

# The protein net electric charge determines the surface rheological properties of ovalbumin adsorbed at the air–water interface

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## Abstract

Adsorption of purified diphosphorylated AI-ovalbumin at the air–water interface was studied by ellipsometry, surface tension, and shear elastic constant measurements. The value of pH did not significantly affect the final value of surface concentration. It affected slightly the kinetics of surface pressure increase and the final value of surface pressure. The interfacial rheology was affected strongly by pH. The interface exhibited a maximum of the shear elastic constant at a pH close to the isoelectric pH of ovalbumin. The bulk protein concentration also had a more pronounced effect on the surface rheology when the protein net charge was low. At a pH where the protein net charge is negative, an increase of the ionic strength increased the final value of the shear elastic constant. The results suggest that interactions between adsorbed ovalbumin molecules, which form slowly in the adsorbed layer upon conformational rearrangements, impart rigidity to the interface, and that these intermolecular associations are hindered at high protein net charge. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Protein; Ovalbumin; Air–water interface; Adsorption; Surface rheology; Charge

## 1. Introduction

Proteins spontaneously adsorb from aqueous solutions to air–water or oil–water interfaces, essentially due to the hydrophobic properties of these interfaces (Walstra & de Roos, 1993). The main driving force is the entropy increase resulting from dehydration of the hydrophobic interface and of hydrophobic regions of the protein surface (Dickinson & McClements, 1996; Kinsella & Phillips, 1989). Adsorption then results in the decrease of the interfacial tension and the interfacial Gibbs free energy. The decrease in surface tension facilitates the formation of a large interface area in foams and emulsions. In the case of food proteins, this ability is of high technological importance, since many food systems are dispersed, multiphase systems. Upon adsorption, globular proteins unfold to an extent depending on their intrinsic structural stability, and constitute progressively an interfacial film exhibiting viscoelastic properties. The relationships between the rheological parameters of the interface and the functional qualities of the dispersed

systems, e.g. foam or emulsion stability, are not established clearly, even though an increased interfacial viscoelasticity is widely believed to be essential for the stability of dispersed systems (Dickinson & McClements, 1996). Recently, correlations have been shown to exist between viscoelastic surface properties and egg-white foaming behavior (Hammershøj, Prins & Qvist, 1999). However, the phenomena that generate macroscopic rheological modifications of the film are far from fully understood at the molecular level.

In addition to surface tension measurements, protein adsorption, i.e. the increase of surface concentration was studied by ellipsometry (de Feijter, Benjamins & Veer, 1978) and radiolabelling techniques (Damodaran, Anand & Razumovsky, 1998; Graham & Phillips, 1979a). Various rheological techniques were used for the study of shear and dilatational properties of the film (Benjamins & Lucassen-Reynders, 1998; Blank Lucassen & Van den Tempel, 1970; Graham & Phillips, 1980a,b).

These experiments have shown that the interfacial behavior is influenced mainly by the protein structural rigidity. Flexible macromolecules, such as  $\beta$ -casein, are very surface-active, but form films with a very low viscoelasticity (Graham & Phillips, 1980b). Globular proteins (lysozyme,  $\beta$ -lactoglobulin, bovine serum-albumin, ovalbumin)

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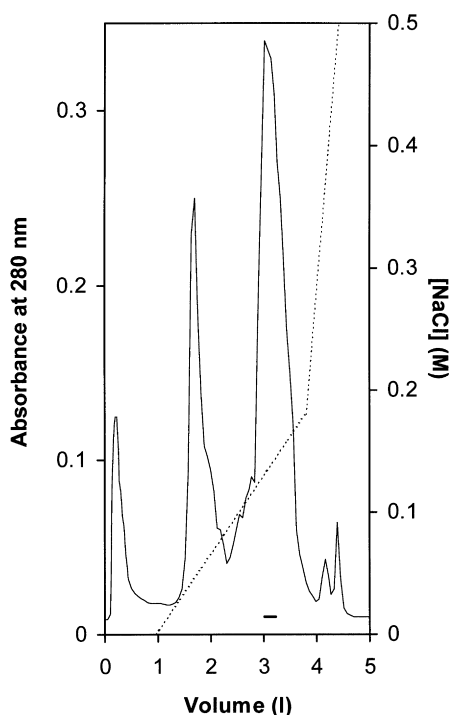


Fig. 1. Preparation of ovalbumin by anion-exchange chromatography. Egg white, prepared as described in experimental, was injected into a Q Sepharose Fast Flow column ( $13.5 \times 5 \text{ cm}^2$ ) equilibrated with 50 mM Tris-HCl, pH 8.0. Proteins were eluted by linear gradients of NaCl concentration in the same buffer as indicated by the dotted line, at a flow rate of  $30 \text{ ml min}^{-1}$ . Proteins were detected by absorbance at 280 nm. Pooled fractions are indicated by the horizontal solid bar.

form films with considerably higher rigidity (Blank et al., 1970; Graham & Phillips, 1980b). Internal structural parameters, such as the presence and number of disulfide bonds or the initial state of denaturation, strongly affect the behavior of globular proteins at the interface (Xu & Damodaran, 1993). Additional information on protein behavior at the air-water interface could be obtained by the structural study of the film itself. Recently, it has been demonstrated by X-ray and neutron reflectivity that the adsorbed film consist of a thin layer (0.8–1 nm), with a very high protein density, attributed to adsorbed trains, and a lower-density layer expanding in the aqueous subphase, probably constituted by hydrophilic loops (Atkinson, Dickinson & Horne, 1995; Harzallah, Aguié-Béghin, Douillard & Bosio, 1998; Horne, Atkinson, Dickinson, Pinfield & Richardson, 1998).

