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Review

Small molecular ion adsorption on proteins and DNAs revealed by separation techniques

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Abstract

Ion binding is a term that assumes that the ion is included in the solvation sphere characterising the biomolecule. The binding forces are not clearly stated except for electrostatic attraction; weak forces (hydrogen bonds and Van der Waals forces) are likely involved. Many publications have dealt with ion binding to proteins and the consequences over the past 10 years, but only a few studies were performed using high-performance liquid chromatography (HPLC: ion exchange, reversed phase without the well-identified immobilised metal affinity chromatography) and capillary zone electrophoresis (CZE). This review focuses on the binding of proteins and DNAs mainly to the oxyanions (phosphate, borate, citrate) and amines used as buffers for both the HPLC eluent and the background electrolyte of CZE. Such specific ion adsorption on biomolecules is evidenced by physico-chemical characteristics such as the mobility or retention volume, closely associated with the net charge, which differ from the expected or experimental data obtained under the conditions of an indifferent electrolyte. It is shown that ion binding to proteins is a key parameter in the electrostatic repulsion between the free protein and a fouled membrane in the ultrafiltration separation of a protein mixture.

Keywords: Reviews; Small molecular ion adsorption; Proteins; DNA

Contents

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1. Introduction

“Ion binding” is a general term assuming that an ion present in the solvation sphere of a biomolecule is included in the physico-chemical characterisation of the biomolecule.

The chemical nature of the bond between a small ion and a biological target molecule is not well identified. One can consider that electrostatic interactions are the driving forces and that other weak forces, such as hydrogen bonds or polar and apolar Van der Waals forces, may also be involved. The chemical nature of the bond between the small ion and the biological molecule will be different according to the chemical nature of both species. The behaviour of inorganic and organic ions of similar charge towards a charged molecule is not exactly the same. Fraaije and Lyklema [1] showed that the electrolyte binds largely in the diffusive layer, especially when the protein surface charge is high. Ninham and co-workers [2] take into account the ionic dispersion effects between charged interfaces within the double layer force. They assume that ion binding to micelles is due to an excess of physisorption without involving any actual bond. They report that this is probably the case for Ca^{2+} binding to proteins.

Many high-performance liquid chromatography (HPLC) studies are focused on the immobilised metal cation in order to perform affinity chromatography, initially proposed by Porath [3]. Basically, complexation occurs between the metal cation as Cu^{2+} and the histidine group of the protein. This type of HPLC, called IMAC, is out of the scope of this review, and the reader is referred to the literature for details (see, for instance, Refs. [4–10]).

In the literature the final combination between the charged biological molecule and the small ion is often called a “complex”. As a donor–acceptor Lewis bond with a metal cation is not involved in such a combination, we avoid using such a term in the following. We prefer to refer to “ion binding”. The term “specific adsorbed ion” will be used when forces other than electrostatic interactions due

to the counter-ions of the charged biological molecule are evidenced or suspected. Moreover, two different kinds of electrolyte have to be considered according to the classification used in colloid science [11]:

(i) Indifferent electrolyte ions, which interact with the protein through attractive electrostatic interactions. Counter-ions (opposite charge) surround the protein and, accordingly, reduce its effective net charge. In other words, the electrophoretic mobility decreases with increasing ionic strength (diffuse double electric layer compression). This property is commonly used in ion-exchange chromatography to cancel interactions between a protein and the stationary phase in order to elute the protein.

(ii) Specifically adsorbed electrolyte ions that interact with proteins through electrostatic and non-electrostatic interactions. Consequently, when the ionic strength increases, the protein charge is screened and, in some cases, charge reversal of the protein occurs. The isoelectric point is then quite different from the theoretical value (p*I*) based on the amino acid content.

Over the past 10 years many publications have dealt with ion binding to proteins and the consequences [12–68]. In general, binding is evidenced by techniques such as X-ray diffraction of crystallised proteins, equilibrium dialysis, potentiometry with specific electrodes, NMR, FTIR or circular dichroism studies. Only a few studies have been concerned with separation techniques such as HPLC [69–85] or capillary zone electrophoresis (CZE) [69,70,72,86–91,131].

