosphate buffered saline at pH 7.4 to measure water content and degree of swelling. Water extracted materials are equilibrated in distilled water for the measurement of air and octane contact angles underwater. These 2 measurements allow us to calculate the polar and dispersion components of the fully hydrated solid surface free energy and the solid-water interfacial free energy. This surface characterization protocol enables one to determine the interfacial energetics, the interface potential, and the surface chemistry, as well as to obtain a crude measure of surface mobility through the bulk glass transition temperature measurement.

It is important to note that in this family of materials, as in virtually all materials, it is not possible to vary one surface parameter without simultaneously affecting the other parameters. For example, incorporating negative or positive charge into a material generally increases its bulk water content and therefore its hydrophilicity, which influences its interfacial energetics.

In order to evaluate the blood interactions of these materials, a blood evaluation protocol was devised which consists of a multi-parameter analysis of the coagulation system. The well characterized materials are evaluated for their effect on the intrinsic system through the measurement of whole blood clotting time using coated test tubes and partial thromboplastin times using a parallel plate cell. The extrinsic system is monitored for control purposes by the prothrombin time using a parallel plate cell. Platelet function is monitored by measurement of the Russell's viper venom time which determines platelet factor 3 activity in a parallel plate cell. Platelet adhesion is monitored in a parallel plate cell using platelet poor plasma. Platelet morphology is also evaluated in these same experiments. The blood evaluation protocol therefore consists of a multi-parameter blood function assay which, together with the multi-parameter surface characterization assays, allows one to develop a matrix which enables us to begin to draw correlations between surface properties and blood materials interactions.

The data suggest that a single parameter correlation either does not exist for most materials or, for most of the hypotheses examined, is at best grossly misleading. For example, a good correlation exists between platelet adhesion and interfacial free energy, showing that as the interfacial free energy decreases, platelet adhesion decreases, in apparent support of the minimum interfacial free energy hypothesis. However, if one plots whole blood clotting time as a function of interfacial free energy, increased interfacial free energy shows a longer whole blood clotting time, indicating more optimal blood compatibility. Therefore, one measure of blood function,

platelet adhesion, correlates in one direction with interfacial free energy; a second measure of blood function, whole blood clotting time, correlates in roughly the opposite direction. Both platelet adhesion and whole blood clotting time have been used by many investigators as normally accepted measures of blood compatibility.

If one examines the polar character of the surface and attempts to correlate polar character with whole blood clotting time, we find that whole blood clotting time decreases with increasing polar character, thus suggesting that high polar character is not desirable or making an argument for apolar surfaces. However, if we look at platelet adhesion, we find that platelet adhesion decreases with increasing polar character. Therefore, the 2 correlations again are in opposite directions.

The effect of alkyl chain length, i.e., surface mobility in the methacrylates as the side chain varies from methyl to octadecyl methacrylate, shows no correlation between whole blood clotting time and alkyl chain length. Yet a strong correlation between platelet adhesion and alkyl chain length was observed, showing decreased platelet adhesion with increasing alkyl chain length.

The major conclusion that one can draw from such studies is that a multi-parameter approach is necessary to fully understand blood-materials interactions. It is expected that such an approach will lead to a thrombogenicity index for materials which consists of a number of parameters, each one representing the surface properties of the material and various measures of blood function. One might envision the development of a thrombogenicity index somewhat analogous to the multi-parameter toxicity index developed by Autian et al.

A preliminary report of the study discussed here has appeared (Coleman and Andrade, 1980). The complete study will be available shortly (Coleman, in preparation).

Complement Activation. It has become clear to us that we have to discuss the clinical importance of complement activation when we consider blood foreign material interactions, for it does appear that such activation occurs in most blood material interactions and that this activation may well be of clinical significance and result in adverse side effects. Finally, it may be that complement activation is only part of a general blood material interaction that needs to be looked at more closely under the heading of that general term biocompatibility.

The complement system refers to a complex group of proteins in normal blood serum which, when working together with other body systems, play an important role as mediators of both immune and allergic reactions. When the complement system is activated and acts against foreign cells, it is called immunity. When it acts against the host cells, such reactions are known allergic or hypersensitive reactions. Thus, in order to minimize the damage to the host's own cell, the activation and the activity of the complement system must be limited in time and scope. This is done via 3 mechanisms: 1) The complement system has a specific recognition site of its own so that it can respond to the appropriate stimulus. 2) It has receptor sites that will enable it to combine with the surface of the foreign cells when it is activated. 3) Most important, it has a short active half life which is partly due to spontaneous decay of activated complement systems and partly due to inactivation by inhibitors and destructive enzymes. To date we know of 21 distinct proteins that are considered constituents of the complement system. Thirteen may be considered components proper and 8 are regulatory proteins. The components of the complement system are divided into 2 different pathways of activation which at one point become a common pathway for membrane attack. One of the pathways is called the classical pathway and the other is called the alternative pathway. Activation of the classical pathway occurs primarily by immune complexes and sometimes by some types of virus. The alternative pathway is an older system and responds to more basic stimuli. It may be activated by plant, fungal and bacterial polysaccharides and the saccharide moiety of lipopolysaccharides that constitute the wall of gram negative bacteria. Experimentally in the lab, inulin, which is a polyfructose, or zymosan, which is a carbohydrate of the yeast cell membrane, are used to activate the alternative pathway. There is no specific chemical structure common to the various activating substances, but there seems to be a need for the material to have a repeating subunit that is polymeric in nature. Inulin, for example, is not capable of activating the pathway in its monomeric form, but is a potent activator in its polymeric form. It is the alternative pathway of complement activation that is the predominant system of complement activation in blood materials interaction. Perhaps this is not surprising when one considers the polysaccharide structure of cellophane which was very early on noted to be a potent complement activator. The evidence for this comes from various experiments - some in vitro some in vivo, involving animals, and some recent data that has been described on dialysis patients. If one measures the function of components of complement during dialysis, as did Craddock in his early work, one quickly finds evidence for activation of several components of complement. For example, Craddock found C3 activation within 10 mins of the institution of blood flow from the efferent line of a cellophane dialyzer, and at 20 mins C3 activation was evident in both afferent and efferent samples. In vitro activation by simply incubating a piece of cellophane in serum also showed a significant reduction of total hemolytic complement C3 activation. By doing various other experiments on heat treated plasma and C2 deficient plasma, he eventually showed that complement activation does occur and primarily by the alternative pathway. In our own data from the Peter Bent Brigham Hospital, we have shown that by 15 mins into dialysis, samples taken from a dialyzer arterial line showed a 35% reduction in the total hemolytic complement, thus supporting the work of Craddock.

So what then is the evidence that this complement activation is clinically significant? It would appear that the clinical significance rests on the ability of activated complement components to lead to transient leukopenia

during dialysis. This has been shown by infusion of autologous cellophane-incubated rabbit plasma into rabbits, which caused significant neutropenia and the degree of neutropenia was proportional to the rate of infusion of this activated plasma. When the activated serum was heated so that complement became inactivated, then the infusion did not result in leukopenia. Again, from our own work at the Peter Bent Brigham Hospital, we have shown that the extent of total hemolytic complement fall is directly proportional to the degree of leukopenia. Furthermore, different dialysis membrane will cause different degrees of activation of complement and those which cause the most activation of complement also result in the most profound leukopenia. The leukopenia so induced is so severe that for a period of about 30-45 mins there is almost 90% depletion of all neutrophils from the systemic circulation. Furthermore, there is considerable evidence that neutrophil function during this period is also greatly impaired. For example, we have shown that the chemotactic ability of neutrophils during the period of neutropenia is down to 5% of its predialysis value and we have also demonstrated decreased phagocytic activity of these neutrophils. Although there is no clear evidence that these laboratory studies of neutrophil function have adverse clinical correlates, it is well known that neutrophil adherence, chemotactic and phagocytic abilities are essential mechanisms in the complex process for combating infections. Dialysis patients are prone to bacteremic infections and, in fact, infection is the principal cause of hospitalization in dialysis patients, as was recently reviewed by Lazarus, Lowrie and their colleagues, and it is strong circumstantial evidence that the cyclical variation in the number and function of neutrophils may well contribute to this high rate of morbidity.

The other relationship that is of clinical significance is the relationship between dialysis-associated hypoxemia and leukopenia. Although this has been a subject of debate, there is strong animal evidence that the leukopenia is due to the sequestration of neutrophils in the lung parenchyma. The specific reason that neutrophils aggregate in the lungs is due to the activation of complement and, specifically, the activation of the C5 component, which is strongly chemotactic to leukocytes and causes aggregation of neutrophils. Although there is probably more than one reason for the dialysis-associated hypoxemia, such as acetate metabolism and CO_2 diffusion across dialyzers, there is evidence that the sequestration of neutrophils in the lungs leads to a transient but significant V/Q abnormality that results in hypoxemia. In fact, some authors had found a linear correlation between the hypoxemia and the neutropenia, and our own work has shown that dialyzer membranes that cause more neutropenia also cause more hypoxemia. Again, the animal studies by Craddock are much more conclusive. The harmful effects of this hypoxemia became evident when studies by Chen and Freidman and others showed that in patients on dialysis who do not use supplemental oxygen to blunt hypoxemia, there was a decrease in contractility and ejection phase indices that result in a significant incidence of hypotension. There is also some evidence that long-term dialysis patients have a high incidence of fibrosis and calcinosis of the lung parenchyma. How general are these post-mortem findings to the dialysis population is not clear at present, but it is something worth looking at.

Recently Jacob, Craddock and their colleagues have suggested that many other disease states, such as sudden blindness in severely traumatized patients and those with acute pancreatitis and the initial stages of the adult respiratory distress syndrome, may well be due to leukocyte aggregation secondary to complement activation, causing what was termed leukoembolic phenomena. This was based on animal studies in which complement activation caused leukocyte aggregation that occluded vessels up to 60 μm in diameter. There is also some evidence that components of complement play a predominant role in the pathogenesis of hypotension and some of the other adverse symptoms of dialysis such as nausea, vomiting and itching.

Finally, it is worthy to note that complement activation occurs as part of a more general activation. We have talked about the activation of complement during the dialysis procedure. Along with the activation of complement there is also activation of the coagulation pathway that is only aborted with large doses of heparin. There is also preliminary work to show that kinin levels are significantly elevated during dialysis, and it is highly likely that the activation of these 3 pathways are probably interrelated. Therefore, it would make perfect sense in biocompatibility studies not just to consider the coagulation mechanism, but to consider these other pathways as well as they must play a role in the general interrelationship of the patient and his device.

SUMMARY

From the discussion which followed the presentations of the panelists, it was clear that the bioengineer must, in future, look beyond blood clotting upon a foreign surface as the sole index of biocompatibility. He must consider the whole interrelationship between the device he is designing and the living organism it is to be used on and, in doing so, should consider systems beyond the coagulation process. One system that requires further study is complement and researchers are urged to study: a) how surface modifications, physical and pharmacological, can influence complement activation, b) the interrelationship between complement activation and the coagulation process, and c) what clinically adverse effects can occur because of such system activation. Hopefully, a further report on progress in these fields will be presented to ASAIO in the future.

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Human haptoglobin adsorption by a total internal reflection fluroescence method

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Haptoglobin (Hp) is one of the major protein constituents of plasma. Three different forms are found in the population. The 1-1 and 2-2 forms adsorb similarly onto hydrophobic silica [treated with dimethyl dichlorosilane (DDS)] and onto clean silica, although the affinities on the silica surface are lower at 60 minutes contact time. The two forms desorb differently from silica, but desorb similarly from DDS-silica. Adsorption is less reversible on the hydrophobic surface. Due to its adsorbtion tendencies and its high concentration in plasma, the adsorption of Hp may be important in blood interaction at solid–liquid interfaces.

Keywords: Adsorption, haptoglobin, silica, plasma, dodecyl silane, TIRIF

The difficulty in maintaining patency of venous implants is well known. The different interactions between venous and arterial environments has been attributed to physical or chemical parameters. We now know that transient haemolytic events can result in haemoglobin (Hb) release[1, 2]. If the oxy form of haemoglobin in the arterial system is released from the red cell, the oxyHb is rapidly and irreversibly bound to haptoglobin (Hp)[3, 4]. The deoxy form of Hb in the venous system is not bound by Hp[3, 4]. DeoxyHb and oxyHb adsorb differently onto artificial surfaces[5]. We also know that protein adsorption is related to subsequent blood interaction. If surfaces containing oxy or deoxyHb, Hp, or Hp–oxyHb complexes prove to show different blood interactions, then the surfaces designed for arterial compatibility may require different properties than those destined for venous application.

Hp is a serum glycoprotein that exhibits polymorphism[6, 7]. The physiological function of Hp is to bind strongly and irreversibly with Hb that has been released from red blood cells. This larger complex prevents undue loss of Hb iron through urinary excretion.

All three types of Hp consist of two kinds of polypeptide chains, the α and β chains, linked by interchain disulphide bonds. The genetic variability of Hp is based on structural differences of a polypeptide controlled by the Hp α locus[6, 8]. The α_1 chain has a molecular weight of about 9300 (84 amino acid residues) and the α_2 chain has a molecular weight of approx. 17 300 (134 amino acid residues)[9, 10].

The three common Hp types are given in *Table 1* (from References 11–13). The distribution of Hp types amongst the common populus is described in *Table 2* (from References 11–13). Hp types 2-2 and 2-1 both exhibit a polymeric series mediated by disulphide bonds that can be formaulated as $(\alpha_2\beta)_n$ (n = 3, 4, 5 . . .) for Hp 2-2 and $(\alpha_1\beta)_2$ $(\alpha_2\beta)_n$ (n = 0, 1, 2 . . .) for Hp 2-1[14].

The normal abundance of Hp is expressed as Hb binding capacity (HbBC). HpBC is the amount of haemoglobin (mg) that can be bound by Hp in a 100 ml solution. For men the HbBC = 113 ± 43 mg/100 ml, whereas for women HbBC = 94 ± 30 mg/100 ml. One mg Hb is bound by 1.3 mg of Hp[10].

ADSORPTION

Many methods have evolved to study interfacial adsorption, but no single independent method seems adequate. The ideal technique should produce quantitative, real time, *in situ* data concerning the amount, activity and con-

Table 1 The different types of haptoglobin (from References 11–13)

Hp Type	α chains	β chains	Average *MW*
Hp 1-1	$\alpha_1\alpha_1$	ββ	100 000
Hp 2-1	$\alpha_2\alpha_1$	ββ	220 000
Hp 2-2	$\alpha_2\alpha_2$	ββ	400 000

Table 2 Distribution of Hp types (from References 11–13)

Population	Hp 1-1	Hp 2-1	Hp 2-2
White American	15%	48%	37%
Chinese	11%	34%	54%
Japanese	6%	36%	58%
Blacks	40%	40%	20%

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formation of proteins adsorbed on well-characterized surfaces.

Total internal reflection intrinsic fluorescence (TIRIF) is a method for determining adsorbed protein at solid–liquid interfaces[15–19]. TIRIF has been used to study the adsorption of albumin[15, 19], IgG[19], fibronectin[16] and insulin[17]. When excited at 285 nm, proteins with tryptophan residues fluoresce maximally in the 320–350 nm range. This intrinsic fluoresence is sufficient to permit sensitive detection of the protein in the vicinity of the solid–liquid interface[19]. The use of intrinsic tryptophan avoids the problem of extrinsic labels, which may alter the adsorption properties of the protein.

The fluorescence signal can be quantitated by direct calibration to establish a surface concentration. The method used here is based upon the assumption that the quantum yields of protein in the adsorbed state and in the bulk state are identical. This assumption may be justifiable for Hp as many tryptophans are uniformly distributed throughout the molecule. It is unlikely that adsorption of a Hp molecule could significantly alter the average microenvironment of the tryptophans.

METHODS AND MATERIALS

The TIRIF technique, the apparatus, and the methods for quantitation have been described[15–19]. Variations and adaptations in the apparatus and experimental procedure will be noted.

All solutions were made from analytical grade reagents and low conductivity water. Phosphate-buffered saline (PBS) (0.145 M NaCl, 2×10^{-4} M KH_2PO_4, 8×10^{-4} M Na_2HPO_4) had a pH of 7.3 and osmolarity of 310 mosmol.

The hydrophilic, amorphous silica surface ('silica') was cleaned in 70°C chromic acid followed by water and ethanol rinsing and Freon TES-ethanol azeotrope vapour degreasing. The 'silica' surface was negatively charged under the pH and ionic strength conditions that were used: pH 7.3 phosphate-buffered saline and 0.145 M NaCl. The cleaned surface exhibited complete wetting and no contact angle hysteresis. This silica surface is considered to be clean and hydrophilic.

To obtain a hydrophobic surface, the silica was treated with dimethyl dichlorosilane (DDS). This procedure consisted of a 10 min soak in 10 vol. % DDS in dry toluene, followed by three rinses in absolute ethanol, one rinse in glass-distilled water, and a final ethanol rinse. The surface was air-dried, then cured in an 80°C oven with nitrogen flow for 3 h. The DDS surface typically exhibited a 90 ± 5°C water advancing contact angle and a 75 ± 5°C receding water angle.

The prism surface was cleaned prior to each experiment in the following sequence: (1) Thorough rinse in low conductivity, filtered water; (2) rinse in filtered, absolute ethanol, and (3) a 5 min vapour degreasing in Freon TES-ethanol azeotrope vapour. All surface cleaning was carried out under Class 10 000 clean room conditions to minimize particulates.

A digital shutter was incorporated into the TIRIF apparatus enabling the control of light exposure to the sampling area.

Hp isolation and purification techniques were adapted from the work of Hamaguchi[20] with a number of variations[8,20,21]. Desalted human serum was fractionated in batches of 25–30 ml with saturated ammonium sulphate [$(NH_4)_2SO_4$] adjusted to pH 7.0 with conc. NaOH. The precipitate obtained between 0.4 and 0.6 saturation was dissolved with a minimum vol. of distilled water. This protein solution was dialysed against distilled water for 1 h. The solution was then passed through a Sephadex G-100 column, 3.2 × 25 cm packed and equilibrated with 0.05 M phosphate buffer, pH 7.0. Each fraction was tested for absorbance at 280 nm; 20–25 ml of the first fraction was active in Hp. This fraction was applied to a DEAE–Sephadex column, 3.2 × 25 cm, equilibrated with 0.05 M phosphate buffer, pH 7.0. The fractions were collected and dialysed against sucrose for 3 h.

Hp concentration determination was achieved using the Bradford dye-binding method[22]. Characterization consisted of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)[23, 24]. A 10% polyacrylamide, 1% SDS vertical gel enabled confirmation of the Hp purity as well as its type.

Transmission fluorescence experiments showed nearly identical spectra and quantum yields for both the 1-1 and 2-2 solutions.

RESULTS

Figure 1 illustrates a typical TIRIF protein adsorption experiment with fluorescence intensity plotted as a function of time. Fluorescence was generated by exciting at 285 nm and observing at 330 nm. Region A is a series of control and calibration data. The cell is carefully pre-filled with PBS so that trapped air is avoided. Background counts (B) are recorded and interpreted as the baseline for the experiment.

Tryptophan solutions, 0.02, 0.05, 0.075 and 0.10 mg/ml were used for calibration purposes (C,D,E and F, respectively). At time zero, Hp was introduced by rapid injection at the rate of 2.0–2.5 ml/s for 1 s. The solution was then static (no flow) for the remainder of the adsorption process. Within the first 10 to 30 s after injection, protein arrives at the interface and begins to adsorb. After 2 min the shutter was closed. Thereafter, the shutter was opened for a period of 6 s every 5 min to give 2% excitation light exposure. Adsorption was allowed to proceed for 60 min (G).