Ovalbumin is a phosphorylated and glycosylated globular protein of molecular weight 43 000, of known sequence (Nisbet, Saundry, Moir, Fothergill & Fothergill, 1981) and three-dimensional structure (Stein, Leslie, Finch & Carrell, 1991), which constitute 54–60% of the egg-white protein fraction. It carries one disulfide bond and four free sulfhydryl groups. This protein contributes to a large part of the functionality of egg-white, which is widely used in the food industry as a foaming agent. The kinetics of ovalbumin adsorption to the air-water interface and the influence of bulk protein concentration on the kinetics have been inves-

tigated already (de Feijter & Benjamins, 1987). The dilatational rheology of the adsorbed films has been studied recently (Benjamins et al., 1998).

The aim of the present work was to investigate the adsorption behavior of ovalbumin and its interfacial properties as a function of its net electric charge. We describe the effect of pH and ionic strength on the adsorption of purified A1-ovalbumin at the air-water interface. To characterize and understand the adsorption kinetics and also the state of the protein film after adsorption, we have developed an approach combining optical, thermodynamical and mechanical techniques. The process of ovalbumin adsorption was investigated by surface pressure measurements and ellipsometry. The latter technique is very sensitive to variations of the molecule concentration at the interface, but does not provide information about lateral organization within the film. Therefore, we developed an experiment to measure the shear elastic constant of the interface which allowed us to monitor in real time the increase of rigidity of the interface. The shear elastic constant measurements were performed upon very low periodic deformations of the film, by the mean of a highly sensitive device involving no mechanical link with the interface. The results suggest that the adsorbed ovalbumin layer undergoes structural rearrangements, much slower than the adsorption itself, which are strongly influenced by the protein net charge.

## 2. Experimental

### 2.1. Materials and chemicals

Hen eggs were purchased from a local market. Standard ovalbumin (grade V, lots 76H7045 and 14H7035) and bis-Tris propane (1,3-bis-(tris-(hydroxymethyl)-methylamino)-propane) were purchased from Sigma (Saint-Quentin-Fallavier, France). Tris-(2-amino-2-(hydroxymethyl)-1,3-propanediol) was purchased from Merck (Nogent sur Marne, France). Other chemicals were of analytical grade. Ultrapure water with  $18 \text{ M}\Omega \text{ cm}$  resistivity (MilliQ, Millipore, Saint-Quentin-en-Yvelines, France) was used for all buffers and dialyses.

### 2.2. Purification of diphosphorylated A1-ovalbumin

Preparative chromatography was performed using a BioPilot system (Pharmacia, Saclay, France). Analytic HPLC was performed using a Waters model 600 solvent delivery system (Waters, Saint-Quentin-en-Yvelines, France), equipped with a Rheodyne injection valve, a  $50 \mu\text{l}$  injection loop and a Waters model Lambda Max 481 spectrophotometer.

The albumen from a single egg was diluted ten-fold in 50 mM Tris-HCl, pH 8.0 (Buffer A), and gently stirred overnight at  $4^\circ\text{C}$ . Precipitated material, consisting essentially of ovomucin (Kato, Nakamura & Sato, 1970; Young & Gardner, 1972), was discarded by centrifugation ( $26\,000g$ , 75 min,  $4^\circ\text{C}$ ) and filtration (filter paper Whatman

541). Ovalbumin from diluted, buffered egg white was purified by anion-exchange chromatography on a  $13 \times 5 \text{ cm}^2$  Q Sepharose Fast Flow column (Pharmacia, Saclay, France) equilibrated with Buffer A. After injection of a 200 ml sample of diluted egg white, the column was washed with Buffer A until the effluent exhibited no absorption at 280 nm. Proteins were eluted at a flow rate of  $30 \text{ ml min}^{-1}$  ( $92 \text{ cm h}^{-1}$ ) by linear gradients of NaCl concentration in Buffer A, from 0 to 0.18 M in 95 min and from 0.18 M to 0.5 M in 20 min, and were detected by absorption at 280 nm. Fractions corresponding to an eluate volume from 3019 to 3235 ml were pooled (see Section 3, Fig. 1) and proteins were precipitated by  $(\text{NH}_4)_2\text{SO}_4$  at 75% saturation. The precipitate was collected by centrifugation ( $11\,000g$ , 25 min,  $4^\circ\text{C}$ ) and suspended in a minimum volume of water. The resulting suspension was extensively dialyzed against ultrapure water. The insoluble fraction was discarded after centrifugation ( $12\,000g$ , 50 min,  $4^\circ\text{C}$ ). The resulting ovalbumin solution was lyophilized, and kept at  $4^\circ\text{C}$  until use. Ovalbumin concentration was measured by absorption at 280 nm, using a specific extinction coefficient  $E_{1\text{cm}}^{1\%} = 7.12$  (Glazer, Mckenzie & Wake, 1963). Purified ovalbumin was analyzed by anion-exchange HPLC on a Q HyperD column ( $0.46 \times 10 \text{ cm}^2$ ) (BioSepra, Villeneuve-la-Garenne, France). Protein samples (approximately 0.5 mg) were injected in the column equilibrated with Buffer A and eluted by linear gradients of NaCl concentration in Buffer A, from 0 to 0.18 M in 24 min and from 0.18 M to 0.5 M in 5 min, at a  $1 \text{ ml min}^{-1}$  flow-rate. Proteins were detected by absorption at 280 nm.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970), using a 12% acrylamide separation gel and a 4% stacking gel containing 0.1% SDS. SDS–protein samples were heated at  $95^\circ\text{C}$  for 4 min. Gels were silver-stained (Tunon & Johansson, 1984).