In this review we focus on two major separation techniques: CZE and HPLC. Some results of ultrafiltration (UF) are also reported. While the first two techniques are mostly performed on an analytical scale, the latter is usually performed on a preparative or industrial scale. Our aim is to show how ion binding provides in-depth information on biological molecules and contributes to our knowledge on the biological activity of DNA, for example. Ion binding is also a key physico-chemical parameter for the ultrafiltration separation of a mixture of several proteins.

After a brief recall of the theory of separation techniques, experimental methodologies for ion binding to charged biological molecules are outlined. A few results are from HPLC, particularly ion exchange and reversed phase. The main results on ion binding to proteins and DNAs are from electrophoretic methods such as CZE and zetametry. Zetametry is a reliable technique for the determination of the electrophoretic mobility. This technique allows the measurement of the electrophoretic mobility of a solute or a particle between two platinum electrodes in an alternating electric field. It is a non-separative technique [137]. Finally, some results on the effect of “ion binding” on protein transport through an ultrafiltration membrane (a separation technique with a single theoretical plate) will be given, where both size exclusion and electrostatic exclusion are involved.

2. Theory

2.1. Liquid chromatography

2.1.1. Size-exclusion chromatography (SEC)–HPLC

In SEC the solute is eluted according to its size, i.e. its hydrodynamic volume. The capacity factor K_D or partition coefficient (dimensionless) is defined as

$$K_D = (V_e - V_0)/(V_t - V_0) = (1 - \lambda)^2 \quad (1)$$

where V_t is the total volume or mobile phase volume of the column, V_0 is the void volume of the column, V_e is the elution volume of the solute, and λ is the ratio of the solute radius (R) to the pore radius (r_p). Eq. (1) gives the fraction of the porous volume accessible to the solute. K_D ranges from 0 (large solute, no permeation within the pores) to 1 (small solute, total permeation within the pores).

2.1.2. Reversed-phase (RP)-HPLC

In RP-HPLC, the protein is eluted according to its hydrophobicity. The capacity factor k' (dimensionless), defined according to Eq. (2), allows an easy comparison between components of different hydrophobicity under isocratic elution conditions: the smaller k', the less hydrophobic the protein:

$$k' = (V_e - V_m)/V_m \quad (2)$$

where V_e is the elution volume of the protein and V_m is the volume of the mobile phase (no interaction between the low-molecular-mass solute and the stationary phase).

2.1.3. Ion-exchange chromatography (IEC)–HPLC

Electrostatic interactions are mainly involved in the exclusion or retention of charged solutes. IEC is commonly used for the separation of solutes (proteins) of opposite charge to that of the exchanger group. Regnier and co-workers [80,85] have proposed a stoichiometric displacement model (SDM), allowing the number of contacts between the solute and the chromatographic ion-exchange groups of the support to be determined:

$$\mathrm{Ln}\, k' = \mathrm{Ln}\, K_n + (Z_c/n') \log C \quad (3)$$

where k' is the retention factor, K_n a constant, Z_c is the number of contacts between the protein and the stationary phase, n' is the valency of the displacement ion, and C is the concentration of the displacement ion (co-ion of the protein). Displacement of the adsorbed protein occurs more efficiently using a multivalent co-ion of the protein to be desorbed [81].

A non-stoichiometric model based on attractive electrostatic interactions and the double electric layer was proposed by Stahlberg et al. [79]. They related k' to the reciprocal of the square root of the ionic strength (I):

$$\mathrm{Ln}\, k' = [-A_p \sigma_2^2 / F(2RT\varepsilon_0\varepsilon_r)^{0.5}] I^{-0.5} + \mathrm{Ln}\, \phi \quad (4)$$

where A_p is the area of the protein surface that interacts with the stationary phase, σ_p is the charge density, ε_r is the dielectric constant, ε_0 is the permitivity in vacuum, I is the ionic strength and ϕ is the column phase ratio (ratio of the area of the stationary phase to the volume of mobile phase).