After 60 min, 30 ml of PBS was quickly injected at 15 ml/min to remove the protein solution. For a 30 min period, desorption of the adsorbed protein occurred as PBS was injected at 1 ml/min. Again, using the shutter, the surface was excited with 2% light exposure (H).

The time scale in *Figure 1* is compressed in order to show the whole experiment. The time resolution of the technique is of the order of 1 s and fairly rapid kinetics can be followed. The background counts, as well as the tryptophan calibration standards, serve as indicators of light scattering by the prism/flow cell apparatus.

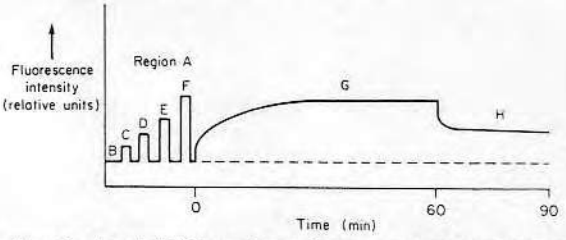

Figure 1 A typical TIRIF experiment. Fluorescence intensity monitored as a function of time.

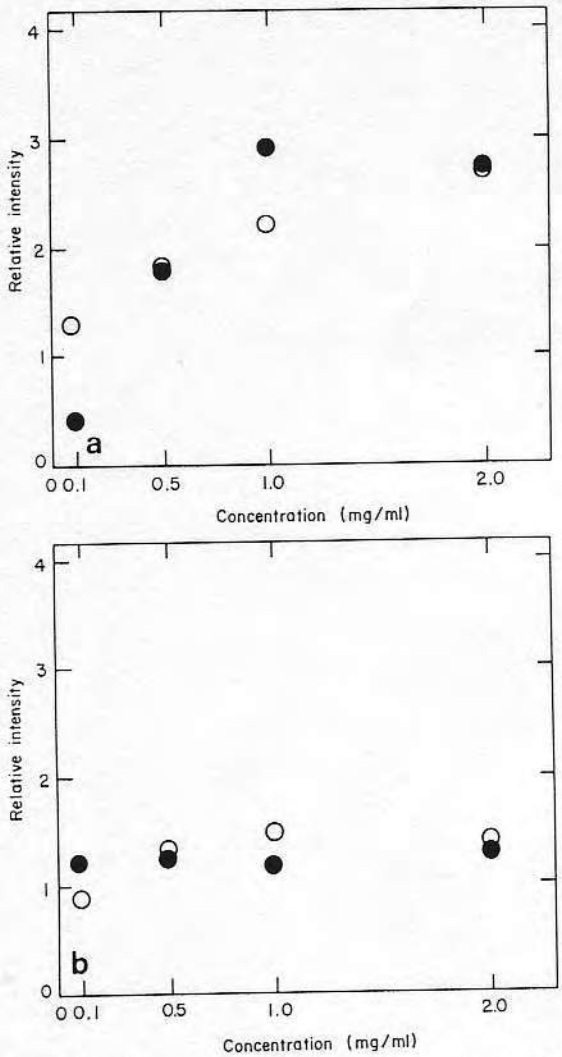

Figure 2 Haptoglobin 1-1 and 2-2 step adsorption isotherms at 60 min for conc of 0.1 to 2.0 mg/ml. Both proteins adsorbed similarly on both surfaces. (a) Onto hydrophilic silica; (b) onto DDS-hydrophobic silica. ●, Hp 1-1; ○, Hp 2-2.

Figure 2a shows a step isotherm for Hp 1-1 and Hp 2-2 adsorption onto hydrophilic silica. *Figure 2b* shows a step isotherm for Hp 1-1 and Hp 2-2 adsorption onto the hydrophobic (DDS-treated) silica surface. The adsorbed level is expressed as the adsorbed intensity, subtracting out the background. The background-subtracted data are plotted as post-flush, bound protein fluorescence intensity *versus* bulk protein conc. Bulk protein concentrations evaluated ranged from 0.1 to 2.0 mg/ml.

Figure 3a shows a step isotherm for Hp 1-1 and Hp 2-2 desorption from hydrophilic silica. *Figure 3b* shows a step isotherm for Hp 1-1 and Hp 2-2 desorption from the DDS-treated silica. The desorbed amount is expressed as the percentage of the total signal desorbed as a result of a 30 min, 1 ml/min buffer flush.

DISCUSSION AND CONCLUSION

Intrinsic u.v.-TIRIF has been used to study the adsorption and desorption of Hp 1-1 and Hp 2-2 on clean hydrophilic silica and hydrophobic dimethyl dichlorosilane-treated silica.

On clean, hydrophilic silica Hp adsorption increased with conc. up to the normal plasma conc. (~ 1.0 mg/ml). On dimethyl dichlorosilane-treated silica, this increase was less prominent. In addition, adsorption levels were greater on hydrophilic silica than on hydrophobic DDS-treated silica. Quantitation of the fluorescence signal would yield a direct relation to surface conc., but this method is still under development[19].

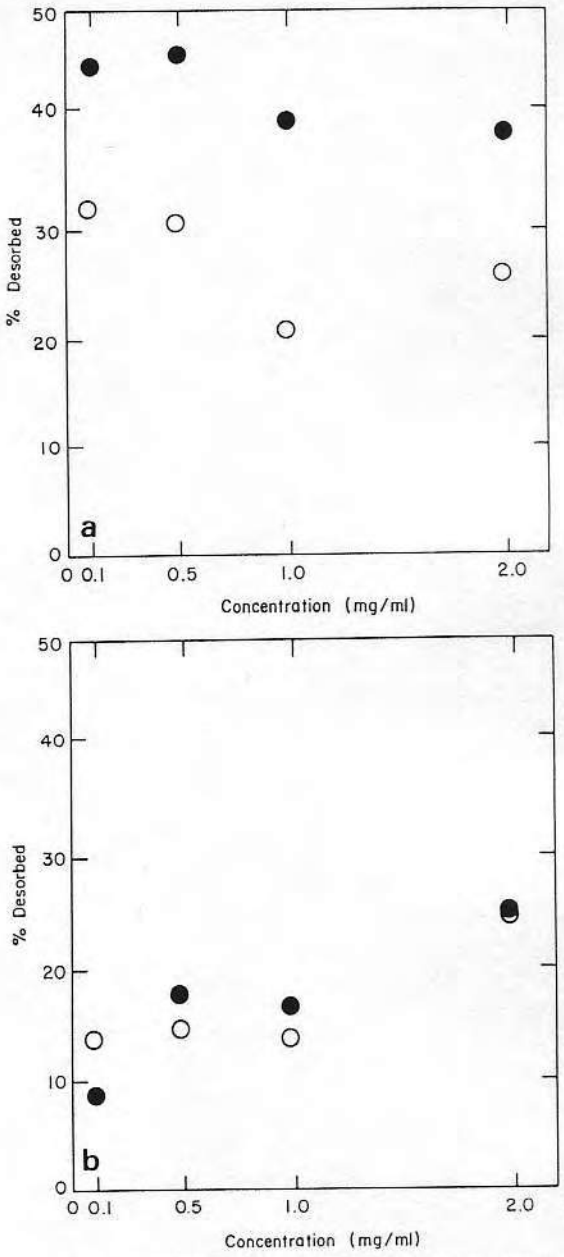

Figure 3 Desorption after 30 min of washout. (a) From hydrophilic silica; (b) from hydrophobic silica. Only about 20% of the protein was desorbed from the hydrophobic surface suggesting highly irreversible adsorption. Roughly 20–50% was desorbed from the hydrophilic material with Type 2-2 being more irreversibly bound. ●, Hp 1-1; ○, Hp 2-2.

Desorption of Hp types 1-1 and 2-2 from hydrophilic silica and hydrophobic silica had different concentration responses. Desorption from silica was about twice that from the DDS-treated surface.

As Hp is a major plasma constituent, its interaction with foreign surfaces should be more thoroughly studied.

Note: The quantitation of TIRIF adsorption data and the quantum yield assumption has been investigated for albumin and IgG[25].

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Cooperative Adsorption of Proteins onto Hydroxyapatite

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Protein adsorption isotherms are often described by the Langmuir equation. Protein adsorption is generally a cooperative process. Although the simple Langmuir equation does not normally include cooperativity, the Hill plot can represent cooperativity, including the degree of cooperativity. Our studies of the adsorption of lysozyme, albumin, transferrin, and lactoferrin onto hydroxyapatite suggest that cooperativity can be described as positive ($n >$ 1.5), partially positive ($1 < n <$ 1.5), partially negative ($1 < n <$ 0.5), and negative ($n <$ 0.5). There are three stages (i, ii, and iii) as protein concentration increases, similar to the cooperativity observed in the interaction between oxygen and hemoglobin. The number of stages is determined by the structural rigidity of proteins and by the lateral interactions between proteins and between protein and adsorbent. Our Hill plots show that stage ii has the lowest degree of cooperativity, stage iii has the highest, and stage i is in between, for most cases. In stage i the low degree of cooperativity is highly consistent with the linear range in the adsorption isotherm when protein solution concentration is low. These results provide useful information for the design and optimization of analytical adsorption chromatography. The factors determining the degree of cooperativity include protein conformation, pI, and solution pH. We now quantitatively understand the cooperative adsorption of these proteins on hydroxyapatite, the degree of cooperativity, and how to control the adsorption process.

INTRODUCTION

Studies on protein interactions at interfaces have been common since the early part of the century, and these studies have provided much of the information in this field and most of the general ''rules of thumb'' (1, 2, 53, 54). Protein adsorption is of interest in manufacture of medical devices, pharmacy and drug delivery, adsorption chromatography, biotechnology and cell culture, filtration and fouling, and manufacture of sensors or diagnostic products (1). Protein adsorption can result in serious technical problems for dental restoratives, cardiovascular implants, artificial kidneys, contact lenses, food processing equipment, and ships' hulls (3).

Proteins are long chains of amino acids (29, 45). Some of these amino acids carry side-chain carboxyl or amino groups, and these may remain free and exposed to the solvent when the protein is in solution. Therefore, they can dissociate in aqueous solution at a suitable pH, resulting in COO^- and NH_3^+ ions covalently attached to the protein macromolecule. The degree of ionization of these groups is not greatly influenced by their incorporation into the large molecule (62). The carboxyl group tends to ionize at pH values over about 4 and the amino group at below about 12 (46). Thus, in acid solution, a typical protein becomes positively charged because of the presence of NH_3^+ and COOH groups, and in basic solution it is charged negatively because of NH_2 and COO^- groups. When pH equals pI, the net charge is zero. This corresponds to the presence of equal numbers of oppositely charged groups on the protein. Such a neutral structure of charged groups is called a dipolar ion, or a zwitterion (47). At pH's near the pI, both NH_3^+ and COO^- groups are present, so that the net charge is small (Fig. 1).

Calcium hydroxyapatite (HA), $Ca_5OH(PO_4)_3$, is the mineral prototype for both bones and teeth, and it is often considered a suitable material for making medical implants. HA has been used as a support for the chromatographic separation of biological macromolecules (4–9). Of interest in biotechnology, it is used as a carrier for genes and for enzyme immobilization (10). There has been substantial work on the adsorption of proteins on HA (11–20). Most such work has focused on albumin of various sources. Quantitative relationships between the adsorption parameters and the properties of the surface, protein, and suspension solution are not yet available.

The classical Langmuir theory for gas adsorption is often applied to qualitatively treat protein adsorption from solution. This application is inappropriate because of the large difference between macromolecules and small molecules in the mechanisms of adsorption at interfaces. The differences result mainly from (a) multiple-site binding for proteins, which often results in irreversible adsorption, (b) the heterogeneous nature of most solid surfaces, and (3) lateral and other cooperative interactions (cooperativity).

Cooperativity originates from the macromolecular nature and from multiple functional groups, which usually result in multiple interactions. In enzymes, the term allostery often

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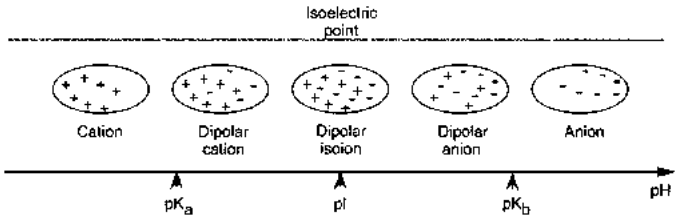

FIG. 1. Schematic representation of the variation of charge with pH for a protein (modified from Ref. 47).

refers to cooperativity in substrate or ligand interactions. Several different definitions of cooperativity lead to confusion (27). In order to avoid unnecessary confusion, ''cooperativity'' will be defined for different situations.

Schulz and Schirmer (28) said that cooperativity refers to the modes in which the components of a protein or a DNA act together to switch from one stable state to another (see Fig. 2a). This definition is often used for intramacromolecular processes (29). In the case of enzymes, cooperativity, or allostery, is defined as the binding of a ligand to one site on a macromolecule influencing the affinity of other sites — such binding is said to be ''cooperative'' (30). Such cooperativity can be positive if binding at one site increases the affinity of other sites, or negative if the affinity of other sites is decreased (see Fig. 2b). In enzymology, multiple interactions generally refer to the interaction of macromolecules (enzymes) with small molecules (ligands). The formation of dimers in the Langmuir treatment of a description is also defined as cooperativity (1) (see Fig. 2c). If a sorbent surface like HA contains multiple binding sites, and the proteins have multiple groups with affinity for the HA sites, cooperative adsorption results (31) (see Fig. 2d). Generally, a Hill plot is used to analyze such cooperativity.

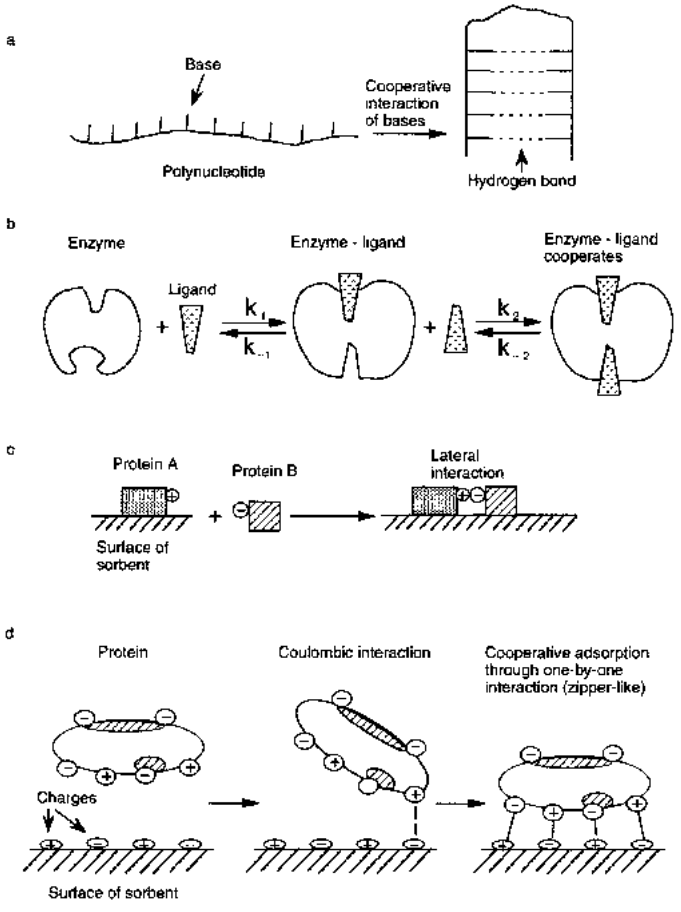

FIG. 2. Various models of cooperative interaction: (a) Intramacromolecular in polynucleotides (Ref. 31). (b) Enzyme and ligands: if $k_2 > k_1$, positively cooperative (Ref. 30); if $k_2 < k_1$, negatively cooperative. (c) Formation of protein dimers. (d) Cooperative adsorption between protein and surface.

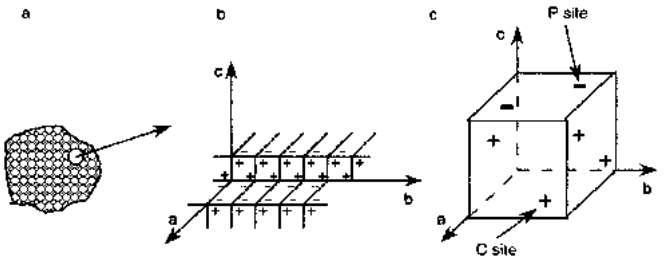

FIG. 3. Schematic representation of the surface charge of hydroxyapatite in crystal state: (a) Hydroxyapatite particle; (b) expanded area of hydroxyapatite; (c) crystal unit cell of hydroxyapatite.

The purpose of this paper is to study the adsorption of hen-egg-white lysozyme (LYZ), bovine serum albumin (BSA), human holo transferrin (HHT), and bovine milk lactoferrin (BML) onto HA. These proteins have different isoelectric points, molecular weights, carbohydrate compositions, and metal binding properties. Hill plots will be used to describe cooperativity in protein adsorption onto hydroxyapatite. Pertinent parameters in the adsorption process will be analyzed and discussed. Hill plots will be compared with the adsorption isotherm described using the simple Langmuir equation. In addition, through the study of cooperative adsorption of protein onto HA, we will deduce optimum conditions for the separation of these four proteins by adsorption liquid chromatography.

MATERIALS AND METHODS

Materials

Hydroxyapatite is a support used for liquid chromatography. Bio-Gel HTP, purchased from Bio-Rad (Richmond, CA), has a specific surface area of 50 m^2/g (by N_2 gas adsorption method at 77 K) and a Ca/P ratio of about 1.67 (supplied by Bio-Rad).

Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is in the space group $P6_3/m$, and its unit cell dimensions are $a = b = 9.432$ Å and $c = 6.881$ Å (39). There are two different binding sites (40, 41), called the P and C sites, in the crystal surface of the primitive unit cell (see Fig. 3). A P site is constructed

with six oxygen ions belonging to three crystal phosphates; P sites are arranged hexagonally on the (a, b) crystal face of HA with a minimal distance of 9.432 Å. A C site is on the (a, c) or (b, c) crystal face; C sites are arranged in a rectangular manner with the interdistance in the a or b direction equal to 9.432 Å and the interdistance in the c direction equal to 3.441 Å $(c/2)$. P sites lack calcium ions or positive charge, and therefore bind basic groups in proteins, but C sites are rich in calcium ions or positive charge, and thus bind with acidic groups in proteins. The above properties are for HA in the crystalline state.

When HA is suspended in aqueous solution, the surface properties are dramatically changed because the ions are hydrated. The charge on HA arises as a result of various dissolution and hydrolytic reactions which occur when the solid is suspended in aqueous solution (42, 43). The Ca/P of HA is fixed; the surface charges of HA are dependent on the pH of the suspension solution. The pH determines the ζ potential of HA particles suspended in solution when the ratio of Ca/P is fixed without any other determinant ions in suspension. The relation of the ζ potential to the pH of the suspension solution is demonstrated in Fig. 4. The intrinsic point of zero charge (pzc) of HA is dependent on the Ca/P ratio and the physical state. When pH $>$ pzc, the ζ potential is negative and the negative charge on the HA surface will dominate; when pH $<$ pzc, the ζ potential is positive and positive charge on the HA surface will dominate. We should point out that Ca^{2+} at the C site can strongly bind with COO^- in protein (44), but the affinity of the negative charge in the P site for NH_3^+ in proteins is not high. So the affinity of the C site for negative charge (COO^-) is larger than that of the P site for positive charge (NH_3^+). These properties of HA make protein adsorption onto HA complicated.

