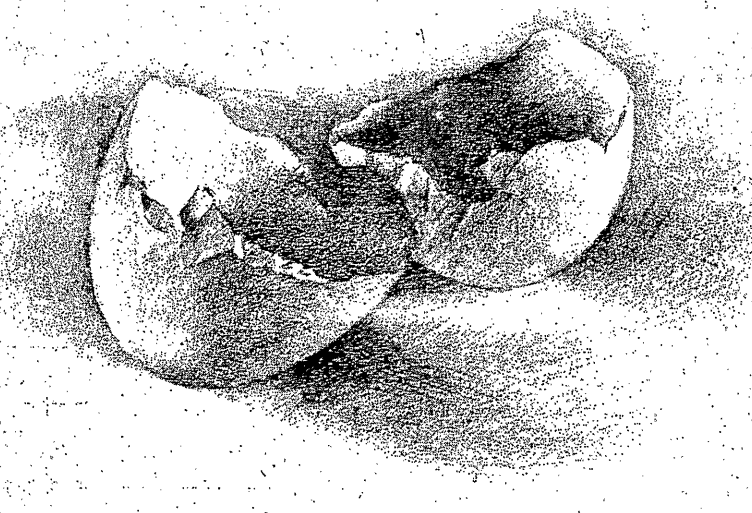
adsorption of plasma proteins

adsorption behaviour on apolar surfaces and effect on colloid stability



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ADSORPTION OF PLASMA PROTEINS

ADSORPTION BEHAVIOUR ON APOLAR SURFACES AND EFFECT ON COLLOID STABILITY

PROEFSCHRIFT

ter verkrijging van de graad van doctor
in de technische wetenschappen
aan de Technische Hogeschool Twente,
op gezag van de rector magnificus,
prof. dr. I.W. van Spiegel, volgens
besluit van het College van Dekanen in het
openbaar te verdedigen op donderdag
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CONTENTS

CHAPTER I

INTRODUCTION

I.1 GENERAL

Centuries ago man already made use of the properties of proteins at interfaces. The old Egyptians used proteins to stabilize their inks, and our ancestors in the countries along the North Sea were very familiar with the beautifull foams on well brewed beer. Life without proteins cannot be imagined e.g. think about bleeding of wounds if fibrinogen would not be available to produce a protecting clot.

In the last century scientists started to study the behaviour of proteins at interfaces. In 1840 Ascherson[1] noticed that oil droplets suspended in albumin solutions had a tough skin or film formed spontaneously around them and he suggested such droplets as models for living cells. In 1851 Melsens[2] showed that an insoluble coagulum of protein was formed when protein solutions were shaken. From then on up to now numerous scientists studied the adsorption and behaviour of proteins at interfaces. The knowledge born from these studies is applied in food industry, cosmetic industry, pharmaceutical industry etc. Recently, the last 10-15 years, protein adsorption got a revival of interest from the side of bioengineers. With the increasing use of prosthetic materials in the body and the accompanying undesired blood-clotting it is essential to know how nonbiological surfaces will interact with biological media[3]. It is believed by many investigators that adsorption of proteins is the initial event that happens when blood is contacted with foreign surfaces and that all subsequent events are determined at least in part by the properties of the adsorbed protein-layer. In particular it has been found that adsorption of different proteins has a different effect on platelet adhesion[4,5], which in itself is believed to be a prerequisite to thrombus formation. Adsorption of albumin has been shown to give a reduced platelet adhesion, while fibrinogen greatly enhances it[5], γ globulin activates the so called release reaction[6]. The stability of protein coated colloidal particles

got attention from immunologists. Dispersion of γ-globulin coated polystyrene latex particles have been employed in agglutination procedures for the detection of "Rheumatoid Factor" macroglobulins[7].

Although a lot of work has been done on protein adsorption and the properties of the proteins at interfaces, literature still contains many articles contradicting on essential points. A few of these points are: the reversibility of the adsorption, the conformation of the molecules at the interface, the adsorption kinetics, the preference in adsorption from mixtures of proteins etc. In this introduction we will give a short review of the existing literature on the behaviour of proteins at interfaces. We will confine ourselves mainly to the literature about albumin and fibrinogen. The behaviour of these proteins at air/water, oil/water and solid/water interfaces will be discussed.

I.2 PROPERTIES OF THE PROTEINS

I.2.1 Albumin

Albumin is the most abundant plasma protein. Human and bovine plasma for instance contain about 70 grams of protein per litre and about half of this amount consists of albumin. Albumin is not very specific but it has a lot of physiologic functions[8] such as regulation of the osmotic pressure and the pH, transport of metal-ions and phospholipids etc. It is known for about one century now. The easy way of purification, the relatively high homogeneity, the high concentration level in blood, the osmotic and transport functions and the slow denaturation made it an often used model protein in biochemical and physico-chemical research. The high stability of albumin permits the use of conditions in which most plasma-proteins have denatured. Owing largely to the deligent work of Brown et al[9] the amino acid sequences of both bovine and human albumin are known.

HSA consists of 8 amino acids and has a theoretical mol. weight of 66.248. Higher values are often obtained because it may contain some dimers[8,10] or because some phospholipids are

attached to the molecules. Although albumin is relatively pure, about 20 genetic variants are known and the composition may vary upon illness[8,11]. In particular with commercially available albumin prepared from pooled blood, microheterogeneity will occur. The secundary and tertiary structure of the albumin molecule are only partly known and they are pH-dependent. Between pH = 4.3 and 10.5 the molecule has its "compact" shape[12] and contains about 60% α helices and β-structure and 40% random coil[8,10] it consists of 3 parts of about equal size[8]. Outside this pH-region the molecule swells due to internal electrostatic repulsion and the α helices become partially unfolded[10,12].

Measurements of the electrophoretic mobility around pH = 4 show two distinct mobilities. Upon further decrease of pH, one of these mobilities disappears. This is called the N - F (Normal - Fast) transition of the albumin. Upon this reversible transition a lot of physical parameters change such as: intrinsic viscosity optical rotation, electrophoretic mobility, percentage of α helix and the solubility[10]. Between pH = 7 and 9 there should be a transition too, the neutral transition, quite similar to the N - F transition, but changes are much more gradually[10] then.

From hydrodynamic measurements it is known that the overall shape of the molecule is a prolate ellipsoid. The exact lengths of the minor and major axes are not known[8,10,13]. They are assumed to be about 4 and 14 nm. Champagne [13] measured and calculated the dimensions of the ellipsoids as a function of pH. He argues that at the isoelectric point (i.e.p.), when the molecule contains equal numbers of positive and negative charges, the molecule should be spherical. At very extreme pH-values the molecule may denature[14].

I.2.2 Fibrinogen[15,16].

Fibrinogen has a much lower concentration in plasma (2 - 4 grams per litre) than albumin. It is one of the least soluble of the proteins in plasma. Fibrinogen plays an important role in the blood-clotting process; In models proposed for the mechanism of blood coagulation it is called coagulation Factor I. Under the influence of thrombin smaller peptides are splitt from

the fibrinogen molecules, the remaining fibrin monomers do polymerize spontaneously to fibrin polymer which forms a clott that stops the bleeding.

The primary structure of about one third (500) of the 1500 fragments in which fibrinogen can be splitt is known. It is expected[15] that within a few years the total primary strcuture is elucidated. Fibrinogen consists of about 2900 amino acids and contains about 33% α-helices. About the tertiary and quaternary structure there is not very much agreement. Generally it is assumed that the molecule consists of two identical parts which each contain three chains. They are held together with S=S bonds. Each of the chains has a molecular weight of about 50.000. A very well known model is the three nodular model of Hall and Slayter[17]. Perhaps the best study on the shape of fibrinogen has been carried out by Lederer and Bachmann[18,19,20]. They concluded that the shape of a fibrinogen molecule should be cylinderlike with minor and major dimensions of 9 and 45 nm. The overall shape of the fibrinogen molecule is of course pH dependant. About 18 genetic variants of the molecule are known.

Fibrinogen and fibrin as foam and bioplast are applied during operations as blood-clotting agents. It can be implanted without danger and it is broken down within a few weeks by the body.

TABLE I

Some physical parameters and contstants of HSA and HFb

	HSA	HFb
Concentration in plasma ($kg.m^{-3}$)	35 - 40	2 - 4.5
Molecular weight	66.248 (calc)	340.000
Minor and Major axes (nm)	4 and 14	9 and 45
Isoelectric point (pH units)	4.5 - 5.0	5.5
Diffusion coefficient in H_2O, 293 K ($m^2.s^{-1}$)	$6.1 * 10^{-11}$	$1.5\text{-}2.0 * 10^{-11}$
Intrinsic viscosity ($m^3.kg^{-1}$)	$4.2 * 10^{-3}$	$25 * 10^{-3}$
Percentage α-helix	48	33
Percentage β-structure	15	-
Half life time in vivo	19 days	6 - 8 hours
Extinction coefficient at 280 nm ($kg.m^{-3}$)	0.53	1.55

I.3 AIR/WATER INTERFACES

I.3.1 Spread protein layers.

Almost all studies of proteins at the air/water interface contain measurements of the surface pressure π ($mN.m^{-1}$) of the protein films. The films can be obtained by spreading on an aqueous phase containing no protein or by adsorption from a bulk aqueous phase containing protein. Neurath and Bull[21] made the same distinction in an extensive review on the surface activity of proteins. Other important early reviews are from Langmuir and Schaeffer[22] and Bull[23]. These authors already found evidence to conclude that spread monolayers of proteins are irreversibly adsorbed at the air/water interface, a situation for which Gibbs' adsorption equation cannot be applied.

Muramatzu[24], using Trurnit's[25] method of spreading, found a complete unfolding of bovine serum albumin (BSA) molecules at the A/W interface. He could calculate a molecular weight of 65000 $\pm$ 5000 for these molecules by extrapolation of the πA vs π curve to π is zero (A = surface area for a fixed amount of spread protein). This indicates that at extremely low π-values the surface film can be described by the two dimensional ideal gas law, $\pi A = nRT$.

Collapse of monolayers

Many authors[22,24,26-30] find a linear relation between π and A at albumin surface pressures of 5-20 $mN.m^{-1}$. Above this pressure, collapse of the films occurs. MacRitchie[31] showed that collapse occurred reversibly at low surface pressures. At higher pressures a coagulum of protein could be seen to separate out on the surface which did not respread on decompression and could not be dissolved in water. Later experiments[32] showed that upon shaking solutions of BSA in 3 or 6 M salt also an insoluble coagulum was formed whereas solutions in distilled water showed no coagulum on shaking. The insolubility of the coagulum shows that the protein in this coagulum has a conformation which differs from the native form. Spreading experiments[30] showed that monoloayers of HSA become more extended upon adding NaCl to the

substrate; this finding agrees with the experiments discussed on shaking solutions[32]. Ageing of the spread layer causes a shift of the π-A curve to larger A values[30]. The surface viscosity of the films appeared to increase with decreasing expansion of the film.

Equations of state

Ruyssen[28] applied the Singer[33] equation

$$\pi = \frac{kT}{\sigma_o} \ln \left(1 - \frac{A_o}{A}\right) + \frac{kT}{\sigma_o} \frac{t-1}{t} \frac{z}{2} \ln \left(1 - \frac{2}{z} \frac{A_o}{A}\right)$$

wherein: t: the total number of segments (amino acid units)
z: the surface coordination number
For a completely rigid chain z=2 and for a completely random chain z=4 thus $2 \leq z \leq 4$

to describe the π-A curve for different proteins which presupposes, analogous to Lucassen-Reynders and Van den Tempel[34] Flory and Huggins[35] and Frisch and Simha[36], that the limiting areas occupied by a solvent molecule (water) and a surfactant molecule or a segment of the polymer chain (amino acid) are equal. For BSA (pH = 4.9) Ruyssen found a surface coordination number z = 2.15, indicating rather rigid chains. At lower surface areas the π-A behaviour of BSA could not be described satisfactorily due to irreversible callapse. Birdi[29] found a linear relation between the work of compression

$$W_c = \int_{A_{initial}}^{A_{final}} \pi . d A$$

and the amount of protein at the surface for both, completely unfolded (BSA) and partially unfolded (Transferrin) proteins. From these plots he also determined the amount of protein lost in the subphase. Using Huggin's[37] virial type equation of state and the Frisch and Simha[36] equations he found that f_m (the avarage fraction of polymer segments in the surface monolayer) is always less than one (from 0.4 for myoglobin to 0.97 for BSA) for proteins[38]. High f_m values gave high values for

W_c and relatively low collapse pressures. The value of f_m decreased upon increasing the amount of protein at the surface indicating less unfolding.

Structure of the monolayer

Evans et al[43] and Mitchell et al[44] used π-c and π-A curves to study spread protein layers; π-c curves are obtained by successive addition of protein to a constant area ($c = A^{-1}$ = quantity per unit of area) and π-A curves are obtained by compression of an initially spread layer. They found that the π-c^{-1} and π-A curves for β-casein coincide and that they differ considerably for BSA. This shows that the conformation of a compressed, initially completely spread BSA layer differs from that of a not completely spread layer. Birdi[39] found a qualitative relation between the ratio of polar and apolar amino acids in the proteins and the degree of unfolding. Proteins with a low polar/apolar ratio (< 1.3) should unfold and those with a high ratio should not completely unfold where BSA with a ratio of 1.56 is an exception and unfolds completely in spite of its 17 S=S bonds. The secundary structure of proteins is not completely destroyed by surface denaturation because irradiation of egg-albumin layers causes a further spreading of the proteins[40]. The limiting area per residue in a helical structure is not significantly different from that in the β-structure[41], indicating that a value of 0.17 $(nm)^2$ is no proof of the degree of unfolding. Scheller et al[42] found that not all reducible groups of adsorbed proteins (at the mercury/water interface) come into contact with the electrode indicating incompletely unfolding. So the proteins may be unfolded but helical structure and interchain bond may remain intact to some extent.

I.3.2 Absorbed protein layers

It is clear that the constitution of an adsorbed protein monolayer is different from that of a spread one. Proteins adsorbing at a surface which already contains protein molecules will have to spread against their own surface pressure. Spreading

experiments[43,44] showed, as discussed, a different π-A behaviour for proteins spread against a surface pressure compared to initially completely spread layers. MacRitchie et al[45] studied the adsorption of different proteins at the air/water interface by measuring the surface viscosity and elasticity. They estimated the adsorbed amount in terms of monolayer thickness using previous results with spread layers which is not allowed because of the different conformation. MacRitchie and Alexander[55,56,57] also studied the kinetics of protein adsorption at the A/W interface. Again they estimate the adsorbed amount from spread film measurements. At low surface pressures (< 0.1 mNm^{-1}) they found that the kinetics could be described by $n = 2\, C_b \left(\frac{Dt}{\pi}\right)^{\frac{1}{2}}$ [58,59] indicating diffusion controlled adsorption without adsorption barrier. (n = number of molecules per unit area which are adsorbed at a time t after creation of a fresh surface, C_b = the bulk concentration of protein, D = the diffusion coefficient of the protein, π = 3.14). When a certain surface pressure has been built up, the adsorption is retarded. Introduction of an energy-term $\pi.\Delta A$ (π = surface pressure, ΔA = the mean clean surface area necessary to facilitate adsorption of one molecule) leaded to the conclusion that protein molecules need a clean surface area of 1 - 1.75 $(nm)^2$ for adsorption. They[57] also found that salt addition at pH-values away from the i.e.p. accelerated the adsorption, indicating the role of an electrical adsorption barrier. Ghosh and Bull[46] determined π as a function of pH and salt concentration; they found a maximum in π at the isoelectric point of BSA which becomes less pronounced with increasing salt concentrations. The lower adsorption of proteins at pH values far away from the isoelectric point, without salt may be due to electrostatic repulsion between the molecules or between the molecule segments. Addition of salt will diminish this repulsion.

Equations of state

Joos[47] and Ruyssen[28] applied the Frisch and Simha[40] equations to the linear part of the π - log c (c = concentration of protein in the bulk) curve and found that BSA molecules at the i.e.p. (pH = 4.9) are adsorbed with only 11.3 (Joos) or 9.5

(Ruyssen) amino acid segments at the surface when adsorbed from 0.1 M NaCl solutions and with 16.3 (Joos) or 13.7 (Ruyssen) segments when adsorbed from distilled water. The application of the Frisch and Simha equations is very questionable because of the irreversible character of the adsorption. The decrease in number of adsorbed segments is not in line with the finding that proteins spread further upon salt addition to the bulk[30]. Joos[60] obtained an equation of state for polymers at interfaces by equating the chemical potentials of the solvent in bulk and surface phases. This approach in itself is much better than the assumption of total equilibrium, but no surprising results were obtained.

Adsorption steps and reversibility

Bull[48] studied surface films of egg albumin adsorbed from 1M Na_2SO_4 and found a two step film pressure curve with respect to protein concentration. The first step extends to a concentration of $4 * 10^{-2}$ kg.m^{-3} at a pressure of 12 m Nm^{-1} followed by a transition region up to c = 0.2 kg.m^{-3} where π = 25 m Nm^{-1}. The same type of 2 step film pressure - protein concentration curve was obtained for BSA in 0.1 M NaCl. He argued that the 2 step curve is characteristic of the transformation of a surface denatured protein film to a film containing native protein molecules. The egg albumin (max. adsorption $4 * 10^{-6}$ kg.m^{-2}) appeared to be adsorbed reversibly except for the first $1.8 * 10^{-6}$ kg. m^{-2}, which was found to agree with the two step surface pressure concentration curve. It appeared from the adsorbed π-A curve for egg albumin that the adsorbed film is significantly more compressed than the corresponding spread film. This finding partially invalidates the calibration of the film-transfer technique which was used to determine the amount of protein adsorbed at the surface.

In 1947 Neurath and Bull[21] already found evidence to conclude that egg albumin adsorbed in two layers, a first completely denaturated layer of 1 nm thickness and below it a layer of more or less spherical, native molecules which is about 4 nm thick.

Gonzales and MacRitchie[61] reported surface pressure measurements with adsorbed BSA layers from which

they concluded that the adsorption of the protein at the A/W interface was reversible. They found desorption from spread monolayers to be dependent on the protein concentration of the bulk-phase, and also that adsorbed layers, after compression on decompression, returned to the "Equilibrium" surface-pressure. It should be stressed here that all experiments were performed at rather high (> 20 m Nm^{-1}) pressures. Earlier MacRitchie[31,32] explained the loss of surface area by reversible collapse or interfacial coagulation. Applying Gibbs' adsorption isotherm to adsorbed protein layers yielded a statistical kinetic unit of 2,5 $(nm)^2$ for BSA. Miller and Bach in a review article[41] also raised some questions to the conclusions of MacRitchie and stressed the posibility that at least part of the protein molecules was adsorbed irreversibly.

Surface viscosity measurements

El-Shimi and Izmailova[49] found that the maximum strength of an adsorbed egg albumin layer increased with increasing temperature and, at constant temperature, is independent of the concentration in the range of 0.5 - 10 $kg.m^3$, which is in agreement with Bull[48]. Adsorbed layers with an age of 900 seconds showed a dependence on pH with maximum strength at the i.e.p. This pH dependency was diminished by KCl addition and by further ageing of the films. The further strengthening with time was ascribed to denaturation and formation of intermolecular hydrophobic bonds. Chasovnikova and Trapeznikov[30] found that at HSA concentrations of $2 * 10^{-2}$ $kg\ m^{-3}$ the surface viscosity reached a constant value within 2 hours. At HSA concentrations of 0.98 $kg.m^{-3}$ the surface viscosity was much higher and a stationary value is not attained even in 6 hours. They concluded that at low concentrations a surface film of unfolded molecules was formed whereas at high HSA concentrations the adsorption layer contains a large number of native molecules. Tulovskaya et al[50] also studied the behaviour of absorbed HSA layers and found that the "Equilibrium" value of the surface tension was reached within 30 minutes while the "equilibrium" surface strength was reached in 3 hours. This indicates that the surface-strenght

method is very sensitive to conformational changes and the formation of intermolecular bonds, The higest strength was obtained at the i.e.p. although the molecules are less uncoiled They also concluded that hydrophobic bonds participate in the formation of a strong two-dimensional structure.

Special techniques

Graham and Phillips[51] studied protein adsorption using radiotracer techniques although it was shown by Adams et al[52] that acetylation of just one residue per molecule may significantly alter the surface properties of a protein. They found[51,53] that at low surface converage, theories based on polymer statistics describe the relation between filmpressure, dilatational modulus and surface coverage satisfactorily for a random coil protein like β-casein. Graham et al[51], Benjamins et al[53] and de Feyter et al[54] used ellipsometry as a tool to study protein adsorption at air/water interfaces. They found that the kinetics in the beginning of the adsorption process are fully diffusion controlled.

I.4 THE OIL/WATER INTERFACE

The oil/water interface is particularly studied in relation with emulsion stability and its determining factors. Further it is of interest for those who are dealing with biological systems where oil/water interfaces play an important role.

Structure and orientation

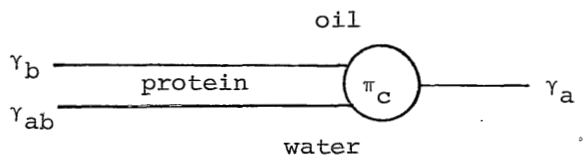
Bull[33] using the pendant drop method, studied the interfacial tension between n-octadecane and aqueous solutions of BSA. He observed that at the O/W interface the interfacial tension sooner reached a constant value than at the A/W interface. It is evident that the protein molecules in an interface are oriented with the polar groups directed towards the aqueous phase and the nonpolar side chains towards the air or the oil-phase. At the O/W interface the cohesion between the non-polar side chains may be reduced by the oil, thus increasing the rate at which the protein molecules relax. Analogous to the finding

at the A/W interface he found π to be dependent on the pH, which effect was diminished by salt addition. From measurements at different temperatures he calculated enthalpy and entropy changes per unit of interface. This is a very questionable thing to do regarding the irreversible character of protein adsorption. From the similarity of the compressibility coefficients of the protein film for a given film pressure irrespective if the concentration of the BSA solutions, he concluded the structure of the film to be a function of the film pressure only and not of the protein concentration. Gosh et al[62] found that proteins gave higher interfacial pressures at paraffin-water than A/W interfaces.

Model applications

Ruyssen[28] and Joos[47], applying Frisch and Simha's equations[36] calculated the number of adsorbed segments of BSA at high surface coverage at the heptane/water interface. They calculated a number of 3.3 (Ruyssen) or 4 (Joos) adsorbed segments per molecule compared to respectively 9.5 and 11.3 segments per molecule under the same conditions at the A/W interface. From this result one should conclude that contrary to other observations the protein molecules are more denaturated at the A/W than at the O/W interface. Hence the application of Frisch and Simha's equations is very questionable regarding the irreversible character of protein adsorption.

MacRitchie and Owens[32] applied a duplex film model to the coagulation pressure π_c of adsorbed egg albumin layers: $\gamma_a = \gamma_b + \gamma_{ab} + \pi_c$ with γ_a = initial free energy of the O/W interface, γ_b = the initial free energy between the nonpolar groups of the monolayer and the non-aqueous phase, γ_{ab} = the interfacial free energy between the polar groups and the aqueous phase.



It appeared that π_c decreased with decreasing γ_a. From this observation one cannot conclude that at interfaces having a low interfacial tension a molecule will not denature. It should be remembered that Birdi[38] found low collapse pressures (π_c) for high f_m values. Ghosh and Bull[46] found no decrease in the amount of BSA adsorbed on n-octadecane emulsions upon addition of octadecylamine (which lowers γ_a).

Interphase strength

El-Shimi and Izmailova[49] reported that the strenghtening of an adsorbed egg albumin layer was faster at a benzene/water (B/W) interface than at an A/W interface. Further the adsorbed layer at a B/W interface was stronger and showed a dependence on the protein concentration up to 10 $kg.m^{-3}$, particularly at 55^oC, a temperature close to the volume denaturation of egg albumin solutions. The dependence on pH was the same as already discussed in the A/W section. They[49] concluded that the protein molecules uncoil further at the B/W than at the A/W interface due to penetration of the hydrocarbon in the nonpolar region of the protein molecules, making them more flexible. Study of the solubilization of hydrocarbons by proteins[63,65] learned that at the interface with the hydrocarbons showing the highest solubilization, the strongest protein films were formed. Tulovskaya et al[50] found a more extensive uncoiling of HSA at a B/W interface than at an interface with air which was also reported by Motomura[64]. From the temperature dependence of the interphase strength they[50] concluded that the strengthening of the adsorbed HSA films is due to hydrophobic bonds between the molecules in the interface.

I.5 THE SOLID/WATER INTERFACE.

The adsorption of proteins at S/L interfaces was studied initially in relation with column chromatography. Landsteiner and Uhlirz[66] reported in 1905 that inorganic powders removed a high percentage of protein from horse serum.

Langmuir adsorption and reversibility

Neurath and Bull[21] reviewed the early literature on this subject. Among others they mentioned the work of Lindau and Rhodius[67] who found that egg albumin adsorbed on quartz powder followed Langmuirs adsorption isotherm. Maximum adsorption was found[67] at the i.e.p. of the protein. The reviewers[21] considered it rather remarkable that protein adsorption, which is apparently irreversible, can be described in terms of the Langmuir adsorption equation.

Bull[68,69] studying BSA and egg albumin adsorption on powdered Pyrex glass, using a depletion technique, found that protein adsorption was partially reversible upon dilution. Maximum adsorption was obtained at the i.e.p. of the protein. He assumed that the amount of protein that could not be washed off was unfolded at the glass surface.

Lyman et al[70] reported a complete reversibility of protein adsorption on cellulose (Cuprophane) and a decreasing adsorption of proteins with increasing molecular size. The dependence on molecular weight in particular is very unusual because normally one expects a more tightly binding of larger molecules due to more adsorption sites per molecule. Lyman used infrared internal reflection spectroscopy (IRIS) to measure the amount of protein adsorbed on a surface. Brash and Lyman[71], using the same technique, found that proteins (HSA, human fibrinogen (HFb), human γ globulin (HGG)) adsorb irreversibly on hydrophobic surfaces. From the amounts adsorbed and the molecular dimensions of the proteins they conclude that the protein molecules retain their native globular form. They stressed this behaviour in an extensive review article[3] on protein adsorption. Other authors[72,73] also reported that protein adsorption onto hydrophobic polymers is completely irreversible upon dilution. MacRitchie[74] found that BSA adsorption at hydrophobic and hydrophilic silica was irreversible at the i.e.p. of the protein and reversible at pH-values away from the i.e.p. Dezelić et al[75] performed a HSA-adsorption study on polystyrene latices (PSL) and described their results as a kind of reversible

interaction between the protein molecules and the detergent onto the polystyrene particles. Oreskes and Singer[76] reported that the adsorption of HGG at PSL fitted the Langmuir adsorption isotherm with a two step adsorption. They gave two interpretations for the phenomenon that two binding constants exist. The first being a double layer adsorption and the second interpretation being in terms of side-on adsorption for the first step and end-on adsorption in the interstices between the side-on oriented molecules for the second step. Lee and Kim[77] reported that bovine albumin, γ globulin and fibrinogen adsorption isotherms on hydrophobic polymer surfaces were consistent with Langmuir type adsorption. It should be realised that applying the Langmuir adsorption isotherm to describe adsorption, implies that the adsorption is completely reversible and that no interactions take place between adsorbed molecules. We therefore agree with Neurath and Bull[21] that it is remarkable that protein adsorption on hydrophobic surfaces which is apparently irreversible can be described by the Langmuir adsorption equation. We will discuss this in more detail when we report our own results on protein adsorption. Up to now it is not clear if and under which circumstances protein adsorption is reversible or not. Of interest with respect to this question are the experiments reported by Brash et al[78] who found that HSA molecules adsorbed at polymer surfaces did not desorb upon exposure to protein free solutions but that, upon exposure to concentrated not labeled HSA solutions, exchange occurred of the adsorbed radiolabeled proteins (I^{131}-label) with the nonlabeled molecules from solution. However it should be kept in mind that Reed and Rossall[79] already noticed that iodine introduced in the position ortho to the phenolic hydroxyl group of tyrosine residues could change the properties in general and surface behaviour in particular of the labeled protein. We will report[80] on the difference in adsorption behaviour between labeled and unlabeled HSA in this thesis. Brash et al[78] did not really proof that there was no preferential selection of labeled or unlabeled molecules by the surface because their results scatter to much. The posibility of label exchange in stead of HSA-

molecule exchange should not be omitted to.

Conformation of adsorbed proteins

Almost all authors mentioned in the fore-going concluded from the maximum amounts of protein adsorbed and the molecular dimensions of the protein molecules that the protein molecules remain their native shape upon adsorption at S/L interfaces. Morrissey et al.[81] and Morrissey and Stromberg[82] concluded from infrared bound fraction measurements and ellipsometry that the internal bonding of HSA and prothrombin molecules is strong enough to prevent changes in conformation while adsorbed, even at low surface coverage. The bound fraction of HFb however increased with increasing adsorption, suggesting possible interfacial aggregation.

Adsorption kinetics

The kinetics of protein adsorption are not studied very well although several authors[77,83,84] show that kinetics are dependent on the type of protein and the substrate. It is likely that the initial adsorption on uncharged surfaces will be diffusion controlled whereas at higher surface concentrations interactions between the adsorbed and the adsorbing protein molecules will play a role. Kim and Lee[77,85] showed that the rate and the amount of protein adsorbed was dependent also on the flow rate of the solution. It is obvious that stirring will affect the rate of adsorption but it is not immediately clear why it should affect the amount of protein adsorbed.

Competitive adsorption

Vroman et al[86] did a lot of work on protein adsorption at S/L interfaces. The authors mostly studied adsorption from very complex systems like platelet rich or poor plasma or whole blood or serum in order to elucidate the mechanism of contact activation of the clotting. Using antisera in ellipsometric studies they found that HSA was not adsorbed at silicon surfaces from plasma. The adsorbed matter, from plasma consists

of HFb and 7s Gamma Globulins. It is striking that using mixtures of purified proteins, the albumin could compete effectively with HFb in adsorption. Having a solution containing four times as much HSA as HFb, both proteins have an equal chance of adsorption. Horbett and Hoffman[71] using radiolabeled proteins also found that the ratio HSA/HFb should be about four in order to get equal adsorptions of HSA and HFb from a mixture of these proteins. Lee et al[87] performed protein adsorption studies with a mixture of BSA, BFb and bovine gamma globuline (BGG) with a concentration ratio of 4 : 2 : 1. Their results are comparable with those mentioned. Additionally they found that the kinetics of adsorption of BFb and BGG on FEP (fluorinated ethylene/propylene copolymer) are influenced by the presence of BSA. It must be remarked that the adsorption results of Kim and Lee[85] obtained with single protein solutions using ^{125}I-labeled proteins are completely different to those reported by the same authors using the IRIS technique. This difference may be due to systematic errors in the techniques or to differences in the used protein samples. Horbett and Hoffman[84] showed that adsorption results obtained with different albumin samples show considerable changes in amount adsorbed. Recently Brash and Uniyal[88] studied competitive adsorption from mixtures of HSA and HFb. They found a more pronounced preferential adsorption of HFb compared to HSA than the authors cited above. It should be kept in mind however that they used both proteins in the labeled (^{125}I or ^{131}I) form and it is very questionable whether this might not change the adsorption behaviour (particularly of the unstable HFb).

I.6 OBJECTIVES OF THE PRESENT STUDY

This study was undertaken to get more information about the behaviour of proteins at interfaces. In chapter II adsorption experiments with HSA and HFb using surface tension measurements, ellipsometry and depletion techniques are described. These experiments give information about kinetics, reversibility upon dilution and increase of concentration and pH changes on

the protein adsorption. Chapter III deals with a study about the applicability of radiolabeled proteins in adsorption experiments. Chapters IV and V describe the influence of protein adsorption on the wettability of polystyrene surfaces and especially the difference between adsorbed HSA and HFb layers. Chapters VI and VII finally deal with the influence of HSA and HFb on the stability of polystyrene latices at different pH values, and for various concentrations of indifferent electrolyte.

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CHAPTER II

ADSORPTION OF HUMAN SERUM ALBUMIN AND HUMAN FIBRINOGEN AT HYDROPHOBIC SURFACES

ABSTRACT

The adsorption of proteins (human serum albumin (HSA) and human fibrinogen (HFb)) at hydrophobic surfaces was studied by different techniques: measurement of the interfacial pressure of adsorbed protein layers at the paraffin oil/water (O/W) interface, measurement of adsorption isotherms onto polystyrene latex (PSL) using a depletion technique and ellipsometric measurements of adsorbed protein on polystyrene (PS) coated chromium surfaces. For both proteins a pH-dependence of the adsorption was found, with maximum adsorption at the isoelectric point (i.e.p.) of the protein. This pH-dependence was greatly diminished by salt addition. The adsorption of HFb onto P.S. at low concentrations was found to be diffusion controlled (ellipsometry). The adsorption of HSA onto hydrophobic surfaces is found to be semi-reversible with respect to protein concentration and pH: additional adsorption after a first adsorption step at low protein concentration (with finite residual solution concentration) was possible by increase of the protein concentration in solution; when the protein concentration in solution was decreased after a first adsorption step at higher concentration, no desorption of the already adsorbed protein molecules was found. It was shown that changing the pH after an adsorption step in the direction of the i.e.p. caused additional adsorption; when the initial adsorption took place at the i.e.p. no desorption was observed upon changing the pH. The adsorption of HFb showed a more genuine irreversible character (high affinity type of isotherm). The thickness of adsorbed HFb layers which was measured by ellipsometry agreed very well with earlier calculations of this thickness from flocculation experiments.

II.1 INTRODUCTION

The adsorption of proteins at interfaces is a very intensively studied subject in science. These studies have their importance for the food industry, the cosmetic industry, health industry etc. The last 10 to 15 years protein adsorption got the interest from the side of bioengeneers. With the use of prosthetic materials in the body they met the problem of undesired blood clotting due to the interaction between blood and the non biological surfaces. The first thing to happen upon contacting blood with foreign surfaces will be adsorption of blood proteins at these surfaces. The so called contact activation of the clotting mechanism which then follows will at least in part depend on the character of the adsorbed protein layer. It has been found that surfaces precoated with albumin show a reduced platelet adhesion[1,2] (presumably platelet adhesion is a prerequisite to thrombus formation) while precoating with fibrinogen greatly enhances it and γ-globulin activates the release reaction[2,3].

Some authors tried to find rules to predict the blood compatibility of non biological surfaces. First Lampert[4] postulated the rule that the more hydrophobic the material is, the more blood compatible it is ('Lampert's rule'). Later on Lyman[5] stated that materials with the lowest surface energy should be the most blood compatible ones. Andrade[6] at last postulated that blood compatibility should increase with decreasing interfacial energy between the material and the aqueous phase. He suggested that hydrogels should be favourable prosthetic materials.

In order to get a better sight on the first events occurring when blood contacts foreign materials it is necessary to study protein adsorption at interfaces. The literature contains extensive reviews on protein adsorption[7,8,9,10] and its relation to the activation of the clotting mechanism[11]. As we already showed[12], there is still a lot of confusion about protein adsorption. A general finding is that protein adsorption is pH-dependent and that maximum adsorption occurs at the iso-

electric point (i.e.p.) of the protein, whereas this maximum becomes less pronounced upon salt addition. The reversibility of the adsorption phenomenon itself and the influence of changing bulk conditions on adsorbed protein layers are still open for discussion. From the literature[12] there seem to be indications that the adsorption at hydrophilic surfaces is more of a reversible type than that at hydrophobic surfaces.

In this article we will confine ourselves to adsorption from aqueous protein solutions onto hydrophobic surfaces. We studied the adsorption of proteins by three different techniques: 1) Measurements of the interfacial tension between protein solutions and paraffin oil. 2) Adsorptions measured by a depletion technique, using polystyrene latex as the substrate. 3) Ellipsometry, to monitor the adsorption of proteins on polystyrene coated surfaces.

It should be noted here that measurements of the interfacial tension cannot be interpreted in a quantitative way. The relation between the interfacial tension, the bulk concentration and the adsorbed amount of protein is not clear because of the irreversible nature of the adsorption. The well-known Gibbs equation cannot be applied. Other theories giving relations between bulk and surface concentrations in terms of distributions of the size of 'loops' and 'trains' cannot be applied either, because they all concern the adsorption of random polymers and the proteins which we use (human serum albumin (HSA), human fibrinogen (HFb)) are highly structured. Silberberg[13] states that it is likely that the adsorbed segment layer predominates in determining the value of the interfacial pressure (π). We shall confine ourselves to conclusions in terms of segment densities in the interface. High values of π (low values of the interfacial tension) indicating high densities and low values of π indicating low densities.

With the adsorption measurements by depletion it is possible to measure the absolute amounts of protein adsorbed under certain conditions. The use of ellipsometry allows us to follow the adsorption as a function of time which cannot be done by the depletion technique because each measurement takes too much

time.

In this paper we will study the reversibility of the adsorption upon concentration variations, the influence of pH-changes on already adsorbed layers, the influence of salt addition on the adsorption and the kinetics of the adsorption.

