

# **Protein Adsorption and Materials Biocompatibility: A Tutorial Review and Suggested Hypotheses**

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*A comprehensive review of protein adsorption at solid-liquid interfaces is presented, including a brief review of protein structure and the principles of protein adsorption. Adsorption-based biocompatibility hypotheses and correlations are discussed, including surface charge, interface energetics, passivation, protein-resistant surfaces, and the role of adsorbed immunoglobulins and complement. New methods for the study of protein adsorption are discussed, including total internal reflection techniques (absorbance, fluorescence, and Raman) and ellipsometry. Qualitative "rules of thumb" of protein adsorption are also presented.*

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## 1 First Observable Event?

It is commonly stated that the first readily observable event at the interface between a material and a biological fluid is protein or macromolecule adsorption. Clearly other interactions precede protein adsorption: water adsorption and possibly absorption (hydration effects), ion bonding and electrical double layer formation, and the adsorption and absorption of low molecular weight solutes — such as amino acids. The protein adsorption event must result in major perturbation of the interfacial boundary layer which initially consists of water, ions, and other solutes.

Body fluids have a rich and complex composition, permitting a wide range of interactions and competitive processes.

The great majority of the available work has focused on blood/plasma/serum applications. In recent years the interaction of tear components with contact lenses have become very important.

Much of the early work is reviewed by Brash and Lyman<sup>1)</sup> and by Vroman<sup>2)</sup>. Protein adsorption is a very old field — the earliest studies probably being monomolecular film observations using Langmuir-Blodgett troughs<sup>3)</sup>. A comprehensive monograph which reviews protein adsorption is now available<sup>4)</sup>.

Early observations showed that blood clotted faster in clean glass tubes than in siliconized glass. When Mr. Hageman's blood failed to clot *in vitro*, it was speculated that a "Hageman Factor" was responsible for the *in vitro* "activation" of blood coagulation (see Ref.<sup>2)</sup> for a delightful account).

Early observations, particularly the work of Vroman<sup>2,5)</sup>, showed that proteins adsorb on practically any surface. Vroman first showed<sup>5a)</sup> that adsorption of protein on a hydrophilic surface rendered it less hydrophilic and also that protein adsorption on a hydrophobic surface rendered it hydrophilic.

Knowledge and understanding of protein structure and properties in the 1950's was rapidly evolving. The unique secondary, tertiary, and even quaternary structures of proteins were becoming understood<sup>6-8)</sup> and the delicateness of protein three-dimensional conformation was recognized, including the possibility for "denaturation" at liquid/air and solid/liquid interfaces<sup>2,3)</sup>.

The fact that Hageman Factor, a plasma protein, circulated free in blood without inducing coagulation, but when exposed to glass or silicate clay surfaces did induce coagulation, led to a unique and novel hypothesis: changes in conformation induced by the adsorption of Hageman Factor result in enzymatic activity which initiates a sequence of reactions leading to fibrin formation. Although interface-induced denaturation of proteins at liquid/air interfaces was well-known, the interest in *in vitro* blood handling and the early development of medical devices provided an impetus for understanding and controlling surface-induced coagulation.

At about the same time, the field of protein separation and purification was undergoing rapid development. The introduction of materials for protein chromatography, such as cross-linked dextran, agarose, and polyacrylamide, provided a means to study protein-surface interactions, as well as to dramatically advance knowledge in protein biochemistry.

## 2 Protein Structure

The treatment and understanding of protein adsorption requires familiarity with modern concepts of protein structure and function, such as provided in Ref. <sup>6-8)</sup>. A concise review is available in Chapter 1 of Ref. <sup>4)</sup>.

Proteins are biological macromolecules constructed for specific and unique functions. They are high molecular weight polyamides produced by the specific co-polymerization of up to 20 different amino acids. The amino acid composition, called the primary structure, is generally unique and specific to each particular protein. The hydrogen bonding characteristics of the polyamide bond in the backbone of proteins result in various secondary structures, such as the well-known  $\alpha$ -helix and  $\beta$ -sheet. Intramolecular associations, including ionic interactions, salt bridges, hydrophobic interactions, hydrogen bonding, and covalent disulfide bonds, result in a unique tertiary structure for each polypeptide chain. Finally, two or more polypeptide chains, each with its own primary, secondary, and tertiary structure, can associate to form a multi-chain quaternary structure. Most proteins contain short, carbohydrate sequences off the main polypeptide chain and are therefore called glycoproteins.

The fundamental principles of protein structure and function are available in modern biochemistry textbooks <sup>6-7)</sup>. The current edition of Stryer is particularly recommended <sup>7)</sup>. The books by Dickerson and Geis <sup>6,9)</sup> are excellent tutorials on the fundamental principles of protein structure. The modern text by Schulz and Schirmer <sup>8)</sup>, *Principles of Protein Structure*, is outstanding. In addition, modern physical

**Table 1.** Minimal protein information required for adsorption studies

Quantity	Method	Significance/Information
Molecular Weight	Light scattering, Osmometry, Gel permeation chromatography	Size, shape, mass of molecule.
Size & Shape	Light scattering sedimentation	Size, shape, mass of molecule.
Amino Acid Composition	Amino acid analysis	Basic.
Electrophoretic Mobility	Gel electrophoresis	Related to net charge.
Isoelectric Point	Isoelectric focusing	Number of charged groups.
UV-Visible Absorption Spectrum	Absorption spectroscopy	Tyrosine-Tryptophan environments, presence of absorbing ligands or impurities. <sup>a</sup>
Fluorescence Spectrum	Fluorescence spectroscopy	Tryptophan environment, presence of fluorescent ligands or impurities. <sup>b</sup>
CD Spectrum	Circular Dichroism	Secondary structure, $\alpha$ -helix, $\beta$ -sheet, ...
Subunits	SDS-gel electrophoresis	Quaternary structure components & their molecular weight.
Subunits & —S—S—	Gel electrophoresis in mercaptoethanol	Tertiary structure — breaks —S—S— bonds.
Solubility — Salting Out	Salting out fractionation	Solubility under different solvent conditions, pH, ionic strength, hydrophobic interactions.

<sup>a</sup> For example, heme in myoglobin & hemoglobin;

<sup>b</sup> For example, bilirubin in albumin

biochemistry texts, in particular Freifelder <sup>10)</sup> and Cantor and Schimmel <sup>11)</sup>, are essential to the understanding of protein structure, function, and interfacial behavior. Bull's *Physical Biochemistry* is still an outstanding introduction to the subject <sup>12)</sup>. Walton's books on biopolymers are also highly recommended <sup>13,14)</sup>, as is Tanford's book <sup>15)</sup>.

Table 1 lists those characteristics of a protein which are normally considered essential for a minimal characterization. Most of the information desired is available for practically all proteins which may be of interest to the biomaterials investigator. Generally, all of this information is available in the literature, and the investigator need only to check for purity, homogeneity, and perhaps activity of the protein preparation.

**Table 2.** Additional protein information desired for adsorption studies

Quantity	Method	Information/Significance
Molecular Vibrations	Raman and infrared spectroscopy	Secondary & tertiary structure.
Thermal Denaturation	Raman, CD, or fluorescence	Conformation (secondary/tertiary) & structure as f (temperature).
Monolayer Properties	Langmuir trough or pendant drop	Air/water interfacial properties, $\pi$ -A curves, can deduce closepacked monolayer dimensions.
Binding Properties	Equilibrium dialysis for lower molecular weight ligands — affinity chromatography for others	Ligand binding characteristics, such as $\text{Ca}^{++}$ binding to fibrinogen, and prothrombin, fatty acid and bilirubin binding to albumin, heparin binding to antithrombin III and fibronectin.
Amino Acid Sequence	Usually cleavage fragments plus sequencing	Sequence important in structure and function.
3-D Structure	X-ray diffraction	Complete stereo-chemistry of tertiary or quaternary structure.
Enzymatic or Specific Biochemical Activity	Substrate turnover, antibody binding, ligand binding	Is protein (enzyme) functional? Does it bind its specific ligands?

For a complete adsorption analysis, the amino acid sequence and secondary, tertiary, and quaternary structure of the protein should be known (Table 2). This is usually only available if a complete X-ray diffraction analysis has been done. Some information on ligand-binding characteristics of the protein should be available: Does it bind low molecular weight solutes? Does it have specific macromolecular binding characteristics, etc.? For example, the physical properties of albumin are different depending on its fatty acid and bilirubin composition. The properties of hemoglobin are different if its heme ligand is in the deoxy or oxy form or in the met ( $\text{Fe}^{+3}$ ) form <sup>9)</sup>. The activity of many proteins is enhanced or potentiated when bound to appropriate ligands. For example, heparin binding to antithrombin III greatly increases the binding of thrombin. All such information is useful in predicting and interpreting protein interactions with surfaces.

The general solubility characteristics of the protein, including its behavior in different pH and ionic strength environments, its behavior in urea solutions, its behavior

Table 3. Major Non-Covalent Forces and Interactions Important in the Organization and Stabilization of Protein Structure in Aqueous Solutions<sup>8, 16, 17)</sup>

Interaction	Description	Directional?	Effect of Increasing		Binding energy (kcal/mol)	Free energy change $H_2O \rightarrow EtOH$ (kcal/mol)
			Temperature	Ionic Strength or Dielectric Constant		
Ionic	Coulombic interaction due to opposite charges.	None — spherical symmetry	Decreases	Decreases	-5 -COO <sup>-</sup> ... N <sup>+</sup> H <sub>3</sub> <sup>-</sup> - <sub>3</sub> -NH...O=C—	-1
H-bonding	A donor-acceptor effect involving the H-atom of the donor — essentially an ionic interaction. Water participates in H-bonds.	Yes, linear bond lengths ~ 3 Å, bond energies ~ 3 to 6 kcal/mol	Decreases	Decreases		
Charge-Transfer (Donor-Acceptor)	In water this is mainly due to $\pi - \pi$ interactions. Trp interaction strongest as it is strong electron donor.	Yes — analogous to H-bonding	Decreases	Increases		
Hydrophobic or Entropic	Water cannot interact with apolar groups via H-bonding, resulting in water adjacent to such apolar groups being more organized or more ordered. If such apolar groups come together, some ordered water is released, increasing entropy — hence apolar groups tend to "interact."	None	Increases	Increases; effect is ion-specific		-2.4 (phe)

in solutions containing small amounts of methanol, ethanol, or glycerol, and related information on solution characteristics are all important in interpreting and predicting interactions at interfaces.

The fundamental classes of forces and interactions important in protein structure and function are summarized in Table 3. The four general classes are: (1) ionic or electrostatic interaction, due to the attraction or repulsion of two or more groups carrying a net charge; (2) hydrophobic interaction, a largely entropically driven process due primarily to water structure effects adjacent to hydrophobic interfaces; (3) hydrogen bonding, a unique type of dipole/dipole interaction which results in interaction energies comparable to very weak covalent bonds; (4) and other interactions, primarily those of a charge-transfer or partial electron donor/acceptor type, which in aqueous solutions are often dominated by  $\pi - \pi$  interactions. It is important to point out that all of these interactions take place in an aqueous medium, and that the hydrogen bonding and dielectric properties of water play a very major role (see Ref. <sup>8, 16, 17)</sup> for details).

There has been considerable effort on the prediction of secondary and tertiary structures of protein from the amino acid sequence using computer-aided minimal potential energy calculations <sup>8)</sup>. The question as to how a primary amino acid sequence begins to produce secondary and super-secondary structures and fold into its equilibrium tertiary structure and functional domains is a very active field of structural biochemistry. A related problem is the mechanism by which a protein unfolds or denatures <sup>18-20)</sup> which is of fundamental interest in the protein adsorption process.

A polypeptide chain in water has a specific pattern of polar and non-polar groups. There is a tendency to minimize the surface area between non-polar groups and the aqueous phase. This can be treated in terms of a surface-free energy argument or in terms of the classical hydrophobic interaction argument. The non-polar groups tend to be excluded from the surface of the polypeptide globule with the highly polar and charged amino acids on the surface interacting strongly with water. There are some regions on the surface which are non-polar, and there are some regions in the interior which contain polar groups, either through hydrogen bonding or salt bridge associations. About 90% of all internal polar groups form hydrogen bonds. The solvent entropy (hydrophobic interaction) effect contributes significantly to overall protein structural stability. The intramolecular interactions are highly efficient and effective.

Disulfide bonds confer additional stability. A frequently encountered structural component is the sequence —Cys—Cys— with both residues forming disulfide bonds with other cystines. This is a useful architectural unit and forms the basis for linking three different chain segments in close proximity. This structure is found in serum albumin.

The interior of the protein has a very high packing density, comparable to that in the protein crystal. The average packing density of a protein is about 0.75, which is incredibly high when one considers that a close-packed structure of spheres has a packing density of 0.74. Thus the packing density of proteins in terms of volume utilization is even more efficient than most close packed metals <sup>8)</sup>. One often hears the criticism that the structure of a protein in the crystal must be very different than in the solution. This is not the case, as the solution packing of a protein is essentially identical to that in the crystal. It is important to note that active centers in reactive sites of proteins tend to be much more loosely packed than the rest of the molecule,

thereby providing the increased flexibility required for chemical reactions and recognition processes.

Careful study of the structure of available proteins suggests regions of amino acid homology and structural homology in very different protein molecules. This has led to the concept of the structural and functional domain. One can consider large globular proteins as being made up of a set of functional domains, a more important structural concept than the chain composition of the protein. Active sites are often located at the interface between two structural domains. One can, therefore, consider proteins as being modular structures with the structural or functional domains as the modules. A good example is the immunoglobulin molecule shown in Fig. 1 (an IgG).

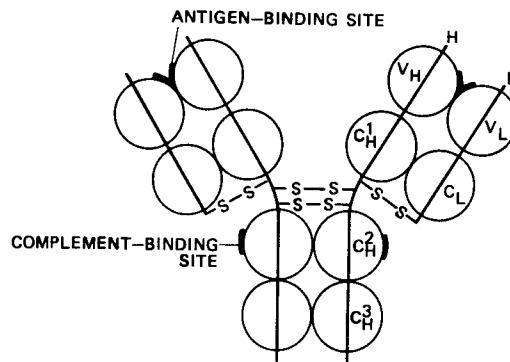


Fig. 1. Structure of an IgG molecule showing the light (L) and heavy (H) chains, the antigen-binding sites (in the Fab regions), the complement-binding site (in the Fc region), and the constant (C) and variable (V) amino acid regions (from Ref. <sup>8</sup>).

There are both functional and structural domains. A functional domain may consist of one or more structural domains. A functional domain is a functionally autonomous region of the molecule. In the case of the IgG, the Fab portion is the functional antigen recognition domain while the Fc region is an effector domain. Structural domains are geometrically separate.

Knowing the binding characteristics of the various domains in the molecule allows the investigator to begin to deduce certain types of adsorption behavior. For example, it is known that human plasma fibronectin contains several heparin binding sites, with pK's in the 8–9 range and with a high, positive charge density. It would be reasonable to suspect that when fibronectin interacts with negatively-charged surfaces it may do so through such binding sites, just as it does with heparin.

The large globular proteins of blood plasma are monomers — that is, they consist of only one chain or a set of covalently linked chains. An oligomer is an aggregate of monomers whereby the aggregate is held together by non-covalent bonds. Most intracellular proteins are oligomers; proteins in blood plasma are large monomers, generally consisting of several functional domains.

A structural domain can be defined as a region of locally high electron density separated by regions of low electron density or clefts in the electron density map. Amino acid residues that are far apart along the chain tend to be far apart in the three-dimensional structure. The concept of neighborhood correlation suggests, perhaps based on kinetic folding considerations, that those amino acids which are close together in a sequence tend to stay close together in the three-dimensional structure. Such neighborhood correlation is probably a consequence of the chain-folding process.

The domains are probably those pieces of a chain which fold independently. Most globular proteins have domains of 100–150 residues and are roughly 25 Å in diameter.

Five different classes of structural domains have been identified, based on the secondary structural makeup <sup>8</sup>). Structural symmetry is often evident, particularly in aggregate or oligomeric proteins. The more contacts, the more stable the interaction.

Depending on the fit, the number and strength of contacts, and other factors, a monomer-oligomer equilibrium will exist. For example, in the case of Concanavalin A the tetramer is in equilibrium with dimer and monomer under normal conditions. In the case of insulin the monomer, dimer, and hexamer are all in equilibrium. In hemoglobin the dimer and tetramer are in equilibrium. In the case of the adsorption of such proteins, one must not only know what is the aggregation state of the protein in solution, but must be able to deduce the adsorbed state. Do the molecules adsorb as dimers <sup>21</sup>? Do they adsorb as dimers and then associate as trimers or hexamers on the surface, etc.?

Some of the free-energy considerations used to probe protein oligomerization and aggregation are also applicable to adsorption. Protein-protein interactions usually involve surface complementarity, which can provide significant specificity. The specificity of a surface is determined by its shape, its pattern of hydrogen-bond donors and acceptors, its pattern of charges, and even its pattern of hydrophobic patches. Based on immuno globulin studies, a 100 Å<sup>2</sup> surface can form on the order of a thousand patterns showing different binding specificities <sup>8</sup>.

An important question is how the linear, amino acid sequence spontaneously folds into the three-dimensional structure of the native form. There has been considerable work on the folding process, as well as on the unfolding or denaturation process. It is possible to take a protein in its native form, and by subtly changing its micro-environment (pH, ionic strength, temperature, addition of denaturants such as urea, addition of other ions, etc.), induce the protein to undergo a conformational change. The energetics of that change can be measured. In a number of such studies, summarized briefly in Table 4, one can conclude that the native state is only marginally stable and is in equilibrium with a variety of other states under normal conditions. One can write the equilibrium equation N ⇌ D. The total free energy change in going from the

Table 4. Estimates of the stability of globular proteins under physiological conditions\*

Protein	Denaturant	$\Delta G_D^{H_2O}$ (kcal/mol)
Lysozyme	GdnHCl or Urea	9.5
	T-pH	10.0
Ribonuclease	GdnHCl or Urea	8.5
	T-pH	6.0
Myoglobin	pH	14.0
	T-pH	14.0
α-Chymotrypsin		
Immunoglobulin light chain (Wes) domain	GdnHCl	5.5

\* (from Ref. <sup>20</sup>, p. 38)

native (N) state to one of the available denatured (D) states is only in the vicinity of 10 kcal/mole for an average globular protein of 150 residues<sup>20)</sup>. This is an energy corresponding to only two to five hydrogen bonds per molecule! It is clear that protein conformations, therefore, are very fragile and truly only marginally stable. For example, the burying of just one surface tryptophan residue provides an energy of 3.4 kcal/mole. Thus, when a protein finds itself in the vicinity of an interface, its microenvironment is altered, and clearly its equilibrium will be altered, probably altering the protein conformation in the adsorbed state.

*In vitro* folding and denaturation studies have established that the process occurs in the time span of 0.1 to 100 seconds. It is commonly assumed that there exist folding nuclei — that is local regions of alpha helices which are stable and which function as nuclei for additional folding or organization<sup>8)</sup>. This provides a domain, therefore, with strong local neighborhood correlation. It is expected that there are various folding pathways and intermediate structures which are used during the folding process. Some of the theoretical methods which are now being applied to the simulation of folding processes may, in modified form, have application to the simulation of the adsorption process.

A protein and its associated water molecules constitute an extremely small thermodynamic system subjected to the continual Brownian motion or buffeting of the solvent molecules<sup>22)</sup>. Such transient changes result in fluctuations in the local thermodynamic properties of the system. A protein with a molecular weight of 25000 experiences internal energy fluctuations of about 40 kcal/mole<sup>22)</sup>. This is much larger than the energy changes involved in ligand-induced conformational transitions. These thermal fluctuations can result in volume changes which provide transitory cavities or channels in the protein. These dynamic fluctuations are a consequence of the thermodynamic uncertainty of small systems. They exist within a single macromolecule and are not correlated with similar fluctuations in other molecules. It is clear from these thermodynamic fluctuations that conformational mobility must be expected. It is now known from NMR studies that even such large and rigid structures as the planar aromatic amino acid side-chains can flip in and out of the protein surface<sup>23)</sup>.