### 2.3. Ellipsometry and surface tension

The ellipsometric measurements were carried out with a conventional null ellipsometer using a He–Ne laser operating at 632.8 nm (Berge & Renault, 1993). The variation of the ellipsometric angle is a relevant probe for changes occurring at the interface. Ellipsometric angles ( $\delta$  and  $\Psi$ ) and surface pressure were recorded simultaneously. The surface pressure was measured with the Wilhelmy system. The Teflon sample cell has a volume of 8.4 ml. The protein was diluted in the buffer and poured into the cell directly after preparation. All the experiments were done at room temperature in the range  $20\text{--}21^\circ\text{C}$ .

### 2.4. Shear elastic constant

The rheometer set-up (Vénien-Bryan et al., 1998; Zakri, Renault & Berge, 1998) uses the action of a very light float applying a rotational strain to the surface through magnetic couple (with a pair of Helmholtz coils and a small magnet pin deposited in the float). Practically, at the center of a 48 mm

diameter Teflon trough, a 10 mm diameter paraffin-coated aluminum disc floats at the air–water interface, surrounded by the surface whose rigidity is measured. The subphase is 5 mm deep. The float carries a small magnet and is kept centered by a permanent field of  $B_0 = 6 \times 10^{-5} \text{ T}$ , parallel to the Earth's field, created by a little solenoid located just above the float. Sensitive angular detection of the float rotation is achieved using a mirror fixed on the magnet and reflecting a laser beam onto a differential photodiode. A sinusoidal torque excitation is applied to the float in the 0.01–100 Hz frequency range, by an oscillating field perpendicular to the solenoid one. The latter field acts as a restoring torque equivalent to a surface having a  $0.16 \text{ mN m}^{-1}$  rigidity. The device behaves like a simple harmonic oscillator. Amplitude and phase of the angular response are measured, and considered to reflect directly the rotational strain of the surface.

For each sample, ellipsometric and shear elastic constant measurements are performed with the same solution and started simultaneously. Initial time points of all figures correspond to the moment of first possible measurements, after the magnetic float becomes centered and stable (few minutes after the deposition). Typical error values given in Tables were estimated from systematic kinetic measurements of ellipsometric angle, surface pressure and shear elastic constant performed on ultrapure water

### 2.5. Buffers and sample solutions

For the study of the effect of pH value, the protein concentration was adjusted to  $1 \text{ g l}^{-1}$ . The working solution was prepared in specific buffers at 50 mM: sodium citrate for pH 3.5 (ionic strength  $I \approx 40 \text{ mM}$ ) and pH 4.6 ( $I \approx 90 \text{ mM}$ ), sodium phosphate for pH 6.0 ( $I \approx 60 \text{ mM}$ ), and bis-Tris propane–HCl for pH 8.0 ( $I \approx 50 \text{ mM}$ ). In order to evaluate the ionic strength effect, additional experiments were performed at  $\text{pH } 6.0 \pm 0.1$  using a  $100 \text{ mg l}^{-1}$  ovalbumin concentration in 20 mM sodium phosphate where NaCl was added at concentration 0–70 mM (total ionic strength 22–92 mM).

The effect of ovalbumin concentration was studied in the  $0.1\text{--}1 \text{ g l}^{-1}$  range at pH 4.6 (50 mM sodium citrate,  $I \approx 90 \text{ mM}$ ) or pH 6.0 (50 mM sodium phosphate,  $I \approx 60 \text{ mM}$ ).

## 3. Results

### 3.1. Characterization of purified ovalbumin

For interfacial behavior studies, we considered essential to use a fully characterized, maximally purified, ovalbumin preparation. Fig. 1 represents the chromatogram resulting from preparative anion-exchange separation of egg-white proteins. Pooled fractions, corresponding to the main peak, are indicated by the horizontal solid bar. This purification procedure yielded about 500 mg protein.

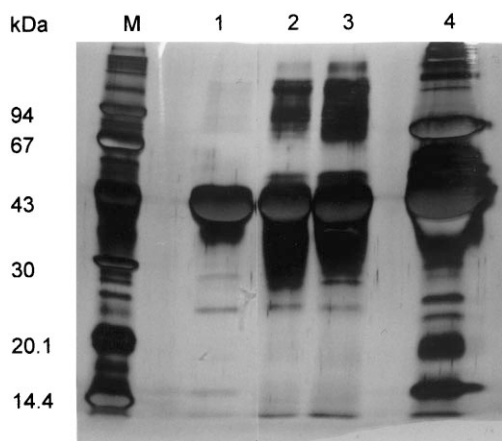


Fig. 2. SDS-PAGE analysis of purified and commercially available ovalbumin. Proteins were separated using a 12% acrylamide gel. The gel was silver-stained. The samples are: M, molecular weight markers; Lane 1, purified ovalbumin (this work); Lane 2, commercially available ovalbumin, lot 14H7035; Lane 3, commercially available ovalbumin, lot 76H7045; Lane 4, egg white.