The net charge of the protein can be determined from the slope of the $\mathrm{Ln}\,k'$ vs. $I^{-0.5}$ plot with the assumption (as for Regnier's SDM model) that only half the protein surface area is involved in the ion-exchange mechanism. Consequently, the net charge of the protein is twice the number of protein contacts with the ion-exchange groups of the stationary phase. Such values are mostly in good agreement with those obtained from protein titration.

Guillaume et al. [71] recently proposed the use of immobilised cation affinity chromatography, initially focused on metal cations, to study the binding of calcium and magnesium on an immobilised protein used as stationary phase. The theoretical approach of the retention mechanism is based on electrostatic interactions between the cation and the protein as in the Gouy–Chapman theory. The retention factor (k') is related to $I^{-0.5}$ according to a relationship similar to that used by Stahlberg et al. [79] in IEC–HPLC:

$$\mathrm{Ln}\,k' = [-2\sigma/(8RT\varepsilon_0\varepsilon)^{0.5}]I^{-0.5} + \mathrm{Ln}\,\chi \tag{5}$$

where σ is the charge density, ε is the dielectric constant, ε_0 is the permitivity in vacuum, I is the ionic strength and χ is a constant equivalent to the effective concentration of the protein. An estimation of the charge density of the protein, taking into account divalent cation binding, can be obtained from the slope of the $\mathrm{Ln}\,k'$ vs. $I^{-0.5}$ plot.

2.2. Capillary zone electrophoresis

2.2.1. Electrophoretic mobility (μ)

The electrophoretic mobility (μ) is roughly the charge-to-size ratio. Electrophoretic mobility occurs when a charged species is placed in an electric field E. According to the Debye–Hückel theory, in a conducting medium (electrolyte), charged solutes are surrounded by ions of the electrolyte. The thickness of the solvation sphere (or double electric layer) is roughly the Debye length κ^{-1}, which depends on the ionic strength (I) of the medium [11,92,93]. The effect of the ionic strength was taken into account by including a correction term $X(\kappa R)$, and μ can be expressed by Henry's equation (in SI units) [93,94]:

$$\mu = (2\varepsilon\zeta/3\eta)X(\kappa R) \tag{6}$$

where ζ is the zeta potential of the particle (V), depending on ionic strength, η is the viscosity of the medium (Pa s), ε is the dielectric constant (C/Vm), κ is the reciprocal Debye length (m^{-1}), R is the solute radius (m) and $X(\kappa R)$ is Henry's correction term. For theoretical calculations [$\log(\kappa R) < 1$], the following expression can be used for $X(\kappa R)$:

$$X(\kappa R) = 1 + \frac{1}{16}(\kappa R)^2 - \frac{5}{48}(\kappa R)^3 - \frac{1}{96}(\kappa R)^4 + \frac{1}{96}(\kappa R)^5 - \left[\frac{1}{8}(\kappa R)^4 - \frac{1}{96}(\kappa R)^6\right]\exp(\kappa R)\int_{\infty}^{\kappa R} \mathrm{e}^{-t}\,\frac{\mathrm{d}t}{t} \tag{7}$$

Mathematically, Henry's equation varies from 1.0, when $\kappa R < 0.1$, to 1.5, when $\kappa R > 100 - 300$. The term $\int_{\infty}^{\kappa R} \mathrm{e}^{-t}\,\mathrm{d}t/t$ can be calculated by means of the Romberg method [95]. For κR in the range 0.1–300, the zeta potential can be expressed as a function of the protein size with the assumption of a spherical shape. Thus, Eq. (6) can be expressed as (in SI units)

$$\mu = (Ze/6\pi\eta R)\cdot[X(\kappa R)/(1+\kappa R)] \tag{8}$$

where Z is the net charge number of the solute (C). The term $[X(\kappa R)/(1+\kappa R)]$ varies from 1 to 0 when I increases from 0 to infinity. When the ionic strength approaches zero (infinite dilution), κ^{-1} is large with a small charge density and the surface charge of the solute is at its maximum value. At high ionic strength, κ^{-1} is small, but, with a large charge density which more efficiently balances the solute surface charge, the net charge of the solute remains minimal.