Proteins are therefore dynamic, flexible objects whose physical and chemical properties are dominated, not only by their conformation, but also by the continual changes in conformation which are a consequence of their microscopic size<sup>22)</sup>. The marginal stability of most protein conformations suggests that processes at one point in the protein might well have an effect on a portion of the molecule far removed.

The process of ligand binding to a protein may also involve conformational change. The process might be viewed as follows<sup>22)</sup>:

Freely diffusing ligand approaches the protein. Assume that the ligand binding site is not a rigid structural feature, but involves transitory conformations. If the protein is in a transitory "open" conformation, the ligand may diffuse in and bind. The binding interactions may now stabilize a conformational state which is optimum for ligand-protein interaction, a state which previously, and in the absence of ligands, would be unstable.

One can envision a conformational fluctuation spectrum with the native state being a mean conformation. Since that mean conformation is only marginally stable, it is

clear that any local microenvironmental change, such as a solid surface, may well stabilize other conformations.

In order to begin to understand and appreciate how a protein may interact with a surface, it is important to be able to "see" the protein in three dimensions. This usually requires the use of models which, in the case of proteins, are terribly cumbersome and expensive. In many institutions molecular models have been replaced by three-dimensional molecular computer graphics. There are some 70 major molecular graphics installations throughout the world which have the capabilities of imaging large macromolecular structures in three dimensions.

Ideally, one should be able to predict a three-dimensional structure from the amino acid sequence. There has been considerable activity along these lines. Current methods can predict the structural class of a protein or domain. Although about two-thirds of all residues in a polypeptide chain can be assigned to the correct secondary structure, the three-dimensional structure cannot be predicted.

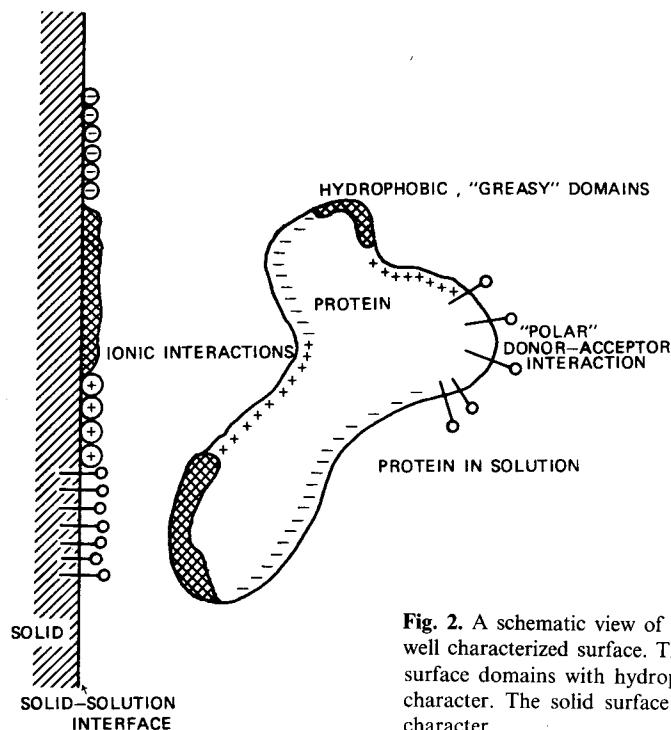
If one independently knows the three-dimensional structure, for example, from X-ray crystallography, i.e., all of the atoms are identified and their atomic coordinates known, then this information can be fed into a computer and the three-dimensional structure displayed. Such coordinates and related information are available for over a hundred proteins from the Protein Data Bank (Brookhaven National Laboratory, Brookhaven, New York). The structure can be displayed as a wire or stick model or as a space-filled model. With space-filled models, one sees only the outer surface of the protein. Using one of a number of algorithms for defining a protein surface<sup>27)</sup>, and by color coding the different amino acid residues or atom types present on the surface, one can quickly "see" which regions of the molecule are hydrophobic, positively or negatively charged, non-charged but polar, and other features.

Feldmann and others have developed a series of teaching tools for macromolecular structure using color-coded molecular graphics-derived images<sup>24-28)</sup>. Connolly and others have improved upon this approach by clearly showing which portions of the protein surface are indeed accessible to a water molecule, as opposed to those portions which are inaccessible, such as in clefts, etc<sup>27)</sup>. Images based on the Connolly methods directly show those regions of the protein surface which can be expected to interact with other molecules. It provides immediate, comprehensible information about steric complimentarity.

Another major advantage of molecular graphics is that it allows real time manipulation of several interacting molecules while quantitatively monitoring the stereochemistry and even the interaction energies. These methods are only beginning to be used for studies of interactions between molecules and only in the most preliminary way for the simulation of protein adsorption<sup>29)</sup>.

It is recommended that any reader seriously interested in protein adsorption obtain *Teaching Aids for Macromolecular Structures*<sup>28)</sup>, which is commercially available for about \$20.00. These aids clearly show the dramatic potential of surface protein structural visualization for the development of hypotheses of protein-surface interactions.

How can all this protein information help our understanding of the adsorption process? Figure 2 is a schematic and idealized view of a single protein interacting with a single, well-characterized solid surface. Assume that the solid surface is well-characterized<sup>30)</sup>. Presume that we know precisely the location of hydrophobic regions



**Fig. 2.** A schematic view of a protein interacting with a well characterized surface. The protein has a number of surface domains with hydrophobic, charged, and polar character. The solid surface has a similar domain-like character

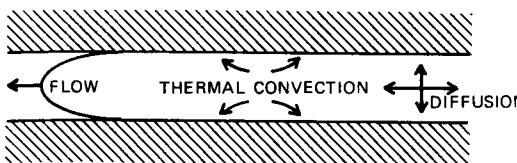
on the surface, the location of positively and negatively charged regions, neutral hydrophilic regions, etc.; i.e., the solid surface properties are thoroughly mapped on a submicroscopic level. We also presume that we have comparable information for the protein; we know its three-dimensional surface structure, the distribution of functional groups, and related properties. We now begin to allow the protein to approach the solid surface (by molecular graphics?) and sample the variety of potential interaction orientations. Clearly, one or more of these interaction orientations will tend to dominate on certain areas of the surface, depending on the interactions present.

It is clear, given the protein and solid surface in Fig. 2, that the protein can interact with the solid surface in a variety of different ways, depending on the particular orientation by which it approaches and the overall binding energetics. Such mental gymnastics, aided by three-dimensional graphics and at least crude estimates of interaction-free energies, should aid in the formulation of definitive hypotheses and in the explanation of data<sup>29</sup>.

### 3 Mass Transport

All adsorption and desorption processes depend on transport of solute to and from the interface. There are basically four major transport mechanisms (Fig. 3):

- a) diffusion;
- b) thermal convection;



**Fig. 3.** The parabolic flow profile in a thin wall channel. In addition to flow, mass transport can occur by molecular diffusion and by thermal convection

- c) flow (commonly called convective transport);
- d) coupled transport, such as convective-diffusion processes.

Turbulent or stirred solutions may incorporate all of the processes noted.

Assume that a freshly prepared interface, such as an air/solution interface in a Langmuir trough, is optimally thermostated to minimize temperature gradients and thus minimize thermal convection. Assume there is no energy barrier to adsorption during the initial stages — every molecule which hits the interface sticks and adsorbs. All molecules near the interface will be rapidly adsorbed, resulting in a depletion of solute in the volume adjacent to the interface, called the sublayer. This concentration gradient drives diffusion from the bulk of the solution towards the depleted sublayer. Under these conditions, the rate of adsorption is equal to the rate of diffusion,  $dn/dt$ <sup>31</sup>:

$$\frac{dn}{dt} = C_0 \left( \frac{D}{\pi t} \right)^{1/2} \quad (1)$$

where  $n$  = number of molecules;

$C_0$  = bulk solution concentration;

$D$  = diffusion coefficient;

$t$  = time.

Thus the *rate* of adsorption is proportional to  $D^{1/2}$  and  $t^{-1/2}$  (Fig. 4).

Integrating gives the total number of molecules,  $n$ , adsorbed at the elapsed time ( $t$ ):

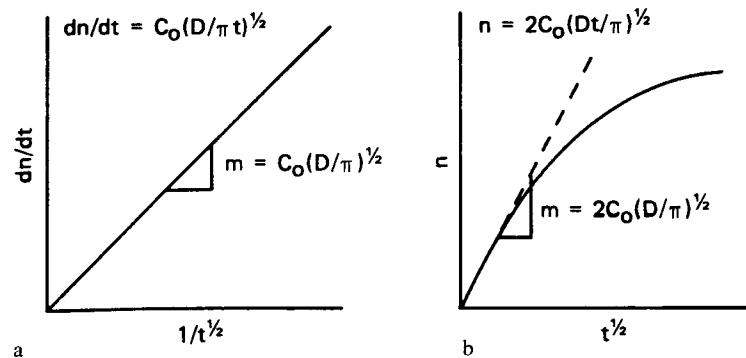
$$n = 2C_0 \left( \frac{Dt}{\pi} \right)^{1/2} \quad (2)$$

The total surface concentration at time,  $t$ , is proportional to  $D^{1/2}$  and  $t^{1/2}$  (Fig. 4).  $D$  for proteins is in the range of  $10^{-6}$  to  $10^{-7} \text{ cm}^2/\text{s}$ .

All fluid interfaces contain an undisturbed layer of solution adjacent to the interface. Mass transport in this boundary layer occurs only by diffusion. The thickness of the boundary layer depends on temperature, stirring, and the interface itself. It is up to 0.1 cm thick in unstirred systems and approaches  $10^{-3} \text{ cm}$  in vigorously stirred systems<sup>3, 31, 32</sup>.

Once the interface is partially saturated with adsorbed solute molecules, then the rate of adsorption falls below the rate of diffusion, suggesting an energy barrier to adsorption.

Molecules at or near the interface may diffuse back into the bulk solution, particularly if the free energy of adsorption is not very high. Mass transport equations which account for back diffusion are available<sup>3, 31, 32</sup>.

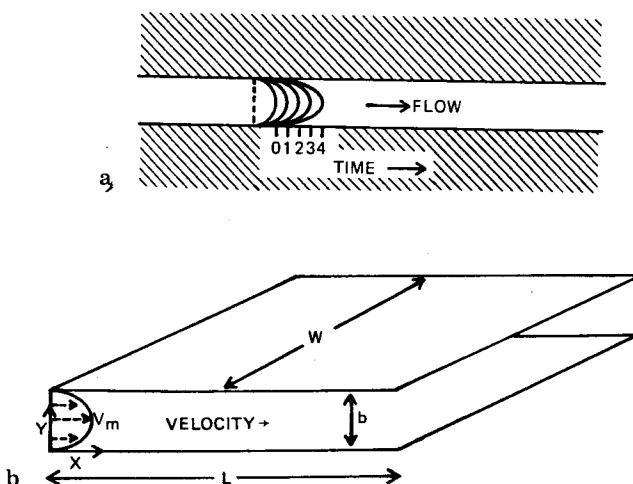


**Fig. 4a and b.** Plotting of diffusion-limited adsorption data (see Ref. <sup>3)</sup>). **a.** rate of adsorption,  $dn/dt$ , is proportional to  $t^{-1/2}$ ; **b.** the total amount adsorbed,  $n$ , in the initial stages is proportional to  $t^{1/2}$

Adsorbed molecules may diffuse laterally at the interface. Although surface diffusion is well-known in classical surface chemistry <sup>33)</sup>, data on adsorbed macromolecules is sparse. Burghardt and Axelrod <sup>34)</sup>; and Michaeli et al. <sup>35)</sup> have both demonstrated rapid interface diffusion of adsorbed albumin.

In many situations, the system is primed with a buffer solution which is displaced by the protein solution of interest (Fig. 5a). Assuming constant, laminar established flow, the velocity ( $V$ ) in a rectangular flow channel of width ( $w$ ), thickness ( $b$ ), and length ( $l$ ), where  $b \ll w$  has a characteristic parabolic profile, given by <sup>36)</sup>

$$V = 6\bar{V} \left( \frac{y}{b} - \left( \frac{y}{b} \right)^2 \right) \quad (3)$$



**Fig. 5a.** The development of the concentration profile due to a plug of protein solution entering a buffer-primed, thin plate flow channel. Note that a "bullet-shaped" concentration profile develops with time (assuming no diffusion); **b.** the geometry and coordinate system used in the convection-diffusion treatment

where  $\bar{V}$  is the average velocity and  $y$  is the  $y$  coordinate (see Fig. 5b).

The volumetric flow rate,  $Q = A\bar{V}$ , where  $A$  is the cross sectional area of the channel, i.e.,  $A = b \cdot w$ , therefore,

$$V = \frac{6Q}{b^2 w} y \left( 1 - \frac{y}{b} \right) \quad (4)$$

It can be shown that the wall shear rate,  $\gamma$ , is

$$\gamma = \frac{6Q}{b^2 w} \quad (5)$$

thus

$$V = \gamma y \left( 1 - \frac{y}{b} \right) \quad (6)$$

which is the equation for the parabola shown in Fig. 5.

Physically, the shear rate,  $\gamma$ , is the change in fluid velocity over a change in distance,  $dV/dy$ , or the velocity gradient. The units of  $\gamma$  are

$$\frac{\text{cm/s}}{\text{cm}} \text{ or } \text{s}^{-1}$$

The shear stress is also of interest. Shear stress ( $\tau$ ) for Newtonian fluids is linearly related to  $\gamma$ ,

$$\tau = \mu\gamma \quad (7)$$

where  $\mu$  is the viscosity.

Note that the velocity at the wall ( $y = 0$  and  $y = b$ ) is zero, meaning nothing moves at the wall, according to classical fluid mechanics.

Now imagine that a protein solution enters the flow chamber at time  $t = 0$ . The protein solution (of uniform concentration and flow velocity) begins displacing the buffer solution (Fig. 5a). Given a parabolic velocity profile, Fig. 5b shows the development of the concentration profile in the cell at various times after entrance. A "bullet-shaped" concentration profile develops. This is easily observed using a dye solution or blood. No protein reaches the surface by convective flow alone. Protein is transported to the interface by diffusion.

The flow system can be designed to produce mixing which minimizes concentration profiles. Such processes are discussed in any basic fluid mechanics or flow injection analysis text book <sup>36, 37)</sup>.

A complete treatment of mass transport to interfaces requires combining convective (flow) and diffusion processes <sup>36–38)</sup> as the molecules present in a flowing stream are transported by flow (convection) and by diffusion simultaneously. Fortunately, this mass transport problem is well-treated <sup>38)</sup>, particularly in the chemical engineering literature. Robertson <sup>39, 40)</sup> and Leonard <sup>41)</sup> are chemical engineers who have been

very productive in the area of protein adsorption — they and their students have presented and used convective diffusion treatments extensively.

For the flow direction and coordinate system given in Fig. 5b, and assuming diffusion perpendicular to the solid-liquid interface, the general convective diffusion equation in Cartesian coordinates is<sup>38–40)</sup>:

$$\frac{\partial C}{\partial t} + V(y) \frac{\partial C}{\partial x} = D \frac{\partial^2 C}{\partial y^2} \quad (8)$$

where

$$V(y) = \gamma y \left(1 - \frac{y}{b}\right) \quad (9)$$

C = concentration,

t = time,

D = diffusion coefficient.

The origin of the coordinate system is the entrance to the flow chamber (far left of Fig. 5b). Assuming there is no adsorption at the interface, the boundary conditions are<sup>40,42)</sup>:

- at  $t = 0$ ,  $C = 0$  for all  $y, x > 0$ ;
- at  $x = 0$ ,  $C = C_0$  for all  $y, t > 0$ ;
- at  $y = b/z$ ,  $C = 0$  for  $t < x/V_m$ ;
- $C = C_0$  for  $t \geq x/V_m$ ;
- at  $y = 0$ ,  $\partial C/\partial y = 0$  for all  $x > 0, t > 0$ .

The equation can be solved using various numerical methods.

As the protein solution moves into and through the rectangular flow channel, a parabolic concentration profile develops (Fig. 5a). Diffusion washes out the flow profile, eventually equalizing the concentration at  $C_0$ .

In the absence of diffusion, the injected material would never reach the wall due to the laminar nature of the flow. In the presence of diffusion, the concentration at the wall rises with time in an S-curve fashion. Lok generated a dimensionless equation which defines the time course of diffusion<sup>40)</sup>:

$$\tau = ty^{2/3} \frac{D^{1/3}}{l^{2/3}} \quad (10)$$

where  $l$  is the distance from the entrance to the midpoint of the flow chamber and where  $\tau = 3$  to reach a plateau value (i.e. the concentration at the wall is  $C_0$ ). Solving for  $t$ , we find that

$$t = \tau \frac{l^{2/3}}{\gamma^{2/3} D^{1/3}} \quad (11)$$

Using the typical values  $D = 4.0 \times 10^{-7}$  cm<sup>2</sup>/s for gamma globulin,  $l = 2.5$  cm,  $\gamma = 47.4$  s<sup>-1</sup> for a volume flow rate of 2.0 ml/min and  $\tau = 3$  to reach equilibrium,

the diffusion time is 57 seconds from the time the newly injected fluid enters the cell.

The study of adsorption or desorption kinetics in the first minute or so must take full account of convection-diffusion processes. Fortunately this has been well-modeled by Lok, et al.<sup>40)</sup> and by Watkins and Robertson<sup>39,42)</sup>.

In any adsorption situation, the adsorption process itself is involved in the boundary conditions, thus affecting the solution of the equation<sup>39–41)</sup>.

Limiting cases of the general convective-diffusion equation are often helpful. If the time dependence is ignored, i.e.,  $\partial C/\partial t = 0$  (for example, at low bulk protein concentration, at long times, and/or when the rate of adsorption is much greater than the transport to the surface), then we have

$$V(y) \frac{\partial C}{\partial x} = D \frac{\partial^2 C}{\partial y^2} \quad (12)$$

This simpler expression can be solved numerically with the appropriate boundary conditions. This is the so-called transport limited or Leveque case<sup>38,41)</sup>.

Another limiting case is when  $V = 0$  (no flow), then we get the common diffusion expression

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial y^2} \quad (13)$$

Techniques which allow one to monitor the boundary layer as a function of time, such as total internal reflection fluorescence (TIRF) spectroscopy<sup>4,43)</sup>, permit a quantitative evaluation of interfacial mass transport processes using, for example, fluorescently-tagged macromolecules which do not adsorb, such as fluorescein-labeled dextran<sup>40)</sup>.

Vroman<sup>44)</sup> has recently demonstrated the great importance of volume and concentration in limiting interfacial transport and thus in influencing the adsorption process (see also Sect. 4.6).

## 4 Protein Adsorption Principles

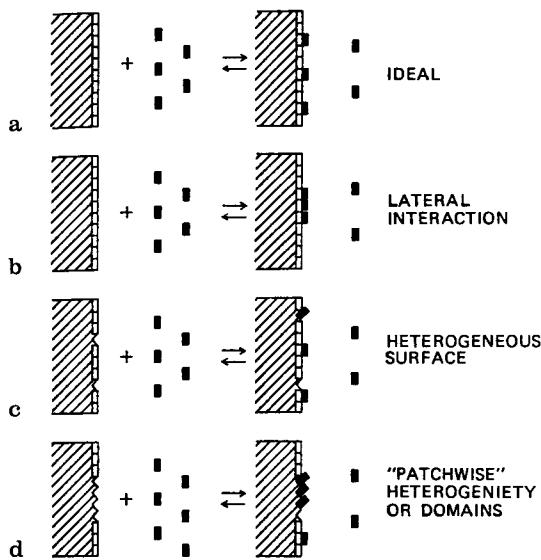
### 4.1 Background

The classical Langmuir theory for gas adsorption can be applied to adsorption from solution, if the solution is sufficiently dilute<sup>45,46)</sup>. The surface is considered to consist of sites of about the same area as the projected area of the solute of interest (Fig. 6).