Ovalbumin molecular mass is 43 kDa. As shown by SDS-PAGE analysis (Fig. 2, Lane 1), our ovalbumin preparation is highly purified. Two distinct lots of a commercial ovalbumin preparation which has been used frequently for the interfacial

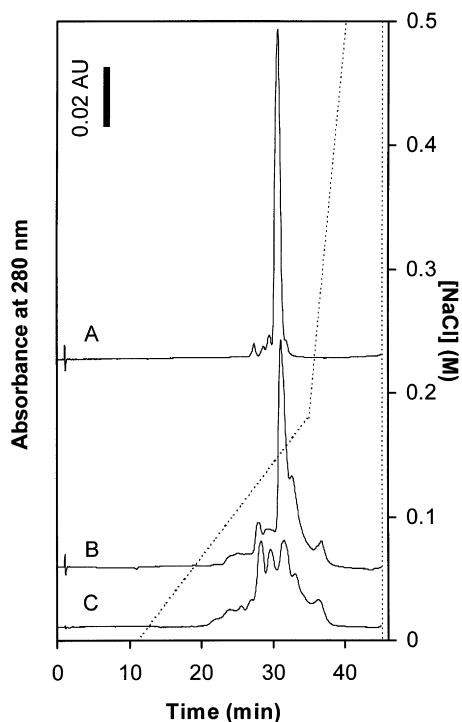


Fig. 3. Anion-exchange HPLC of purified and commercially available ovalbumin. Proteins (0.5 mg) were separated using a Q HyperD column ( $0.46 \times 10 \text{ cm}^2$ ), equilibrated with 50 mM Tris-HCl, pH 8.0, by linear gradients of NaCl concentration in the same buffer as indicated by the dotted line, at a flow-rate of  $1.0 \text{ ml min}^{-1}$ . Proteins were detected by absorption at 280 nm. (A) Purified ovalbumin (this work). (B) Commercially available ovalbumin, lot 14H7035. (C) Commercially available ovalbumin, lot 76H7045.

behavior studies exhibited a much higher degree of heterogeneity (Fig. 2, Lanes 2 and 3). Moreover, the protein composition of the commercial preparation did not appear to be identical from one lot to another.

In order to further characterize our ovalbumin preparation, and to compare its purity with commercial material, we analyzed it by anion-exchange HPLC. The results shown in Fig. 3 reveal the heterogeneity of commercial ovalbumin preparations also from the ionic point of view. In contrast, our preparation consists of one major form of ovalbumin, well defined from the ionic point of view. Enzymatic dephosphorylation followed by similar anion-exchange analysis shows that this major form is diphosphorylated A1 ovalbumin (data not shown).

In summary, our biochemical characterization reveals that the commercially available preparation may exhibit considerable heterogeneity in the charge properties, and that this heterogeneity does not reflect proportions of ovalbumin isoforms in egg white. Therefore, the studies of interfacial behavior reported in this paper were performed using a highly purified A1-ovalbumin preparation, with homogeneous, well-defined ionic characteristics.

### 3.2. Effect of protein net charge

The results obtained at various pH are summarized in Table 1. For all pH values, the ellipsometric angle at saturation had almost the same value ( $10.2 \pm 0.4^\circ$ ). Using the measured ellipsometric angles  $\Delta$  and  $\Psi$  and knowing the refractive index increment of the protein ( $0.180 \text{ ml g}^{-1}$  for ovalbumin) (Sober, 1968), the surface concentration  $\Gamma$  of adsorbed protein was calculated using the relationship between  $\Gamma$  and  $\Delta$  found in the data of de Feijter et al. (1978). Its value remained constant after 4 h. The value obtained is similar to that calculated by de Feijter and Benjamins (1987) on ovalbumin. Fig. 4 shows that the final value of surface pressure changed with pH, with a maximum value close to the isoelectric point of ovalbumin ( $pI_{\text{ova}} = 4.75$ ) (Kitabatake, Ishida & Doi, 1988). Kinetics of surface pressure increase and time for half-maximum magnitude were also pH dependent (Fig. 4, inset).

Fig. 5 shows the variation of the final value of the shear elastic constant  $\mu$  with pH. A maximum was also observed close to  $pI_{\text{ova}}$ . The rigidity decreased down to almost zero when pH increased above  $pI_{\text{ova}}$ . The results suggest that  $\mu$  reflects the degree of intermolecular association in the adsorbed layer (see Section 4). At pH 4.75, the protein is neutral and the hydrophobicity of the molecule probably drives the lateral organization. At alkaline pH, the protein net charge is negative and the electrostatic repulsion hinders contacts between ovalbumin molecules at the interface.

Previously, we had performed the same experiment with commercially available ovalbumin (not shown). We observed a similar  $\mu$  versus pH curve. The only difference was observed around the isoelectric point, where for pure A1-ovalbumin  $\mu$  was higher than for the commercially

Table 1

Effect of pH on adsorption parameters measured 8 h after the beginning of adsorption. Ovalbumin concentration was  $1.0 \text{ g l}^{-1}$  in the indicated buffer

pH	Buffer, 50 mM	$\Delta$ ( $^\circ$ ) ( $\pm 0.5$ )	$\Gamma$ ( $\text{mg m}^{-2}$ ) ( $\pm 0.1$ )	$\pi$ ( $\text{mN m}^{-1}$ ) ( $\pm 0.3$ )	$\mu$ ( $\text{mN m}^{-1}$ ) ( $\pm 1$ )
3.5	Sodium citrate	10.3	2.1	23.2	24
4.6	Sodium citrate	9.7	1.9	25.6	29
6.0	Sodium phosphate	10.0	2.0	24.0	8
8.0	Bis-Tris propane-HCl	10.7	2.1	22.2	3

available preparation. At pH 4.75, we obtained  $\mu$  values of  $30 \text{ mN m}^{-1}$  and  $22 \text{ mN m}^{-1}$ , respectively.