In an insulating medium ($I = 0$), Eq. (8) reduces to the well-known Stokes' relationship:

$$\mu = (Ze/6\pi\eta R) \tag{9}$$

From Eq. (6) and for $X(\kappa R) = 1$ at 25 °C in water the electrophoretic mobility of a solute (μ) is proportional to its zeta potential (ζ):

$$\zeta(\mathrm{mV}) = 12.85\mu(10^{-8}\mathrm{m^2V^{-1}s^{-1}}) \tag{10}$$

2.2.2. Theoretical calculation of mobility: choice of parameters

To calculate the theoretical mobility using Eq. (8), the net charge and the radius of the protein have to be determined.

2.2.2.1. Solute radius. The actual size of the protein in solution is often discussed in the literature. The use of the gyration radius, from light scattering, as for a polymer has sometimes been shown to be satisfactory for describing the diffusive behaviour of proteins in solution [96]. Most workers have used the Stokes' radius for globular proteins (Ref. [97] and references cited therein). The use of the Stokes' radius for a globular protein (lysozyme) was shown to be the appropriate choice in CE [72].

2.2.2.2. Net charge. The theoretical net charge number (Z) of a protein can be calculated from the amino acid composition of the protein and the pK_a values of the side-chain amino acids (the pK_a values used were those of free amino acids [72]).

The net charge of a protein can be determined by titration [92]. Whitesides and co-workers described an original

method for the determination of the effective charge number (Z) of an unknown protein based on the "charge ladder concept" [98–100]. A protein charge ladder is a family of derivatives of the protein obtained after chemical modification of the charge of the protein by means of chemical bonding (typically by acetylation). The observed Z values match well with the net charge calculated from the amino acid sequence for a set of 12 proteins.

Finally, when comparing theoretical calculations from amino acid sequences and experimental results from charge ladders, despite the possible denaturation of the protein at pH 12 during chemical modification, it is quite satisfactory to use the primary sequence of the protein (when known). Many reports have used the charge ladder concept, as it can be used for proteins with an unknown primary sequence [101–104].

2.2.3. Empirical correlation between mobility and molecular mass

The electrophoretic mobility μ is often expressed as an empirical function of the ratio Ze/M^n [Eq. (11)] via a relationship between the friction coefficient (f) and the molecular mass [Eq. (12)]:

$$\mu \propto Ze/M^n \tag{11}$$

$$f \propto 1/M^n \tag{12}$$

where Ze is the net charge, Z is the net charge number, M is the molecular mass, and f is the friction coefficient.

The pioneering work on peptides ($n = 2/3$) was carried out by Offord with 2 to 50 amino acid residues at quite acidic pH values, when polypeptide coils are in a fully extended conformation [105], probably due to their positive net charge. The reported values of n were mostly 1/3, 1/2 and 2/3 for proteins and peptides [97,106,107] and 0.8–1.0 for DNA fragments [91]. A relationship between molecular mass and gyration radius was proposed from viscosity measurements [96,108,109]:

$$V \propto [\eta]M \propto \phi(R_G^2)^{3/2} \propto M^{1+a} \tag{13}$$

where V is the hydrodynamic volume of the protein, η is the intrinsic viscosity, R_G is the gyration radius, M is the molecular mass, and a is the coefficient of the Mark–Houwink viscosity relationship [92]. Coefficient a accounts for the thermodynamic quality of the solvent, i.e. of the protein shape: $a = 0.5$ for a globular protein, i.e. in a theta solvent (bad solvent, close to precipitation), compact hard sphere, according to Flory's theory [92]; $a = 0.8$ in a good solvent, expanded Gaussian coil; and $a = 1$ for a rod (helical protein or DNA), fully extended coil, polyelectrolyte.

Substituting R by R_G in Eq. (9) leads to

$$\mu \propto ZeV^{-1/3} \propto ZeM^{-(1+a)/3} \tag{14}$$

The conformation of the solute is probably the bottleneck for a comprehensive relationship with molecular mass, and, in fact, with molecular size as in SEC–HPLC.