II.2 EXPERIMENTAL

II.2.1 Materials

Human serum albumin (HSA), crystalline from Sigma (no. A9511) was used without further purification. *Human fibrinogen (HFb)* from Kabi, Stockholm (grade L, 90% clottable) was used after dialysing overnight against twice distilled water at pH = 9, T = 278 K). *Polystyrene Latex (PSL)*, was prepared by the method of Goodwin *et al.*[14], particle diameter $0.56.10^{-6}$ m, ζ-potential -72 mV in distilled water (pH = 7.0). *Polystyrene (PS)*, Mw = 670.000, Mw/Mn = 1.15, was obtained from Pressure Chemical Company, Pittsburgh, Mellon Institute, Special Polystyrene Standard, lot. no. 13A. *Paraffin Oil (P.O.)* was obtained from Merck, DAB 7, NF XIV. Before use it was twice purified by percolating over an Al_2O_3 column. The initial value of the interfacial tension between P.O. and twice distilled water was 51.0 ± 1.0 $mN.m^{-1}$ and this value did not decrease more than 1.5 $mM.m^{-1}$ after one day of standing. Buffer solutions were prepared by adding an aqueous solution of 0.01 M KH_2PO_4 to an aqueous solution of 0.01 M NaOH until the desired pH value was reached.

The protein solutions were stored at a temperature of 277 K and were used within one day after preparation. All chemicals used were analytical grade.

II.2.2 Methods

II.2.2.1 *Measurements of interfacial tensions at O/W interfaces*

The interfacial tension was measured by the static Wilhelmy-plate method. Glass-plates were used instead of the more

commonly used roughened platinum plates in order to avoid specific interactions between amine groups and the Wilhelmy-plate [15,16]. For correct measurements the aqueous phase has to wet the Wilhelmy-plate (W.P.) completely. Therefore we performed contact angle measurements with oil drops on W.P.'s immersed in water and in protein solutions. It appeared that even after more than 24 hours of contact between the oil drop and the W.P., the contact angle measured through the oil phase was 180^{o}.

The W.P. was attached to an automatically recording Cahn electro balance which kept the W.P. at a constant height during the measurements. Normal interfacial measurements were performed as follows. Buffer solution ($150 * 10^{-6}$ m^3) was brought in a well cleaned (chromic acid) thermostated beaker. A layer of paraffin oil was poured on top of this aqueous phase. The thickness of this layer was somewhat more than the height of the W.P. After temperature equilibration a W.P. wetted with the aqueous phase was brought through the paraffin layer to the interface, by lifting the beaker with a lab-jack. Upon contacting the aqueous phase the W.P. is pulled into the aqueous solution and the force with which this happens is automatically recorded. Then $0.2 * 10^{-6}$ m^3 of protein solution with the same pH and salt concentration as the buffer is injected into the aqueous phase which is gently stirred for 60 seconds to get a homogeneous solution. The stirring time required was found by ink addition and measurement of the time necessary to get an equal distribution of the ink through the solution. The stirring does not disturb the interface. From then on the registration of the interfacial tension is completely automatic. The reproducibility of the measurements was within 0.5 $mN.m^{-1}$. Final values of the interfacial tension (γ_{∞}) were always taken after 16 hrs.

Alteration of the pH or of the protein concentration to higher concentrations can be achieved very easily during the measurements by injection of a certain volume of HCl, NaOH or protein solution and subtracting the same volume from the aqueous phase after 60 seconds of gentle stirring. For the dilution of the aqueous phase without disturbing the interface a special set up was designed which is shown in Figure 1. This

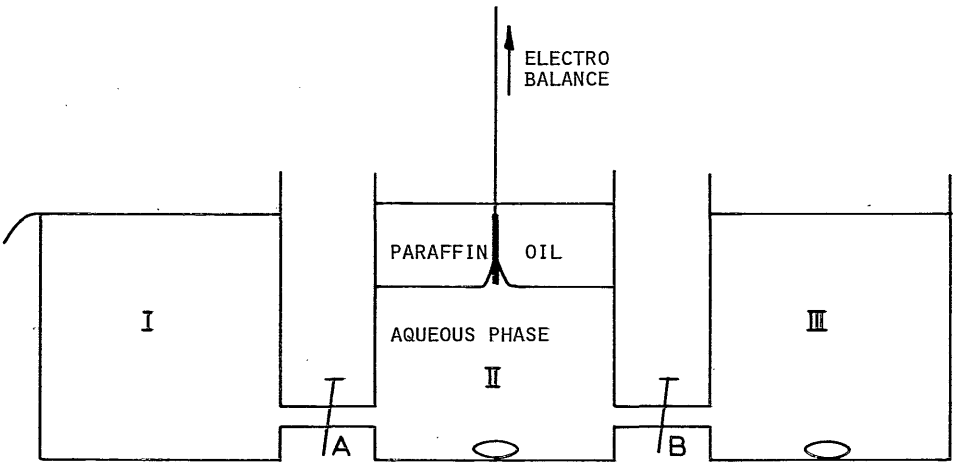

Figure 1: Schematic representation of the experimental set-up used for dilution experiments during the interfacial pressure measurements.

set-up consists of three beakers which are connected with each other. At the start of the measurement the system is filled with the aqueous phase until the overflow of beaker I works. Then the valves A and B are closed and after protein addition a normal measurement can be carried out in beaker II. When the protein solution has to be diluted the valves A, B are opened and,using a roller pump,buffer solution of the desired constitution is added to beaker III. The overflow of beaker I keeps the oil water interface at a fixed place during this dilution step. In this way it is possible to measure the interfacial tension continuously during dilution.

II.2.2.2 *Adsorption on Polystyrene latices by the depletion technique*

This method consists of the measurement of protein concentrations before and after the addition of a known amount of PSL

to a protein solution. After the addition of $1.00 * 10^{-6}$ m^3 PSL to $5.00 * 10^{-6}$ m^3 protein solution, both having the same pH and salt concentration, the test tube containing the PSL-protein solution mixture was rotated end over end during one night. Then the mixture was centrifuged during about 2.10^3 seconds at 2.10^4 g in a Sorvall Superspeed RC2-B centrifuge. The protein concentrations of the clear supernatant and the initial protein solution were determined using a modification of the method of Lowry[17], developed further by Roozen[18]. From the change in protein concentration due to adsorption on the PSL and the known surface area of the added latex the adsorbed amount per unit of surface area could be calculated.

II.2.2.3 *Ellipsometry*

Ellipsometry is an optical technique which allows the measurement of the thickness and refractive index of films at interfaces. This technique is based on the change in polarization of a light beam upon reflection. The theoretical base for this technique has been formulated by Drude[19,20]. The exact equations which he obtained for the relationship between wavelength, optical constants of the system, thickness of the layers and angle of incidence, were too complex to be used without approximations at that time. Nowadays it is possible to use them with the aid of computer calculations.

The experimental set up which we used*) has been described extensively in the thesis of Cuypers[21]. This ellipsometer has the advantage that it measures automatically the values of ψ and Δ which characterize the change in polarization due to reflection. Normally each measuring point takes 5 to 10 minutes while this automatic set-up measures once in every 3-5 seconds. This

*) All ellipsometer measurements have been performed at the Medical University Limburg, Maastricht, The Netherlands. We are very grateful to dr. P.A. Cuypers who placed the instrument at our disposal and to Miss R. Janssen who performed the measurements.

apparatus allows the continuous monitoring of adsorption processes at interfaces.

We measured the adsorption of HSA and HFb from aqueous solutions on polystyrene coated chromium surfaces. The polystyrene coating was obtained by spraying $1.0 * 10^{-6}$ m^3 of polystyrene solution in toluene with a syringe on a fast rotating chromium surface. The speed of rotation was 60 $r.s^{-1}$ (rotations per second). The optimal concentration of the polystyrene solution was found experimentally by using a range of concentrations. The coated surfaces obtained were tested by contact angle measurements with a drop of paraffin oil on the surfaces immersed in distilled water. Fig. 2 shows the obtained contact angles measured through the oil phase as a function of the polystyrene concentration used. The lowest concentration that yielded a contact angle equal to that of polystyrene (30^{o}), was used in the experiments. Using this concentration of 8 $kg.m^{-3}$ polystyrene in toluene gave polystyrene coatings with a refractive index of 1.66 ± 0.03 and a thickness of 18 ± 2 nm.

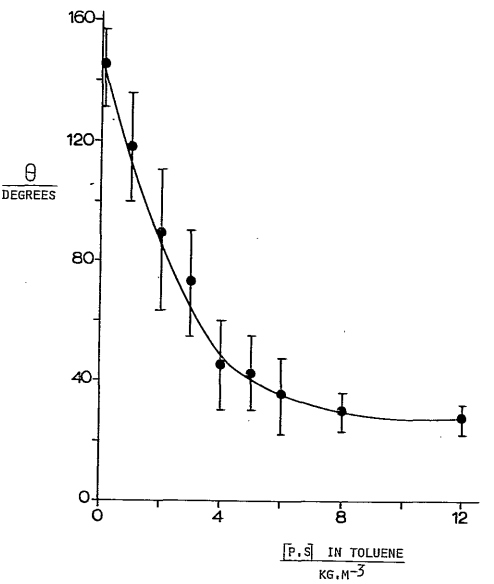

Figure 2: Contact angles of a drop of paraffin oil on polystyrene coated chromium surfaces immersed in water as a function of the polystyrene concentration used in the coating procedure (T = 298 K).

The wavelength of the laser light used for the ellipsometry was 632,8 nm and the angle of incidence was 68.00°. The optical constants of the bare chromium slide in buffer were determined from the ψ and Δ values of that slide with the use of the computer program 'Virgin'[21]. Then the ψ and Δ values of the polystyrene coated slide were measured and (using the exact Drude equations) the thickness and refractive index of the polystyrene layer were calculated with the use of the programs 'Ellis' and 'Ellip'[21]. Then protein was added to the buffer solution which could be stirred by a magnetic stirrer. Using the optical constants of the chromium slide, the thickness and refractive index of the polystyrene coating and the changing values of ψ and Δ (due to protein adsorption) it is possible to find the thickness and refractive index of the protein layer at subsequent stages of the adsorption. Because of the small differences between the refractive indices of the buffer solution (n_D = 1.3348), the protein layer (n_D = 1.34-1.52) and the polystyrene coating (n_D = 1.66) it is not possible to get very accurate values for the thickness and refractive index of the protein layers. Changes of 0,01° in ψ and Δ cause considerable differences in the calculated optical constants of the protein layer. The amount of protein adsorbed per unit of surface, Γ[kg.m^{-2}], showed much less uncertainty. This was also found by de Feyter *et al.*[22] when they evaluated their ellipsometric protein adsorption study at the air/water interface.

II.2.2.4 *Calculation of Γ from the ellipsometric measurements*

The value of Γ can be calculated from the average thickness (h) and the refractive index (n) of the adsorbed protein layer when the refractive index is a linear function of the protein concentration[22]. Figure 3 shows the refractive index of HSA solutions in 0.15 N NaCl as a function of the HSA weight fraction W_{HSA}. In Figure 4 the density of HSA solutions (ρ_{HSA}) in 0.15 N NaCl is shown as a function of W_{HSA}. Both figures show linear relations, indicating no irregularities of the protein solution up to a protein weight fraction of 0.36. Therefore Γ can be cal-

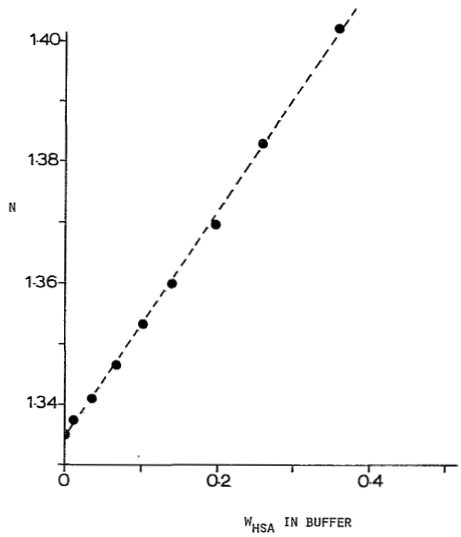

Figure 3: The refractive index of aqueous solutions of human serum albumin as a function of the weight fraction of albumin (T = 298 K).

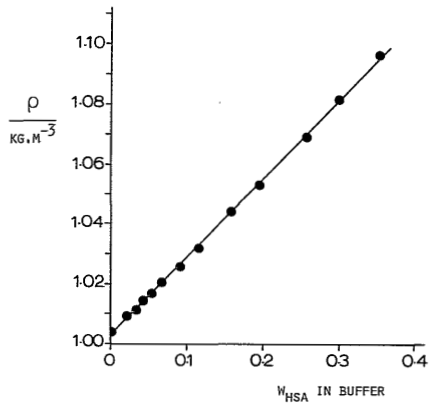

Figure 4: The density of aqueous solutions of human serum albumin as a function of the weight fraction of albumin (T = 298 K).

culated from these data as follows.

From the refractive index of the adsorbed HSA layers, W_{HSA} for this layer can be obtained using Figure 3. Subsequently the matching density of the layer can be obtained using Figure 4. These values together with the obtained average thickness of the layer allows the calculation of Γ

$$\Gamma = W_{HSA} \cdot \rho_{HSA} \cdot h \qquad (1)$$

In order to get proper values for the refractive index and the thickness of the layer it is necessary to do the computer simulations using the optical constants of the four layers of the system, viz. the protein solution, the adsorbed protein layer, the polystyrene layer and the bare chromium surface. We also tried to treat the system as a 3-layer system, the protein solution, the adsorbed protein layer and the polystyrene-coated chromium surface. The optical constants for the polystyrene coated chromium surface were obtained from the ψ and Δ values of this surface using the Drude equation for bare surfaces (program 'Virgin'). Using these 'effective' optical constants in a 3-layer simulation we could only find ψ and Δ values which matched the experimental points when we assumed that the adsorbed protein layers were very loose and very extended. These values indicated that the 3-layer procedure was not right. When, however, these unrealistic results were used in equation (1), it appeared that the calculated value of Γ was within 5% of the Γ-values obtained with the proper 4-layer simulation. The 3-layer system was much easier to simulate and the experimental procedure for the 3-layer case was much easier (no measurement of the thickness of the polystyrene coating). Therefore, when we were only interested in the value of Γ, our experiments have been treated as a 3-layer system.

In the calculation of Γ-values from experimental results (n and h) for HFb we used the values of dn/dw and $d\rho/dw$ which we measured for HSA (Figures 2 and 3). It was not possible to get proper values of these ratios for HFb because this protein

is much less soluble than HSA. The values obtained from the refractive index and the thickness of the layer do not depend on those ratios and have real meanings. The Γ-values obtained for HFb thus have a more approximate character.

II.3 RESULTS

II.3.1 Isotherms at different pH and salt concentrations

II.3.1.1 *Interfacial pressure of HSA layers adsorbed at the O/W interface*

Final values of the interfacial pressure ($\pi_\infty = \gamma_o - \gamma_\infty$) between aqueous HSA solutions and paraffin oil at 298 K were measured as a function of the protein concentration at four different pH-values. These values, all obtained after 16 hrs, are shown in Figure 5. The protein solutions in these experiments all contained fysiological concentrations of NaCl (0.15 kmol.m^{-3}).

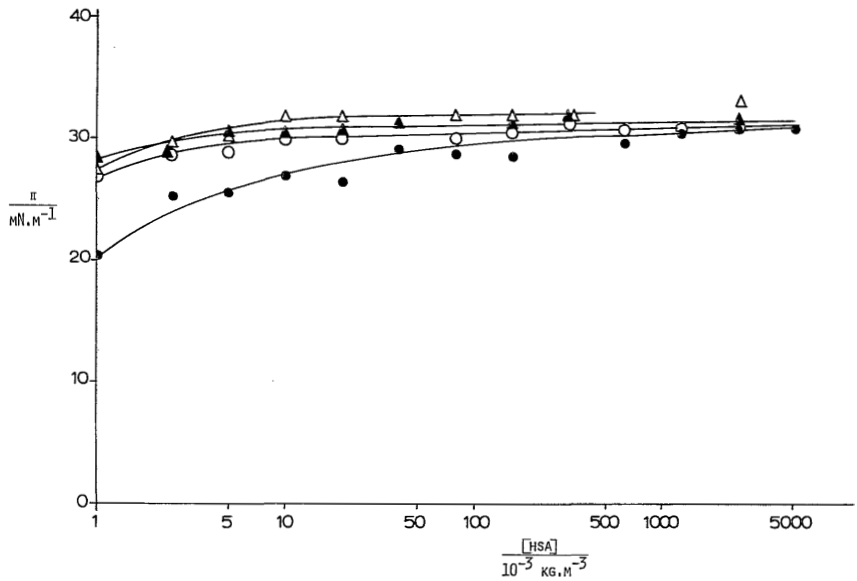

Figure 5: Final values of the interfacial pressure ($\gamma_o - \gamma_\infty$) of albumin layers adsorbed at the PO/W interface as a function of the protein concentration at different pH-values, ●, pH = 7.4; o, pH = 4.9; ▲, pH = 3.7; Δ, pH = 2.5. ([NaCl] = 0.15 kmol.m^{-3}, T = 298 K).

The influence of pH and salt concentration is shown very clearly in Figure 6 where the π_∞-values of solutions with equal protein concentrations ($1.0 * 10^{-2}$ kg.m^{-3}) are plotted against pH for two different salt concentrations. It is clear that the presence of the salt (NaCl) diminishes the influence of the pH on the surface pressure.

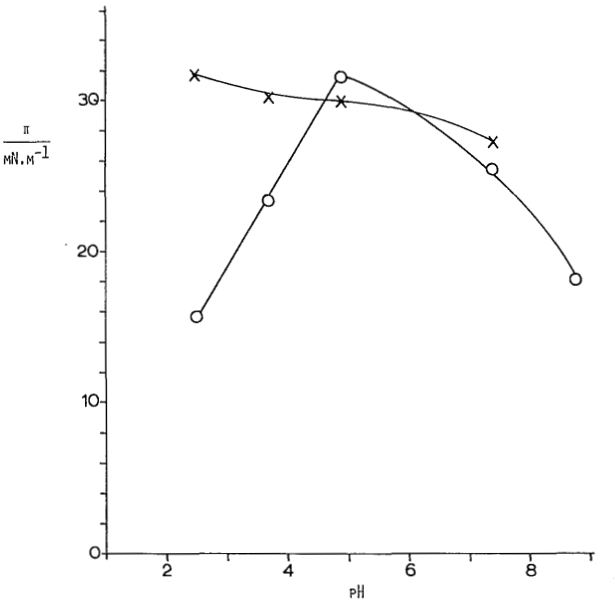

Figure 6: pH dependence of π_∞ due to HSA layers adsorbed from HSA solutions (0.01 kg.m^{-3}) at the PO/W interface, o, in the absence of NaCl; x, with 0.15 kmol.m^{-3} NaCl. (T = 298 K).

II.3.1.2 *Adsorption isotherms at polystyrene latex*

The adsorption isotherms of HSA on PSL at different pH-values in the absence of salt are shown in Figure 7. All isotherms have been measured at 298 K. It appears that at pH-values above the i.e.p. (pH = 4.9) the maximum adsorption is reached at higher bulk concentrations than at pH-values at or below the i.e.p.

Figure 8 shows isotherms for HFb at pH = 3.5 and 8.9 without salt (i.e.p. HFb = 5.5). These isotherms show that at least

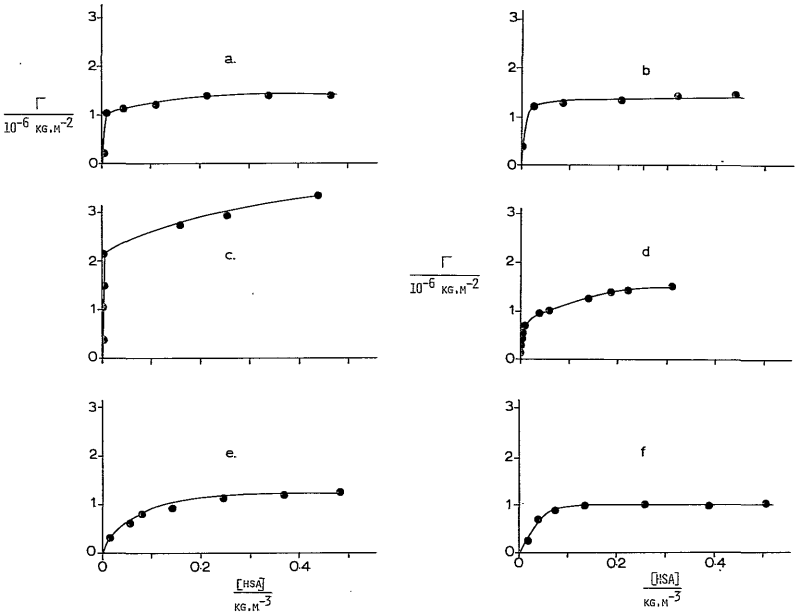

Figure 7: Adsorption isotherms of HSA onto PSL at different pH values: a, pH = 2.6; b, pH = 3.5; c, pH = 4.9; d, pH = 7.4; e, pH = 7.8; f, pH = 11.0; ([NaCl] = 0 kmol.m^{-3}, T = 298 K).

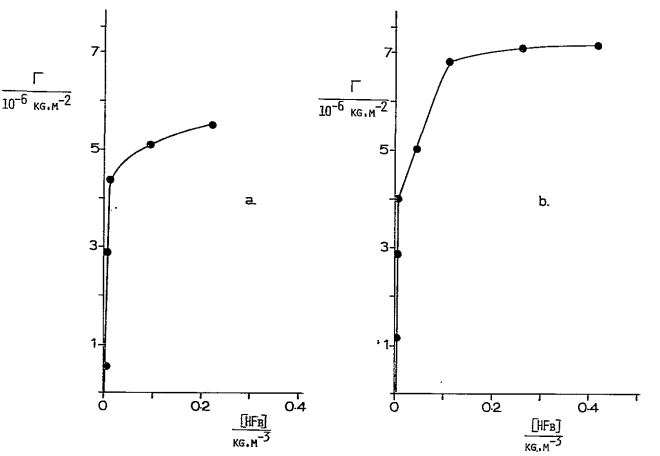

Figure 8: Adsorption isotherms of HFb onto PSL. a, pH = 3.5; b, pH = 8.9; ([NaCl] = 0 kmol.m^{-3}, T = 298 K).

up to surface concentrations of $4 * 10^{-6}$ kg.m^{-2} the solution is completely depleted by the adsorption. Isotherms of HFb can only be obtained in a limited pH-range because the protein is not stable in solution in a pH-range around its i.e.p. (pH = 5.5) and at low pH values (< pH = 2), these instability regions also depend on the salt concentration[23].

The dependence of the adsorbed amount (Γ) on the pH has been studied at protein concentrations where maximum adsorption occurs (plateau region). Figure 9 shows the Γ-pH curves for HSA and HFb with and without salt.

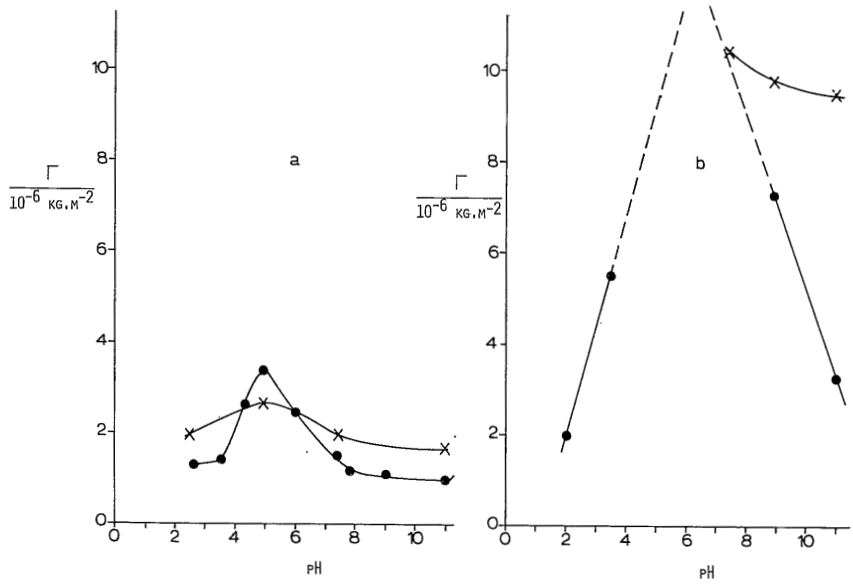

Figure 9: pH dependence of the maximum adsorbed amount of protein. a, HSA; b, HFb; • [NaCl] = 0 kmol.m^{-3}; x [NaCl] = 0.15 kmol.m^{-3}. (T = 298 K).

Again it is shown that maximum adsorption occurs at the i.e.p. (for HFb drastic interpolation) and that the presence of salt tends to nullify the pH-effect.

II.3.2 Time dependence of the adsorption

II.3.2.1 *From interfacial pressure measurements*

The time dependence of the interfacial pressure has been measured for all interfacial pressure measurements performed. This is inherent in this type of measurements for which the interfacial pressure is measured continuously. Figure 10 shows the π-t curves for HSA solutions with different concentrations at pH = 7,4, T = 298 K and [NaCl] = 0.15 kmol.m^{-3}. This figure shows that the interfacial tension, due to the initial adsorption, changes faster with increasing protein concentrations.

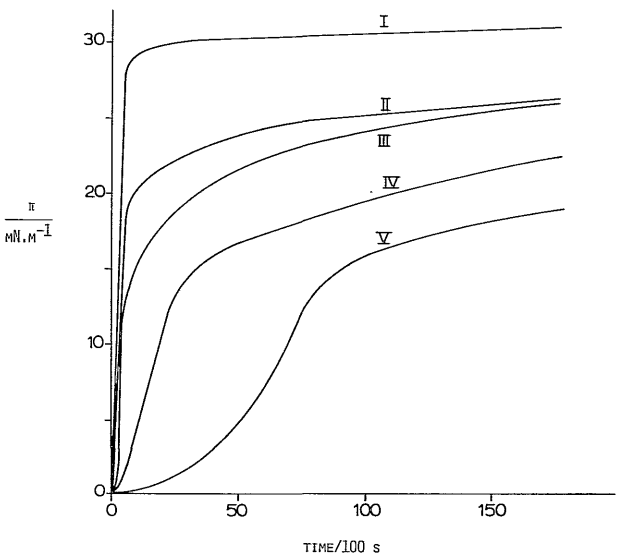

Figure 10: Time dependence of the interfacial pressure at the PO/W interface due to HSA adsorption from solutions with different protein concentration. I, [HSA] = 0.64 kg.m^{-3}; II, [HSA] = 0.020 kg.m^{-3}; III, [HSA] = 0.005 kg.m^{-3} IV, [HSA] = 0.0025 kg.m^{-3}; V, [HSA] = 0.001 kg.m^{-3} (pH = 7.4, [NaCl] = 0.15 kmol.m^{-3}, T = 298 K).

The influence of pH on the π-t curves is shown in Figure 11 for HSA solutions with a protein concentration of $1.0 * 10^{-2}$kg.m^{-3}.

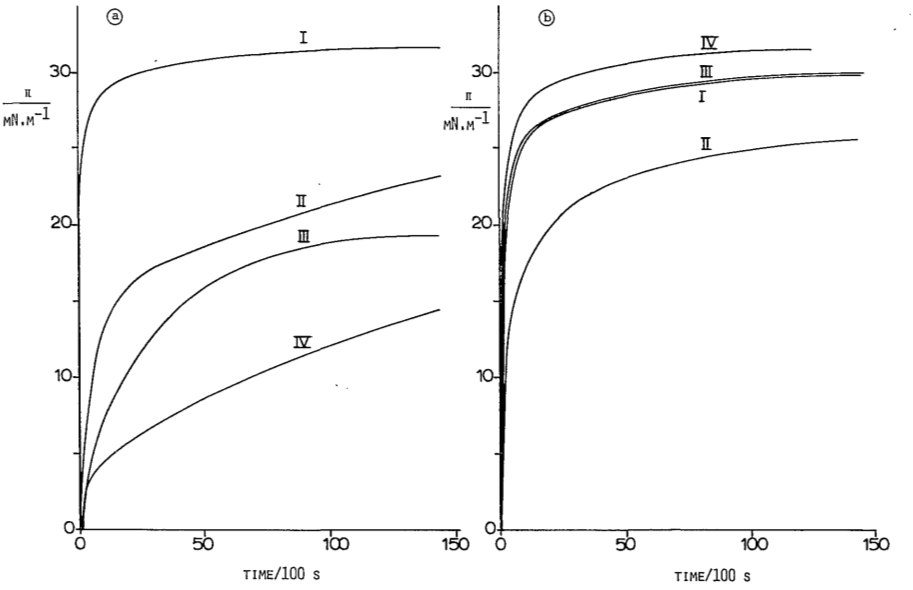

Figure 11: Time dependence of the interfacial pressure at the PO/W interface due to HSA adsorption from solutions with different pH; a, in the absence of salt; b, in the presence of 0.15 kmol.m^{-3} NaCl; I, pH = 4.9; II, pH = 7.4; III, pH = 3.7; IV, pH = 2.5. ([HSA] = 0.010 kg.m^{-3}, T = 298 K).

Figure 11a shows that the π-t curves are strongly pH dependent in the absence of salt; Figure 11b shows that in the presence of salt (0.15 kmol.m^{-3} NaCl) the influence of pH is less pronounced.

II.3.2.2 *From ellipsometry*

Some Γ-t curves of HSA onto PS (pH = 7.4, [NaCl] = 0.15 kmol.m^{-3}, 298 K) are shown in Figure 12. The low adsorption of HSA at low solution concentrations and the impossibility of measuring Γ-changes of less than 0.08 * 10^{-6} kg.m^{-2} (corresponding with a change of 0.01^{o} in the analyser position) did not allow a quantitative analysis of the results in terms of a diffu-

sion controlled adsorption mechanism. The lowest curve in Figure 12a will be described in the part dealing with reversibility upon concentrating the protein solution.

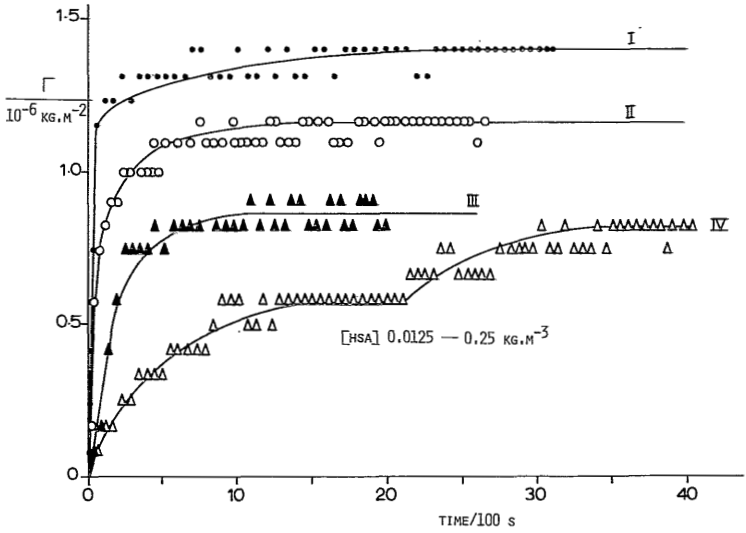

Figure 12: Time dependence of the HSA adsorption at the PS/W interface from solutions with different HSA concentrations. I, [HSA] = 1 $kg.m^{-3}$; II, [HSA] = 0.5 $kg.m^{-3}$; III, [HSA] = 0.25 $kg.m^{-3}$; IV, first adsorption step [HSA] = 0.0125 $kg.m^{-3}$, then [HSA] is increased to 0.25 $kg.m^{-3}$, (pH = 7.4, [NaCl] = 0.15 $kmol.m^{-3}$, T = 298 K).

Contrary to HSA, HFb already shows high adsorptions at low solution concentrations (Figure 8), this makes it possible to get more quantitative information about the role of duffusion in the adsorption process. When the adsorption is irreversible (or when desorption may be neglected) and adsorption is diffusion-controlled then the rate of adsorption onto a flat surface can be described by the following equation[24]:

$$\Gamma = 2c(Dt/\pi)^{\frac{1}{2}} \qquad (2)$$

where c is the constant protein concentration ($kg.m^{-3}$), D is the diffusion coefficient of the protein ($m^2.S^{-1}$), t is the time during which the adsorption takes place (S). Figure 13 shows the plot of Γ vs $(t-t_o)^{\frac{1}{2}}$ for the adsorption from a 0,032 $kg.m^{-3}$ HFb solution (pH = 7.4, [NaCl] = 0.15 $kmol.m^{-3}$, 298 K) where t_o is the initial time interval (60 seconds) after addition of the protein, during which the solution is stirred in order to get a homogeneous protein solution.

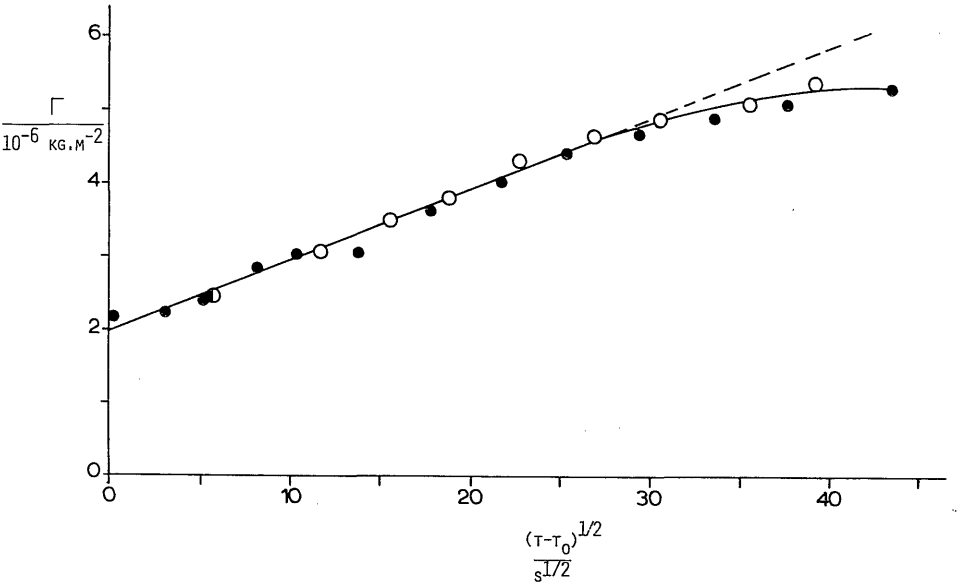

Figure 13: HFb adsorption from ellipsometry at the PS/W interface as a function of the square root of the time of adsorption after homogenization of the solution.([HFb] = 0.032 $kg.m^{-3}$, pH = 7.4, [NaCl] = 0.15 $kmol.m^{-3}$, T = 298 K). • and o are two separate experiments under identical circumstances.

II.3.3 Reversibility upon dilution

II.3.3.1 *Interfacial pressure measurements*

The reversibility of the interfacial pressure upon dilution was studied using the experimental set up shown in Figure

1. Dilution of the aqueous phase (0.100 $kg.m^{-3}$ HSA, [NaCl] = 0.15 $kmol.m^{-3}$, pH = 298 K) after 16 hrs of adsorption, to a HSA concentration of 0.001 $kg.m^{-3}$ did not show any significant decrease in π. This measurement was performed by continuously measuring π and additionally by introducing a new clean W.P. after the dilution step. The new W.P. was applied because in accordance with Blank *et al.*[25] we found that it is often not possible to measure a decreasing π with the Wilhelmy plate method[25]. Both methods did not show any decrease in π upon dilution.

II.3.3.2 *Adsorption of PSL*

No detectable desorption took place when a latex fully covered with HSA, after centrifugation, was resuspended in a protein-free solution, and left as such for 16 hrs.

II.3.3.3 *Ellipsometry*

When after adsorption the contents of the measuring cell was displaced by a protein-free solution, no decrease in the amount of protein (HSA or HFb) adsorbed at the PS-coated chromium substrates was found.

II.3.4 Reversibility upon concentrating the protein solution

II.3.4.1 *Interfacial pressure measurements*

The reversibility upon concentrating was studied in the following way. The interfacial pressure between an aqueous solution containing 0.001 $kg.m^{-3}$ HSA was allowed to reach its final value in 16 hrs of adsorption time. Then the protein concentration was increased to 0.160 $kg.m^{-3}$ in the way described above. The interfacial pressure was measured continuously during this process. Figure 14 shows the part of the π-t curve where the protein concentration is increased and the resulting increase of π. The final value of π, 16 hrs after the change of the HSA concentration, was 28.2 $mN.m^{-1}$ which is very near to the normal π_∞ for a 0.160 $kg.m^{-3}$ HSA solution, being 28.6 $mN.m^{-1}$.