The studies of pH effects were performed at variable ionic strength  $I$ . In order to check the influence of ionic strength at a given pH, and to test the hypothesis that the protein net charge plays an essential role in determining intermolecular associations, the value of  $I$  was varied in the range 20–90 mM at pH 6.0. As a matter of fact, at this pH, we had observed previously intermediate final values of  $\Delta$ ,  $\pi$  and  $\mu$ , and electrostatic repulsion was assumed to have a notable influence.

No significant variation of  $\Delta$  and  $\pi$  values was observed for the different ionic strengths. At saturation, all values converged to  $\Delta = 6.0 \pm 0.4^\circ$  and  $\pi = 23.0 \pm 0.3 \text{ mN m}^{-1}$ .

Nevertheless, we observed differences between surface pressure measurements performed at the same ionic strength ( $I \approx 60 \text{ mM}$ ) in the original buffer (50 mM sodium phosphate) and in the buffer at a lower concentration (20 mM) with NaCl added. These differences could be explained mainly by the fact that ion–protein interactions, and probably the structural properties of proteins in the adsorbed layer, depend on the nature of the involved ions. Thus, the surface pressure  $\pi$  seems to be sensitive to the nature of ion and buffer species.

On the contrary, the final value of  $\mu$  increased with ionic strength up to 50 mM NaCl added ( $I \approx 70 \text{ mM}$ ), and then stabilized at  $6.0 \pm 0.5 \text{ mN m}^{-1}$  for higher  $I$  values. This asymptotic value is equal the one obtained in our previous experiments in 50 mM sodium phosphate buffer

( $I \approx 60 \text{ mM}$ ). In contrast with the surface pressure, the shear elastic constant measurement is only sensitive to protein–protein interactions and lateral organization in the adsorbed protein layer.

### 3.3. Influence of protein concentration

In a first set of experiments, the influence of protein concentration was studied near the isoelectric point of ovalbumin, where the strongest values of surface rigidity are obtained. A second set of experiments was performed at a pH value where the protein charge is negative. Fig. 6A and B show the ellipsometric angle  $\Delta$  versus time at different concentrations for pH 4.6 and pH 6.0, respectively.

The variations of  $\Delta$  with time show that the adsorption kinetics depend on the initial protein bulk concentration. The rate of adsorption increases with increasing protein concentration.

Generally, at pH 4.6 and for any concentration above  $40 \text{ mg l}^{-1}$ ,  $\Delta$  reached a saturation value of  $9.2 \pm 0.4^\circ$  after 4 h. At pH 6.0,  $\Delta$  reached a saturation value of  $9.9 \pm 0.4^\circ$  after 4 h, which is close to the one obtained at pH 4.6, indicating that the amount of protein adsorbed is roughly the same in both cases.

Fig. 6C and D show plots of surface pressure  $\pi$  as a function of the adsorption time for pH 4.6 and 6.0, respectively. Table 2 summarizes all results concerning values obtained after 6 h. Unlike  $\Delta$ , the final value of  $\pi$  is significantly higher at pH 4.6 than at pH 6.0 for any given concentration, the difference being even higher at low protein concentrations. These results suggest that for the same amount of protein adsorbed to the interface, lateral interactions between proteins are different, depending on pH.

The increase of rigidity started immediately after the beginning of the adsorption for concentrations higher than  $10 \text{ mg l}^{-1}$ . The  $\mu$  value quickly rose to a plateau value during the first hour. Fig. 7 represents  $\mu$  as a function of initial bulk protein concentration after an adsorption time of 6 h. The strong dependence of lateral interactions on the pH value, suggested by surface pressure measurements, is clearly confirmed by these measurements of surface rigidity.

It was not possible to perform experiments with ovalbumin concentrations higher than  $1 \text{ g l}^{-1}$  as the too high starting rigidity made the alignment of the set-up difficult. However, it seems that  $\mu$  values of  $8 \text{ mN m}^{-1}$  and  $29 \text{ mN m}^{-1}$  for pH 6.0 and 4.6, respectively, obtained at

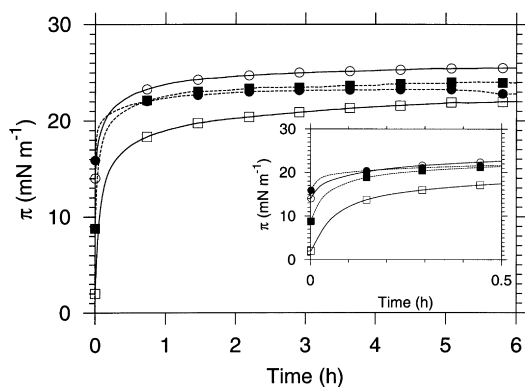


Fig. 4. Influence of pH on kinetics of surface pressure increase. Bulk valbumin concentration was  $1 \text{ g l}^{-1}$  in 50 mM sodium citrate, pH 3.5 (●); pH 4.6 (○); sodium phosphate, pH 6.0 (■); bis-Tris propane-HCl, pH 8.0 (□). Inset: same data, restricted to the time range 0–0.5 h.

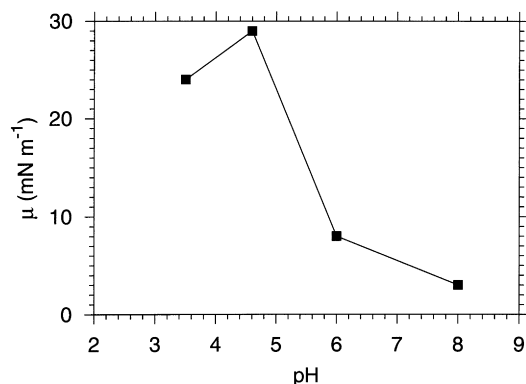


Fig. 5. Influence of pH on the shear elastic constant  $\mu$  at  $t = 8$  h. Bulk ovalbumin concentration was  $1 \text{ g l}^{-1}$ . Buffers: see the legend of Fig. 4.