2.2.4. Empirical correlation between mobility and ionic strength

As Eq. (8) is quite difficult to handle and the radius and Z are not always known, a simplified empirical relationship has been proposed to take into account the decreasing effect of the ionic strength on mobility. For multivalent organic anions, μ can be expressed as

$$\mu = \mu_0 \exp(-AZ^n I^{0.5}) \tag{15}$$

where μ_0 is the mobility in an insulating medium (higher value) and Z the valency of the organic ion.

(i) $A = 0.5$ and $n = 1.8$ for Z in the range 1–3 for ionic strength (I) <10 mmol L^{-1} [110,111];

or (ii) $A = 0.77$ and $n = 0.5$ for Z in the range 2–6 for ionic strength in the range 1–100 mmol L^{-1} [112].

Li et al. [113] and Pitts et al. [114] considering a term with μ_0 for multivalent organic ions proposed the expression

$$\mu \approx \mu_0 - [IZIKI^{0.5}/(1 + BRI^{0.5})] \tag{16}$$

where Z is the net ion charge, R is the ion radius, B is a solvent-dependent parameter and K a constant.

2.2.5. Ion binding to proteins

Solute mobility depends on the nature of the electrolyte ions and two kinds of electrolytes can be distinguished [11,93]:

(i) indifferent electrolytes, for which only electrostatic interactions occur between the protein and the ion (electrical double layer compression);

(ii) specifically adsorbed ions, for which electrostatic and non-electrostatic interactions between the protein and the electrolyte ions occur. For specifically adsorbed ions, it is possible to reverse the net charge of the protein according to the electrolyte concentration at a fixed pH value.

A decrease of the mobility of indifferent ions can be predicted from Eq. (8) due to the ionic strength screening effect, whereas the behaviour of specifically adsorbed electrolyte ions cannot [72]. Other calculations based on the ion condensation of Manning's theory [115] have been used for DNA [87].

2.3. Ultrafiltration

A parallel between the retention mechanism in HPLC and ultrafiltration was recently reported [69]. Molecular sieving, or size exclusion, is the sole mechanism for the ultrafiltration (UF) of neutral solutes, as in SEC. When the solute is charged, one has to consider additional repulsive electrostatic interactions between the membrane and the solute. The retention mechanism in UF involves size exclusion, electrostatic repulsion and hydrophobic interactions with supports (membranes) bearing both charged and hydrophobic moieties. Recently, the CDE model, taking into account convection, diffusion and electrophoretic migration, has been proposed for the UF of charged proteins [116].

The performance of UF is expressed as permeate flux, J (m s^{-1}), and solute retention (*Ret*) or transmission (*Tr*) by the membrane:

$$Tr = 1 - Ret = C_p/C_r \quad (17)$$

where C_p is the concentration of the solute in the permeate (passing through the membrane), and C_r is the concentration of the solute in the retentate (remaining over the membrane).

3. Methodology

3.1. Ion "binding" to proteins in HPLC

3.1.1. IEC–HPLC

The HPLC determination of molecular interactions is generally carried out by the addition of the interacting molecule, at various concentrations, to the mobile phase.

3.1.2. Reversed-phase HPLC

Chaufer and co-workers [73] have proposed another procedure to highlight the interactions of ions with globular proteins: the molecule of interest was dissolved in solutions containing various concentrations of the interacting molecule at a fixed pH, and eluted in a given eluent, different from the solvent used for sample dilution. They showed that the pH, ionic strength and the chemical nature of the electrolyte in the sample solution have an effect on k' when the ions are specifically adsorbed on the target biomolecule, even if the eluent has a pH of ~2.

3.2. Ion binding to proteins and DNAs in CZE

3.2.1. Chemical nature of the capillary and the background electrolyte (BGE)

Generally speaking, do interactions between proteins and the capillary surface modify the measured electrophoretic mobility? First, the adsorption of proteins in the case of a silica capillary is so strong that, in some cases, no detection of proteins has been reported [117–119]. For analytical purposes, in order to avoid this adsorption phenomenon, additives are added to the BGE, such as rare-earth ions [120] and polyethyleneglycol (PEG) [121], or the silica capillary is modified [118,120,122,123].

