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Title: The Effect of pH and Ionic Strength on the Adsorption of β–Lactoglobulin onto Well-Characterized Silicon

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The effect of pH and ionic strength on the equilibrium adsorptive behavior of β-lactoglobulin onto hydrophobic and hydrophilic silicon surfaces was studied using ellipsometry. Plots of amount adsorbed (μg protein/cm^2) as a function of protein concentration (mg/ml) exhibited attainment of plateau values beyond a protein concentration of 0.250 mg/ml. At a given pH and ionic strength, plateau values associated with hydrophobic surfaces were observed to be greater than those associated with hydrophilic surfaces.

The Langmuir adsorption isotherm was chosen as the most appropriate model to represent the data and was used to compare results obtained under different experimental conditions. Effects of pH and ionic strength on protein adsorption at hydrophilic surfaces indicate that electrostatics played a major role, while pH and ionic strength effects on adsorption to hydrophobic surfaces reflect a greater importance of nonelectrostatic interactions.

The Effect of pH and Ionic Strength on the Adsorption of β-Lactoglobulin onto Well-Characterized Silicon

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE AND ABBREVIATIONS

A_p - Molar refractivity of a protein

C_{eq} - Equilibrium protein concentration (mg/ml)

d - Film thickness (nm)

Δ - Delta, change of phase of reflected light

Γ - Gamma, mass adsorbed ($\mu g/cm^2$)

HPA - Human Plasma Albumin

HPB - Hydrophobic silicon surface

HPL - Hydrophilic silicon surface

i.e.p. - Isoelectric point

m - mass adsorbed ($\mu g/cm^2$)

M_p - Molecular weight of a protein

n_i - Refractive index of material i

Ψ - Psi, arctangent of change in amplitude ratio of reflected light

RNase - Ribonuclease

V_{20} - Partial specific volume at 20°C

The Effect of pH and Ionic Strength on the Adsorption of β-Lactoglobulin onto Well-Characterized Silicon

INTRODUCTION

Protein adsorption is a phenomenon observed when a biological fluid comes into contact with a foreign surface. In the food industry, heating of biological fluids often causes deposition (fouling) on the process equipment (1). Proteins play a major role in the fouling of heat exchangers because of their heat sensitivity and high content in food stuffs. Protein adsorption is also of interest to the medical industry since it is a primary event when foreign surfaces are exposed to blood or living tissue (2). Medical problems, such as blood coagulation, often occurring at the sites of implantation of foreign devices, are intimately correlated with the course of the initial protein adsorption. Much work has been carried out to determine the parameters affecting protein adsorption as well as to determine a model that accurately describes protein adsorption.

Some factors that affect protein adsorption are temperature, conformation of the protein in solution, pH, ionic strength and the surface characteristics of the material onto which adsorption occurs. Many workers have studied the effect of pH (3-10) and in general have shown that maximum adsorption occurs at or near the isoelectric point. The isoelectric point (i.e.p.) is that pH at which the net charge on the protein molecule is zero. A protein molecule will be in its most compact form at the i.e.p..

The degree to which ionic strength affects protein adsorption is a function of the role electrostatics play in the adsorption driving force. As ionic strength is increased, the surface charge of the protein becomes increasingly shielded, thus reducing electrostatic effects and leading to a more globular protein configuration (5). Previous work has generally seen an increase in the amount of protein adsorbed as ionic strength increases (3, 5, 7, 8, 11), but decreases in adsorbed amount have also been observed (6, 11). Electrostatics are not the only important interactions that take place during protein adsorption, and such varying observations for the adsorbed mass as a function of ionic strength are not unexpected.

A surface property that has been shown to be of importance in protein adsorption is the degree of hydrophilicity (1, 2, 5, 8, 11-13). Surfaces that do not have a strong affinity for water molecules are termed "hydrophobic" and those that strongly bind water molecules are termed "hydrophilic". A water drop placed on a hydrophilic surface will spread while a drop of water placed on a hydrophobic surface will bead. Several research groups have shown that protein adsorption is greater on hydrophobic surfaces than onto hydrophilic surfaces (2, 7, 11).

The degree to which pH, ionic strength, and surface characteristics affect protein adsorption is determined to a large extent by the conformational stability of the particular protein molecule. Norde and Lyklema (3) have observed that the plateau amounts of protein adsorbed are virtually independent of pH for bovine pancreas ribonuclease (RNase), whereas those for human plasma albumin (HPA) have a variation up to a factor of two. Relative to RNase, HPA

exhibits a high degree of conformational adaptability, which allows this protein to change its structure as conditions in the solution change. The changes in structure for HPA are reflected in the observed changes in amounts adsorbed onto a given surface as a function of pH. Conversely, the lack of change in the RNase protein structure in response to changing pH leads to the amount adsorbed being independent of changing solution conditions. Goat and rabbit immunoglobulin G's have a known structural form in the shape of a "T", with pH affecting adsorption by changing the degree of repulsions in the protein's "arms" (4). Triple-helical collagen monomers are a fairly stiff rod over most of their length; as a result of this stability, pH and ionic strength have little effect on its adsorptive behavior (7). To further complicate interpretation of pH effects, protein molecules such as β-lactoglobulin exist as dimers at pH=6.0 and monomers at lesser and greater pH values (1).

The objective of the present work was to study the equilibrium adsorption of the milk protein β-lactoglobulin onto modified silicon oxide surfaces. Both hydrophilic and hydrophobic silicon surfaces were used, with adsorption occurring at a constant temperature under varying conditions of protein concentration, pH, and ionic strength. Dried films, as opposed to films *in situ*, were analyzed by ellipsometry, a nondestructive optical technique useful for determining the optical properties of thin films as well as their adsorbed mass.

GENERAL BACKGROUND

Proteins are complex macromolecules that may constitute 50% or more of the dry weight of living cells. They fall into the general class of polymers, linear molecules built up from simple operating units called monomers. A protein molecule is unique in that it can be synthesized from up to 20 different monomer units (amino acids), whereas many polymers have a single type of monomer unit. The simple linking of amino acids into random sequences refers to formation of a polypeptide chain ("peptide" refers to the type of bond formed between amino acids). The term "protein" is usually reserved for chains with a specific sequence, length, and folded conformation. A protein molecule acquires a very specific folded three-dimensional conformation, which is determined by a protein's specific sequence of amino acids as well as the environment the protein is in.

The number of amino acid units that make up a protein usually number between 50 and 1000 units. With 20 different building blocks, one can imagine a vast number of combinations which could make up the composition of different protein molecules. These many different combinations affect the degree to which physical forces determine the properties of a protein. Ionization of amino acids within a protein are pH-dependent since most have an acid dissociation constant value in the pH range studied. The degree to which groups are ionized affects the interaction between charged species, a phenomenon referred to as electrostatic interaction.

Hydrogen bonding is the primarily electrostatic interaction that occurs between water molecules (or other polar molecules such as alcohols). This interaction is intermediate between ionic interactions and covalent bonding because a hydrogen atom is shared between an acid (proton donor) and a base (proton acceptor). Another type of interaction involves van der Waals forces, which result from the net attracting force caused by the oscillating dipoles of neighboring atoms. Van der Waal forces are not as strong as hydrogen bonding or electrostatic interactions, but can become important in nonpolar, neutral systems.

A major factor in the stability of proteins are hydrophobic interactions. These interactions refer to the absence of hydrogen bonding between nonpolar molecules and water, rather than a favorable nonpolar molecule interaction with another nonpolar molecule. Regions or surfaces which show this lack of affinity for water molecules are thus termed hydrophobic. A protein molecule will adjust its conformation in such a way so as to minimize this unfavorable interaction with water molecules.

The surface of a protein molecule's three-dimensional conformation cannot be described completely (e.g., hydrophobic, positively charged, neutral, or negatively charged surface) because the characteristics of a protein's surface are a function of the types of amino acids exposed. A protein molecule's surface is usually a combination of the mentioned possibilities, a fact which makes a protein molecule very surface active when in contact with a foreign surface. This interaction with a surface is most commonly referred to as protein adsorption.

The occurrence of proteins at solid/liquid interfaces is of great biological, medical and technical significance. Some proteins that have been used for studying the adsorption at surfaces are human plasma albumin (3, 6, 8), bovine serum albumin (5, 2, 9, 10, 19), immunoglobulin G's (2, 4, 11, 13) and β-lactoglobulin (1, 19). The proteins used for adsorption studies are usually well characterized so that interpretations of the effects of changing environmental conditions on adsorption can be better done. Conditions that are usually varied are protein concentration, pH, ionic strength, type of surface and temperature.

A wide variety of methods have been used to determine the amount of protein adsorbed onto a surface. An indirect method for determining adsorbed mass is ultraviolet spectroscopy (4), which involves measuring the amount of protein left in solution after adsorption and subtracting this from the initial amount. Knowing the contact area allows determination of mass adsorbed per unit area. Direct methods include radiolabeling (5), ellipsometry (1, 2, 11, 13, 17), and internal reflection spectroscopy (12). In radiolabeling the protein molecules are labeled with radioactive iodine molecules. Once a surface has protein adsorbed onto it, the mass adsorbed is calculated by measuring the radioactivity of the surface (a calibration is done to relate the number of radioactive counts per unit mass of protein).

Ellipsometry uses the changes in the properties of light reflected from a surface and the optical properties of the bare surface to determine the amounts adsorbed. This method is limited in that the surface of interest must reflect the light and the film formed on the

surface must have a refractive index different from that of the medium through which the laser beam must pass. For example, if a film is being measured in air and has the same refractive index as air, then the ellipsometer will not "see" the film. The same would hold true if the film was being measured such that the ellipsometer laser beam was going through water and the film had the same refractive index as water. A strength of the method is that a specular material can be chemically treated to "mimic" another surface (e.g., silicon can be treated so that the surface behaves as a polymer).

Internal reflection infrared spectroscopy measures the difference between ratios of the amide I (C=O stretching) band at 1640 cm^{-1} to a standard band for each polymer before and after adsorption to give a net adsorption value. This value is then related by a calibration curve to the actual surface concentration. An obvious limitation of this technique is the need for a surface that is infrared active. An example of a class of useful surfaces are polymers, as used by Lee and Kim (12).

The choice of experimental method is dependent upon the system of interest and the equipment available. A comparison of ellipsometry with a radiolabeling technique has shown that the two methods give results in good agreement (19). Comparison of ellipsometry with internal reflection infrared spectroscopy is difficult because most polymers are not specular enough to work with ellipsometry.

THEORY

The equilibrium relationship between adsorbed mass and protein concentration often follows that described by the Langmuir isotherm (3, 4, 6, 8, 12). Plots of adsorbed mass versus protein concentration show a steep initial slope at low concentrations and a plateau value beyond some critical protein concentration. Mathematically, a Langmuir isotherm is of the form

$$\Gamma = \frac{a\, C_{eq}}{b + C_{eq}} \qquad (1)$$

where Γ = amount adsorbed ($\mu g/cm^2$), C_{eq} = equilibrium protein concentration (mg/ml), and a and b are function constants (a = plateau value for Γ and (a/b) = initial slope of Γ vs. C_{eq} curve). Although an isotherm may be adequately described by a mathematical equation, the physical interpretation holds only if the system follows the premises of the associated model.

Langmuir's theory assumes a homogeneous surface and no adsorbate-adsorbate interaction (14). Norde (3) points out that, for this reason, application of Langmuir's theory to protein adsorption is not appropriate. Moreover, he notes that theories that describe adsorption of randomly coiled polymers also do not apply to proteins, although proteins can be roughly classified as polymers. A protein molecule has a definitive three-dimensional conformation which is determined by the sequence of amino acids that make up the protein chain.

Although the theories of Langmuir may not be rigorously applied to protein adsorption, the fact that data may fit a Langmuir equation is useful when trying to compare relative adsorptive behavior of a single protein under varying conditions.

Another equation that has some use for protein adsorption is the Freundlich equation

$$\Gamma = a\,(C_{eq})^{b} \qquad (2)$$

This power function equation is strictly experimental i.e., it has no theoretical basis, but sometimes is very useful in modeling adsorption isotherms where the Langmuir relationship fails. An example where the Langmuir equation fails is the adsorption of Bovine Serum Albumin onto glass and siliconized glass (5). Plots of Γ versus protein concentration do not reach well defined plateau values and do not show a linear relationship at low concentrations.