Let  $\bar{v}_s$  be the number or moles of solute molecules adsorbed per area of surface. The subscript on  $\bar{v}$  refers to the case of adsorption on a *solid surface* as contrasted with  $\bar{v}$  used in the case of protein-ligand equilibria<sup>4,47–49)</sup>. Let  $[A]$  be the equilibrium solute concentration.

The classical Langmuir adsorption isotherm is then

$$\bar{v}_s = \frac{kA}{1 + kA} \quad (14)$$



**Fig. 6a-d.** Schematic view of adsorption from solution onto smooth, planar surfaces where the surface "sites" are considered to have the same area as the projected area of the solute of interest. **a.** Top, the ideal (Langmuir) case; **b.** clustering of adsorbed solute due to attractive lateral interactions or positive cooperativity; **c.** heterogeneous surface, i.e., two sets of binding sites; **d.** "patchwise heterogeneity" or surface domains of different adsorptive properties

which assumes:

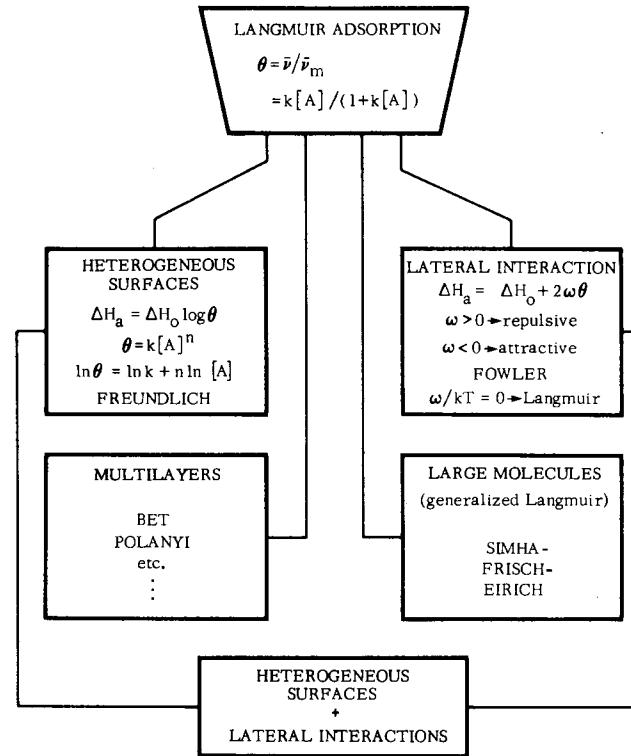
1. only one molecule can be adsorbed per site (commonly called the monolayer assumption);
2. only one type of site is present (homogeneous surface);
3. the adsorption of one molecule does not affect the adsorption energy of other molecules (no lateral interactions or cooperativity);
4. only one adsorbing species is present (no competitive adsorption);
5. dilute solution; and
6. reversible adsorption.

These assumptions are identical to those in the ideal theory of multiple equilibria used to analyze low molecular weight ligand binding to proteins<sup>4,47-49</sup>. Figure 7 shows how these assumptions can be considered in more refined treatments of adsorption from solution<sup>45</sup>.

The case of the heterogeneous surface (Fig. 6) is treated as

$$\bar{v}_s = \sum_i \left( \frac{k_i A}{1 + k_i A} \right) \quad (15)$$

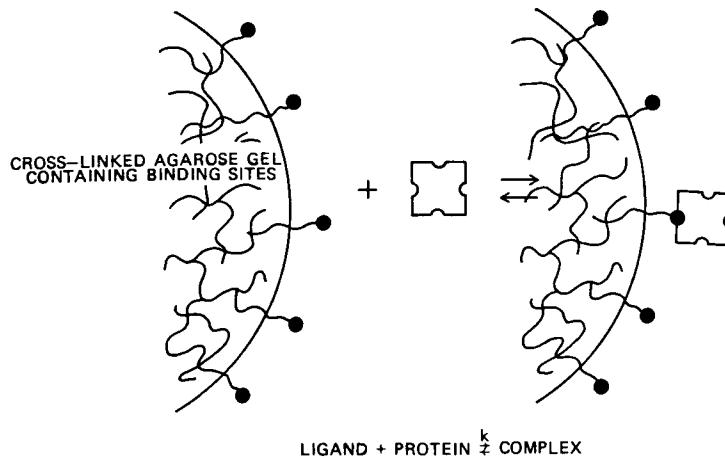
The case of patchwise heterogeneity versus "homogeneous" heterogeneity cannot be distinguished by adsorption data using low molecular weight solutes<sup>45</sup>. The case of lateral interactions can also be treated using assumed functions for the lateral interaction energy (Figs. 6 and 7)<sup>45</sup>.



**Fig. 7.** The relationships between the different models of adsorption. The various assumptions involved in the classical Langmuir treatment can be considered explicitly in other, more complex models (see Ref.<sup>45</sup> for further details)

The adsorption theories and protein-ligand equilibria theories are formally identical. If one simply treats the protein molecule as a heterogeneous surface (two or more classes of binding sites), interacting with identical low molecular weight ligands, the equations are identical. The assumptions involved are generally identical — for example, one ligand per site, which is the same as one adsorbed molecule/site or the monolayer assumption of Langmuir adsorption. Lateral interaction in the Langmuir treatment becomes cooperativity in the ligand equilibria treatment; heterogeneous surfaces are the same as two or more classes of binding sites; etc.

There is a class of adsorption or ligand equilibria data which is of particular importance and relevance to our discussion — the literature of protein chromatography. Proteins can be separated by ion exchange, hydrophobic, and charge-transfer processes on suitable chromatographic supports. Generally, a support is used which has very low interactions with protein ( $k$  is very low). A common one is cross-linked agarose. The support is then derivatized with the proper type and density of binding site (immobilized ligand). The ligand may be a carboxylic acid group, a quaternary ammonium group, a charge-transfer (electron donor or electron acceptor) group, or a hydrophobic group. We shall only consider the hydrophobic case as an example here. The underderivatized agarose is a high water content gel which consists mainly



**Fig. 8.** Schematic of a hypothetical protein interacting with a crosslinked agarose gel containing certain bonding sites. The bonding sites on the gel are considered to be immobilized ligands

of  $-OH$  groups, which are considered to have a negligible interaction with most proteins in water.

Consider Fig. 8 where the binding equilibrium is expressed diagrammatically. Clearly the binding groups on the agarose bead surface play the same role as ligand in a multiple equilibria treatment. Jennissen<sup>50</sup> has used this approach to study the interaction of protein with alkyl-substituted agaroses of different degrees of substitution (alkyl group densities or surface concentrations). His data<sup>51</sup> suggests a cooperative binding process, where about four butyl residues (binding sites) are involved in the adsorption. In the case of the methyl derivative of Sepharose<sup>1</sup>, 6 to 7 methyl groups are required for adsorption<sup>51</sup>.

The usual adsorption experiment consists of a fixed surface and a variable protein concentration, thus, the "ligand" concentration is constant and the protein concentration is varied. The expressions can be derived in terms of  $[P]$  rather than  $[A]$ , where  $[P]$  is the equilibrium protein concentration<sup>4</sup>:

$$\bar{v} = \frac{\text{moles bound protein}}{\text{total moles ligand}} = \frac{[PA]}{[A_T]} = \frac{[PA]}{[PA] + [A]} \quad (16)$$

For  $n$  identical, non-interacting sites

$$\bar{v} = \frac{nk[P]}{1 + k[P]} \quad (17)$$

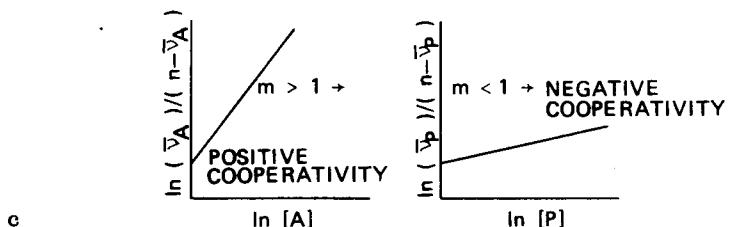
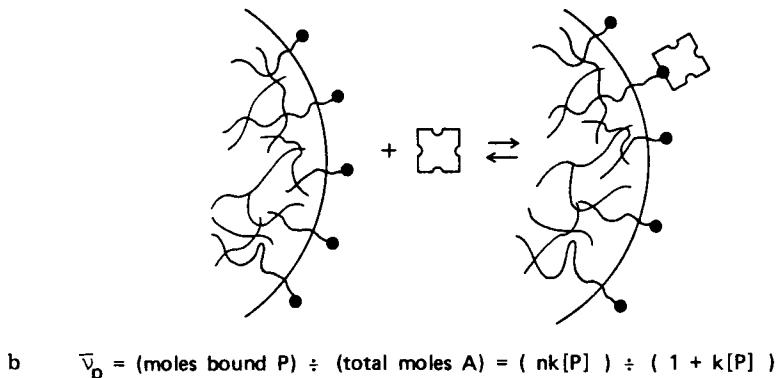
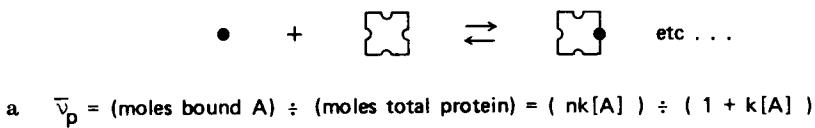
Due to steric considerations,  $n$  will generally be a lower number than in the free ligand case. For the adsorption of proteins on a solid surface, the total ligand concentration is usually represented as the actual surface area of the adsorbent. In studies utilizing

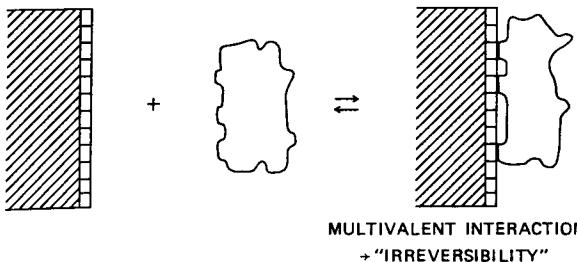
<sup>1</sup> Sepharose is the commercial cross-linked agarose produced by Pharmacia, Inc.

cross-linked gel chromatographic adsorbents, ligand concentration is often given as moles/ml packed gel. Units of  $[k]$  are reciprocal concentration units.

Jennissen's data<sup>50, 51</sup> shows that in terms of ligands bound (for example, butyl groups), the process is positively cooperative. Binding of protein to the first butyl group increases the probability of interaction with a second butyl group, etc. However, in terms of protein binding or protein adsorption, binding of one protein to the butyl groups decreases the probability that a second protein can interact, or the system exhibits negative cooperativity. Figure 9 summarizes these concepts in simple, diagrammatic form.

The adsorption or interaction of proteins with chromatographic supports discussed in the previous section made one overriding assumption: the underderivatized support does not significantly interact with proteins; i.e., the underderivatized agarose has such





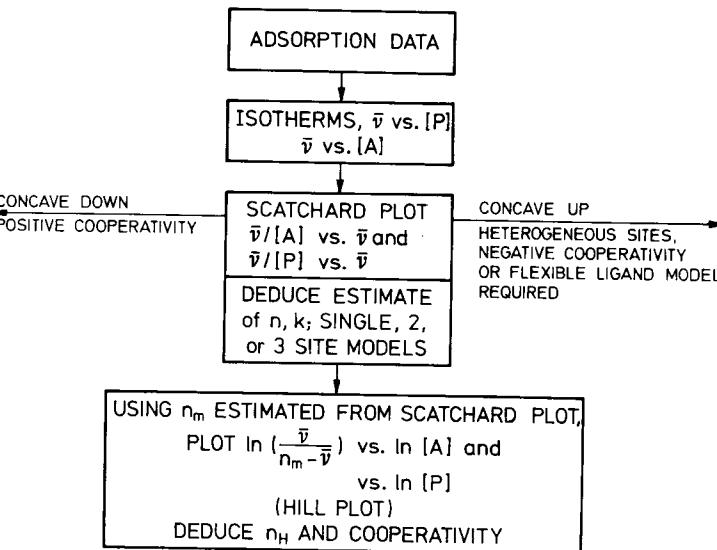
**Fig. 10.** Typical adsorbent surfaces can be considered to have very high binding site ("ligand") densities, resulting in multivalent interactions with adsorbed protein. If the multivalent interactions are of sufficient number and energy, the adsorptive interactions is "irreversible"

a low interaction energy per site with proteins that for all practical purposes that interaction is negligible. This is not the case with most other surfaces with which proteins come in contact. Using our earlier definition of surface site from Fig. 6, Fig. 10 schematically shows what happens when a typical surface of very high site density interacts with a protein. This is the case with hydrophobic surfaces such as, for example, polyethylene, polypropylene, polystyrene, and related biomedical polymers. One can consider every monomer unit exposed at the surface in such polymers to be a surface site interacting with the hydrophobic domains or patches on a protein by strong hydrophobic interactions. A large number of such interactions per individual protein molecule results in irreversible adsorption. This is, of course, also experienced in conventional protein adsorption chromatography, whether by hydrophobic or ionic (electrostatic) mechanisms. When the site density on the chromatographic support is too high, the protein is irreversibly bound. Sometimes this condition can be overcome by changing the elution medium. In the case of ion exchange chromatography, one would go to a higher ionic strength eluant, which would decrease the magnitude of the electrostatic interactions. Under these new ionic strength conditions, that protein would then be reversibly bound and could be eluted. The same is true of irreversible hydrophobic adsorption. If one elutes with an eluant which destroys or decreases the magnitude of the hydrophobic interaction, then what was an irreversible process is reversible in the new solution environment<sup>52)</sup>. Most studies of protein adsorption onto solid surfaces in the literature are under irreversible or at best semi-reversible conditions.

Ideally, protein adsorption experiments should be conducted on surfaces of low binding site density so that the interaction remains reversible and can be analyzed by multiple equilibria and related models. This will then allow the estimation of the number of interaction sites and information on binding constants and interaction energies. This is not only useful for the characterization of the surface, but for the characterization of the protein itself, i.e., the accessible regions on the protein surface which are involved in the adsorption process. There are cases and conditions in which proteins can be studied on hydrophobic surfaces in a reversible fashion — for example, proteins which appear to be very weakly hydrophobic or at the bottom of the scale in hydrophobic chromatography experiments<sup>52)</sup>. Albumin is highly hydrophobic and near the top of the hydrophobic chromatography scale and would be expected to adsorb irreversibly to most low energy surfaces. On highly hydrophilic surfaces,

such as clean, wettable quartz, it is known that some proteins adsorb in a highly reversible fashion, including albumin<sup>53)</sup>. Here, the hydrophobic interactions are probably minimal, and the major adsorptive interaction is probably ionic.

The most useful information on the interactions of proteins with surfaces will come from studies analogous to those of protein chromatography, where well-characterized and understood proteins are studied with well-characterized surfaces of known functional group type and density. The information obtained is then analyzable in such a way as to deduce interaction site densities and interaction energies. Only with such data in hand will we be able to begin to quantitatively treat and understand protein adsorption.



**Fig. 11.** Scheme for the analysis of protein adsorption data using the methods of multiple equilibria of proteins

Given the assumptions and limitations outlined in this section and elsewhere<sup>4)</sup>, conventional adsorption isotherm data can be analyzed in terms of multiple equilibria models. The general scheme is outlined in Fig. 11. First, the adsorption data is taken with sufficient care to get a wide range of points over a wide range of concentrations, preferably as both a function of protein solution concentration and immobilized ligand concentration, as outlined by Weber<sup>54, 55)</sup>. In many cases, the ligand surface concentration will be kept constant or will be largely unknown. The adsorption isotherm is then plotted. It is then most convenient to perform a Scatchard plot<sup>4)</sup>; if the plot is approximately linear, an estimate of  $n$  and  $k$  can be derived. If the plot has a strong concavity, then one can use the approximation techniques to derive a set of  $n$  and  $k$  for two different assumed site populations. The shape and concavity of the Scatchard plot immediately indicates several things. If it is concave-down, one can assume that this is due primarily to a positive cooperativity process. For example, the papers by Jennissen make such conclusions<sup>50, 51)</sup>. If it is concave-up, one can assume two or more populations, i.e., heterogeneous sites. One can also assume

negative cooperativity. The concavity may be due to the fact that one is using a flexible ligand, and a flexible ligand model is needed to analyze such data<sup>56</sup>. However, from the Scatchard plot or from the original isotherm, one can generally obtain an estimate of the upper value for  $n$ . Using this value for  $n$ , one can perform a Hill plot, i.e.  $\log \bar{v}/(n-\bar{v})$  versus  $\log [P]$ <sup>4,48,49</sup>. From the slope of the Hill plot one can deduce some measure of cooperativity.

For many proteins and many surfaces, the adsorption will be essentially irreversible, which will result in a Scatchard plot with a shape which is not easily analyzed. The models and methods presented here are not adequate for treating such data. It is important under such conditions to redo the adsorption experiment, preferably on a modified surface of lower binding site density or energy. Figure 12 outlines in schematic form what one might expect if this is done, although no such data are at present available in the literature.

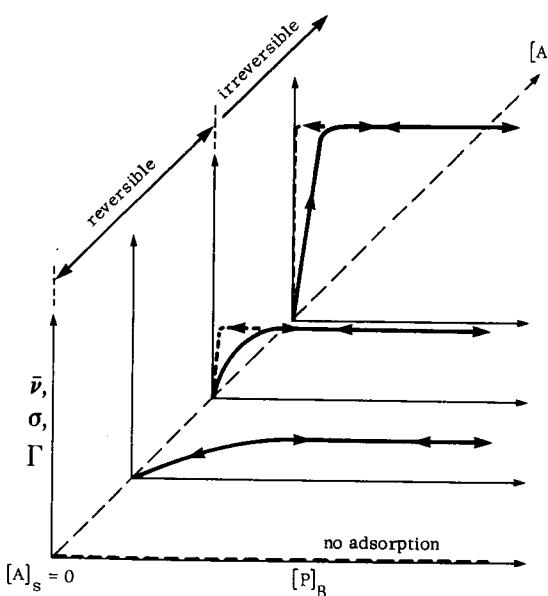


Fig. 12. Hypothetical 3-D plot of protein adsorption isotherm ( $\bar{v}$  plotted against  $[P]_B$ ) as a function of surface ligand concentration,  $[A]_s$ . Note that the system is reversible only up to a critical  $[A]_s$  and then behaves irreversibly for higher ligand surface concentrations. The right arrows ( $\rightarrow$ ) denote adsorption; the left-facing ones ( $\leftarrow$ ), desorption

Assume that we have a surface — for example, a substituted agarose analogous to the chromatography supports discussed earlier — in which the ligand surface density is allowed to vary. Further assume that each of these different supports of different ligand group density is studied with respect to different protein solution concentrations, and the amount adsorbed is determined. One then has two sets of isotherms, basically  $\bar{v}$  vs.  $[P]$ , as a function of ligand group density or  $\bar{v}$  vs.  $[A]$ , i.e., ligand group density or concentration, as a function of  $[P]$ . These can be plotted in a three-dimensional form as indicated in Fig. 11 and analyzed by the methods described herein. This has been described briefly by Weber<sup>55</sup>. Note from Fig. 12 that we are assuming

that as the ligand group density  $[A]$  increases, the adsorption isotherm becomes irreversible, which is no longer within the domain of analysis of the models and methods presented here. However, the adsorption behavior can be shifted to a reversible domain if the solution conditions are changed as discussed earlier.