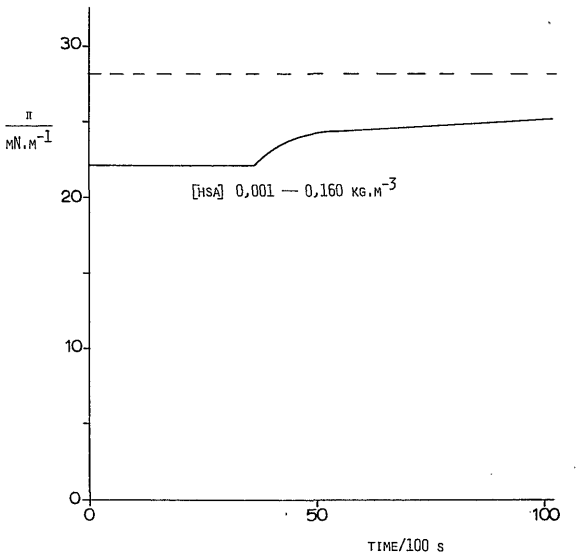

Figure 14: Change in the interfacial pressure due to an increase of the HSA concentration from 0.001 $kg.m^{-3}$ to 0.160 $kg.m^{-3}$. (pH = 7.4, [NaCl] = 0.15 $kmol.m^{-3}$, T = 298 K).

The π_∞ value for the 0.001 $kg.cm^{-3}$ HSA solution was 22.1 $mN.m^{-1}$. These measurements were performed at pH = 7.4, [NaCl] = 0.15 $kmol.m^{-3}$, 298 K.

II.3.4.2 *Adsorption on PSL*

The reversibility of adsorption of HSA upon concentrating was measured with the depletion technique by performing three sets of experiments. The first experiment started with such a HSA concentration that no plateau adsorption could be reached. A second experiment started from this same low concentration, and after 16 hrs of adsorption an additional amount of protein was added. The concentration in solution was measured 16 hrs later. The third experiment started with such a concentration of HSA, that the same amount of HSA was present in the adsorption mix-

ture as in the second experiment after addition of the extra protein. The different adsorption values were measured in the usual way after 16 hrs of adsorption time. The adsorption in the first experiment was less than the plateau adsorption value for HSA although still some HSA was present in solution. The adsorption values in the second and third case were identical, indicating that the preadsorption of a small amount of HSA (30% of the plateau value) does not influence the maximum amount of HSA that can be adsorbed on the surface.

II.3.4.3 *Ellipsometry*

The reversibility of adsorption upon concentrating was also studied by ellipsometry. First the adsorption of HSA onto the P.S.-coated chromium surfaces from a 0.0125 $kg.m^{-3}$ HSA solution was followed, then the protein concentration in solution was increased to 0.25 $kg.m^{-3}$ and the successive adsorption was followed. The result of this experiment is shown in the lower curve of Figure 12. It is clear that after increasing the protein concentration additional adsorption occurs, up to the same final amount as in the experiment with an initial concentration of 0.25 $kg.m^{-3}$.

II.3.5 Reversibility upon pH-changes

II.3.5.1 *Interfacial pressure measurements*

The reversibility upon pH-changes was studied in the absence of salt because the influence of pH is much more pronounced then. The interfacial pressure between P.O. and a protein solution (0.01 $kg.m^{-3}$) was allowed to reach its final value (after 16 hrs). Then the pH of the aqueous phase was changed by addition of NaOH or HCl. A step-wise pH change was performed with time intervals of one hour.

It appeared that starting at a pH of 4.9 (the i.e.p. of HSA) the final value of the interfacial pressure could not be changed by NaOH or HCl addition. This was measured as well continuously as with a new Wilhelmy plate introduced after the pH

change.

When the first adsorption took place from solutions with a pH far away from the i.e.p. of the HSA, it appeared that changing the pH in the direction of the i.e.p. caused an increase in the interfacial pressure. Figure 15 shows the influence of pH-changes on the interfacial pressure of a HSA solution starting from a pH value above the i.e.p. (Figure 15a) and below the i.e.p. (Figure 15b).

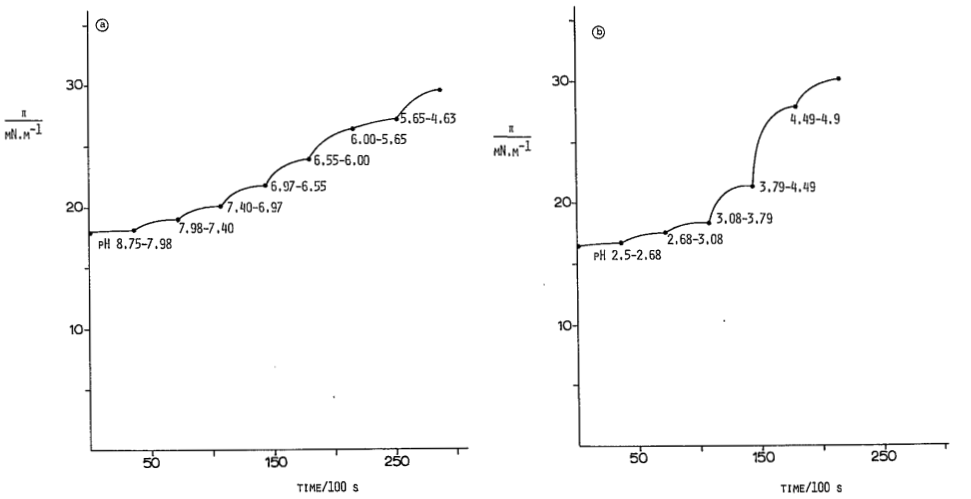

Figure 15: Effect of pH-changes in the direction of the i.e.p. on the interfacial pressure of HSA solutions starting at a), a pH of 8.75; b), a pH of 2.5. [HSA] = 0.010 kg.m^{-3}; [NaCl] = 0, T = 298 K.

II.3.5.2 *Ellipsometry*

The ellipsometric measurements showed the same behaviour upon pH-changes as the interfacial pressure measurements. After adsorption of HSA (0.5 kg.m^{-3}, no salt) at pH = 4.9 changes of the pH to lower or higher values hardly caused any desorption (Figure 16 a, c). After adsorption of HSA (0.5 kg.m^{-3},

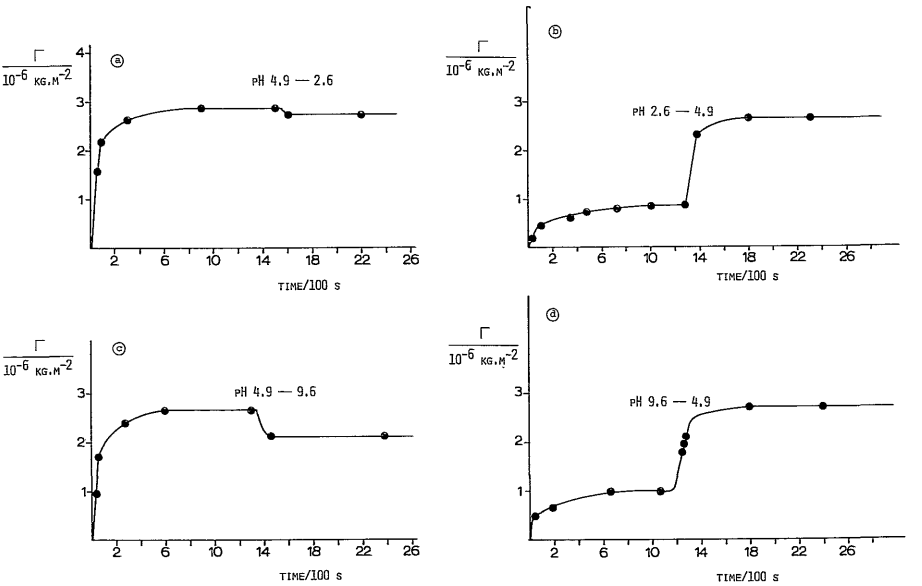

Figure 16: Effect of pH-changes of HSA solutions on the adsorbed amount at PS/W interfaces from ellipsometry, a) pH 4.9 → 2.6; b) pH 2.6 → 4.9; c) pH 4.9 → 9.6; d) pH 9.6 → 4.9. ([HSA] = 0.5 kg.m^{-3}, [NaCl] = 0, T = 298 K).

no salt) at pH-values below or above the i.e.p., changes of the pH in the direction of the i.e.p. showed additional adsorption occurring up to amounts which were observed for experiments starting at the i.e.p. (Figure 16 b, d).

II.4 DISCUSSION

II.4.1 Adsorption isotherms at different pH and salt concentrations

The results of the interfacial pressure measurements, Figures 5 and 6, give only qualitative information. From Figure 5 it can be seen that the interfacial pressure increases with increasing protein concentration. This figure also shows that $\frac{d\pi_\infty}{d\ \ln[HSA]}$ decreases with increasing protein concentration. In

case of reversible, positive adsorption the value of $\frac{d\pi_\infty}{d\ \ln[HSA]}$ would increase with increasing protein concentration, according to Gibb's law which says that $\frac{d\ \pi_\infty}{d\ \ln\ c} = \frac{\Gamma}{RT}$ (c = concentration of the surfactant and Γ is the excess concentration of the surfactant at the interface). The shape of the observed π_∞-ln[HSA] curves is a first indication that protein adsorption is not an ideal reversible process.

The influence of pH and salt concentration on the adsorption is shown very clearly in all three types of measurements which we performed, interfacial pressure measurements (Figures 6; 15), adsorption measurements on PSL (Figures 7, 8, 9) and ellipsometric measurements (Figure 16). The general trend is that at the i.e.p. of the protein, maximum adsorption or maximal values for the interfacial pressure are observed. It is also shown that the presence of 0.15 kmol.m^{-3} NaCl tends to diminish the influence of the pH on the adsorbed amount. This influence of the ionic strength is a general feature for the adsorption of polyelectrolytes. At higher ionic strengths the charge of the molecules will be screened resulting in a decrease of the inter- and intramolecular electrostatic repulsions . In this respect it is surprising that at the i.e.p. the adsorption of HSA is higher at low ionic strength (Figure 9a). This might indicate that the HSA molecules at the i.e.p. have a more compact shape at low ionic strength due to a net electrostatic intramolecular attraction. Norde[26], performing HSA adsorption on PSL with the depletion technique, measured similar adsorption values as we did, but he did not observe this increased adsorption in the absence of salt at the i.e.p.

The ellipsometric measurements (Figure 16) yielded somewhat lower HSA adsorption values than the PSL adsorption measurements but it should be noted that these ellipsometrically monitored adsorptions took place at uncharged polystyrene substrates, whereas the polystyrene latex used contains many charged SO_3^- groups. Norde[26] also found a decrease in adsorption with decreasing surface charge density of the polystyrene. Brash *et al.*[27], using internal reflection spectroscopy, reported HSA adsorption values of 5 * 10^{-6} kg.m^{-2} at polystyrene surfaces. These

values are much higher than those reported here and by Norde. This difference may be due to the method which they used to measure the adsorptions. Another possibility is that differences in the HSA-preparates used are responsible for the observed discrepancies[28].

As is shown in Figure 7, the pH does not only affect the maximum adsorption but it also influences the shape of the isotherms. At pH values ≤4.9 plateau values are attained at very low protein concentrations whereas the initial slopes of the isotherms at pH-values >4.9 are much less steep. When HSA adsorption onto polystyrene is irreversible and the size of the molecules does not change upon adsorption then isotherms of the shape shown in Figure 7d to f cannot be expected. It should not be possible that after prolonged adsorption times significant protein concentrations are observed in solution, while at the same time the adsorption has not reached its plateau value. This point will be stressed further in the discussion about the reversibility of adsorption with respect to the protein concentration.

The adsorption isotherms of HFb onto PSL (Figure 8) show a much more 'irreversible' character than those of HSA. The initial slope of the isotherms is practically infinite which should be expected for irreversible adsorption. At low bulk concentrations where adsorption leads to surface concentrations up to $4 * 10^{-6}$ kg.m^{-2} the protein solution is completely depleted. When more protein is available, a residual amount of protein will stay in solution. At higher initial HFb concentrations more HFb can be adsorbed than at lower initial concentrations. This is shown very pronounced in Figure 8b where after the very steep part of the isotherm a second part is observed which shows increased adsorption with increasing residual HFb concentrations (and consequently higher initial concentrations). This feature may be due to a different orientation of the molecules onto the surface at higher concentrations, resulting from the faster adsorption and consequently less chance for reconformation of the adsorbed molecules. Other experiments[29] showed that contrary to HSA, the HFb molecules are associated at a polystyrene-water interface indicating a strong intermolecular interaction between the ad-

sorbed molecules. Ellipsometric measurements of the adsorption of HFb from a 0.1 $kg.m^{-3}$ HFb solution onto polystyrene at pH = 8.9 in the absence of salt, yielded a protein layer thickness of 20 ± 3 nm and a refractive index of 1.352 ± 0.004. This layer thickness is in very good agreement with the monolayer thickness calculated from flocculation experiments of PSL with HFb[23] and with ellipsometric measurements on chromium surfaces[31]. The layer thickness and density of adsorbed HSA layers were much more difficult to establish. The much lower adsorption of HSA and consequently the small changes in ψ and Δ values allowed only very rough estimates about layer thickness and refractive index of the HSA layers. At pH 4.9 without salt the thickness of a HSA layer adsorbed from a 0.5 $kg.m^{-3}$ HSA solution onto polystyrene is 4.5 ± 1.5 nm and the refractive index is 1.418 ± 0.03 (W_{HSA} = 0.42 ± 0.16). At pH 2.6 and 9.6 even poorer estimates of thickness and refractive index were possible, because of much lower adsorption values. The tendency is that those layers are more extended (9 ± 5 nm) and have a much lower protein density W_{HSA} = 0.1 ± 0.05.

II.4.2 Time dependence of the adsorption

The influence of protein concentration on the time dependence of the adsorption is shown in Figures 10 and 12. Figure 10 shows that the time dependence of the interfacial pressure is mainly determined by the HSA concentration in the aqueous phase. This indicates that the rate of arrival of the molecules at the interface is the rate determining factor in the presence of 0.15 $kmol.m^{-3}$ NaCl. Figure 12 also shows an increased rate of adsorption with increasing HSA concentration. From these results it may be concluded that there are no important energy barriers counteracting the adsorption of HSA at pH = 7.4 and [NaCl] = 0.15 $kmol.m^{-3}$.

From the linear part of the Γ vs $(t-t_o)^{\frac{1}{2}}$ curve in Figure 13 it may be concluded that the adsorption of HFb onto polystyrene from a 0.032 $kg.m^{-3}$ HFb solution is a diffusion controlled process. The diffusion coefficient of HFb calculated from the slope

of this linear part is: $D_{HFb} = 0.8 * 10^{-11}$ $m^2.S^{-1}$. This value is somewhat lower than the values given in the literature for HFb ($1.5\text{-}2.0 * 10^{-11}$ $m^2.S^{-1}$)[30]. This discrepancy may be due to the fact that we used the values of $\frac{dn}{dw}$ and $\frac{d\rho}{dw}$, which we measured for HSA, in the calculation of the adsorbed amounts of HFb.

The influence of the pH on the time dependence of the interfacial pressure and the rate of adsorption is shown in Figures 11 and 16. Figure 11a shows that the time dependence of the interfacial pressure between paraffine oil and a 0.01 $kg.m^{-3}$ HSA solution without salt, depends strongly on the pH of the aqueous phase. The increase of the interfacial pressure with time of adsorption is much faster at the i.e.p. of the protein than at pH values below or above the i.e.p. This result is in agreement with James *et al.*[32]. It is likely that at pH-values away from the i.e.p., when the protein molecules bear a net charge, the adsorption of the protein molecules will be hampered by the charge of the already adsorbed molecules whereas at the i.e.p. this electrostatic adsorption barrier will not exist because the molecules do not bear a net charge.

Figure 11b shows that the addition of 0.15 $kmol.m^{-3}$ NaCl eliminates the strong pH-dependence on the rate of change of the interfacial pressure. It should be noticed that at pH values ≤ 4.9 the pH dependence is fully eliminated whereas at pH 7.4 (above the i.e.p.) the change of the interfacial pressure is still slower than at the i.e.p.

II.4.3 Reversibility of the protein adsorption

The question whether protein adsorption is reversible or not is still open to discussion. Most authors state that protein adsorption is an irreversible process but many of them apply normal thermodynamics in discussing the adsorption results. Oreskes *et al.*[33] and Lee *et al.*[34] found that protein adsorption was consistent with Langmuir type adsorption, implying reversible adsorption and no interactions between adsorbed molecules. MacRitchie[35] reported reversible adsorption of albumin at pH-values away from the i.e.p. Gonzales *et al.*[36] reported revers-

ible adsorption of albumin at the air-water interface, and so on. We studied the reversibility of adsorption of HSA onto O/W and polystyrene/W interfaces upon concentration and pH-changes of the solution.

When after adsorption onto O/W or polystyrene/W interfaces the protein concentration in the solution was decreased or even completely washed away, no indications were found that any desorption of HSA from the interface took place. This feature was general, for the interfacial pressure measurements, the adsorption measurements on PSL and the ellipsometric measurements. When after adsorption at low HSA concentrations the concentration of HSA in the aqueous phase was increased, all results showed that additional adsorption took place up to the amount that would adsorb when the adsorption was performed from solutions having this increased HSA concentration. Figure 11 shows such an experiment for the interfacial pressure measurements. Also the adsorption studies on PSL showed additional adsorption. The two lower curves of Figure 12 show that also by ellipsometric measurements one observes additional adsorption upon increasing the HSA concentration in solution. Study of the reversibility with respect to pH-changes of the aqueous phase yielded the same semi-reversible character. When the initial adsorption was performed at the i.e.p. of HSA (yielding the highest adsorption and π-values), pH changes away from the i.e.p. did not show any significant desorption (Figure 16a, c) or decrease in π. When the initial adsorption, however, took place at pH-values above or below the i.e.p. then an increase in the adsorption (Figure 16b, d) and the interfacial pressure (Figure 15a, b) was observed upon changing the pH of the aqueous phase in the direction of the i.e.p.

All these results, about reversibility with respect to concentration and pH, have in common that changing conditions which should increase the π-values or the Γ-values do increase these parameters and that those changes in conditions that should cause a decrease in the π-values or Γ-values do not give this decrease. In other words, a HSA molecule, once adsorbed will not desorb but additional adsorption is quite possible. From these

results it is understandable that some authors describe protein adsorption in terms of Langmuir-type adsorption. Most adsorption isotherms are measured with increasing protein concentrations, in that case the adsorption indeed behaves like a completely reversible adsorption. The feature that protein adsorption is semi-reversible upon pH-change can be explained as follows. When an interface is completely covered by a HSA layer adsorbed at a pH far away from the i.e.p. than this layer consists of HSA molecules which are swollen due to intramolecular repulsions. Changing the pH in the direction of the i.e.p. will cause a decrease of this electrostatic repulsion and the molecules will get a more compact shape[37]. This shrinkage of the layer in three dimensions will cause new adsorption sites at the interface to become available which will be filled up by molecules arriving from the bulk. When initial adsorption takes place at the i.e.p. then the change of pH will cause a swelling of the adsorbed molecules. This swelling can only take place in one direction, perpendicular to the interface, because the close packing of the molecules at the interface does prevent swelling in directions parallel to the interface. The feature of semi-reversibility of the adsorption with respect to the protein concentration is not well understood. It is also not understood that the adsorption isotherms of HSA doe not show the 'high affinity' character that should appear regarding the irreversibility of the adsorption (once adsorbed no desorption). This deviation from high affinity character itself could be explained if the protein molecules should unfold upon adsorption. In that case the deviation from plateau values at lower protein concentration can be explained in terms of competition between the rate of arrival of the proteins at the interface and the rate of unfolding of the molecules already adsorbed. If this, however, should be true then the increase in adsorption on further increase of the protein concentration cannot be explained in turn. Solution of this problem might be possible after more extensive studies of the conformation of the protein molecules at the interface.

II.5 CONCLUSIONS

This study of the adsorption of proteins at hydrophobic interfaces shows that:

1. The adsorption of proteins is strongly dependent on the pH of the solution from which adsorption takes place, showing maximum adsorption at the i.e.p. of the protein.
2. Salt addition diminishes the pH-dependence of the adsorbed amounts by giving higher adsorptions at extreme pH-values and lower adsorptions at the i.e.p. of the protein.
3. At low ionic strengths the rate of adsorption depends strongly on the pH, with high rates at the i.e.p. and lower rates for other pH-values resulting from an electrostatic adsorption barrier. Salt addition diminishes this pH-dependence.
4. The adsorption of HSA onto hydrophobic interfaces is semi-reversible with respect to pH and protein concentration (once adsorbed no desorption; additional adsorption sometimes possible).
5. It is possible to perform ellipsometric measurements of protein adsorption at polymer substrates.

These experiments show that the combination of interfacial pressure measurements and ellipsometry is a useful tool in protein adsorption studies. More extensive measurements may result in an equation of state for adsorbed protein layers, which allows useful comparisons between adsorption theories and practice. Further refinements of the ellipsometric technique, like e.g. the development of substrates with very reproducible optical constants, will allow accurate studies of layer thicknesses and densities in relation with parameters like protein concentration, pH and ionic strength of the solution.

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CHAPTER III

THE FEASIBILITY OF RADIOLABELING FOR HUMAN SERUM ALBUMINE (HSA) ADSORPTION STUDIES "PROTEIN ADSORPTION"

ABSTRACT.

Human serum albumin (HSA) was labeled in various ways and with different radioactive labels (^{99m} Technetium and ^{125}Iodine). Following the usual characterization methods (electrophoresis on polyacryl gel and immuno electrophoresis) no difference between the labeled and non-labeled HSA molecules could be detected. Two methods testing adsorption behaviour were developed to see whether the radiolabeled HSA molecules were identical to the non-labeled HSA molecules. It appeared that the labeled HSA molecules always showed a preferential adsorption onto polystyrene (and silastic rubber) substrates. The ^{99m}Tc-labeled molecules showed a high value of the preferential adsorption factor ($\phi >> 1$). The ϕ-value for ^{125}I-labeled molecules was around 1.4. We also considered the stability of the bond between the label and the HSA molecules which was found to be low for Tc-labeled preparations and high for the other ones. It was concluded that it is always necessary to determine whether preferential adsorption of radio labeled molecules compared to non-labeled molecules of the same type occurs, before using labeled molecules in adsorption studies. The methods developed to determine preferential adsorption between labeled and non-labeled molecules are also generally applicable for different types of molecules.

III.1 INTRODUCTION.

The development of improved blood-compatible materials requires a better insight in the events, which occur when a foreign material is contacted with blood. One of the initial events is the adsorption of a protein layer at the blood material interface. This layer modifies the original surface and has a strong influence on subsequent phenomena such as platelet adhesion and blood coagulation[1,2,4].

Several methods are available to perform protein adsorption studies. These are depletion techniques[5-10]; spectroscopic techniques, such as colorimetry[1-13], ATR/IR[14-17], CD[18] and ellipsometry[19-25], and radio labeling[7,26-28]. This last method seems to be a very elegant one, which offers the possibility to determine the simultaneous adsorption of different proteins onto a surface.
The work published up to now however, hardly contains any information about the influence of the label and the labeling techniques on the protein adsorption data.
Protein adsorption studies with labeled proteins are only useful when the bond between the label and the protein molecule is stable under adsorption conditions and when the adsorption behaviour of the labeled protein is identical with that of the non-labeled protein. These aspects have been investigated for ^{99m}Tc and ^{125}I labeled HSA (Human Serum Albumin), prepared by different labeling procedures.
Two methods have been used to study the adsorption behaviour of labeled HSA. The first method comprises the measurement of the adsorption isotherms of labeled proteins on PS (polystyrene) and SR (silicon rubber) from solutions containing different concentration ratios of labeled to non-labeled protein. The second method consists of the simultaneous determination of the adsorption of labeled and non-labeled protein on a PS latex, with a high specific surface area.

III.2 CONSIDERATIONS ON ADSORPTION BEHAVIOUR.

The application of radio-labeled protein in adsorption studies is only allowed when the labeling method or the label itself does not change the adsorption properties of the protein. Therefore the adsorption behaviour of labeled protein molecules A has to be compared with non labeled protein molecules B, which differ only with molecule A in the label.
Once it has been established that there is no difference in adsorption between the labeled molecule A and the non-labeled molecule B, one can use the labeled molecules A to use them in preferential adsorption studies with a completely different molecule C. In the derivations given below B can then be replaced by C.

A simple model, derived from adsorption/desorption kinetics and assuming monolayer adsorption is used for the description of the possible difference in adsorption behaviour of A and B.
The rates of adsorption of A and B are given by

$$n_A = k_A [A] . L/M_A (1-\theta) \quad (1)$$

$$n_B = k_B [B] . L/M_B (1-\theta) \quad (2)$$

n_A, n_B = the number of effective collisions of molecules A and B per unit of surface area and time, $m^{-2}.s^{-1}$
k_A, k_B = proportionally constants, $m.s^{-1}$
$[A]$, $[B]$ = the concentrations of A and B in the solution, $kg.m^{-3}$
M_A, M_B = molecular weights of A and B, $kg.mole^{-1}$
θ = fraction of total available surface area, covered with A and B
L = Avogadros number, $mole^{-1}$

and

$$n_A/n_B = (k_A.M_B/k_B.M_A) [A]/[B] \quad (3)$$

The rates of desorption of A and B are given by

$$P_A = (1/t_A). L . A_s/M_A \quad (4)$$

$$P_B = (1/t_B). L . B_s/M_B \quad (5)$$

P_A, P_B = number of molecules A and B leaving the surface per unit surface area and time, $m^{-2}.s^{-1}$
t_A, t_B = mean residence time of A and B on the surface, s
A_s, B_s = the amount of A and B adsorbed onto the surface, $kg.m^{-2}$

and

$$P_A/P_B = (t_B.M_B/t_A.M_A) A_s/B_s \quad (6)$$

Three cases can be considered:

1. The adsorption of A and B is irreversible.
   This means that molecules once adsorbed, do not leave the surface and the ratio A_s/B_s will depend entirely on the ratio of the number of collisions. For constant [A] and [B] we find

$$A_s/B_s = (k_A M_B / k_B M_A) \cdot [A]/[B] \qquad (7)$$

2. The adsorption of either A or B is irreversible.
   In this case the surface will be covered completely by the molecules which adsorb irreversibly.

3. The adsorption of A and B is reversible (Langmuir).
   Equilibrium will be reached when $n_A = P_A$ and $n_B = P_B$, so

$$n_A/n_B = P_A/P_B \qquad (8)$$

   From equations (3), (6) and (8) the ratio A_s/B_s is found to be

$$A_s/B_s = (t_A k_A / t_B k_B) \; [A]/[B] \qquad (9)$$

   Hence we find in general

$$A_s/B_s = \phi \; [A]/[B] \qquad (10)$$

The proportionality factor ϕ will be a constant in the first and the third case mentioned above and ϕ will be infinite or zero in the second case.
In order to get information about the value of ϕ for a given system, measurements must be carried out with a constant concentration ratio, [A]/[B] in the solution.

$$[A]/[B] = C \qquad (11)$$

The surface will be covered completely with adsorbed molecules when the sum of the surface fractions occupied by molecules A and B equals one.

$$S_A \cdot A_s + S_B \cdot B_s = 1 \tag{12}$$

S_A, S_B = the specific surface areas of A and B when the surface would be occupied by one of these species, $m^2.kg^{-1}$

Assuming that the specific surface area of A is x times that of B

$$S_A = x\, S_B \tag{13}$$

the following expressions can be derived, using (12) and (13)

$$B_s = (1 - x\, S_B A_s)/S_B \tag{14}$$

$$A_s/B_s = A_s S_B/(1 - x\, S_B A_s) \tag{15}$$

An expression for A_s is found from equations (10), (11) and (15)

$$A_s = C\, \phi/(S_B\, (1 + C\, \phi\, x)) \tag{16}$$

This expression shows the relationship between the directly measurable quantuty A_s and the parameters ϕ and x. Equation (16) is of course only valid when the exposed surface is completely covered with molecules A and B. Using this expression it is also possible to determine the quantity $\phi\, x$, without knowing ϕ or S_B.
This can be done by performing two series of measurements (adsorption isotherms) with different concentration ratios C_1 and C_2 ($C_2 > C_1$). Each of these adsorption isotherms yields a maximum adsorption (plateau value) for A_s, illustrated by figure 1.

The ratio of these two values is

$$A_{s_2}/A_{s_1} = y = (C_2/C_1)(1 + C_1\, \phi\, x)/(1 + C_2\, \phi\, x) \tag{17}$$

or

$$\phi\, x = (1 - y\, C_1/C_2)/C_1(y - 1) \tag{18}$$

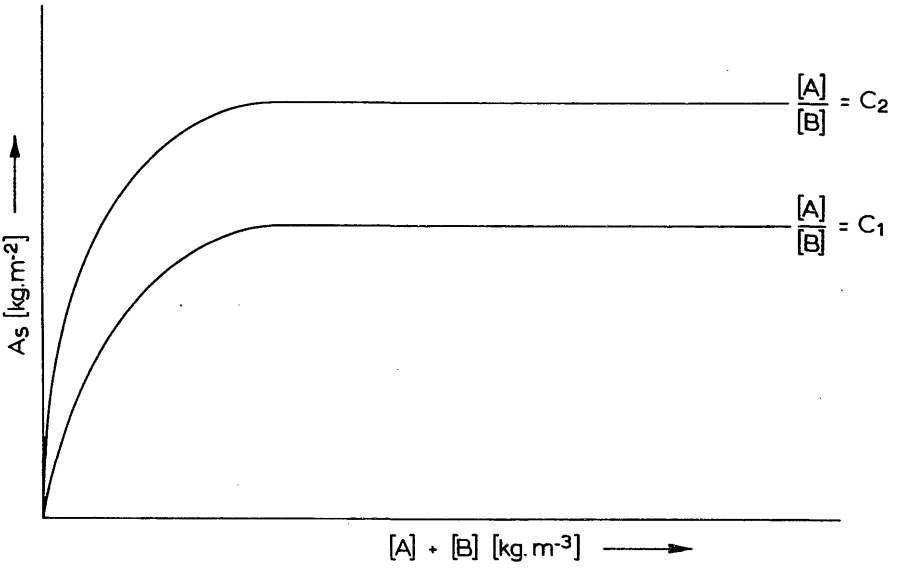

Figure 1: Two hypothetical adsorption isotherms of labeled protein measured with concentration ratios C_1 and C_2.

The specific surface area S_A can also be calculated from the two adsorption isotherms, via

$$S_A = (C_2 - y\,C_1)/((C_2 - C_1)\,A_{s_2}) \qquad (19)$$

When $\phi\,x$ is determined by the method described above we have to evaluate the uncertainty in $\phi\,x$, caused by the experimental error in y. Because the adsorption isotherms measured with radio active proteins have an uncertainty in plateau value of about 5%, the estimated error in y becomes 0.1 y.
The error in $\phi\,x$ which may arise from experimental error will be

$$\Delta\,\phi\,x = \left|\left(\frac{\partial \phi x}{\partial C_1}\right)_{C_2,y}\right| . \Delta C_1 + \left|\left(\frac{\partial \phi x}{\partial C_2}\right)_{C_1,y}\right| . \Delta C_2 + \left|\left(\frac{\partial \phi x}{\partial y}\right)_{C_1,C_2}\right| . \Delta y \qquad (20)$$

Taking ΔC_1 and ΔC_2 zero, we obtain

$$\Delta \phi x = \left| \frac{C_1 - C_2}{C_1 C_2 (1 - y)^2} \right| \Delta y \qquad (21)$$

For practical reasons, C_2 will not exceed 0.1 or $|C_1 - C_2| < 0.1$. Since for values of ϕx not too far from 1, y will be of the same orc as C_2/C_1 and since $\Delta y \gtrsim 0.1y$ one finds that $\Delta \phi x \geq 1.0$.

This analysis shows that when only the adsorbed amount of radio labeled protein can be measured, ϕx cannot be determined accurately enough to exclude prefential adsorption (no preferential adsorption means $\phi x = 1$).
When larger surface areas (PS latex) are used the uncertainty in the determination of A_s will decrease. Moreover it is possible to measure in addition the adsorbed amount of non-labeled protein (depletion of protein in the solution). Up to this point we can conclude that ϕ can be measured directly but not very accurately, using equations (10), (16) and (17), in a system with irreversible adsorption where the protein concentrations in solution do not change significantly upon adsorption, when S_B in equation (16) is known from a separate experiment.
In the case of depletion experiments one should realize that protein concentrations decrease upon adsorption so that the ratio A/B changes during the adsorption when $\phi \neq 1$. In the case of reversible adsorption with depletion there is no problem in using equation (10) to find ϕ. When the adsorption of A and B is irreversible and depletion in solution occurs one can still find ϕ using a different approach. The rates of adsorption can be expressed as follows:

$$n_A = -\frac{dA}{dt} \cdot \frac{L}{M_A} \cdot \frac{1}{S_L} = k_A [A] \cdot \frac{L}{M_A} (1-\theta) \qquad (22)$$

$$n_B = -\frac{dB}{dt} \cdot \frac{L}{M_B} \cdot \frac{1}{S_L} = k_B [B] \cdot \frac{L}{M_B} (1-\theta) \qquad (23)$$

S_L = specific surface area of the latex, $m^2.m^{-3}$.

From equation (22) and (23) we obtain

$$\frac{dB}{dA} = \frac{k_B[B]}{k_A[A]} \tag{24}$$

Integration gives

$$\frac{\ln \frac{A}{A_o}}{\ln \frac{B}{B_o}} = \frac{k_A}{k_B} \tag{25}$$

Using equation (7) and (10) this expression for experiments with depletion in solution is related to the preferential adsorption factor ϕ without depletion:

$$\frac{\ln \frac{A}{A_o}}{\ln \frac{B}{B_o}} = \phi \cdot \frac{M_A}{M_B} \tag{26}$$

A_o, B_o = initial concentrations of A and B, $kg.m^{-3}$.

Although in the case of irreversible adsorption with depletion the straightforward use of equation (10) is not allowed to find ϕ, one can define an experimental parameter $\phi_{depl.}$ to be found from

$$A_s/B_s = \phi_{depl.} \cdot A/B \tag{27}$$

The relation between ϕ and $\phi_{depl.}$ can be given by

$$\phi_{depl.} = \frac{1 - (B/B_o)^{\phi M_A/M_B}}{(B/B_o)^{\phi M_A/M_B}} \cdot \frac{B/B_o}{1 - B/B_o} \tag{28}$$

and

$$|1 - \phi_{depl.}| \geq |1 - \phi| \tag{29}$$

From equation (29) one sees that $\phi_{depl.}$ gives an over-estimation of the preferential adsorption factor. Only in cases when $\phi \to 1$

will $\phi_{depl.} \rightarrow \phi$. For reversible adsorption $\phi_{depl.} = \phi$.
It can be derived both from equation (26) and (27) that the possible error in ϕ or $\phi_{depl.}$ due to experimental errors, is about 8%, on the basis of relative errors of 2% in the determined concentrations.

III.3 EXPERIMENTAL

III.3.1 Materials

Human serum albumin (HSA, crystalline) was obtained from Sigma, no. A 9511.
^{99m}Tc was obtained from Philips Duphar.
^{125}I (carrier free) was purchased from Amersham (IMS-30).
Polystyrene (PS), M_w = 670.000, M_w/M_n = 1.15, was obtained from Pressure Chemical Company, Pittsburgh, Mellon Institute Special Polystyrene Standard, lot.no. 13A.
Stainless steel squares (total surface area 3.2 * 10^{-4} m^2) were coated with PS by dipping in a solution of PS in toluene (7%). The dipping procedure was repeated after several hours and the surfaces were dried during 15 hours at 20°C temperature.
PS latex was prepared by the method of Goodwin **et** al.[29]. Particle diameter 0.56-10^{-6} m, ζ-potential -72 mV in distilled water (pH 7.0).
Silastic Rubber (SR), Silastic sheeting, non reinforced. Dow Corning Medical Grade Silicone Rubber, Subdermal Implant Material, lot.no. H 0 583.
The sheeting was cut in square pieces (total surface area 2.0 * 10^{-4} m^2).
Buffer was prepared by adding a solution of 0.01 M NaH_2PO_4 and 0.15 M NaCl to a solution of 0.01 M NaOH and 0.15 M NaCl until a pH of 7.4 was obtained.
All chemicals used were analytical grade.

III.3.2 Labeling of HSA.

Labeling of HSA with ^{99m}Tc was carried out by using:
a. an incubation method[30,31]; this product is indicated as ^{99m}Tc-HSA (i);

b. a modified incubation method. This method is similar to method a, except for the pH during the incubation step (pH = 7.4 instead of 2); ^{99m}Tc - HSA (im);
c. an electrolytic method[30,32,33]; ^{99m}Tc - HSA (e);

Labeling of HSA with ^{125}I was carried out by using:
d. an electrolytic method[34]. This labeling was carried out at pH 7.4 in buffer solution at I^- concentrations lower than one I^--ion per HSA molecule and a potential difference between the cathode and the anode of less than 900 mV; ^{125}I - HSA (e);
e. an electrolytic method (micro). In principle this method is similar to method d. This labeling was carried out in a micro reaction cell designed by the IRI*. In the cell the bottom (Pt) acts as the cathode and a stirrer (Pt) as the anode. The reaction volume is about 50 µl, and the electrolysis is completed within two minutes instead of 4 hours (method d); ^{125}I - HSA (em).
f. the lactoperoxidase method[35]; ^{125}I - HSA (l).