Electrostatic and hydrophobic interactions are important factors affecting the adsorption of protein onto a surface (15). The interaction between electrically charged bodies is described by the DLVO theory developed by Derjaguin and Landau (1941) and Verwey and Overbeek (1948). Norde (16) reviewed pertinent features of the DLVO theory, which can be briefly summarized as follows. Two components comprise the total free energy associated with an adsorbate-adsorbant interaction: (i) an electrostatic interaction and (ii) a dispersion contribution to the van der Waals interaction. Ionic strength affects the electrostatic interactions to a greater extent than it does the van der Waals interactions. As ionic

strength is increased, the adsorbed amount increases, with the effect being greater for adsorbate-adsorbant systems of the same charge relative to opposite charge.

Hydrophobic interactions are a contributing factor to the entropy driving force observed for the adsorption of proteins from an aqueous solution (5). Entropy is increased due to freedom gained by water molecules resulting from partial dehydration of the solid surface and the protein "surface" and in part to structural changes that occur in the less rigid, partially dehydrated protein molecule. Since hydrophobic surfaces have a low affinity for water molecules, the protein will change conformation to remove contact of these regions with the aqueous environment by either "burying" the hydrophobic region internally or associating with similar regions on the surface.

Hydrophobic and electrostatic interactions are phenomena that occur very near a solid/liquid interface or on the solid surface itself. This interface may or may not have the same properties as measured in the bulk solution (e.g., the microenvironmental pH and ionic strength in the region of the interface may be dissimilar to bulk pH and ionic strength). As a consequence, interpretation of the effects caused by changing solution parameters in terms of these surface interactions should be done with this possible difference in mind.

Prediction of the effect that pH and ionic strength will have on protein adsorption is dependent upon which interactions are predominant (e.g., electrostatic, hydrophobic, or van der Waals interactions). If electrostatics comprise the major interactions, then for a negatively charged surface, adsorbed mass should be greater at pH

values below the i.e.p. relative to pH values above the i.e.p.. Below the i.e.p., the protein and surface are of opposite charge, while both the protein and solid surface are negatively charged at pH values greater than the i.e.p.. As ionic strength increases, the electrostatic interaction is reduced due to "shielding" by counterions; consequently, increasing the ionic strength should decrease adsorbed mass at pH values less than the i.e.p. and increase the adsorbed mass at values of pH greater than the i.e.p..

When hydrophobic and general nonelectrostatic interactions are taken into account, the relationship between adsorbed mass and changes in pH and ionic strength becomes intimately tied to a protein's conformational stability. In general, pH and ionic strength conditions that lead to a less stable conformation for the protein in solution will lead to an increased adsorbed mass, assuming that the protein molecule is more stable on the solid surface.

ELLIPSOMETRY

Ellipsometry involves analysis of the change in the state of polarized light that accompanies reflection from a surface. The state of polarization is defined by the phase and amplitude relationships between the two component plane waves into which the electric field oscillation is resolved. These waves are denoted p, for the wave in the plane of incidence, and s, for the wave normal to the plane of incidence. In general, reflection causes a change in the relative phases of the p and s waves and a change in the ratio of their amplitudes. Reflected light is characterized by the angle Δ, defined as the change in phase, and the angle Ψ, the arctangent of the factor by which the amplitude ratio changes.

Figure 1 shows a schematic layout of the optical system of an ellipsometer. Analysis of a sample is done with and without the compensator. The value of Δ obtained with the compensator is used if Δ recorded is within 45° of 0° or within 45° of 180°. The value of Δ obtained without compensator is used if its value is greater than 45° from either 0° or 180°. The technique may be applied to any substratum-film combination that provides reasonably specular reflection of the incident light beam. Although suitable for measuring both dry films and those in solution (*in situ*), work done with protein adsorption using ellipsometry has relied mainly on *in situ* analysis (1, 2, 11, 13, 17).

Resolution of the measured Ψ and Δ for a film into a refractive index and thickness is done by methods based on the Drude equations, as detailed by McCrackin (18). This resolution is most com-

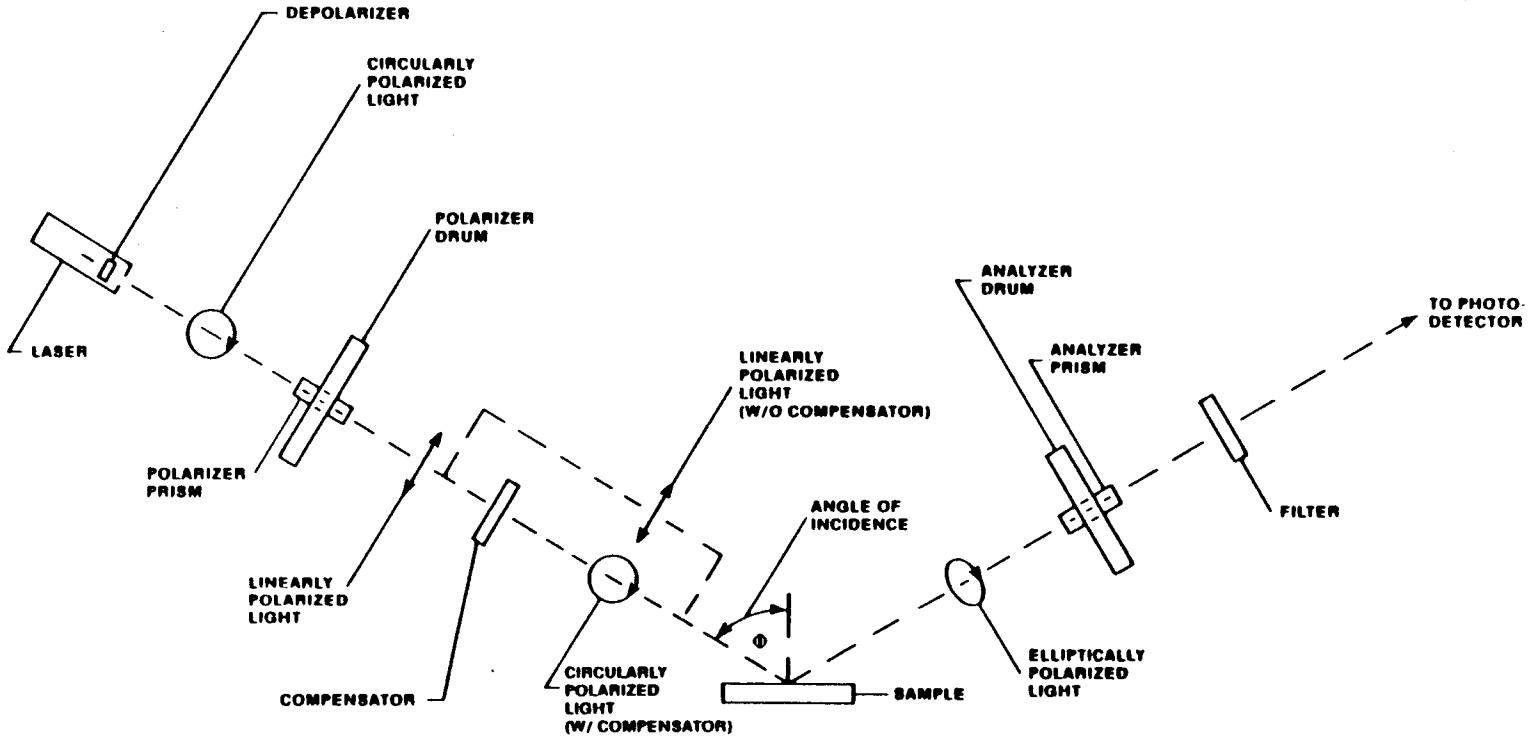

Figure 1. Schematic of an Ellipsometer. From the users manual for a Gaertner Ellipsometer L116B.

monly done with a computer. The film's refractive index, n_f, and thickness, d, can be converted into a value of adsorbed mass using the following relationship as developed and verified by Cuypers (17):

$$m = \frac{0.3\ d\ f(n)}{\frac{A_p}{M_p} - V_{20}\frac{n_b^2 - 1}{n_b^2 + 2}} (n_f - n_b) \qquad (3)$$

$$f(n) = \frac{n_f + n_b}{(n_f^2 + 2)(n_b^2 + 2)} \qquad (4)$$

where m = amount adsorbed ($\mu g/cm^2$), M_p = molecular weight of protein, A_p = molar refractivity of the protein, d = film thickness (nm), V_{20} = partial specific volume of protein at 20°C, n_f = film refractive index and n_b = refractive index of buffer. For β-lactoglobulin, $M_p/A_p = 3.796$. When the buffer phase denoted by "b" is air, n_b becomes 1, thus simplifying the equations to:

$$m = \left(\frac{M_p}{A_p}\right) 0.3\ d\ f(n)\ (n_f - 1) \qquad (5)$$

$$f(n) = \frac{n_f + 1}{(n_f^2 + 2)\ 3} \qquad (6)$$

The basis of these equation is the Lorenz-Lorentz relationship for the refractive index of a mixture of substances.

MATERIALS AND METHODS

Materials

Hydrophobic and Hydrophilic Silicon. Silicon wafers (Wacker Siltronic Corporation, 1-0-0 orientation, 0.1-0.16 resistivity) were cut into plates of approximately 1 x 2 cm using a tungsten knife. The preparation of modified silicon surfaces was done in a fume hood to avoid contact with vapors from the reacting solutions. Hydrophilic surfaces were prepared by reaction with a basic solution (ammonium hydroxide:hydrogen peroxide:water, 1:1:5) for 5 minutes at 80°C, a short rinse with distilled water, reaction with an acidic solution (hydrochloric acid:hydrogen peroxide:water, 1:1:5) for 5 minutes at 80°C, another short rinse with distilled water, and storage in 50% aqueous ethanol. Hydrophobic surfaces were prepared by reacting hydrophilic surfaces for 10 minutes in a solution of 10% dichlorodimethyl-silane in trichloroethylene. Surfaces were subsequently rinsed with trichloroethylene, acetone and ethanol and then stored in a desiccator. Contact angles were measured to check whether the silicon samples, as treated above, became hydrophobic and hydrophilic. The optical constants obtained for the dry surfaces were: Ψ = 10.45, Δ = 169.36 for hydrophilic silicon and Ψ = 10.50, Δ = 168.19 for hydrophobic silicon.

β-Lactoglobulin Solutions. Protein solutions were prepared from a mixture of β-lactoglobulin A and B obtained from Sigma Chemical Company. Solutions containing protein were prepared immediately prior to use to minimize the possibility of denaturation as a result of allowing the protein solution to sit for long periods of

time. A solution of desired protein concentration was made by dissolving a weighed amount of protein into 500 ml of buffer solution. A stirrer was used to assure complete solubilization of protein in a short period of time.

Buffer Solutions. Buffer solutions were prepared using chemicals of analytical grade and water that was both distilled and deionized. Four buffer solutions were prepared and consisted of (per 1 L):

(1) 0.0265 g $Na_2HPO_4 \cdot 7H_20$, 6.8500 g $NaH_2PO_4 \cdot H_20$, 4.383 g NaCl, 0.5 ml H_3PO_4 (pH=3.00, 0.1 M Na^+)

(2) 6.080 g $Na_2HPO_4 \cdot 7H_20$, 7.500 g $NaH_2PO_4 \cdot H_20$ (pH=6.60, 0.1 M Na^+)

(3)13.232 g $Na_2HPO_4 \cdot 7H_20$, 0.076 g $Na_3PO_4 \cdot 12H_2O$ (pH=8.90, 0.1 M Na^+)

(4)13.232 g $Na_2HPO_4 \cdot 7H_20$, 0.076 g $Na_3PO_4 \cdot 12H_2O$, 23.376 g NaCl (pH=8.90, 0.5 M Na^+).

Equipment

Ellipsometric measurements were done using a model L116B ellipsometer (Gaertner Scientific Corporation). The light source was a 1 mW, helium-neon laser having a beam wavelength of 6328 Å. An angle of incidence of 70° was used. Software supplied by Gaertner called "SubCA" was used to determine the optical constants Ψ and Δ for bare surfaces as well as adsorbed films.