There are some inherent limitations and assumptions in the multiple equilibria treatment. One is the fact that the treatment was devised primarily for ion interactions with proteins, that is small, spherically, symmetric rigid solutes. There are models available for non-rigid solutes<sup>55-59</sup>, which are more appropriate to fatty acid binding to proteins, particularly through the hydrophobic alkyl chains. Such models would be more appropriate to the study of protein interactions with highly mobile surfaces, such as those of elastomers and low glass transition materials, and probably even the substituted agaroses. Finally, both the rigid solute and the soft solute models<sup>55-59</sup> assume that the protein is conformationally rigid, although the rigid model has been treated in terms of allosteric phenomena or conformational coupling of the binding process<sup>60</sup>. In most cases of particular interest, the protein cannot be assumed to be conformationally rigid, and further methods, models and means of obtaining data will be required in order to interpret and understand such processes. The allosteric treatments of conventional rigid ligand-protein equilibria<sup>60</sup>, coupled with the treatments of mobile ligand-rigid protein equilibria<sup>56,57</sup> should allow such problems to be approached. The methodologies and discussion in this section, therefore, are but a first step in the analysis of protein interactions at solid-liquid interfaces.

## 4.2 Thermodynamics

What is the driving force for protein adsorption? Is the adsorption driven by overall energetic (enthalpic) interactions or does the entropic contribution prevail? Do both entropic and enthalpic contributions play a major part in the adsorption process, the extent of each depending on the particular protein and surface in question? An illuminating thermodynamic analysis given by Norde and Lyklema<sup>62,66</sup> for the adsorption of two different globular proteins (human serum albumin, HSA, and bovine pancreatic ribonuclease, RNase) on polystyrene latices will be presented. We believe this analysis has general validity.

Two parts of the adsorption isotherm can be distinguished: the initial part and the plateau region. At very low protein solution concentration, the adsorbed amounts are so low that lateral interaction between protein molecules can be neglected. This part of the isotherm (or its initial slope) reflect only the interaction between protein and adsorbent. In the case of HSA adsorption on negatively-charged polystyrene latices at pH = i.e.p., the adsorption was found to be independent of temperature, T, while at pH greater or less than the i.e.p., adsorption increases with increasing temperature<sup>68</sup>. The positive values of  $(\partial\Gamma/\partial T)$  suggest that the process is enthalpically unfavorable and is entropically driven. The more the pH differs from the i.e.p., the larger  $(\partial\Gamma/\partial T)$  becomes, implying that unfolding of HSA on the surface is a process which is not favored enthalpically.

At the plateau region direct calorimetric measurements of  $\Delta_{ads}H$  (adsorption enthalpy) showed that for a number of protein-surface combinations there is a part of the pH range where  $\Delta_{ads}H > 0$ . Since  $\Delta_{ads}G < 0$  for the process to occur,  $T\Delta_{ads}S$

> O. The positive sign of the entropy change indicates that there are conditions where the process of adsorption is entropically favorable, if not driven solely by positive  $\Delta_{ads}S$ . For rigid proteins like RNase, unfolding is opposed by strong intramolecular enthalpic forces. If the RNase molecule cannot gain entropy by adsorption or cannot find other enthalpy sources, it can not and does not adsorb. This was found in the case of interactions between positive-charged haematite and RNase; no adsorption took place due to electrostatic repulsion<sup>68)</sup>. Equal signs of the surface charges and the protein charge does not necessarily imply an absence of adsorption, however. It was found in a number of cases that negatively-charged HSA adsorbs on negatively-charged surfaces with its negative groups oriented toward the surface. This was due to counterions incorporated in the adsorbed protein layer between the surface and protein molecule providing an electrostatic bridging mechanism<sup>69)</sup>. It is an important part of the adsorption process since it contributes both to  $\Delta_{ads}H$  and  $\Delta_{ads}S$ .

Following are the contributions to enthalpy and entropy of protein adsorption that have to be considered, according to Norde and Lyklema<sup>61, 62)</sup>:

1. Hydrophobic dehydration results from bonding of the protein's hydrophobic patches to the hydrophobic regions on the adsorbent. The enthalpic part of this interaction is small; the entropy change is positive. Hydrophobic dehydration is relatively unimportant for hydrophilic surfaces and/or rigid hydrophilic proteins.
2. Overall electrostatic interactions depend on the surface charge and protein charge, both of which are usually functions of pH and solution ionic content. It is a more decisive parameter for rigid protein because of the ion-incorporation and possible conformational changes of flexible proteins.
3. Protein conformational changes contribute positively both to the enthalpy and entropy of adsorption. Such contributions are pH-dependent.
4. Ion incorporation has two important aspects: a) due to the transport of ions from solution to the adsorbed protein layer, contributions to enthalpy and entropy are usually negative; b) in the case of charge redistribution in the electrical double layer, the ion contribution to the enthalpic forces depends on the protein-surface charge difference, pH, and solution ionic content. Its entropic contribution is positive.
5. Overall, Van der Waals interactions are relatively insignificant due to the similar Hamaker constant of proteins and water.
6. Specific binding may also play an important role and its contributions to both enthalpy and entropy of the adsorption depend on the specific protein-surface combination.

Apparently, no single factor can be used to predict the process of adsorption; there are always several different properties of protein and adsorbent that determine the protein-surface interaction. As a summary, the following general guidelines can be given:

1. The affinity of a negatively-charged surface for a given protein increases if the surface is more hydrophobic or/and has smaller electrokinetic potential.
2. The affinity of protein towards a given negatively-charged surface increases the more hydrophobic the protein is and/or the smaller the amount of negatively-charged parts of protein occupy the inner region of the adsorbed protein layer.

3. Adsorption is enhanced by higher concentration and valency and smaller chaotropicity of the indifferent ions.
4. The adsorption process is often entropically driven with the gain in entropy arising from dehydration of the adsorbent surface and structural rearrangements inside the protein molecule (the state of hydration and field overlap changes inside the adsorbed protein included).

#### 4.3 Kinetics

The kinetics of single protein adsorption have been discussed and modeled by several groups<sup>70–73)</sup>. The models are summarized in Figs. 13a-f. These models assume the process is surface reaction limited, i.e. protein transport to the interface is not rate limiting. The nomenclature is:

- $C_0$ : bulk solution concentration [g/l]
- $C_s(t)$ : adsorbed surface concentration at time  $t$  [kg/m<sup>2</sup>]
- $(C_s)_\infty$ : plateau surface concentration at long  $t$  [kg/m<sup>2</sup>]
- $k_j$ : rate constants [min<sup>-1</sup> or 1/g min]
- $n_i$ : number of molecules in state  $i$  per unit area of surface
- $a_i$ : surface area fraction occupied by molecules in state  $i$
- $n_s$ : total number of adsorbed molecules per unit area of surface

Figure 13a shows basic Langmuir reversible adsorption, discussed earlier.

The Lundström model<sup>70)</sup> is given in Fig. 13b. He assumes that protein adsorbs with a rate constant  $k_a$  into State 1. Upon adsorption, some of the adsorbed proteins in State 1 (native) may conformationally change (via rate constant  $k_r$ ) to State 2 (denatured). Letting  $n_1$  and  $n_2$  be the number of molecules per unit area in States 1 and 2, and  $a_1$  and  $a_2$  be the area fractions occupied in each state, he says (noting that the unoccupied area fraction =  $(1 - a_1n_1 - a_2n_2)$ ) that<sup>70)</sup>:

$$\frac{dn_1}{dt} = (k_a C_0 - k_r n_1)(1 - a_1 n_1 - a_2 n_2) \quad (18)$$

The build-up of proteins in State 2 is given by

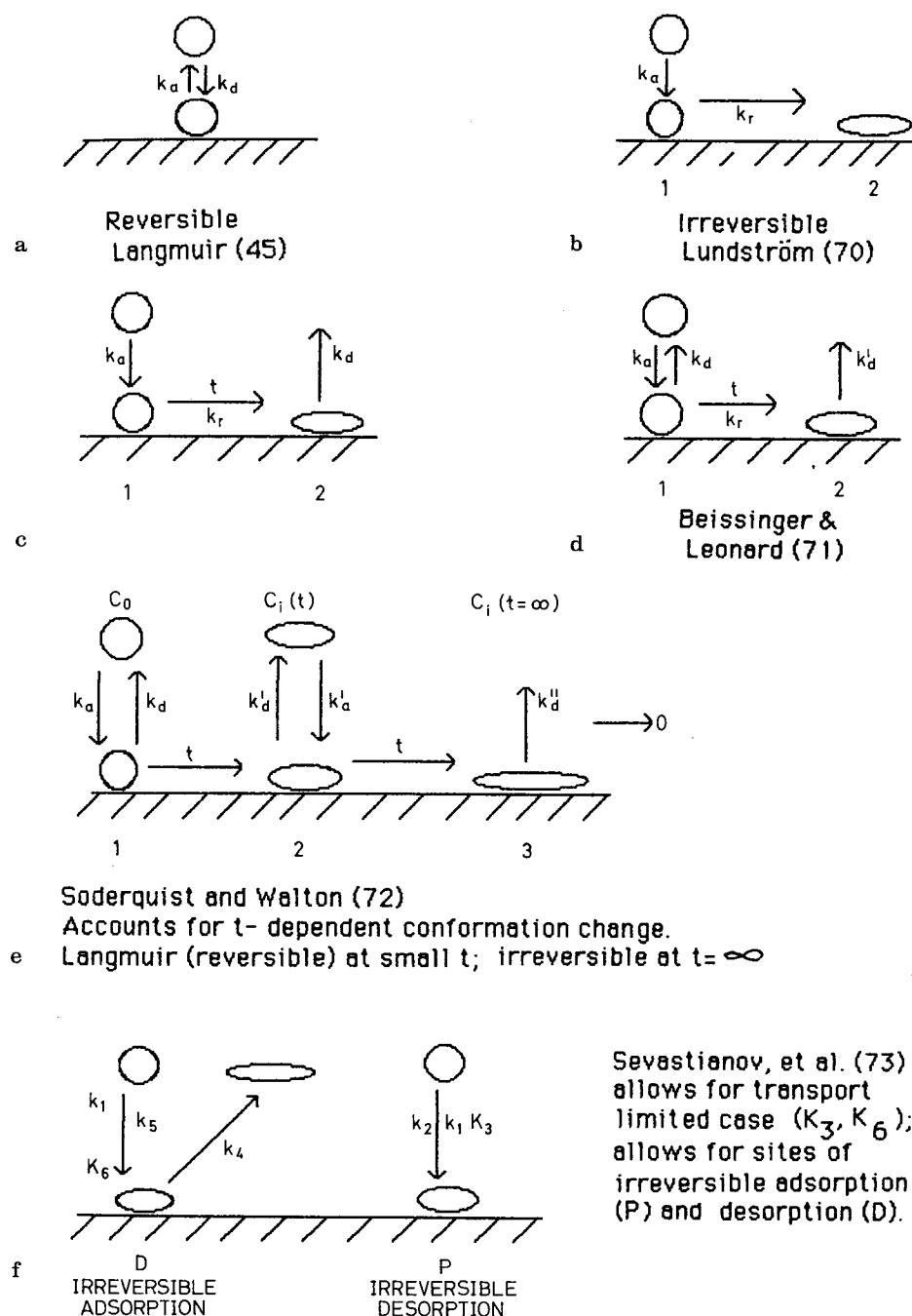
$$\frac{dn_2}{dt} = k_r n_1(1 - a_1 n_1 - a_2 n_2) \quad (19)$$

Lundström shows that<sup>70)</sup>:

$$n_2 = \frac{k_a C_0}{k_r} \ln \left( \frac{k_a C_0}{k_a C_0 - k_r n_1} \right) - n_1 \quad (20)$$

He goes on to determine relations for  $a_2/a_1$  at steady state (assuming monolayer coverage) and for  $n_1 + n_2$ , given experimentally by radioisotope or ellipsometry studies.

The Lundström model allowing for desorption is given in Fig. 13c, although it has not been treated in the literature.



**Fig. 13.** Most of the kinetic models which might be applicable to protein adsorption (see Refs. <sup>70-73</sup>);  $k$  is rate constant, subscript a and d are adsorption and desorption respectively, 1 and 2 are adsorption states — usually native and denatured

Beissinger's and Leonard's model (Fig. 13d) accounts for desorption of both native and denatured adsorbed species (States 1 and 2, respectively). They used the classical Langmuir-Hinshelwood model for catalytic reactions (the surface is the catalyst for conformational change or denaturation of the adsorbed protein), which assumes equilibrium at a steady state between adsorbed and solution molecules. They show:

$$\frac{da_1}{dt} = C_0 k_a (1 - a_1 - a_2) - a_1 k_r (1 - a_1 - a_2) - a_1 k_d \quad (21)$$

$$\frac{da_2}{dt} = a_1 k_r (1 - a_1 - a_2) - k'_d a_2 \quad (22)$$

$$n_s = n_1 + n_2 \quad (23)$$

The equations were best fit to albumin on quartz adsorption data by a nonlinear regression method to obtain values of  $k_a$ ,  $k_d$ ,  $k'_d$ ,  $k_r$ , and  $n_s$ . They then extended the model to consider the competitive adsorption of two proteins <sup>71</sup>.

Soderquist and Walton <sup>72</sup> added time dependence of the surface reaction to the model. They also allowed for readsorption of desorbed material (Fig. 13e). This model in principle takes into account the time dependence of the conformational change of adsorbed protein. They consider three distinct processes or states:

1. Rapid and reversible adsorption reaching a pseudo-equilibrium during the first minute;
2. Surface-induced change in conformation which optimizes protein-surface interaction and decreases the probability for desorption;
3. At  $t \rightarrow \infty$ , adsorbed material is fully denatured and if desorbed can not readsorb; desorption from State 3 is irreversible.

**Process 1:** Rate of adsorption,  $\frac{dn_1}{dt}$ , is proportional to solution concentration,  $C_0$ , but desorbed material readsorbs at a different rate.

$$R_{ads} = (k_a C_0(t_0) + k'_a C_1(t_1) + \dots) A = A \sum k_a^{(i)} C_i(t_i) \quad (24)$$

where  $i$  refers to solution species which had been on surface for  $t_i$ ;  $A$  is available surface area;  $k_a^{(i)}$  is the rate constant for species  $i$ .

**Process 2** is difficult to model.

**Process 3:** The desorption rate is much slower, particularly after long contact times. They assume that fully denatured protein does *not* desorb.

$$R_{des} = \sum k_d^{(i)} (C_s)_i (t_i) \quad (25)$$

where  $(C_s)_i$  is the adsorbed concentration which has resided for a period  $t_i$  on the

surface. If we consider material incubated for a definite time,  $t$ , and all desorbed material is continuously removed, then

$$R_{des} = k_d C_s(t) \quad (26)$$

which can be written in the form

$$R_{des} = k_d C_s(1 + k_r t^{n-1}) \quad (27)$$

where  $k_r$  is the rate constant for conformational change of the adsorbed protein.

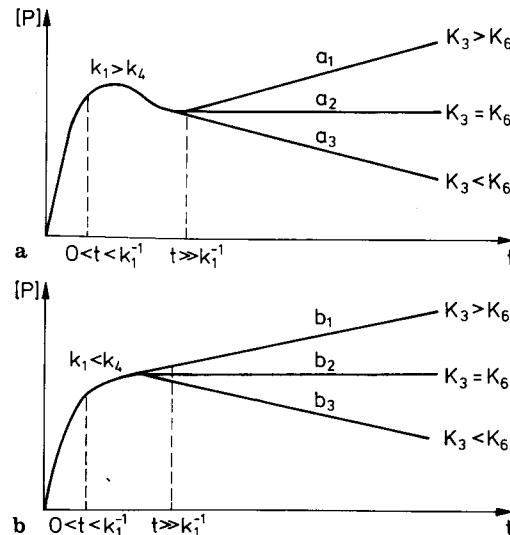
Combining (24) and (27) by noting that at steady state  $R_{ad} = R_{des}$ , they get

$$A \sum k_a^{(i)} C_i(t_i) = k_d C_s - (1 + k_r t^{n-1}) \quad (28)$$

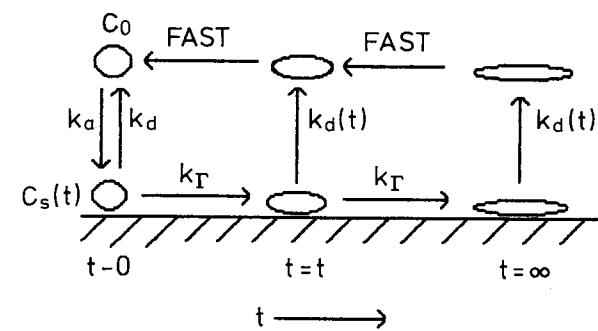
at  $t \rightarrow 0$ , this reduces to the Langmuir equation for reversible adsorption; as  $t \rightarrow \infty$ , the process becomes fully irreversible (see Fig. 19 which will be discussed later). The model reconciles essentially all of the physical evidence according to Soderquist and Walton<sup>72</sup>, although several of its assumptions will be questioned later.

Sebastianov et al.<sup>73, 74</sup> have developed a model which considers the effect of surface heterogeneity on the adsorption process. They define "centers of irreversible adsorption", labeled P, and "centers of irreversible desorption", labeled D. They argue, in agreement with Soderquist and Walton, that desorbed material is conformationally altered and thus cannot readsorb — hence desorption is irreversible. The results of this model are given as Fig. 14, taken from Ref.<sup>73</sup>. The model also includes the case where adsorption may be transport limited. The model fits commonly observed adsorption data, including the "overshoot" phenomenon (Fig. 14, top) (discussed in Ref.<sup>72</sup>) to be discussed later.

Although these models are ambitious, complex, and tend to fit experimental data



**Fig. 14a and b.** Results of the Sebastianov, et al., kinetic model for protein adsorption<sup>73, 74</sup>;  $k_1$  is an adsorption rate constant [ $s^{-1}$ ];  $k_2$ ,  $k_4$ ,  $k_5$  are "velocity" constants in [ $M/s$ ]; and  $K_3$  and  $K_6$  are transport constants in [ $kg/M^2 \cdot s$ ]. Curves  $a_1$ ,  $a_2$ ,  $a_3$  (top) are for  $k_1 > k_4$ ;  $C_1 > 0$ ,  $C_2 < 0$ ; curves  $b_1$ ,  $b_2$ ,  $b_3$  (bottom) are for  $k_1 < k_4$  and for  $C_1$ ,  $C_2 > 0$  (from Ref.<sup>73</sup>)



**Fig. 15.** Suggested general kinetic model for protein adsorption in the absence of any covalent bond formation or disruption. Any protein desorbed in a denatured state is assumed to rapidly renature in solution. If the surface is heterogeneous, then two or more such scenarios can be formulated, with appropriate account of the area fractions of each type of surface present

reasonably well, in many cases, some of the assumptions are not reasonable. Although proteins do indeed undergo time-dependent conformational changes on a surface, if those proteins desorb, they probably "renature" in solution, becoming indistinguishable from proteins in bulk solution. Protein denaturation is generally reversible unless covalent bonds have been formed or disrupted, which could happen if the surface is catalytically active or if proteolytic enzyme activity is present.