$1 \text{ g l}^{-1}$ , were close to the maxima which would have been obtained with higher ovalbumin concentrations.

According to Eq. (1), the initial part of the  $\Gamma - t^{1/2}$  plot is straight:

$$\Gamma(t) = 2c_0(Dt/\Pi)^{1/2}, \quad (1)$$

where  $c_0$  is the initial bulk protein concentration and  $D$  the diffusion coefficient. This relation was only followed at the lowest protein concentration used ( $1 \text{ mg l}^{-1}$ ) and in the initial part of the plots. Under these conditions,  $D$  can be calculated from the slope of the initial linear region. For pH 4.6 and 6.0, we found  $D$  values between  $0.5 \times 10^{-10}$  and  $1.0 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ .

## 4. Discussion

### 4.1. Parameters of ovalbumin adsorption

In this work, the aim was to investigate the effects of the protein net charge on its interfacial properties. We performed experiments at different ovalbumin concentrations. Our results may be compared with those obtained a few years ago by ellipsometry and surface pressure measurements on commercial ovalbumin by Benjamins and Lucassen-Reynders (1998) and de Feijter and Benjamins (1987).

At least in the first 10 min and at low initial concentrations, ovalbumin adsorption, as measured by ellipsometry, was diffusion-driven. The apparent diffusion coefficients calculated for the lowest bulk concentrations ( $1 \text{ mg l}^{-1}$ ) are in the range  $0.5\text{--}1.0 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ , consistent with values of literature for ovalbumin (de Feijter & Benjamins, 1987; Sober, 1968), and with data of photon correlation spectroscopy (Pezennec & Michel, unpublished results).

The value of surface protein concentration at saturation (about  $2.0 \text{ mg m}^2$  for a  $1.0 \text{ g l}^{-1}$  bulk concentration) would indicate a molecular area of the order of  $35\text{--}40 \text{ nm}^2$ , a figure that is compatible with a close-packing of adsorbed molecules, considering molecular dimensions of ovalbumin ( $7 \times 4.5 \times 5 \text{ nm}^3$ ) (Stein et al., 1991). This value is similar to those found for ovalbumin by Benjamins and Lucassen-Reynders (1998) and de Feijter and Benjamins (1987). For bovine serum-albumin (de Feijter et al., 1978), a plateau value of  $2.9 \text{ mg m}^2$  and a layer thickness close to one

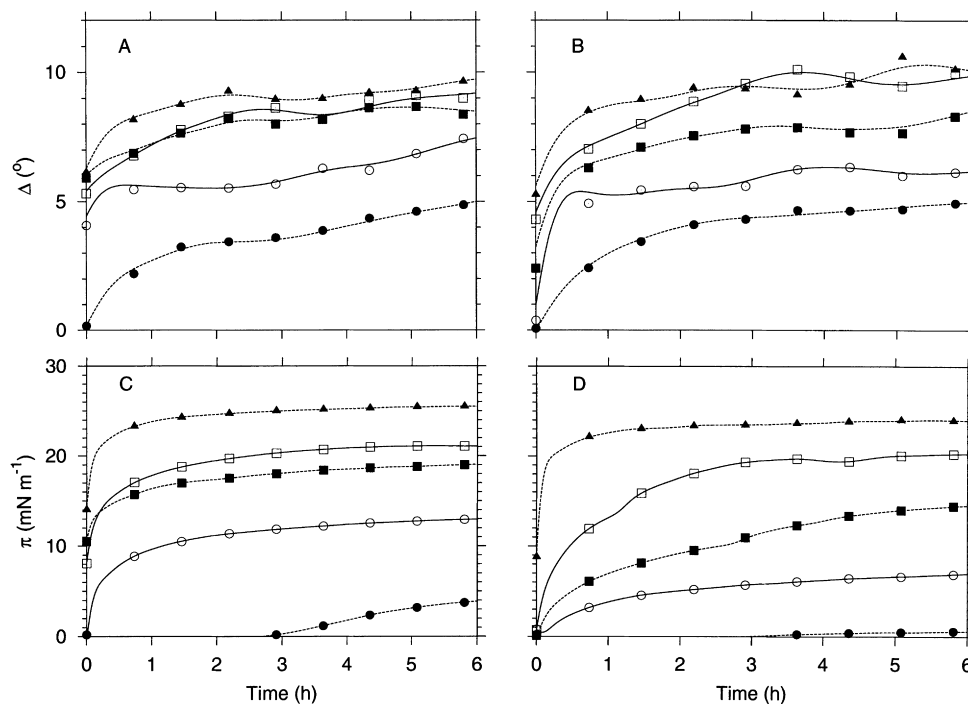


Fig. 6. Influence of ovalbumin concentration on the kinetics of adsorption and surface pressure increase, at pH 4.6 and pH 6.0. The results for five different ovalbumin concentrations (in  $\text{mg l}^{-1}$ ) are shown: 1 ( $\bullet$ ); 10 ( $\circ$ ); 40 ( $\blacksquare$ ); 100 ( $\square$ ); 1 ( $\blacktriangle$ ). A, B: Ellipsometric angle  $\Delta$ ; C, D: Surface pressure  $\pi$ ; A, C: 50 mM sodium citrate, pH 4.6; B, D: 50 mM sodium phosphate, pH 6.0.