A model 2001 VWR Orbital Shaker was used to agitate the solutions during an adsorption experimental run. The stirrer used

for preparation of buffer solutions and rinsing of contacted surfaces was a Corning Stirrer/Hot Plate model PC-520.

Adsorption Procedure

Hydrophobic and hydrophilic samples were attached to a glass slide with double sided mounting tape. This allowed the surfaces to be treated identically and provided consistency through each adsorption run. To assure a similar contacting geometry among runs, the glass slides were attached to stainless steel holders that were specially designed for the 400 ml beakers used (see Figure 2). Using a controlled temperature room set at 15.6°C, samples were equilibrated in 300 ml of buffer for one hour and then contacted with 300 ml of protein solution for three hours. Parafilm® was used as a cover to prevent dust and other materials from contaminating the solution during the experiment. Both the equilibration and adsorption steps were performed with the shaker set at 100 rpm. After adsorption of protein, samples were rinsed for one minute in a 400 ml beaker containing 300 ml of distilled water. The rinse step was done with stirring by a 1" magnetic stir bar on a stirrer set at "2". Rinsing is an important step that removes loosely bound protein that is not actually bound to the surface. Samples were then allowed to dry in a desiccator for at least 12 hours before ellipsometric measurement.

Ellipsometric Procedure

The optical properties Ψ and Δ were measured at several locations on each sample, with care taken to avoid spots on the surface exhibiting visible signs of contamination. To be sure that a given

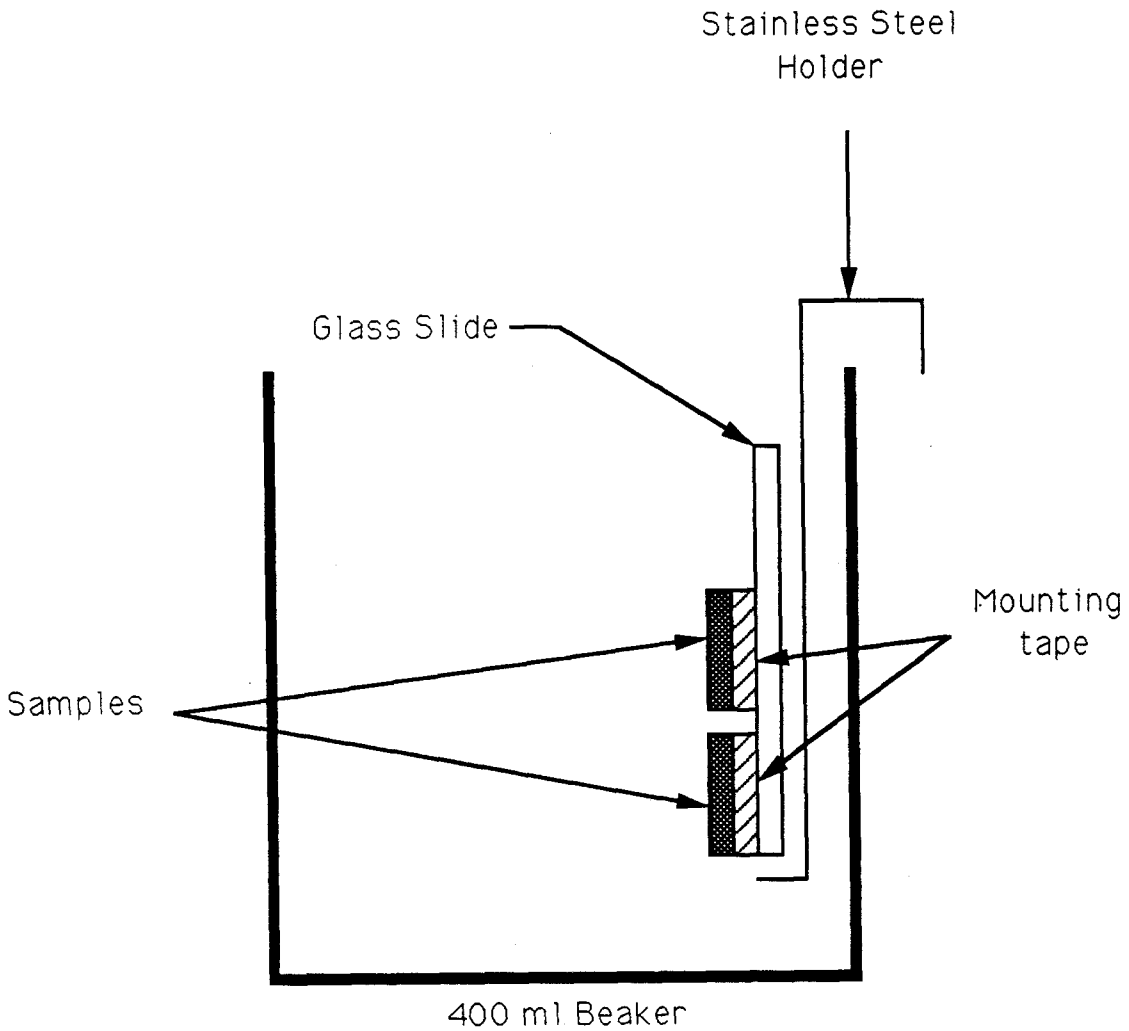

Figure 2. Adsorption Apparatus. Glass slide attached to holder with a paper clip.

pair of optical parameters was correct, the same spot was measured twice and values kept if the separate measurements were equivalent. If successive measurements were not consistent, then the ellipsometer was realigned to obtain a higher intensity and the measurement retaken. Ψ and Δ were resolved into n_f and d using a computer program written in our laboratory, and the amount adsorbed calculated according to the equations presented earlier. The adsorbed mass, m, is typically referred to as Gamma ($\mu g/cm^2$) and shall be hereafter be referred to as Gamma.

RESULTS AND DISCUSSION

Duplicate samples were run simultaneously and denoted "A" and "B". Under each experimental condition, the value of gamma used to represent adsorbed mass present on a particular surface was calculated from the average Ψ and Δ measured for the film upon that surface. This average value of gamma models the adsorbed layer of protein as uniform. Measurements of gamma for a surface shows "surface deviation" caused by the fact that protein does not form a uniform layer. Duplicates run under the same conditions are expected to give average gamma values that are not separated by more than this "surface deviation". The surface deviation was thus used as a type of error criterion for eliminating data that showed discrepancies with developed models (the normalized surface deviation used is 0.10, as determined from experimental data).

A "combined" gamma was calculated by averaging Ψ and Δ for both samples A and B. Tables 1-4 tabulate the values of gamma calculated as described above. Figure 3 provides a comparison of the A, B and combined gamma values for an adsorption run onto a hydrophilic surface. Discrepancies between gamma values indicate experimental error or possible contamination. The "combined" gamma was deemed an adequate representation of the true amount adsorbed onto a surface under a given set of conditions and was used for modeling purposes.

The protein concentrations used in Tables 1 - 4 are the initial protein concentrations. This concentration was deemed an adequate representation of the equilibrium protein concentration based on the

Table 1. Average Gamma Values - pH=3.00, 0.1 M Na^+, T=15.6 °C.

Hydrophobic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.050	1.058	1.117	1.099
0.051	0.446	0.245	0.330
0.100	1.630	1.287	1.456*
0.250	0.927	0.539	0.729
0.500	0.902	0.377	0.587
0.755	0.372	0.244	0.296
1.001	0.740	-	0.740
1.252	0.537	-	0.537
1.501	1.050	1.095	1.046

Hydrophilic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.050	0.310	0.561	0.406
0051	0.288	0.167	0.232
0.100	1.821	1.380	1.027*
0.250	0.395	0.244	0.296
0.500	0.549	0.325	0.404
0.755	0.802	0.155	0.387
1.001	0.553	-	0.553
1.252	0.630	0.409	0.420
1.501	0.554	0.433	0.493

* More than three surface deviations away from the developed model; not used in estimating final Langmuir regression constants.

Table 2. Average Gamma Values - pH=6.60, 0.1 M Na^+, T=15.6 °C.

Hydrophobic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.051	0.217	-	0.217
0.099	1.206	0.503	0.844*
0.100	0.712	0.605	0.717*
0.101	0.945	-	0.945*
0.250	0.765	-	0.765
0.500	0.968	-	0.968
1.001	0.655	-	0.655
1.249	1.103	1.126	1.081
1.499	0.382	0.429	0.398*

Hydrophilic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.051	0.324	-	0.324
0.099	0.233	0.289	0.200
0.100	0.371	0.606	0.387
0.101	0.812	-	0.812
0.250	0.564	-	0.564
0.500	1.183	-	1.183*
0.749	0.275	-	0.275
0.999	0.553	0.275	0.302
1.001	0.871	-	0.871*
1.499	0.516	-	0.516

* More than three surface deviations away from the developed model; not used in estimating final Langmuir regression constants.

Table 3. Average Gamma values - pH=8.90, 0.1 M Na^+, T=15.6 °C.

Hydrophobic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.050	0.426	-	0.426
0.101	0.744	0.505	0.660
0.252	0.424	-	0.424*
0.501	1.037	0.836	0.855
0.503	1.193	0.806	1.140
0.753	0.950	-	0.950
1.001	1.125	-	1.125
1.501	0.734	0.913	0.810

Hydrophilic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.050	0.177	-	0.177
0.101	0.244	0.368	0.232
0.252	0.262	-	0.262
0.501	0.201	0.335	0.231
0.503	0.975	0.371	0.950*
0.753	0.191	-	0.191
1.501	0.321	-	0.321

* More than three surface deviations away from the developed model; not used in estimating final Langmuir regression constants.

Table 4. Average Gamma values - pH=8.90, 0.5 M Na^+, T=15.6 °C.

Hydrophobic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.053	0.406	0.755	0.551
0.076	0.475	-	0.475
0.103	1.047	0.351	0.400
0.252	0.799	0.297	0.521
0.501	0.263	1.059	0.370
0.502	0.603	0.491	0.561
0.757	1.192	0.566	0.680
0.999	0.368	-	0.368
1.489	0.665	-	0.665

Hydrophilic Silicon

[Protein] (mg/ml)	Gamma ($\mu g/cm^2$) A	B	Combined
0.053	0.228	0.539	0.344
0.076	0.289	-	0.289
0.103	0.400	0.174	0.281
0.252	0.200	0.109	0.099*
0.501	0.205	0.743	0.498
0.502	1.339	0.371	0.921*
0.757	0.380	0.288	0.335
0.999	0.141	-	0.141*
1.489	0.261	-	0.261

* More than three surface deviations away from the developed model; not used in estimating final Langmuir regression constants.