Therefore, a more general kinetic model is suggested in Fig. 15, based on the pioneering studies reviewed in Fig. 13. This is essentially a simplified form of the Soderquist and Walton<sup>72</sup> treatment, except we have assumed that any protein desorbed in a denatured state rapidly renatures in solution. This model could be combined with two or more classes of surface sites (heterogeneous surfaces) to treat more complex materials. To our knowledge, no modeling or data fitting to such a model is presently available.

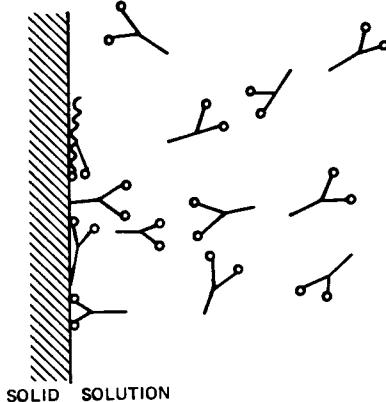
#### 4.4 Conformation Effects

We have already established that the protein adsorption process may result in significant conformational changes. In addition to adsorbed amounts and rates, the orientation and conformation of the adsorbed protein are critical (Fig. 16). Conformation refers to the secondary ( $\alpha$ -helix,  $\beta$ -sheet), tertiary, and quaternary structures.

Certain orientations may make a specific site on the protein inaccessible to ligand, substrate, or antigen. For example, consider the adsorptive immobilization of a specific IgG for a solid-phase immunoassay. The procedure will be optimal if the Fab domains are free to bind antigen (Figs. 1 and 16).

If the protein's structure is changed due to the adsorption process and/or the new local micro-environment, then it is said to be fully or partially "denatured", meaning that its properties are no longer those of the native protein. Conformational changes can occur due to ligand binding (for example, hemoglobin), substrate binding (many enzymes), heparin binding (antithrombin III), and surface binding (Hageman Factor, fibronectin, albumin, etc.). Although there have been many hypotheses relating to protein adsorption conformational effects, there is little direct data available.

## PROTEIN ADSORPTION



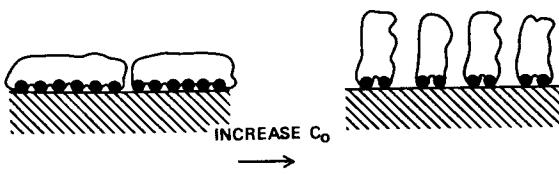
**Fig. 16.** The adsorption of an IgG molecule (shown for convenience as Y-shaped) may result in several orientations. The molecule may adsorb through one or both of the Fab ends or through the Fc tail — or lie on its "side". In addition to such orientation effects, the molecule may be conformationally altered or denatured

The surface itself may also be conformationally altered or "denatured", due to its interaction with the protein, particularly polymer surfaces which tend to relax or change in response to new environments<sup>30</sup>.

We have already established that proteins are highly dynamic structures, which are constantly "sampling" different conformations due to local thermodynamic fluctuations. Some such transient conformations can be stabilized in new micro-environments, such as at a solid-liquid interface.

Many studies of proteins at air-solution interfaces have indirectly established that the adsorbed proteins undergo detectable conformational changes. Similar studies at solid-liquid interfaces are few. We review here only several key studies.

Morrissey<sup>53</sup> used transmission infrared spectroscopy to study protein adsorption onto silica particles in a heavy water ( $D_2O$ ) buffer. By observing the shift in the amide I absorption band, he could deduce the fraction of protein carbonyl groups involved in bonding to the silica surface. He found that bovine IgG had a bound fraction of 0.20 at low bulk solution concentrations, but only about 0.02 at high solution concentrations. However, neither prothrombin nor bovine serum albumin exhibited a change in bound fraction with concentration. Parallel experiments with flat silica plates using ellipsometry showed that the IgG-adsorbed layers had an optical thickness of 140 Å and a surface concentration of 1.7 mg/m<sup>2</sup> at low bulk solution concentration — in concentrated solutions the surface amount was 3.4 mg/m<sup>2</sup> with a thickness of 320 Å (Fig. 17).



**Fig. 17.** At low solution concentration, the protein has no neighbors on the surface and thus can optimally adapt to the surface, maximizing the number of binding interactions. At high solution concentration, any one adsorbed protein is immediately surrounded by neighbors, minimizing the probability that it can conformationally adapt to the interface. This behavior leads to the differences in adsorbed amount and adsorbed protein thickness (determined by ellipsometry), as discussed in the text

Morrissey suggested that at low solution concentrations the adsorbed protein has sufficient time and "elbow room" to accommodate to its new microenvironment by conformational change resulting in significant hydrogen bonding to the silica surface. At high solution concentrations, the collision frequency with the surface is so high that an adsorbed protein has neither the time nor the room to optimize its interaction with the surface. This general interpretation is common in the air/solution field as well. Air/water studies show that the protein only needs to get a sufficient "foothold" on the surface to minimize the probability for desorption<sup>3</sup>. Once attached to the surface, the rest of the protein is "dragged" to the interface, thereby optimizing the interaction. The foothold need only be roughly 100–200 Å<sup>2</sup>, in contrast to the cross sectional areas of typical proteins, 1000–10000 Å<sup>2</sup>. Morrissey's high bulk concentration case may represent a minimum foothold.

Walton and his students have also pioneered the study of protein conformational changes, using fluorescence and circular dichroic spectroscopies<sup>72, 75, 76</sup>. Transmission CD spectra of Hageman Factor, adsorbed on a stack of quartz plates, suggested conformational changes upon adsorption. CD spectra of proteins eluted after different contact times with the surface show that the degree of conformational change is directly related to the contact time for periods of up to 10 days. It is now generally accepted that protein conformational change can be a rather slow process.

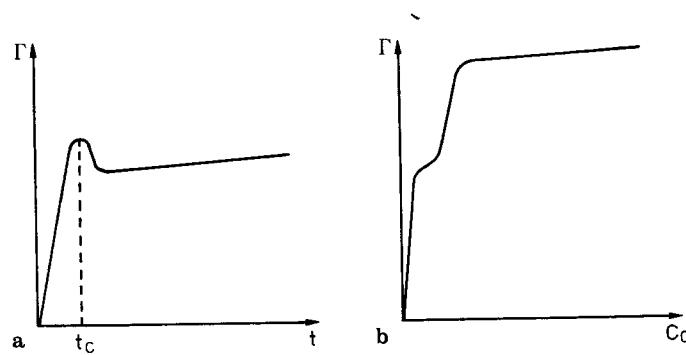
Brash has also used CD to study eluted proteins and finds large changes in  $\alpha$ -helix content of fibrinogen, perhaps due to enzymatic fragmentation produced by the surface-induced activation of plasminogen to plasmin<sup>77, 78</sup>.

Transmission fluorescence studies of adsorbed Hageman Factor show changes, which can be interpreted in terms of conformational and other micro-environmental effects<sup>75</sup>, although such studies must consider substrate effects on fluorescence<sup>76</sup>.

Adsorption may lead to an increase or decrease in titratable groups. Titration data can thus be interpreted in terms of conformational changes.

Soderquist and Walton<sup>72</sup> showed an "overshoot" in adsorbed amount as a function of time and a kink or inflection in the isotherm at about half saturation (Fig. 18, see also Fig. 14). They proposed:

1. Within the first minute of contact, adsorption is rapid and reversible — a pseudo-



**Fig. 18a.** A schematic of kinetic "overshoot" adsorption data, as discussed by Soderquist and Walton<sup>72</sup>, Van Dulm and Norde<sup>65</sup>, and others. See text for explanations. **b.** The "kink" at about half-saturation often observed in protein adsorption isotherms

- equilibrium is present. The protein is adsorbed in a random arrangement at coverages less than 50%.
2. At coverages greater than 50%, surface transitions and ordering may develop which lead to more efficient packing and an increase in adsorbed amount, hence the kink in the isotherm.
  3. Given sufficient time, adsorbed proteins undergo conformational changes which lead to increased surface interaction. During this process, proteins less optimally adsorbed undergo desorption, hence the overshoot in the time curve.
  4. The desorption rate decreases with increasing residence time.
  5. Desorbed protein may be permanently denatured, suggested by CD results.

Although this last point may not be general, the others are now accepted as rough "rules of thumb" for protein adsorption, though clearly each protein-surface-solution system is unique and may not exhibit all of the features noted.

Van Dulm and Norde<sup>65)</sup> (in a study of human plasma albumin on negatively-charged polystyrene latices) showed a fast initial adsorption followed by desorption, probably due to conformational change of the adsorbed albumin, which induces the release of less tightly bound protein. This result was observed at pH 4, where the albumin has a net positive charge and was not observed at pH 7.4, where it is highly negative.

The attenuated total reflection (ATR) Fourier transform infrared spectroscopic (FT-IR) studies of Gendreau, Jakobsen, and others<sup>79)</sup> have the potential for direct determination of conformational changes during the adsorption process due to shifts in the infrared absorption bands. Sakurai et al.<sup>80,81)</sup>, have used ATR-FTIR, as well as CD, to probe conformational changes upon adsorption.

Total internal reflection fluorescence (TIRF) spectroscopy has recently been applied by several groups<sup>4,39-42)</sup> and complete reviews are now available<sup>4,43)</sup>. The method can easily follow the kinetics of adsorption, using proteins labeled with extrinsic fluors, such as fluorescein or rhodamine. The intrinsic UV fluorescence of tryptophan (Trp) can be used to follow adsorption. The UV approach has the advantage that the tryptophan fluorescence is sensitive to the local micro-environment and no label is required. The major disadvantage of the UV method is the UV photochemical changes which occur, although such changes can be minimized by working at low light levels.

The intrinsic UV fluorescence of proteins is dominated by the tryptophan indole rings. The absorption maximum is 280–290 nm with the fluorescence maximum ranging from 315–355 nm, depending on the local environment of the indole side-chains. Quantum yields range from 0.04 to 0.50; 0.10 is a common value. As the local environment polarity or dielectric constant increases, the fluorescence maximum shifts up to 355 nm, such as for an indole ring in water or buffer. Trp moieties in highly hydrophobic environments fluoresce at 315–320 nm. Thus the fluorescence emission maximum (and the quantum yield) provide indirect information as to the local environment of the Trp fluors.

Although a number of proteins of interest (human serum albumin, for example) contain a single Trp, most contain two or more. Thus the spectrum observed is the sum of all active Trp fluors, making it difficult to deduce the local environment of each fluor. Nevertheless, the UV fluorescence emission spectrum is useful in deducing orientation and/or conformation changes upon adsorption.

Fibronectin (Fn) adsorption from 0.05 mg/ml solution showed very different adsorption kinetics on hydrophobic and hydrophilic surfaces. The data for static and flow adsorption and desorption have been reported<sup>82)</sup>. The quantity of interest here is the fluorescence maximum, which for Fn on hydrophilic silica is identical to that in bulk solution, suggesting no major conformational change upon adsorption. The Fn-silica surface interactions probably involve the charged groups on the surface of the molecule, most likely the highly positively-charged heparin binding regions of the molecule ( $pK = 8-9$ ).

Fn adsorbed on hydrophobic silica, however, fluoresces at 326, suggesting a slight denaturation of the molecule. Fn interactions with the hydrophobic surface may involve some of the apolar residues in the protein interior, suggesting a partial denaturation. Clearly, studies on surfaces of a range of charge, polarity, and apolar character would be of interest.

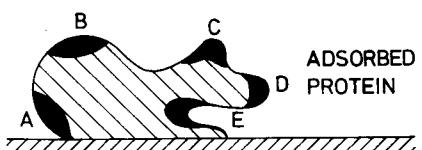
Bovine albumin has been studied to date via intrinsic TIRF only on hydrophilic quartz<sup>92,159)</sup>. The fluorescence maximum (1 mg/ml BSA in PBS) is 342 nm and shifts to 333 nm upon adsorption. These results suggest that the adsorption of BSA onto silica changes the conformation of the molecule such that the two Trp fluors are in a more hydrophobic environment.

Studies using radio-iodinated proteins in the TIRF apparatus permit a direct measurement of the amount adsorbed, thus allowing one to deduce if the quantum yield is also changed on adsorption<sup>84)</sup>.

Hydrophobic chromatography studies of proteins intramolecularly crosslinked to minimize unfolding suggest that "... the effective hydrophobicity of proteins is due in part to the extent to which buried hydrophobic residues are exposed by protein unfolding" (Ref.<sup>83</sup>, p. 97).

Both Vroman<sup>44)</sup> and Chuang<sup>85-87)</sup> have shown that specific antibodies can be used as probes of adsorbed protein orientation or conformation. Chuang showed that fibrinogen adsorbed on Cuprophane surfaces could be measured quantitatively, using a  $^{125}\text{I}$ -labeled antifibrinogen-IgG<sup>85</sup>, however fibrinogen adsorbed on polyvinyl chloride "... was not readily accessible for reaction with  $^{125}\text{I}$ -antifibrinogen-IgG."

Clearly specific antibodies, and particularly monoclonal antibodies, may be very useful in probing the properties of adsorbed proteins. Specific antibodies have been used to probe the structure of antigens in solution<sup>88)</sup>. Consider the adsorption of a simple protein with a small number of reasonably well-defined epitopes (surface sites with antibody binding activity), as in Fig. 19. Clearly epitopes E and A are not accessible for binding, while B, C, and D would be sterically accessible. One could also envision a conformational change upon adsorption which produces an epitope



**Fig. 19.** Schematic of a protein with five different antigenic sites (epitopes). Each epitope may have one or more specific monoclonal antibodies. A set of such antibodies can be used to probe which epitopes are accessible or not, allowing the investigator to deduce the orientation of the adsorbed protein

normally unavailable. Antibodies to such "hidden" epitopes may be generated if the protein is first denatured in solution to expose the normally hidden site.

It is important to note that antibodies are very large molecules (Fig. 1) and may be sterically unable to bind to an adsorbed protein, even if the proper epitopes are indeed exposed to the solution (Fig. 19).

Chuang and coworkers observed that a specific antibody to thrombin showed very low reactivity with prothrombin in solution but very high reactivity with adsorbed prothrombin, suggesting that adsorbed prothrombin exposes a new binding site equivalent to one of the thrombin epitopes<sup>87)</sup>. The reactivity of adsorbed prothrombin for the thrombin antibody was surface specific, suggesting that adsorbed prothrombin has different orientations and/or conformations on the surfaces examined. After a thorough study, Chuang concluded<sup>87)</sup>, "The data from this study appear to demonstrate that adsorption of prothrombin to artificial surfaces, such as PVC, had induced some conformational changes of the macromolecule that resembled antigenically the domain(s) of adsorbed thrombin . . . Monoclonal antibodies specific either to prothrombin or thrombin may be useful to pinpoint the exact domain(s) involved in such changes."

Chuang further cautions that "In solid phase immunoassay, it is generally assumed that antigens adsorbed to a surface, such as polystyrene microtiter dishes, will react with specific antibody in a manner similar to that antigen-antibody reaction in solutions such as occur in immune precipitation. However, our evidence and others<sup>89)</sup> seem to point out that data obtained from solid phase immunoassays should be interpreted with caution since adsorption of a nonantigen to a polymer surface could render it immunoreactive to previously unreactive antibodies."

Clearly a set of monoclonal antibodies may help elucidate the nature of adsorbed protein orientation and conformation. Such studies are in progress by several groups.

#### 4.5 Desorption, Exchange, Hysteresis

The adsorption of macromolecules is rarely an equilibrium process. Just as the properties of synthetic polymers are often dependent on non-equilibrium processes and relaxation phenomena<sup>30)</sup>, so do the properties of adsorbed proteins depend on time, metastable states, and hysteresis processes.

As the adsorption site density (Fig. 12) or total free energy of adsorption increases, one moves from the realm of "reversibility" to that of "irreversibility." As proteins can undergo conformational and orientational changes on a surface, they can optimize their interfacial interactions so as to provide the maximum free energy of adsorption. Such conformational alterations are relatively slow and hence very time-dependent.

Soderquist and Walton<sup>72)</sup> showed that the desorption rate is a function of residence time. The adsorption process can be characterized in three stages:

1. Short times where adsorption is reversible; presumably little or no time is available for conformational changes.
2. At longer times where slow conformational changes occur, the process is semi-reversible and desorption occurs very slowly.
3. At long times where the conformational change is completed, adsorption is now irreversible, and desorption is improbable.

Those proteins which do not undergo any significant conformational change at a particular interface may not show any significant time-dependence.

Soderquist and Walton proposed that the kinetic "overshoot" (Fig. 18a) and the kink at around half-saturation (Fig. 18b) often observed in protein adsorption studies may be due to time-dependent, surface-induced transitions. The conformational changes probably result in some unfolding and an increased number of protein sites contacting the surface. Conformationally changed protein requires greater surface area — those proteins further along in the conformational change process "consume" surface at the expense of their neighbors, which are less tightly adhered to the surface. Therefore, the amount of protein adsorbed can go through a maximum as noted in Fig. 18. Van Dulm and Norde have made similar observations<sup>65)</sup>.

The isotherm ("equilibrium") case follows a similar argument (Fig. 18b). At low  $C_0$ , the surface is not saturated, and the adsorbed proteins are randomly oriented on the surface. At greater than 50% coverage, lateral interactions among neighbors become important, possibly producing an ordering of the adsorbed proteins, which can be thought of as a surface phase transition. The more ordered adsorbed layer requires less area per molecule, hence the surface has "room" for more proteins to adsorb — thus the kink in the isotherm (Fig. 18b). Direct evidence of two-dimensional protein crystallization and ordering is now available for the case of antibodies deposited on haptenated phospholipid monolayers<sup>90)</sup>.

Soderquist and Walton's<sup>72)</sup> model of the protein adsorption process (discussed earlier, Fig. 13) reduces to the classical Langmuir (small-molecule) adsorption as  $t \rightarrow 0$ . As  $t \rightarrow \infty$ , their model predicts that adsorption is irreversible. A model which reduces to Langmuir adsorption at short contact time and to irreversible adsorption at very

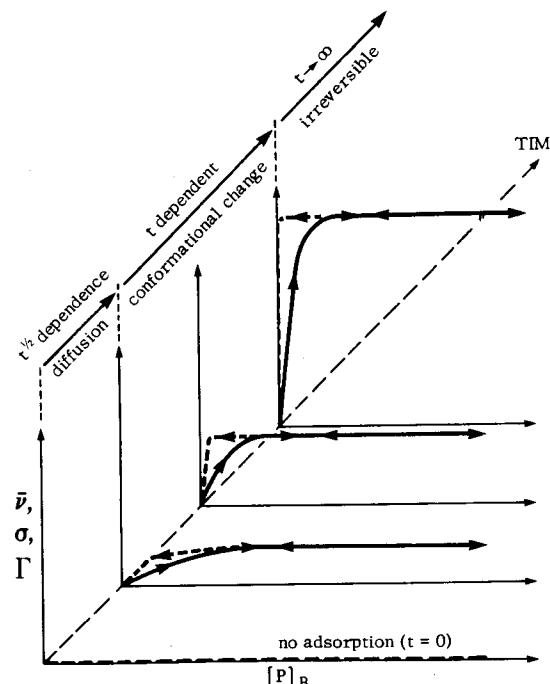


Fig. 20. Schematic adsorption "isotherms" with a constant surface site concentration ( $[A]_s$  in Fig. 12 is here constant), but with adsorption time as a variable. At very short times, adsorption is diffusion controlled. At short times, the protein has insufficient time to conformationally adjust to the interface, thus adsorption can be reversible and of the Langmuir type. At longer times, conformational adjustments begin leading to the commonly observed semi-ori-reversible behavior of protein adsorption. Other nomenclature same as Fig. 12

long contact times is certainly consistent with much of the data in the literature. Walton and Koltisko<sup>91</sup> have examined both protein stability and surface-induced, time-dependent conformational changes. Such treatments deserve to be more fully developed and investigated.