The surface of HA is heterogeneous, with both negative and positive charge, but with the charge density dependent on pH and on the salt ions present in the suspension solution. The binding strength of the HA positive charges is stronger than that of its negative charges.

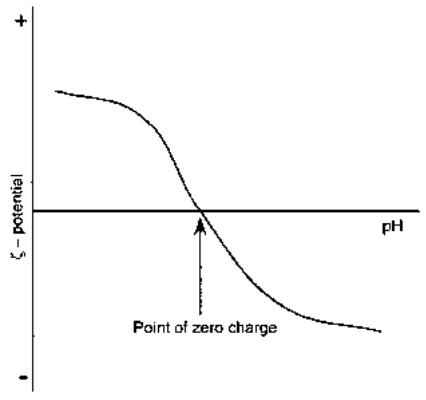

FIG. 4. The relation of ζ potential to pH (modified from Ref. 18).

The surface properties of proteins are even more heterogeneous. Not only are there positive and negative charges on the surface of protein particles, but there are also hydrophobic patches. Jennisen (48) used hydrophobicity to describe the cooperative adsorption of protein on hydrophobic agaroses. The surface properties of HA can be considered more complicated than hydrophobic agaroses, as several factors affect the degree of cooperativity in the HA–protein system.

The proteins LYZ, BSA, HHT, and BML (a small fraction saturated by iron) were supplied by Sigma Chemical Co. (St. Louis, MO). Here, we should point out that the degree of saturation of iron ions in lactoferrin influences its stability. Generally, the stability for the holo-state (metal-containing) is the greatest and that for the apo-state is the least. Important properties of proteins are listed in Table 1. All other chemicals used were of analytical grade.

Methods

a. Adsorption isotherms. The adsorption isotherm experiments were carried out in 1.5-ml polypropylene micro centrifuge tubes (Bio Plas, Inc., San Francisco, CA). Three different suspension solutions (SS) were used (Table 2): 30 mM NaCl (pH 6.76) (termed SSI), 30 mM Tris buffer salt including 30 mM NaCl (pH 8.50) (termed SSII), and 30 mM Tris buffer containing 150 mM NaCl (pH 8.50) (termed SSIII). The ratio of solid (hydroxyapatite) to liquid (SS) is 1 mg/1 ml. The range of protein concentration is 0–0.24 mg/ml. After the hydroxyapatite had been hydrated in suspension solution for 1 h, the protein solution was added to the hydroxyapatite dispersion to make 1 ml total volume. Then the tubes were rotated end over end for 18 h. The samples were then centrifuged for 5 min at 13,000 rpm and the clear supernatant was collected. Protein concentration in the supernatant was measured by intrinsic UV fluorescence (32). The samples of diluted supernatant were analyzed using a PCI photon counting spectrofluorometer (ISS, Inc., Champaign, IL) at $\lambda_{ex.} = 280$ nm and $\lambda_{em.} = 340$ nm. The maximum protein concentration was limited to less than 20 μg/ml in order to maintain a pseudo-linear relation between protein concentration and fluorescent yield. The sample solutions and standard solutions were identical except for the presence of protein. Temperature was controlled at 21 ± 1°C.

b. Hill plot. Hill (33) first developed an equation to describe the binding between hemoglobin and oxygen. Jennissen (1, 34, 35) used the ''Hill plot'' in his analysis of multivalent interactions of proteins with hydrophobic agaroses,

$$\Theta/(1 - \Theta) = K[P]^n, \qquad [1]$$

where Θ is the fractional saturation of hydrophobic agaroses, K is the Hill constant, and n is the Hill coefficient. $[P]$ is

TABLE 1
Important Properties of Proteins

Protein	Molecular weight (21, 22)	p*I* (21, 22)	Dimensions (Å) (23–26)	Projected area (Å^2) (side-on)
LYZ	14,300	10.7	40 × 40 × 19	760
BSA	66,400	4.6	94 × 94 × 40	3760
HHT	79,600	5.0	64 × 64 × 72	4634
BML	80,800	7.8	78 × 97 × 56	5432

Note. LYZ, hen-egg-white lysozyme; BSA, bovine serum albumin; HHT, human holo-transferrin; BML, bovine milk lactoferrin.

bulk protein concentration. Equilibration occurs via the bulk protein phase. Generally, Eq. [1] is used in the logarithmic form

$$\log \Theta/(1 - \Theta) = \log K + n \log[P]. \qquad [2]$$

From Eq. [2], the relation of $\log \Theta/(1 - \Theta)$ to $\log[P]$ is linear. The slope of the straight line is the Hill coefficient, n. Generally, if $n > 1$, cooperativity is defined as positive; if $n < 1$, cooperativity is negative. Considering that the surface of hydroxyapatite is heterogeneous and has multiple binding sites, we can redefine Θ. In this paper, Θ is the ratio of the HA surface covered by adsorbed proteins to the total HA surface. The specific surface area of HA is 50 m^2/g. The protein adsorbed on HA is assumed to be in side-on form, thus permitting an estimate of surface area covered by proteins (see Table 1).

Reproducibility

Measurements were carried out in duplicate. Measurements which were not within 8% (relative error) were repeated until the error was less than 8%. Relative error is defined as

$$|d_1 - d_2|/(d_1 + d_2)/2 \times 100\%,$$

where both d_1 and d_2 are the protein adsorption value of the duplicate. A blank sample (corrected for protein adsorbed onto the tube) was used.

TABLE 2
Suspension Solutions

Name	SSI	SSII	SSIII
NaCl (mM)	30	30	150
Tris (mM)	0	30	30
pH	6.76	8.50	8.50

RESULTS AND DISCUSSION

Hill plots of the four different proteins are presented in Figs. 5–8. We can state frankly that the data are not sufficient for rigorous or quantitative plots. Measurement of the adsorption isotherms of these four proteins is necessary to determine parameters for displacement chromatography of proteins. However, using the Hill plot to describe the adsorption, these figures suggest interesting and useful information. The adsorption of protein onto HA can be divided into three stages, with different Hill coefficients n, reflecting the degree of cooperativity in each of the three stages. These phenomena, shown in the figures, are similar to the binding behavior of O_2 and normal hemoglobin at 25°C in 0.1 M NaCl at pH 7.4 (36). Although in both situations there is cooperativity, the mechanism is completely different.

When Fair and Jamieson (37) studied protein adsorption on polystyrene latex surfaces, they discussed three-stage ad-

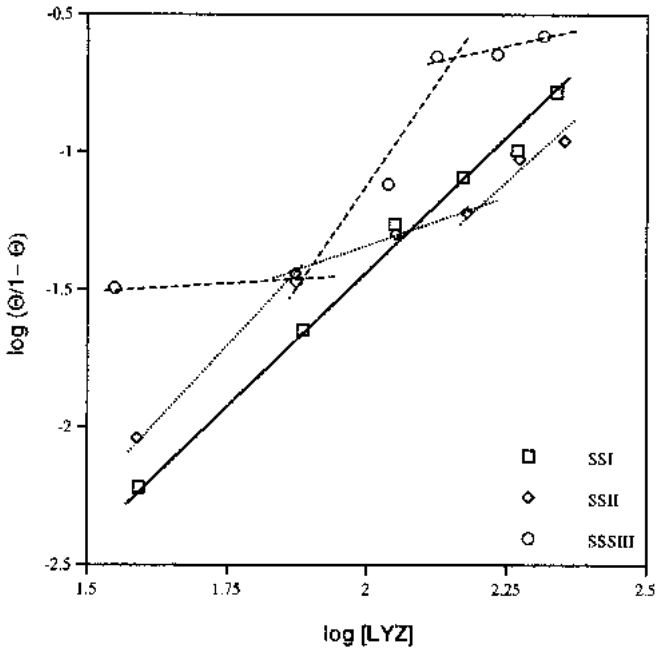

FIG. 5. Hill plot of hen-egg lysozyme (LYZ).

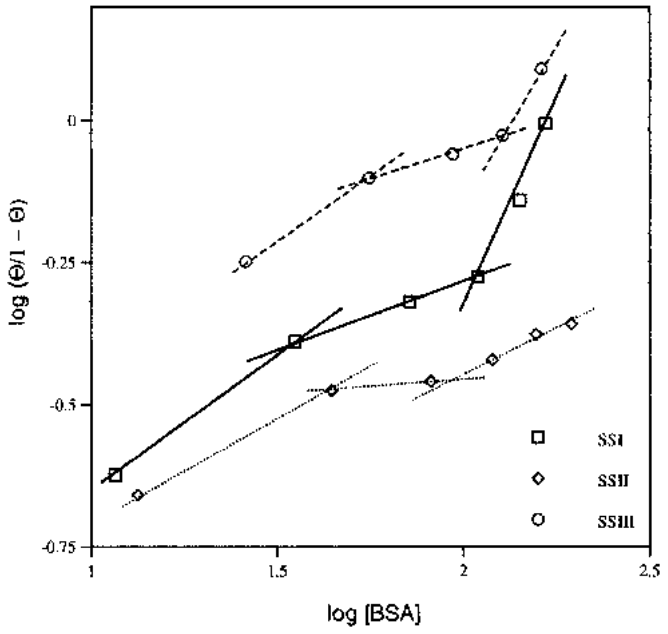

FIG. 6. Hill plot of bovine serum albumin (BSA).

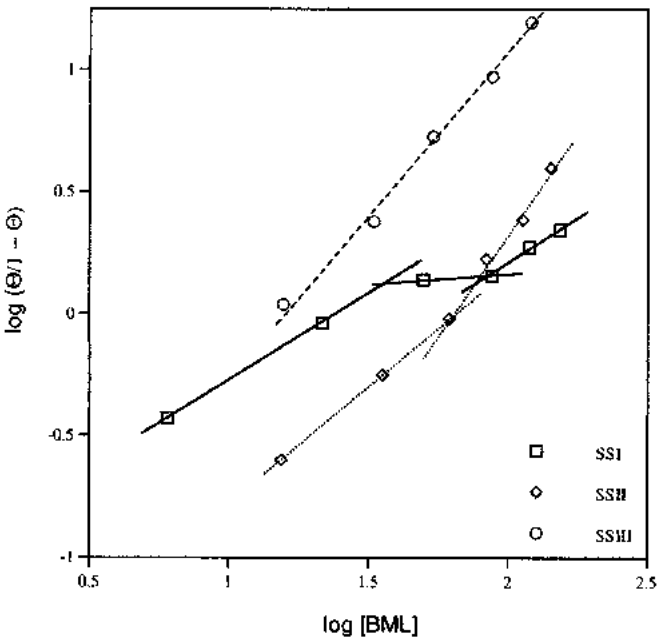

FIG. 8. Hill plot of bovine milk lactoferrin (BML).

sorption isotherms. Hearn *et al.* (65) worked on adsorption isotherms for BSA on PEI-bonded silicas of varying pore diameter and found that the Hill plots from the data are S-shaped, indicative of three-stage cooperativity, similar to our observations. It seems there is cooperativity of protein adsorption onto HA sorbent.

In adsorption liquid chromatography of proteins, if the elution is isocratic, the elution mode often is ''none'' or ''all'' (38), due to the cooperative adsorption and desorption processes. This is the reason that gradient elution is utilized in adsorption liquid chromatography (ALC) of macromolecules. A low degree of cooperativity is preferred for ALC, because high degrees of cooperativity lead to difficulty in elution.

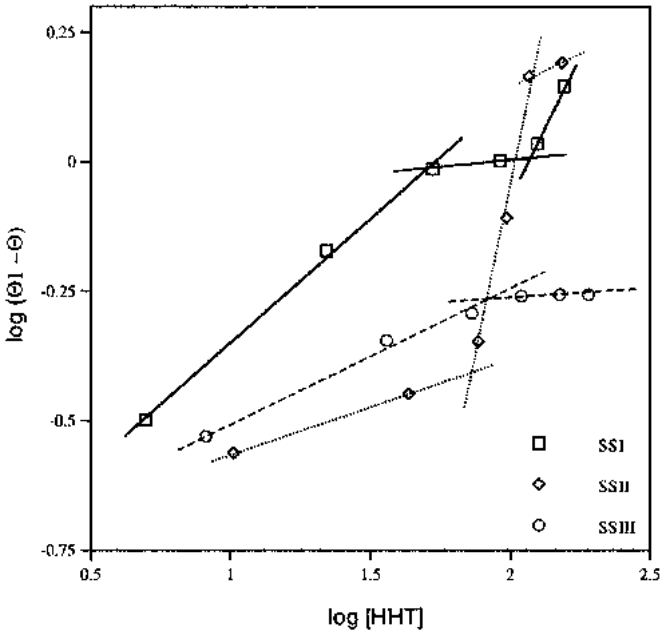

FIG. 7. Hill plot of human holo-transferrin (HHT).

The Hill coefficient, n, represents the degree of cooperativity. Figures 5–8 show that n depends on adsorbed protein concentration at the surface of HA. However, the change of n is really caused by the interactions both between protein and adsorbent (HA) and between adsorbed proteins, due to the lateral interaction when adsorbed protein concentration at the HA surface increases. When Myers and Prausnitz (66) studied the thermodynamics of mixed-gas adsorption, they introduced the concept of the adsorbate phase activity coefficient, γ, which is a function of spreading pressure, temperature, and composition. Talu and Zwiebel (67) further developed the theory of the adsorbate phase activity. They thought that the value of γ can predict the nonidealities caused by the lateral interactions between adsorbates. Li and Pinto (68) applied the concept of adsorbate phase to protein adsorption in preparative protein chromatography. They found that the lateral interactions between adsorbates (adsorbed proteins) influence preparative protein chromatography. In preparative protein chromatography, the adsorbed protein surface concentration is relatively high, which often results in the deviation of the linear adsorption range. When Ryan *et al.* (69) and Grasberger *et al.* (70) studied the interaction between proteins on the cell surface, they concluded that lateral interactions between proteins localized in membranes exist. These workers did not identify the sources of the lateral interactions. The lateral interaction force is complex and

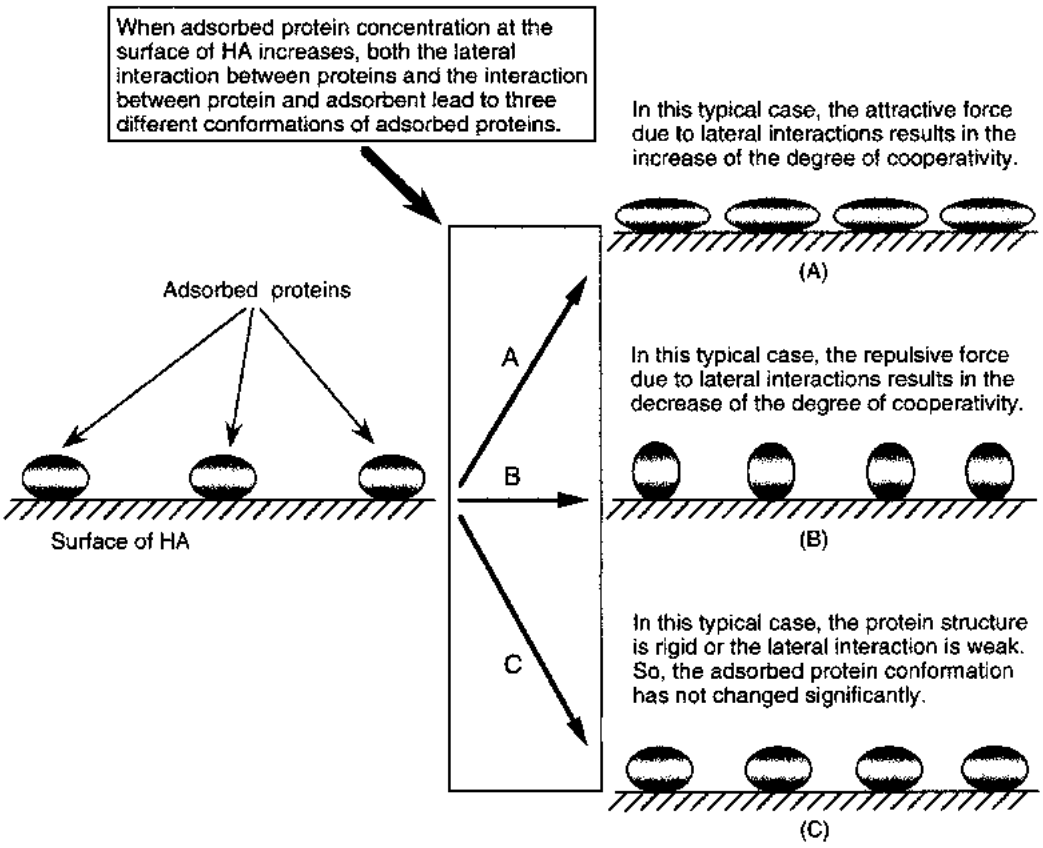

FIG. 9. Schematic representation of the three different conformation states of adsorbed proteins due to the lateral interaction between the proteins in surface phase and the interaction between protein and adsorbent.

includes components contributed by coulombic interaction and van der Waals interactions. The overall force can be repulsive or attractive.

Figure 9 schematically demonstrates three different conformations (A, B, and C) of adsorbed proteins resulting from both the lateral interaction between adsorbed proteins and the interaction between adsorbed protein and adsorbent. Figure 10 diagrammatically suggests a mechanism for the conformation change process of adsorbed proteins. Figures 10A and 10B, corresponding to states A and B in Fig. 9, respectively, clearly show the increase (Fig. 9A) or decrease (Fig. 9B) of the degree of cooperativity, likely due to the interactions between proteins and between protein and adsorbent. Lundström gave an example similar to Fig. 9B in his model of protein adsorption on solid surfaces (71).

Generally, if the lateral interaction force is attractive, the protein coverage on the adsorbent would be increased due to protein conformational change, which results in the increase of the degree of cooperativity; the reverse may also be true. The Hill coefficient, n, gives a quantitative measure of the degree of cooperativity. The n values measured and calculated from Figs. 5–8 are listed in Table 3. So we assume that the degree of cooperativity can be considered in four states (Fig. 11): positive ($n > 1.5$), partially positive ($1 < n < 1.5$), partially negative ($0.5 < n < 1$), and negative ($n < 0.5$). From Table 3, the values of n under most different suspension conditions are less than 2 for most suspension environments, so the degree of cooperativity is not high. In a few environments, n can be very large (HHT on HA in SSII).