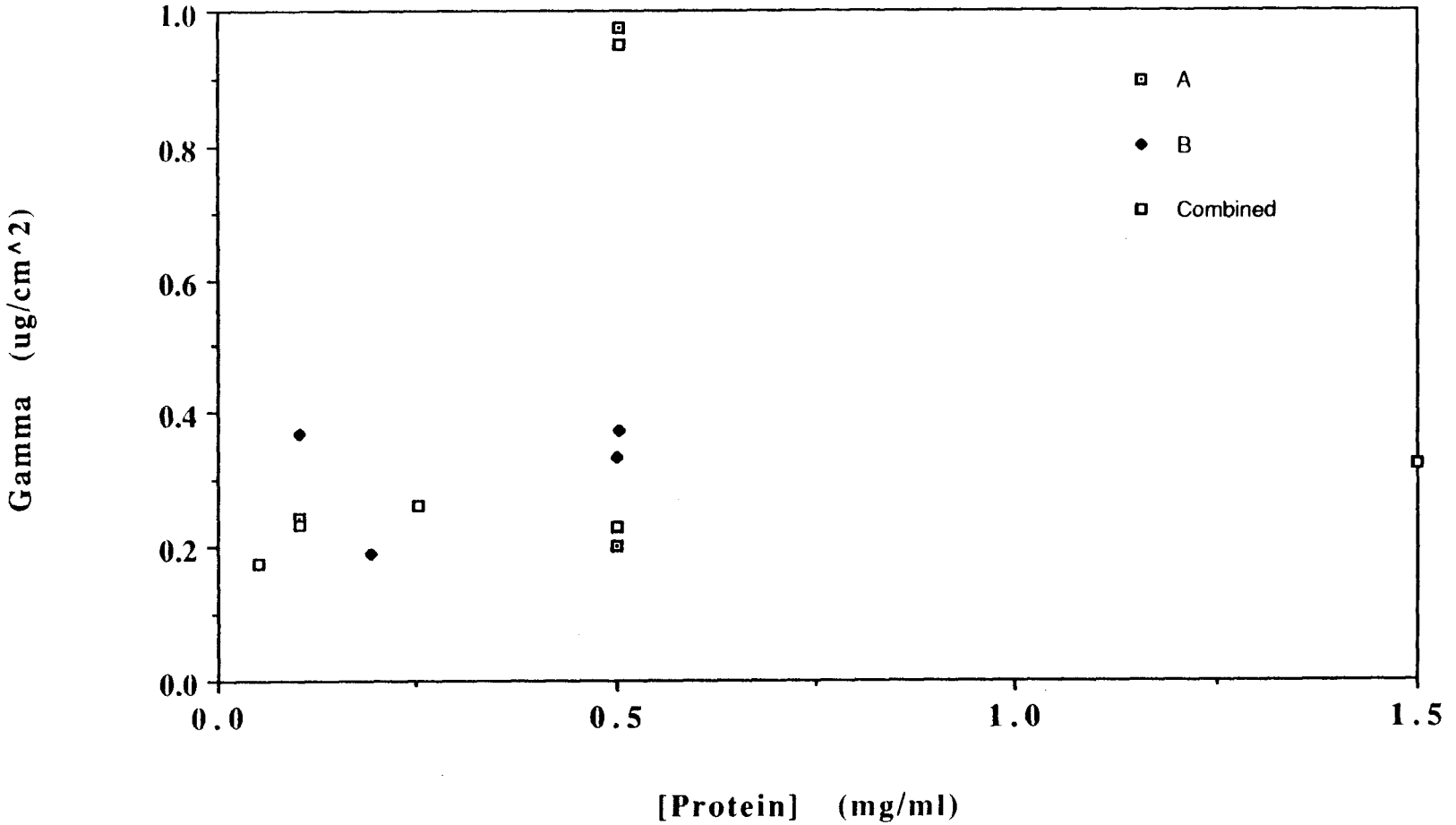

Figure 3. Comparison of Gamma Values. Hydrophilic Silicon - pH=8.90, 0.1 M Na^+, T=15.6°C.

following. Assuming that the slides and holder add 25% to the wetted surface area in a 400 ml beaker (filled with 300 ml of solution) leads to a contact area of approximately 250 cm^2. Using a gamma value of 2 $\mu g/cm^2$ (which is higher than any gamma value recorded) leads to a mass removed from solution of 0.50 mg. The lowest initial concentration used was 0.05 mg/ml, which gives an initial protein mass of 15 mg (for 300 ml of solution). At this concentration, the error in assuming initial [Protein] equal to final [Protein] is 3.3%.

Parameters for the Freundlich equation were obtained from regression of the data in the linear form: ln(gamma) = bln[Protein] + ln(a). The fitted curve was forced through the origin by letting 1 x 10^{-5} approximate zero. Langmuir constants were obtained through regression of the data in the form $(gamma)^{-1}=(b/a)[Protein]^{-1}$ +(1/a). Forcing the Langmuir fit through the origin lead to negative constants, which, according to equation 1, would give a physically meaningless negative adsorbed mass. The data were therefore regressed without forcing the regressed line to go through the origin. Data that was more than three surface deviations away from the developed model curve (starred points in Tables 1-4) were deleted from the regression data set and new parameters calculated.

Figure 4 is representative of the consistent observation associated with comparison of the Freundlich and Langmuir equations to approximate the data. Modeling the data with the Freundlich equation would suggest a continuing increase in gamma as the protein concentration is increased; whereas, gamma levels off in the Langmuir model, which is the case with the experimental data at

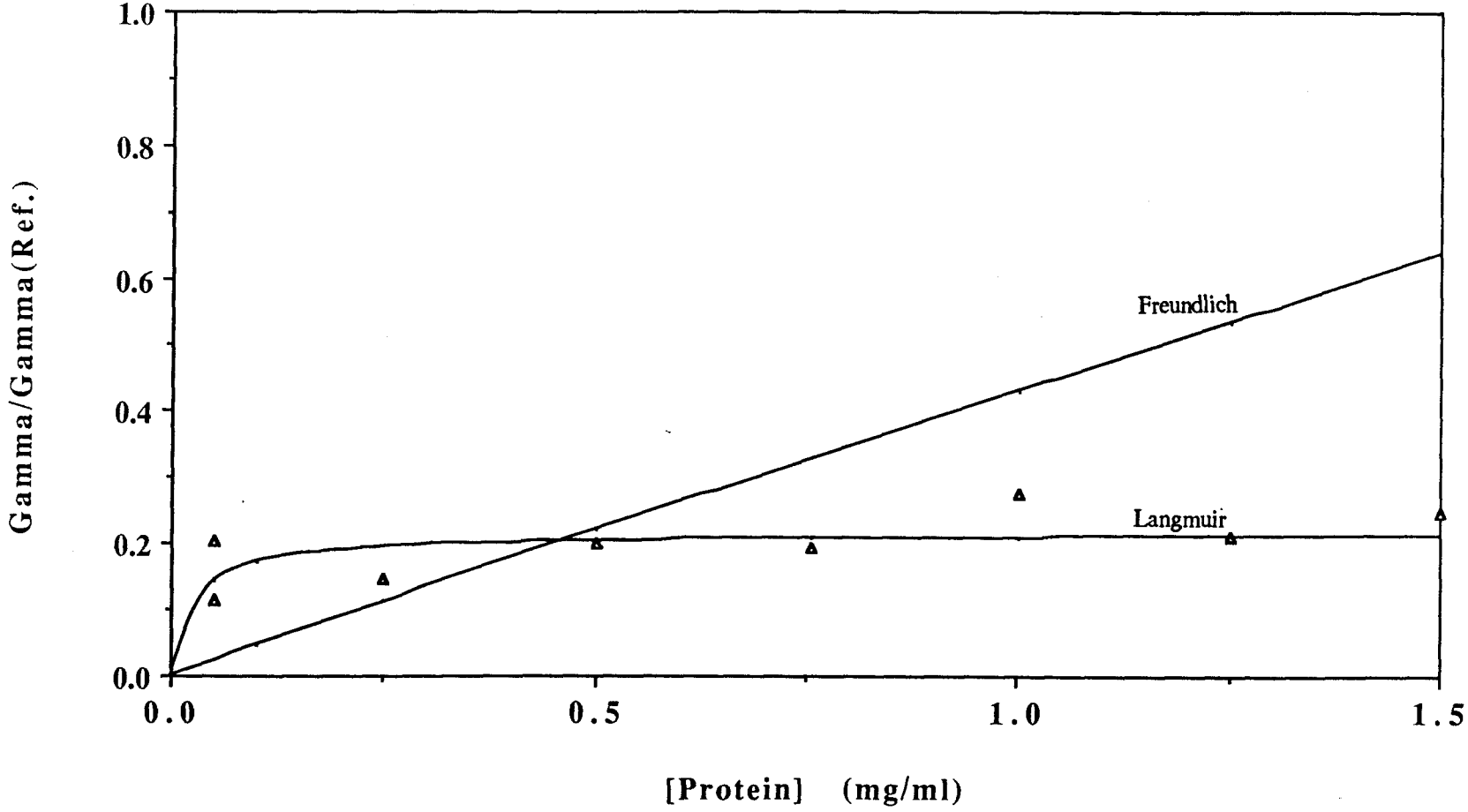

Figure 4. Comparison of Langmuir and Freundlich Equations. Hydrophilic Silicon - pH=3.00, 0.1M Na^+, T=15.6°C. Gamma(Ref.)=2μg/cm^2.

higher protein concentrations. The Langmuir equation is clearly a better representative equation for the data and hence, has been used for comparison of different adsorption conditions. The regression constants for each surface and at each of the four conditions of pH and ionic strength are shown in Table 5. Figures 5 and 6 show how the data for hydrophobic and hydrophilic surfaces fit the Langmuir equation at various experimental conditions.

Figures 7 - 10 use the derived Langmuir equations to compare the relative amount adsorbed onto hydrophobic (HPB) and hydrophilic (HPL) silicon surfaces under varying solution conditions. At all conditions, the plateau value for gamma is greater for HPB than for HPL surfaces. This can be attributed to an entropic driving force

Table 5. Langmuir Regression Constants

Conditions	Surface	a	b
pH=3.00, 0.1M Na^+	Hydrophobic	0.5651	0.0049
	Hydrophilic	0.4292	0.0243
pH=6.60, 0.1M Na^+	Hydrophobic	1.1857	0.2247
	Hydrophilic	0.4054	0.0146
pH=8.90, 0.1M Na^+	Hydrophobic	1.0612	0.0717
	Hydrophilic	0.2547	0.0195
pH=8.90, 0.5M Na^+	Hydrophobic	0.4863	0.0001
	Hydrophilic	0.3273	0.0030

due to the greater hydrophobic interaction supported by HPB as opposed to HPL surfaces. One would expect that at all protein concentrations the amount adsorbed onto HPB surfaces would be greater

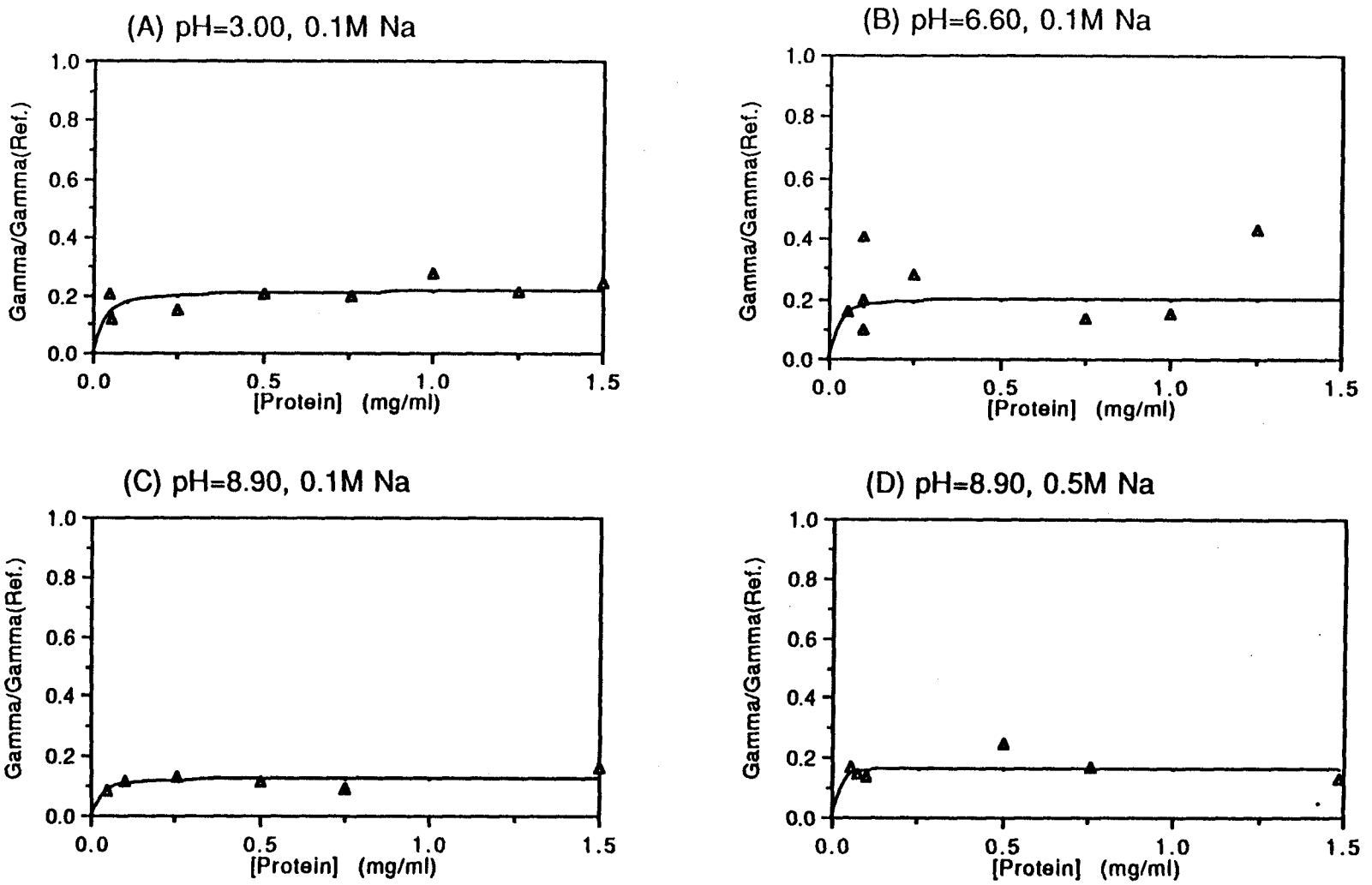

Figure 5. Fit of Data to Langmuir Equation - Hydrophilic Silicon. T=15.6°C. Gamma(Ref.)=2μg/cm^2.