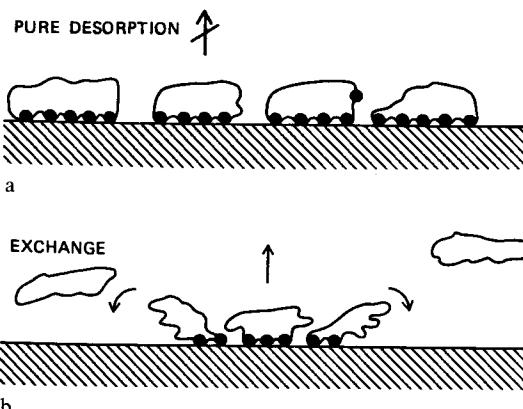
The tendency for an adsorbed protein to undergo conformational change is protein- and interface-specific, as well as time-dependent. Total internal reflection fluorescence (TIRF) studies on IgG adsorption and desorption on hydrophobic and hydrophilic surfaces as a function of residence time show clearly both the time-dependence, as well as the surface-dependence, of desorption<sup>92</sup>.

Figure 20 summarizes the situation in schematic form. Assuming a constant surface binding site concentration (fixed surface properties), we examine the amount bound as a function of time and bulk solution concentration. At very short times and particularly at low bulk concentrations, adsorption is diffusion-controlled and shows a  $t^{1/2}$  dependence. At longer times the sluggish conformational changes begin to become important. A significant hysteresis now begins to appear on the adsorption-desorption isotherm. At long contact times the conformational adjustments are complete, adsorption-free energy is maximized, and adsorption is irreversible, producing maximum hysteresis.

Clearly, proteins with a high concentration of binding sites on their surfaces may adsorb in the proper orientation, resulting in a multipoint attachment and high adsorption-free energy, even without any conformational adjustment. Thus, hysteresis can be present in some systems even at very short contact times. Proteins with very stable tertiary or quaternary structures may not show significant time-dependence due to the low probability for conformational change.

It is commonly observed that protein desorption can be very slow or even non-existent, but protein exchange can be rapid. This "anomaly" has been pointed out many times<sup>93, 94</sup>. Fortunately Jennissen's studies<sup>95-97</sup> and the arguments and work discussed in this chapter lead to a reasonable explanation.

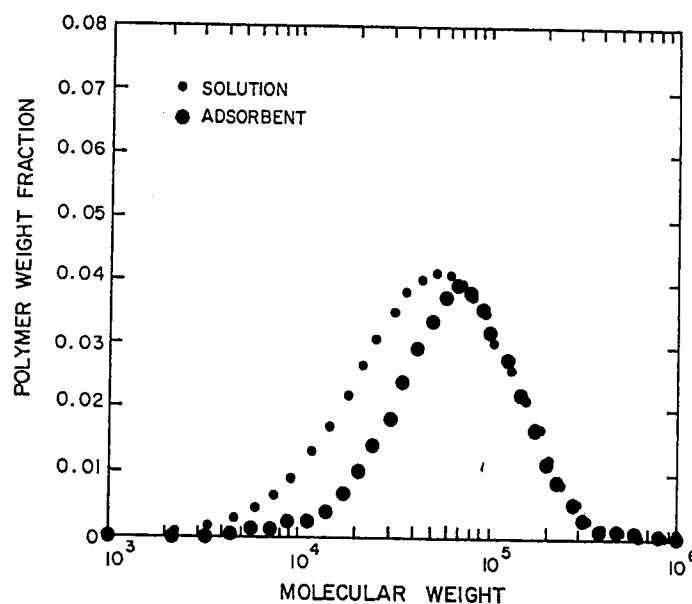
Consider the cartoon in Fig. 21. Imagine some adsorbed proteins with multiple



**Fig. 21a.** Cartoon showing the improbability of desorption for a protein adsorbed by 4 or 5 binding sites; **b.** exchange of adsorbed protein with protein in the bulk solution is probable, however, due to the fact that as one foot releases, a foot from a different protein may attach. See text for further discussion. Explanation due to Jennissen<sup>95-97</sup>

binding sites. Call each protein binding site a "foot". Statistically, a protein foot may lift off every now and then. Clearly, all feet must be lifted off in order for the protein to desorb. This is statistically improbable, thus desorption cannot occur. We say the protein is irreversibly bound. Exchange is a different matter. Now we have proteins present in the local environment, diffusing to and colliding with the surface. If one of these proteins should statistically place a foot on the space vacated by a "desorbing" foot of an adsorbed protein, then a "competition" is present. If the new protein then puts several feet down and becomes anchored, it may indeed induce the removal of the first protein. Therefore, one protein can, in essence, be lifted off by a number of proteins adsorbing and attempting to accommodate with the surface. This is a very schematic and simplistic explanation, but it appears to at least qualitatively explain the situation.

The adsorption of polydisperse synthetic polymer shows similar behavior in that the average molecular weight of the adsorbed polymer increases with time — high molecular weight material is more tightly bound (more "feet" attached), thus inducing desorption of the low molecular weight molecules (see Fig. 22).



**Fig. 22.** Molecular weight effect of synthetic polymer adsorption. Molecular weight (MW) distribution of poly(vinyl chloride) in solution and in the adsorbed layer at equilibrium. Note that the adsorbed material has a higher average MW than the bulk solution (from Ref.<sup>100</sup>, p. 120)

This is probably the basis of the complex protein exchange processes observed by Vroman and coworkers<sup>98</sup>. The more feet and energy per foot (total adsorption-free energy), the lower the probability that exchange can occur. Hence in a competitive adsorption process, one finds that those molecules present in the highest amounts and of smaller size bind first (due to diffusion and collision arguments) and are then exchanged by the more strongly adsorbing components. Vroman and Adams have shown<sup>98</sup> for plasma proteins adsorbing on glass and certain metal oxides that the

exchange hierarchy is: albumin < gamma globulin < fibrinogen < fibronectin < Factor XII (Hageman Factor) < high molecular weight kininogen (HMWK) (albumin adsorbs first).

#### 4.6 Competitive Adsorption — the “Vroman Effect”

The adsorption of protein from single component solutions is qualitatively understood, although a quantitative understanding and models or theories with predictive character are not yet available. If the structure and solution properties of the protein are known and if the solid-buffer interface properties are known, then by careful examination of the “surface” of the protein (ideally via molecular computer graphics), we can indeed predict what orientation of the protein is “preferred” on that particular surface.

Recently, considerable progress has been made on the calculation of electrostatic and hydrophobic interactions in biochemical systems<sup>189–191</sup>. We can expect such calculations to become common for protein-solid surface interactions. Thus we can expect approximate values for the adsorption energy in selected systems to appear in the near future. The problem of time-dependent conformational adaptation of the protein to the surface (and vice versa) will be much more difficult. Initially, we will have to resort to crude measures of the structural stability of a protein, such as the temperature at which thermal denaturation occurs, the urea molar concentration for solution denaturation, etc. One or more of the models given in Fig. 13 should apply.

But what happens if we have two or more proteins in solution? Clearly there will be a competition and the resultant surface concentration of the two proteins at some time, t, will be a complex function of protein, surface, and solvent properties.

The general problem involves, for example, three different proteins (A, B, C) in solution and interacting with a surface. The key variables are bulk solution concentration ( $C_A, C_B, C_C$ ), diffusion coefficients ( $D_A, D_B, D_C$ ), and the adsorption free energy, which is a function of time,  $\Delta G(t)$  for each protein. The free energy of adsorption at initial contact,  $\Delta G_0$ , is related to the final free energy state,  $\Delta G_\infty$ , through the “denaturability” of the protein. The denaturability is a function of the stability of the protein in solution, the interactions available at the solid surface, and the surface occupancy (fraction of sites occupied). The problem is to take these qualitative concepts and develop a quantitative model useful for predicting adsorption.

Although there have been many experimental studies of protein adsorption from binary and trinary mixtures, little or no modeling has been attempted.

Beissinger and Leonard<sup>71</sup> modeled the competitive adsorption of two proteins, albumin (A) and IgG (G), permitting each protein to occupy part of the surface in two different states, and also allowing for desorption of each protein from each of its two allowed states. The model uses 12 adjustable parameters, making it difficult to apply for predictive purposes.

Adsorption from plasma is considerably more complex, as literally hundreds of different proteins are present<sup>99</sup> — all competing for the surface.

Vroman has shown by antibody methods that plasma interactions with solid surfaces result in a hierachial adsorption process<sup>98</sup>. The high concentration proteins dominate the surface at short times due to the higher collision rates. As time passes

various exchange processes occur and proteins with higher surface affinities dominate the surface. Finally at very long times only the highest affinity proteins are present on the surface, even if their bulk solution concentration is very low.

This effect is well-known in synthetic polymer adsorption and results in low molecular weight species (fast diffusion) adsorbed initially, but the high molecular weight fractions are preferentially adsorbed at very long times (higher adsorption free energy) (see Fig. 22).

Adams, et al.<sup>101</sup> used a curved disc on a flat surface to study the effect of solution volume at constant surface area on competitive plasma protein adsorption. Although the experiment was qualitative, it elegantly demonstrated the importance of exchange, bulk solution concentration, and surface-volume ratio on competitive adsorption<sup>98</sup>.

Recently Horbett<sup>102</sup> and Brash and ten Hove<sup>103</sup> have quantitatively demonstrated the “Vroman effect” in a series of experiments studying competitive adsorption of fibrinogen, albumin, IgG, and hemoglobin from diluted plasma.

The adsorption values at 5 minutes onto glass and polyethylene as a function of plasma dilution are given in Fig. 23. Fibrinogen adsorption on glass is maximal at about 1.5% plasma. From 0 to 1.5% plasma, a “typical” protein adsorption isotherm is observed. On polyethylene, the result for fibrinogen is similar. Albumin adsorbs in high amounts on the hydrophobic polyethylene surface showing a “normal” concentration dependence. The kinetics of fibrinogen adsorption show clearly that fibrinogen does indeed adsorb at the high concentration, but is removed or exchanged within a minute or so — exactly what Vroman observed many years ago.

Other workers have observed concentration-dependent competitive adsorption, including Grinnell and Feld<sup>89</sup> (fibronectin), and Breemhaar et al.<sup>104</sup> (fibrinogen, IgG, albumin).

What are these high affinity plasma components which compete so effectively for certain surfaces? Vroman suggests high molecular weight kininogen (HMWK), based on studies with HMWK-deficient plasma<sup>98</sup>. Breemhaar et al.<sup>104</sup> suggest it could be a lipoprotein. Another explanation may lie in Brash’s observation<sup>77, 78</sup> that plasminogen can be activated by contact with glass to plasmin, which can then degrade adsorbed fibrinogen. Clearly there are many possibilities.

Clearly studies are needed which monitor the adsorbed protein population from plasma as a function of time. Only two methods come to mind:

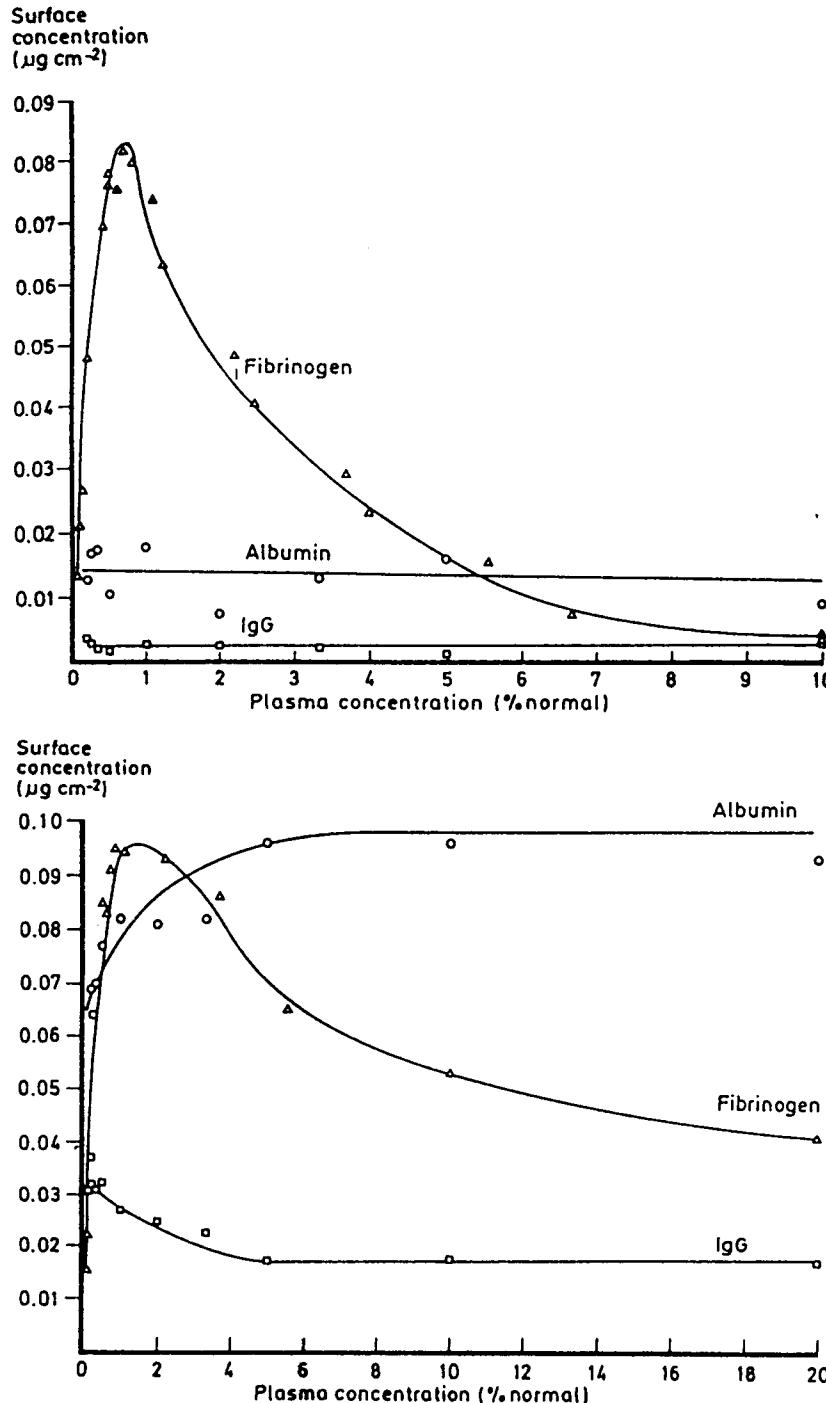
1. Elute all adsorbed material from a sample and then do an ultrasensitive electrophoretic separation;
2. Use a large set of specific labeled antibodies to specifically and quantitatively bind with the adsorbed proteins.

Both approaches are fraught with problems.

## 5 Adsorption Based Biocompatibility Hypotheses and Correlations

### 5.1 Surface Charge

The surface charge concept goes back to the original observations that blood clots more rapidly in a glass tube than in hydrophobic glass or plastic tubes. This difference



**Fig. 23.** Brash and ten Hove's results on the adsorption of three plasma proteins from diluted plasma as a function of total plasma concentration. Up: adsorption on glass showing a maximum adsorption of fibrinogen at about 1% plasma; Down: adsorption on polyethylene; plasma was diluted with isotonic Tris, pH 7.35. Adsorption time was 5 minutes (reprinted from Ref. <sup>103</sup>)

was attributed to the negative surface charge of glass and is still quoted in hematology textbooks as the mechanism for surface-induced activation of coagulation. With the discovery of Hageman Factor and the intrinsic contact activation mechanism of coagulation, the negative surface charge theory was given some credibility. It was shown that Hageman Factor could indeed bind to negatively-charged surfaces: quartz, glass, and various silicate minerals. The counter theory argued that negatively-charged surfaces were more blood compatible because heparin, a common anti-coagulant, was highly negatively charged. Indeed, early studies by Lovelock and Porterfield <sup>105</sup> on the sulfonation of polystyrene to produce sulfonic acid groups analogous to those on heparin showed that such surface increased static blood coagulation times.

Sawyer's pioneering studies to measure the surface potential of the vascular surface by electrokinetic methods demonstrated that the vascular surface was negatively charged <sup>106</sup>. The mechanism suggested is that plasma proteins are generally negatively charged and therefore are repelled from a negatively-charged surface. This simplistic approach was very satisfying and dominated the blood compatibility field for several decades, despite the contradiction that Hageman Factor was known to be activated by negatively-charged surfaces.

It is now known that negatively-charged proteins are not repelled from negatively charged surfaces for a variety of reasons. One is that although a protein may exhibit a negative charge it may have localized regions or domains where negative charge is not present, where positive charge may be present, or where no charge is present. We demonstrated earlier in this chapter that proteins can adsorb to surfaces by a variety of interaction mechanisms. It is important to note that under normal physiologic conditions, the negative charge is screened by counterions beyond about 10 Å. By the time a protein is within 10 Å of the surface, specific intermolecular interactions are already dominant and gross electrostatic repulsion is not a significant effect.

Clearly, proteins can adhere to surfaces by electrostatic mechanisms, particularly at low ionic strength where the electrostatic field of the surface and the protein is much more extended. Indeed, this is the basis of ion exchange chromatography, so widely used for the separation, purification, and characterization of proteins. However, by the time one reaches the 0.15 M salt concentration of the physiologic environment, general electrostatic processes are no longer dominant.

## 5.2 Interfacial Energetics

With the wide availability of synthetic plastics in the late 50's and early 60's, there was considerable interest in relating the surface properties of plastics to their blood interactions. The only method of measuring a surface property was via wettability, i.e., contact angle measurements. Zisman and coworkers developed the critical surface tension concept <sup>107</sup>, which permitted researchers to obtain empirical measures of the surface energy of polymeric materials. This approach was widely applied by Baier and eventually led to the hypothesis that surfaces with a critical surface tension in the range of 20–25 dyne/cm have optimal blood compatibility <sup>108</sup>. Such surfaces do indeed adsorb proteins. Baier argues that the critical surface tension of passive adsorbed protein films, and indeed of the vascular intima itself, is in the range of

20–25 dynes/cm. No satisfying mechanism has been proposed for this correlation. One suggestion is that the apolar component of the surface tension of water is 22 dynes/cm, within the range postulated by Baier.

An important hypothesis related to interface energetics is that of Nyilas, who said that the free energy of adsorption basically drives conformational change<sup>132</sup>. He probed this approach by measuring the enthalpy of adsorption using micro calorimetry and attempted to relate surfaces with low heats of protein adsorption with increased blood compatibility.

Shortly after the development of the critical surface tension concept Fowkes<sup>109</sup> and Girifalco and Good<sup>110</sup> developed means to estimate the surface-free energy and the surface-free energy components of solids by judicious application of probe liquids for contact angle studies and by intermolecular interaction approximations. These developments led Lyman and others to attempt to correlate the surface-free energy of polymers with their blood compatibility<sup>111</sup>. Lyman argued that as the surface-free energy increases, it increases the probability for protein binding and activation, such as by the Hageman Factor mechanism, and thereby decreases blood compatibility.

Lyman's ideas were extended by Andrade who argued that the governing parameter is not the surface-free energy of the polymer, but the interfacial-free energy of the polymer-water interface<sup>112</sup>. He argued that as the solid side of the interface begins to look more and more like water in energetic terms, there is a decreased driving force for protein adsorption and denaturation. This approach provided a semi-quantitative rationale for the rapidly developing interest in hydrogels as blood compatible surfaces.

With the development of neutral hydrophilic methacrylates in Prague, originally for contact lens applications in the early 60's<sup>113</sup>, considerable interest was generated in the application of these materials in the cardiovascular environment. The qualitative argument was that such soft, water-rich surfaces must be relatively non-traumatic to proteins and cells. The development of neutral hydrophilic polysaccharide-based particles for protein chromatography in the late 60's provided evidence that such surfaces do indeed show minimal binding of proteins.