Nashabeh and El Rassi [124] reported that the electrophoretic mobility of lysozyme and ribonuclease A was independent of the nature of the coating with various kinds of PEG-modified capillaries. Rabiller-Baudry and co-workers compared uncoated silica capillaries and different coatings with cross-linked polyethyleneimine (PEI) adsorbed on silica [72], or organo titanate bearing either amino groups or pyrophosphate groups grafted onto the silica (unpublished results). Filled with a similar BGE, α-lactalbumin and β-lactoglobulin (A + B) mobilities were the same, within an accuracy of 15%, regardless of the capillary. Therefore, mobility determination proved independent of the chemical nature of the capillary surface, contrary to quantitative determinations, where strong adsorption of the protein onto the capillary wall leads to poor area reproducibility.

3.2.2. Experimental mobility/apparent mobility

When the electroosmotic flow (EOF) is zero, the apparent mobility measured is the electrophoretic mobility of the solute [as expected according to Eq. (8)]. This is true when silica capillaries are coated with an uncharged polymer and when charged protein adsorption on the capillary wall is avoided. Plotting the EOF versus the run number can readily demonstrate the latter.

For a charged capillary wall (silica, charged coating or charged protein adsorption on the wall) the EOF has to be measured for each run and the experimental solute mobility (μ^{exp}) can be deduced from the apparent mobility (μ^{app}) according to Eqs. (18) and (19):

$$\mu^{app} = L_t L_d / t_m V \quad (18)$$

where L_t is the total length of the capillary, L_d is the distance of the capillary from the detector, t_m is the migration time of the solute, and V is the potential applied for the separation

$$\rightharpoonup \mu^{app} = \rightharpoonup \mu^{exp} + \rightharpoonup \mu^{EOF} \quad (19)$$

where μ^{EOF} is the experimental mobility corresponding to the EOF and is measured as the apparent mobility of a neutral marker co-injected with the protein of interest [be careful of the sign when using Eq. (19) as control of the sign is of prime importance].

3.2.3. Evidence of ion binding to proteins

Ion binding is studied by following the measured electrophoretic mobility versus the ionic strength of the BGE. Variation of the BGE is only due to the concentration of the ion of interest with an indifferent monovalent counter-ion. The ion of interest can be either a co-ion or a counter-ion of the protein.

3.2.3.1. Indifferent ion. The ion of interest acts only through the ionic strength effect, i.e. the electrophoretic mobility decreases with increasing I according to Eq. (8). When the experimental value matches the theoretical value calculated according to Eq. (8), it is concluded that the BGE acts as an indifferent electrolyte [72].

3.2.3.2. Specific adsorbed ion. The electrophoretic mobility of a protein is plotted against the ionic strength of a specifically adsorbed electrolyte. The effective net charge number (Z^{eff}) of the protein is then obtained by substituting the experimental mobility (μ^{exp}) for μ in Eq. (8) to take into consideration effects additional to the ionic strength [70]. The theoretical net charge number (Z) is compared with the

experimental effective charge (Z^{eff}) in order to obtain the number of specific adsorbed ions. Two types of behaviour can be observed [70,72]:

(i) an abnormal (low) electrophoretic mobility is measured compared with the calculated value (or titrated value);

(ii) a constant electrophoretic mobility is measured versus increasing ionic strength, which is unexpected on the basis of the double layer compression.

When only an abnormal (low) mobility is measured, no conclusion can be drawn when the primary sequence of the protein is unknown. On the contrary, a constant mobility over a wide range of ionic strength indicates a variable ratio of adsorbed ion to protein and evidences ion-specific binding to the protein.

From a practical point of view, measurements can be performed either with a single protein or a mixture of proteins. Such measurements are time consuming as the data from many runs cannot be used due to the fact that no buffer is present in the BGE to stabilise the system, particularly at an ionic strength of <10 mmol L^{-1} (heating, electrical discharge, etc.).

3.2.4. Ion binding to DNAs

The experimental mobility of the studied DNA fragment was measured using a coated capillary without any EOF. The mobility of the DNA of interest is compared to that of a reference sample [Eq. (20)]. The reference component was either (i) a large DNA fragment, as it was previously reported that, over 400 bp, all DNAs exhibit the same mobility ($\mu = 3.75 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} at 25 °C) [88]; or (ii) the DNA of interest in another non-interacting buffer:

$$\Delta\mu = \mu - \mu^{ref} \quad (20)$$

where μ is the mobility of the DNA of interest and μ^{ref} is the mobility of the reference sample. $\Delta\mu$ is plotted against the variation of the physico-chemical environment of the BGE (ionic strength, concentration of additive ions, etc.) [86–91].