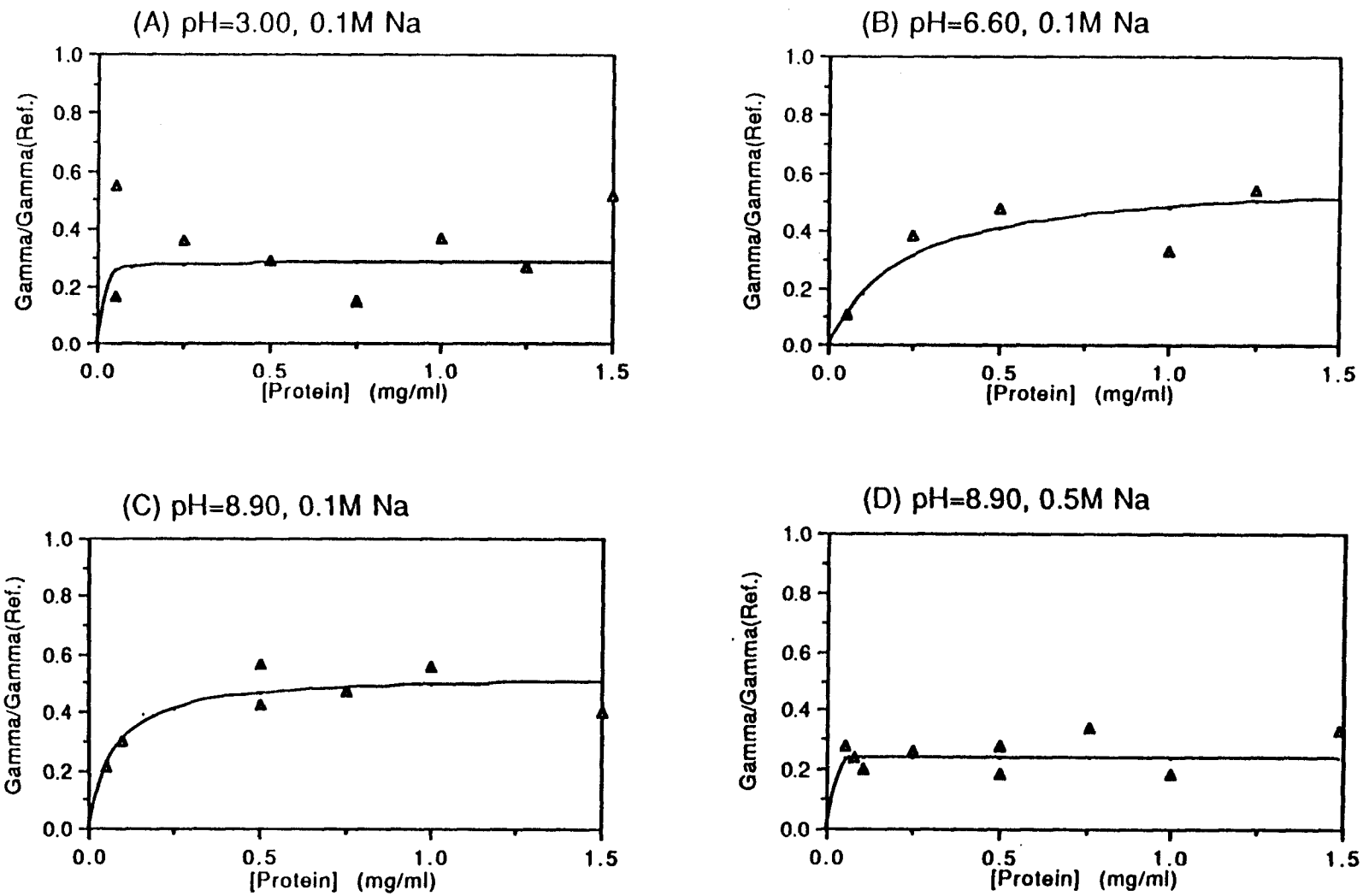

Figure 6. Fit of Data to Langmuir Equation - Hydrophobic Silicon. T=15.6°C. Gamma(Ref.)=2μg/cm^2.

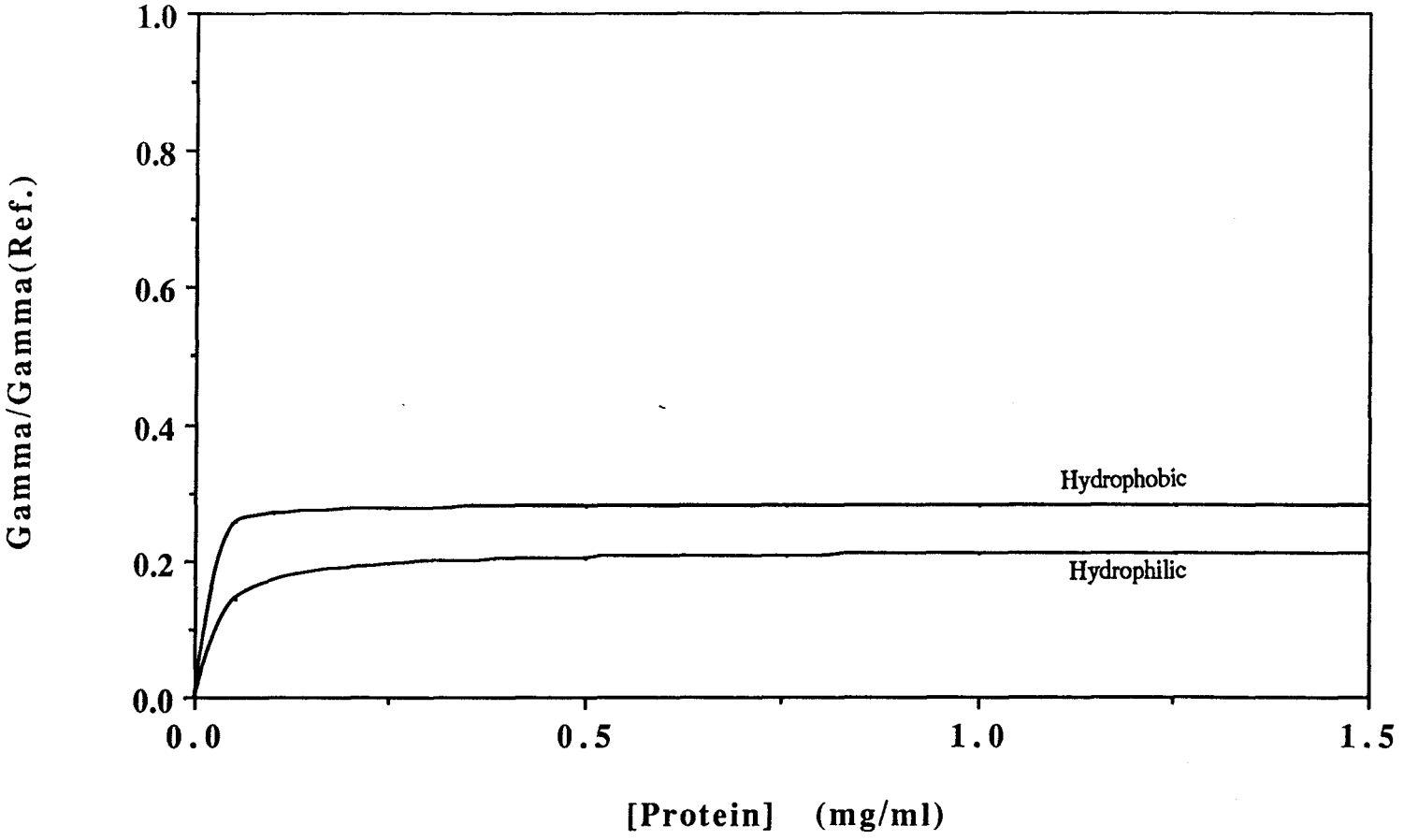

Figure 7. Comparison of Surfaces, pH=3.00, 0.1M Na^+, T=15.6°C. Gamma(Ref.)=2μg/cm^2.

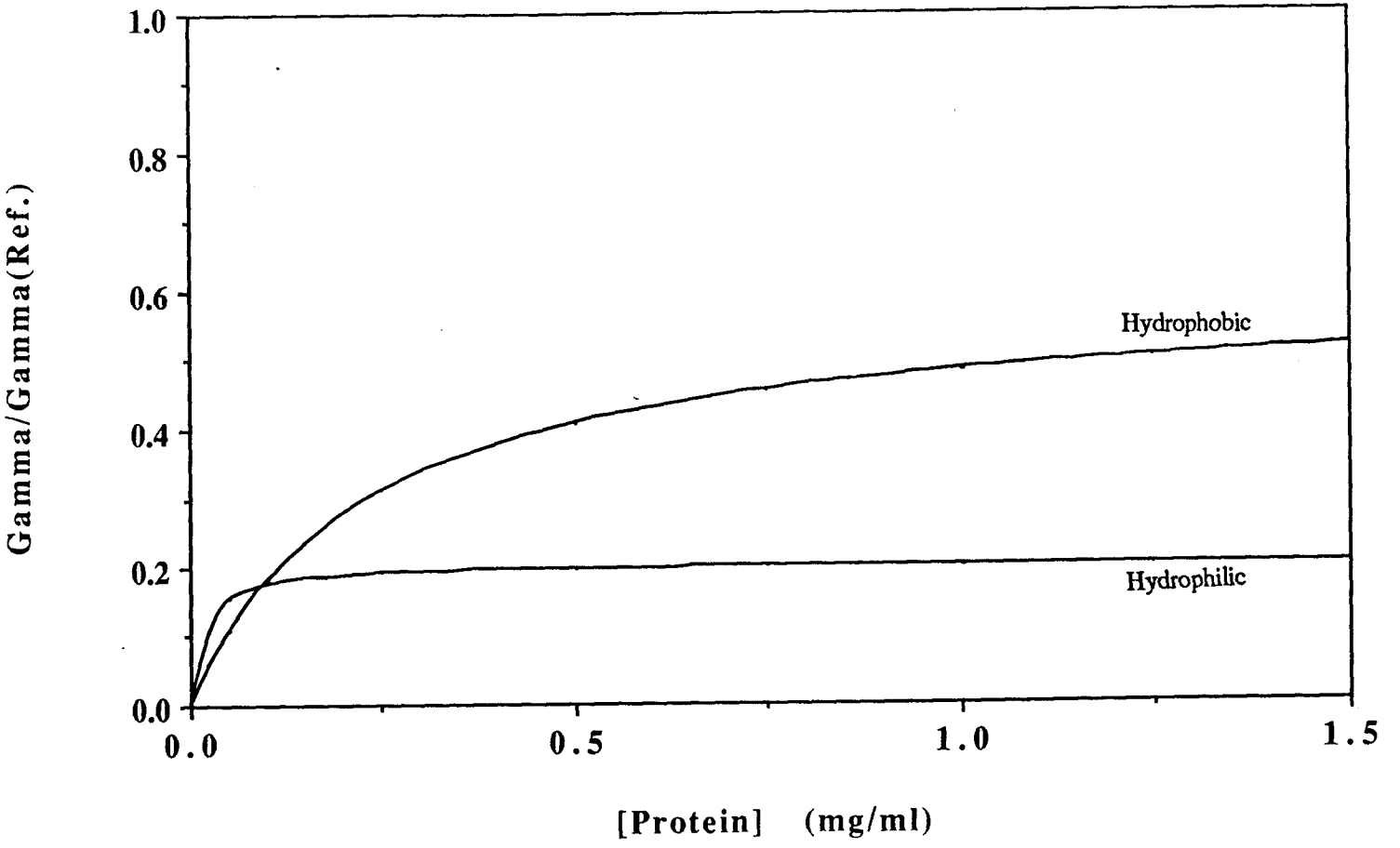

Figure 8. Comparison of Surfaces, pH=6.60, 0.1M Na^+, T=15.6°C. Gamma(Ref.)=2μg/cm^2.