Basically, the interfacial-free energy hypothesis said that as the interfacial-free energy goes to zero, the driving force for protein adsorption goes to zero, and adsorption cannot occur<sup>112</sup>. If adsorption cannot occur, no mechanism is present by which to activate coagulation, and therefore the surface is blood compatible. Unfortunately, the contact angle methods and approximations for deducing interfacial free energy could not discriminate between materials ranging from about 40% water, such as poly(hydroxyethyl methacrylate), to over 95% water, such as the lightly cross-linked agaroses<sup>114</sup>. Thus the hypothesis could not be rigorously tested. Also, the highly hydrophilic surfaces required were not always stable or longlived in the cardiovascular environment.

### 5.3 Protein Passivation

In the mid to late 60's, considerable interest in the pre-adsorption of proteins evolved. The basic idea was that if one could saturate the surface with a layer of proteins, then the surface would not be available for the binding and activation of Hageman Factor

or other contact activation proteins. Basically, albumin-treated surfaces were resistant to platelet adhesion, while other proteins — especially fibrinogen — promoted adhesion. This work has been well reviewed recently<sup>115–117, 192</sup>.

The result was that albumin passivation came into vogue for sometime. In fact, artificial kidneys and blood oxygenators were often treated with albumin solutions prior to clinical use<sup>118, 119</sup>. There is considerable evidence that such pre-treatment did indeed result in decreased platelet adhesion and activation for short periods, perhaps up to several hours, but that the effect was relatively short-lived.

Matsuda et al. have argued<sup>127</sup> that practical blood compatible surfaces, such as the polyether urethanes, function by tightly adsorbing a layer of protein, which is highly denatured but well adhered to the surface. A second protein layer deposits on the first, also well adhered and partially denatured. The process continues as in Fig. 24 until an adsorbed protein film, perhaps a micron thick, eventually develops. Inner layers of the film are very tightly adhered and stable. The outer reaches of the film are basically in reversible equilibrium with circulating proteins in the blood. The problem occurs when such a film de-laminates or de-adheres from the surface thereby exposing bare surface, which can then, depending on the competitive adsorption and cellular processes present, activate coagulation and related processes. This model is reminiscent of that developed by Moacanin and Kaelble in which they argue that optimum blood compatibility is related to strong bio-adhesion events<sup>128</sup>.

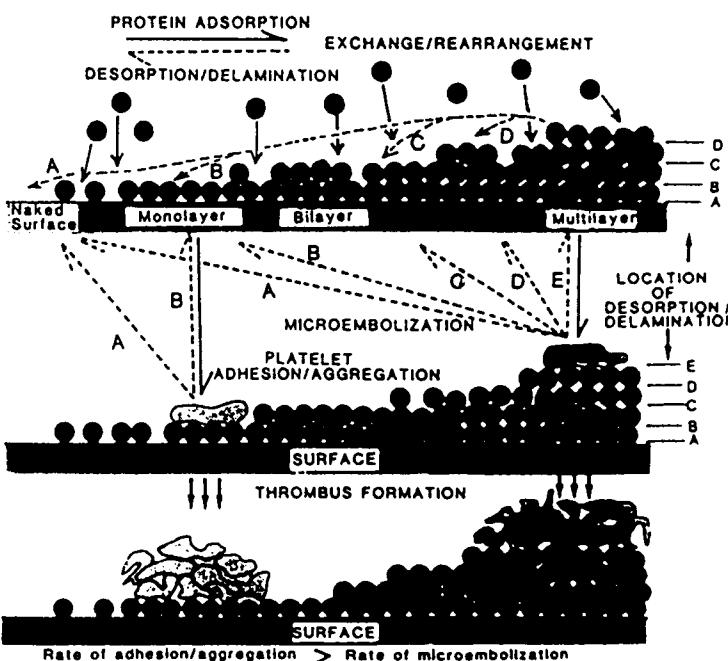
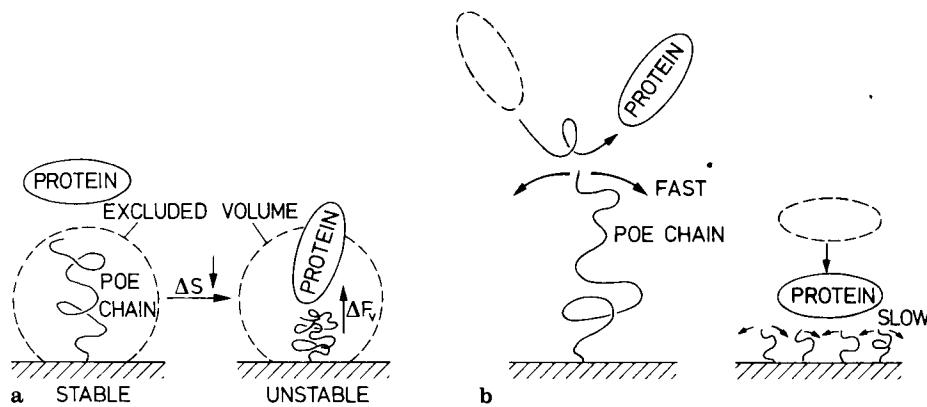


Fig. 24. Matsuda, et al.'s model of the protein adsorption/denaturation/aggregation/desorption/delamination process involved in the blood interactions of materials (from Ref. <sup>127</sup>, p. 357)

## 5.4 Protein Resistant or Repulsive Surfaces

A current hypothesis, which is receiving considerable attention, is that one can indeed produce a surface which actively repels proteins and other macromolecules<sup>123, 124, 133</sup>. The basic idea is presented in Fig. 25, which shows that a neutral hydrophilic polymer, which exhibits considerable mobility or dynamics in the aqueous phase, can actively repel macromolecules from the interface by steric exclusion and interface entropy methods. This method has been well-known and applied in the field of colloid stability for many years<sup>120</sup>. The most effective polymer appears to be polyethylene oxide, probably because of its very high chain mobility and only modest hydrogen bonding tendencies<sup>121–123</sup>.



**Fig. 25a and b.** A protein resistant surface based on the steric repulsion argument commonly used in the colloid stability field<sup>120</sup>. The interaction between a polyethylene oxide grafted surface and a protein solution is shown. **a.** suggests an excluded volume or steric repulsion mechanism; **b.** the surface dynamics or polymer chain motion mechanism (from Ref.<sup>133</sup>)

Merrill and Salzman have developed PEO soft segment polyurethanes and indeed have demonstrated minimal adsorption of blood proteins and minimal platelet adhesion on such surfaces<sup>121</sup>. Nagaoka et al. have studied various methacrylate copolymers with PEO side-chains of varying lengths and showed a direct correlation between minimization of platelet adhesion with increased PEO side-chain length and surface mobility<sup>123, 133</sup>.

Gregonis et al. have shown that PEO bound to quartz surfaces greatly minimizes the adsorption of proteins from plasma and from singly component protein solutions<sup>122</sup>. Bell and coworkers at Los Alamos have developed a theory of cell-cell adhesion based on the steric repulsion characteristics of the hydrophilic macromolecules present on cell surfaces<sup>124</sup>.

## 5.5 Immunoglobulins and Complement

Within the last ten years or so there has been considerable interest in the activation of the Complement system by surface-induced processes. Immunoglobulin of the IgG or IgM class, adsorbed at a surface with the right orientation and spatial ordering

and distribution, can activate Complement<sup>125</sup>. Certain hemodialyzer membranes, particularly of the cellulose type, are known to activate Complement via the alternate pathway, resulting in a transient, white cell depletion in the early stages of hemodialysis<sup>125</sup>. Sevastianov and Tseytlin recently reported the Complement activation properties of a variety of biomedical polymers<sup>126</sup>. There is therefore considerable interest on the adsorption of Complement components Cl and C3, as well as IgG, on surfaces, not only on cardiovascular materials, but also on intraocular lens implants, and at other sites of demonstrated Complement activity.

## 5.6 Other

Hoffman<sup>129</sup> and Baier<sup>130</sup> have reviewed most of the hypotheses and mechanisms suggested for blood compatibility in general and for the role of protein adsorption in particular. The safest statement one can make is that protein adsorption is indeed important in the blood compatibility process, in the compatibility of soft contact lenses, in the stability and acceptance of intraocular lenses, in the soft tissue foreign body reaction<sup>131</sup>, and in virtually all situations where solid surfaces come into contact with physiologic environments.

It is also safe to say that, because of the great complexity of proteins, even of the simplest, most well-characterized proteins, such as insulin, lysozyme, and myoglobin, and because of the very wide range of proteins present in most physiologic environments, very simplistic hypotheses and mechanisms are generally not very applicable.

## 6 New Methods

### 6.1 Background

Techniques and methods for the study of protein adsorption have been well reviewed<sup>4</sup>. It is now generally recognized that it is not necessarily the type and amount of protein present at the surface which is most important, but rather the orientation and conformational state of those proteins. At present it is virtually impossible to predict the specific conformation of an adsorbed protein at a particular interface. The techniques used in the determination of protein conformation in solution or in the solid state do not usually apply to adsorbed proteins. Hence, the difference between adsorbed and bulk solution protein conformation has to be inferred indirectly.

Protein adsorption studies are performed either on high surface area material dispersed in a liquid phase containing dissolved protein, or on low surface area material, often flat, which is in contact with protein solution. Both approaches complement each other and can provide valuable information about adsorbed protein layers.

In the case of adsorbents with high surface area, changes in protein bulk solution concentration before and after adsorption are usually large enough for independent determination of the amount of protein adsorbed, either via solution depletion meas-

urements or directly at the surface after the solid phase has been separated from the solution phase. Such studies are usually performed using various colorimetric methods of quantitative protein analysis or by measuring the radioactivity of proteins labelled with  $^{125}\text{I}$ ,  $^{14}\text{C}$ , or  $^3\text{H}$ . In some cases, labelling *per se* cause changes in protein adsorbability to a certain surface<sup>134)</sup>, producing different surface concentrations of labeled and nonlabeled protein and erroneous results. Similar problems apply also to protein labeled with fluorophores, such as fluorescein, rhodamine-B, and other dyes.

Due to the importance of the blood coagulation process, the adsorption of protein is often studied in buffer solutions at conditions similar to "physiological" (pH = 7.4, 0.15 M NaCl, 37 °C). Analysis of the adsorption process starts with the protein surface concentration presented as a function of equilibrium protein solution concentration, i.e., the adsorption isotherm. Most protein adsorption isotherms display a well-defined plateau in the dilute concentration range ( $C_b < 1 \text{ mg ml}^{-1}$ ). The adsorbed amount is usually up to the equivalent of a close packed monolayer, indicating an absence of multilayer adsorption. The adsorption isotherm is often claimed to be a "Langmuirian type", although the premises of Langmuir adsorption are rarely fulfilled, almost never investigated (with the exception of dilution effects), nor experimentally confirmed in the protein adsorption. The adsorbed amount usually changes only slightly with dilution, indicating apparent irreversibility<sup>135)</sup>. However, it has been shown that proteins on the surface can exchange both with the proteins from the solution<sup>136)</sup>, as well as laterally with the neighboring protein molecules via surface diffusion<sup>137)</sup>. The shape of the adsorption isotherm depends on experimental parameters; it can provide crude information about protein-adsorbent interaction. The initial isotherm slope reflects the affinity of protein towards the surface; the (apparent) adsorption isotherm plateau values can be related to the molecular dimensions of the adsorbed protein molecule. In some cases, the adsorption isotherms were found to display kinks which are thought to reveal distinct steps of interfacial protein rearrangement as the concentration of protein in the solution increases<sup>138-140)</sup>. All of these effects were discussed earlier (Sect. 4).

In most cases, the adsorbing surface is tacitly assumed to be completely inert and nonresponsive to protein attachment. This assumption may be valid for surfaces of crystalline material but in the case of "hairy" polymer surfaces, changes in the polymer surface conformation due to the presence of adsorbed protein may be expected.

The major advantage of protein adsorption studies on high surface area materials is that changes of some extensive properties which accompany the process of adsorption are large enough to be directly measured: heat of adsorption through microcalorimetry<sup>141)</sup>, uptake or release of small ions by a combination of electrokinetic methods and titration<sup>142)</sup>, thickness of adsorbed layer or an increase of the volume fraction of solid phase by a hydrodynamic method like viscometry<sup>143)</sup>. Chromatographic-like analysis can also be applied to protein adsorption<sup>144)</sup>.

For many areas of interest the most valuable information describing the interactions between a protein and a surface is conformational change of the adsorbing protein molecule as it passes from solution to the interface. Low surface area samples in combination with some form of spectroscopic method are generally used in the evaluation of protein conformation. Recent advances in this area warrant a more detailed description of the experimental approaches.

## 6.2 Spectroscopy by Evanescent Surface Waves<sup>92, 154, 155)</sup>

As a rule these methods are based on the concept of evanescent surface waves caused by total internal reflection at a solid/liquid interface. In order to understand the so-called "evanescent" spectroscopy, a simplified theory of total internal reflection is given below. More complete treatments can be found in various specialized monographs and reviews<sup>145-147)</sup>.

An electromagnetic wave reflection at an interface forms a standing wave, due to superposition of the incident and reflected waves. This also occurs in the case of total internal reflection, where the standing wave forms in the optically more dense medium (medium 1). The nature of the standing wave is a function of both the optically more dense medium and the less optically dense medium (medium 2). In the familiar case of metallic reflection, the standing wave is aligned with its node at the reflecting metallic surface. In the case of the interface between two dielectric media, the electric field amplitude right at the interface, but in the denser medium, has a nonzero value. Since the boundary conditions for electromagnetic wave reflection do not allow any discontinuity in tangential field across the interface, the electric field amplitude at the interface in the less dense medium 2 will be equal to the electric field amplitude at the interface in denser medium 1. The solution of Maxwell's wave equation for total internal reflection shows the existence of a surface wave which propagates along the interface<sup>148)</sup>. Its electric field amplitude decays exponentially into the less optically dense medium 2. In general,

$$E^t = E^{t,0} \exp\left(-\frac{z}{d_p}\right) \quad (29)$$

where  $E^t$  is the transmitted electric field amplitude at distance  $z$  normal to the interface;  $E^{t,0}$  is the electric field amplitude right at the interface in less dense medium 2; and  $d_p$  is defined by:

$$\frac{1}{d_p} = \left(\frac{2\pi}{\lambda_2}\right) \left[ \left(\frac{n_1}{n_2}\right)^2 \sin^2 \theta_1 - \nu \right]^{1/2} \quad (30)$$

where  $n_1$  and  $n_2$  are refractive indices of the optically more dense and less dense media, respectively;  $\lambda_2$  is the wavelength of the electromagnetic wave in medium 2; and  $\theta_1$  is the angle of incidence of the electromagnetic wave measured from the interfacial normal. One can arbitrarily define  $d_p$  as the distance from the interface where the electric field amplitude decreases to  $e^{-1}$  of its interfacial value (often called the "depth of penetration of the evanescent wave"), i.e.  $\exp(-z/d_p) = \exp(-1)$ . An this case it can be shown that:

$$d_p = \lambda / 2\pi (n_1^2 \sin^2 \theta_1 - n_2^2)^{1/2} \quad (31)$$

The magnitude of  $E^{t,0}$  can be calculated from Fresnel's law. If the electric field amplitude as a function of distance  $z$  is expressed per unit of incident electric field amplitude,  $E^{i,0}$ , of the perpendicular polarized electromagnetic wave, then:

$$E_1^e = (E_1^{i,0}/E_1^{i,0}) \exp(-z/d_p) = (2 \cos \theta_1)/[1 - (n_2/n_1)^2]^{1/2} \exp(-z/d_p) \quad (31)$$

From an experimental point of view it is important to recognize that the profile of  $(E_1^e)^2$  as a function of  $z$  is proportional to the profile of the intensity of electromagnetic radiation in the proximity of the interface in medium 2. Such a profile will determine the "surface sensitivity" of the evanescent wave; the "depth of penetration" is smaller if:

- there is greater mismatch between the refractive indices of two media,
- the wavelength of electromagnetic radiation is shorter, and
- the incident angle of electromagnetic wave is closer to its critical value.

Thus, choosing the experimental parameters one can design a sensing profile to a particular need. It has to be mentioned that in this simplified derivation of evanescent spectroscopy theory two implicit assumptions were made:

1. Both media are nonabsorbing, only real refractive indices were being used,<sup>1</sup> and
2. Any species adsorbing to the interface is not distinguished as an optically separate layer.

What is even more relevant to the present subject is that a thin dielectric layer of polymer can be situated between two media without grossly distorting the optical nature of the interface, i.e. total internal reflection would occur as if no polymer layer is present even if its refractive index is unmatched to both media. Such a layer will, however, affect the intensity of the evanescent wave and particularly its "depth of penetration."

One can distinguish between methods in which absorption of the evanescent surface wave in different wavelength regions is measured (these are often called "attenuated total reflection" methods), and methods which use the evanescent wave to excite other spectroscopic phenomena, like fluorescence and Raman scattering or light scattering. As the methods of conventional fluorescence spectroscopy have been shown to be exceptionally successful in studies of proteins and other biopolymers, their "evanescent" surface-sensitive counterparts will be reviewed first.

### 6.3 Total Internal Reflection Fluorescence (TIRF)

TIRF at solid/liquid interfaces was introduced by Hirschfeld<sup>149</sup>. Although this first use of TIRF was the study of bulk dissolved fluorescein in the vicinity of a fused silica-electrolyte interface, a number of advantages over the conventional transmission technique were demonstrated:

1. The intensity-concentration relationship was linear up to concentrations a hundred-fold higher;
2. Multiple total reflections increased the sensitivity of TIRF; and
3. Adsorption of dye to the surface increased the local concentration, enhancing the TIRF sensitivity.

<sup>1</sup> In this sense, the name "total internal reflection" is a misnomer since depending on the extent of the absorption of medium 2, there will be an electromagnetic energy "leakage" across the interface making rigorous critical angle definition not applicable. However, for weakly absorbing medium 2 the concept of total internal reflection is useful. The reader is referred to Ref. 146 and 147 for the derivations of evanescent wave equations for absorbing multilayered interfaces.

Further application involved collected of fluorescence from dansyl-labeled bovine serum albumin via TIRF optics<sup>150</sup>, TIRF-immunoassay for specific dye-labeled antibodies binding from the solution to an antigen-coated surface<sup>151</sup>, and a "viro-meter" — a optical sensor for viruses treated with a fluorescent probe bound to the virus nucleic acid<sup>152, 153</sup>.

In TIRF protein adsorption experiments, it is desirable to correlate the intensity of excited fluorescence with excess protein concentration at the interface. Such an adsorbed layer is often in equilibrium with bulk-nonadsorbed protein molecules which are also situated inside the "evanescent volume" and thus contributing to the overall fluorescence. Various calibration schemes were proposed, using external nonadsorbing standards<sup>40, 154</sup>, internal standard in a form of protein solution together with a type of evanescent energy distribution calculation<sup>154</sup>, and independent calibration of protein surface excess<sup>155</sup>. Once the collected fluorescence intensity is correlated with the amount of adsorbed protein, TIRF can be applied in the study of various interactions between surface and protein.

Two different sources of fluorescence are possible:

1. Proteins containing amino acids like tryptophan and tyrosine which intrinsically fluoresce upon excitation in the ultraviolet range can be employed, hence, the name total internal reflection intrinsic fluorescence-TIRIF, or
2. Protein can be covalently labeled with an extrinsic fluor like fluorescein, rhodamine, etc.