After the labeling procedures all protein solutions (a-f) were dialyzed against buffer solution to remove any unbound radioactive label. Then the percentage of label bound to the protein was determined by paper chromatographic methods as used by Stern et al.[36] and as described in a technical report of the IAEA[37]. These percentages were determined at the end of each labeling. It appeared in all cases, that more than 98% of the label was bound to the protein. The release of the label from the labeled proteins in buffer solution at 310 K (fig. 2) was studied as a function of time. From this figure, it can be concluded that for the ^{99m}Tc labeled proteins only the ^{99m}Tc-HSA(e) showed no significant release. Consequently protein labeling by methods a and b was not further investigated.
The ^{125}I labeled proteins showed no release of the label.

* Interuniversity Reactor Institute, Delft, Netherlands. Thanks are due to Dr. D. v.d. Hamer for assistance in the experiments.

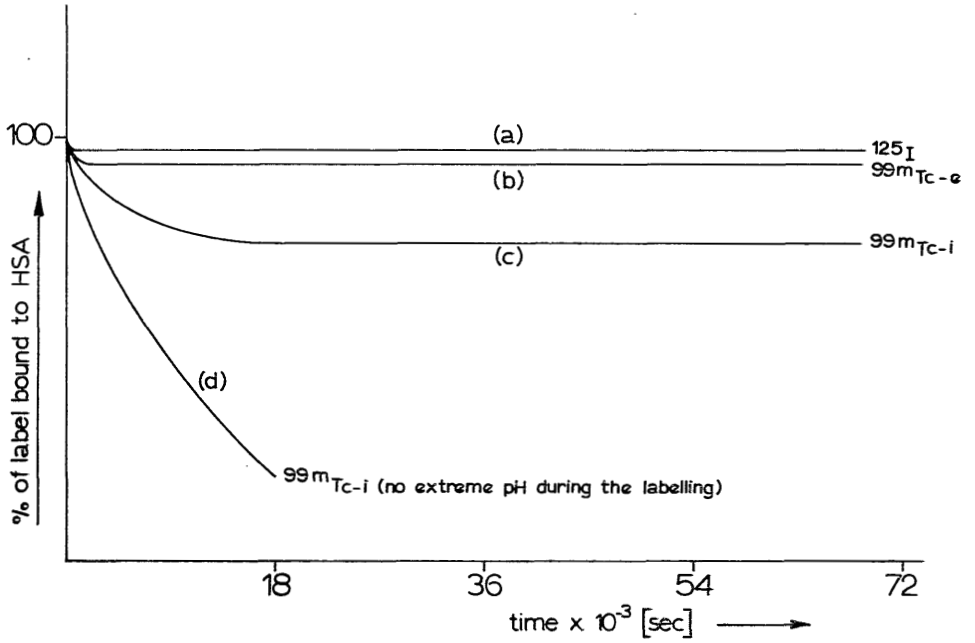

Figure 2: Percentage of label bound to HSA as a function of time measured in buffer solution at 310 K, pH = 7.4, [NaCl] = 0.15 M.

III.3.3 Characterization of proteins.

HSA (sigma, A 9511) was characterized by disc-electrophoresis on polyacryl amide gel with a Shannon apparatus; three bands were observed (fig. 3), which correspond with HSA monomer, dimer and trimer.

Chromatography using a column packed with Sephadex G-100 only showed the presence of monomer and dimer.

Immuno electrophoresis using rabbit anti-human serum did not reveal the presence of other proteins.

Labeled HSA (^{99m}Tc - HSA(e), ^{125}I - HSA(e), ^{125}I-HSA(em) or ^{125}I - HSA(l)) was mixed with HSA (non-labeled) and the different mixtures were investigated by disc-electrophoresis. It was shown that the radio-activity was only present in the three coloured bands, and the amount of radio-activity correlates with the intensity of the colour of the bands, indicating that the electrophoretic behaviour of the labeled proteins has not changed significantly by the labe-

ling methods. Chromatography of a micture of ^{125}I - HSA(em) and HSA using a sephadex G-100 column, yields two fractions (monomer and dimer) with activities in accordance with the relative protein concentrations.
Immuno electrophoresis of the same mixture showed that the immuno activity was not changed by the labeling method.

Figure 3: Electrophoretic pattern of HSA (Sigma, A 9511). Electrophoresis was carried out on polyacryl amide gel at pH = 7.4, [NaCl] = 0.15 M in phosphate buffer.

III.4 METHODS

The adsorption experiments were carried out by using two methods:

1. Adsorption onto PS and SR platelets. With this method only the amount of adsorbed radio-labeled protein can be measured. The total protein concentration in the solution during each adsorption experiment remains practically constant.
2. Adsorption onto PS latex. In this case the adsorbed amounts of labeled as well as non-labeled protein can be measured. The total protein concentration in the solution during each adsorption experiment decreases as a result of the large surface area of the latex.

A PS latex was added to a partially ^{125}I-labeled protein solution of known concentration. After 24 hours adsorption time, the mixture was centrifuged at 20.000 g. The protein concentration in the supernatant was determined by two methods:

a) folin-phenol method[11], and

b) by measuring the radio-activity in the solution.

The adsorption experiments with the PS surfaces (3.2×10^{-4} m^2) and the SR surfaces (2.0×10^{-4} m^2) were carried out in a thermostated adsorption cell, containing four trays in which the samples immersed in buffer solution, can be separated from the main solution (fig. 4).

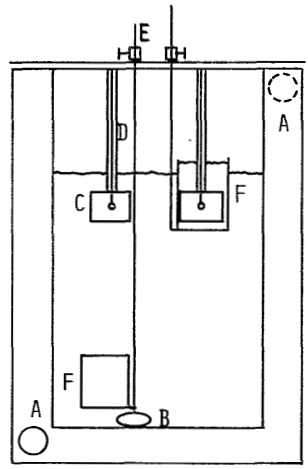

Figure 4: Thermostated adsorption cell, containing four trays in which samples immersed in buffer solution can be separated from the main solution. A: connection with the thermostated bath; B: magnetic stirrer; C: surfaces under investigation; D: nylon rope in glass tubing; E: glass cover; F: trays.

Contact of the solid surfaces with the liquid-air interface was avoided during the adsorption experiments and the rinsing procedures. This is important because "denaturated" protein layers might be deposited on the surfaces which are studied.

A typical adsorption experiment was performed as follows: the cell (pyrex-glass) was cleaned in a "chromic acid" cleaning mixture and rinsed thoroughly, first with tap water and then with double distilled water. After drying, the cell was connected with a thermostated bath (310 K), placed on a magnetic stirrer and filled with buffer solution. The surfaces were attached with nylon ropes to the glass cover and brought into the buffer solution. After temperature equilibration the surfaces were drawn several times through the liquid-air interface in order to remove any air-bubbles. Then the trays were lifted and a concentrated, partially labeled protein solution was injected into the stirred solution. During the addition, special care was taken to make sure that the needle was situated well below the liquid-air interface. After complete mixing, stirring was stopped and the trays were lowered to the bottom, which allows the surfaces to contact the protein solution.
This procedure was developed to avoid a possible contact of the surfaces with a non homogeneous protein solution, which might cause deviations in the adsorption data.
After the desired adsorption time, the trays were lifted and the glass cover with trays and sample surfaces was placed on a beaker. Then the solution in the trays was displaced by buffer solution until the solution in the trays was free of activity. The trays were lowered and the surfaces were placed into counting tubes. The measured activity on the surfaces was not affected by further rinsing. The amount of radio active protein on the surfaces is calculated by comparing the measured radio activity in counts per 180 sec. with the activities of the solution samples. The activity was determined with a scintillation counter (Baird Atomic, Spectrometer model S 30).

III.5 RESULTS.

III.5.1 Adsorption onto PS and SR platelets.

Figure 5 shows the results of the adsorption of ^{99m}Tc-HSA(e) using two concentration ratios of labeled to non-labeled protein, $C_1 = 1.15 \times 10^{-2}$ and $C_2 = 4.76 \times 10^{-2}$ onto PS.

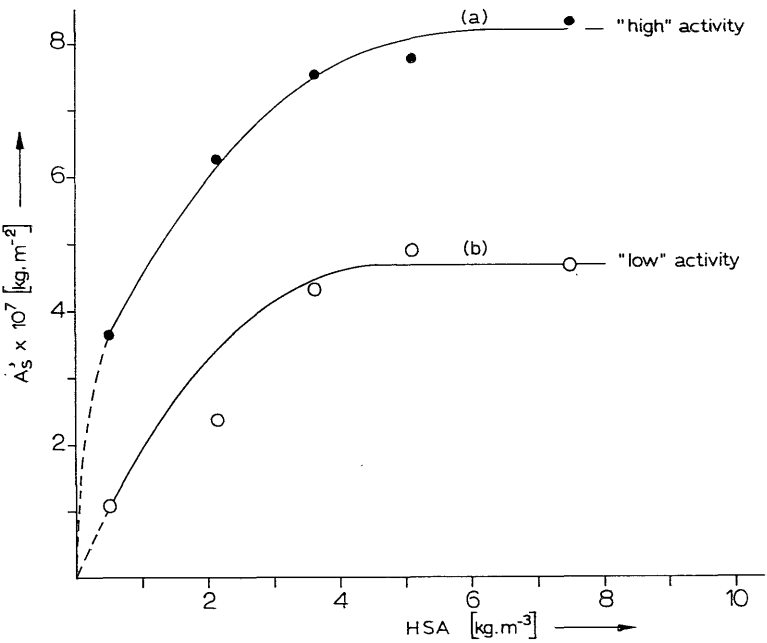

Figure 5: Surface concentration of ^{99m}Tc-HSA(e) (A_s) on PS as a function of total HSA concentration in solution (A+B). Concentration ratios of labeled to non-labeled protein; $C_1 = 1.15 \times 10^{-2}$ and $C_2 = 4.67 \times 10^{-2}$.

Using equations (18) and (21) we can calculate that in this case $\phi x = 66 \pm 20$. This means that the ^{99m}Tc-HSA(e) adsorbs preferentially as compared to non-labeled HSA. The results of a similar experiment with SR are shown in figure 6. Now $\phi x = 5.4 \pm 3.5$. This implies that proteins labeled in the same way show different adsorption behaviour upon exposure to different surfaces. ^{125}I-HSA(em) was tested in the same way on PS covered platelets (fig. 7). In this case ϕx was 1.0 ± 2.9 (equations (18) and (21)).

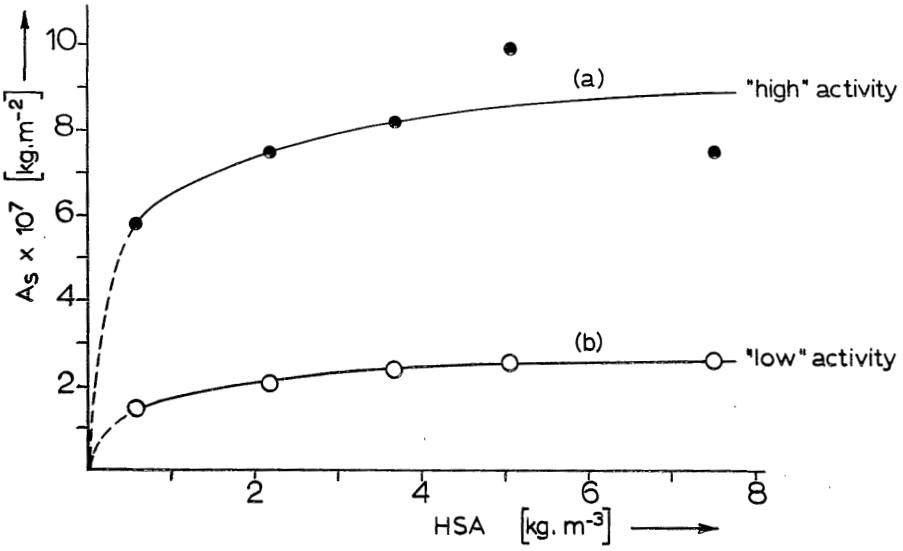

Figure 6: Surface concentration of ^{99m}Tc-HSA(e)(A_s) on SR as a function of total HSA concentration in the solution (A+B). Concentration ratios of labeled to non-labeled protein; $C_1 = 1.15 \times 10^{-2}$ and $C_2 = 4.67 \times 10^{-2}$.

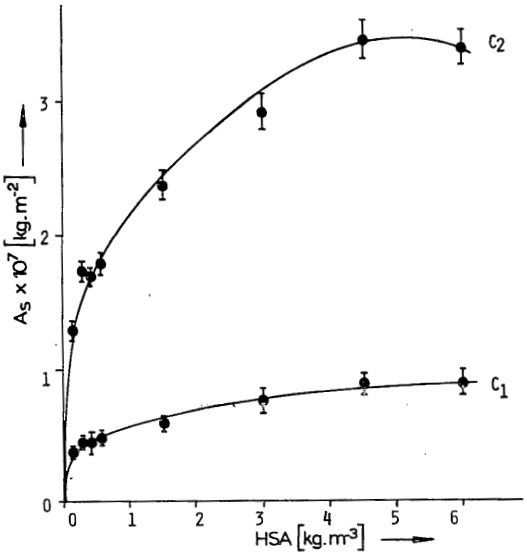

Figure 7: Surface concentration (A_s of ^{125}I-HSA(em) on PS as a function of total protein concentration in solution (A+B). Concentration ratios of labeled to non-labeled protein; $C_1 = 1.6 \times 10^{-2}$ and $C_2 = 6.4 \times 10^{-2}$.

III.5.2 Adsorption onto a PS latex.

These experiments were carried out with ^{125}I-HSA(e), ^{125}I-HSA(em) and ^{125}I-HSA(1).
Using equations (26) and (27) values of ϕ and $\phi_{depl.}$ for the different cases can be calculated. These data are given in table 1.

Protein	A/A_o	B/B_o	ϕ	A_s/B_s	A/B	$\phi_{depl.}$
^{125}I-HSA(e)	0.362	0.488	1.42	0.0119	0.0199	1.67
^{125}I-HSA(em)	0.421	0.520	1.32	0.0129	0.0193	1.50
^{125}I-HSA(1)	0.438	0.528	1.29	0.0134	0.0192	1.43

Table 1: Experimental data for the adsorption of differently labeled proteins onto PS-latex (adsorption after 24 hours, T = 298 K, pH = 7.4, [NaCl] = 0.15 M, phosphate buffer.

III.6 DISCUSSION.

When labeled proteins are used for adsorption experiments it is necessary to investigate the stability of the bond between the label and the protein molecules. This was done with the different ^{99m}Tc- and ^{125}I-labeled HSA preparates.
Figure 2 shows that ^{99m}Tc-HSA (incubation methods, i and im) are not stable under adsorption conditions. Consequently these compounds were not further investigated.
The remaining labeled proteins were characterized by electrophoretic methods. These methods all indicate that the labeled proteins resemble the non-labeled ones in their electrophoretic behaviour and no preferential labeling of HSA monomer, dimer or trimer was observed.

Subsequently adsorption experiments were carried out to detect any possible different adsorption behaviour of the labeled protein as compared to the non-labeled.
Adsorption experiments of ^{99m}Tc-HSA(e) onto PS and SR platelets (figures 5 and 6) show a preferential adsorption of the labeled protein. The preferential adsorption, expressed by ϕ x (equation (18)) was different for the two polymer surfaces (on PS: ϕ x = 66 $\pm$ 20 and on SR: ϕ x = 5.4 $\pm$ 3.5).
This means that the difference in adsorption behaviour between labeled and non-labeled protein is a function of the substrate used. Because ^{99m}Tc-HSA(e) shows preferential adsorption it was not used in further experiments.
For ^{125}I-HSA(e) on PS (figure 7), ϕ x is 1.0 $\pm$ 2.9, indicating that in this case the test method is not sensitive enough to conclude whether preferential adsorption takes place. This method was also used by Brash et al.[27] who measured the adsorption of ^{125}I-HSA (labeled by the iodine monochloride method of MacFarlane[38]) onto polyethylene (PE). Also in that case the method is not sensitive enough to exclude preferential adsorption.
Horbett et al.[39] measured the preferential adsorption of hemoglobin compared to other proteins onto PE, using radio-labeled proteins.
Lee et al.[15,26] and Dezelič et al.[7] also used different ^{125}I labeled proteins for competitive and kinetic adsorption experiments. The possible influence of the labeling on the adsorption behaviour of the proteins was not investigated. When ϕ x is close to 1, a more accurate method for the determination of possible preferential adsorption is required. This was achieved by using as the substrate a PS latex with a high specific surface area.
Now the adsorption causes a significant decrease of the protein concentration in the solution. The decrease of the total protein concentration [A+B] in the solution after 24 hours can be measured spectrophotometrically and the decrease of the concentration of the labeled protein [A] by measuring the radio-

activity of the solution. The preferential adsorption parameter ϕ and $\phi_{depl.}$ can now be calculated by using equations (26) and (27). Table 1 shows values of ϕ and $\phi_{depl.}$ for ^{125}I-HSA, labeled in different ways, which can be measured with an accuracy of about 10%.

All labeled proteins show values of ϕ and $\phi_{depl.} \neq 1$ indicating slight preferential adsorption. It is also shown that $\phi \neq \phi_{depl.}$, indicating irreversible adsorption. In conclusion, when labeled proteins are used for adsorption studies it is necessary to determine whether preferential adsorption occurs, even when the bond between the label and the protein is stable under experimental conditions and when the labeled proteins show the same electrophoretic behaviour as the non-labeled ones.

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CHAPTER IV

DYNAMIC ASPECTS OF CONTACT ANGLE MEASUREMENTS ON ADSORBED PROTEIN LAYERS "PROTEIN ADSORPTION"

ABSTRACT.

Contact angle measurements using drops of parafin oil have been performed on polystyrene (PS) substrates, coated with human serum albumin (HSA) or human fibrinogen (HFb), immersed in buffer solution. The contact angle appeared to be time dependent. The final value for HSA-coated substrates was $50^{o} \pm 5^{o}$ and for HFb-coated substrates $130^{o} \pm 10^{o}$ (measured through the oil phase). From measurements of the interfacial tension at oil-buffer interfaces on passing HSA coated substrates and from measurements using radiolabelled HSA in such experiments, it may be concluded that an adsorbed layer of HSA on PS can turnover from the PS substrate to the oil/buffer interface. The difference in behaviour between PS substrates coated with HSA and PS surfaces coated with HFb is attributed to association of the HFb molecules upon adsorption at the PS/buffer interface, whereas the stronger intra molecular forces in HSA do prevent this behaviour.

IV.1 INTRODUCTION.

When blood is contacted with the surface of an artificial material, adsorption of proteins will occur instantaneously. At the same time blood coagulation will be initiated by a contact activation mechanism.
Andrade[1,2] postulates that this activation is a function of the interfacial free energy between blood and the exposed surface. This implies that it is important to investigate how the nature of the exposed surface and the balance of intermolecular forces will be altered upon adsorption of protein molecules. Once the protein is adsorbed the other blood components might only interact with the adsorbed protein layer, masking the underlaying material.

Vroman[3] studied the effect of the nature of the exposed surface on the adsorbed protein layer. He used a roughly estimated contact angle value of water on different surfaces, coated with human fibrinogen, as an indication for the state of the protein layer. The protein coated surfaces were dried before judging their wettability.
McMillin[4] studied the influence of drying on the conformation of the adsorbed protein. It appears that an adsorbed layer of a certain protein (Hageman factor) shows entirely different ORD (Optical Rotation Dispersion) spectra for coated substrates which were dried and for those which were non-dried. These observations are not surprising, considering the important role of water in the tertiary structure of proteins (Lumrey[5], Bachmann[6]), and of possible irreversible changes in conformation upon drying.
In this study, contact angle measurements have been performed with paraffin oil drops, brought in contact with protein coated substrates immersed in buffer solutions. The buffer solution, which contains no protein, has been used to replace the original protein solution from which adsorption took place.
In this way the possible effect of drying of the coated substrates was prevented. The polar interactions between adsorbed protein and water will be investigated as well as dynamic aspects of the wetting behaviour of the oil drops on the immersed protein coated substrates.
As substrate material, polystyrene has been chosen which is a very apolar material. The proteins under investigation are human fibrinogen and human serum albumin.

IV.2 EXPERIMENTAL.

IV.2.1 Materials.

Human serum albumin (HSA), crystallized, was obtained from Pierce Chemicals. Prod. No. 30430.
^{125}*I-HSA* (batches with varying concentrations $\sim$ 7 kg.m^{-3}) was obtained from I.R.E. Fleurus, Belgium, No. Sari 125-B.
Human fibrinogen (HFb), > 90 % clottable, was obtained from AB Kabi, Stockholm.

Polystyrene (PS) was obtained from Pressure Chemical Company, Pittsburgh, Mellon Institute, Special Polystyrene standard. M_w = 670.000 M_w/M_n = 1.15, lot No. 13a.
Paraffin oil was obtained from Baker Chemicals, Deventer, Holland. Before use it it was twice purified by percolating over an Al_2O_3 column.
Buffer solution was prepared by adding an aqueous solution of 0.01 M KH_2PO_4 and 0.15 M NaCl to an aqueous solution of 0.01 M NaOH and 0.15 M NaCl until a pH of 7.4 was reached.
All protein solutions were stored at a temperature of 277 K, under sterile conditions, and they were used within one week after preparation.
All chemicals used were analytical grade.

IV.3 METHODS.

IV.3.1 Contact angle measurements.

Polystyrene (PS) substrates were obtained by dipping glass surfaces in a solution of PS in toluene (7%) and drying them afterwards. The dipping procedure was repeated three times. After the third time, the substrate was dried during 1 day at room temperature. Drying during a week at a temperature above the glass transition of PS did not give different contact angle results. Subsequently the substrates were conditioned in a buffer solution during 1 day.
Then the substrate was placed in a fresh buffer solution (pH = 7.4) after which a concentrated protein solution was added until the desired protein concentration in the range from 0.002 to 0.5 $kg.m^{-3}$ was reached. After three hours the protein solution was washed away by buffer solution containing no protein at all. The replacement of protein solution is a necessary step in the procedure, first to be sure that the oil-water interfacial tension during contact angle measurements does not change due to adsorption of protein from the solution and secondly to prevent contact between the substrate and the protein solution/air interface after the adsorption step. If this contact occurs the adsorbed and probably denaturated protein layer at the air/solution interface may be deposited on the

substrate (Gosh and Bull[7]) as a kind of Blodgett[8] layer. Subsequently the substrate was taken out, put directly into a cell containing buffer solution, and placed on a platform in the contact angle measurements set up. Using a glass capillary, a small drop of paraffin oil ($\sim 10^{-11}$ m^3) was brought under the substrate. The contact angle θ of this drop with the substrate was determined from the dimensions of the drop (Bargeman[9]), see fig. 1.

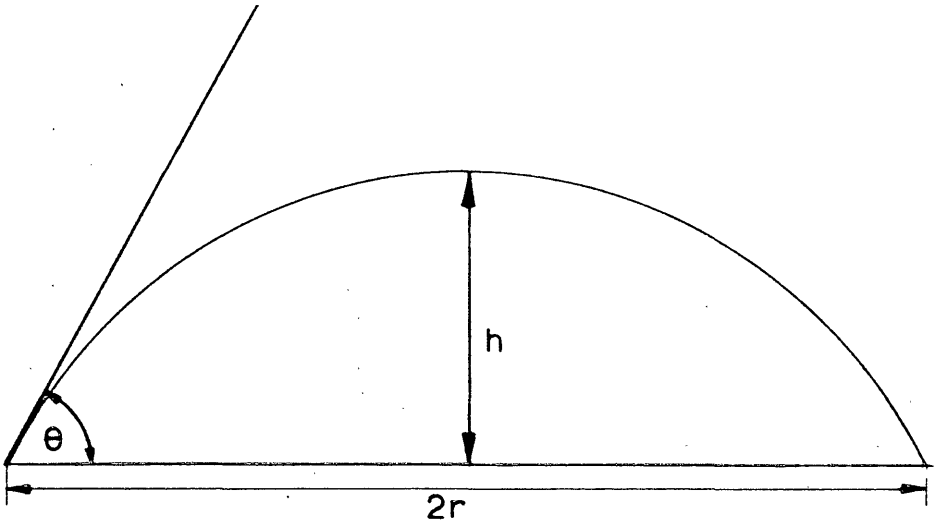

Figure 1: $\sin \theta = 2\,hr/(h^2 + r^2)$.

A necessary condition for this method is that the shape of the drop is not influenced by gravity (small drops should be used).

IV.4 RESULTS.

The contact angle θ of paraffin oil measured through the oil phase upon clean PS substrates immersed in buffer solution containing no protein appeared to be:

$$\theta = 30^{o} \pm 3^{o}$$

Measurements of the contact angle of paraffin oil with PS substrates immersed in a buffer solution deliberately containing 0.5 $kg.m^{-3}$ protein yielded an angle of contact being:

$$\theta = 180^{o}$$

In this system no wetting of the substrate by the oil drop was observed at all, not even after prolonged waiting (> 1 day).

Substrates were prepared carrying protein layers adsorbed from different protein solutions as described before. Buffer solutions containing 0.5, 0.05, 0.01 and 0.002 $kg.m^{-3}$ HSA and in a second series, buffer solutions containing 0.5, 0.05 and 0.002 $kg.m^{-3}$ HFb were used as adsorption liquids. After replacement of the protein solutions, contact angles were measured as a function of time of contact of the oil drop.
Results obtained with HSA coated substrates are given in fig. 2. Immediately after dispositing the paraffin oil drop onto the surface, the contact angle is 180^{o}. This situation could be maintained for longer periods if the substrate was kept in vibration by tapping against it. Without this mechanical influence the angle of contact decreased as a function of time. The rate of decrease and the final value of the contact angle seemed to be quite independent of the protein concentration in the solution from which the protein layer had been adsorbed.
After 2×10^{3} seconds of contact, θ was about 65^{o} and after 10^{4} seconds the ultimate value of $\theta = 50^{o} \pm 5^{o}$ was practically reached.
Results obtained with HFb-coated substrates are given in fig. 3. The contact angle measured on these substrates decreased much less than was observed for the HSA-coated substrates. The initial value was also 180^{o}, which situation also could be prolonged by tapping, but the final value of θ was 120^{o} to 140^{o}. Again this value was shown to be independent of the protein concentration in the solution from which the protein had been adsorbed.

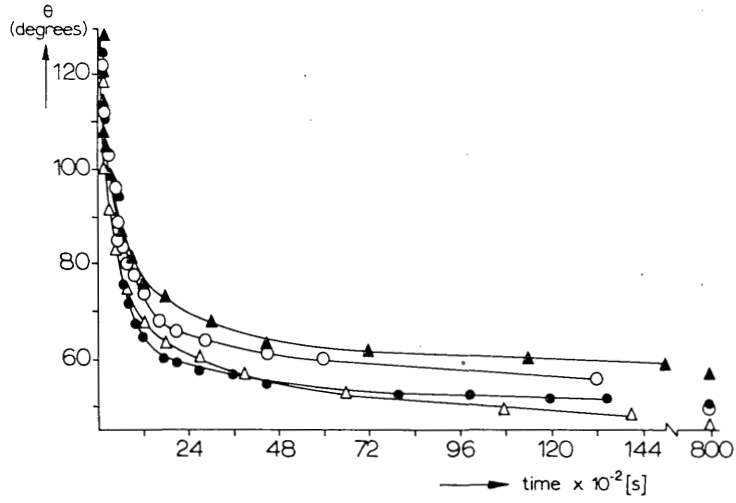

Figure 2: The time-dependence of the contact angle θ on HSA-coated PS substrates in buffered salt solutions (pH = 7.4, T = 293 K).
The protein was adsorbed from buffered solutions containing 0.5 kg.m^{-3} (•), 0.05 kg.m^{-3} (o), 0.01 kg.m^{-3} (▲) and 0.002 kg.m^{-3} (Δ) HSA respectively.

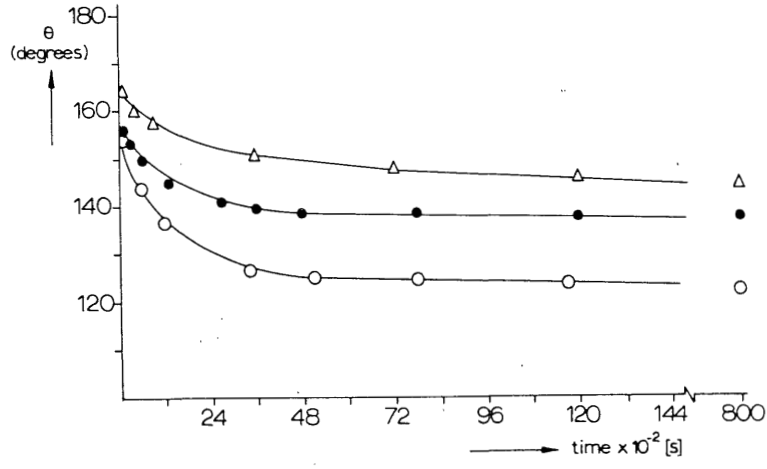

Figure 3: The time-dependence of the contact angle θ on HFb-coated PS substrates in buffered salt solution (pH = 7.4, T = 293 K).
The protein was adsorbed from buffered solutions containing 0.5 kg.m^{-3} (•), 0.05 kg.m^{-3} (o) and 0.002 kg.m^{-3} (Δ) HFb respectively.

In order to investigate the influence of drying of the adsorbed protein layer on the wetting behaviour of paraffin oil some protein coated surfaces were dried during 1 day at room temperature. Contact angle measurements on these substrates were performed, as soon as possible after their immersion in buffer solution. The results of this type of measurements carried out with substrates obtained originally by adsorption from a buffer solution containing 0.5 $kg.m^{-3}$ HSA are given in fig. 4 and from buffer solutions containing 0.5 $kg.m^{-3}$, 0.05 $kg.m^{-3}$ and 0.002 $kg.m^{-3}$ HFb resceptively in fig. 5.

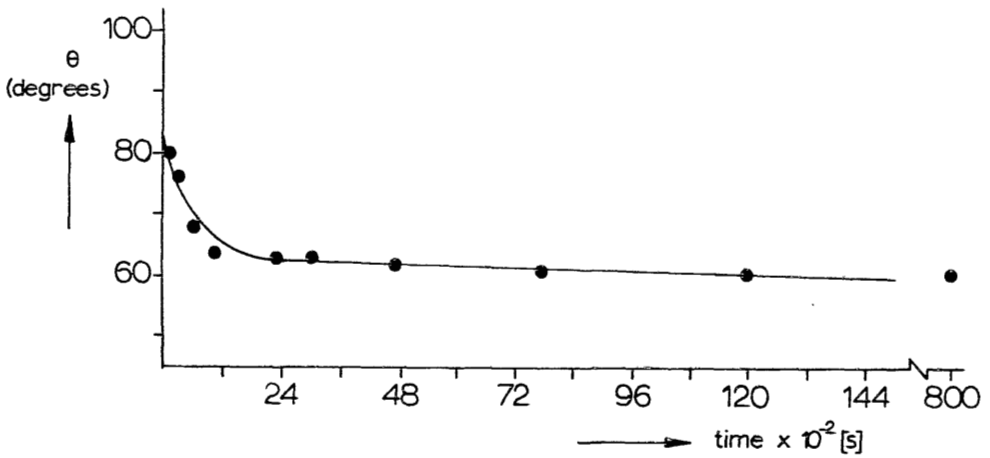

Figure 4: The time-dependence of the contact angle θ on intermediately dried HSA-coated PS substrates in buffered salt solution (pH = 7.4, T = 293 K). The protein was adsorbed from buffer solutions containing 0.5 $kg.m^{-3}$ HSA.

In contradistinction to the situation for non-dried protein layers, the initial contact angles are smaller than 180^{o} here. The contact angle on the intermediately dried HSA surface still shows a time dependence (fig. 4) whereas the fibrinogen substrates obtained

from 0.5 kg.m^{-3} and 0.05 kg.m^{-3} solutions immediately show constant contact angles. The HFb coated substrates obtained from 0.002 kg.m^{-3} solutions show a decrease of θ as a function of time (fig. 5).

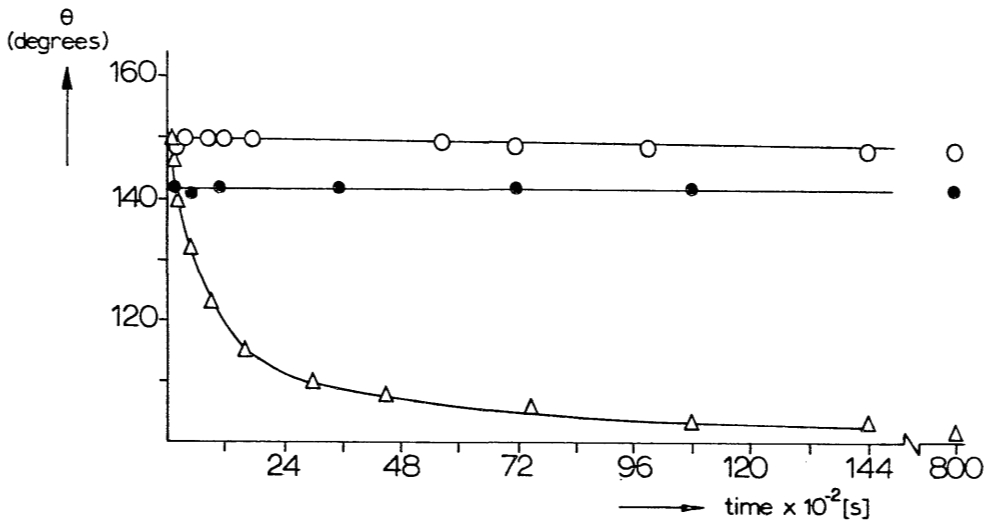

Figure 5: The time-dependence of the contact angle θ on intermediately dried HFb-coated PS substrates in buffered salt solution (pH = 7.4, T = 293 K). The protein was adsorbed from buffered solutions containing 0.5 kg.m^{-3} (•), 0.05 kg.m^{-3} (o) and 0.002 kg.m^{-3} (Δ) HFb respectively.

IV.5 DISCUSSION.

For a three phase system, paraffin oil/water/solid, the relation between the contact angle of an oil drop on the solid surface which is immersed in the aqueous phase and the acting interfacial tensions is given by the Young-equation:

$$\gamma_{o,w} \cos \theta = \gamma_{s,w} - \gamma_{s,o} \qquad (1)$$

where γ is the interfacial tension [mN.m^{-1}], θ is the contact angle measured through the oil phase and s,w and o indicate the solid, water and oil phase respectively.