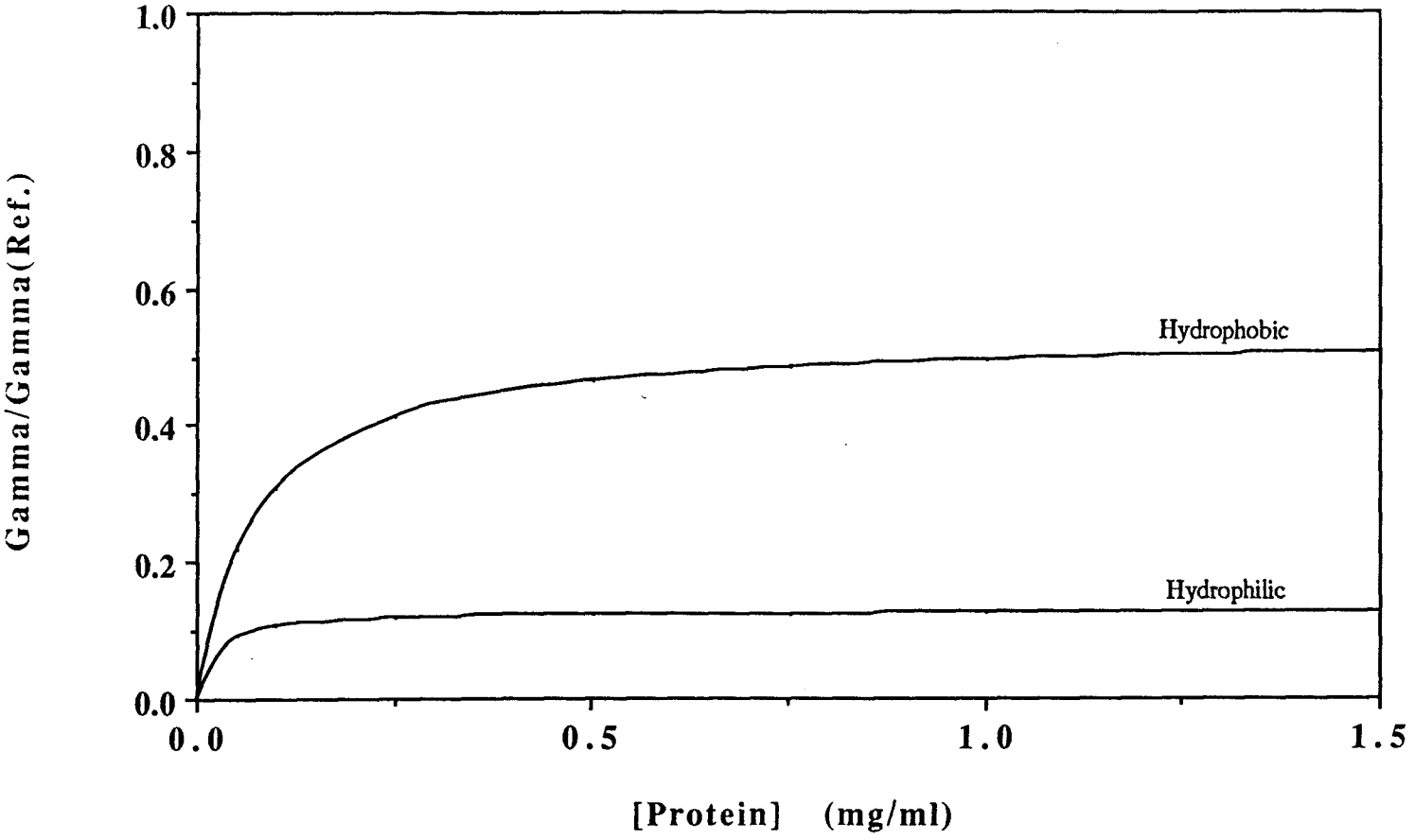

Figure 9. Comparison of Surfaces, pH=8.90, 0.1M Na^+, T=15.6°C. Gamma(Ref.)=2μg/cm^2.

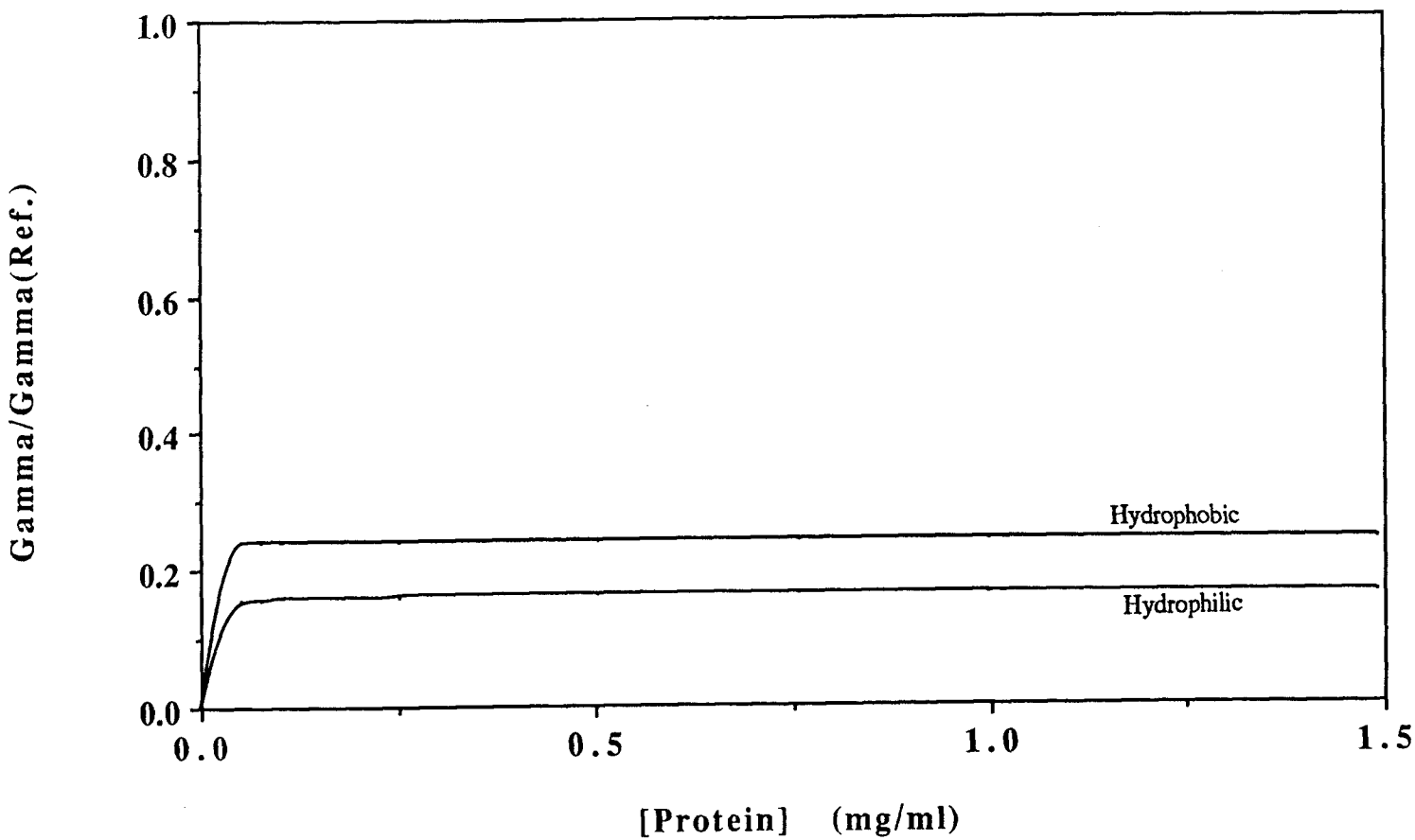

Figure 10. Comparison of Surfaces, pH=8.90, 0.5M Na^+, T=15.6°C. Gamma(Ref.)=2μg/cm^2.

than that onto HPL surfaces. The apparent anomaly seen at low values of concentration in Figure 8 is likely due to experimental error at these concentrations, thus yielding parameters that lead to a lower prediction of gamma for HPB surfaces at low concentrations.

As pH is increased the ratio of plateau values for gamma HPB relative to gamma HPL increases. This phenomenon can be explained by considering the effect of pH on both the electrostatics of protein and its conformation in solution. β-Lactoglobulin has an isoelectric point near 5.1; below this pH the protein has a net positive charge and above this pH the protein has a net negative charge. Based on electrostatics alone, gamma should be expected to decrease as pH increases due to increased repulsion between a protein molecule and a negatively charged surface.

β-Lactoglobulin has three distinct conformations over the pH range of 3.0-8.90. At pH values between 3.5 and 7.5, the protein molecule self-associates to form a dimer. Below pH=3.5 and above pH=7.5, the protein exists as a monomer, with the monomer being more dissociated at the higher pH. These pH induced conformational effects are expected to have a greater impact on hydrophobic interactions as opposed to electrostatic; this is reflected in the greater values of adsorbed mass recorded on HPB surfaces at increasing pH.

Figure 11 illustrates the effect that pH has on gamma for a hydrophilic surface. The significant lowering of gamma for pH=8.90 as compared to lower pH's can be attributed to a substantial increase of negative surface charge attributed to the protein as a result of being at a pH much higher than the isoelectric point. Although the protein molecule has a net positive charge at pH=3.00 and, therefore,