Lysozyme. Lysozyme has a pI of 10.7, a rigid structure (49), and the lowest molecular weight of the four model proteins. When HA is suspended in SSI (in which the buffer salt is absent), the pH of SSI is near the point of zero charge (pzc) of HA, meaning that the magnitudes of positive and negative charges on the surface of HA are the same. The net charge of lysozyme is positive because p$I >$ pH. Table 3 shows that the adsorption is positively cooperative because $n = 1.9$ and there is only one stage in the experimental range of protein concentration. The reason may be that the distribution of charge in LYZ is not even (50) and that there is a patch of negative charge. So in this suspension solution, the degree of cooperativity is relatively high (see Fig. 11a). Protein LYZ structure is rigid (72). The meaning of one-stage cooperativity corresponds to the typical case shown in Fig. 9C. When HA is suspended in SSII in the presence of 30 mM Tris buffer salt and pH is increased to 8.5, pH $>$ pzc and pH is nearer to pI; thus, the surface of HA has

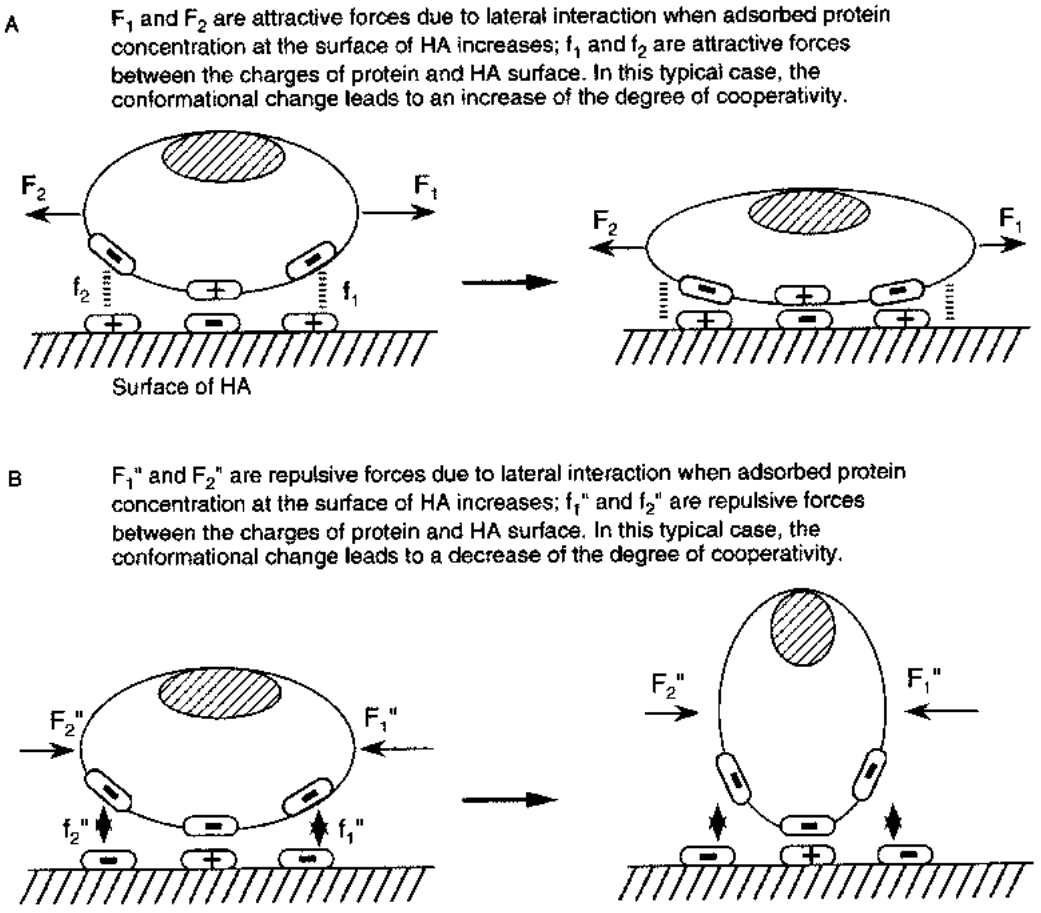

FIG. 10. Illustration of a possible mechanism for the conformation change of adsorbed proteins, due to lateral interaction between proteins in surface phase plus the interactions between protein and adsorbent.

more negatively charged sites, and there are fewer positive charges on the protein. The degree of cooperativity is reduced from positive to partially negative (see Fig. 11c), with three stages of cooperativity apparent, likely caused by lateral interaction between proteins. In SSIII, there is more NaCl in the suspension solution (see Table 2), reducing stage i and stage iii cooperativity. The reason is that the surface charge of both protein and adsorbent (HA) is suppressed. But the middle stage (ii) is relatively high, possibly due to appropriate conditions for the formation of LYS dimers (51).

TABLE 3
Hill Coefficients Collected from Figs. 2–5

Protein	Suspension solution	n_i	n_{ii}	n_{iii}
LYZ	I	1.9		
	II	1.0	0.4	0.8
	III	0.1	1.5	0.2
BSA	I	0.6	0.2	1.6
	II	0.5	0.1	0.5
	III	0.7	0.3	2.0
HHT	I	0.8	0.1	2.0
	II	0.3	4.9	0.4
	III	0.5	0.1	
BML	I	0.5	0.1	0.7
	II	0.8	1.4	
	III	1.3		

Albumin. Albumin has a p*I* of 4.6 (52), a flexible structure, three domains, and a medium size. Figure 6 shows that albumin adsorption on HA under three different suspension conditions demonstrates three stages of cooperativity (i, ii, and iii). In SSI, the solution contains only NaCl salt but no buffer salt. The pH is higher than the p*I* but almost equals pzc. So albumin has a net negative charge; the positive charge on the surface of HA dominates. The degree of cooperativity is partially negative (see Fig. 11c) at stage i, negative (see Fig. 11d) at stage ii, and positive (see Fig. 11a) at stage iii. This is a typical example, as shown in Figs. 9A and 10A. When surface protein concentration is increased, the distance between proteins is reduced, which leads to increased lateral interaction. Thus an attractive force causes

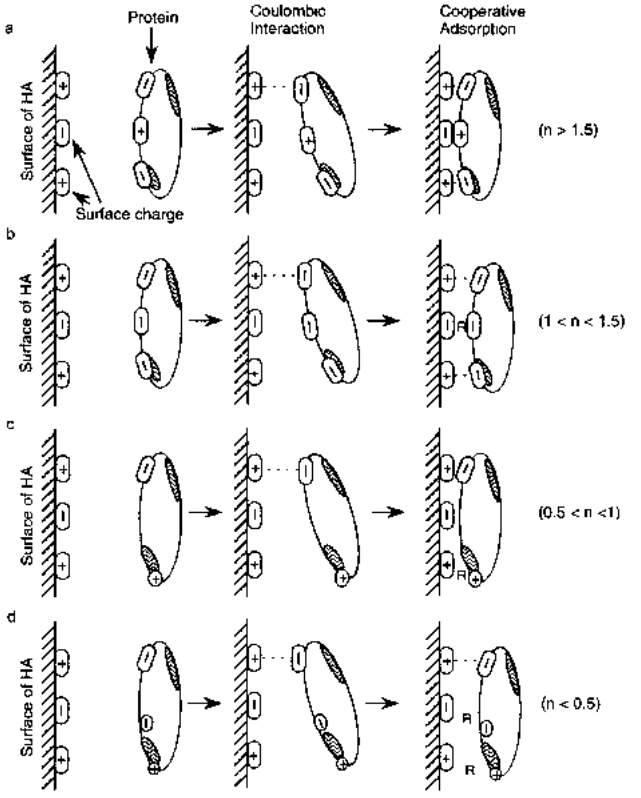

FIG. 11. Schematic representation of the different degrees of cooperativity for protein adsorption onto the surface of hydroxyapatite in different environments: (a) positively cooperative; (b) partially positively cooperative; (c) partially negatively cooperative; (d) negatively cooperative. Both ''– – –'' and ''R'' represent attractive and repulsive interactions, respectively. See text.

change in the conformation of the proteins on the adsorbent and increase in the degree of cooperativity. In SSII, the buffer salt, Tris, was added to adjust the pH to 8.5. Negative charge on both protein and HA is increased. This leads to a large decrease in the degree of cooperativity, especially in stages ii and iii. In SSIII, the ionic strength increases five times (from 0.03 to 0.15 M NaCl). The surface negative charge of HA is greatly suppressed but the positive charge is not, resulting in interaction between the calcium sites and COO^- (55–57). In SSI and SSIII, the cooperativity is greatly increased in stage iii, in which the protein concentration is relatively high, beyond the linear range in the adsorption isotherm. In adsorption liquid chromatography this often leads to difficult elutions. So in ALC the degree of cooperativity should be low. The bulk protein concentration in stage i can satisfy such a requirement. This phenomenon in ALC is well explained through the study of cooperative adsorption of protein onto HA.

Transferrin. Transferrin is an acidic protein (pI 5.0); the size is relatively large, there are four domains, and holoion transferrin is more rigid than apolactoferrin. In addition, transferrin is a glycoprotein (58). In SSI, the cooperativity is clearly divided into three stages and is very similar to that of albumin. Transferrin has a pI value slightly higher than that of albumin and a slightly higher molecular weight. The three stages of SSII are shown in Fig. 7. A comparison between SSI and SSII suggests that the degree of cooperativity in stage ii is very high ($n_{ii} = 4.9$), but that it is very low ($n_{ii} = 0.1$) in SSI. The reason is not very clear. It may be related to the carbohydrate playing a key role in adsorption. In SSIII, there are only two stages in cooperative adsorption in the experimental range of protein concentration. The degree of cooperativity is very similar to that in SSI. The explanation seems to be the same as that for albumin in SSIII.

Lactoferrin. Lactoferrin is also a member of the family of iron-binding proteins known as the transferrins. It is a monomeric glycoprotein of 80,800 Da, approximately 8% of this being due to two heterogeneous sugar chains (59). However, a key difference between transferrin and lactoferrin is that transferrin is acidic and lactoferrin is basic (pI = 7.8). Lactoferrin is more hydrophobic than transferrin (60). From Fig. 8, in SSI only, there are three stages in cooperative adsorption. In stage i, the cooperativity is partially negative, in stage ii it is negative, and in stage iii it is partially negative. So the degree of cooperativity is low. The reason for this is that in SSI the pH is near the pzc and the pI is higher than the pH; thus positive charge, which has less affinity for the surface of HA, dominates in BML. In SSII, there are only two stages (i and ii) for this limited protein concentration. Stage i is partially negative and stage ii is partially positive. The degree of cooperativity is increased in stage i and stage ii. This results from the fact that in SSII, pI > pH; thus the net surface charge of protein is slightly negative and the surface of HA is negatively charged because pH > pzc. Thus COO^-, which has a higher affinity for HA, dominates over the increase of negative charge on HA. In SSIII, there is only one stage in the experimental range of protein concentration. Stage i has a partially positive cooperativity because of $n = 1.3$. This phenomenon is very similar to lysozyme in SSI. There is a common phenomenon when the cooperativity has only one stage in the experimental range of protein concentration; i.e., the degree of cooperativity is relatively high like lactoferrin in SSIII and lysozyme in SSI. Both lysozyme and lactoferrin are basic proteins. These facts suggest that when the protein concentration of the surface phase increases, the conformational change of adsorbed protein is determined by the structural rigidity of the protein (e.g., LYZ), because a rigid structure can resist conformational change induced by lateral interaction. The conformational change is affected by the coulombic forces (e.g., BML in SSIII); suppression of coulombic interaction causes a decrease in the degree of cooperativity.

We conclude from the above analyses that protein adsorption is cooperative and that in most cases the protein concen-

tration increases in three stages. Our four model proteins show low cooperativity under most suspension conditions in stage i. In this stage, the protein concentration is lower than that in stage iii. Low cooperativity helps in the elution of proteins in adsorption liquid chromatography. This is quite consistent with the linear adsorption isotherm described by the Langmuir equation, because the low degree of cooperativity leads to more possibilities for reversible adsorption and desorption when the protein concentration is low. A linear range of protein concentration in the adsorption isotherm is preferred in analytical adsorption liquid chromatography. Through the selection of the pH, the ionic strength of the suspension solution, and the protein concentration, we can obtain conditions under which cooperativity is low enough for ALC.

Hay's group studied the adsorption thermodynamics of acidic proline-rich human salivary proteins on HA (61). They concluded that adsorption of proteins on HA is driven by an increase in entropy. We think that protein adsorption on HA is driven by coulombic interactions between the charges on the protein and on HA, as does Gorbunoff (63, 64). After adsorption, of course, the entropy of the protein is changed. The change in protein entropy depends mainly on the degree of cooperativity and the protein structure. Here we have emphasized cooperativity. If the degree of cooperativity is high, the change in protein entropy may be large.

SUMMARY

Hill plots were used to describe protein adsorption onto HA. It was found that the adsorption is cooperative, and the degree of cooperativity can be divided into four states: positive ($n > 1.5$), partially positive ($1 < n < 1.5$), partially negative ($0.5 < n < 1$), and negative ($n < 0.5$). There are three stages of cooperativity in most adsorption environments. In stage i, the degree of cooperativity is low compared to that in stage iii, but relatively high compared to that in stage ii under most suspension conditions. It seems that stage ii represents a transition, as protein concentration is increased from low to high, for most suspension environments. The linear range of protein concentration in the adsorption isotherm is often used for analytical adsorption chromatography. This is consistent with the relatively low degree of cooperativity required by analytical adsorption chromatography.

For different proteins and different solutions, the degree and the stages of cooperativity are different. For albumin, there are three stages under the three different solution conditions. For lysozyme, there are three stages only in two different solutions. Lactoferrin shows behavior similar to that of lysozyme. The cooperative adsorption of transferrin onto HA is not regular. The interesting thing is that although lactoferrin and transferrin belong to the same protein family, our lactoferrin is apo-iron but transferrin is holo-iron, and transferrin is acidic but lactoferrin is a basic protein. These differences make the cooperative adsorption process complicated. Protein conformations are likely key factors affecting the degree of cooperativity and the cooperative stages. Other main factors are the pI of proteins and the pH of the suspension solution, which determine the number and sign of charges on protein and on HA. The initial adsorption is driven by coulombic interaction. After adsorption, the protein conformation may be changed, caused by lateral interaction between adsorbed proteins or between protein and adsorbent, which may partially contribute to the entropy change of proteins and often results in an increase in the degree of cooperativity.

The optimum conditions for ALC are for SSIII to be used as mobile phase and for equilibrium protein concentration to be limited to less than 0.1 mg/ml.

ACKNOWLEDGMENTS

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Thin-layer ion-exchange chromatography of proteins

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Abstract

Thin-layer chromatography (TLC) is one of the simplest and most convenient techniques to separate small molecules. Of a variety of TLC separation modes, only size-exclusion was successfully used to separate proteins. In this paper, adsorption-TLC was used to separate proteins. The net charges were calculated for four model proteins, albumin, transferrin, lactoferrin and lysozyme, under different pH values. The suitable pH values for separation were determined according to the results from such calculations. Then, the adsorption isotherms of the four proteins were measured to deduce the ionic strength for appropriate elution conditions. Optimal conditions, 0.01 *M* bicine and pH 8.50, and a three-step elution process (1st step 0.01 *M* NaCl, 2nd 0.025 *M* NaCl, and 3rd 0.10 *M* NaCl), were obtained. Finally, the four model proteins were successfully separated under these elution conditions.

Keywords: Adsorption isotherms; Proteins; Albumin; Transferrin; Lactoferrin; Lysozyme

1. Introduction

Protein separation techniques, including sedimentation, gel electrophoresis (GE), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE), are highly developed [1,2]. However, a separation method which is simple, relatively inexpensive, utilizes little or no electrical energy, creates minimal waste, is applicable to diagnostics, and is gentle enough to isolate complex protein systems is still lacking [3].

Thin-layer chromatography (TLC), also called planar chromatography, is one of the simplest, the most popular, and widely used methods of separating small molecules [4]. TLC generally utilizes natural capillary forces to propel the mobile phase along a plate coated with a thin-layer sorbent. Pumps and related devices are not needed. TLC requires minimal amounts of sorbents and solvents and thus produces minimal waste. TLC is easier and more convenient to run than most other separation techniques [5]. Many chromatographic modes, including adsorption, size-exclusion, hydrophobic interaction, reversed-phase and affinity, and isoelectrofocusing and chromatofocusing have been used for separating both small and large molecules using column liquid chromatography (CLC). In TLC, most modes have been used only for separating small molecules. There have been few reports on the various modes in TLC used for separating proteins, except for size-exclusion [4,6,21].

Proteins vary in a number of their physical and chemical properties as a result of their amino acid sequences [7]. The amino acid residues attached to the polypeptide backbone may be positively or negatively charged, neutral and polar, or neutral and hydrophobic. In addition, the polypeptide is folded in definite secondary and tertiary structures to create a unique size, shape, and distribution of residues on the surface of the protein. By exploiting the differ-

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ences in properties between proteins in the mixture, a rational technique to separate them can be designed. Differences in surface charge properties are particularly effective due to the fact that the surface charges are strongly affected by pH and ionic strength (I) in solution [8–10]. There is a relation between isoelectric point (pI), pH, and net charge of protein [24].

Ion-exchange chromatography (IEC), one of the adsorption modes, has been the most widely used technique for the fractionation and purification of proteins, since the introduction of cellulosic ion exchangers in the 1950s [9]. IEC uses the charged groups on the surface of a protein to bind to an insoluble matrix of opposite charge. More precisely, the protein dipolar ion displaces the counterions of the matrix functional groups and will itself be displaced with an increasing proportion of counterion. This is usually done by increasing the concentration of ions in the elution buffer. The most important parameters in IEC are the choice of ion-exchange matrix and the initial conditions, including buffer type, pH, and ionic strength [11].

At equilibrium, the protein concentration in the stationary phase (C_s) is related to that in the mobile phase (C_m) and both are a function of the initial protein concentration and of the ionic strength in the mobile phase when the pH is fixed:

$$C_s = K(C,I)C_m \quad (1)$$

where K is a distribution coefficient between C_s and C_m [12]. Eq. (1) shows that if the protein is strongly adsorbed onto sorbent, in other words C_s is large, the K value will be large; the reverse is also true. In equilibrium theory, if zone spreading effects are ignored, the migration velocity of the protein zone along the sorbent layer can be expressed as:

$$dZ_p/dt = u/[1 + K(V_s/V_m)] \quad (2)$$

where Z_p, u, V_s and V_m are the peak position of the protein, velocity of mobile phase, the stationary volume, and the mobile phase volume, respectively [12]. We should point out that K ($=C_s/C_m$) in Eq. (2) is valid if linear elution conditions exist. For analytical purposes such conditions can be easily satisfied because the capacity of the ion exchanger used is very high [700 mg bovine serum albumin (BSA)/1 dry g anion exchanger (Whatman, Clifton, NJ, USA)] and the deposited sample is very low (8 μg/deposited spot). Generally, the slope $(C_s-0)/(C_m-0)$ of the isotherms are roughly equal to the tangent, dC_s/dC_m, in the low protein sample range (see Fig. 2B). Eq. (2) only roughly describes the migration velocity decrease with an increase in the distribution coefficient K when K equals $(C_s-0)/(C_m-0)$. Our purpose in using Eq. (2) is only to roughly predict the migration differences between different proteins, not to exactly describe protein band migration in TLC. K values differ from protein to protein, reflecting the interaction forces between each protein and the stationary phase. This is the underlying principle of the separation and one of the most important factors affecting separation in liquid chromatography [12].

In this paper, we calculated the net charges for four model proteins, albumin, transferrin, lactoferrin and lysozyme, under different pH values to estimate optimum pH for separation. By measuring adsorption isotherms, the appropriate ionic strength ranges for optimal mobile phase conditions are obtained from the distribution coefficients. We developed a new, convenient, and efficient method to measure adsorption isotherms. The distribution coefficient is here defined as the initial distribution coefficient ($K_{ini.}$), which is different from the more common distribution coefficient, which is defined as $K_{equ.}$ in this paper (see Fig. 2). This new method works well. The separation of the four model proteins were carried out using such optimal elution conditions.