The interfacial tension between two mutually non dissolving phases 1 and 2 equals the sum of the surface tensions of the phases in vacuum minus twice the free energy of interaction ($\gamma'_{1,2}$) between the two phases.

$$\gamma_{1,2} = \gamma_1 + \gamma_2 - 2\,\gamma'_{1,2} \qquad (2)$$

This free energy of interaction can be subdivided into two parts,
1) a contribution due to dispersive interaction ($\gamma'^{d}_{1,2}$) and
2) a contribution due to non dispersive interactions ($\frac{1}{2}\, I_{1,2}$).
or

$$\gamma'_{1,2} = \gamma'^{d}_{1,2} + \tfrac{1}{2}\, I_{1,2} \qquad (3)$$

Fowkes[10] showed semi-empirically that $\gamma'^{d}_{1,2} = \sqrt{\gamma_1^d\, \gamma_2^d}$, where γ_1^d and γ_2^d are the dispersive contributions to the surface tensions of phase 1 and phase 2 respectively.
Substitution into equation (2) gives:

$$\gamma_{1,2} = \gamma_1 + \gamma_2 - 2\sqrt{\gamma_1^d\, \gamma_2^d} - I_{1,2} \qquad (4)$$

It is assumed that the surface tension of paraffin oil results from Van der Waals forces only, so $\gamma_o = \gamma_o^d$.
In this study, contact angle measurements have been performed with paraffin oil drops on clean and protein coated substrates immersed in buffer solution; these surfaces will be indicated respectively by the indices s and s(p). Young's equation for system a (paraffin oil/buffer/PS) is:

$$\gamma_{o,w} \cos\theta_a = \gamma_{s,w} - \gamma_{s,o} \qquad (5)$$

and for system b (paraffin oil/buffer/protein coated PS)

$$\gamma_{o,w} \cos\theta_b = \gamma_{s(p)w} - \gamma_{s(p)o} \qquad (6)$$

Using (4), (5) and (6)

$$\gamma_{o,w}\ (\cos\theta_b - \cos\theta_a) = \gamma_{s(p)} + \gamma_w - 2\sqrt{\gamma^d_{s(p)}\ \gamma^d_w} - I_{s(p)w}$$

$$-\gamma_s - \gamma_w + 2\sqrt{\gamma^d_s\ \gamma^d_w} - \gamma_{s(p)} - \gamma_o + 2\sqrt{\gamma^d_{s(p)}\ \gamma_o} + \gamma_s + \gamma_o - 2\sqrt{\gamma^d_s\ \gamma_o}$$

or

$$\gamma_{o,w}\ (\cos\theta_b - \cos\theta_a) = 2(\sqrt{\gamma^d_{s(p)}} - \sqrt{\gamma^d_s})(\sqrt{\gamma_o} - \sqrt{\gamma^d_w}) - I_{s(p)w} \quad (7)$$

Equation (7) has been obtained, on the basis that the clean PS substrate has no nondispersive interactions with the aqueous phase[9] and also that the clean and the protein covered PS surface show no polar interaction with the oil phase. Introduction of $\gamma^d_w = 21.8\ mN.m^{-1}$ and $\gamma_o = 32\ mN.m^{-1}$ simplifies the first term on the right, yielding

$$\gamma_{o,w}\ (\cos\theta_b - \cos\theta_a) = 2(\sqrt{\gamma^d_{s(p)}} - \sqrt{\gamma^d_s}) - I_{s(p)w} \quad (8)$$

Values for $\gamma^d_{s(p)}$ are not available, but considering that the value of $\gamma^d_{s(p)}$ might be somewhere between 46 $mN.m^{-1}$ and 21.8 $mN.m^{-1}$ (the values of γ^d for nylon 6-6[11] and water respectively), it can be stated that

$$4.5 < \sqrt{\gamma^d_{s(p)}} < 7$$

Introduction of this value and the γ^d_s-value for polystyrene[12] (44 $mN.m^{-1}$) in equation (8) yields

$$\gamma_{o,w}\ (\cos\theta_a - \cos\theta_b) = I_{s(p)w} - (0 \text{ to } 5)\ nM.m^{-1} \quad (9)$$

From the final contact angle $\theta_b = 50^o \pm 5^o$ (fig. 2) measured on HSA coated substrates and $\theta_a = 30^o \pm 3^o$ measured on clean PS substrates it may be concluded that:

$$0 < I_{s(p)w} < 20\ mN.m^{-1} \quad \text{for HSA coated surfaces}$$

From the final contact angle $\theta_b = 120^o$-140^o (fig. 3) measured on HFb coated substrates and $\theta_a = 30^o \pm 3^o$ it may be concluded that

$$65 < I_{s(p)w} < 85 \text{ mN.m}^{-1} \quad \text{for HFb coated surfaces}$$

The difference between the calculated values for the non-dispersive interactions of HSA and HFb with water is much greater than can be expected from the differences in the chemical composition of these molecules. It is known that both proteins are well soluble in water. When, for instance HFb is dissolved in an aqueous solution it contains water up to 4 times its own weight[6]. From these data it is to be expected that there is a strong interaction between these proteins and water. Since the I-term acting across a hypothetical water/water interface can be calculated to be $\sim$ 100 mN.m^{-1} ($I_{ww} = \gamma_w + \gamma_w - 2\sqrt{\gamma_w^d \gamma_w^d} \approx 100$ mN.m^{-1}), it might be concluded that the value of $I_{s(p)w}$ must be of the same order. This means that the $I_{s(p)w}$ found for HFb/water has an acceptable value (65-85 mN.m^{-1}). It also means that in the case of HSA-coated substrates something has happened which causes an unlikely low value of $I_{s(p)w}$ to be calculated.

A different behaviour of HSA-coated substrates compared to HFb-coated substrates has been observed already with the dried substrates (figs. 4 and 5). Fig. 4 shows that the contact angle on the intermediately dried HSA substrates still shows a time dependence, whereas fig. 5 shows no time dependence for the contact angles measured on intermediately dried HFb substrates.

Three possible explanations can be given for the difference in the contact angle vs time behaviour of the adsorbed protein layers under investigation:

1) The conformation of the adsorbed protein layer changes drastically under the influence of the oil drop.
2) The adsorbed protein layer dissolves in the buffer solution.
3) The adsorbed protein layer is "stripped" off from the PS surface by the paraffin oil drop. This will result in the presence of protein at the oil/water interface.

The second possibility can be ruled out since the behaviour of an oil drop on a protein coated substrate did not change when this protein coated substrate was incubated for several hours in a protein-free buffer solution. So we only have to consider the first and the last mentioned possibility. Reconformation of protein molecules situated between PS and the oil drop cannot be ruled out, but it does not seem to be a very obvious process. When the last mentioned possibility occurs, the value of $\gamma_{o,w}$ will decrease. It is not possible to directly measure this decrease of $\gamma_{o,w}$ at the advancing interface of a very small drop of paraffin oil.
However, the situation of a slowly advancing contact angle can be simulated by pulling protein coated PS platelets very slowly through an oil/buffer solution interface. When "stripping" occurs, the interfacial tension ($\gamma_{o,w}$) will decrease, resulting from the presence of "stripped" protein at the interface. This experiment has been performed as follows:

Glass plates (0.024×0.040 m) were coated with polystyrene and HSA was adsorbed from a buffered solution containing 0.5 $kg.m^{-3}$ HSA. After the replacement of the protein solution by buffer solution without protein, the platelets were taken out all wet, and placed in a beaker containing buffer solution. Then a layer of paraffin oil was carefully brought on top of the buffer solution and the interfacial tension $\gamma_{o,w}$ was measured using the Wilhelmy-plate method (check on wetting angle). Subsequently the protein-coated platelets are pulled very slowly (1-2 $\mu m.s^{-1}$) through the oil/buffer interface, after which the interfacial tension was measured again.

The interfacial tension had decreased from

$$50\ mN.m^{-1} \text{ to } 43\text{-}45\ mN.m^{-1}$$

From these experiments it is concluded that at least a part of the time-dependence of the contact angle measurements on HSA substrates, dried and non-dried, results from the "stripping" of the adsorbed HSA layer by the oil drop.

In order to verify this feature in still a different way, experiments with radiolabeled HSA have been performed. For these experiments HSA labeled with ^{125}I (^{125}I-HSA) was used.

Polystyrene substrates were coated with a protein layer by adsorption from a buffer solution containing ^{125}I-HSA and HSA with a total protein concentration of 0.5 $kg.m^{-3}$. The adsorption procedure has been described elsewhere[13]. The HSA coated substrates were placed in a beaker containing buffer solution only. Some of the plates were connected to a mechanism which could pull them upwards with a rate of 1 or 2 $\mu m.s^{-1}$, and a layer of paraffin oil was carefully brought on top of the solution; the others were placed in another beaker with buffer solution. After 20 hours the substrates were taken out: the first series of substrates had passed the oil/buffer interface by means of the mentioned mechanism and the other series of substrates were taken out directly from the buffer solution.

The surface concentration of HSA on the substrates was measured with a scintillation counter (Baird Atomic Spectrometer model 530). The results are given in table 1).

Rate of emersion $[\mu.m.s^{-1}]$	Surface conc. of HSA on PS substrate without passing the oil/buffer interface $\times 10^6$ $[kg.m^{-2}]$	Surface conc. of HSA on PS substrate after passing the oil/buffer interface $\times 10^6$ $[kg.m^{-2}]$
1	1.6	0.4
2	1.6	0.8

Table 1: The amount of protein present on PS substrates which have been or have not been drawn through an oil/buffer interface.

By these experiments it has been confirmed that an oil drop can "strip" an adsorbed layer of HSA from a polystyrene surface. With an increased effectivity of this "stripping" upon a decrease in the transport rate of the boundary line.

A similar effect has been found by Bikerman[14] for deposited multilayers of bariumstearate; the phenomenon was called boundary corrosion. The adsorption of bariumstearate however is reversible whereas for an explanation of this stripping effect it is necessary to realize that the adsorption of macromolecules like HSA and HFb is generally assumed to be irreversible and that each adsorbed protein molecule will be adsorbed at the substrate by a number of apolar segments. The assumed irreversibility does not necessarily mean that each binding site shows irreversibility in adsorption. There is, however, statistically an extremely small possibility that desorption occurs at all the contact places at the same time.
The observed "strip effect" proves that each of the trains of segments with which the HSA molecules are adsorbed onto the surface is bound in a reversible way. This can be visualised schematically as follows (fig. 6):

A drop of paraffin oil has been brought in contact with a protein coated surface immersed in buffer solution. Initially the observed contact angle (measured through the oil phase) will remain 180^{o} (fig. 6a), possibly resulting from a retarded reorientation of hydrophilic segments of the adsorbed protein molecules in the thin waterfilm next to the protein layer. This non-wetting was shown to be a dynamic effect; it could be prolonged when the substrate was kept in vibration causing displacement on the drop. Hence the adhesional forces are very weak in the beginning and there is no spontanious tendency for the water layer in contact with the protein layer to break away. The phenomenon that on intermediately dried surfaces the initial contact angle was smaller than 180^{o} might indicate that, upon drying the adsorbed protein layer, reconformation occurs from a hydrophilic outer layer to a (at least partially) hydrophobic outer

layer. When the water layer breaks, the oil-protein contact will be realized and a finite contact angle (fig. 6b) will be establishe At the three phase boundary the protein may adsorb at the paraffin oil/water interface also. Then the protein molecules can be peeled off from the PS (fig. 6b), because the interaction of the protein molecules with the liquid oil can be stronger than with the solid PS, resulting from the flexibility of liquid-liquid interfaces. Now the oil phase may be able to reach the underlying apolar substrate at some places (fig. 6c). The protein molecules are supposed to be long ellipsoids connected to the surface by a number of apolar segments (the dots in fig. 6a-d).
When such a segment next to the oil phase contact line desorbs, the oil will proceed spreading on the PS and the train will readsorb onto an apolar substrate, now the paraffin oil (fig. 6d). In this way the protein molecules might slide over from the PS/buffer inter face to the paraffin oil/buffer interface (fig. 6e), and the contac angle will show a time dependent decrease.

Obviously the "strip effect" takes place on albumin coated substrates only and not on the fibrinogen coated substrates. If the proposed mechanism of "stripping" is right then this difference cannot result from the higher molecular weight. It may result from the fact that fibrinogen molecules denaturate much easier than albumin molecules do. Hence the adsorbed fibrinogen molecules may show association at the surface resulting in an extended, associated fibrinogen layer which cannot be "stripped" from the surface.
Morrisey and Stromberg[15] found that upon adsorption the infrared bound fraction of fibrinogen increased, whereas this did not happen for albumin. They concluded that the internal binding of albumin is sufficient to prevent changes in the structure while adsorbed, even at low surface concentration. The increase of the bound fraction of fibrinogen upon adsorption may result from interfacial aggregation, which is in line with our measurements.

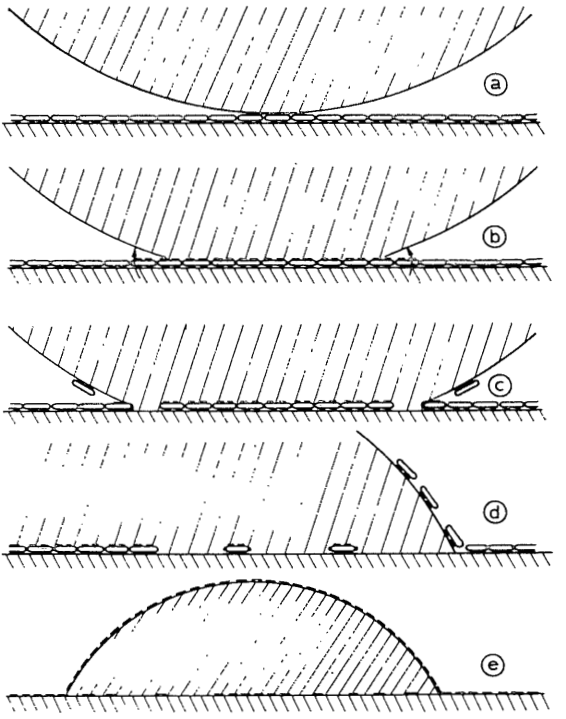

Figure 6: Schematical wetting process of an oil drop onto a protein coated PS substrate.
a) initial situation
b) finite contact angle on the protein layer and the first turnover of the protein molecules
c) wetting of the PS substrate by the paraffin oil
d) "stripping" of the protein molecules
e) final situation.

The time dependence of the contact angle on the intermediately dried HFb substrate, adsorbed from a 0.002 $kg.m^{-3}$ HFb solution (fig. 5) may be the result of an incomplete coverage of the surface so that complete association cannot occur.
The absolutely non-wetting behaviour of an oil drop on a PS substrate, immersed in a protein solution (0.5 $kg.m^{-3}$) can be explained by entropic repulsion, caused by the adsorbed protein layers at the PS/solution and the paraffin oil/solution interfaces, or the lowering of the $\gamma_{o,w}$ by protein adsorption. This can be shown by the following calculations on for instance

fibrinogen coated substrates.

From the experiments without protein in the buffer solution we know that the final contact angle is about 130^{o}. Further it is known that $\gamma_{o,w} = 50\ mN.m^{-1}$. So equation (6) becomes

$$-32 = \gamma_{s(p)w} - \gamma_{s(p)o}$$

The values of $\gamma_{s(p)w}$ and $\gamma_{s(p)o}$ will not change upon addition of protein to the buffer solution. The value of $\gamma_{o,w}$ however, will decrease from 50 $mN.m^{-1}$ to 20 $mN.m^{-1}$ (pH = 7.4 [NaCl] = 0.15 $kmol.m^{-3}$, 298 K, Wilhelmy Plate Method). This means that when a finite contact angle would occur

$$22 \cdot \cos\theta = -32$$

Because $\cos\theta$ never is smaller than -1, the conclusion must be that no wetting occurs at all and $\theta = 180^{o}$.

IV.6 CONCLUSIONS

From contact angle measurements on polystyrene (PS) substrates coated with human fibrinogen (HFb), immersed in buffer solution, information can be obtained about the non-dispersive (polar) interaction of a protein layer with water (65-85 $mN.m^{-1}$).

The time dependence of the contact angle of a drop of paraffin oil on a human serum albumin (HSA)-coated PS substrate immersed in buffer solution appeared to show two subsequent features.

1) The disruption of a hydrated water layer on the protein layer (which is stable under dynamical conditions).
2) The turnover of part of the adsorbed protein molecules, from the PS/buffer interface to the oil/buffer interface.

From this last feature it may be concluded that the binding sites between the protein molecules and the substrate are reversible.

The influence of intermediate drying the protein-coated substrates on the final contact angle appeared to be negligible, whereas the initial contact angle was influenced strongly; this suggests that the difference results from an incomplete hydration of the dried protein layer.
From the inability of adsorbed human fibrinogen to turn over from a PS substrate immersed in buffer to an oil drop it may be concluded that the adsorbed fibrinogen molecules show interfacial association.

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CHAPTER V

ADSORBED LAYERS OF ALBUMIN AND FIBRINOGEN ON POLYSTYRENE, PROBED BY CONTACT ANGLE MEASUREMENTS

ABSTRACT.

Earlier measurements of paraffine oil (PO)-water contact angles on protein layers (human serum albumin (HSA) or human fibrinogen (HFb)) adsorbed on polystyrene (PS) showed that HSA could be transferred from the PS/water to the PO/water interface by a passing PO-water front, while HFb could not.
Interfacial association of adsorbed HFb molecules was adopted as an explanation for the irreversible localization of HFb at the PS surface.

In the present work it is shown that aggregation by a heat-treatment of adsorbed HSA molecules also causes an irreversible localization of the HSA at the PS surface. It is shown that advancing and receding PO-water contact angles on HSA-coated PS substrates have practically the same value. Variation of pH and NaCl concentration hardly shows any effect on these contact angles, indicating that in all cases the HSA coating behaves the same.

The advancing and receding PO-water contact angles on HFb coated substrates differ greatly. In analogy with El-Shimi and Goddard we conclude that the natural HFb coating is able to adopt and retain a configuration compatible with the immediate environment.

V.1 INTRODUCTION.

Early work by Vroman[1] suggests that there is a difference in wetting behaviour of different proteins, adsorbed on glass or polymer surfaces. Vroman's treatment was a more qualitative one: adsorbed protein layers had been dried in air completely and wetting was judged by vapour (droplet) condensation.

In a previous paper[2] we have worked out protein layer wettability in a more quantitative way. It was shown that the behaviour of an oil drop on a protein coated polystyrene (PS) substrate immersed in buffer solution depends on the type of protein used for the coating. On a PS substrate coated with human serum albumin (HSA) the contact angle, measured through the oil phase, decreased slowly from $\sim 180^{o}$ to $50^{o} \pm 5^{o}$. The contact angle on a PS substrate coated with human fibrinogen (HFb) reached a final value of $130^{o} \pm 10^{o}$. The difference between the contact angles on the protein-coated substrates appeared to be the result of a difference in the transfer of the proteins when a three phase contact line is passing. HSA molecules are transferred from the PS/water interface to the oil/water interface leading to a large decrease of the θ value in that case. The irreversible localisation of HFb at the PS surface, possibly due to interfacial association, opposes such a large decrease in contact angle for the oil drop.

In the present manuscript further evidence is presented for the statement that a layer of associated protein molecules will not be transferred from the PS/water interface to the oil/water interface by a passing oil/water front. From adsorption studies[3] it is known that protein adsorption is influenced by pH and salt concentration. The influence of these parameters on the contact angle is studied for both proteins. The results hitherto obtained have been measured for an advancing oil/water interface. Measurements of contact angles for a receding oil/water interface have now been performed to get information about the character of the adsorbed protein layers.

V.2 EXPERIMENTAL.

V.2.1 Materials

Human serum albumin (HSA), crystallized, was obtained from Pierce Chemicals. Prod. No. 30430.
^{125}*I-HSA* (batches with varying concentration ~ 7 kg.m^{-3}) was obtained from I.R.E. Fleurus, Belgium, No. Sari 125-B.
Human fibrinogen (HFb), > 90% clottable, was obtained from AB Kabi, Stockholm.

Polystyrene (PS) was obtained from Pressure Chemical Company, Pittsburg, Mellon Institute, Special Polystyrene standard. M_w = 670.000 M_w/M_n = 1.15, lot No. 13a.
Paraffin oil (PO) was obtained from Baker Chemicals, Deventer, Holland. Before use it was twice purified by percolating over an Al_2O_3 column.
Buffer solutions were prepared by adding an aqueous solution of 0.01 M KH_2PO_4 and the proper amount of NaCl to an aqueous solution of 0.01 M NaOH and the same amount of NaCl until a pH of 7.4 was reached. Other pH values were obtained by adding NaOH or HCl until the desired value was reached.
All protein solutions were stored at a temperature of 277 K, under sterile conditions, and they were used within one week after preparation.
All chemicals used were analytical grade.

V.3 METHODS.

V.3.1 Contact angle measurements

Polystyrene (PS) substrates were obtained by dipping glass surfaces in a solution of PS in toluene (7%) and drying them afterwards. The dipping procedure was repeated three times, After the third time, the substrate was dried during 1 day at room temperature. Drying during a week at a temperature above the glass transition of PS did not give different contact angle results. Subsequen ly the substrates were conditioned in a buffer solution during 1 day Then the substrate was placed in a fresh buffer solution after which a concentrated protein solution was added until the desired protein concentration of 0.5 $kg.m^{-3}$ was reached. After one hour the protein solution was washed away by a buffer solution containing no protein. The replacement of protein solution is a necessary step in the procedure, first to be sure that the oil/water interfacial tension during contact angle measurements does not change due to adsorption of protein from the solution and secondly to prevent contact between the substrate and the protein solution/air interface after the adsorption step. If this contact occurs the adsorbed and probably denaturated protein layer at the air/

solution interface may be deposited on the substrate as a kind of Blodgett layer[4,5].
Subsequently the substrate was taken out, put directly into a cell containing buffer solution, and placed on a platform in the contact angle measurements set up. Using a glass capillary, a small drop of paraffin oil (diameter $\sim 0.3.10^{-3}$ m) was brought under the substrate. The contact angle θ_a of this drop with the substrate was determined from the dimensions of the drop (Bargeman[6], see fig. 1).

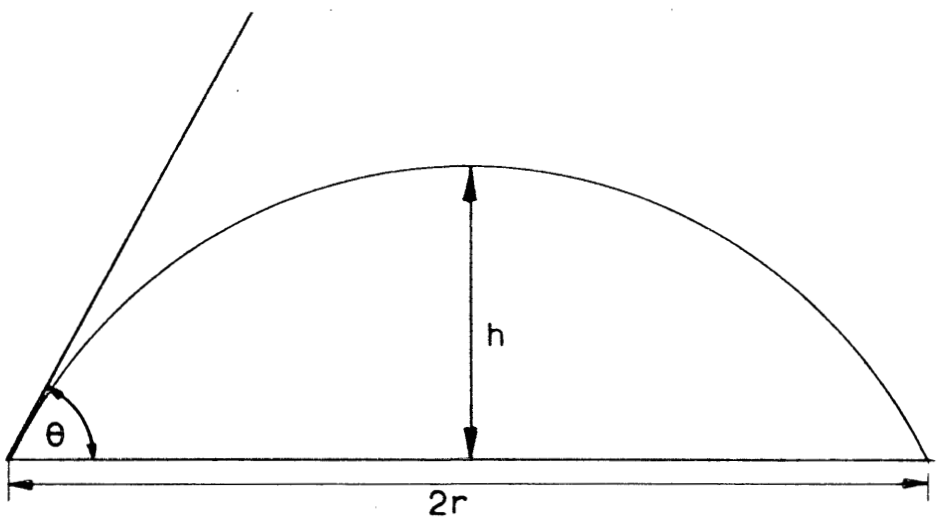

Figure 1: $\sin \theta = 2\,hr/(h^2 + r^2)$.

A necessary condition for this method is that the shape of the drop is not influenced by gravity (small drops should be used).
For measurements of the receding contact angle through the oil phase θ_r the substrate was taken out of the buffer solution swepped off and then placed in a cell containing paraffin oil. Using a glass capillary a small drop of buffer solution (diameter $\sim 0.3.10^{-3}$ m) was dropped on the substrate. The contact angle of this drop, which is equal to $180^{\circ} - \theta_r$, was determined in the same way as described above.

Heat denaturation of adsorbed protein layers was performed prior to immersion in the cell, by placing the substrate in a buffer solution at 343 K for $\sim 10^3$ sec.

V.4 RESULTS

All adsorption experiments have been performed at protein concentrations of 0.5 $kg.m^{-3}$ in buffer solutions at different pH-values and NaCl concentrations.
Advancing contact angles θ_a obtained with paraffin oil drops on HSA coated substrates immersed in buffer solutions at different pH-values and NaCl concentrations are given in table 1.

pH	[NaCl] $kmol.m^{-3}$	θ_a (initial) (within 30 sec.)	θ_a (final)
2.5	0	45^o	45^o
4.9	0	145^o	65^o
9.0	0	130^o	45^o
2.5	0.15	142^o	42^o
4.9	0.15	145^o	62^o
7.4	0.15	160^o	55^o
9.0	0.15	160^o	75^o

Table 1: Advancing contact angles of oil drops on HSA coated substrates.

In this table θ_a (initial) is the first value of the contact angle that could be measured after deposition of the PO drop on the substrate (10-30 sec. after deposition). The final value of the contact angle θ_a (final) has been measured after $\sim 6.10^4$ sec. Actually this last value has been reached generally within 10^3 sec. after deposition. Results obtained on HFb coated substrates for different pH-values and NaCl concentrations are shown in table 2.

pH	[NaCl] kmol.m^{-3}	θ_a (initial) (within 30 sec.)	θ_a (final)
3	0	140	102
7.4	0	165	160
9.5	0	165	162
3	0.05	150	120
7.4	0.15	155	140
9.5	0.15	165	155

Table 2: Advancing contact angles of oil drops on HFb coated substrates.

Measurements on heat denaturated HSA coatings on PS substrates have been performed at different pH values. The θ_a-values obtained are shown in table 3.

pH	[NaCl] kmol.m^{-3}	θ_a (initial) (within 30 sec.)	θ_a (final)
2.5	0.15	60°	45°
4.9	0.15	150°	120°
7.4	0.15	150°	140°
9.0	0.15	140°	120°

Table 3: Advancing contact angles of oil drops on heat treated HSA coatings.

The contact angles θ_r for the oil phase obtained with drops of buffer solution on HSA-coated substrates immersed in PO ("inverse system") at different pH-values and NaCl concentrations are given in table 4.

pH	[NaCl] $kmol.m^{-3}$	θ_r (initial) (within 30 sec.)	θ_r (final)
2.5	0	60^o	60^o
4.9	0	75^o	65^o
9.0	0	67^o	50^o
2.5	0.15	63^o	63^o
4.9	0.15	72^o	70^o
9.0	0.15	58^o	58^o

Table 4: Receding contact angles for the oil phase on HSA coated substrates ("inverse system").

The results from the measurements in the inverse system on HFb coated substrates at different pH-values and NaCl concentrations are collected in table 5.

pH	[NaCl] $kmol.m^{-3}$	θ_r (initial) (within 30 sec.)	θ_r (final)
3.0	0	80^o	85^o
7.4	0	82^o	83^o
9.0	0	90^o	90^o
3.0	0.05	80^o	80^o
7.4	0.15	90^o	90^o
9.0	0.15	86^o	86^o

Table 5: Receding contact angles for the oil phase on HFb coated substrates ("inverse system").

The contact angle θ_a was also measured in the inverse system by decreasing the volume of the drops of buffer solution until the PO/water boundary on the substrate started to move. For the HSA-coated substrate this contact angle θ_a was practically the same as the observed value of θ_r. On the other hand the volume of a drop of buffer solution (pH = 7.4) on a HFb coated substrate could deliberately be decreased without the effect that the PO/water boundary started to move.

This could be continued until a θ value < 20^{o} measured over the droplet phase, was reached, which means that for this measuring technique $\theta_a > 160^{o}$.

V.5 DISCUSSION.

V.5.1 The effect of pH and salt on results for HSA-coatings.

From the influence of pH and salt concentration on the maximum adsorption of HSA on PS[3] it was expected that there might be a difference in behaviour between a layer of HSA adsorbed at its isoelectric point (pH = 4.9) and layers adsorbed at pH-values far away from this i.e.p.
Table 1 shows the influence of pH and NaCl concentration on the θ_a-values on HSA-coated PS-substrates. It appears that the influence on the final θ_a-values is very small.
In a previous paper we derived an equation to estimate the value of the polar interaction term, $I_{s(p)w}$, between an adsorbed protein layer and the aqueous phase.
It was assumed that no turnover of the protein molecules from the PS substrate to the PO/water interface takes place.

$$I_{s(p)w} = \gamma_{o,w} (\cos \theta_1 - \cos \theta_2) - (0 \text{ to } 5) \text{ mN.m}^{-1} \qquad (1)$$

θ_1 = $30^{o} \pm 3^{o}$ contact angle for an oil drop on the bare PS substrate immersed in water (buffer)

θ_2 = contact angle for an oil drop measured on the protein coated substrate in water (buffer)

$\gamma_{o,w}$ = 50 mN.m^{-1} interfacial tension at the PO/water interface.

Introduction of the θ_a (final) values in equation (1) gives a polar interaction term of

$$0 < I_{s(p)w} < 25 \text{ mN.m}^{-1} \quad \text{for HSA coatings.}$$

This value is much lower than expected for the polar interaction between a highly swollen protein layer and water.

This would indicate that in these measurements turnover of the HSA molecules from the PS/water interface to the PO/water interface takes place.
From the difference between θ_a (initial) and θ_a (final) it is clear that at pH = 4.9 and 9.0 θ_a is time dependent and at pH = 2.5 the final value of θ_a has been reached immediately. The very fast turnover of adsorbed HSA molecules at this pH might be due to the fact that the HSA molecules have a different conformation at this pH. At pH values between 4.9 and 2.5 the so-called N-F transition of the molecule is observed[7].

V.5.2 Effect of pH and salt on results for HFb-coatings.

The values for θ_a on HFb coated substrates at different pH values and NaCl concentrations are shown in table 2. In this case no experiments have been performed at the isoelectric point of the HFb (pH = 5.5) because HFb solutions are not stable at their i.e.p. The experimental conditions were chosen such that HFb adsorption took place from clear solutions.

It is shown (table 3) that there is hardly any influence of pH and salt concentration on the θ_a values on the HFb coated substrates. Using equation (1) and the θ_a (final) values the polar interaction term between the protein and the aqueous phase is

$$80 < I_{s(p)w} < 90 \text{ mN.m}^{-1} \quad \text{for HFb coatings}$$

for all measurements, exept those at pH = 3.0.

At this pH = 3.0 the polar interaction is calculated to be

$$50 < I_{s(p)w} < 70 \text{ mN.m}^{-1}$$

The somewhat lower value of $I_{s(p)w}$ at low pH may be due to a reduced polar contribution of the HFb coating to the PO/HFb interfacial tension resulting from reconformation of the HFb molecules.

In our first paper[2] we postulated that the difference in behaviour between an oil drop on a HFb-coated and a HSA-coated substrate is caused by association of the adsorbed HFb molecules, whereas HSA molecules do not associate. The interfacial association of the HFb molecules prevents the protein layer from being transferred from the PS-substrate to the PO/water interface. It is known[7] that at temperatures above 333 K HSA molecules do associate in solution. Therefore HSA-coated substrates were incubated for about 10^3 sec. at 343 K in protein-free buffer solutions in order to produce a substrate coated with associated HSA molecules.

The results of contact angle (θ_a) measurements on these substrates are shown in table 3.
The observed θ_a values are similar to those found for HFb-coated substrates. This indicates that adsorbed, associated HSA molecules are not transferred either from the PS substrate to the PO/water interface by a passing oil/water front. HSA-coated substrates which have been incubated at pH = 2.5 show a different behaviour. These substrates still show the same θ_a value as untreated HSA-coated substrates. Some qualitative experiments have been performed to obtain an explanation for this phenomenon. HSA solutions at the investigated pH-values (2.5, 7.4, 9.0) were incubated for about 10^3 sec. at 343 K. After incubation, the solutions with a pH value of 7.4 and 9.0 were cloudy due to HSA association, but the solution at pH = 2.5 was still clear, indicating that at this pH aggregation does not take place. More accurate experiments on the determination of the aggregation (poly-acryl gel electrophoresis) also showed that after incubation only the solution of pH = 2.5 contained not-aggregated HSA.

V.5.3 Receding contact angles (inverse systems)

Tables 4 and 5 show results for contact angle measurements in inverse systems. These contact angles θ_r are obtained from a receding PO phase.
Comparison of contact angles on HSA coated substrates in table 1 and table 4 shows an insignificant difference between θ_a (final) and θ_r (final). The large difference between the initial values of

θ_a and θ_r results from the fact that in the inverse system there is no time dependency for θ_r.
The instantaneously obtained θ_r (final) value can be explained by assuming that upon immersion of the protein-coated substrate in PO the thin aqueous film on the PS substrate breaks up in tiny droplets.
The oil phase thus is in direct contact with the PS surface for the greater part of the interface and most of the HSA molecules are accumulated at the oil/water interface of the droplets. When a drop of water is deposited on such a substrate it will be obvious that the tiny droplets will merge instantaneously with the advancing water drop and the HSA molecules will be accumulated at the oil/water interface.
In this way the final situation at the three phase boundary will be almost identical to that in the normal system, after turnover of the protein molecules. The observation that the contact angle in the inverse system does not change significantly upon decreasing the volume of the water drop is in agreement with the above picture.
Table 5 shows the results of measurements on HFb coated substrates in the reverse system. Comparison of these θ_r values with θ_a measurements in table 2 on HFb coated substrates, shows that in both cases hardly any time dependence occurs. However, there is a large difference between θ_a and θ_r on these substrates. A similar feature has been found by El-Shimi and Goddard[8] for contact angles on bovine hoof keratin. For advancing oil phase they observed a θ_a of $> 170^o$.
A θ_r value of 30^o was found when the substrate was first treated with mineral oil.
They conclude that this behaviour is governed by the history of exposure to the contacting liquid, which seems to be a specific feature of natural surfaces. In analogy we conclude that the HFb coating is able to adopt and retain a configuration compatible with the immediate environment. The observation that the contact angle in the inverse system changes drastically (from 90^o to $> 160^o$) when decreasing the volume of the drop, is in agreement with this conclusion.

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CHAPTER VI

THE INFLUENCE OF ADSORBED PROTEINS ON THE STABILITY OF POLYSTYRENE LATEX PARTICLES

ABSTRACT.

Flocculation experiments of polystyrene latex (PSL) with human serum albumin (HSA) and human fibrinogen (HFb) have been performed above and below the iso-electric points (i.e.p) of the proteins. The stability of the proteins (HFb, HSA) in solution has been determined as a function of salt concentration (NaCl, $BaCl_2$) and pH. Using a stopped flow spectrophotometer the rate constant of flocculation (or coagulation) k_{11} has been measured at different protein and salt concentrations ($BaCl_2$, NaCl). A model is proposed and tested to explain the enhancement of k_{11} above the value for bare PSL when bridging occurs at pH-values above the i.e.p of the proteins. The observed enhancement of k_{11}, being 20-30% for HSA and 50-60% for HFb, is a result of two effects: reduced hydrodynamic interaction between the flocculating particles and increased effective collision radius of the latex particles when they are partially covered with the protein. It is shown that steric stabilization by proteins occurs only at "good solvent" conditions for these proteins. At pH-values below the i.e.p. of the proteins flocculation is observed in the absence of salt. Measurements of electrophoretic mobilities of the latex particles as a function of the protein concentration learned that this flocculation is mainly due to charge neutralization by adsorbed protein molecules. Restabilization by charge inversion of the latex particles occurs at relatively low protein concentration.

VI.1 INTRODUCTION.

This paper deals with the influence of adsorbed proteins on the stability against flocculation of hydrophobic colloids, here a polystyrene latex. The processes which we are concerned with play a role in food technology, cosmetic

industry, biological systems etc. In immunology[1] protein coated polystyrene latices are used as test materials and it is important to study the stability of those latices. Organic water soluble polymers are used in industry for the flocculation of a wide range of aquous suspensions. Despite the long history of application of stabilization or destabilization by adsorbed polymers (Rembrandt and his friends already used egg albumin to stabilize their paints) it is only in the last decades that some of the mechanisms involved have been studied. Reviews concerning the influence of polymers on colloid stability have been written by La Mer[2], Lyklema[3], Vincent[4] and Napper[5]. Although a lot of work has been done on stabilization of colloidal systems by non-ionic macromolecules[6-11], cationic[12-16] and anionic polyelectrolites[17-21], only little attention has been paid in the last years to stabilization by amphoteric macromolecules like proteins.
Some work has been done on gelatins [22,23] and Singer[24] studied the flocculation of latex by γ-globulin.
In this paper we shall study the influence of human serum albumin (HSA) and human fibrinogen (HFb) on the stability of a negatively charged polystyrene latex (PSL). Following the definitions first proposed by La Mer[4], aggregation due to mechanisms in which protein molecules play a role will be called flocculation, aggregation by London-Van der Waals forces will be called coagulation.

Experiments at two distinct pH-values have been made: at pH = 8.9. i.e. above the iso-electric point (i.e.p.) of the proteins (i.e.p. HSA at pH = 4.9, i.e.p. HFb at pH = 5.5) and at (pH = 3.5) i.e. below their i.e.p. At pH = 8.9 we are dealing with protein molecules and PSL both negatively charged. At pH 3.5 the initial charges of PSL and protein are opposite.

The influence of the protein on the stability of the PSL at pH 8.9 can be two-fold. First at incomplete coverage of the PSL by the protein molecules, the protein may act as a destabilizing agent. The instability cannot be caused by charge

neutralization because protein and latex bear the same charge. According to earlier studies[19,20] it may be necessary to add small amounts of electrolyte for flocculation. These amounts will be smaller than those needed for coagulation of the bare PSL. This so-called sensitization is caused by bridging of the protein molecules between the latex particles. Secondly, at high surface coverage, the PSL will be sterically stabilized by the adsorbed protein layer. Steric stabilization occurs when the adsorbed layer prevents the colloid particles from approaching each other so close that coagulation by Van der Waals forces can take place. The stabilizing mechanism is characterized by two effects[25]: the osmotic pressure effect; and the volume restriction effect. The adsorption of the macromolecules must be strong and the stabilizing chains should be in "good" solvent environment[26]. Therefore we studied the stability of the proteins themselves in solution as well.

At pH = 3.5 we are dealing with positively charged macromolecules and negatively charged PSL. In contrast with the above systems at pH 8.9 it may be possible to get flocculation by charge neutralization, resulting from protein adsorption.
Gregory[12] showed this effect by measuring the electrophoretic mobility of PSL as a function of the positively charged flocculant concentration, he observed neutralization and even charge reversal.

From these observations several types of destabilization in our system as a function of salt and protein concentration can be expected.

1. Coagulation of unprotected latex particles resulting from double layer suppression by indifferent electrolyte as predicted by the Derjagin, Landau, Verwey and Overbeek (DLVO)-theory.
2. Flocculation by bridging of protein molecules between the latex particles. This is only possible when the electrical double layer is suppressed by electrolite addition (pH=8.9) or by charge neutralization (pH=3.5) so that its thickness

is smaller than half the thickness of the adsorbed protein layer.