Both approaches have distinct advantages and disadvantages: the first approach provides the possibility of recording intrinsic protein fluorescence emission and excitation spectra, which in turn can provide information about conformational changes in adsorbed proteins. It is also directly comparable with the methods of conventional fluorescence spectroscopy of proteins in solution. An interesting combination of TIRF and <sup>125</sup>I-labeled protein  $\gamma$ -detection was recently developed for determination of both the amount of protein and its fluorescence quantum yield in the adsorbed state<sup>26</sup>. It was shown recently that with appropriate apparatus the intrinsic fluorescence lifetimes of adsorbed protein can be determined<sup>27</sup>. Some proteins may show very weak fluorescence due to low quantum yield, have only a small number of tryptophanyl and/or tyrosinyl residues per molecule, and may be particularly photosensitive and unstable upon exposure to ultraviolet light<sup>92</sup>.

The extrinsic probe approach is more suitable to competitive protein adsorption studies and to kinetic studies, provided that protein labeling by an extrinsic fluor does not influence protein adsorbability. The uptake of fluorescein-labeled albumin,  $\gamma$ -globulin and fibrinogen onto silicone rubber coated surfaces has been followed as a function of time and flow rate<sup>39</sup>; it was demonstrated that adsorption was diffusion limited<sup>40</sup>. A combination of total internal reflection with either fluorescence photo-bleaching recovery (TIR/FRP) or fluorescence correlation spectroscopy was described<sup>137, 155, 157</sup>. With this variation of TIRF, it is possible to determine surface desorption rates and surface diffusion coefficients without unnecessary perturbation of chemical equilibrium, like bulk protein concentration changes or changes in the composition of the buffer solution. Energy transfer between multi-labeled BSA (donor/acceptor pairs: dansyl/eosin and 4-chloro-7-nitro-2,1,3-benzoxadiazole/rhodamine) has found to decrease upon the adsorption of BSA, as detected by TIRF<sup>158</sup>,

a feature that can be interpreted as a conformational change of adsorbed BSA. Fluorescent probes, used in probing membrane protein properties, have recently been employed in the evaluation of conformational changes upon adsorption<sup>159)</sup>.

Fluorescence from labeled adsorbed protein has also been excited with the evanescent surface wave created by integrated optics. Both optical fiber<sup>160)</sup> and flat rectangular waveguides<sup>193)</sup> have been used. Interesting use of optical fiber as a remote protein sensor was demonstrated; the excitation light was sent down the fiber whose tip was immersed in protein solution, evanescently excited fluorescence was collected by the same fiber and delivered to a scanning monochromator<sup>160)</sup>.

Changing the incident excitation beam angle and/or the angle of fluorescence observation provides other interesting information: a fluorophore concentration profile from the interface to distances which are within an order of magnitude of the wavelength used<sup>161)</sup>. In the case of adsorbed protein, variable angle total internal reflection fluorescence (VATIRF) would not only give as a result an average thickness of the protein layer but its spatial distribution.

In conclusion, TIRF promises to be exceedingly useful in the study of protein-substrate interactions. It gives *in situ*, possibly remote, real-time information about protein adsorption-desorption parameters, conformational changes upon adsorption and hopefully, nanosecond time-resolved fluorescence lifetime information about adsorbed proteins<sup>156)</sup>.

#### 6.4 Absorbance Spectroscopy of Adsorbed Proteins

Both conventional transmission absorbance spectroscopy and its evanescent counterpart (often called attenuated total reflection (ATR) spectroscopy) are based on measuring the light characteristics after it has propagated through the sample. Such techniques are classified according to the wavelength of the light used. Ultraviolet wavelengths are absorbed by all proteins. This feature has been used in the transmission absorbance mode in the evaluation of molar absorptivity of  $\beta$ -lactoglobulin films on the surfaces of quartz plates<sup>162)</sup>. The absorption of visible light by rhodamine-BSA adsorbed to a number of stacked quartz plates was measured as a mean to calibrate extrinsic fluor TIRF experiments<sup>155)</sup>. The use of attenuated total reflection both in ultraviolet and visible wavelength range for protein adsorption studies has to our knowledge not been reported, although a UV-TIR absorbance study of synthetic polymer adsorption has been reported<sup>163)</sup>.

ATR spectroscopy in the infrared has been used extensively in protein adsorption studies. Transmission IR spectra of a protein contain a wealth of conformational information. ATR-IR spectroscopy has been used to study protein adsorption from whole, flowing blood *ex vivo*<sup>164)</sup>. Fourier transform (FT) infrared spectra (ATR-FTIR) can be collected each 5–10 seconds<sup>165)</sup>, thus making kinetic study of protein adsorption by IR possible<sup>166)</sup>. Interaction of protein with soft contact lens materials has been studied by ATR-FTIR<sup>167)</sup>. The ATR-IR method suffers from problems similar to TIRF; there is no direct quantitation of the amount of protein adsorbed, although a scheme similar to the one used for intrinsic TIRF has been proposed<sup>168)</sup>; the “depth of penetration” is usually much larger than in any other evanescent method, i.e. up to 1000 nm; water absorbs strongly in the infrared and can overwhelm the protein signal, even with spectral subtraction applied.

#### 6.5 Raman Spectroscopy of Adsorbed Proteins

Raman spectroscopy is another powerful method used for the study of solution protein conformation. Its application at the solid/liquid interface is conceivable<sup>169)</sup>, but to our knowledge it has not been applied to protein adsorption on polymer surfaces. One of the problems is that most proteins have a small Raman scattering cross-section. In order to enhance the weak Raman scattering signal, use of an evanescent streak propagating through a thin polymer waveguide layer with adsorbed protein has been demonstrated<sup>193)</sup>. Total internal reflection optics were used to induce Raman scattering of 5  $\mu\text{m}$  thick dry BSA films on a sapphire internal reflection element<sup>170)</sup>. Another possibility to enhance the signal is to induce resonance Raman scattering of a protein absorption band; in the visible wavelength range this would apply mainly to proteins containing heme in their structures. Two other ways of enhancing the Raman scattering signal from surfaces were devised: use of surface plasmon excitation at the surfaces of thin silver films<sup>171)</sup> and surface enhanced Raman scattering (SERS) by silver island films and finely dispersed silver colloids<sup>172)</sup>. The Raman spectra of 75 Å thin phospholipid monolayers has been obtained by the first method<sup>173)</sup>. SERS has been used to induce resonance Raman spectra from cytochrome-c and myoglobin adsorbed at a silver electrode<sup>174)</sup>. The full potential of Raman scattering techniques applied to the adsorption of proteins has yet to be exploited.

#### 6.6 Ellipsometry

Ellipsometry is another powerful tool in solid/liquid interface analysis. While evanescent spectroscopy has the capability of sensing the adsorbed protein molecules by interaction with the evanescent surface wave, ellipsometry “sees” a protein layer on the reflecting surface as a distinct optical medium. Ellipsometry is based on the calculation of the optical properties of the reflecting surface, given the change in the state of the polarization of the reflected light. Once the optical properties of the bare surface are known, the ellipsometric analysis can be applied to any dielectric film deposited on the surface. The exact relations between wavelength, optical constants, thickness and incident angle were obtained in exact form in the 19th century, but they could not be solved in the closed form. Early work on ellipsometry used only approximate equations, neglecting the higher order terms of the Taylor expansions of the Drude equations. The introduction of computers made routine analysis of the equations readily available. The use of ellipsometry in protein-surface interaction studies advanced rapidly after Trurnit<sup>175, 176)</sup> introduced an automated recording ellipsometer and measured the activity of the proteolytic enzyme, chymotrypsin, at a solid/liquid interface. Bovine serum albumin was deposited on the solid phase and depending on the condition used (ionic strength and pH) chymotrypsin was either removed or adsorbed to the albumin layer.

Studies of the role of protein-surface interactions in blood coagulation were done by Vroman<sup>56)</sup>. The plasma proteins were adsorbed onto various hydrophilic or hydrophobic surfaces. Vroman showed that fibrinogen was an important component of the plasma protein layer adsorbed to the solid/liquid interface.

A complete review of the early work applying ellipsometry to biomedical problems is available<sup>177)</sup>.

The surface of silicon has optimum optical properties and the adsorption of proteins

was followed on both hydrophobic and hydrophilic silicon surfaces<sup>178, 179)</sup>. A fibronectin adsorption study showed, for example, that the adsorption is partially reversible on the hydrophilic surface, but much less so on the hydrophobic surface<sup>179)</sup>. Interaction of antibodies with preadsorbed fibronectin layers suggest that fibronectin adsorbs in different orientation or conformation on the two surfaces<sup>179)</sup>.

Ellipsometry can follow the interactions between two types of biological macromolecules, the first of those two bound physically to the surface, the other acting from the solution. The binding of concanavalin A to adsorbed mannose<sup>180)</sup> and of cholera toxin to adsorbed ganglioside<sup>183)</sup> are examples. The adsorption of complement factors to an antibody-coated surface was monitored by ellipsometry and a modification of the same method was used for quantification of migration inhibition of human polymorphonuclear leucocytes<sup>182)</sup>. Interaction of proteins and cells with affinity ligands covalently coupled to silicon surfaces has been also studied<sup>183)</sup>.

Recent development in the application of ellipsometry to protein/substrate interaction studies is due to the introduction of the "dynamic ellipsometer", capable of recording both analyser and polariser positions automatically with time, which significantly improves the time resolution of the method<sup>184)</sup>. The Lorentz-Lorenz equation is usually applied in order to calculate the mass of adsorbed proteins<sup>185)</sup>. In particular, molecular weight, molar refractivity and partial specific volume are needed to calculate adsorbed mass from the experimentally determined thickness and refractive index of deposited film. While in most of the previous studies the assumption was made that the refractive index of the adsorbed layer was constant, the experiments done by Cuypers showed that such an assumption is not justified<sup>186)</sup>. The refractive indices of protein layers varied as a rule with the time of adsorption<sup>187)</sup>. So far, no general rules emerged about the course of refractive index changes. The refractive index of fibrinogen adsorbed on hydrophilic chromium oxide surface, for example, remained almost constant ( $n = 1.4$ ) while the thickness increased to 12 nm. On the contrary, the refractive index of fibrinogen adsorbing on hydrophobic chromium was found to change significantly with time of adsorption; on the onset ( $t < 150$  s) it was 1.8, only to drop to 1.48 while the thickness of the layer continued to increase to 7 nm. From this point on the fibrinogen layer became optically denser and thinner. Final values for the fibrinogen layer deposited from 10 µg/ml solution (0.01 M Tris-HCl buffer, pH = 7.0) onto hydrophobic chromium surface were: thickness,  $d = 3.5$  nm, refractive index,  $n = 1.72$ . Such changes directly reflect some conformational changes and packing or ordering of the adsorbing protein. It is, however, difficult to explain such a high value of refractive index of the protein layer. It was reported that the refractive indices of prothrombin layers adsorbed on two phospholipids (di-C14:0 phosphatidylserine and di-C18:0 phosphatidylserine) were 1.46 and 1.90, respectively<sup>185)</sup>. In order to account for this discrepancy, the authors speculated that refractive index values higher than the values for the pure protein reflected an interaction between the protein and the adsorbing surface and that in order to compensate for higher refractive indices of the protein film, a decrease of the refractive index of adsorbing surface has to be introduced. This assumption cannot be proved experimentally, but it was supported by finding that the adsorption of protein onto more swollen phospholipid layers, (i.e. onto a layer with a lower refractive index) resulted in higher refractive indices of protein film<sup>187)</sup>. Clearly, more detailed modeling and computer simulation studies would be helpful.

## 7 Some "Rules of Thumb" (See also Sect. 4.2)

### 7.1 Area Required For Initial Adsorption

At an air/water interface, the two-dimensional interfacial pressure ( $\Pi$ ) can be easily monitored using an instrumented Langmuir trough. The initial adsorption rate at a clean surface is simply the rate of diffusion

$$\frac{dn}{dt} = K_a C_0 \quad (32)$$

where  $K_a$  is the adsorption rate constant. Assume that the interface is partially occupied and the interfacial pressure is  $\Pi$ . "In order for a molecule to adsorb, it must compress molecules already adsorbed against the interfacial pressure  $\Pi$  to create an area of interface,  $\Delta A$ , equal to that required for the molecule to move into (Ref.<sup>3)</sup>, p. 289)."

The work required is  $\int_0^{\Delta A} \Pi dA$  or  $\Pi \Delta A$  if  $\Pi$  is approximately constant. Then

$$\frac{dn}{dt} = K_a C_0 \exp\left(-\frac{\Pi \Delta A}{kT}\right) \quad (33)$$

where  $k$  is Boltzman's constant,  $T$  is absolute temperature, and no desorption has been assumed (valid for  $\Pi < 15$  mN/m). Equation 33 can be expressed as:

$$\ln\left(\frac{dn}{dt}\right) = \ln(K_a C_0) - \frac{\Pi \Delta A}{kT} \quad (34)$$

A simple linear plot of the data allows  $\Delta A$  to be obtained. The results of a set of experiments (Table 5) are surprising.  $\Delta A$  is independent of the size or molecular weight of the protein. Although the cross-sections of the proteins studied range from ~1000 to 10,000 Å<sup>2</sup>,  $\Delta A$  is nearly constant at 100 to 200 Å<sup>2</sup>. Conclusion: "...only a small portion of the protein molecule needs to enter the interface in order for adsorption to then proceed spontaneously (Ref.<sup>3)</sup>, p. 290)." It is as if only a small "foothold" or "handhold" is required to stabilize the molecule against desorption. Now firmly planted at the interface, the molecule can optimize its interfacial interactions by time-dependent orientation and perhaps conformational changes. The size of the "foot" is obviously relevant to the exchange discussion in Sect. 4.5.

**Table 5.** Values of  $\Delta A$  for various proteins (from Ref.<sup>3)</sup>)

Protein	Concentration (g l <sup>-1</sup> )	$\Delta A$ (Å <sup>2</sup> ) <sup>-3</sup>	Molecular Weight
Myosin	0.03	145	600000
Human $\gamma$ -globulin	0.01	130	180000
Human albumin	0.02	100	70000
Ovalbumin	0.03	175	44000
Lysozyme	0.01	100	15000

An alternative interpretation of the data is that  $\Delta A$  is proportional to the number of water molecules per protein participating in the adsorption and denaturation process and is related to water activity at the interface.

## 7.2 Electrical Potential Barrier

A similar experiment to that noted above can be performed, but now let the interface be populated by a molecular layer at constant  $\Pi$  and known interface electrical potential. A molecule adsorbing at such an interface must do work against the electrical potential barrier, as well as against the interfacial pressure. We get

$$\frac{dn}{dt} = K_a C_0 \exp - \left( \frac{(\Pi \Delta A + q\Psi)}{kT} \right) \quad (35)$$

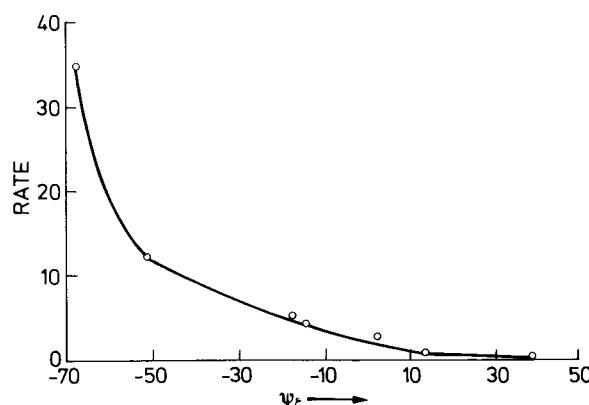
where  $q$  is the charge on the adsorbing molecule and  $\Psi$  is the interface electrical potential (3). Table 6 and Fig. 26 present data on lysozyme adsorption into different monolayers. Clearly the *electrostatic effects are very significant*.

**Table 6.** Relative initial rates of adsorption of lysozyme into different monolayers<sup>a</sup>

Monolayer	Rate	$\zeta$ -Potential (mV)
Cephalin	34.5	-68.1
Polyglutamic acid	13.2	-51.5
Pepsin	5.4	-16.7
Serum albumin	4.5	-14.1
Octadecanol	5.0	Not measured
Trypsin	3.0	+3.2
Lysozyme	(1.0)	+12.2
Polylysine	0.3	+38.5

pH 6.5, I.S. = 0.01, charge on lysozyme  $\approx +9$  units.

<sup>a</sup> from Ref. 3



**Fig. 26.** Plot of the data of Table 6 showing rate of lysozyme adsorption at air/water monolayers of different zeta potentials. Refer to Eq. (35) and text for details (from Ref. <sup>3</sup>).

Because of the large size of the protein and the  $\Delta A$  required for initial adsorption, only a small region of the molecule need participate in the initial stage of the process.

It is well-known that *protein adsorption tends to be at a maximum at the isoelectric point* because the protein has zero net charge. Under such conditions, electrostatic barriers to adsorption are minimized.

Protein adsorption is dependent on the nature and concentration of the electrolyte. Norde and Lyklema have shown <sup>61</sup> that adsorption generally increases with decreasing charge on the protein and on the surface, although counterion effects can often override this generalization.

## 7.3 Hydrophobicity

The now classic studies by Norde and Lyklema and their detailed thermodynamic analysis (Sect. 4.2) have established that the *interaction between a protein and a surface increases with increasing hydrophobicity of the surface and increases with increasing hydrophobicity of the protein*. “Desorption from hydrophobic surface usually does not occur whereas proteins can often be removed from hydrophilic surfaces by exposure to extreme pH, high ionic strength, or by extensive rinsing <sup>61</sup>.”

## 7.4 Diffusion and Mass Transport

Proteins have a low diffusivity:  $D \sim 2$  to  $6 \times 10^{-7}$  cm<sup>2</sup>/s. Therefore, the *initial stages of adsorption are generally diffusion limited* and the amount adsorbed is proportional to  $t^{1/2}$ . If the adsorption rate,  $dn/dt$  is:

1.  $\sim 1/t^{1/2}$ , diffusion is rate limiting;
2.  $> 1/t^{1/2}$ , then convection or other mass transport effect is present;
3.  $< 1/t^{1/2}$ , the adsorption is reaction limited; i.e., there are energy barriers to the adsorption process.

## 7.5 Time

As contact or residence time increases, the protein tends to orientationally and conformationally adjust to the interface, leading to stronger bonding, and greater irreversibility of adsorption.

## 7.6 Surface

Surface adsorption site energy and density are very important. Most *biomaterial surfaces have very high site densities*, making it difficult to study the mechanisms governing adsorption. Low site density surfaces are available. Heterogeneous surfaces, such as block copolymers and polymer blends, may have very unique adsorption properties. If one of the phases or domains tends to dominate the surface, it may act as a homogenous surface. If both phases are present on the surface, then two or more

very different classes of adsorption sites will be present. Protein adsorption on each of the two phases can be very different<sup>80, 81</sup>.

### 7.7 Protein

The protein's intrinsic properties (size, molecular weight, 3-D structure, surface site density, conformational stability) are all very important and must be fully characterized and understood in order to interpret adsorption data.

## 8 Summary/Conclusions

The general principles of protein adsorption are beginning to be identified and understood. New techniques and methods are now available which, together with well-established methods, allow one to thoroughly probe the adsorption process.

In the past, the solid surface and the solid/solution interface were often poorly characterized and poorly understood. A range of new concepts and tools are now available for the study of solid/solution interfaces<sup>30</sup>. A new monograph on protein adsorption is now available<sup>4</sup>. An international conference on protein and polyelectrolyte adsorption was recently held and proceedings will soon be available<sup>188</sup>.

Protein surface structure and conformational dynamics are now much better understood. In the near future, we can expect extensive application of computer molecular graphics to better visualize and understand protein-surface interactions.

Very qualitatively — protein adsorption is roughly understood. The challenge now is to take what we know about proteins, surfaces, and adsorption and to begin to quantitatively model the protein adsorption process.

The future looks bright!

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