2. Experimental

2.1. Materials

The sorbent, diethyl aminoethyl (DEAE)-derivatized microgranular and preswollen type cellulose (anion exchanger), was manufactured by Whatman. Proteins, including BSA, human holo-transferrin (HHT), bovine milk lactoferrin (BML), and hen egg-white lysozyme (LYZ) were purchased from Sigma (St. Louis, MO, USA). The degree of saturation with iron ions in lactoferrin is about 20%. Generally, the stability of the holo-iron state is higher than that for the apo-iron state. Important

Table 1
Important properties of the proteins used in this study

Abbreviation	Protein	p*I* [18,19]	M_r [18,19]
BSA	Bovine serum albumin	4.6	66 400
HHT	Human holo-transferrin	5.0	79 600
BML	Bovine milk lactoferrin	7.8	80 800
LYZ	Hen-egg white lysosyme	10.7	14 300

properties of proteins are listed in Table 1. All other chemicals used were of analytical grade.

2.2. Solutions

Solutions used for equilibration of ion exchanger, for adsorption isotherms, and for chromatographic development (elution) contained sodium phosphate, bicine and sodium chloride (Table 2).

2.3. Preparation of resin

Before use, DEAE anion exchanger was equilibrated with the desired solutions. Five grams of ion exchanger is dispersed in 40 ml solutions. After manual stirring for about 1 min, pH is adjusted to the desired values (6.50 for phosphate buffer and 8.50 for bicine buffer) with 6 *M* HCl, the suspension is allowed to settle, and the supernatant liquid is decanted off. This process is then repeated three times. For isotherm adsorption use, the final suspension is filtered through Whatman filter paper No. 1, and the equilibrated ion exchanger dried in the air on the filter paper. The dried ion exchanger was stored in a covered container. For chromatographic use, supernatant fluid is then removed until the ratio of settled ion exchanger to supernatant volume is about 4:1. Such anion exchanger is stored for coating the plates.

2.4. Adsorption isotherm

The adsorption isotherm experiments were carried out in 1.5-ml polypropylene micro centrifuge tubes (Bio Plas, San Francisco, CA, USA). The ratio of solid (prepared anion exchanger) to liquid (suspension solution) is 1 (mg):1 (ml). The initial protein concentration was fixed at 800 μg/ml for all samples. NaCl concentration (ionic strength) range is 0–0.20 *M*. The different NaCl concentrations were obtained by different ratios of two different suspension solutions (PSI and PSII or BSI and BSII). After hydration of prepared ion exchanger in aqueous suspension for 5 min, the protein solution was added to the ion exchanger dispersion to make a total volume of 1 ml. Then the tubes were rotated end-over-end for 4 h. The samples were then centrifuged for 5 min at 13 000 rpm. The clear supernatant was measured by intrinsic UV fluorescence. The samples of diluted supernatant were analyzed using a PCI photon counting spectrofluorometer (ISS, Champaign, IL, USA) at $\lambda_{ex.} = 280$ nm and $\lambda_{em.} = 340$ nm. The maximum protein concentration was limited to less than 20 μg/ml to maintain a pseudolinear state between protein concentration and the fluorescence

Table 2
The compositions and properties of suspension solutions

Name	$NaH_2PO_4 \cdot H_2O$ (*M*)	$Na_2HPO_4 \cdot 7H_2O$ (*M*)	Bicine (*M*)	NaCl (*M*)	pH
PSI	0.00685	0.00315			6.50
PSII	0.00685	0.00315		0.20	6.50
BSI			0.01		8.50
BSII			0.01	0.20	8.50

intensity. The additives and pH in the sample solutions and standard solutions were identical. Temperature was controlled at 21±1°C.

2.5. Coating of plates

Glass plate sections 6.9 cm×8.8 cm in dimension were cleaned by soaking in chromic sulfuric acid solution for 6 h. They were then scrubbed by sponge, then rinsed by double deionized and filtered water. Such plates were placed in a vacuum oven and dried before coating with ion exchanger slurry. In order to control the thickness of sorbent, two narrow plastic strips (total 0.6 cm wide) were bound on the two-sides of the plate with two-sided tape. The thoroughly suspended ion exchanger slurry was poured into the plate at a thickness setting of 1 mm. After making the slurry even and smooth, the plate was dried overnight at room temperature. The thickness of dried sorbent on the plate is about 0.4 mm. The plate was stored in the open air.

2.6. Sampling

Five samples, including BSA, HHT, BML, LYZ, and a mixture composed of BSA, HHT, BML, LYZ were used on one plate. The concentration for all five samples was 4 mg/ml. The amount per sample is 2 μl. Every deposited spot on the plate contained 8 μg of protein. Before all samples are ready for depositing, the plate to be eluted is placed in the development container and the mobile phase is allowed to ascend about 1 cm in order for the samples to be deposited on a wet surface. After sampling, the plate was quickly returned to the container for elution.

2.7. Development (elution)

In these experiments ascending development was applied in a beaker covered with transparent plastic film. The mobile phase level in the beaker was about 0.4 cm. The rate of development, which varied to a small extent from plate to plate, averaged about 0.8 cm/min.

2.8. Detection

Fluorescamine was used as a label. Fluorescamine reacts with primary amine groups on proteins, unbound dye is nonfluorescent, and its sensitivity depends on the number of amines present [13]. The developed plate was dried in the air at room temperature. The dried plate was dipped into the solution of 0.05% fluorescamine in acetone. After about 1 h, the plate was placed under UV light of multiband UV-254/366 nm of Model UVGL-58 (^{U}UVP, Upland, CA, USA). The fluorescent spots were recorded as chromatograms with a Spectra AF camera (Polaroid, Cambridge, MA, USA), then the chromatograms were copied onto the transparent papers in order to make the chromatograms more clear.

3. Results and discussion

3.1. Adsorption isotherm

The batchwise method was used to measure adsorption isotherms in order to quickly screen the conditions for development (elution). The advantages of this method are convenience, rapidity and simplicity. In addition, this method is thrifty because only a very small amount of protein sample is required, which is very important for expensive proteins. The disadvantages are that the exact elution condition is not identical with that obtained from adsorption isotherms. So, such measurement can only predict the rough range of ionic strength required for elution.

Since ion exchanger was used as sorbent, it is necessary to analyze the properties of proteins and sorbent and to characterize the surface charge which plays an important role in ion exchange chromatography. The program ''p*I* protein 1.0v1'' (Internet: iho@biobase.aau.dk) was used to calculate the net charge of proteins under different pH values (Fig. 1). From Fig. 1, pH values 6.50 and 8.50 were selected because at these two pH values the different proteins have different net charge values. At the same time this is an optimal choice for the DEAE anion exchanger because the working pH range for anion exchanger is less than 9.00 [20].

The real aim in measuring adsorption isotherms is to establish the relation between distribution coefficient (K), ionic strength and pH. However, there are two methods to express K. One has the same initial

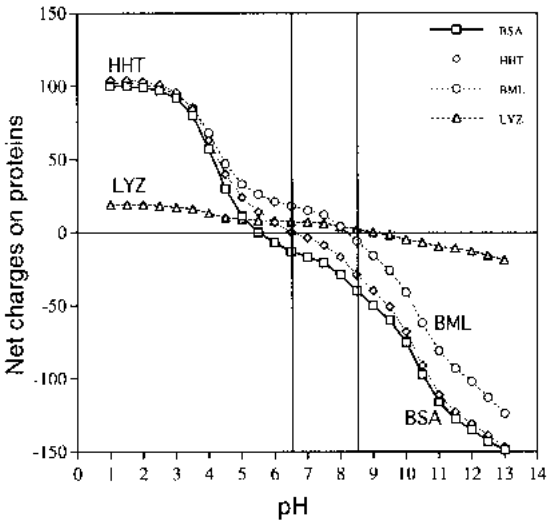

Fig. 1. The calculated relationship between charge number and pH of albumin, transferrin, lactoferrin and lysozyme.

protein concentration in the mobile phase ($C_{m,I.}$); such a K is called the initial distribution coefficient and termed as $K_{ini.}$ (Fig. 2A). The other is the more common K, which has the same equilibrated protein concentration in mobile phase ($C_{m,e.}$); such a K is called the equilibrium distribution coefficient and termed as $K_{equ.}$ (Fig. 2B). From our experiments, we found that it is more convenient and efficient to measure $K_{ini.}$. In such adsorption isotherms the sample is of low concentration and is near the linear adsorption range. So, the ratio, C_s/C_m, in the isotherms can roughly replace the tangent, dC_s/dC_m, which also avoids the minus values sometimes obtained with dC_s/dC_m. It seems more suitable to call such $K_{ini.}$ the partition coefficient [22,23]. In Fig. 2A, every point corresponds to a different ionic strength, which is very important for the step elution processes. However, in Fig. 2B, every curve corresponds to an ionic strength.

With pH fixed, the adsorption isotherm is measured at various sodium chloride concentrations, range 0.00–0.20 M, realized by changing the ratios of PSI/PSII or BSI/BSII. Phosphate and bicine were used as buffer salts for pH values 6.50 and 8.50, respectively (Table 2). Figs. 3 and 4 demonstrate the relations of initial distribution coefficients ($K_{ini.}$) of four proteins with ionic strength under different buffer salts and pH values. Figs. 3 and 4 show that

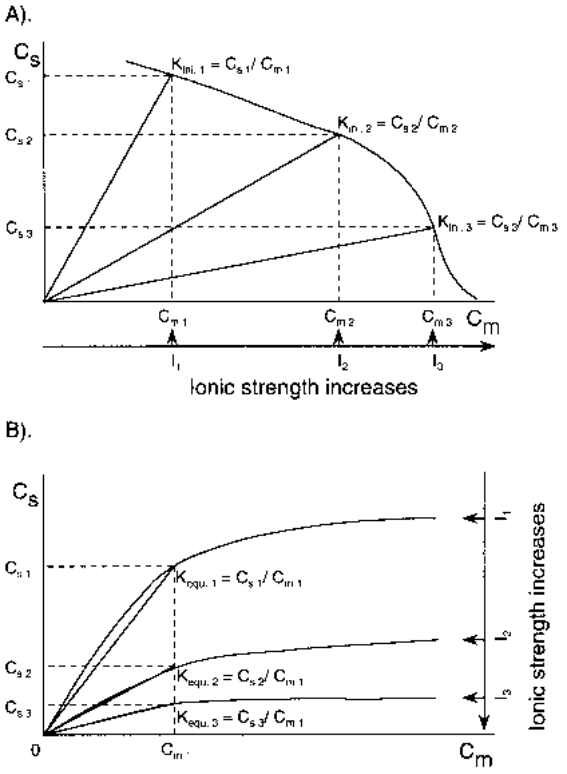

Fig. 2. Schematic diagrams of how to obtain the two different distribution coefficients. (A) $K_{ini.}$ is the initial distribution coefficient. Every point (C_s, C_m) in the isotherm corresponds to an ionic strength. When ionic strength increases, C_s decreases and thus $K_{ini.}$ reduces. C_s almost equals zero, or no protein is adsorbed, when ionic strength reaches a higher value. (B) $K_{equ.}$ is the equilibrating distribution coefficient.

for acidic proteins (albumin and transferrin), $K_{ini.}$ values under the conditions studied are very sensitive to NaCl concentrations or ionic strength of aqueous suspension, because Coulombic interactions dominate between acidic proteins and anion exchanger when pH>pI values (Table 1). For basic proteins (lactoferrin and lysozyme), the sensitivity to ionic strength is low. These are expected results because anion exchanger is more appropriate for separation of acidic proteins. For basic proteins, the adsorption is not strongly controlled by the Coulombic interaction force, therefore $K_{ini.}$ values of lactoferrin and lysozyme are small and do not change very much when ionic strength is changed. However, both lactoferrin and lysozyme are more hydrophobic than albumin and transferrin [14]. Also the fact that

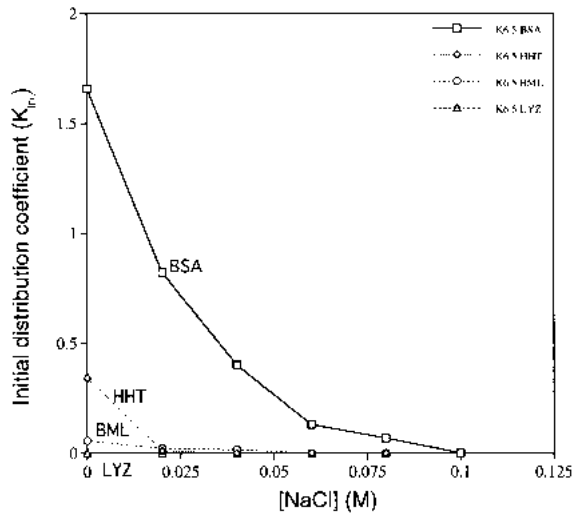

Fig. 3. The relation of initial distribution coefficient with ionic strength. Conditions for adsorption isotherms: 0.01 *M* sodium phosphate and pH 6.5.

lysozyme has a patch of negative charge [15] makes the behavior of lysozyme and lactoferrin on anion exchangers not easily predictable.

$K_{ini.}$ values of acidic proteins (albumin and transferrin) are strongly affected by buffer salt types. In sodium phosphate buffers $K_{ini.}$ values are small (less than 2). However, in bicine buffer the $K_{ini.}$ value sometimes is very large and the maximum reaches 137. Phosphate ions have the ability to complex with positively charged groups on the anion exchanger, plus multivalent charges on phosphate increase the ability to shield the surface charge on anionic exchanger, thus greatly reducing the ζ-potential of the charged groups on the anionic exchanger. This leads to a decrease of Coulombic interactions between acidic proteins and anion exchanger. Bicine is a zwitterionic buffer which is a dipolar ion molecule and does not complex with the anionic charges on the ion exchanger, as does phosphate. So in bicine buffer solution, the ζ-potential of the charged groups on the anionic exchanger was not strongly affected by bicine buffer ions.

Generally, if $K_{ini.}$ values are very large and $K_{ini.}$ is not sensitive to ionic strength, the elution of the adsorbed protein by increased ionic strength is difficult because the interaction between protein and sorbent is too strong. In addition, if $K_{ini.}$–I curves of two proteins overlap or the gap between curves is too narrow, these two proteins can not be separated or separation is difficult because they have the same or similar elution properties under this mobile phase condition. Based on these criteria, the conditions in Fig. 4 satisfy the elution requirements for separation. In thin-layer ion exchanger chromatographic development, the conditions in Fig. 4 were utilized.

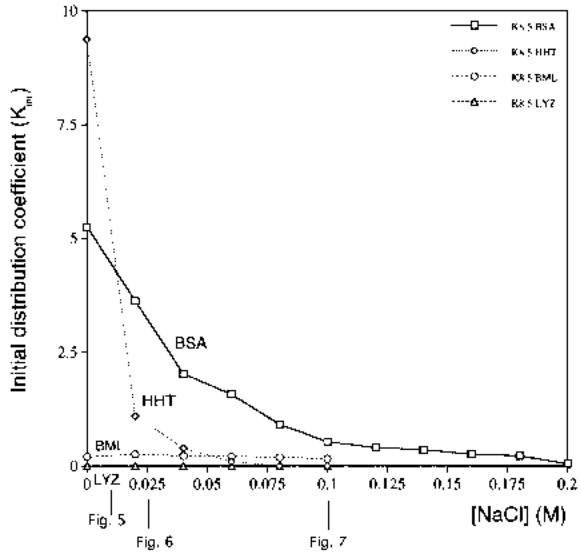

Fig. 4. The relation of initial distribution coefficient with ionic strength. Conditions: 0.01 *M* bicine and pH 8.5. Elution conditions are based on this figure.

3.2. Chromatograms

Fig. 1 shows that when pH = 8.50 lysozyme and lactoferrin have small numbers of net positive charge and net negative charge, respectively; albumin has maximum net negative charge number, and the net negative charge number of transferrin is in between lactoferrin and albumin. Generally, the order of Coulombic interaction force is the same as that of the net charge number when the anionic surface charge is fixed. So, for the four model proteins, the elution strengths (ionic strength) are different for different proteins. The larger the net charge number of protein, the higher the elution strength [16]. This is consistent with the results in Fig. 4. Eq. (2) demonstrates that the larger the $K_{ini.}$ value, the smaller the protein migration velocity (dZ_p/dt). It can be

expected that in chromatograms lysozyme should be in the front, followed by lactoferrin, followed by transferrin, with albumin last.

At the same time, the most important differences between small molecules and proteins arise from the polyelectrolyte character of the latter. The polyelectrolyte properties of proteins often result in multiple binding between the solute and the stationary phase. Multibinding tends to result in very high or very low distribution coefficients between phases, with an abrupt transition between extremes [17]. The retention mechanism behaves as ''all or none''. In addition, the gradient method may possess higher resolution at the cost of considerable tailing, while the discontinuous method will provide sharp zones at the expense of resolution, especially if the changes in effluent concentration are large. That is why discontinuous elution is required in TLC ion-exchange chromatography of proteins.

Figs. 5–7 are the chromatograms from a three-step elution. When ionic strength is 0.01 *M* NaCl (Fig. 5), only lysozyme migrates in the same step with effluent; when ionic strength is 0.025 *M* NaCl, not only does lysozyme continue to move together with effluent but lactoferrin begins to migrate (Fig. 6); when ionic strength in the third step was increased to 0.10 *M* NaCl, only albumin was kept in the sampling area, the other three proteins migrate at their own velocities (Fig. 7). The mixture sample in the middle (Fig. 7) was separated into four proteins. This discontinuous elution method clearly showed that the elution of one protein corresponds to one ionic strength and different proteins require different ionic strengths. It should be pointed out that the ionic strength required for elution is much higher than that shown in Fig. 4. These differences are caused by the different ratios (20 times) of solid volume divided by liquid volume of suspension solution or mobile phase between the chromatographic and batchwise methods, which was introduced simply in Section 3.1.

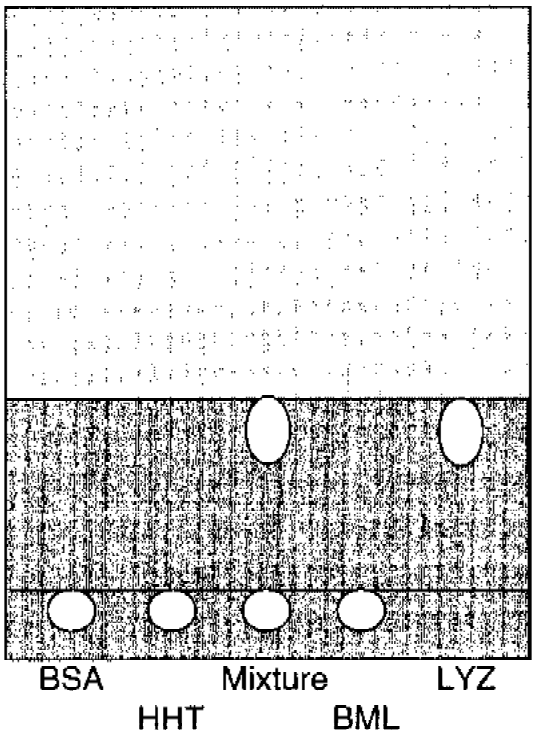

Fig. 5. The chromatogram after second elution step.