3. Hetero coagulation, in the case that neither the protein nor the PSL is stable under the chosen circumstances.

The aggregation has been studied by "stopped flow" measurements. This technique allows us to follow the transmittance of the colloidal dispersions in the earliest stages of the aggregation. Lipps and Willis[27,28] and later Lichtenbelt[29] showed the relation between the change of adsorbance and the rate constant of flocculation in its earliest stages. We used the method of Lichtenbelt in our calculations. This offers the opportunity of calculating real rate constants of flocculation instead of the well-known relative stability factor W[30].
Hitherto only a few authors did report in literature about kinetic flocculation studies[11,14], although they did not use an absolute rate constant but the slope of the adsorbance-time curve. From the use of real rate constants of aggregation we expect to get a better view on phenomena such as the hydrodynamic interaction between latex particles[31,32] and the influence of the dimensions of the protein molecules on the rate of flocculation[33].

VI.2 CONSIDERATIONS ON THE RATE CONSTANT OF AGGREGATION

1. Experimental determination of k_{11}.
   According to Lichtenbelt et al.[29] the change in turbidity of a latex in the initial stage of coagulation or flocculation can be given by

$$\left(\frac{d\tau}{dt}\right)_{t=0} = \left(\frac{C_2}{2} - C_1\right) 2\, k_{11}\, N_1^{\,2} \qquad (1)$$

   where
   N_1 = the initial concentration of singlet particles
   k_{11} = the rate constant of flocculation (coagulation) for singlets ($m^3.s^{-1}$) ($-d\,N_1/dt = 2\,k_{11}.N_1^{\,2}$).

C_1 and C_2 are the optical cross sections of singlets and doublets respectively

Using the relations T = exp. $(-\tau L)$ with T transmittance and L the length of the cuvet and $\tau_{t=0} = C_1 N_1$, equation (1) can be rewritten as

$$k_{11} = \frac{\left(\frac{dT}{dt}\right)_{t=0}}{2\,T_{t=0}\,\ln T_{t=0}\,\left(\frac{C_2}{2C_1} - 1\right)\,N_1} \qquad (2)$$

The factor $\left(\frac{C_2}{2C_1} - 1\right)$ has been called the optical factor and can be conceived as the relative change in absorbance that would happen when a colloidal system consisting of a number of singlets only, would be replaced by a system consisting of half of that number of doublets only. This factor can be calculated with the Rayleigh Gans-Debeye theory and has been tabulated by Lichtenbelt for different values of α.

$$\alpha = \frac{2\,\pi\,a}{\lambda} \qquad (3)$$

a = radius of the colloid particle (nm)

λ = wavelength (nm) of the light used in the continuous phase $\frac{\lambda_{air}}{n_{water}}$

In this work colloidal particles with a radius of 117 nm are used, and light with a wavelength of 546 nm.
The particle concentration N_1 at t=0 is always 8.6×10^{15} m^{-3}.
From these data α can be calculated and the matching value of the optical factor is found to be 0.21.

2. Theoretical expressions for fast and hindered coagulation.

Already in 1916 Von Smoluchowski[34] developed a theory of the rate of coagulation. He described coagulation as a bimolecular

reaction, obeying the equation (forinitial stages of coagulation of a monodisperse colloid)

$$-\frac{d N_1}{dt} = 2 k_{11} N_1^2 \qquad (4)$$

k_{11} = 8 π Da = $5.4 * 10^{-18}$ ($m^3.s^{-1}$) (in water at 298 K)
D = diffusion coefficient ($m^2.s^{-1}$)
a = particle radius (m)

In 1934 Fuchs derived an equation for the flux of particles towards a central particle in a field of force. In the absence of electrostatic repulsion (fast coagulation) this leads to

$$J_s = \frac{8 \pi D N_1 a}{\int_0^\infty \frac{\exp. \frac{V_A}{kT}}{(u+2)^2} du} \qquad (5)$$

J_s = flux of particles towards a central one (fast coagulation) (s^{-1})
V_A = energy of attraction between two equal spheres ($J.K^{-1}$)
$u = \frac{H}{a} = \frac{R - 2a}{a}$
R = distance between the centers of two particles (m)

In 1966 Deryaguin[36] noted that at very short distance the viscous resistance between two approaching particles would become infinite so that Brownian-motion never could cause coagulation. Spielman[31] and Honig[32] developed, analogous to work by Brenner[37], a correction on the diffusion coefficient as a function of the distance between the particles.

$$D = D(u) \cdot \beta(u) \qquad (6)$$

D = diffusion coefficient at infinite separation ($m^2.s^{-1}$)
D(u) = diffusion coefficient at relative distance u ($m^2.s^{-1}$)
$\beta(u)$ = diffusion correction factor at relative distance u.

Honig et al.[32] gave a usefull approximation for $\beta(u)$

$$\beta(u) = \frac{6u^2 + 13u + 2}{6u^2 + 4u} \tag{7}$$

Introduction of this hydrodynamic interaction in equation (5) gives:

$$J_s = \frac{8 \pi D N_1 a}{\int_0^\infty \frac{\beta(u)}{(u+2)^2} \cdot \exp. \left(\frac{V_A}{kT}\right) du} \tag{8}$$

According to Hamaker[38], the energy of attraction between two equal spheres can be described as follows

$$V_A = - \frac{A_{1(3)1}}{6} \left(\frac{1}{u^2+4u} + \frac{2}{u^2+4u+4} + \ln \frac{u^2 + 4u}{u^2+4u+4}\right) \tag{9}$$

$A_{1(3)1}$ = Hamaker constant for two bodies of material 1 embedded in medium 3 (Joule)

Analogous to Roebersen[40] a dimensionless rate constant of flocculation k^* is defined

$$k^* = \frac{J_s}{16 \pi D N_1 a} \tag{10}$$

This constant k^* is equal to one for the theoretical Von Smoluchowski rate ($\beta(u)=1$ and $V_A=0$). So the dimensionless rate constant k^* can also be written as

$$k^* = \frac{k_{11} \text{ (observed)}}{k_{11} \text{ (Von Smoluchowski)}} \tag{11}$$

Figure 1 shows the inverse of k^* as a function of the Hamaker constant $A_{1(3)1}$ for particles with a radius of 117 nm.

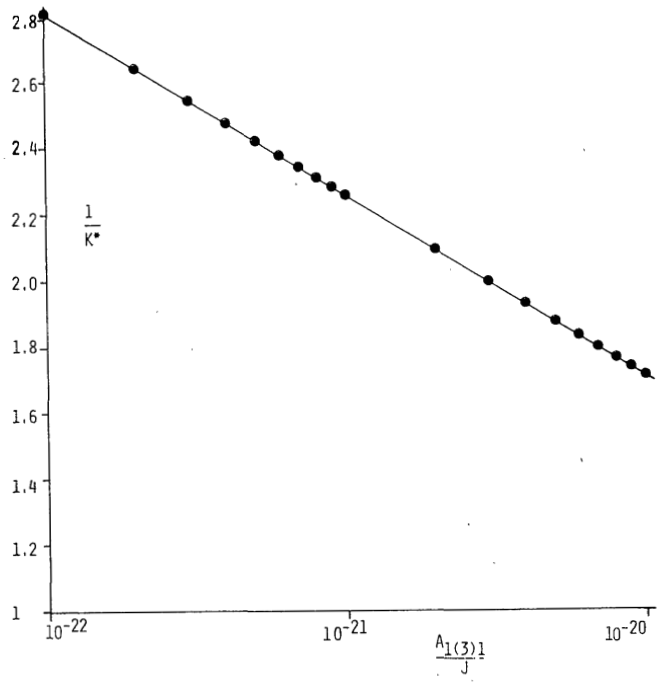


Figure 1: The inverse of the dimensionless rate constant k^* as a function of the Hamaker constant $A_{1(3)1}$ for particles with a radius of 117 nm.

When two particles are connected firmly by a bridge between them their probability of aggregation becomes 1. This means that neither hydrodynamic effects nor other interactions can prevent them from aggregation and the integration of the hydrodynamic factor $\beta(u)$ and the field of force effect $\frac{1}{(u+2)^2} \cdot \exp.(\frac{V_A}{kT})$ must not be done from u = 0 to ∞ but from u = x = h/a (where h is bridge length) to ∞.
Following Walles[33] it is assumed that the presence of the tails on the surface of the particles does not influence the diffusion coefficient of the particle. The larger effective collision radius of the particle will enhance the flux with a factor $\frac{a+h}{a}$.

For the flux in this case we can write

$$J_H = (8 \pi D N(a+h))/(\int_x^\infty \frac{\beta(u)}{(u+2)^2} \cdot \exp.(\frac{V_A}{kT})\, du) \qquad (12)$$

From these considerations it can be seen that the rate of aggregation of colloidal particles will be enhanced by bridging. This enhancement will be optimal when the diffusion coefficient of the particles is not influenced by the presence of the tails and when there are still enough bare places left on the surface for adsorption of tails of approaching particles. The maximal accelleration factor γ can be expressed by

$$\gamma = \frac{a+h}{a} \frac{\int_0^\infty \frac{B(u)}{(u+2)^2} \cdot \exp.(\frac{V_A}{kT})\, du}{\int_x^\infty \frac{B(u)}{(u+2)^2} \cdot \exp.(\frac{V_A}{kT})\, du} \qquad (13)$$

We calculated γ for particles with a radius of 117 nm as a function of the length (h) of the tails for different values of $A_{1(3)1}$. The results of these calculations are shown graphically in figure 2.

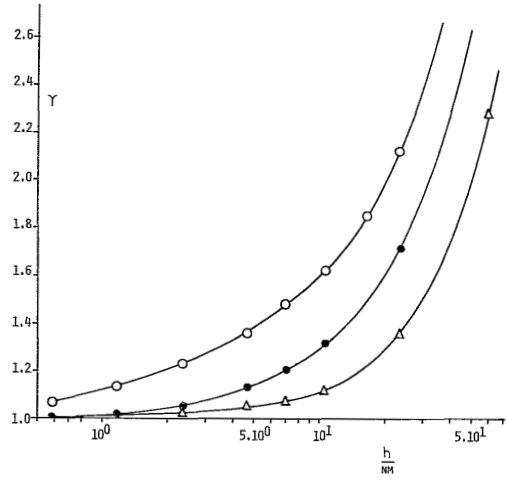


Figure 2: The maximal accelleration factor γ for particles with a radius of 117 nm as a function of the length (h) of the tails for different $A_{1(3)1}$; o, $A_{1(3)1} = 10^{-22}$ J, •, $A_{1(3)1} = 10^{-21}$ J; Δ, $A_{1(3)1} = 10^{-20}$ J.

VI.3 EXPERIMENTAL.

VI.3.1 Materials.

Polystyrene latex (PSL), purchased from Dow (Dow LS-1047-E) a monodispersed latex with particle diameter of 243 ± 2.6 nm.
Human Serum Albumin (HSA), crystalline from Pierce Chemicals (nr. 30430), was used without further purification.
Human Fibrinogen (HFb), from Kabi, Stockholm (grade L, 90% clottable) was used after dialyzing against twice distilled water at pH = 9, T = 278 K during 8.10^4 sec.
All chemicals used were analytical grade.
The water used in the experiments was always twice distilled and degassed.

VI.3.2 Apparatus.

1. *Micro electrophoresis apparatus.*
   The measurements of the electrophoretic mobility of bare and protein coated latex particles have been performed with a Rank Micro Electrophoresis Apparatus MK II, equipped with a cylindrical capillary cell and reversible platinum electrodes.

2. *Stopped flow apparatus.*
   All flocculation experiments have been carried out in a Durrum-Gibson Stopped Flow Spectrophotometer, Model D-110, with a 20 mm path length cell. In this apparatus the solutions to be mixed are contained in two syringes. By a pressure (5×10^5 $N.m^{-2}$) operated actuator, equal volumes are reproducibly mixed within a few milliseconds. The transmittance of the mixture is recorded on a storage oscilliscope. The wavelength of the light used was 546 nm using a tungsten iodide light-source.

VI.3.3 Methods.

1. *Cloud point measurements.*
These experiments have been carried out as a function of pH and salt concentration. Solutions with protein concentrations of 1 $kg.m^{-3}$ and the desired amount of salt were devided into two equal portions. The pH of one of these portions was slowly increased by NaOH addition and the pH of the other portion was slowly decreased by HCl addition. In this way "instability areas" in the pH-salt diagram were determined when the solution was cloudy.

2. *Flocculation and coagulation measurements.*
Coagulation experiments have been performed with the stopped flow apparatus, using one syringe for the bare latex and the other for the salt solution.
Flocculation experiments without salt are carried out by using one syringe for the bare latex and the other for the protein solution.
Flocculation experiments in the presence of salt are performed by using one syringe for the PSL-protein mixture and the other for the salt solution . The PSL-protein mixtures are prepared by adding the PSL slowly to the desired protein solution.
Concentrations where always chosen such that after mixing in the stopped flow apparatus the latex concentration was 6.10^{-2} $kg.m^{-3}$ (8.6×10^{15} particles m^{-3}). The value for the rate constant of aggregation k_{11}, is calculated using equation (2), from the initial change of the transmission $(\frac{dT}{dt})_{t=0}$ after mixing.
The initial transmission T_o in our experiments was always 36%. From this value and the value of the optical factor (0.21) it can be calculated that a 1% decrease of the transmission indicates that 12% of the single particles have been converted to doublets (assuming that only singlet-singlet collisions occur). From the measured value of k_{11} it can be calculated how much time it takes to reach 1% decrease of the

transmission. We found that the calculated time was equal to the observed time, indicating that up to a decrease of 1% in T the assumptions are valid. At higher conversions of the singlets a difference between calculated and observed time has been observed. From this fact it is concluded that the value of $\frac{dT}{dt}$ always has to be measured in the region where the decrease in T is < 1%.

3. *Electrophoretic mobility measurements.*
The electrophoretic mobilities of the latex particles are measured as a function of the protein concentration. For these measurements it was important to carry them out in the following way.
The PSL is mixed with protein solutions in the same concentrations as used in the flocculation experiments. These flocculation experiments however, always take about 10 seconds, so we are interested in the mobility after 10 seconds of contact between the PSL and the protein solution. In order to obtain this, the mixture of PSL and protein solution was diluted 10^4 times after 10 seconds of contact. Because the proteins are bound very tightly to the surface[39] it may be expected that the coverage of the particles by the protein will not change upon dilution. On the other hand, further adsorption of protein at the latex particles can be neglected after the drastic dilution. When the dilution step was done a few minutes after the mixing, it was difficult to measure the electrophoretic mobility for some experiments where flocculation occurs, because too many aggregates are present at that time.
All experiments have been performed at 298 K.

VI.4 RESULTS AND DISCUSSION

VI.4.1 Instability areas of the proteins in pH-salt diagrams.

Figures 3 and 4 show the instability areas (shaded areas) of respectively HSA and HFb in pH-salt diagrams for three different salts, NaCl, $BaCl_2$ and $La(NO_3)_3$.

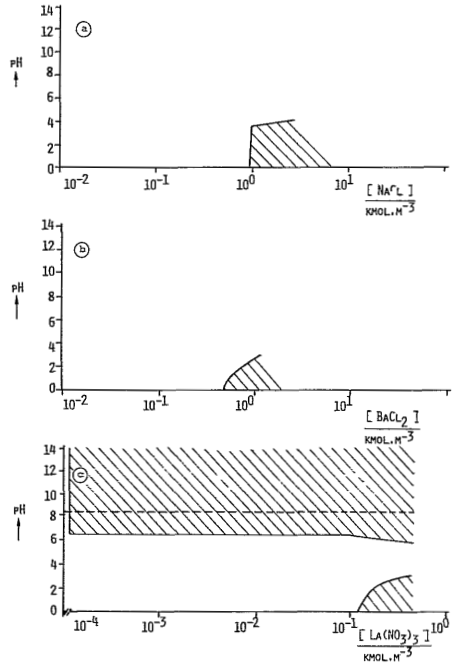


Figure 3: Instability areas of HSA in pH-salt diagrams: a) with NaCl; b) with $BaCl_2$; c) with $La(NO_3)_3$

For HSA, figure 3, instability areas have been observed at low pH only. Under these circumstances the protein molecules are positively charged (pH below i.e.p. (pH = 4.9)) and the Cl^- or NO_3^- ions will act as counterions. It is shown that for NaCl and $BaCl_2$ the solutions get cloudy at Cl^- concentrations above 0.9 $kmol.m^{-3}$. For $La(NO_3)_3$ this happens at NO_3^- concentrations above 0.45 $kmol.m^{-3}$.

The instability area with $La(NO_3)_3$ at pH-values > pH = 6.5 is mainly caused by hydrolysis of the $La(NO_3)_3$. At pH-values above the dotted line in figure 3c, solutions of $La(NO_3)_3$ without protein are cloudy. The instability just below this dotted line may be due to the interaction of HSA molecules with primary flocs of the salt.

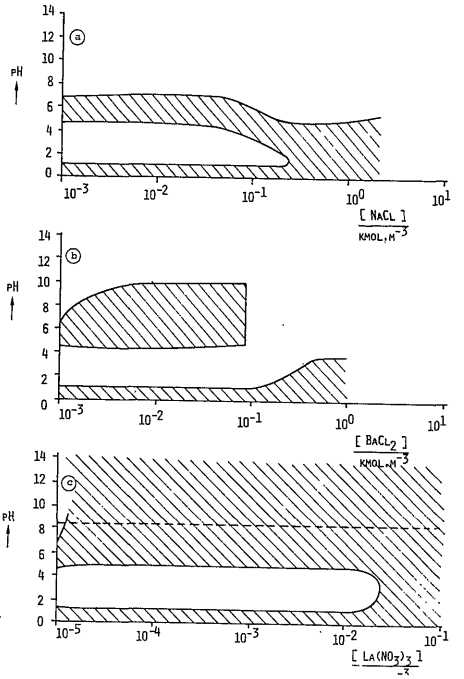


Figure 4: Instability areas of HFb in pH-salt diagrams: a) with NaCl; b) with $BaCl_2$; c) with $La(NO_3)_3$.

For HFb, figure 4, it is shown that in the pH region around the i.e.p. (pH = 5.5) the protein is less stable in solution. Under these conditions the molecule hardly bears any net charge and it has its most compact shape[41]. Proteins always show a decreased solubility around their i.e.p.[42] resulting from a decreased coulombic repulsion which favours aggregation. It is striking that at physiological conditions (pH $\tilde{\sim}$ 7.4 and [NaCl] = 0.15 kmol.m^{-3}) the upper boundary of the instability area goes to a somewhat lower pH, indicating a higher stability under these conditions.

At pH-values below 1 the HFb solution gets cloudy too; at higher salt concentration this instability area extends to higher pH values. In this region the molecule has a resultant positive charge, so the negative ions will act as counterions. The extension of the low instability region using NaCl and $BaCl_2$ occurs

at the same Cl^- concentrations namely 0.2 kmol.m^{-3}, which is about 5 times lower than observed for HSA. With $La(NO_3)_3$ this extension happens at $[NO_3^-]$ = 0.1 kmol.m^{-3}, about 5 times lower than for HSA.

The difference between the instability region around the iso-electric point and that below pH = 1 is, that the clouding of the protein solution around the i.e.p. is reversible and below pH = 1, it is not. The latter indicating a complete denaturation of the protein. A difference between figure 4a and figure 4b is that the high and low instability region in the first case are connected and in the second case not. For $BaCl_2$, a protein solution at pH = 8.9 will get cloudy upon a first addition of $BaCl_2$ whereas it will clearify again on further $BaCl_2$ addition.

A second difference between figure 4a and figure 4b is that the high pH instability region extends to higher pH values for $BaCl_2$ than for NaCl, indicating that above the iso-electric point, the valency of the positive counterions plays a role in the mechanism of destabilization. One might think here about a kind of physical crosslinks of Ba^{2+} between the protein molecules. Perhaps the repeptization at high Ba^{2+} concentration can be seen as a complete saturation of the available crosslink sites by Ba^{2+}.

At pH values below the i.e.p. of the proteins, the destabilization by NO_3^- ions is twice as effective as that by Cl^- ions for both proteins. This indicates that the same mechanism plays a role although for HSA much higher concentrations are needed than for HFb. Comparison of figures 3 and 4 leads to the conclusion that HSA is a much more stable protein than HFb.

VI.4.2 Coagulation of the latex with salt.

Coagulation experiments of PSL with salt only have been performed at pH = 3.5 and pH = 8.9. Figure 5a shows the value of the rate constant of coagulation k_{11} as a function of the $BaCl_2$ concentration. It is clear that k_{11} increases with increasing salt concentration. The coagulation results from the decrease in double-layer repulsion between the negatively charged PSL particles due to the Ba^{2+} ions.

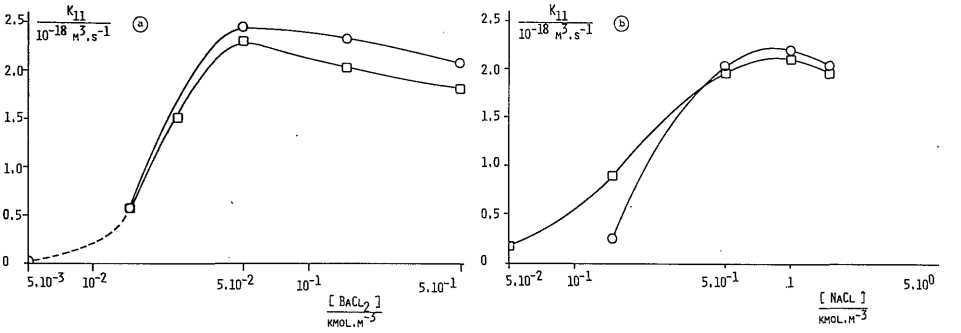


Figure 5: The rate constant of coagulation k_{11} for bare PSL as function of the salt concentration.
a) with $BaCl_2$ at o pH = 8.9 and □ pH = 3.5;
b) with NaCl at o pH = 8.9 and □ pH = 3.5.

For a $BaCl_2$ concentration of 0.05 kmol.m^{-3} a maximum value of $k_{11} = 2.45 \times 10^{-18}$ $m^3 s^{-1}$ at pH = 8.9 and $k_{11} = 2.3 \times 10^{-18}$ $m^3 s^{-1}$ at pH = 3.5 is observed. The decrease of k_{11} at higher $BaCl_2$ concentration is mainly due to a higher viscosity of the continuous phase resulting in a lower value of the diffusion coefficient D of the latex particles ($D = kT/(6 \pi \eta a)$). Up to $BaCl_2$ concentrations of 0.05 kmol.m^{-3} the viscosity of the salt solutions is almost constant (1% deviation from η_{water}). At higher $BaCl_2$ concentrations the viscosity increases drastically until at 1 kmol.m^{-3} an increase of 28.5% is reached[43]. The difference between k_{11} at pH = 8.9 and at pH = 3.5 is not understood. The rate constant of Von Smoluchowski at 298 K is 5.38×10^{-18} $m^3 s^{-1}$.

Taking k_{11} (observed) = 2.38×10^{-18} $m^3 s^{-1}$ the dimensionless rate constant from equation (10) appears to be

$$k^* = 0.44$$

Figure 5b shows the dependence of k_{11} on the NaCl concentration at pH = 8.9 and pH = 3.5. This figure shows that for NaCl the maximum value of $k_{11} = 2.2 \times 10^{-18}$ $m^3 s^{-1}$ at pH = 8.9 and $k_{11} = 2.1 \times 10^{-18}$ $m^3 s^{-1}$ at pH = 3.5 is reached at NaCl concentrations of 1 $kmol.m^{-3}$. At this salt concentration the viscosity of the continueos phase is 1.094 times that of water[43]. This results in a k_{11} (Von Smoluchowski) = 4.94×10^{-18} $m^3 s^{-1}$. Taking $k_{11} = 2.15 \times 10^{-18}$ $m^3 s^{-1}$ the dimensionless rate constant (equation (10)) is

$$k^* = 0.44$$

Using figure 1 which shows the relation between $1/k^*$ and the Hamaker constant $A_{1(3)1}$, the value of the Hamaker constant for our system can be calculated

$$A_{1(3)1} = 1.0 \ (\pm 0.1) \ . \ 10^{-21} J$$

Visser[44] tabulated Hamaker constants of different materials in water. He gives values obtained with the Lifshitz theory ($A_{1(3)1} = 3.5 \times 10^{-21}$) and values obtained from colloid chemistry ($10^{-21} < A_{1(3)1} < 10^{-19}$ J) for PS in water.
Comparison with these values shows that the value obtained from our experiments is somewhat low but not unrealistic.
The salt concentrations at which fast coagulation occurs are higher than those reported for classical test tube experiment. This should be expected since in our experiments coagulation times are in the order of seconds and ortho kinetic coagulation is absent[11].
From these experiments we conclude that our apparatus fulfills the conditions for the type of measurements we are doing.

VI.4.3 Flocculation experiments with HSA at pH = 8.9.

When PSL and HSA solutions were mixed in the absence of electrolyte, no flocculation could be detected at HSA concentrations between 0.00 and 0.25 $kg.m^{-3}$ after mixing.

Fig. 6a,b shows the dependence of k_{11} on the $BaCl_2$ and NaCl concentration for curves with constant HSA concentration.

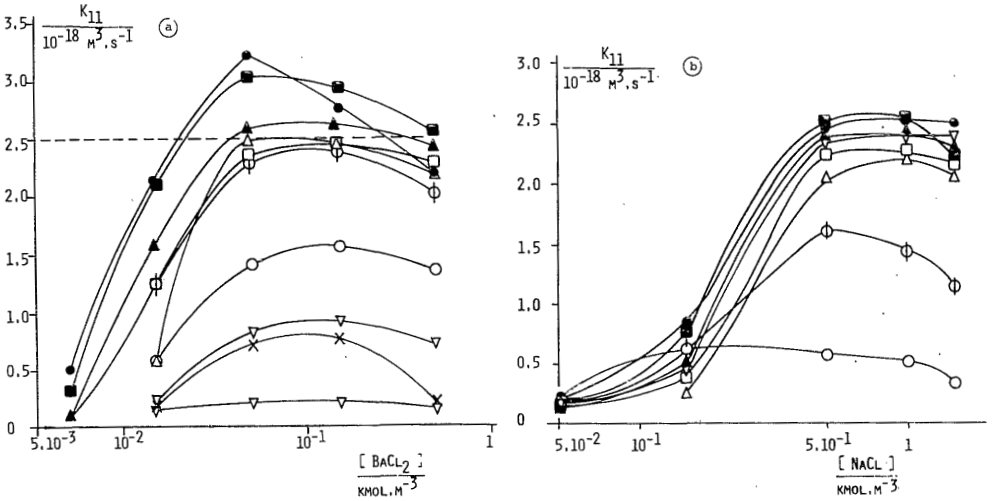


Figure 6: The dependence of k_{11} on the salt concentration (pH = 8.9) for curves with constant HSA concentration:
a) with $BaCl_2$ at HSA concentrations of Δ, 0; □, 0.17; ▲, 0.33; ■, 1.67; ●, 3.33; ϕ, 6.66; o, 13.33; ▽, 26.67; x, 50.0; ▼, 250 x 10^{-3} $kg.m^{-3}$;
b) with NaCl at HSA concentrations of Δ, 0; □, 0.5; ▲, 0.8; ■, 1.9; ●, 2.3; ϕ, 4.0; o, 8.0 x 10^{-3} $kg.m^{-3}$.

It is clear that at HSA concentrations < 5.10^{-3} kg.m^{-3} smaller amounts of salt are needed to start the flocculation than for PSL without protein. This increased sensitivity for electrolyte addition is called sensitization. It is not caused by a decreased electrostatic interaction due to protein adsorption, because protein and latex are both negatively charged. The sensitization results from a well known mechanism called bridging: molecules which are already adsorbed on one latex particle absorb on the surface of another latex particle. This mechanism can take place when the double layer interaction between the particles has been suppressed by electrolyte addition so that particles can approach each other closely. It is also necessary that the latex particles still have adsorption sites available for adsorption of the bridging molecules.

Figure 7a, b shows k_{11} as a function of the HSA concentration for curves of constant $BaCl_2$ and NaCl concentration. It is shown that at higher HSA concentrations k_{11} decreases. This is the result of steric stabilization of PSL by adsorbed HSA layers. When the latex particles are fully covered by protein, no adsorption sites are available for bridging molecules any more. When particles are approaching, the adsorbed protein layers must penetrate into each other, resulting in a repulsion which we described in the introduction. At HSA concentrations > 0.1 kg.m^{-3} electrolyte addition hardly causes any flocculation. This concentration to reach a fully shielding monolayer, is in good agreement with adsorption isotherms of HSA on PSL[39,45], showing that maximum adsorption occurs at this concentration.

It is shown in figure 7a, b that at HSA concentrations of about $1.7*10^{-3}$ kg.m^{-3} a maximum of k_{11} excists. The maximum values of k_{11} in these figures exceed the maximum value of k_{11} for the bare latex.
Gregory[13,14] found an enhanced rate of flocculation using negatively charged PSL and cationic polyelectrolytes. He explained this in terms of an uneven distribution of negative and positive charges on the PSL surface. This could lead to

an extra attractive contribution to the interaction between the particles, due to "oriented approach". In our case this explanation is not possible, because latex particles and protein molecules both are negatively charged. Another argument is that the enhancement to k_{11} values above those for bare PSL only occurs at high salt concentrations, in other words electrostatic interactions cannot play a role. We propose that the increase of k_{11} results from the diminished hydrodynamic interaction through bridging of the protein molecules between the latex particles.

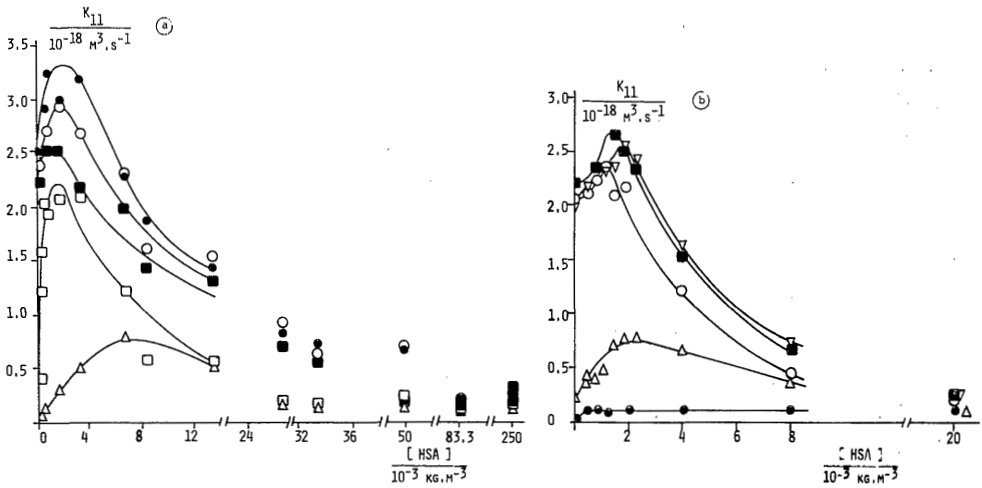
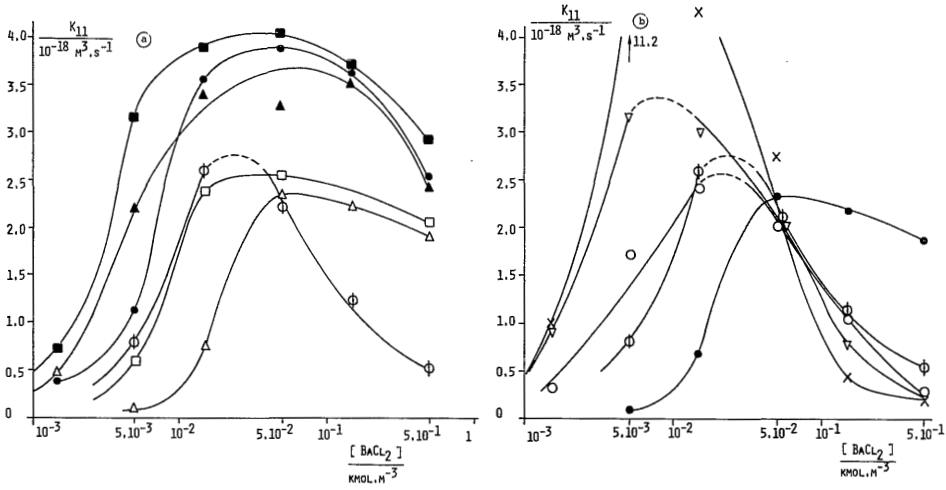


Figure 7: The dependence of k_{11} on the HSA concentration (pH = 8.9) for curves with constant salt concentration:
a) with $BaCl_2$ concentrations of ■ , 0.5; o, 0.15; ●, 0.05; □ , 0.015; Δ, 0.005 kmol.m^{-3};
b) with NaCl concentrations of o, 1.5; ■ , 1.0; ▽, 0.5; Δ, 0.15; x, 0.05 kmol.m^{-3}.

From figure 7a, b the maximum enhancement of k_{11} at respectively $[BaCl_2]$ = 0.05 kmol.m^{-3} and $[NaCl]$ = 1.0 kmol.m^{-3} appears to be 30% and 20%. Figure 2 shows the maximal accelleration γ (equation (13)) as a function of the length of the tails on a particle for different values of the Hamaker constant. Using a Hamaker constant of 10^{-21} J it can be seen that the observed accelleration factor γ which is 1.20-1.30 can be caused by tails with a length of 7-10 $\times 10^{-9}$m. This is in excellent agreement with the dimensions of the albumin molecule,which is assumed to be a prolate ellipsoid with major and minor axes of 14 and 4 $\times 10^{-9}$ m[46].

VI.4.4 Flocculation experiments with HFb at pH = 8.9.

When PSL and HFb solutions were mixed in the absence of electrolyte, no flocculation was observed at HFb concentrations between 0.00 and 0.25 kg.m^{-3} after mixing. Figure 8a,b,c shows the dependence of k_{11} on $[BaCl_2]$ and $[NaCl]$ for several lines of constant protein concentration. From these figures it can be seen that analogous to the results with HSA, HFb also causes sensitization at low protein and salt concentrations. In figure 8b it is shown that the sensitization at low concentrations of $BaCl_2$ also occurs at high HFb concentrations up to 0.25 kg.m^{-3}. This phenomenon will become clear later on in the discussion in relation with the instability area of HFb.
The figure 9a,b,c shows the relation between k_{11} and the HFb concentration for curves of constant $[BaCl_2]$ and $[NaCl]$. It is shown that analogous to HSA, the HFb causes an increase of k_{11} at low protein concentration. The maximum value of k_{11} is reached at a HFb concentration of 2.5 $\times 10^{-3}$ kg.m^{-3}. From our own work on adsorption isotherms of HFb on PSL we know that countrary to HSA all HFb molecules in solution will be adsorbed on the latex particles at low initial HFb concentrations (high affinity isotherms). The latex surface available for adsorption in our experiments is 1.48$\times 10^{3}$ $m^2.m^{-3}$. This means that the surface concentration of HFb on the PSL at maximum flocculation rate is 1.7 $\times 10^{-6}$ kg. m^{-2}.



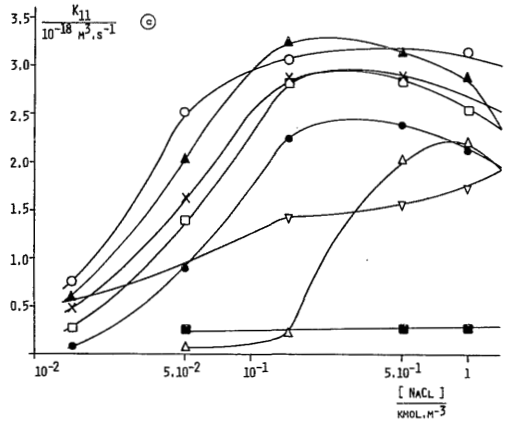
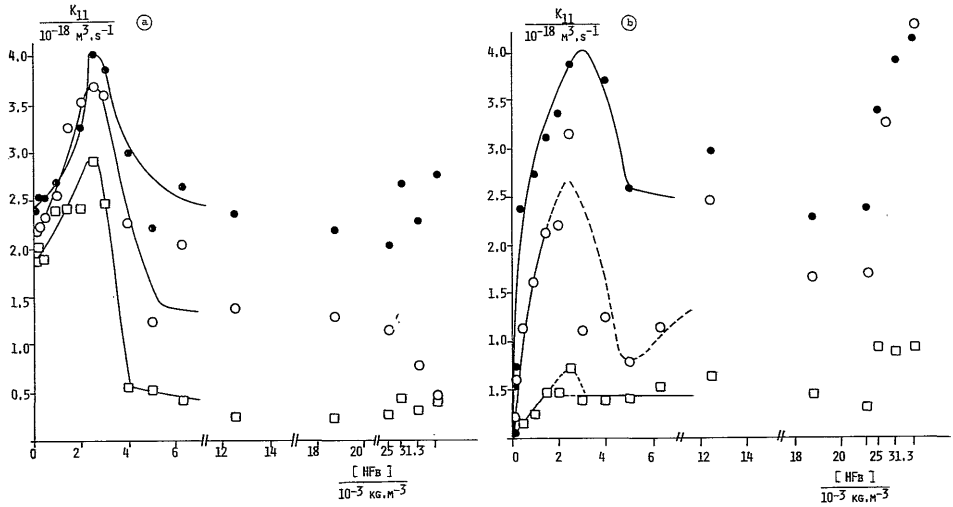


Figure 8:
The dependence of k_{11} on the salt concentration (pH=8.9) for lines with constant HFb concentrations:

a) with $BaCl_2$ at HFb concentrations of Δ,0; □ , 0.25; ▲, 2.0; ■ , 2.5; ●, 3.0; ϕ, 5.0 x 10^{-3} kg.m^{-3}.

b) with $BaCl_2$ at HFb concentrations of ●,0; ϕ, 5.0; o, 25.0; ∇, 62.5; x, 250 x 10^{-3} kg.m^{-3}.

c) with NaCl at HFb concentrations of Δ,0; ●, 0.5; □ , 1.3; x, 1.6; ▲, 1.9; o, 2.5; ■ , 8.0 x 10^{-3} kg.m^{-3}



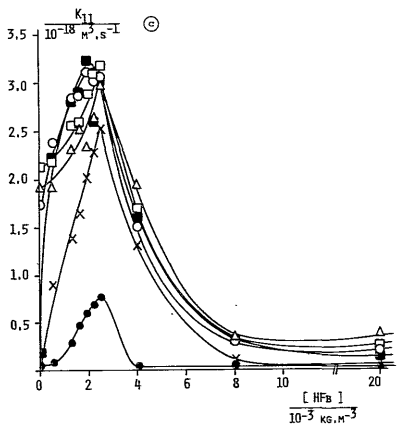


Figure 9:
The dependence of k_{11} on the HFb concentration (pH=8.9) for curves with constant salt concentration:

a) with $BaCl_2$ concentrations of ■ , 0.5; o, 0.15; ●, 0.05 kmol.m^{-3}.
b) with $BaCl_2$ concentrations of ●, 0.015; o, 0.005; ■ , 0.0015 kmol.m^{-3}.
c) with NaCl concentrations of Δ, 1.5; □ , 1.0; o, 0.5; ■ , 0.15; x, 0.05; ●, 0.015 kmol.m^{-3}.