Table 2

Influence of initial bulk ovalbumin concentration on adsorption parameters measured 8 h after the beginning of adsorption, in 50 mM sodium citrate, pH 4.6, or 50 mM sodium phosphate, pH 6.0.

$c_0$ (mg l <sup>-1</sup> )	$\Delta$ (°) ( $\pm 0.5$ )		$\Gamma$ (mg m <sup>-2</sup> ) ( $\pm 0.1$ )		$\pi$ (mN m <sup>-1</sup> ) ( $\pm 0.3$ )		$\mu$ (mN m <sup>-1</sup> ) ( $\pm 1$ )	
	pH 4.6	pH 6.0	pH 4.6	pH 6.0	pH 4.6	pH 6.0	pH 4.6	pH 6.0
1	5.0	5.0	1.0	1.0	3.9	0.6	1	0
10	7.4	6.2	1.5	1.2	13.0	7.0	2	0
40	8.6	8.5	1.7	1.7	19.0	14.6	7	1
100	9.2	9.8	1.8	2.0	21.1	20.2	14	6
1000	9.7	10	1.9	2.0	25.5	24.0	29	8

dimension of the globular molecule were interpreted as a close packing of side on-adsorbed molecules. In the case of lysozyme,  $\beta$ -lactoglobulin and  $\beta$ -casein, a surface concentration of 2–3 mg m<sup>2</sup> was reported to be the maximum monolayer coverage (Graham & Phillips, 1979b; Murray, 1987).

In our work, interfaces were not compressed. Ovalbumin adsorption gave final values of surface pressure in the range 20–30 mN m<sup>-1</sup>, as previously found for ovalbumin (de Feijter & Benjamins, 1987; Kato, Tsutsui, Matsudomi, Kobayashi & Nakai, 1981; Kitabatake & Doi, 1988) or other globular proteins (Graham & Phillips, 1979b).

Depending on concentrations, the interface exhibited progressively resistance to shear as measured by the shear elastic constant  $\mu$ . Recently, we demonstrated the interest of the association between ellipsometry, surface pressure and shear elastic constant measurements in two cases: the first one is the growth of 2D crystals of proteins (Vénien-Bryan et al., 1998) and the second one is the process by which a lipid monolayer at the air-buffer interface could serve as a template for the polymerization of monomeric actin into single filaments (Renault, Lenne, Zakir, Aradian, Vénien-Bryan & Amblard, 1999). In the present case, it is noteworthy that, in a range of bulk ovalbumin concentration where adsorption and surface pressure were already close to their plateau value, the variation of  $\mu$  reflected consider-

able changes of the interfacial rheology. The high final value of  $\mu$  has to be related to previous observations of rheological alterations of the interface upon protein adsorption (Benjamins et al., 1998; Blank et al., 1970; Graham & Phillips, 1980b). The viscoelastic surface properties are considered to depend on rearrangements of protein structure within the film (Dickinson et al., 1988).

Two steps of conformational reorganization upon adsorption of a globular protein may be distinguished roughly. The first step consists of individual, partial unfolding of adsorbed molecules, resulting in increased exposure of hydrophobic regions of protein molecules. This step can be hindered at high surface pressures, in such a way that the unfolding degree is lower in film adsorbed at high subphase concentrations, as in the case of lysozyme (Graham & Phillips, 1979c) or  $\beta$ -lactoglobulin (Wüstneck et al., 1996). Hence, in films exhibiting identical surface concentrations, the degree of denaturation of adsorbed proteins may be quite different depending on initial bulk concentration. In the second step, simultaneously with continuing unfolding, a two-dimensional (2D) network is then formed under the effect of protein–protein interactions (Graham & Phillips, 1980b), which are mainly hydrophobic interactions (Dickinson et al., 1996). This network confers viscoelastic properties to the adsorbed layer.

Polarization-modulated infrared reflection-absorption spectroscopy (PM-IRRAS) allows to characterize the conformation of organic molecules adsorbed at fluid interfaces and, in particular, the secondary structure of adsorbed peptides and proteins (Blaudez, Turllet, Dufourcq, Bard, Buffeteau & Desbat, 1996). Our preliminary results on A1-ovalbumin adsorption at the air–water interface obtained by PM-IRRAS (Renault et al., unpublished results) show that the interface aging involves slow modifications of the secondary structure of adsorbed ovalbumin. They suggest that  $\beta$ -sheets, forming slowly in the adsorbed layer, are involved in the 2D network, which confers cohesion to the interface.

#### 4.2. Charge and elasticity

In our conditions, the net charge of the protein, as determined by pH, had a small if any effect on the surface concentration at saturation. However, the increase of

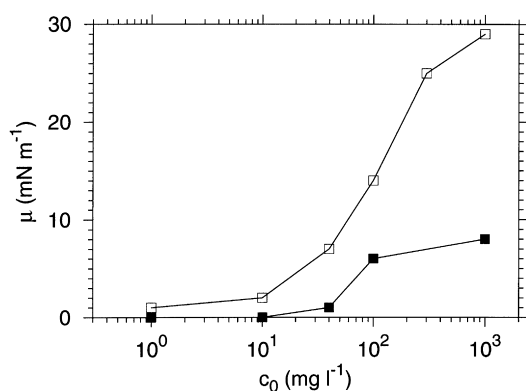


Fig. 7. Influence of protein concentration on the shear elastic constant  $\mu$  at pH 4.6 and 6.0. The final value of the shear elastic constant (measured after an adsorption time of 8 h) is represented as a function of the initial bulk ovalbumin concentration in 50 mM sodium citrate, pH 4.6 (□); sodium phosphate, pH 6.0 (■).

surface pressure appeared faster when the protein net charge was low (i.e. close to  $pI_{\text{ova}}$ ). The principal influence of pH could be observed through the final value of surface pressure and, to a much greater extent, through the long-term shear elastic constant  $\mu$ . At comparable level of surface concentrations, significantly higher  $\pi$  value and much higher  $\mu$  values were reached at pH 4.6 than at pH 6.0.