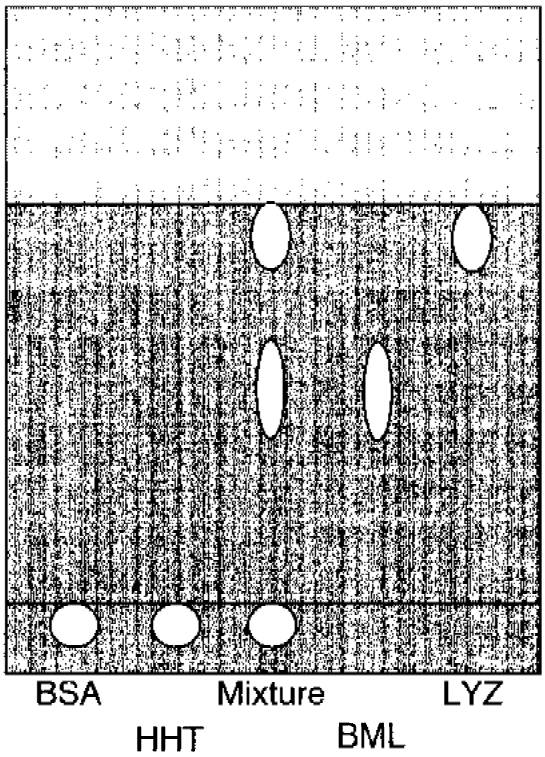

Fig. 6. The chromatogram after first elution step.

The concentration of NaCl in equilibrated solvent for the ion exchanger and in the mobile phase is of critical importance in controlling the migration of

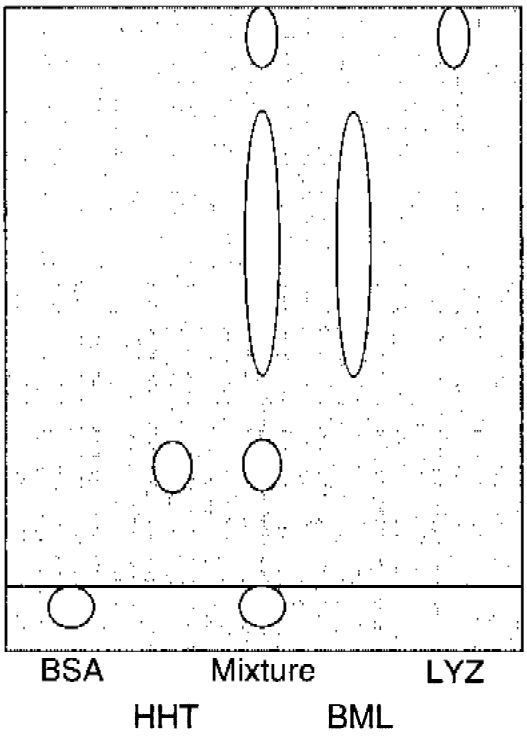

Fig. 7. The chromatogram after third elution step.

proteins. The significant value is the sum of the salt concentration in the ion exchanger and in the mobile phase. As this sum increases, the migration velocity of the samples also increases, but the increase in mobile phase is more effective according to our experiments. The optimal sum was semiempirically obtained. For the plates used in Figs. 5–7, the NaCl concentration in anion exchanger is 0.025 *M*.

4. Conclusion

Our studies were designed to obtain the basic information needed to develop a process for protein separation using adsorption TLC. Through establishing the relation between protein net charge and pH, the suitable pH values for elution were determined. Then by measuring adsorption isotherms for model proteins, the ionic strength ranges for elution were obtained from the initial distribution coefficients of the four proteins. From these data, 0.01 *M* bicine was selected as the mobile phase and a three-step (first step 0.01 *M* NaCl, second 0.025 *M* NaCl, and third 0.10 *M* NaCl) elution process was applied. Finally, the four proteins were thus successfully separated. This process represents a successful separation of proteins by adsorption-TLC. Future work will focus on optimizing the design of a separation device to make the development and visualization more convenient. Adsorption-TLC should be capable of separating proteins in complex mixture samples.

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Electrokinetic Flow through Packed Capillary Columns

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Abstract: This paper is a theoretical study of electroosmotic flow through various model packings to understand the mechanisms of capillary electrochromatography (CEC) and to take full advantage of CEC. Two different equations for electroosmotic flow velocity are described and compared with each other through two- and three-dimensional figures. The optimal channel diameter in the common ionic strength range is determined. The importance of ionic strength and packing microstructure is further verified. The properties of various packing beds are compared and an "ideal" packing, with parallel flow channels and minimum tortuosity, is suggested. The reasons that CEC has not achieved the predicted separation efficiency are pointed out. This study sheds light on approaches to achieve the high separation efficiency and selectivity available by CEC. J Micro Sep 11: 682–687, 1999

Key words: *electroosmotic flow; flow channel; packing microstructure; electrochromatography*

INTRODUCTION

The principles and mechanisms of electrokinetic flow through packed beds are still not completely understood although capillary electrochromatography (CEC) is now widely applied [1–3]. The questions mainly concentrate on (1) the model to describe double layer overlap, (2) the influence of ionic strength on the electroosmotic flow and the column efficiency, and (3) the effect of packing microstructure on the electrokinetic flow.

At present, there are two different mathematical equations to describe electroosmotic flow in a capillary column. Rice and Whitehead [4] applied the zero-order modified Bessel function of the first kind to describe the flow equation. Probstein [6] used Debye-Hückel linearization directly to deduce numerical solutions and thus to get the expression. However, the results from these two different equations are not consistent with each other, especially when the Debye length is large. In addition, it has not been shown which expression more appropriately describes electroosmotic flow.

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The packing microstructure strongly influences the electroosmotic flow since the flow is generated in the channels between the particles [7]. When the Debye length is large or ionic strength is small, the channel diameter becomes important, and the flow along all channels with the same orientation to the axis will be very different, due to their dimensions. Moreover, there will still be dispersion due to the variety of orientations of the channels. One advantage of CEC is that a smaller particle size can be used due to the low pressure drop in electroosmotic flow, and the smaller the particle diameter the higher the column efficiency [2]. A variety of packings, including particles and continuous polymeric or silica gels, have been applied for CEC [8]. However, for the particle packed column, the advantage supplied by CEC cannot be fully realized due to the variety of packing arrangement and tortuous channel structures. Further, wall effects on the fluid flow properties cannot be neglected when the column-to-particle diameter ratio is less than 30:1 [9].

In this study, first two different velocity expressions for electroosmotic flow will be compared and plotted in two and three dimensions using Maple software. Then, the appropriate channel diameter for packings for CEC will be determined. Finally, a

model for an "ideal" packing microstructure for CEC will be set up and discussed.

THEORY AND METHOD

Electroosmosis. Since all surfaces are, in principle, charged, an electrical double layer exists at all surfaces in contact with an electrolyte. When a field is applied parallel to the surface, as is done in CEC systems, the liquid will move under the field [10]. The velocity profile for electroosmotic flow can be expressed. Rice and Whitehead [4] first solved the problem for flow in cylindrical capillaries. Sørensen and Koeford [5] deduced a formula similar to that of Rice and Whitehead. The equation is expressed as

$$u(r) = \frac{\varepsilon_0 \varepsilon_r \zeta E}{\eta}\left[1 - \frac{I_0(\kappa r)}{I_0(\kappa a)}\right] \tag{1}$$

where ε_0, ε_r, ζ, κ, E, η, r, and a represent, respectively, the permittivity of a vacuum, the relative permittivity of the medium, the zeta potential of the surface of the flow channel, the reciprocal of the electrical double-layer thickness, the applied electric field strength, the viscosity of the medium, the distance to the channel center, and the radius of the flow channel, with the zero-order modified Bessel function ($I_0(\kappa r)$) of the first kind and the Debye-Hückel parameter

$$\kappa = 1/\lambda_D = F\sqrt{\frac{2I}{RT\varepsilon_0 \varepsilon_r}} \tag{2}$$

where λ_D, F, R, and T are Debye length, Faraday, gas constant, and absolute temperature, respectively, and the usual definition of ionic strength

$$I = \frac{1}{2}\sum c_i z_i^2 \tag{3}$$

where z_i is the valence of each ion and c_i is the ion concentration.

Grossman [11] performed the derivation in rectangular rather than cylindrical coordinates to improve the clarity of the treatment. The expression through such treatment is the same as that of Probstein [6] who neglected the capillary curvature effect. Probstein first inferred the surface potential equation and then deduced an equation for electroosmotic flow profile through capillary channels using Debye-Hückel linearization

$$u(r) = \frac{\varepsilon_0 \varepsilon_r \zeta E}{\eta}[1 - e^{-\kappa a(1-(r/a))}] \tag{4}$$

Expression (4) appears different from Expression (1). We will see that the differences expressed by Equations (1) and (4) appear especially at low ionic strength or for relatively narrow flow channels.

Packing microstructure and ionic strength. Parameters related to packing microstructure include particle sphericity, particle orientation, tortuosity, connectivity, roughness, and anisotropy. Expressions (1) and (4) show that a/λ_D or $a\kappa$ strongly influences electroosmotic flow. So, channel diameter, a, is one of the important parameters which determine the electroosmotic flow profile. The channel diameter in the packing is a function of packing microstructure. However, if the packing particle is mono-size with perfect sphericity, the channel diameter of the packing microstructure can be determined by the particle size and packing arrangements [12]. Wan [13] tried to define the mean channel diameter d_{mc} to simulate the effect of electrical double-layer overlap on the electroosmotic flow in packed-capillary columns. Knox [14] used a dimensionless flow resistance parameter (ϕ) to determine the minimum particle diameter for CEC. In fact, the porous medium can be supposed to be equivalent to a series of channels. It seems more reasonable to use a hydraulic diameter or equivalent diameter, D_h, to represent channel diameter, a, to provide a clear physical meaning for the channel diameter. This diameter is conventionally defined as four times the flow cross-sectional area (A_x) divided by the wetted perimeter (P_w) and measures the ratio of volume to surface of the pore space [6,12]. In terms of the porous medium characteristics,

$$D_h = \frac{4A_x}{P_w} = \frac{4A_x L}{P_w L} = \frac{4V_{\text{void}}}{A} \tag{5}$$

where L is the channel length, V_{void} is the volume of voids, and A is the total surface area. Since the porosity is defined by

$$\varphi = \frac{V_{\text{void}}}{V} \tag{6}$$

where V is the total volume of the medium. It is common to express the total surface area in terms of an inverse length, termed the specific area, A_v, which is the ratio of the surface area to the volume of the solid's fraction of the porous medium:

$$A_v = \frac{A}{(1-\varphi)V} \tag{7}$$

For uniformly sized spheres, $A = \pi D_p^2$ and $V = \pi D_p^3/6$, the combination of (5) and (6) with (7) gives

$$D_h = \frac{2}{3}\frac{\varphi D_p}{1-\varphi} \tag{8}$$

where D_p is particle diameter. D_h will become an important parameter for designing packing beds with optimum microstructure for CEC.

Wall effect. Except for completely ordered arrangements of spheres or geometrical matrices, the mean void fraction is highest at the wall of the column and then oscillates with decreasing amplitude toward a uniform value [12]. For clarity the wall effects for viscous flow and electroosmotic flow should be discriminated. The wall effect for viscous flow is a barrier due to the increase of eddy diffusion, which results in increasing the dispersion of analyte band in liquid chromatography. The wall effect on electroosmotic flow depends on D_h. If the magnitude of D_h is in the range of the electrical double-layer overlap, the wall effect will destroy the plug-like flow; if D_h is beyond the range of electrical double-layer overlap, wall effect will not be a problem. However, the wall effect can be minimized by designing an appropriate microstructure of the packing bed.

RESULTS AND DISCUSSION

Electroosmotic velocity profile. We have pointed out that two main factors, flow channel diameter and ionic strength, influence the electroosmotic flow in packed capillary column. Maple was used to plot Equations (1) and (4) as Figure 1. Figures 1(A2) and 1(B2) demonstrate the electroosmotic flow velocity profiles under different Debye lengths and positions in the flow channel. From Figures 1(A1) and 1(B1), it can be seen that when $\kappa a > 100$ ($I > 0.1$ or Debye length < 1 nm), the velocity profile is like a plug. When $I < 0.001$ or $\lambda_D > 10$ nm, there is a difference in the relative velocity between the two different Equations (1) and (4). Especially when $\kappa a < 5$, the differences in the velocity profiles of Equations (1) and (4) are very large. Fortunately, for practical CEC systems, κa should be at least larger than 50. It should be noted that Equation (1) is based on the assumption that the surface potential $\psi(a)$ should be less than or equal to 25 mV; Equation (4) is subject to the boundary condition $\psi(a) =$ 70 mV. The solution of Equation (4) is consistent with that solved numerically on a digital computer without resource to Debye-Hückel linearization.

To help understand the effect of Debye length on the electroosmotic flow, Figure 2(A) schematically shows that $\psi(o) = 0$ at the center, as the solution is electrically neutral there for the limiting case of small Debye length. For this case, electroosmotic flow is plug-like. For the limiting case of large Debye length, the entire capillary is within the double layer. If 1 and 4 designate locations in the external solution just outside of the double layer and 2 and 3 designate locations inside the channel entrance, then at equilibrium with no flow $u = 0$. This clearly shows the role of ionic strength in electroosmotic flow [6].

Packing microstructure. For particle packed beds, Equation (8) shows that channel diameter, a, or hydraulic diameter, D_h, is determined mainly by particle diameter and porosity (void fraction). For mono-size particles, the effect of porosity depends on the particle arrangements. According to the study of Martin *et al*. [17], simple cubical packing is the least compact arrangement with 0.48 porosity, rhombohedral packing is the most compact packing with 0.26 porosity, and random packing has 0.40 porosity. Three different porosities, three common particle sizes, and a minimum particle size (0.5 μm) were used to calculate channel diameters with Equation (8). Table I lists the results which will be used for evaluating the limit of particle size by comparison with the data deduced from Figure 1.

Figure 1 demonstrates that when κa is larger than 50, the electroosmotic flow profiles gradually become plug-like, which means that adsorbent particle size for CEC has some limits. We try to set guidelines for how to select the particle size and run conditions to make full use of CEC advantages by showing an example. The permitted channel diameters in the range of ionic strength 0.001–0.1 are calculated and listed in Table II. For reverse-phase CEC, the common ionic strength is between 0.001 and 0.01. Comparing Tables I and II, note that some particle diameters should be excluded in CEC. For example, when I is 0.001, if the packing is random, the particle size should be larger than 2 μm. However, if ionic strength is increased from 0.001 to 0.01, the particle size can be less than 1 μm. This typical example shows that after ionic strength for elution is fixed, the minimum particle size can then be determined.

Knox [15] used the following equation to predict band broadening in CEC:

$$h = 0.6v^{1/3} + \frac{1.6}{v} + 0.1v \tag{9}$$

with dimensionless velocity $v = ud_p/D_m$, linear velocity in column $u = 2$ mm/s, analyte diffusion coefficient $D_m = 10^{-9}$ m^2/s, and reduced plate height

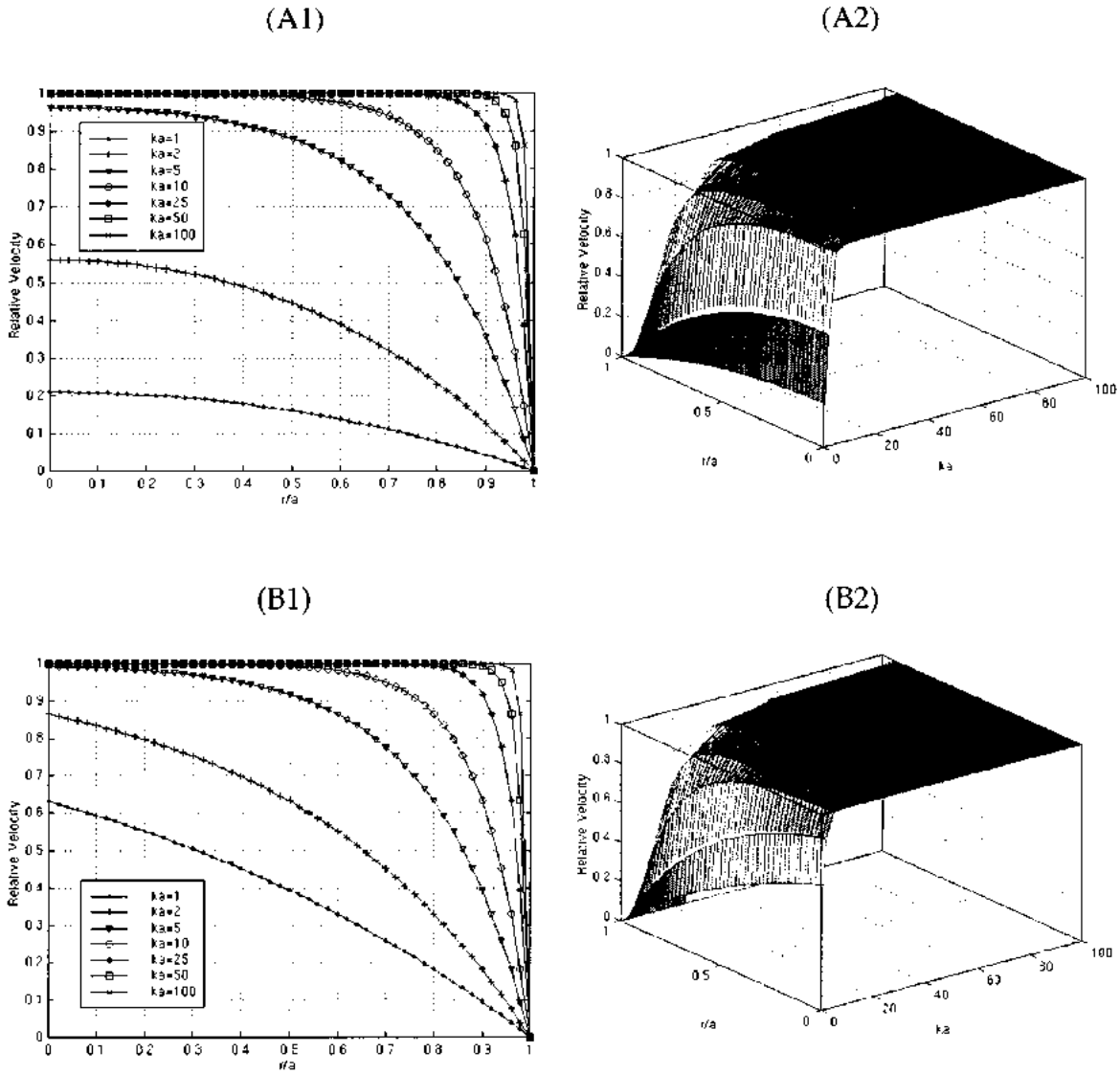

Figure 1. *The profiles of electroosmotic flow velocity in two (A1 and B1) and (A2 and B2) three dimensions. k in the figures means* κ *in the text.*

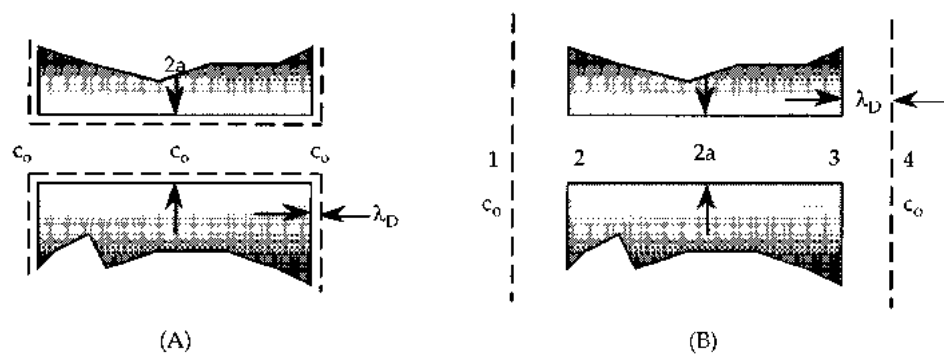

Figure 2. *Debye layer location in a cylindrical pore: (A)* λ_D *small; (B)* λ_D *large [6].* c_o *is electrolyte concentration far from the charged surface.*

Table I. *The hydraulic diameters (D_h) for different void fractions (φ) and particle sizes (D_p).*

φ	D_p (μm)	D_h (μm)
	0.5	0.31
	1	0.62
0.48	2	1.24
	3	1.86
	0.5	0.22
	1	0.44
0.40	2	0.88
	3	1.32
	0.5	0.13
	1	0.26
0.26	2	0.52
	3	0.78

Table II. *The minimum channel diameter for different ionic strengths deduced from Figure 1 and calculated from Equation (2).*

I[a]	λ_D (nm)	a (μm)
0.001	9.61	0.961
0.01	3.04	0.304
0.1	0.961	0.096

[a] Assumed as symmetrical electrolyte and $z^+ : z^- = 1:1$.