From these adsorption experiments[45] it is also shown that at surface concentrations above 4-5 * 10^{-6} $kg.m^{-2}$ a certain amount of HFb stays in solution. Although the maximum surface concentration to be reached at higher concentrations is 9.8 * 10^{-2} $kg.m^{-3}$. From these data we conclude that maximal rates of flocculation occur at surface coverages of 17-38%. This is a somewhat lower coverage than the 50% predicted by La Mer et al.[4], but in agreement with the 35% observed by Singer et al.[24] for γ-globulin on PSL.

The observed accelleration factor γ (equation (13)) is 1.60 for $BaCl_2$ and 1.50 for NaCl. From figure 4 it can be read that using a Hamaker constant of 10^{-21} J this accelleration can be the result of bridges with a length of 17-20 * 10^{-9} m. These values are in between the values of the minor and major axes of the HFb molecules which are 9 and 45 * 10^{-9} m[47-49].

Figure 9c shows that at HFb concentrations above 8 * 10^{-3} $kg.m^{-3}$ the PSL is protected against aggregation on NaCl addition by steric stabilization. Figures 9a,b however, show that at higher HFb concentrations the PSL is not always protected against aggregation on $BaCl_2$ addition. At higher concentrations of HFb even a drastic increase of k_{11} is observed for some concentrations of $BaCl_2$. This phenomenon can be understood after inspection of the instability area of HFb in the pH-$BaCl_2$ diagram (figure 4b). This figure shows that at pH 8.9 the HFb solution is cloudy at $BaCl_2$ concentrations between 0.003 $kmol.m^{-3}$ and 0.1 $kmol.m^{-3}$. At these $BaCl_2$ concentrations the continuous phase has "bad solvent" properties with respect to the HFb molecules. At these conditions no steric stabilization can be expected[26].

The extremely high values of k_{11} at high concentrations of HFb are the result of a kind of co-sedimentation of the free HFb in the continuous phase with the covered PSL particles. In fact it is not correct anymore to speak about a k_{11} in this region. The aggregation that happens under these circumstances will not be a bimoleculair process. The process might be described by a kind of bridging. The bridges should not be regarded as composed of one adsorbed protein molecule, but

rather as an infinite network formed by physical crosslinks between the protein molecules by divalent Ba^{2+} ions, as was already proposed for alginates[18]. At HFb concentrations between 6.10^{-3} kg.m^{-3} and 3.10^{-2} kg.m^{-3}, k_{11} in figure 9a,b is independent of the HFb concentration. This indicates that in this concentration range the free HFb molecules in the continuous phase still do not play a role in the flocculation. In this region the flocculation still may be described as a bimolecular process. From the values of k_{11} in these regions, (figures 9a,b) for different $BaCl_2$ concentrations together with the instability area, figure 4b, one can see the influence of the solvent properties on the steric stabilization. Good solvent means, steric stabilization "bad" solvent means no steric stabilization.

VI.4.5 Flocculation experiments without salt at pH 3.5.

At pH = 3.5 both proteins (HFb and HSA) bear a net positive charge. This offers the possibility of charge neutralization when the positive protein molecules adsorb at the negative latex particles. Results of flocculation measurements of PSL with HSA and HFb are shown in figure 10a,b. It appears that k_{11} increases with increasing protein concentrations. At protein concentrations > 0.7×10^{-3} kg.m^{-3} restabilization of the PSL-protein mixtures was observed after an initial onset of flocculation.

Measurements of the electrophoretic mobility of the PSL particles as a function of HSA and HFb concentration (figure 15a,b) show charge reversal of the particles already at protein concentrations around 0.7×10^{-3} kg.m^{-3}, hence at extremely low surface coverage ($\sim$ 5%). This indicates that the observed restabilization results from electrostatic repulsion at these and higher protein concentrations. When restabilization of the mixture is observed, the value of k_{11} is taken to be zero although at the first moments after mixing flocculation occurs.

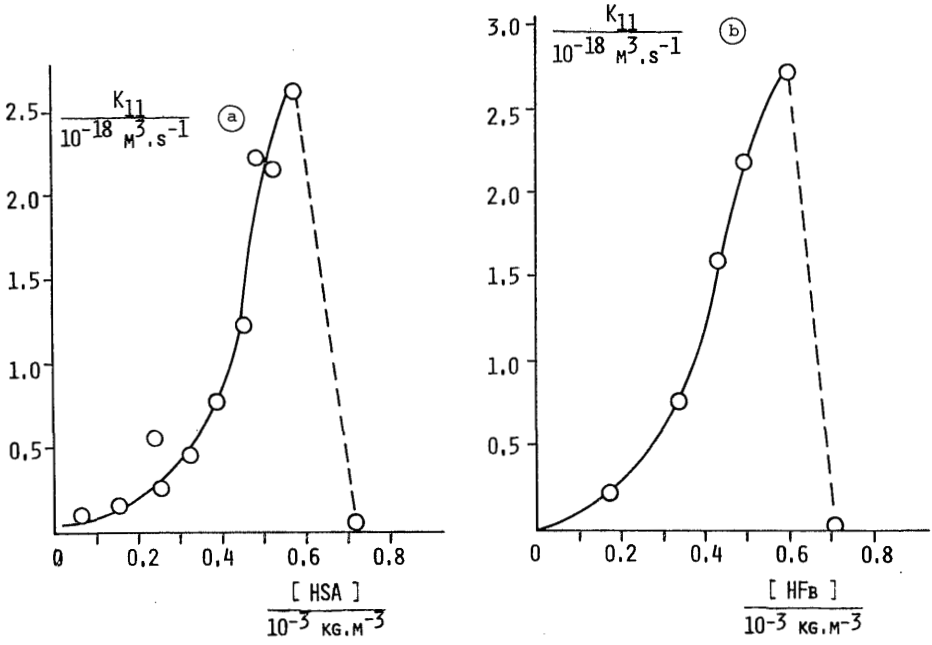


Figure 10: The rate constant of flocculation (k_{11}) as a function of the protein concentration (pH = 3.5) in the absence of salt.
a) dependence on the HSA concentration.
b) dependence on the HFb concentration.

The time during which this flocculation occurs may reflect the time necessary for adsorption and reconformation of the protein molecules on the latex particles. Further experiments on this subject are in progress and will be reported elsewhere. Gregory[13,14] found that the optimum flocculation concentration coincides with the flocculant concentration at which the charge of the colloid particles is just neutralized. Our measurements endorse his conclusions when we ignore the initial flocculation rate which is found immediately before restabilization at concentrations $> 7.10^{-4}$ kg.m^{-3} of protein.

VI.4.6 Flocculation with HSA and salt at pH = 3.5.

When PSL is slowly added to solutions containing HSA concentrations $\geq 1.67.10^{-3}$ kg.m^{-3} the turbidity of the mixture appeared to be equal to that of a PSL of the same concentration without HSA. The PSL-HSA mixtures at these protein concentrations are stable due to the positive charge of the adsorbed HSA molecules. Flocculation experiments of these mixtures with $BaCl_2$ and NaCl are shown in figures 11, 12.

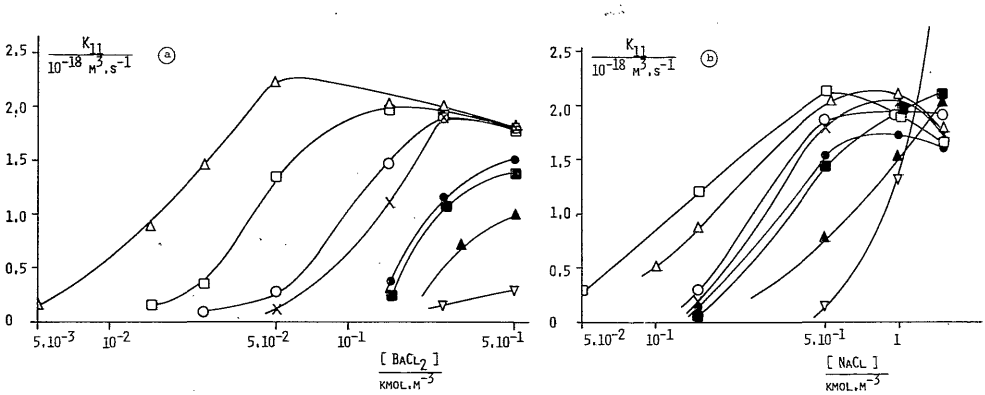


Figure 11: The dependence of k_{11} on the salt concentration (pH = 3.5) for curves with constant HSA concentration:
a) with $BaCl_2$ at HSA concentrations of Δ, 0; □, 1.67; o, 6.67; x, 13.3; ▽, 250 $\times 10^{-3}$ kg.m^{-3}.
b) with NaCl at HSA concentrations of: Δ, 0; □, 2.0; o, 3.0; x, 5.0; ●, 10; ■, 12.5; ▲, 8.0; ▽, 250 $\times 10^{-3}$ kg.m^{-3}

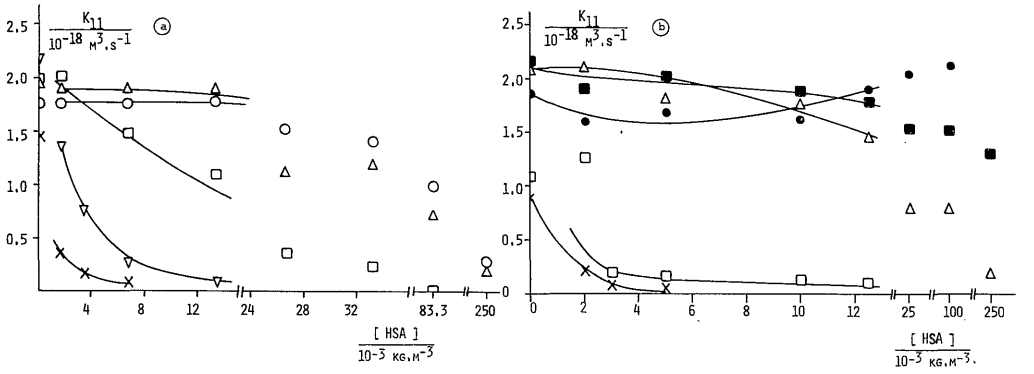


Figure 12: The dependence of k_{11} on the HSA concentration (pH = 3.5) for curves with constant salt concentrations:
a) with $BaCl_2$ concentrations of o, 0.5; Δ, 0.25; □, 0.15; ∇, 0.05; x, 0.025 kmol.m^{-3}.
b) with NaCl concentrations of ●, 1.5; ■, 1.0; Δ, 0.5; □, 0.15; x, 0.05 kmol.m^{-3}.

The charge reversed PSL can still be flocculated by salt addition which confirmes the statement that the restabilization observed without salt, results from electrostatic repulsion and not from steric stabilization. From figure 11a one might conclude that no sensitization is observed because the HSA-PSL mixture needs higher $BaCl_2$ concentrations than the bare PSL does. This conclusion however, may be wrong because the bare PSL is negatively charged and the PSL particles in the mixture are positively charged. Therefore the Ba^{2+} ions act as counterions for the bare PSL and the Cl^--ions for the charge reversed PSL particles. When NaCl is used as electrolyte, the counterions

Na^+ and Cl^- are both mono valent. From figure 11b it can be observed that at a HSA concentration of 2.10^{-3} kg.m^{-3} a slight sensitization occurs.
Figure 12a shows that at $BaCl_2$ concentrations > 0.15 kmol.m^{-3} very high HSA concentrations are needed to obtain steric stabilization. With NaCl (figure 12b) it appears that at salt concentrations > 1.0 kmol.m^{-3} no steric stabilization occurs, and at very high HSA concentrations an increase of k_{11} appears. Inspection of the instability area of HSA with NaCl (figure 3a) shows that at pH = 3.5 and NaCl concentrations > 1.0 kmol.m^{-3} the continuous phase has bad solvent properties for HSA, which explains the flocculation behaviour.
It is remarkable that no "hydrodynamic" accelleration occurs at low protein concentrations. This may be caused by the electrostatic attraction forces between the adsorbed positive protein molecules and the negative latex surface. This attraction may result in a flat orientation of the molecules onto the surface and consequently in very short tails.

VI.4.7 Flocculation with HFb and salt at pH = 3.5.

Stable HFb-PSL mixtures could be obtained at HFb concentrations > 2.10^{-3} kg.m^{-3}. The flocculation behaviour of these mixtures (figures 13 and 14) are analogous to that of HSA-PSL mixtures. Again no "hydrodynamic" accelleration has been observed at low protein concentrations. The denaturated state of the protein molecule at low pH makes it more flexible[50] which facilitates a flat orientation at the latex surface. Using NaCl (figure 14b) it is shown that at higher NaCl (> 0.2 kg.m^{-3}) and HFb concentrations (0.1 kg.m^{-3}) the value of k_{11} is extremely high. At these bad solvent conditions for HFb (figure 4a) the rate of "co-sedimentation" is largely influenced by the concentration of HFb in the continuous phase. Comparison of figures 11a and 12a with figures 11b and 12b and comparison of figures 13a and 14a with figures 13b and 14b shows that the flocculation behaviour of charge inversed PSL is governed by the Cl^--concentration.

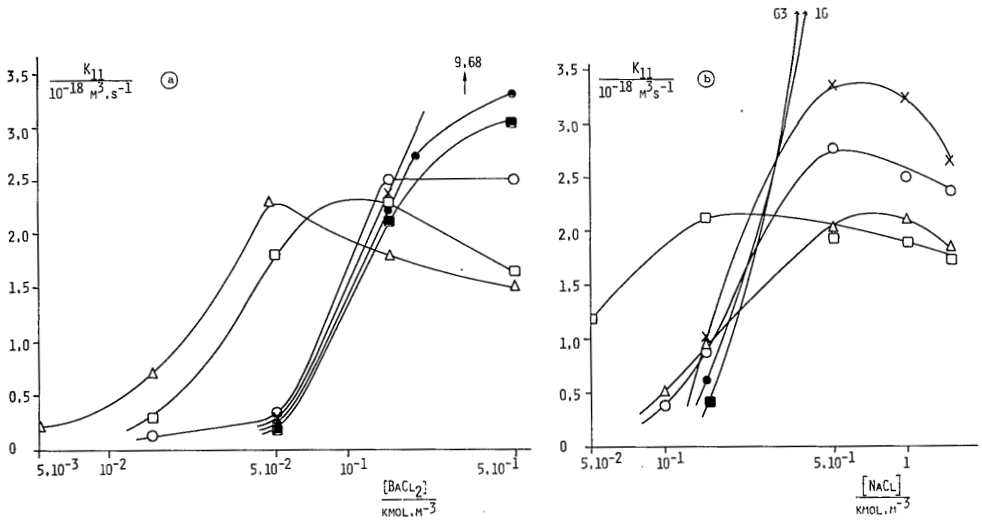


Figure 13: The dependence of k_{11} on the salt concentration (pH = 3.5) for curves with constant HFb concentration:

a) with $BaCl_2$ at HFb concentrations of Δ, 0; □, 2.5; o, 5.0; ●, 10; ■, 20; x, 40 ⁎ 10^{-3} $kg.m^{-3}$.

b) with NaCl at HFb concentrations of Δ, 0; □, 2.0; o, 5.0; x, 10.0; ●, 25; ■, 100 ⁎ 10^{-3} $kg.m^{-3}$.

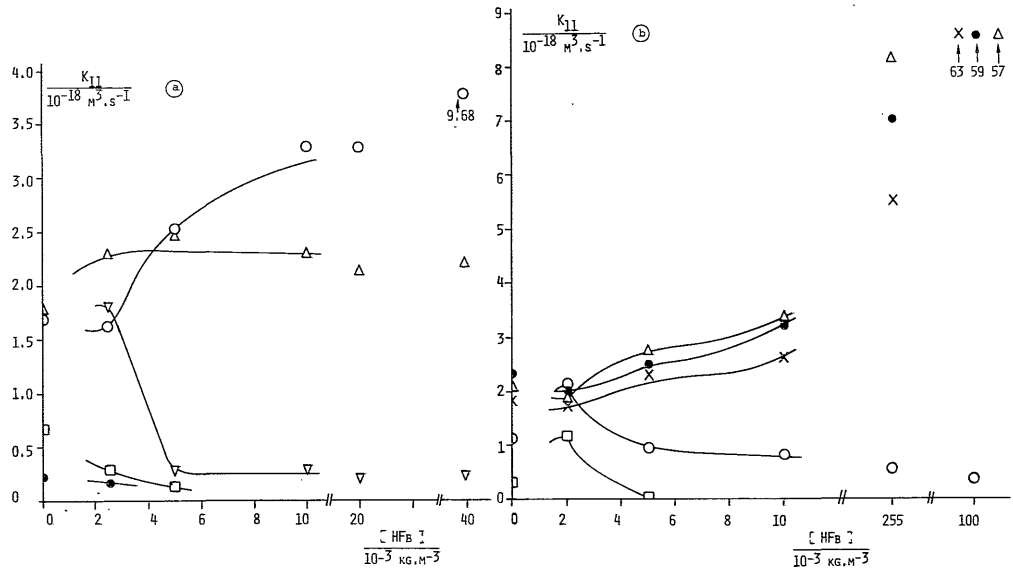


Figure 14: The dependence of k_{11} on the HFb concentration (pH = 3.5) for curves with constant salt concentration:

a) with $BaCl_2$ concentrations of o, 0.5; Δ, 0.15; +, 0.05; □, 0.015; ●, 0.005 kmol.m^{-3}.

b) with NaCl concentrations of x, 1.5; ●, 1.0; ▲, 0.5; o, 0.15; □, 0.05 kmol.m^{-3}.

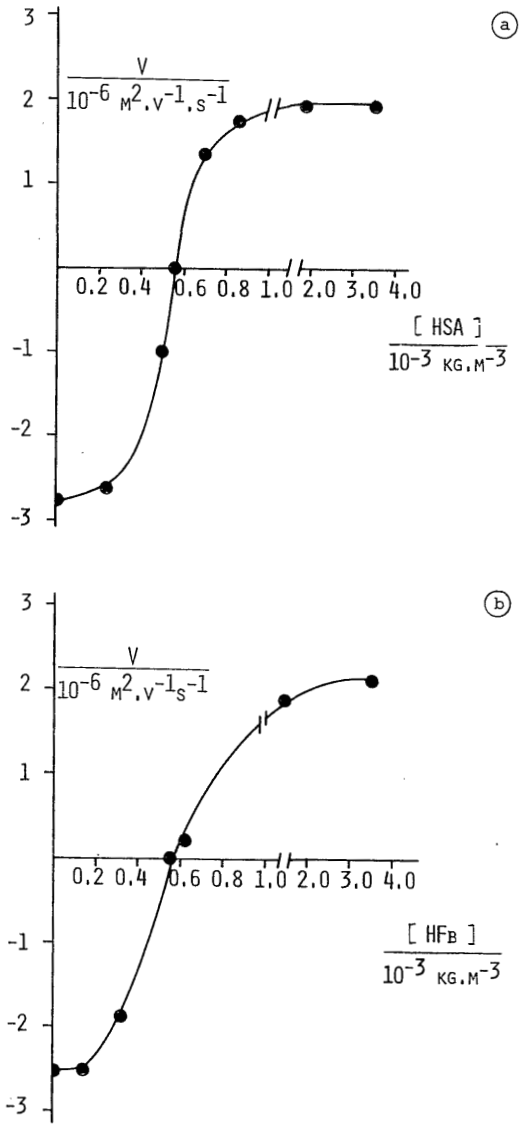


Figure 15: The electrophoretic mobility V of the latex particles as a function of the protein concentration at pH = 3.5.
a) with HSA
b) with HFb.

VI.5 CONCLUSIONS

The influence of the adsorption of the proteins HFb and HSA on the stability of a negatively charged polystyrene latex can be summarized as follows:

1. The adsorbed molecules protect the latex particles against aggregation by electrolyte addition if they occupy the surface of the latex particles completely and if the continuous phase has "good solvent" properties for the protein.

2. No steric stabilization is observed when the continuous phase has "bad solvent" properties for the protein. At "bad solvent" properties for the protein and high protein concentrations the initial aggregation of protein latex mixtures cannot be described by a bimolecular process.

3. Positively charged protein molecules can be induce flocculation of the negatively charged latex by charge neutralization. At relative low protein concentrations the charge of the negative latex particles is reversed by adsorption of positive protein molecules resulting in restabilization of the latex by electrostatic repulsion.

4. The latex cannot be flocculated by negatively charged protein molecules in the absence of salt. At low surface coverage of the latex particles by negatively charged proteins flocculation can be caused by very small additions of electrolyte (sensitization by a bridging mechanism).

5. Bridging of latex particles by protein molecules increases the rate of flocculation compared to the rate observed for protein-free latex. This enhancement of flocculation rate can be explained by a reduced hydrodynamic interaction between the particles and an increased effective collision-radius of the particles, depending on the size of the protein molecules.

6. At "good solvent" conditions, steric stabilization by HFb occurs at much lower protein concentrations than it does using HSA, this indicates a higher affinity of the HFb for the PS-surface.

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CHAPTER VII

THE INFLUENCE OF PROTEIN ADSORPTION ON THE FLOCCULATION KINETICS OF POLYSTYRENE LATEX

ABSTRACT.

Flocculation experiments of negatively charged polystyrene latex (PSL) with human serum albumin (HSA) and human fibrinogen (HFb) have been performed at pH = 3.5, well below the isoelectric points (i.e.p.) of the proteins. Using a stopped flow spectrophotometer the rate constant of flocculation k_{11} is measured as a function of the protein concentration. Flocculation without restabilization was observed at those protein concentrations where the electrophoretic mobility of the latex particles appeared to be zero. At higher concentrations restabilization was observed after initial flocculation. Restabilization resulted from charge reversal by further adsorption of the positively charged protein molecules.

From measurements of the time necessary to get restabilization of the latex as a function of the protein concentration it is concluded that the adsorption of the protein molecules onto the latex particles is a diffusion controlled process. The very high initial rates of flocculation at higher protein concentration are explained by a combination of bridging and electrostatic attraction between approaching particles due to an uneven charge distribution on the latex particle surface. The initial flocculation at protein concentrations several times higher than the concentration where charge neutralization is observed still causes a decrease in the number of single particles of more than 5%, although in this region restabilization occurs in less than one second.

VII.1 INTRODUCTION.

In a previous study[1] on the flocculation of negatively charged polystyrene latex (PSL) by proteins, i.e. human serum albumin (HSA) and human fibrinogen (HFb), it was demonstrated that at pH 3.5, below the iso-electric point (i.e.p.) of the proteins, adsorption flocculation occurs*. Above the i.e.p. (at pH = 8.9) only sensitization was observed. The maximum rate of flocculation for partially protein coated latex particles in that case was larger than the maximum rate of coagulation for the bare latex. We explained this enhancement of the rate of aggregation (k_{11}) by a reduced hydrodynamic interaction between the partially covered latex particles and their increased effective collision radius, both phenomena accompanying the bridging of particles by protein molecules.

Fleer[4] did not observe an increased k_{11}-value when AgI-sols were flocculated by polyvinyl alcohol (PVA). In a simple experiment when mixing the AgI-sol with a PVA solution he found very low values for k_{11}. Using a two-portion method (mixing of PVA covered AgI with bare AgI) he found a value of k_{11} which was 50% of the value found for fast coagulation (k_f) with salt only. This relatively low k_{11} value is not very surprising. The AgI particles which are fully covered by PVA will not only have an increased effective collision radius but the hydrodynamic plane of shear will also be at the top of the adsorbed layer. This means that no decrease in hydrodynamic interaction between a covered and a bare particle will occur and that the decrease of the diffusuon coefficient resulting from the increase in radius of the coated particles will rule out the effect of the increased effective collision radius.

* Following La Mer[2] and Fleer[3] we will distinguish between coagulation (aggregation by low molecular weight electrolytes only) and flocculation (aggregation by polyelectrolytes or polymers). When no low molecular weight electrolyte is needed for flocculation the process is called adsorption flocculation, otherwise it is called sensitization.

Gregory[5,6] was the first to observe an appreciable enhancement of the rate of flocculation compared to the rate of fast coagulation when he flocculated negatively charged PSL with cationic polymers with and without further addition of salt. From electrophoretic mobility measurements he concluded that charge neutralization by adsorption was the main factor involved. He also concluded that the enhancement of k_{11} was not due to bridging but to electrostatic interactions which deminished upon salt addition. Adsorption of large positively charged polyelectrolyte molecules on a negatively charged surface, causes a mosaic pattern of positive and negative places on the surface. The approach of two particles bearing a net zero charge with the described mosaïc pattern should give a favourable interaction between the two mosaïcs probably by oriented approach.

In our measurements[1] at pH = 3.5 we reported that at protein concentrations > 0.7×10^{-3} kg.m^{-3} restabilization of the PSL-protein mixtures was observed, after an initial onset of flocculation. Leaving this initial flocculation (a problem for further study) we took k_{11} to be zero when restabilization was observed.
Gregory[6] also reported an initial flocculation which he ignored by taking the rate of flocculation one to two minutes after mixing. Fleer[4] also found an initial flocculation using higher PVA concentrations in the one-portion method. He reported that this initial flocculation was very slow compared to the fast coagulation of the bare AgI-sols with salt.

In this paper we shall report on the rate of flocculation of PSL with HSA and HFb at pH 3.5 in the absence of low molecular weight electrolytes.
Special attention will be paid to the initial rates of flocculation at concentrations where restabilization occurs in later stages. The flocculation experiments will be carried out with a stopped flow spectrophotometer. This technique allows us to follow the transmission of the colloidal dispersions in the earliest stages of the aggregation.

Lipps[7,8] and later on Lichtenbelt[9] showed the relation between the change in transmission and the rate constant of aggregation (k_{11}) in its earliest stages. We used the method of Lichtenbelt in our calculations.

VII.2 THEORY

The steady-state flux J_o of particles towards a central particle (which is also in Brownian motion), including hydrodynamic interaction can be described by[10]

$$J_o = 2\, k_{11} . N_1 \frac{8\,\pi\, N_1\, D\, a}{\int_0^\infty \frac{\beta(u)}{(u+2)^2} \cdot \exp.\left(\frac{V_T}{kT}\right) du} \qquad (1)$$

k_{11} = the second order rate constant of aggregation ($m^3.s^{-1}$); N_1 = number of particles per unit of volume (m^{-3}); $D = \frac{kT}{6\,\pi\,\eta\,a}$, diffusion coefficient of the particles ($m^2.s^{-1}$); a = effective collision radius of the particles (m); $u = \frac{R-2a}{a}$, the reduced distance between the surface of two particles; R = the distance between the centres of two particles (m); $\beta(u) = \frac{D}{D(u)}$, the diffusion correction factor at reduced distance u; D(u) = the diffusion coefficient at reduced distance u; V_T = the total interaction energy between two particles (Joule); k = Boltzmann's constant 1.38×10^{-23} (Joule.K^{-1}); T = temperature (K).

We defined[1] a dimensionless rate constant of aggregation k^*, for the coagulation in case of bare latex

$$k^* = \frac{J_o}{16\,\pi\, D\, N_1\, a} \qquad (2)$$

This rate constant k^* is equal to one for the theoretical Von Smoluchowski rate when $\beta(u) = 1$ and $V_T = 0$.

Figure 1 shows the inverse of the dimensioless rate constant of aggregation k^* as a function of the Hamaker constant.

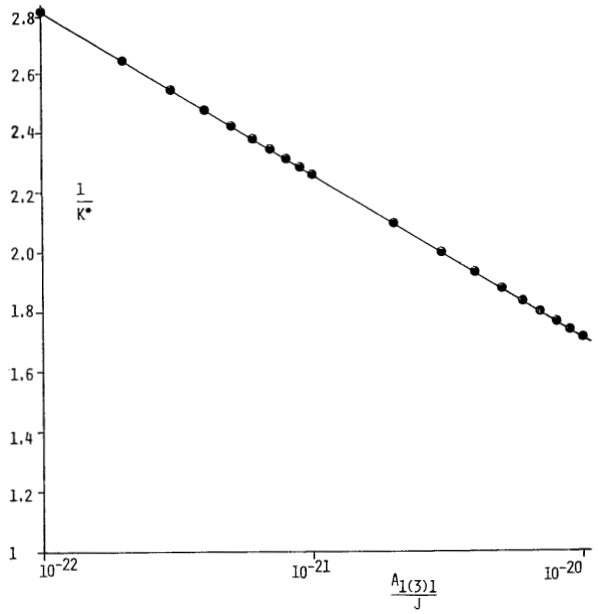

Figure 1: The inverse of the dimensionless rate constant k^* as a function of the Hamaker constant $A_{1(3)1}$

The numerical calculation of k^* was carried out for fast coagulation ($V_T = V_A$) using the Hamaker equation[13]

$$(V_A = -\frac{A_{1(3)1}}{6}\left(\frac{2}{u^2+4u} + \frac{2}{u^2+4u+4} + \ln\left(\frac{u^2+4u}{u^2+4u+4}\right)\right)) \text{ and the expression for } \beta(u) \text{ which is developed by Honig[10]}$$

$$(\beta(u) = \frac{6u^2+13u+2}{6u^2+4u})$$

For flocculation of particles, partially covered by bridge forming tails with length h (reduced length $x = \frac{h}{a}$), in the absence of electrostatic repulsion ($V_T = V_A$ = Hamaker attraction energy between two equal spheres) we developed[1], a formulation for the steady state flux J_H

$$J_H = 8\ \pi\ D\ N_1 (a+h) / \left(\int_x^\infty \frac{\beta(u)}{(u+2)^2} \cdot \exp.\ \left(\frac{V_A}{kT}\right)\ du \right) \qquad (3)$$

Also we defined[1] an accelleration factor γ which gives the relative enhancement in k_{11}, due to bridging by tails with length h

$$\gamma = (1+x) \cdot \frac{\int_0^\infty \frac{\beta(u)}{(u+2)^2} \cdot \exp.\ \left(\frac{V_A}{kT}\right)\ du}{\int_x^\infty \frac{\beta(u)}{(u+2)^2} \cdot \exp.\ \left(\frac{V_A}{kT}\right)\ du} \qquad (4)$$

x = the reduced length of the tails $\left(\frac{h}{a}\right)$

Figure 2 shows the value of the accelleration factor γ as a function of the length (h) of the tails for different values of $A_{1(3)1}$ (= Hamaker constant for two bodies 1 embedded in medium 3 (Joule)) and particles with a radius of 170 nm).

According to Lichtenbelt[9] the relation between the change in transmission $\left(\frac{dT}{dt}\right)_{t=o}$ at the beginning of the aggregation (t=o) and the rate constant of aggregation k_{11} can be described by

$$k_{11} = \frac{\left(\frac{dT}{dt}\right)_{t=o}}{2.T_{t=o}\ \ln T_{t=o}\ \left(\frac{C_2}{2C_2} - 1\right)\ N_1} \qquad (5)$$

The factor $\left(\frac{C_2}{2C_2} - 1\right)$ has been called the optical factor and it can be conceived as the relative change in absorbance that would occur when a colloidal system consisting of a number of singlets only, would be replaced by another colloidal system consisting of half of that number of doublets only.

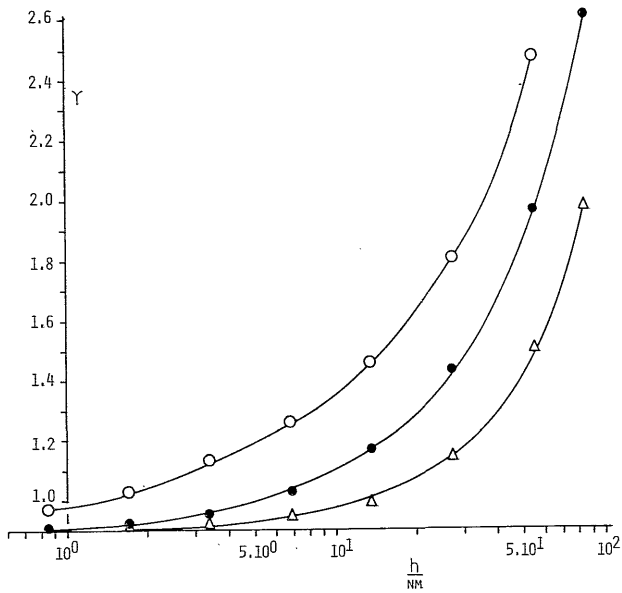

Figure 2: The maximal accelleration factor γ for particles with a radius of 170 nm as a function of the length (h) of the tails for different $A_{1(3)1}$. o, $A_{1(3)1} = 10^{-22}$ J; •, $A_{1(3)1} = 10^{-21}$ J; Δ, $A_{1(3)1} = 10^{-20}$ J.

Lichtenbelt tabulated this factor (calculated with the Rayleigh Gans-Debeye theory) for different values of α.

$$\alpha = \frac{2\ \pi\ a}{\lambda} \qquad (6)$$

λ = wavelength of the light used in the continuous phase (m)

$$= \frac{\lambda_{air}}{n_{water}} .$$

In this work colloidal particles with a radius of 170 nm are used and light with a wavelength of 546 nm.
From these data α can be calculated and the matching value of the optical factor is 0.18.

Equation (5) has been developed for the first stages of aggregation, when only collisions between single particles occur. Defining a degree of conversion y, the fraction of single particles which is converted into double particles after t seconds of aggregation leads to

$$N_{1,t} = (1-y)\ N_{1,0} \text{ and } N_{2,t} = \tfrac{1}{2}\ y \cdot N_{1,0} \qquad (7)$$

$N_{1,t}$ = number of single particles per unit volume after t seconds of aggregation (m^{-3})
$N_{1,0}$ = initial number of single particles per unit volume (m^{-3}).
$N_{2,t}$ = number of double particles per unit volume after t seconds of aggregation (m^{-3}).

The absorbance τ_t after t seconds of aggregation is given by

$$\tau_t = (1-y)\ N_{1,0} \cdot C_1 + \tfrac{1}{2}\ y \cdot N_{1,0}\ C_2 \qquad (8)$$

$$\tau_o = N_{1,0} \cdot C_1 \qquad (9)$$

where C_1 and C_2 are extinction cross sections of single and double particles.
From equation (8) and (9) we find

$$\frac{\tau_t}{\tau_o} = 1 + y\ \left(\frac{C_2}{2C_1} - 1\right) \qquad (10)$$

Because $\tau = -\frac{1}{L} \ln T$, we can easily find the relation between y and T

$$y = \frac{\ln\left(\frac{T_t}{T_o}\right)}{\ln T_o} \times \frac{1}{\left(\frac{C_2}{2C_1} - 1\right)} \qquad (11)$$

This equation only holds when $y < 0.15$ since at higher degrees of conversion the influence of collisions between single and double and between double and double particles may not be ignored anymore.