At constant ionic strength, the variation of $\Delta\mu$ with the concentration of the ion of interest indicates that binding of the ion occurs to the studied DNA. Moreover, "strong" ion binding leads to a distorted peak (fronting or tailing or stronger distortion) and bimodal distributions can be observed. Using the empirical correlation between μ and M^{-n} [Eq. (11)] allows the estimation of the number of ions bound to the DNA.

Measurements were mainly performed in a complex BGE containing buffered species. The pH of most BGEs is between 8.0 and 8.3 (DNA negatively charged) and is based on:

(i) Tris–acetate (TA) or Tris–acetate–EDTA (TAE);

(ii) Tris–borate (TB) or Tris–borate–EDTA (TBE); or

(iii) amine-based buffers such as Tris, histidine (His), and Good's buffer.

The ion of interest can be either one in the BGE or an additive ion such as an alkaline ion.

4. Results

4.1. Liquid chromatography

HPLC is the most widely used analytical technique for proteins and biopolymers, either for identification or quantification. Accordingly, no attention is paid to the control of the biological quality of the protein and denaturing elution conditions are often used such as urea–guanidine in SEC–HPLC or water–organic solvent (methanol, acetonitrile) in RP-HPLC. IEC–HPLC runs are performed under non-denaturing conditions and use increasing salt gradients.

4.1.1. Binding of oxyanions on proteins, by RP-HPLC

An original RP-HPLC method was proposed by Chaufer and co-workers [73] where the ion of interest is present in the sample and not in the eluent.

4.1.1.1. Phosphate and citrate on lysozyme. Lysozyme is a small globular protein (MM=14 300 g/mol, pI = 10.7, R_s = 1.83 nm) positively charged at pH <10.7 and designated a "hard" protein according to Arai and Norde [125]. RP-HPLC of lysozyme dissolved in various environments was performed on a polystyrene stationary phase with isocratic elution by water–acetonitrile eluent at pH 2 (TFA). When lysozyme was dissolved either in water or in potassium chloride solutions of increasing ionic strength from 1 to 775 mmol L^{-1}, the chromatogram remained unchanged and the k' values were constant and close to 5. When lysozyme was dissolved in potassium phosphate solutions (pH 9 and 7) of increasing ionic strength, the peak of the native protein disappeared and was replaced by a less hydrophobic peak, leading to k' values that decreased with increasing ionic strength (Fig. 1). The specific adsorption of phosphate on lysozyme was indirectly checked by RP-HPLC, as the hydrophobic character of lysozyme binding to phosphate decreases at pH 9.0 [73] and pH 7.0 [70,73].

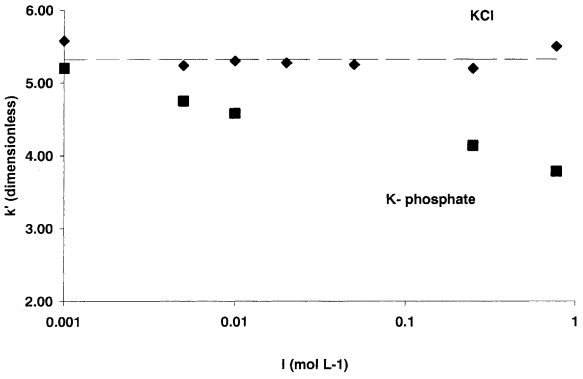

Fig. 1. RP-HPLC—k' of lysozyme versus ionic strength of KCl (pH 7.0, ◆) and potassium phosphate (pH 7.0, ■) prior to injection into the RP-HPLC system (PLRP-S, Polymer Laboratories). Isocratic elution (32.8% acetonitrile, pH 2) (reprinted with permission from the author and publisher) [70].