$h = H/d_p$. Equation (9) is used to calculate the reduced plate height. The plate number is obtained by $N = L/H$ (L is column length). The calculated data are given in Table III, which shows that the smaller the particle, the higher the separation efficiency (N). According to the electroosmotic flow velocity equation, the particle diameter can be smaller without a pressure drop limitation. However, when particle size becomes very small (< 2 μm), the packing will be very difficult, especially for capillary columns. Moreover, the local values of porosity for particle packing are very variable and unpredictable due to the heterogeneous nature of random packing. Giddings [18] recognized that the geometrical complication of pore structure has defied all effort to come to grips with it mathematically, and that our understanding of porosity must be limited to inexact and intuitive concepts supported by experience. According to the above discussion, the advantages supplied by CEC cannot be fully realized for particle packing. In the following section, a model for an "ideal" packing bed will be developed.

Table III. *Plate height (H), reduced plate height (H / d_p), and plate number (N) for different reduced velocities (ud_p / D_m) and particle diameters (d_p).*

d_p (μm)	υ	h	H (μm)	N (/m)
3	6	2.0	6	133,000
2	4	1.8	3.6	280,000
1	2	1.8	1.8	560,000
0.5	1	1.2	1.2	870,000

Wall effect. The mean void fraction is highest at the wall of the column and then oscillates with decreasing amplitude toward a uniform value. If the appropriate ratios of the column-to-particle diameter are larger than 30, the wall effect will be greatly reduced. Is this condition easily satisfied? The answer is no. For a 75-μm inner diameter capillary column, the particle diameter should be smaller than 3 μm. The wall effect can be removed only by designing an optimum packing.

Ideal packing bed. Figure 3 schematically represents three different ideal packings in capillary columns. The packing in Figure 3(A) is formed by many bundles of rigid fibers. In such packing the electroosmotic plug flow can be most efficiently realized and the plate number (N) will be very high, but it is difficult to fabricate such packings. Figure 3(C) shows a completely ordered arrangement of monospherical particles. From the view of the channel line [14], the electroosmotic velocity in a particle packed bed is lower than that in an open tube due to tortuous channel structure, which means the flow properties in Figure 3(C) cannot be compared with those in Figure 3(A). The packing represented in Figure 3(B) is a continuous polymeric bed with submicron size channels. In this structure, the geometric tortuosity is greatly reduced compared with that

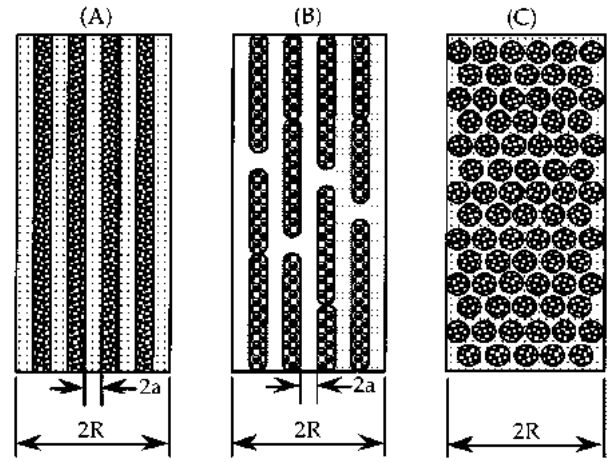

Figure 3. *Schematic representation of three different ideal packings for capillary columns: (A) parallel, multiple channels formed by bundles of rigid fibers; (B) continuous polymeric bed with submicron size channels; (C) an ordered particle packed bed.*

in Figure 3(C). Formation of this packing is more practical than that in Figure 3(A). Although Hjertén, Svec, and Novotny and co-workers [19–21] have worked on synthesizing continuous polymeric beds, they have not purposefully designed the polymeric structure to facilitate electroosmotic flow. In the future, important work in CEC will be how to design a continuous polymeric bed to reach the ideal structure shown in Figure 3(B).

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JOURNAL OF CHROMATOGRAPHY B

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Phytic acid as an efficient low-molecular-mass displacer for anion-exchange displacement chromatography of proteins

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Abstract

Phytic acid, inositol-hexaphosphoric acid, molecular mass 650, a low-molecular-mass compound, has been identified as a nearly ideal displacer in anion-exchange displacement chromatography for the concentration and purification of model protein mixture. The concentration of low-molecular-mass displacer is a very important parameter for successful separation by displacement chromatography. Displacer concentration influences the formation of the isotachic train and the yield and recovery of the displacement chromatographic process. There is an optimum displacer concentration in which the yield and recovery are highest.

Keywords: Displacers; Phytic acid; proteins

1. Introduction

Protein chromatography continues to play a significant role in biotechnology, serving as an effective analytical tool and a powerful method for large-scale separation [1,2]. Displacement chromatography has become popular in recent decade even though its potential was already recognized by Tiselius in the early 1940s [3–9]. This progress has been driven by the development of biotechnology and the specific advantages of the method [10,11], including:

1. the feed concentration generally does not affect process efficiency;
2. products can be concentrated in the column effluent;
3. the concentration of displacer can easily adjust the speed and efficiency of the separation; and
4. peak tailing is greatly reduced.

Kundu and Cramer [12] had pointed out that, even though a great many systems have been tried, the lack of effective and nontoxic displacers hampers the wide application of displacement chromatography in biotechnology. Generally, whether displacement chromatography is successful or not depends greatly on the efficiency of displacer. The displacer, with a higher surface affinity for the adsorbent than any of the feed components, effectively competes with the

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feed components for adsorption sites on the stationary phase under nonlinear conditions [20]. Displacers can be low-molecular-mass compounds or macromolecules. Macromolecular displacers can be synthetic polyelectrolytes or proteins. The displacer should meet a number of requirements: nontoxic, stable, detectable, soluble, inert (no interaction with other feed components), high affinity, and be highly uniform, cheap, and reusable [13]. Generally, it is not easy for a displacer to rigorously satisfy all these requirements.

An important recent advance in displacement chromatography was the discovery that low-molecular-mass compounds can be successfully used as displacers for protein purification [16]. Compared with macromolecular displacers, low-molecular-mass displacers have significant operational advantages:

1. they can be easily separated from the purified protein components;
2. the economics of the process can be improved because of the relatively low prices of low-molecular-mass displacers; and
3. column regeneration is facilitated.

Phytic acid (Fig. 1) is a nearly ideal displacer. When the pH>7.2, there are at least eight negative charges on a small inositol molecule. Phytic acid has a strong affinity for anion-exchangers under appropriate conditions. Phytic acid is abundant plant constituent in cereals and can be easily separated from this food source. Phytic acid meets most of the requirements suggested by Kasper et al. [13] for an ideal displacer. We have evaluated phytic acid as displacer.

In this paper, a strong anion-exchanger was used as adsorbent. A mixture of β-lactoglobulins A and B is employed as model proteins in order to evaluate the separation resolution using phytic acid as a low-molecular-mass displacer. The two forms of β-lactoglobulins A and B differ in p*I* by approximately 0.1 unit. Aspartic acid (64) and valine (118) in A is replaced by glycine and alanine, respectively, in B. The program "p*I* protein 1.0v1" was used to draw titration curves, thus determining the suitable pH. Frontal analysis was used for measuring adsorption isotherms, and then determining the feed concentration. Finally, the column chromatographic separation was carried out with the optimum conditions determined by frontal analysis and titration curves.

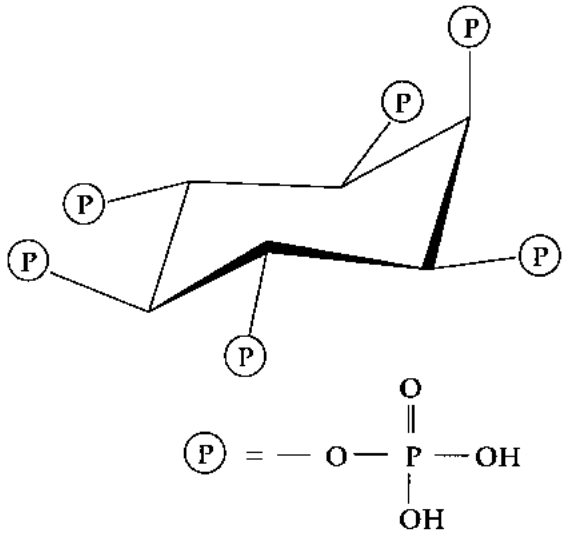

Fig. 1. The chemical structure and titration properties of phytic acid [25].

2. Experimental

2.1. Materials

Two adsorbents, strongly basic, quaternary amine (QA-52) bearing and diethylaminoethyl-derivatized (DE-52) microgranular and preswollen type celluloses (strong and weak anion-exchangers, respectively), were manufactured by Whatman (Clifton, NJ, USA). QA-52 medium is fully ionized and bears constant charges in the pH range 2–12; but DE-52 medium depends on pH. Phytic dodecasodium salt (PN12), phytic dipotassium salt (PK2), β-lactoglobulin A, β-lactoglobulin B, and a mixture β-lactoglobulins A and B, were all purchased from Sigma (St. Louis, MO, USA). Buffer, N,N-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid (free acid and Ultra Grade), N,N′-bis(2-hydroxyethyl)-2-amino-

ethanesulfonic acid, was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). All other chemicals used were of analytical grade.

2.2. Equipment

PCI photon counting spectrofluorometer was manufactured by ISS (Champaign, IL, USA). Fast protein liquid chromatography systems including a fraction collector and columns, HR 100×10 mm I.D., HR 100×5 mm I.D., and HR 50×5 mm I.D., respectively, were obtained from Amersham Pharmacia (Piscataway, NJ, USA).

2.3. Solutions

Solutions used for equilibration of ion-exchanger, package of columns, frontal analysis, thin layer ion-exchange chromatography, column ion-exchange chromatography, and displacement chromatographic developments contained sodium phosphate, phytate, BES, and sodium chloride (Table 1).

2.4. Titration curves

Generally, there are three methods, including chemical titration, calculation, and isoelectric focusing, to get titration properties of proteins. If the amino acid composition of the protein is known, the calculation method is preferred; if not, the chemical titration method is more suitable. However, for complex samples, the isoelectric focusing method is better. In this paper, the calculation method was used to achieve titration curves.

Table 1
The compositions and properties of buffer solutions[a]

Name	$NaH_2PO_4 \cdot H_2O$ (*M*)	$Na_2HPO_4 \cdot 7H_2O$ (*M*)	BES (*M*)	NaCl (*M*)
NPB1	0.0032	0.0168		
NPB2	0.0032	0.0168		0.2
NPB3	0.0032	0.0168		0.1
BES1			0.02	
BES2			0.02	0.2
BES3			0.02	0.1
BES4			0.02	0.25
CAB			0.025	0.025
REB[b]			0.025	0.25
CPB[c]			0.025	0.1

[a] pH for all buffer solutions is 7.50.
[b] Regenerant buffer.
[c] Corrected purpose buffer.

2.5. Frontal analysis

A column (HR 50×5 mm I.D.) packed with 0.04 ml QA-52 medium was used for frontal analysis, which was modified from Ref. [21]. The final bed height was 2 mm. First, the column was equilibrated with carrier buffer (Table 1), then 2, 4, 8, 16, 32 ml protein solutions of corresponding different concentrations, 0.64, 0.32, 0.16, 0.08, 0.04 m*M*, respectively, were flown through the column under the gravity force, and the various whole effluents were collected. Finally, the protein concentrations were measured by intrinsic UV fluorescence. The samples of diluted effluent were analyzed using a PCI photon counting spectrofluorometer (ISS) at $\lambda_{ex.}=280$ nm and $\lambda_{em.}=340$ nm. The maximum protein concentration was limited to less than 20 μm/ml to maintain a pseudo-linear state between protein concentration and the fluorescence intensity. Temperature was controlled at 21±1°C. The subtract between the protein concentrations of original solution and effluent, respectively, multiplied by the effluent volume was the adsorbed protein amount.

2.6. Effluent analysis

Displacement chromatography requires the use of an on-line analyzer because the detectors usually used do not provide sufficient information on the boundary regions. In this work, two separation techniques were used: ion exchange thin layer chromatography (EXTLC) and ion-exchange column chromatography (EXCC).

2.6.1. EXTLC

EXTLC, due to its simplicity, convenience, and speed, was employed to quickly screen the fractions collected from displacement chromatography. The operation processes of EXTLC are in Ref. [14]. Here are given some important development parameters, including DE-52 as adsorbent; sodium (Na) phosphate buffer as equilibrated solution; and the solution made up of 20% NPB2 and 80% NPB3 as eluent,

which eluted β-lactoglobulin B but not β-lactoglobulin A.

2.6.2. EXCC

EXCC was used for quantitatively measuring β-lactoglobulins A and B in the fractions screened by EXTLC. A HR 100×10 mm I.D. column packed with QA-52 was used. The final bed height is 88 mm. The chromatography was carried through FPLC systems (Amersham Pharmacia). Equilibrated solution was BES3. Step gradient elution was used. The solution in the first step is made up of 87% BES2 and 13% BES4 and the solution in the second step made up of 19% BES2 and 82% BES4. The concentrations of β-lactoglobulins A and B in effluent fractions were measured by intrinsic UV fluorescence.

2.7. Displacement chromatography operation

Displacement chromatography was carried out through FPLC systems. The volume of sample loop is 1.5 ml. 1.85 ml of QA-52 medium were packed in a HR 100×5 mm I.D. column at a flow-rate of 1 ml/min. The final bed height was 94 mm. The column was first equilibrated with CAB solution (carrier). The solution of β-lactoglobulins A and B was loaded in the sample loop and pushed into the column by displacer solution (phytic acid in carrier). Effluent fraction, its sizes 0.5 ml or 0.25 ml, respectively, depending on displacer concentrations, was collected through a fractional collector.

3. Results and discussion

3.1. Titration properties of β-lactoglobulins A and B

The titration curves are generally used for determining pH suitable for eluent, or displacer solution. Fig. 2 shows the titration curves of β-lactoglobulins A and B drawn by the program ''p*I* protein 1.0v1'' (Internet: iho@biobase.aau.dk). Fig. 2 shows that the difference in net charge numbers is small in a broad pH range. So, the selection of pH 7.50 is mainly based on the consideration of protein stability and protein binding capacity of adsorbent. β-Lactoglobulins A and B tends to form dimers in pH range 1.8–5.4 [22]. As the pH increases above 5.4, an ionization-linked transition is observed in all variants. The conformation changes and increasing dissociation near pH 7.5 are established immediately on mixing [23]. Groves et al. detected the presence of both reversible and irreversible changes above pH 8.0 [24].

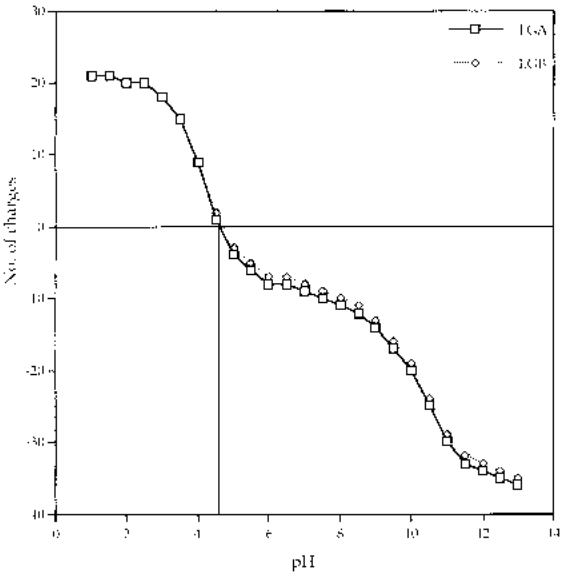

Fig. 2. The relation between the net calculated charges and pH of β-lactoglobulins A and B.

3.2. Adsorption isotherms

The adsorption isotherms are utilized to determine displacer concentrations suitable for displacement separation development. However, we did not measure the adsorption isotherm of phytic acid displacer. For a small molecular mass displacer, such as phytic acid, it is hard to find appropriate units (mole, weight, or equivalent scales) to express displacer concentrations. According to the research of Jen and Pinto [15], the adsorption isotherms will be completely different if the concentration units to express adsorption isotherms are different even for macromolecular displacers. Here we only measured the adsorption isotherms of model protein, β-lactoglobulins A and B. The effects of displacer concentration on the displacement chromatographic separation

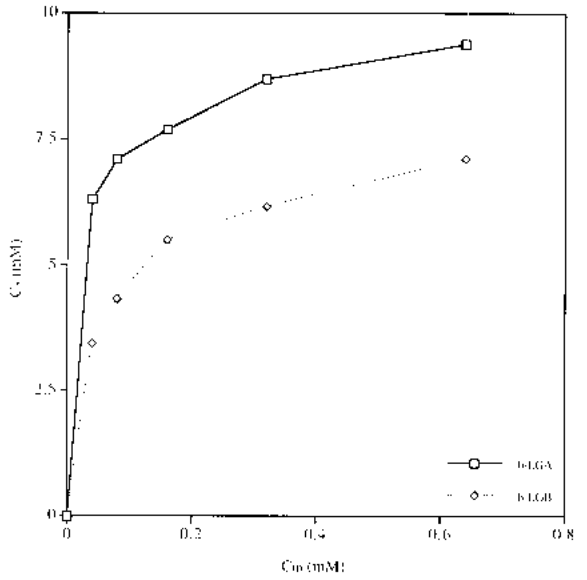

Fig. 3. Adsorption isotherms of β-lactoglobulins A and B at 25 m*M* BES, 25 m*M* NaCl, and pH 7.5 measured by frontal analysis.

were tested directly in the development processes. Fig. 3 demonstrates the adsorption isotherms of β-lactoglobulins A and B. Although there are only two amino acids different between β-lactoglobulins A and B, there is a relatively large difference in adsorption isotherms, which means these two proteins can be separated each other from the view of adsorption isotherms. Moreover, Fig. 3 also shows that the concentrations of β-lactoglobulins A and B can be selected from a relatively broad range.