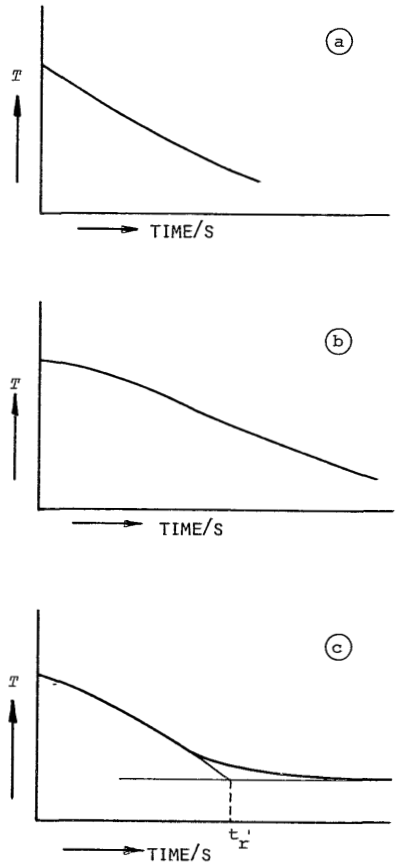

Figure 3: Some typical transmission-time curves.
a) The transmission-time curve for the coagulation of a bare latex with salt.
b) The transmission-time curve for flocculation of latex with protein only, at concentrations to low to get restabilization.
c) The transmission-time curve for flocculation of latex with protein only, at concentrations high enough to get restabilization.

In figure 3 some typical shapes of the transmission-time curves are shown. Figure 3a shows the shape of the transmission-time curve which is obtained in coagulation experiments of the latex with salt. The shape of the transmission-time curve for flocculation experiments of PSL with protein, at protein concentrations where no restabilization is observed, is shown in figure 3b. The typical shape in the case of flocculation at protein concentrations where restabilization occurs is shown in figure 3c. From the type of curves shown in figure 3c we obtained the time necessary for restabilization (t_r). This was performed by extrapolating the steepest part of the curve and the final horizontal part of the curve, the point where these lines intersect is taken as the point of restabilization (by charge reversal, as will be shown later on).

VII.3 EXPERIMENTAL

VII.3.1 Materials.

Polystyrene latex (PS), was prepared by emulsion polymerization, free of added emulsifier, using sodium persulphate (1.5×10^{-3} kmol.m^{-3}) as initiator[11,12]. The styrene was twice distilled prior to use and it represented about 1.5%, by weight of the reaction mixture. Polymerization was carried out overnight in a sealed flask under nitrogen rotating in a waterbath at 343 K. The resulting latex was extensively dialyzed in a well boiled cellulose acetate dialyzing tube against distilled water during one week. The particle radius was measured using electron microscopy and nitrogen adsorption isotherms on the dried latex. These measurements gave particle radii of 170 $\pm$ 4 nm.

Human serum albumin (HSA), crystalline, was obtained from Pierce Chemicals (nr. 30430) and it was used without further purification.

Human fibrinogen (HFb) was obtained from Kabi, Stockholm (grade L, 90% clottable) and it was used after dialyzing against twice distilled water at pH = 9, T = 278 K during one night.

All chemicals used were analytical grade.
The water used in the experiments was always twice distilled and degassed (to prevent gas bubbles which may disturb the transmission measurements).

VII.3.2 Apparatus.

Micro electrophoresis apparatus

The measurements of the electrophoretic mobility of bare and protein coated latex particles have been performed with a Rank Micro Electrophoresis Apparatus MK II, equipped with a cylindrical capillary cell and reversible platinum electrodes.

Stopped flow spectrophotometer

All aggregation experiments have been carried out in a Durrum-Gibson Stopped Flow Spectrophotometer, Model D-110 with a 20 mm path length cell. In this apparatus the solutions to be mixed are contained in two syringes. By a pressure (5×10^5 $N.m^{-2}$) operated actuator equal volumes are reproducibly mixed within a few milli-seconds. The transmission of the mixture is recorded on a storage oscilloscope. The wave-length of the light used was 546 nm using a tungsten iodide light-source.

VII.3.3 Methods

Flocculation and coagulation measurements

Coagulation experiments of the bare PSL with $BaCl_2$ have been performed with the stopped-flow apparatus, using one syringe for the bare latex and the other for the salt solution.
Flocculation experiments of PSL with HSA and HFb have been carried out by using one syringe for the bare latex and the other for the protein solution.
The values for salt and protein concentrations which are given in the different figures always represent the initial concentration after mixing in the stopped flow apparatus.

The initial particle concentration after mixing in all experiments is 2.4×10^{15} m^{-3}. The rate constant of aggregation k_{11} is calculated from $(\frac{dT}{dt})_{t=o}$, the initial change of transmission after mixing, using equation (5). The initial transmission T_o in the experiments is always 31.3%. From the coagulation experiments we found that $\frac{dT}{dt}$ always has to be measured in the region where ΔT is < 1%. At larger ΔT values equation (5) cannot be used to calculate k_{11} because other collisions than those between single particles will then play a role.

Measurements of the electrophoretic mobility.

The electrophoretic mobility of the latex particles is measured as a function of the protein concentration. For these measurements it was important to carry them out in the following way. The PSL is mixed with protein solutions of the same concentrations as were used in the flocculation experiments. These flocculation experiments however, always take about 10 seconds, so we are interested in the mobility after 10 seconds of contact between the PSL and the protein solution. In order to obtain this, the mixture of PSL and protein solution was diluted 10^4 times after 10 seconds of contact. Because the proteins are bound very tightly to the surface[14] it may be expected that the coverage of the particles by the protein will not change upon dilution. On the other hand, further adsorption of protein at the latex particles can be neglected after the drastic dilution. When the dilution step was done a few minutes after the mixing, it was difficult to measure the electrophoretic mobility for some experiments where flocculation occurs, because too many aggregates are present at that time.
All experiments have been performed at 298 K.

VII.4 RESULTS AND DISCUSSION

VII.4.1 Fast coagulation of PSL with $BaCl_2$.

Coagulation experiments of PSL with $BaCl_2$ have been performed at pH = 3.5 and 8.9. In these experiments fast

coagulation was observed at $BaCl_2$ concentrations $\geq$ 0.05 kmol.m^{-3}. The observed rate constant of fast coagulation k_{11} with $BaCl_2$ concentrations of 0.05 kmol.m^{-3} is 2.38 $\times 10^{-18}$ $m^3.s^{-1}$ at pH 3.5 and 2.61 $\times 10^{-18}$ $m^3.s^{-1}$ at pH 8.9. These values are considerably smaller than the value predicted by Von Smoluchowski (5.38 $\times 10^{-18}$ $m^3.s^{-1}$), as a result of the hydrodynamic interaction[10] between the particles. Expressed in the dimensionless rate constant k^* (equation (2)) these values are 0.44 and 0.48. From figure 1 which shows the relation between $1/k^*$ and the Hamaker constant $A_{1(3)1}$, the value of $A_{1(3)1}$ for our system is found to be 10^{-21} J (at pH 3.5) or 2 $\times 10^{-21}$ J (at pH 8.9). These results show the extreme effect of small errors in k_{11} on the calculated $A_{1(3)1}$. The value found here is in agreement with the $A_{1(3)1}$ value which we observed[1] for a commercially available PSL (Dow LS-1047-E). Visser[15] tabulated Hamaker constants of polystyrene in water. He gives values obtained with the Lifshitz theory ($A_{1(3)1}$ = 3.5 $\times 10^{-21}$ J) and from colloid chemistry ($10^{-21} < A_{1(3)1} < 10^{-19}$J). Comparison with these values shows that the values obtained from our experiments are somewhat low but not unrealistic.

VII.4.2 Electrophoretic mobility.

The results of the electrophoretic mobility measurements of the PSL particles as a function of the protein concentration are shown in figure 4a,b. From this figure it appears that due to protein adsorption the charge of the PSL particles decreases and even reverses at protein concentrations of >0.7 $\times 10^{-3}$ kg.m^{-3}.

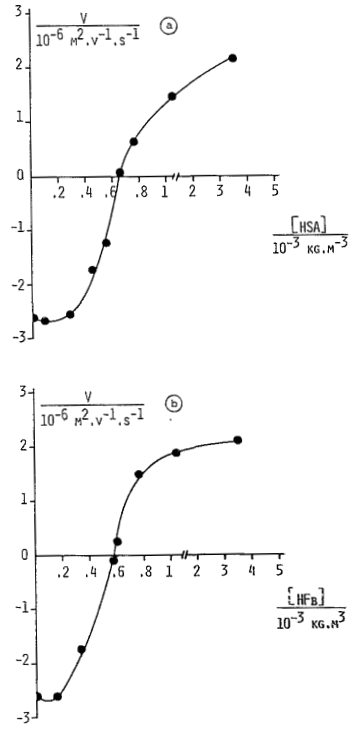

Figure 4: The electrophoretic mobility V of the latex particles as a function of the protein concentration at pH = 3.5.
a) with HSA; b) with HFb.

VII.4.3 Flocculation with HSA and HFb.

The shape of the transmission-time curves after mixing of the PSL with salt or protein solutions is shown in figure 3. When PSL was mixed with salt the initial part of the curve was the steepest part (fig. 3a). When PSL was mixed with protein solutions the shape shown in either figure 3b or figure 3c is observed. The shape in figure 3b can be explained as follows: the first part of the curve is not very steep because there is not enough protein adsorbed onto the latex particles to get an effective destabilization. When time proceeds, more protein will be adsorbed at the particles causing a better destabilization (steeper slope) of the PSL, until further protein adsorption stops because of depletion of the solution. Figure 3c shows the shape of the T-t curve when enough protein is present in solution to get restabilization.

After an initial slow flocculation the rate of flocculation will increase by more effective destabilization. The steepest part of the curve will represent the region where the optimal amount of protein is adsorbed to produce flocculation. Further adsorption of protein causes a decrease (less steep slope) in the rate of flocculation. Finally enough protein has been adsorbed to get a complete restabilization of the PSL (slope zero).

The rate of flocculation, obtained from the steepest part of the T-t curves (which is always reached before ΔT was 1%) is plotted against the initial protein concentration in figure 5.

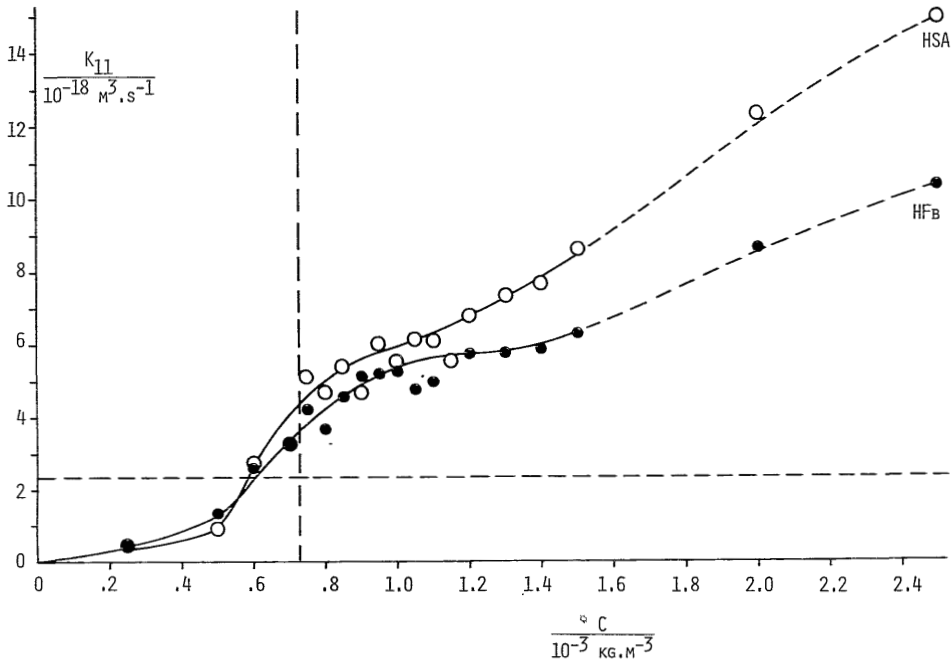

Figure 5: The maximal rate constant of flocculation k_{11} as a function of the protein concentration. At protein concentrations > 0.7 x 10^{-3} kg.m^{-3} restabilization occurs after the initial flocculation: o, HSA; •, HFb.

At protein concentrations $\geq 0.75 * 10^{-3}$ kg.m^{-3} the k_{11} values are obtained from the steepest part of the curves with a shape as shown in figure 3c. If at these concentrations, the value of k_{11} is taken to be zero which value will be reached soon after the initial flocculation, then the results are similar to those obtained by Gregory[5,6]. In that case an optimum protein concentration for flocculation is found at $0.65 * 10^{-3}$ kg.m^{-3}. In the present paper however, we do not ignore the initial flocculation when later on restabilization occurs. From the electrophoretic results (figure 4) it can be seen that at protein concentrations $\geq 0.7 * 10^{-3}$ kg.m^{-3} the charge of the latex particles is reversed by adsorption of the positively charged protein molecules. This protein concentration coinsides very well with the concentrations where restabilization of the PSL takes its starting point. Therefore it is concluded that final restabilization results from electrostatic repulsion between the now positively charged latex particles.

The time (t_r) (figure 3c) necessary to get restabilization will be determined by the rate of adsorption of the protein molecules. This rate of adsorption will be governed by the diffusion rate of the protein molecules and/or their rate of attachment. When the adsorption is irreversible and diffusion controlled, then the adsorption process can be described with the Von Smoluchowski[16] theory of coagulation. In this case we may assume that only the protein molecules do disappear upon colliding with the latex particles, and protein-protein as well as latex particle , latex particle collisions, may be ignored. The rate of disappearance of the protein molecules can now be described by

$$-\frac{dC}{dt} = k \cdot N_1 \cdot C \qquad (12)$$

C = protein concentration (kg.m^{-3}), t = time(s), k = bimolecular rate constant (m^3.s^{-1}), N_1 = number of latex particles per unit of volume (m^{-3}).

Integration of (12) between t=o and $t=t_r$ gives

$$\ln \frac{C_{t_r}}{C_o} = - k \cdot N_1 \cdot t_r \qquad (13)$$

If we suppose that restabilization always occurs when a certain amount of protein is adsorbed at the latex particles then we can write

$$C_{t_r} = C_o - C_r \qquad (14)$$

In this equation C_r will be the lowest concentration where restabilization is observed.

From equations (13) and (14) it follows that plotting $\ln \frac{C_o - C_r}{C_o}$ against t_r gives a straight line with slope $- k.N_1$. Figure 6 shows $-\ln \frac{C_o - C_r}{C_o}$ as a function of t_r. From the observed slopes we found that $k \cdot N_1 = 0.483$ for HSA and $k.N_1 = 0.148$ for HFb. Following Von Smoluchowski k can be expressed by

$$k = 4 \pi (D_1 + D_2)(r_1 + r_2) \qquad (15)$$

For a system with latex particles with radius r_1 and a diffusion coefficient D_1, colliding with protein particles with radius r_2 and a diffusion coefficient of D_2. This equation may be simplified by neglecting D_1 with regard to D_2 and neglecting r_2 with regard to r_1, so

$$k = 4 \pi D_2 \cdot r_1 \qquad (16)$$

Inserting $r_1 = 170 \times 10^{-9}$ m and $N_1 = 2.4 \times 10^{15}$ m^{-3} in the observed values of the slopes in figure 6 gives a value for the diffusion coefficient of $D_{HSA} = 9.5 \times 10^{-11}$ $m^2.s^{-1}$ for HSA and $D_{HFb} = 2.9 \times 10^{-11}$ $m^2.s^{-1}$ for HFb.

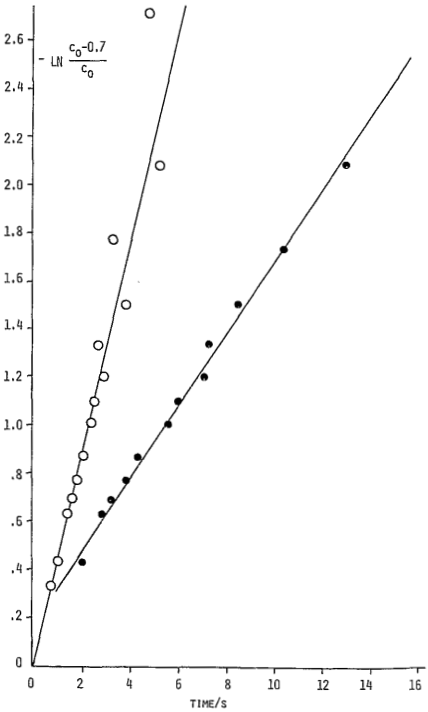

Figure 6: The relation between $-\ln \frac{C_o - 0.7}{C_o}$ and the time necessary yo get restabilization t_r (equation (13) and (14)) for: o, HSA; •, HFb.

These values are somewhat higher than the literature values of $D_{HSA} = 6.1 \times 10^{-11}$ $m^2.s^{-1}$ [17] and $D_{HFb} = 1.5$ to 2.0×10^{-11} $m^2.s^{-1}$ [18].

The difference between the observed values and the literature values may be due to the fact that the protein molecules and the latex particles are oppositely charged.

From the linear relation between $-\ln \frac{C_o - C_r}{C_o}$ and t_r and the calculated diffusion coefficients of the protein molecules we conclude that the rate of adsorption of HFb and HSA onto PSL-particles is diffusion controlled.

The values of k_{11} shown in figure 5 at protein concentrations of 1×10^{-3} kg.m^{-3} are more than two times as large as those observed for fast coagulation with salt only. When these high values would result from bridging only, we could estimate the length of the bridges (tails) using figure 2, which shows the accelleration factor γ (equation (4)) due to bridging. For a Hamaker constant of 10^{-21} J an accelleration factor > 2 can be explained by tail lengths > 50 nm. Comparing this length with the dimensions of the protein molecules leads to the conclusion that bridging alone cannot explain the observed rates of flocculation. (HFb is assumed to be a rod like molecule with minor and major axes of 9 and 45 nm[19-21], HSA is assumed to be a prolate ellipsoïd with minor and major axes of 4 and 14 nm[17]).

Following Gregory[5,6] we must explain part of the observed enhancement of k_{11} compared to k_f (fast coagulation) by a net attraction between the particles due to an uneven charge distribution on the surface of the particles.

The further enhancement of k_{11} at higher protein concentrations might be explained as follows. When the protein concentration increases, the time to reach charge neutralization by adsorption decreases as a result of faster adsorption. This implies that at higher concentrations the adsorbed molecules have less time to reorganize at the surface before flocculation starts, resulting in longer positive tails. These long positive tails will be very effective bridges because they will be attracted by the negative places on other particles. In this way a faster flocculation can be expected, caused by bridging as well as attractive forces due to uneven charge distribution.

Flocculation experiments under the same conditions, but now in the presence of low molecular weight electrolytes are needed to get more information about the influence of the uneven charge distribution on the observed rate constants. Such experiments might confirm the last somewhat speculative remarks on the extreme enhancement of k_{11} at higher protein concentrations.

Using equation (11) the degree of flocculation resulting from initial flocculation, before restabilization occurs, has been calculated from the difference between the initial transmission and the transmission after restabilization.
Figure 7 shows the value of the conversion factor y as a function of the protein concentration. It is seen that above the concentration necessary to obtain charge reversal of the latex particles (0.7×10^{-3} kg.m^{-3}) this y value rapidly drops. Addition of even large protein concentrations > 1.5×10^{-3} kg.m^{-3} cannot prevent that the initial flocculation causes a decrease in single particles of about 5%.

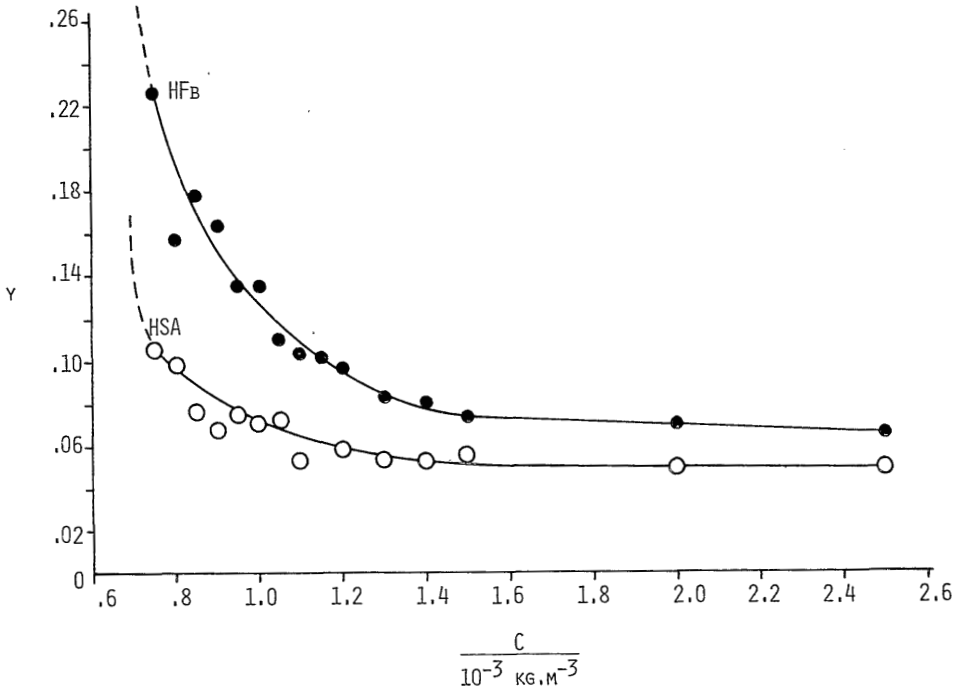

Figure 7: The dependence of the conversion factor y on the initial protein concentration: o, HSA; •, HFb.

VII.5 CONCLUSIONS

This study shows that flocculation measurements may provide information about the rate of adsorption of the flocculant onto the particle surface of the flocculating sol. Using human serum albumin and human fibrinogen as flocculant and polystyrene latex as sol, it appears that the initial adsorption of these proteins onto the latex particles is a diffusion controlled process. It is also shown that the flocculation of the negatively charged latex particles with positively charged protein is mainly caused by charge neutralization by adsorbed proteins molecules. The rate of flocculation is determined by bridging and electrostatic attraction resulting from an uneven charge distribution on the surface of the latex particles.

Initial flocculation causes at least a 5% decrease in the number of single particles, even at protein concentrations which are three times higher than necessary for charge neutralization.

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SUMMARY

In this thesis the adsorption of some plasma proteins (human albumin (HSA) and fibrinogen (HFb)) on non polar surfaces is studied, together with the influence of these proteins on the stability of polystyrene latices. The aim of these investigations is a better understanding of the processes occurring at the surface of polymer materials in contact with blood.

Chapter I, a short review of the literature about protein adsorption at interfaces, shows that many contradictions about protein adsorption exist. Especially the reversibility of the adsorption is still an unsettled question.

Chapter II deals with the adsorption of proteins at the undisturbed paraffin oil/water and the polystyrene/water interface. This was studied by interfacial tension measurements, by adsorption measurements using a depletion technique and by ellipsometry. Maximum adsorption is always observed at the isoelectric point (i.e.p.) of the protein. Salt addition diminishes the influence of pH on the adsorption. Protein adsorption at apolar surfaces is irreversible (no desorption is ever observed once the protein has been adsorbed). From the slope of the isotherms of HSA one is inclined to infer that protein adsorption is reversible, because at low protein concentrations significant amounts of protein remain in solution although the adsorption plateau value has not been obtained. Changes in the bulk conditions which should cause increased adsorption (concentration increase, pH changes in the direction of the i.e.p.), do appear to enhance adsorption. Changes which should cause desorption (dilution, pH changes away from the i.e.p.), do not bring about a decrease in adsorbed amount. This behaviour is called semi-reversible.

Chapter III describes a study of the feasibility of radiolabeled HSA in adsorption studies. A model was developed which enables the evaluation of preferential adsorption of one protein from a mixture of proteins. When this model is used, radiolabeled HSA apparently adsorbs preferentially with respect to non-labeled HSA. Therefore the use of radio-labeled HSA in ad-

sorption studies is not recommended.

The Chapters IV and V deal with contact angle measurements on protein coated polystyrene surfaces. Adsorbed fibrinogen molecules seem to associate; this behaviour is not shown by adsorbed albumin molecules unless after heat treatment in the adsorbed state. The conformation of adsorbed fibrinogen molecules changes with the hydrophilic/hydrophobic character of their environment.

In the Chapters VI and VII the influence of adsorbed proteins and proteins in solution on the stability of polystyrene latex is studied. The absolute rate of aggregation of the latex caused by salt and/or protein is measured with a stopped-flow spectrophotometer.

At pH-values above the i.e.p. of the proteins, where protein and latex both carry negative charges, flocculation of the latex with protein in solution only cannot be achieved. Small amounts of protein do sensitize the latex with respect to aggregation by salt. The maximum rate of flocculation of a partly protein-covered latex is higher than that of a bare latex. The protein molecules form bridges between the colliding latex particles, which diminishes the hydrodynamic interaction at short distances. At higher protein concentration the latex is stabilized sterically by the complete protein layer. Under bulk conditions such that the protein is not stable in solution, cosedimentation of latex and protein takes place.

At pH-values below the i.e.p. of the proteins, where the protein molecules and the latex particles are oppositely charged, small amounts of protein cause flocculation by charge neutralization. After initial flocculation restabilization of the latex occurs by charge reversal of the latex particles when the protein concentration is increased. The time necessary to get restabilization at different initial protein concentrations indicates that the adsorption of HSA and HFb is diffusion-controlled.

SAMENVATTING

In dit proefschrift wordt de adsorptie van enkele plasma eiwitten (menselijk albumine (HSA) en fibrinogeen (HFb) aan apolaire oppervlakken bestudeerd alsmede de invloed van deze eiwitten op de stabiliteit van polystyreen latices. Het doel van dit onderzoek is het verkrijgen van meer inzicht in de processen die zich afspelen aan het oppervlak van polymere materialen in kontakt met bloed.

Een kort literatuuroverzicht in Hoofdstuk I toont aan dat er met betrekking tot de adsorptie van eiwitten aan grensvlakken nog veel tegenstrijdige literatuur bestaat. Vooral het al of niet reversibele karakter van de adsorptie is een vraagstuk dat van groot belang is voor de interpretatie van de meetgegevens.

Hoofdstuk II bevat de resultaten van het onderzoek naar de adsorptie van eiwitten aan het niet verstoorde paraffine-olie/water en het polystyreen/water grensvlak. Dit is verricht met behulp van grensvlakspanningsmetingen en het meten van adsorpties door uitputtingsmetingen en ellipsometrie. Eiwitadsorptie blijkt sterk pH-afhankelijk te zijn met maximale adsorptie bij het isoelektrische punt (i.e.p.) van het eiwit. De invloed van de pH op de adsorptie wordt sterk gereduceerd indien zout aan de eiwitoplossing wordt toegevoegd.

De adsorptie van de eiwitten aan de gebruikte apolaire oppervlakken is irreversibel (eenmaal geadsorbeerde molekulen desorberen niet meer). Uit de vorm van de adsorptie isothermen van vooral HSA zou men evenwel afleiden dat de adsorptie reversibel is, omdat er bij lagere koncentraties signifikante hoeveelheden eiwit in oplossing blijven terwijl de plateau waarde van de adsorptie nog niet is bereikt. Gebleken is dat veranderingen in de bulkomstandigheden die een toename van de adsorptie zouden kunnen bewerkstelligen (koncentratieverhoging, pH verandering naar het i.e.p. toe) dat inderdaad ook doen. Veranderingen die desorptie zouden veroorzaken (koncentratieverlaging, pH verandering weg van het i.e.p.) blijken geen vermindering in

de geadsorbeerde hoeveelheid te geven. Dit gedrag is omschreven als semi-reversibel.

Hoofdstuk III beschrijft een onderzoek naar de toepasbaarheid van radioaktief gelabeld HSA in adsorptie-studies. Er werd een model ontwikkeld dat de kwantificering van preferente adsorptie van een eiwit uit een mengsel van eiwitten mogelijk maakt. Met gebruikmaking van dit model werd gevonden dat, uit een mengsel van wel en niet radioaktief gelabeld HSA, het radioaktief gelabelde HSA (in lichte mate) preferent adsorbeert ten opzichte van het niet gelabelde. Het is dan ook niet zonder meer geoorloofd om radioaktief gelabeld HSA te gebruiken in adsorptie-studies.

In de Hoofdstukken IV en V worden kontakthoekmetingen aan met eiwit bedekte polystyreenoppervlakken beschreven. Uit de metingen blijkt dat geadsorbeerde fibrinogeen molekulen associëren terwijl geadsorbeerde albumine molekulen dit alleen doen na een warmtebehandeling. Verder is gebleken dat de conformatie van de geadsorbeerde fibrinogeen molekulen verandert met het hydrofiele/hydrofobe karakter van de direkte omgeving.

In de hoofdstukken VI en VII wordt de invloed van geadsorbeerde eiwitten en eiwitten in oplossing op de stabiliteit van polystyreen latices (PSL) bestudeerd. De absolute vloksnelheid van de PSL onder invloed van zout en/of eiwit is gemeten met behulp van een stopped-flow spectrofotometer.

Bij pH-waarden boven het i.e.p. van de eiwitten, waar eiwit en latexdeeltjes beide negatief geladen zijn, is het niet mogelijk de latex te doen uitvlokken met behulp van eiwit alleen. Wel blijken kleine hoeveelheden eiwit geadsorbeerd aan de latex deze gevoeliger te maken voor uitvlokken met zout. De maximale vloksnelheid van een gedeeltelijk met eiwit bedekte latex is groter dan die van een kale latex doordat de eiwit molekulen bruggen vormen tussen de botsende latexdeeltjes, waardoor de hydrodynamische wisselwerking op korte afstand geen rol meer speelt. Bij hoge eiwit koncentraties wordt de latex sterisch gestabiliseerd doordat de deeltjes volledig bedekt zijn met eiwit. Indien de omstandigheden zo gekozen worden dat het eiwit zelf niet meer stabiel is in oplossing, heeft het eiwit zelfs een destabiliserende werking. Er treedt dan cosedimentatie van PSL met ei-

wit op.

Bij pH-waarden beneden het i.e.p. van de eiwitten, waar het eiwit positief en de latexdeeltjes negatief geladen zijn, veroorzaken kleine hoeveelheden eiwit vlokking van de latex door ladingsneutralisatie. Bij toenemende eiwitkoncentraties blijkt na een initiële vlokking weer stabilisatie van de latex op te treden door omlading van de latexdeeltjes. Uit de tijd die bij verschillende initiële eiwitkoncentraties nodig is om de latexdeeltjes te restabiliseren blijkt dat de adsorptie van HSA en HFb op de latexdeeltjes een diffusie-bepaald proces is.

IN 'T KORT ALNS BIE MEKAARE

In dit preufbeukske wod de plakkerieje van 'n stuk of wat blood-eiwitten (Albumine en Fibrinogeen van mèènske) an vettige oppervlakten oet'ezocht, samen met de oetwerking van disse eiwitten op 't van mekaare blieven van plastiek böllekes in water.

Disse zeukerieje is bedoeld um meer te begriepen van wat der gebuurt aj plastiek en blood bie mekaare doot.

Aj now bekiekt wat hier al oawer 'eschreven is, dan zeej dat de geleerde leu 't nog nich met mekaare eens zunt hoo dee plakkerieje eig'lek precies in zien werk geet.

Ne vraoge dee vuural belangriek is, dat is of dee eiwitten waor wie 't hier oawer hebt erg good vast'eplakt zit of daj ze der weer of könt kriegen, aans dan weej nich waj mut doon met alns waj op hebt metten.

In 't tweede kapittel staot de oetkomsten van 't onderzeuk naor de plakkerieje van eiwitten an 't röstige keersenölliewater en 't plastiek-water grensvlak. Dit is e'daon duur 't metten van de sterkte van 't grensvlak, duur 't metten van hoovöl der oet 't water geet en hoo dik 't vast'eplakte laogke is. Hoo völ der plakt henk sterk of van hoo zoer de oplössing is en 't meeste plakt der an as der gin stroom op 't eiwit steet. De plakkerieje henk völ minder van 'n zoergraod of aj 'n luk zoalt bie 't eiwit doot.

De plakkerieje van de eiwitten an de vettige oppervlakten is zo good, as ze der eenmaol an zit dan köj ze der nich weer of kriegen.

Aj de grafieken van de plakkerieje van veural albumine good bekiekt zöj dèènken daj 't der toch wa of könt kriegen, umdat as 't oppervlak nog nich helemaol vol is der toch nog eiwit in 't water blif. Wie hebt mèèrkt dat aj de umstandigheden in 't water zó verandert daj meer plakkerieje zollen verwaochten (meer eiwit in 't water of stroom verminderen op 't eiwit) dit ook gebuurt.

Veranderingen waorvan ie zollen verwaochten dat ze de plakkerieje zollen verminderen (minder eiwit in 't water of meer stroom op 't eiwit) könt de vast'eplakte eiwitten nich lös ma-

ken. Wie neumt dit semi-reversibel.

In 't derde kapittel steet 't onderzeuk naor de meuglikheid van 't gebroek van radioaktief gemerkt albumine in plakkeriej-onderzeuk. Wie hebt beschreven hoo aj met ciefers könt anteunen dat één eiwit oet 'n mengsel van eiwitten harder plakt as de andern. Op disse manier hew veunden dat, oet 'n mengsel van wal en nich gemerkt albumine 't radioaktief deel better plakt as 't nich radioaktieve. Ie majt dan ook nich zonder meer radioaktief gemerkt albumine gebroeken aj de plakkerieje van albumine bekiekt.

In 't veerde en viefde kapittel hew kekken hoo of dröppelkes keersenöllie der oet zeet aj ze op 'n plastiek oppervlak doot wat vol'eplakt zit met eiwit. Oet 't metten blik dat vast-'eplakte fibrinogeen molekuuln an mekaare gaot zitten en albumine molekuuln doot dit alleenig as ze opwarmd wod. Wieder is der oetkommen dat de gedaonte van de vast'eplakte fibrinogeen molekuuln zich veraandert naor gelang zee in de buurte van water of öllie komp.

In 't zesde en zeumde kapittel hew oet'ezocht of an plastiek böllekes vast'eplakte eiwitten of eiwitten in 't water disse böllekes könt laoten kloeten. Um te weten hoo rap dee plastiek böllekes an mekaare vleejt aj der zoalt en/of eiwit bie doot hebbe wie metten met 'n masjien waormet iet 't kloeten könt zeen duur'n gloepens dun lichtstraolke.

Aj 'n zoergraod zo nemt dat der ne negatieve stroom op 't eiwit steet, terwiel de plastiek böllekes altied negatieve stroom hebt dan köj de plastiek böllekes nich laoten kloeten duur der alleenig eiwit bie te doon. Aj der 'n klein betje eiwit bie doot dan köj de böllekes laoten kloeten met minder zoalt as daj der gin eiwit bie doot.

Aj 'n betje eiwit an de plastiek böllekes hebt zitten dan könt ze harder kloeten as daj der gin eiwit an hebt zitten. Dat komp umdat de eiwitmolekuuln bruggen vormt tussen de plastiek böllekes as ze vlak bie mekaare komt, waorduur zee der minder last van hebt dat 't water tussen de böllekes stroperig wod at ze vlak bie mekaare komt. Aj meer eiwit gebroekt dan komt de böllekes vol te zitten en dan blievt ze mooi van mekaar umdat

de vast'eplakte eiwitmolekuuln mekaar in de weg zit.

Aj 'n toostand zo nemt dat 't eiwit oet zich zölm a kloet dan kriej dat de plastiek böllekes en de eiwitmolekuuln samen gaot kloeten. Aj 'n zoergraod zo nemt daj ne positieve stroom op 't eiwit kriejt dan köj de negatieve plastiek böllekes laoten kloeten duur der alleening 'n betje eiwit bie te doon. De vast'eplakte eiwitmolekuuln nemt dan 'n stroom van de böllekes vot. Wie hebt 'evunden dat aj der meer eiwit bie doot de plastiek böllekes eerst efkes kloet, meer dat is rap oaver duur dat 'n stroom op de böllekes umslöt. Wie hebt 'emetten hoo rap 't kloeten van de böllekes oawer is aj alverdan aandre porsies eiwitten in 't water doot.

Hieroet hew op'emaakt dat de plakkerieje van oonze eiwitten an de plastiek böllekes duur niks wod teeg'n werkt.

LEVENSBESCHRIJVING

At van der Scheer werd op 28 mei 1949 geboren in Zwolle, waar hij in 1967 zijn HBS-B-diploma haalde aan het Christelijk Lyceum. In datzelfde jaar begon hij zijn studie in de chemische technologie aan de THT.

De baccalaureaatsstudie werd in januari 1971 afgerond met een opdracht over hoge temperatuur oxidatie. Van februari 1971 tot juli 1973 was hij voor halve dagen in dienst bij de leerstoel Anorganische Chemie om in samenwerking met TNO onderzoek te verrichten aan corrosie door vloeibare alkali metalen. Het ingenieursexamen werd in januari 1973 behaald (met lof).

In juli 1973 begon hij als wetenschappelijk ambtenaar in de vakgroep Macromoleculaire Chemie en Materiaalkunde van de THT aan het onderzoek dat in dit proefschrift wordt beschreven. Hij is op 1 november 1977 begonnen als fysisch-chemicus bij het Koninklijke/Shell Laboratorium in Amsterdam.