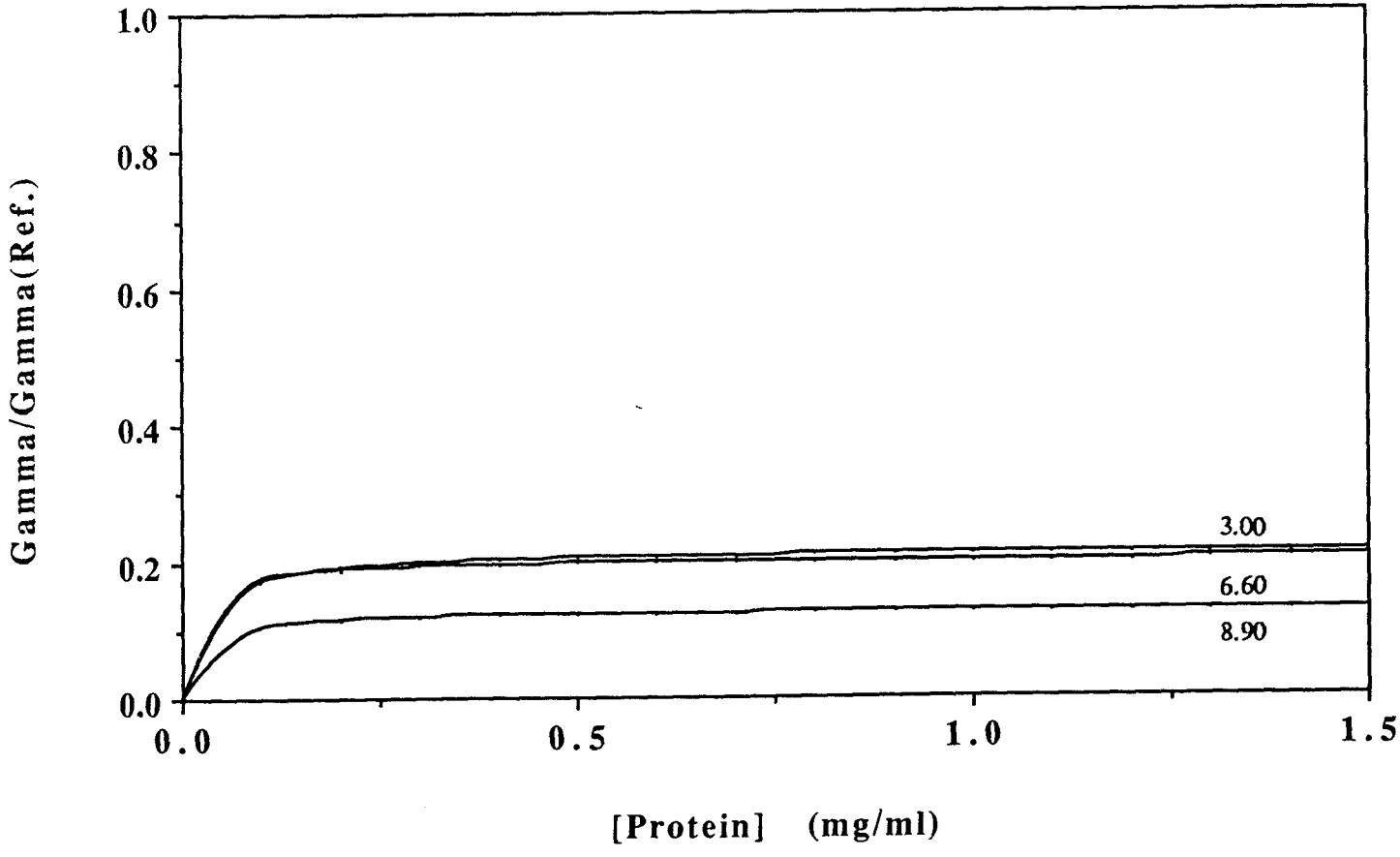

Figure 11. Effect of pH - Hydrophilic Silicon. T=15.6°C.
Gamma(Ref.)=2μg/cm^2.

is attracted to the negatively charged surface, the lateral repulsions may be great enough to lower the plateau value to a value near that of pH=6.60. This might also be explained by an accumulation of protons onto the negatively charged HPL surface; consequently, the positively charged protein molecule may see a "net" positively charged surface. The plateau value at pH=6.60 is expected to be greater than that at pH=8.90 because of a lesser similar charge repulsion with the surface. It should be noted that due to the strong HPL surface affinity for water molecules, the hydrophobic interaction (which would increase the system entropy by displacing water molecules) is not considered to be a major factor in adsorption onto HPL surfaces.

Conversely, the electrostatic interaction on HPB surfaces is minor relative to the hydrophobic interaction. Figure 12 illustrates the effect of pH on adsorbed mass at HPB surfaces. The monomer at pH=8.90 is more dissociated than the monomer at pH=3.00. This higher degree of dissociation leads to more hydrophobic regions of the molecule being exposed to water molecules (a destabilizing effect) and results in a greater amount being adsorbed at pH=8.90. Self-association into a dimer at pH=6.60 should lead to a more stable conformation and a lower value of adsorbed mass. The observed increase of gamma at pH=6.60 relative to pH=3.00 may be due to the surface pH being near the i.e.p..

Figure 13 illustrates the effect of ionic strength on protein adsorption at pH=8.90 for a hydrophilic surface. As cited earlier, pH values greater than the i.e.p. cause a protein molecule to have a net negative charge and thus a lower gamma value due to electrostatic

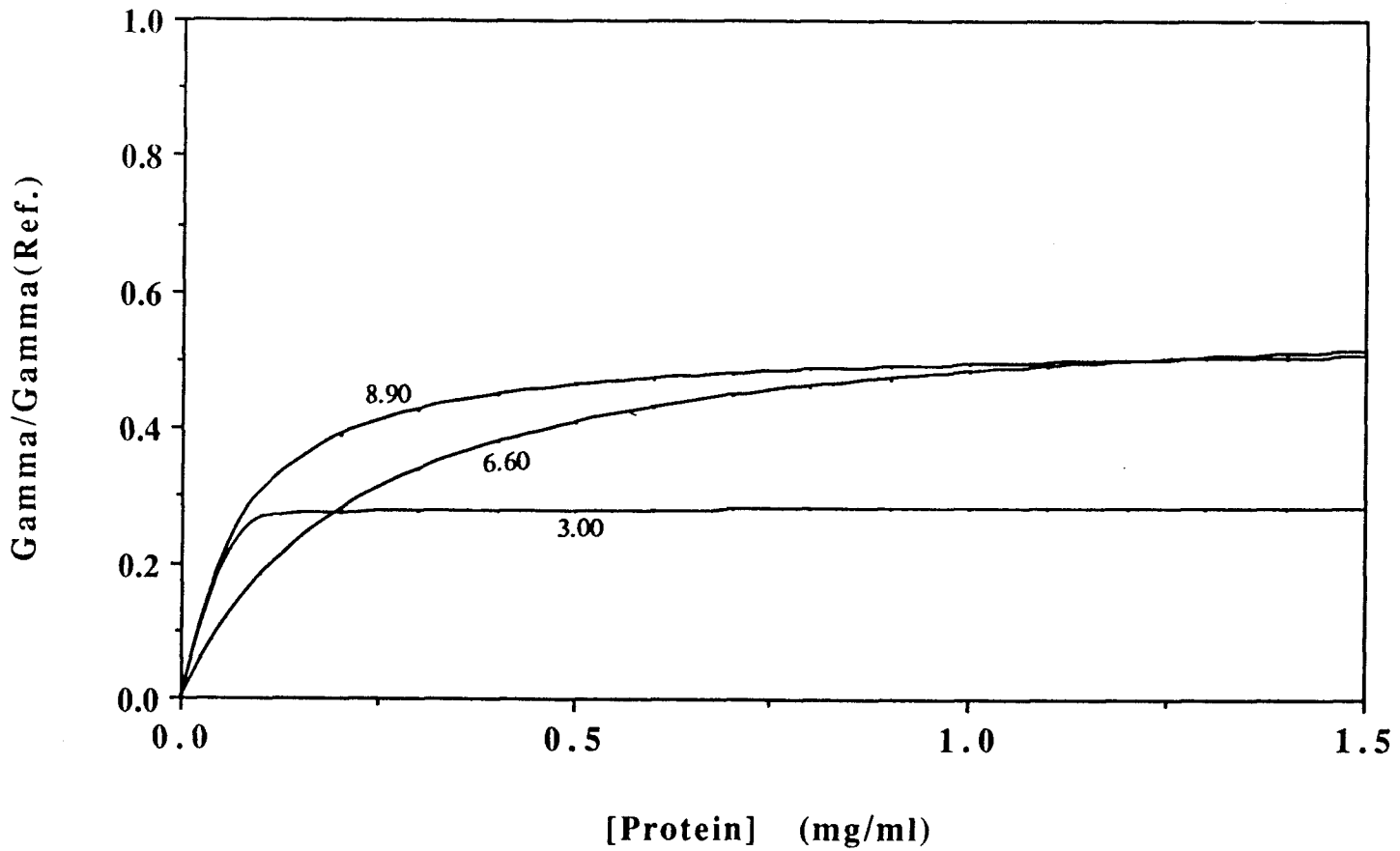

Figure 12. Effect of pH - Hydrophobic Silicon. T=15.6°C.
Gamma(Ref.)=2μg/cm^2

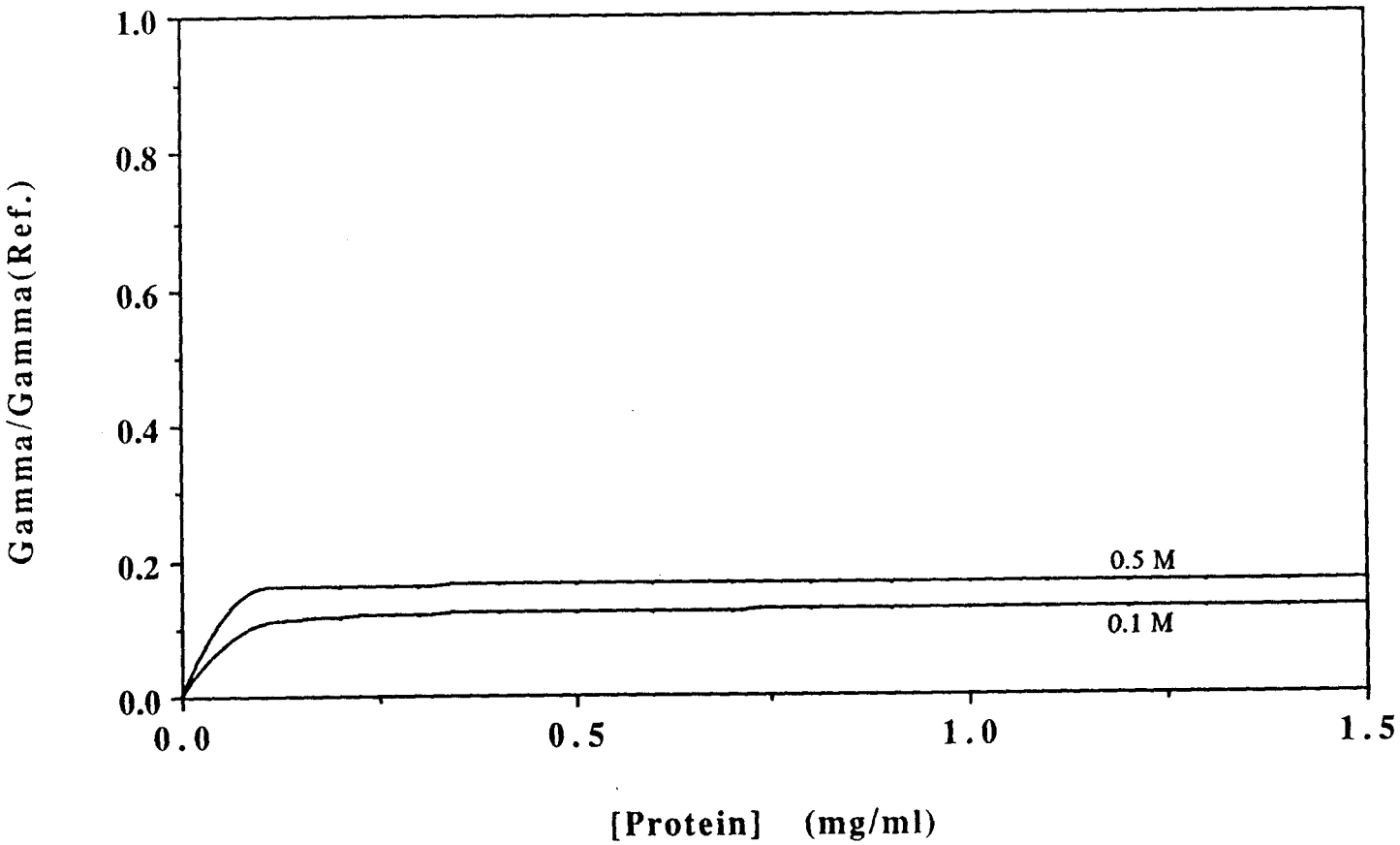

Figure 13. Effect of Ionic Strength - Hydrophilic Silicon.
pH=8.90, T=15.6°C. Gamma(Ref.)=2μg/cm^2.

repulsion with the surface. Increasing the ionic strength of the solution "shields' the protein molecule by increasing the number of positively charged ions surrounding it. Shielding reduces the electrostatic repulsion and is a factor in the observed increase in gamma as ionic strength is increased.

Shielding also has a role in explaining the observed effect of increasing ionic strength on adsorption at HPB surfaces. Figure 14 illustrates a decrease in the plateau amount adsorbed onto a HPB surface as the ionic strength is increased from 0.1 M to 0.5 M Na^+. In this case the observed trend is opposite to that at HPL surfaces, which are influenced more strongly by electrostatics. Because of the high ion content of the solution, it is possible that the protein molecule stabilizes its conformation by displacing water from its surface, and replacing it with sodium ions.