3.3. The development of displacement chromatography

3.3.1. The effect of displacer concentrations

Displacement chromatograms of β-lactoglobulins A and B in Fig. 4 show some interesting phenomena. First, when displacer (phytic acid) concentration increased from 10 m*M* to 40 m*M* in carrier (CAB), the isotachic trains are gradually formed. The formation of an isotachic train is very important, as this gives the highest recovery yield, although it does not permit the highest possible production rate [17]. Second, when the displacer concentration is increased, the elution properties are changed from overload isocratic elution in Fig. 4a to displacement

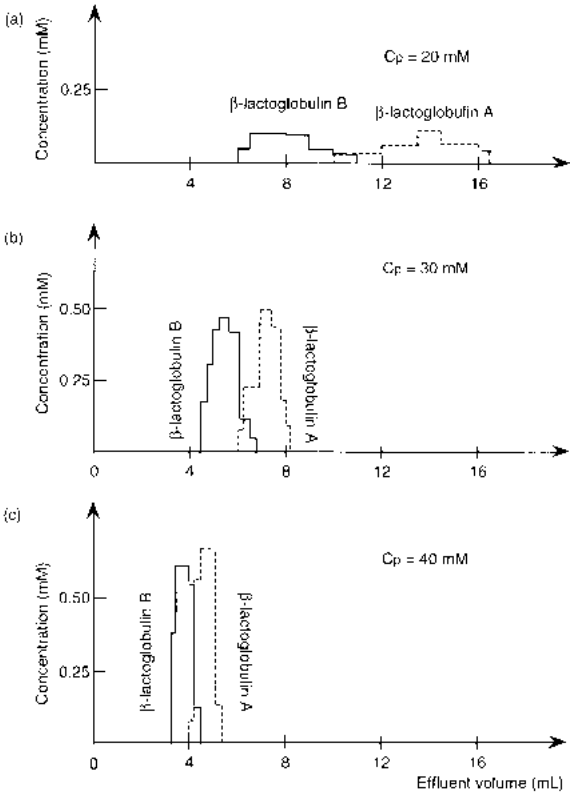

Fig. 4. Displacement chromatograms of β-lactoglobulins A and B. Column, QA-52, HR 100×5 mm I.D.; Carrier, 25 m*M* BES, 25 m*M* NaCl, and pH 7.5 with 0.5 ml/min flow-rate; Feed, 1.5 ml of a mixture of β-lactoglobulins A and B at each concentration of 0.50 m*M* in the carrier with 0.1 ml/min flow-rate; Displacer, phytate composed of PN12 and PK2 in carrier, (a) 20 m*M*, (b) 30 m*M*, and (c) 40 m*M* phytate, respectively; Fraction size, (a) 500 μl, (b) and (c) 250 μl; Regenerant, 25 m*M* BES, 1.0 *M* NaCl, and pH 2.5 with flow-rate 0.5 ml/min.

development in Fig. 4c. This phenomenon is consistent with that reported by Liao et al. [5] although they used macromolecular compound as displacer. It should be pointed out that the characteristic of displacement chromatography is that there is an isotachic train or there is a plateau concentration in displacement chromatogram. In displacement chromatography, it often occurs that the concentration of some of the feed components in the isotachic train is higher than their concentration in the feed. This is in contrast with overload elution chromatography, where dilution of the feed always occurs [19]. From this viewpoint, overload elution chromatography is

shown in Fig. 4a because of the dilution of the feed components; displacement chromatography is shown in Fig. 4(b and c), due to the concentration increase in the feed components.

The results for the 10 m*M* displacer concentration are not shown in Fig. 4 because at this displacer concentration, β-lactoglobulins A and B cannot be eluted or stay in the column. When carrier pH is 7.5, phytic acid has about eight net charges (Fig. 1). Ionic strength is calculated according to the equation,

$$I = \frac{1}{2}\sum_i c_i z_i^2 \quad (1)$$

where I is ionic strength, c_i is ion concentration, and z_i is the charge number of the ions. For phytic acid, when $c = 10$ mM and $z = 8$, $I = 0.32$ M. From the step-gradient elution data [18], if NaCl is used as elution salt, the needed ionic strength is only 0.23 M even for β-lactoglobulin A, which means that phytate cannot be simply considered as the common salt.

Generally, β-lactoglobulins A and B cannot be separated by isocratic elution if a common salt like NaCl is used. However, when a low concentration of phytic acid was used in displacement chromatography, β-lactoglobulins A and B can be separated under overload elution. In displacement chromatography, the mobile phase flow-rate is much lower than that in isocratic elution chromatography, which itself seems to contribute to separation resolution because the mass transfer resistance is greatly reduced through the decrease in mobile phase flow-rate. Cysewski et al. [26] modeled multivalent ion-exchange for mass overload conditions. Their simulation results predicted that peak tailing due to column overloading may be reduced using a counter ion of higher valence; they thought that this conclusion may be useful for optimizing separations in preparative chromatography. Phytate is a counter ion with high valence (at least eight), so phytic acid also made contributions to separation resolution in isocratic overload elution chromatography of proteins. Further research is needed in this respect.

3.3.2. Yield and recovery

Table 2 shows yields and recoveries of the process. The yields and recoveries depend on the displacer concentrations. There is an optimum displacer concentration (30 mM), in which the yield and recovery are highest. When phytic acid concentration is less than 30 mM, it seems that there is protein left in the column perhaps due to the formation of dimer. There is a stronger interaction between protein dimer and adsorbent because the interaction sites in dimers are almost two-times that of monomers. β-Lactoglobulins prefer to form dimers when ionic strength is high, protein concentration is high, and pH is equal or less than 7.5 [27]. When phytic acid concentration is 40 mM, the yield and recovery are also low. As the displacer concentration increases, the hydrophobic interaction between protein and adsorbent also increases. The low yield and recovery may result from protein being retained in the column due to hydrophobic interaction, increase in higher displacer concentrations, and relatively higher hydrophobicity of protein β-lactoglobulins [28].

Table 2
Yields and recoveries of β-lactoglobulins A and B purified by displacement with a purity of 100%

Displacement conditions	Yield (mg)	Recovery (%)
β-Lactoglobulin A		
as in Fig. 4a	6.64	53.6
as in Fig. 4b	10.8	87.1
as in Fig. 4c	6.80	55.0
β-Lactoglobulin B		
as in Fig. 4a	6.11	49.4
as in Fig. 4b	8.23	66.5
as in Fig. 4c	7.39	59.7

4. Conclusions

Phytic acid has been evaluated as an efficient displacer for anion-exchange displacement chromatography. Phytic acid as a small molecular mass displacer has its own intrinsic elution property and displacement properties. When the phytic acid concentration in the carrier is increased, the elution properties are changed from isocratic elution to displacement separation. The yields and recoveries of the displacement chromatographic process also depend on the displacer concentrations. There is an optimum displacer concentration, at which the yield

and recovery are highest and an isotachic train is formed. The concentration of low-molecular-mass displacer in displacement chromatography is the most important parameter.

Acknowledgements

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Platelet Interaction with Protein-coated Surfaces: An Approach to Thrombo-Resistant Surfaces*

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We have recently shown that when polymer surfaces are exposed to flowing whole blood, in the absence of any blood/air interface, there is a direct relationship between platelet adhesion and critical surface tension of uncharged hydrophobic polymers (1, 2). The similarity of this relationship to the previously observed relationship between critical surface tension and surface thrombogenicity (3) does suggest a major role for platelet adhesion and activation as the initiating step in *in vivo* surface-induced coagulation of blood on uncharged, hydrophobic polymers (1, 2, 4).

In our initial studies of blood-polymer interactions, a dipping technique had been used in which the polymer surface was moved through a blood/air interface. Heavy platelet adhesion was observed on all surfaces. Since proteins are known to spread and denature at the blood/air interface, it was suspected that a denatured protein surface was coated on the polymer by Langmuir-Blodgett transfer, and that this denatured protein layer caused the heavy platelet adsorption (1, 2). These observations led us to examine the effect of a native protein layer on platelet adhesion, since it was felt these surfaces would be less damaging to platelets and might represent an approach to a thrombo-resistant surface. Our earlier studies (1, 5) had indicated that such proteinated surfaces are readily prepared by adsorption from solution onto uncharged, hydrophobic polymers, with the proteins forming compact monolayers with no apparent dimensional denaturation.

Independent studies were also begun by one of us (JDA) on the covalent bonding of proteins to polystyrene as a means of rendering this material thrombo-resistant. Albumin was chosen since it appears to function largely in the maintenance of osmotic balance, and therefore, should present a relatively passive surface to blood.

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Platelet Adhesion and Aggregation in Thrombosis: Countermeasures

Transactions of the Eighteenth Annual Symposium on Blood, Wayne State University School of Medicine, Detroit, Michigan, held on January 16 and 17, 1970

E. F. MAMMEN G. F. ANDERSON M. I. BARNHART
EDITORS

203 Figures, 50 Tables

19 70

F. K. SCHATTAUER VERLAG · STUTTGART – NEW YORK

This paper reports the results of surface proteination as an approach to thromboresistant surfaces.

Materials

The polymers used in this study were as follows:

a) Polytetrafluoroethylene, fluorinated ethylene/propylene copolymer (Teflon TEF film and Teflon FEP film), DuPont.
b) Polydimethylsiloxane (Silastic No. 372, medical grade, unvulcanized, nonreinforced sheet), Dow Corning. The Silastic sheet was cured at 93 °C for four hours.
c) Polyethylene (film), Olin Mathison.
b) Fibrinogen (human fraction I, 65% clottable), NBC. The fibrinogen was purified by the method
e) Polystyrene (film and ¼ inch ID tubing), Westlake Plastics, Inc. The tubing was machined into Gott vena cava rings (6).

The proteins used in this study were as follows:

a) Albumin (human 4 × crystallized), NBC; and albumin (bovine, fraction V), Mann Research Labs.
b) Fibrinogen (human fraction I, 65 % clottable), NBC. The fibrinogen was purified by the method of Straughn and Wagner (7).
c) γ-Globulin (human fraction II), NBC.

Plasma was obtained from freshly drawn blood anticoagulated with potassium ammonium oxalate solution with heparin (Dade Reagents, Inc.).

The isotonic phosphate buffered saline (pH 7.4) contained 10.2 g of $Na_2HPO_4 \cdot 7H_2O$, 1.49 g of $NaH_2PO_4 \cdot H_2O$, and 1.48 g of NaCl/liter. The phosphate buffered saline/albumin solution was made from the above stock solution by adding 3.8 g/liter of albumin (bovine, fraction V powder, NBC).

Lillie's modification of Wright's stain was made by the procedure of Conn *et al.* (8).

Method

The polymer films were coated with individual proteins and with plasma by solution adsorption techniques (1,5). In all of these procedures an air/solution (or plasma) interface was avoided. Coated polymer samples were examined by attenuated total reflection infrared spectroscopy (ATR) to ascertain that protein (and plasma) adsorption had been accomplished.

The polymer films, coated with protein or plasma, were then clamped into our closed (flow-through) cell (1,2), and the cell assembly filled with phosphate buffered saline. By a technique described previously (1,2), venous blood was fed directly from the donor to the closed (flow-through) cell, where it displaced the buffered saline solution (thus avoiding an air/blood interface) and was then allowed to flow through the cell for one minute. The cell was immediately rinsed with phosphate-buffered saline solution containing albumin (again avoiding an air/solution interface), then rinsed with absolute methanol to fix the cellular material. The polymer films were removed, stained, and then examined by oil immersion microscopy (1500 ×). A systematic and representative sampling of the fields (approximately 20) were chosen from the central 2 × 0.5 cm rectangle portion of the exposed film, and the number of adsorbed platelets per 20,000 μ^2 area was determined.

A modification of the technique of Jenny (9), using $ZnCl_2$ as the catalyst in an aqueous solution of HCl, was utilized to bind albumin to polystyrene.

Polystyrene film (18 × 50 mm, 10 mils) and polystyrene vena cava rings were washed with 95 % ethanol, then chloromethylated (10) by immersing them in a solution of 5.0 g $AlCl_3$ and 3.6 ml chloromethylmethyl ether in 100 ml nitrobenzene at room temperature for fifteen seconds (15). The salmon-pink samples were immediately plunged into 100 % ethanol. A uniform white surface formed after about twenty seconds. The samples were then soaked and rinsed in 95% ethanol until no further trace of nitrobenzene was evident. ATR spectra of the films indicated extensive

para substitution of the aromatic groups. The albumination solution was prepared by dissolving bovine albumin (fraction V) in a 0.7 % solution of zinc chloride in distilled water, adjusted to pH 4 with HCl, to give a 1 % solution of albumin; the control solution contained NaCl (0.85 %) in place of the $ZnCl_2$. The chloromethyl polystyrene film and rings were immersed in the albumination solution at 40 ° C for eight hours, then thoroughly washed for three days in isotonic saline until there was no gross evidence (frothing) of protein remaining. ATR spectra of the control surfaces showed little evidence of protein on the surface; however, the spectra of the treated samples contained peptide bond absorption bands, indicating a significant amount of protein on the surface; a decrease in intensity of the C-Cl bands was also apparent (11).

Control and treated vena cava rings (6, 12) were implanted in dogs by Dr. Vincent Gott. The acute (two hour) control rings were thrombosed; the treated samples were essentially clot-free after both two hour and two week tests.

Results and Discussion

The results (shown in Table 1) indicate that fewer platelets adhered to a polymer surface precoated with a layer of native protein than to the pure polymer surface, or to a denatured protein surface such as that formed at an air/blood interface. This was not unexpected, since if these proteins are truely in their native state, their interaction with platelets should be no different from the reaction in blood itself. The similarity in adhesion of platelets to the albumin-coated surfaces (average platelet count was 0.2), the γ-globulin-coated surfaces (0.5), the fibrinogen-coated surfaces (0.7), and the plasma-coated surfaces (0.5) indicated essentially complete masking of the substrate. The fact that some adhesion did occur is believed to be due to impurities in the protein material used (other plasma constituents, denatured proteins, etc.), to the nature of the surface resulting from these closely packed arrays, or from imperfections in the coating. The fact that platelet adhesion was essentially

Table 1. Adhesion of platelets from whole blood to various protein-coated surfaces[1]).

Polymer substrate	Coatings				
	None[2])	Albumin	Fibrinogen	γ-Globulin	Plasma
Polyethylene	15.3	0.1 (0.0—0.2)	0.6 (0.3—0.9)	0.5 (0.0—1.6)	0.5 (0.0—0.9)
Fluorinated ethylene/propylene copolymer	5.4	0.5 (0.1—1.2)	0.9 (0.2—1.6)	0.4 (0.2—0.7)	0.5 (0.0—1.0)
Polytetrafluoroethylene	4.6	—	0.7 (0.0—1.5)	0.6 (0.1—1.0)	0.7 (0.1—1.1)
Polydimethyl siloxane	4.6	0.1 (0.0—0.3)	0.4 (0.3—0.6)	0.5 (0.0—1.3)	—
Poly-4,4′-isopropylidene-diphenylene carbonate	15.5	—	—	—	0.5 (0.2—1.3)
Mean for all substrates		0.2	0.7	0.5	0.5

1) Exposure time was 1 minute of flow through our closed (flow-through) cell. Values are number of platelets per 20,000 μ^2 of surface and represent average values from at least five experiments for each surface.

2) See Ref. 2.

zero when the surface was coated with a protein by our solution adsorption technique (1,5), whereas it was high (> 50) when a blood/air interface was present, provides additional evidence that proteins adsorbed on an uncharged, hydrophobic polymer surface in the absence of an air interface are not denatured.

Several other proteins (hemoglobin, myoglobin, and ribonuclease) adsorbed onto polymer surfaces have also shown low platelet adhesion (0.1, 0.1, and 0.7 respectively).

White cells often adhered to the protein coatings (especially albumin and γ-globulin), and platelets were usually found clumped around the white cells. The types of white blood cells adhering appeared to be in proportion to their total peripheral blood content and were often in a lysed condition. To minimize this effect, polymer surfaces were coated with the donor's own plasma, then the films were exposed to this whole blood in the closed (flow-through) cell. As expected, fewer white blood cells adhered, and those that did were in good condition, not lysed.

Platelet adhesion on these plasma-coated surfaces averaged 0.5 platelets per 20,000 μ^2 area. While this value was higher than one would expect if the plasma proteins were adsorbed in proportion to their concentration in the plasma (i.e., more albumin in the coating), it may be that the resulting irregular structure of the coating due to differences in protein molecular weight overrides any proportionality effects. However, it may also be that there is a preferential adsorption of γ-globulin and/or fibrinogen on the polymer surfaces. Attempts to explore this possibility using the antisera for fibrinogen and albumin have indicated that for polyethylene, at least, the test is positive for both proteins.

It was also of interest to see whether or not platelet adhesion on the protein-coated surfaces would increase with longer exposure time. From experiments with a fluorinated ethylene/propylene copolymer coated with albumin, it would seem as though the adhesion did not increase with time. The average number of platelets was 0.1 per 20,000 μ^2 for times ranging from 30 seconds to three minutes on the albumin-coated surfaces, while for the uncoated polymer surface, the number of platelets increased from 2.6 per 20,000 μ^2 at 30 seconds exposure to over 18 per 20,000 μ^2 at three minutes exposure time.

It would appear, therefore, that coating uncharged, hydrophobic polymer surfaces with a native layer of plasma protein or with plasma might represent an approach to achieving a nonthrombogenic surface. Of the proteins studied, albumin appeared to be the best coating material.

These results lead one to consider the covalent bonding of an individual protein to a synthetic polymer as an alternate route to surface proteination. Albumin was coupled to polystyrene film and vena cava rings by a modified Friedel-Crafts reaction using aqueous $ZnCl_2$ solution as the catalyst.

Examination of the films by ATR showed a decrease in the intensity of the C-Cl bands and an increase in the amide I band characteristic of the proteins. While the actual nature of the surface formed here is not known, there appeared to be a large amount of bound albumin. The actual form of the albumin is not known, though it is probably complexed with zinc ions (13). The fact that albumin can undergo a reversible conformation change at about pH 4 (13) must also be considered.

The vena cava rings were implanted in the inferior vena cava of dogs. The proteinated rings were essentially clot-free after both two hour and two weeks implantation in contrast to the control samples which were thrombosed at two hours. This is a very severe test, and all common, unaltered polymers will show severe thrombus formation after two hour implants (12). Therefore, the nonthrombogenic behavior of this albuminated polystyrene thus appeared to be about as effective as that of heparinized surfaces for the conditions described here.

Thus it might appear as though this albuminated surface is similar to the absorbed albuminated surface and its thrombo-resistant behavior due to its inertness toward platelets. Whether or not this inertness toward platelets is due to the physical and chemical nature of the protein itself, or due to the hydrated nature of the overall structure is not yet known. Since the vascular walls are hydrophilic in nature (3, 14), it is reasonable to suspect that the more ideal materials to be used in contact with blood will be hydrophilic (3). The blood compatibility of the non-ionic hydrogels that have been studied is quite good (15) and would tend to support this hypothesis (3).

A mechanism for the interaction between water-containing surfaces and blood is not yet available. Andrade has examined the role of the water-loaded surface in adsorption from aqueous solution (11). His model considered the intermolecular forces responsible for adsorption in certain limited systems and the role of the water content of the surface. The conclusion was that a nonpolar polymeric surface containing a high water content would not tend to adsorb apolar material from aqueous solutions. A molecule in the vicinity of such an interface would »feel« as if it were in bulk water and not under the influence of an interface. Actual adsorption data to prove or disprove this hypothesis are not yet available; work is in progress. If the model and interpretation is correct, it may offer an explanation for the behavior of hydrophilic surfaces.

Summary

Uncharged, hydrophobic polymers coated with a native layer of plasma proteins show little or no platelet adsorption. Studies relating surface thrombogenicity to platelet adsorption suggest that the proteination of polymers might represent an approach to achieving a nonthrombogenic surface. Of the proteins studied, albumin appears to be the best coating material. Albuminated polystyrene, prepared by an

aqueous Friedel-Crafts reaction between bovine albumin and chloromethylated polystyrene, has been shown to be nonthrombogenic. This proteinated surface appears to be as thrombo-resistant as a heparinized surface.

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