Therefore, the protein charge affects mainly the 2D rearrangements of adsorbed protein. It should be noticed that in this range of ionic strength and at pH values far from  $pI_{\text{ova}}$ , the electrostatic repulsion between ovalbumin molecules does not significantly limit the value of  $\Gamma$  at saturation, but it prevents intermolecular associations within the adsorbed film and limits the modification of the interfacial rheology, in such a way that the shear elasticity remains low. At pH values close to  $pI_{\text{ova}}$ , the low charge allows close and effective contacts between neighboring molecules for the formation of intermolecular bonds. This is in agreement with the earlier findings of other authors about the surface behavior of various proteins at the gas–liquid or liquid–liquid interfaces, which highlighted maximal viscoelastic parameters near the isoelectric pH (Biswas & Haydon, 1962; Graham & Phillips, 1980b; Izmailova, 1979; Kim & Kinsella, 1985). Tachibana, Inokuchi & Inokuchi (1957), studying the so-called surface gelation of ovalbumin, found no variation of the critical gelation area between pH 2 and pH 7, but found a maximum value in extremely acidic conditions, attributed to the modification of the molecular shape.

In the present work, experiments on pH effects were performed with an ionic strength in the range 40–90 mM. Influence of ionic strength at pH 6.0 was mainly observable through the interface rigidity. The increase of rigidity with the ionic strength up to a plateau confirms that electrostatic parameters control lateral organization at the interface. However, the increase in ionic strength seems unable to eliminate completely electrostatic effects, since it did not restore the values of  $\mu$  obtained at a pH closer to  $pI_{\text{ova}}$ .

The pH of the subphase also strongly modulated the ovalbumin concentration effects on the interface rheology. At pH 4.6, a two-fold increase of  $\mu$  was observed between  $0.1 \text{ g l}^{-1}$  and  $1.0 \text{ g l}^{-1}$ . At pH 6.0, only a slight increase of  $\mu$  value was observed in the same range of ovalbumin concentration: thus, in these conditions, the increase of  $\mu$  was limited by the protein net charge. At both pH values,  $\Gamma$  reached its asymptotic value at about a  $0.1 \text{ g l}^{-1}$  subphase concentration. The effect of bulk concentration between  $0.1$  and  $1.0 \text{ g l}^{-1}$  at pH 4.6, i.e. the large increase in  $\mu$  at almost constant surface concentration can be explained by the augmentation of the protein–protein interaction density within the film. The shear elastic constant is not a unique function of protein adsorption. This suggests that in conditions of low electrostatic repulsion, the degree of unfolding, which is controlled by the surface pressure at early stages of adsorption, and hence by the bulk concentration, is essential in the development of viscoelastic properties. This is in

agreement with the statement of Graham and Phillips (1979c), that in addition to the contribution of intermolecular associations, the intramolecular cohesion, i.e. the residual native structure of adsorbed globular proteins plays a significant role in the rheological properties of interfacial films.

Ovalbumin carries four free sulfhydryl groups and one disulfide bond. Molecular rearrangements at the interface include sulfhydryl–disulfide exchange, which may contribute to the 2D network formation and to viscoelastic properties of the film, though it does not appear to contribute significantly to foam stability (Doi, Kitabatake, Hatta & Koseki, 1989). Sulfhydryl–disulfide exchange rate is influenced probably also by pH, the  $pK_a$  value of cysteine sulfhydryl groups being close to 9. However, in the pH range where this rate should increase, electrostatic repulsion is high, and the low elasticity exhibited by the film even at high bulk concentrations suggests that covalent protein–protein interactions remain limited.

As shown by earlier studies, surface properties can be correlated with protein hydrophobicity (Kato et al., 1981) and structural stability (Semenova & Gauthier-Jaques, 1997). Adsorption kinetics and foaming power also have been shown to be correlated (Kitabatake & Doi, 1988). In the latter work, the stability of foams was not studied, but in appropriate conditions, increased shear elasticity would probably modify foam stability. As a matter of fact, recently, the dynamic surface properties of whole egg-white proteins have been correlated clearly with their foaming behavior (Hammershøj et al., 1999). At pH 4.8, where the viscoelasticity of the interfacial film was maximum, foamability and foam stability had their highest value, and the bubble size had its smallest one. Our results are in very good agreement with these findings. Thus, it seems that our results about the influence of the protein charge on the interfacial shear elastic constant can be correlated with functional parameters such as foam stability.

## 5. Conclusions

Performing ellipsometric, surface pressure and shear elastic constant measurements allowed us to monitor separately the time-courses of A1-ovalbumin adsorption and surface rheological changes which presumably reflect molecular rearrangement processes within the interfacial film. The surface concentration  $\Gamma$  at saturation is a function of protein concentration in the subphase, and is not significantly influenced by the protein net charge (in our range of ionic strength).

The final value of the shear elastic constant  $\mu$  is strongly influenced by pH. The electrostatic repulsion controls the lateral organization of adsorbed ovalbumin molecules. This lateral organization must be correlated with molecular conformation changes and with intermolecular associations within the film. This raises questions about the nature of



these conformational changes, and of the intermolecular forces, which control the interfacial cohesion.

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