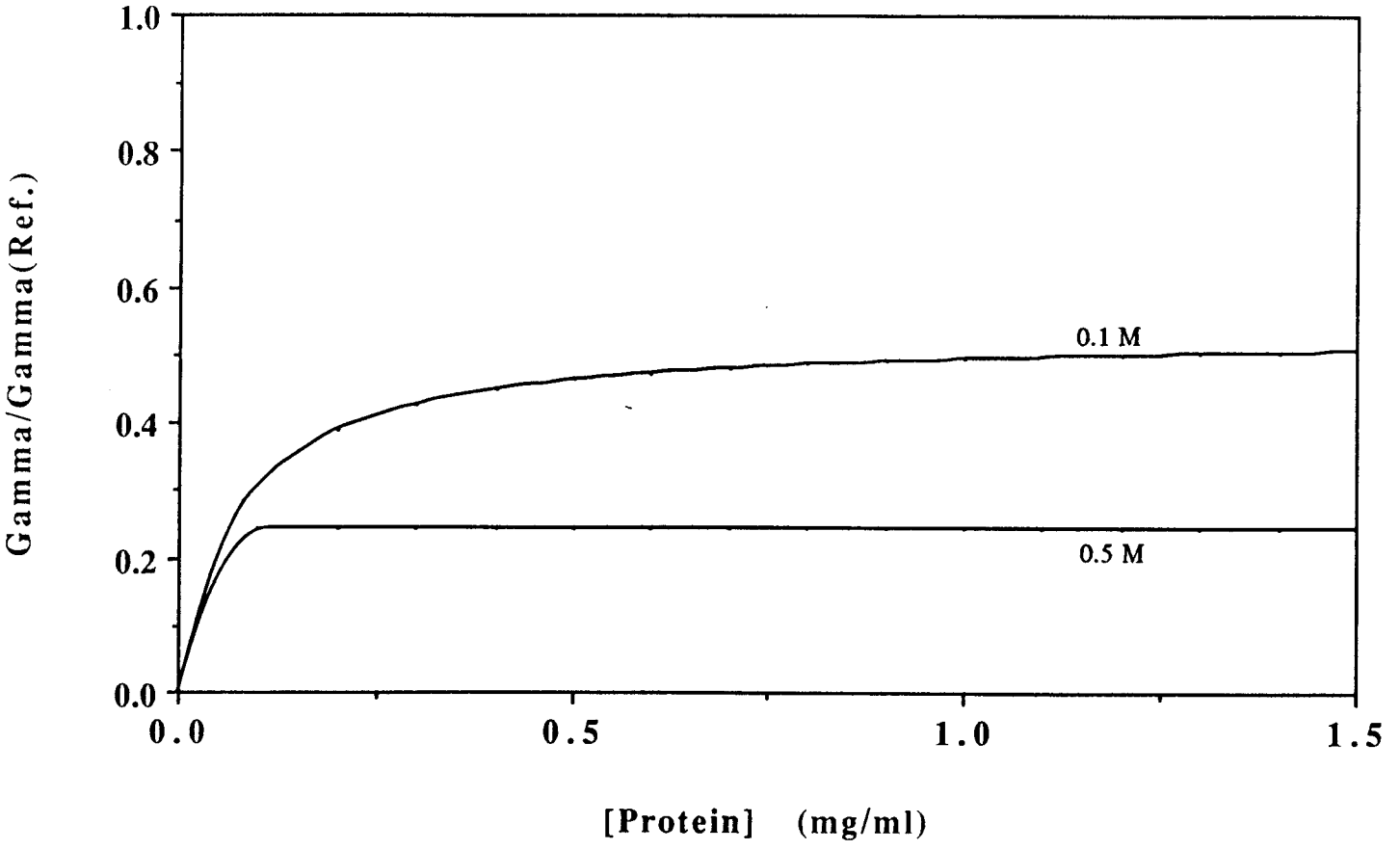

Figure 14. Effect of Ionic Strength - Hydrophobic Silicon. pH=8.90, T=15.6°C. Gamma(Ref.)=2μg/cm^2.

CONCLUSIONS

Ellipsometry is a very useful technique for determining the optical properties of a bare surface or a surface coated by a transparent film. The conversion of these optical properties into adsorbed mass is done quickly and easily by a computer. Adsorbed mass was observed to follow a Langmuir type of relationship with protein concentration. Comparison of the data fitted to Langmuir models at varying experimental conditions led to the following observations:

1) The amount of adsorption onto hydrophobic surfaces was observed to be greater than that at hydrophilic surfaces under all conditions studied.
2) Electrostatic interactions play a more important role than nonelectrostatic interactions in adsorption onto hydrophilic surfaces.
    a) As pH is increased above the isoelectric point, the plateau value of adsorbed mass decreases.
    b) As ionic strength is increased, the plateau value of adsorbed mass increases.
3) Nonelectrostatic interactions play a more important role than electrostatic interactions in adsorption onto hydrophobic surfaces.
    a) Plateau values of adsorbed mass are greatest at pH values where the protein molecule is most unstable.
    b) Effect of ionic strength is opposite to that exhibited by a hydrophilic surface.

RECOMMENDATIONS

The current method rinses off the loose protein from a sample and then lets the sample dry overnight. This method does not remove the majority of the water, as can be seen by the formation of water droplets upon the surface even after attempts have been made to "shake" the water from the surface. These water droplets may become contaminated and leave a film upon drying, or they can be a collection point for protein molecules which are mobile upon the surface. Development of a method to quickly dry the surface may eliminate the formation of foreign films and may result in a more uniform surface coverage.

The use of ellipsometry with dried films should be compared with other methods for determining the adsorbed mass. A method which could work with dried films is radio labeling. This method uses a mixture of labeled and unlabeled proteins and relates the measured radioactivity of a surface to the amount adsorbed onto that surface. Comparison of the values obtained from measurements in solution with those obtained from dried films would also provide a type of internal consistency check.

The specular nature of silicon makes it a very good surface for studies using ellipsometry. Modifications of silicon are very simple and can be easily done without the need for complex experimental equipment. Proposed future work to support the observations seen for the effects of pH, ionic strength, and surface properties on the adsorption of β-lactoglobulin from solution are as follows:

1) For a given pH and ionic strength, vary the degree of hydrophobicity of the solid. For an aqueous solution, it would be expected that plateau values for gamma increase as the surface is made more hydrophobic.
2) At a pH below the isoelectric point, vary the ionic strength. Compared to the observations at high pH, expect the same result for hydrophobic surfaces and the opposite result for hydrophilic surfaces.
3) Vary the polarity of the solvent. Less polar solvents might lead to an observation where the hydrophilic surface adsorbs more than the hydrophobic surface.
4) Increase the number of different pH conditions so as to determine if the isoelectric point gives the maximum amount of adsorbed mass.

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APPENDIX

APPENDIX

Optical Constants of Hydrophobic Silicon

pH=3.00, 0.1 M Na^+, T=15.6C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.050	11.70	144.93	12.02	141.52	11.61	143.23
0.051	10.93	156.79	10.96	158.25	10.95	157.52
0.100	12.72	134.31	12.40	138.13	12.56	136.13
0.250	11.68	145.60	11.02	154.47	11.35	154.47
0.500	11.05	152.39	11.18	154.38	11.12	153.38
0.755	11.01	156.20	10.98	158.23	11.00	157.20
1.001	11.46	148.89	-	-	11.46	148.89
1.252	11.81	148.32	-	-	11.81	148.32
1.501	11.91	142.93	11.86	143.10	11.89	143.03

pH=6.60, 0.1 M Na^+, T=15.6°C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.051	11.01	159.66	-	-	11.01	159.66
0.099	11.53	145.30	11.08	153.94	11.31	149.62
0.100	10.92	155.63	10.94	155.48	10.93	155.56
0.101	10.87	154.04	-	-	10.87	154.04
0.250	11.12	152.30	-	-	11.12	152.30
0.500	11.25	149.08	-	-	11.25	149.08
1.001	10.88	155.53	-	-	10.88	155.53
1.249	11.50	146.02	11.14	149.75	11.32	147.88
1.499	11.26	153.80	11.28	152.92	11.27	153.40

Optical Constants of Hydrophobic Silicon

pH=8.90, 0.1 M Na^+, T=15.6°C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.050	10.87	157.23	-	-	10.87	157.23
0.101	11.29	150.48	11.17	154.14	11.26	151.70
0.252	12.06	153.09	-	-	12.06	153.09
0.501	11.26	148.64	11.30	149.77	11.30	149.35
0.503	10.97	150.75	11.08	152.57	11.01	151.27
0.753	10.98	152.48	-	-	10.98	152.48
1.001	11.19	148.62	-	-	11.19	148.62
1.501	11.45	149.62	11.38	148.15	11.41	148.89

pH=8.90, 0.5 M Na^+, T=15.6°C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.053	11.09	155.19	11.09	152.48	11.09	154.26
0.076	10.97	156.09	-	-	10.97	156.09
0.103	11.16	149.97	11.41	154.23	11.35	152.89
0.252	10.98	154.14	11.14	156.27	11.04	154.98
0.501	11.64	154.62	10.91	152.89	11.28	153.76
0.502	11.17	153.05	10.96	155.56	11.09	154.02
0.757	11.48	146.02	12.03	147.43	11.76	146.72
0.999	11.42	153.67	-	-	11.42	153.67
1.489	11.48	149.04	-	-	11.48	149.04

Optical Constants of Hydrophilic Silicon

pH=3.00, 0.1 M Na^+, T=15.6°C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.050	10.98	157.39	10.99	155.03	10.99	156.21
0.051	10.59	164.17	10.71	162.43	10.65	163.30
0.100	12.77	137.34	12.52	140.11	12.64	138.73
0.250	10.83	159.19	10.75	160.54	10.79	159.87
0.500	11.01	155.37	10.97	157.22	10.99	156.30
0.755	10.56	159.14	10.81	163.02	10.69	161.08
1.001	11.32	151.29	-	-	11.32	151.29
1.252	11.18	152.57	11.55	153.53	11.36	153.05
1.501	11.54	149.51	11.22	153.47	11.38	151.49

pH=6.60, 0.1 M Na^+, T=15.6°C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.051	10.61	163.53	-	-	10.61	163.53
0.099	10.83	165.58	11.49	159.8	11.16	162.69
0.100	11.09	155.64	11.21	152.32	10.93	155.56
0.101	11.08	152.40	-	-	11.08	152.40
0.250	10.87	157.44	-	-	10.87	157.44
0.500	11.57	145.39	-	-	11.57	145.39
0.749	11.02	158.16	-	-	11.02	158.16
0.999	10.81	157.61	11.06	157.99	10.94	157.80
1.001	11.02	152.65	-	-	11.02	152.65
1.499	10.83	158.44	-	-	10.83	158.44

Optical Constants of Hydrophilic Silicon

pH=8.90, 0.1 M Na^+, T=15.6°C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.050	10.57	166.19	-	-	10.57	166.19
0.101	10.95	159.53	10.79	160.13	10.90	159.72
0.252	10.85	159.48	-	-	10.85	159.48
0.501	10.92	160.78	10.62	162.78	10.77	161.78
0.503	10.60	157.95	10.77	160.49	10.60	157.27
0.753	10.89	161.41	-	-	10.89	161.41
1.501	11.00	157.14	-	-	11.00	157.14

pH=8.90, 0.5 M Na^+, T=15.6°C

[Protein] (mg/ml)	Average Ψ(A)	Average Δ(A)	Average Ψ(B)	Average Δ(B)	Average Ψ(Comb.)	Average Δ(Comb.)
0.053	10.57	165.26	10.71	159.71	10.62	163.18
0.076	10.67	163.20	-	-	10.67	163.20
0.103	10.58	162.11	10.75	162.01	10.69	162.04
0.252	10.61	165.08	10.77	165.00	10.68	165.05
0.501	10.91	160.84	10.62	158.54	10.77	159.69
0.502	10.69	152.40	10.79	159.77	10.72	155.44
0.757	11.23	154.41	11.11	157.47	11.17	155.94
0.999	11.13	164.75	-	-	11.13	164.75
1.489	11.29	159.15	-	-	11.29	159.15

Location of Further Information

The raw data, laboratory notebook and complete bibliography for this investigation are in the possession of Dr. Joseph McGuire, Department of Agricultural Engineering, Oregon State University, Corvallis, Oregon 97331. Questions and requests for further information should thus be directed toward Dr. McGuire.