Similar results were observed at pH 7.0 when lysozyme was dissolved in sodium citrate solution in the ionic strength range from 0 to 775 mmol L^{-1} [126].

4.1.1.2. Phosphate on whey proteins. Similar experiments were performed with α-lactalbumin, a small globular protein (MM=14 400 g/mol, pI = 4.2–4.6, R_s = 1.95 nm, a "soft" protein [125]), and lactoferrin (MM=80 000, pI = 8–9, R_s = 2.2 nm), in phosphate solutions of various ionic strength at pH 7.0. It was not possible to determine any variation of the hydrophobic character as k' was constant over a wide I range. Nevertheless, adsorption of phosphate was shown by CZE for both proteins [70].

This method appears to be a complementary tool to CZE in order to demonstrate specific adsorption, but appears to be restricted to certain proteins. The authors assume that the "hard" or "soft" character of the protein has to be taken into consideration, with additional data on the behaviour of the protein for elution at acidic pH.

4.1.2. Ion binding in IEC–HPLC

Most reports deal with the eluted salt, namely identification of the ion of the bound protein displaced from the stationary phase. For binding of the protein to the support, the question of the charge and/or the nature of the buffer have been considered of minor importance regarding separation performance. As a rule of thumb, the binding of a protein to an oppositely charged ion-exchange group of the stationary phase is performed with a buffer similarly charged as the stationary phase. For instance, an acetate buffer for a weak cation exchanger [82], and phosphate at pH 6.5 for a strong cation exchanger [83]. Buffers based on amine groups are widely used for negatively charged protein adsorption on weak and strong anion exchangers (DEAE, quaternary amine). The use of a phosphate buffer has led to unexpected retention times [127].

Few studies have been reported with the buffer similarly charged as the protein for protein binding to the stationary phase. Stout et al. [83] used a phosphate buffer at pH 6.4 for binding negatively charged ovalbumin and conalbumin (close to their pI values) to a weak and strong anion exchanger. Gooding et al. [84] used a Tris buffer to bind several positively charged proteins on a weak cation exchanger.

New trends in affinity chromatography with cations other than metal ions have appeared in the literature [71]. Human serum albumin (HSA, MM = 68 300, pI = 5.3) is well known for its ability to reversibly bind to a number of ions. Guillaume et al. [71] studied the binding of alkaline-earth cations on immobilised HSA versus the ionic strength of phosphate buffer and at various temperatures. At pH 6.5, HSA is theoretically negatively charged. From a plot of $\ln k'$ vs. $I^{-0.5}$ [Eq. (5)] the number of HSA charges interacting with cations was determined. The binding of one magnesium per HSA molecule (2.2 charges) and two calcium per HSA molecule (4.5 charges) was shown. The results were consistent with the literature data.

4.1.3. Critical comments on literature data for IEC–HPLC

Knowing the possible specific adsorption of phosphate and citrate (and likely other carboxylate groups?) on proteins we consider that the results published previously for the IEC–HPLC of proteins can be re-analysed according to this assumption. A close inspection of the literature data can then show the role of the buffer used.

For lysozyme, the net charge from IEC–HPLC was only 9.2 using acetate buffer at pH 4.9, versus 10.6 by titration [79]. It is suspected that the lower charge is due to the specific adsorption of 1.4 acetate groups of opposite sign per protein molecule.

For lysozyme in phosphate buffer at pH 6.5, with three ion-exchange supports, the net charge is twice the number of contacts of the SDM model, i.e. in the range 6.8–10.8 [83]. The charge from titration is 8.6. No conclusion can be drawn concerning the specific adsorption of phosphate, due to interference from the available ion-exchange groups.

For ovalbumin at pH 6.4, the titration charge is about −15.3. It was shown that the number of charges (twice the number of contacts) is between −18.8 and −24.4 in 100 mmol L^{-1} phosphate buffer [83]. The difference is in the range −3.5 to −9.1, indicating that about 3 to 4.5 phosphates (net charge −1.2) can bind to the protein. On the other hand, for runs performed in Tris buffer at pH 7.8, the net charge of ovalbumin is lowered to 5.5–3.5 with respect to the titration charge [79]. One suspects a Tris-specific adsorption on ovalbumin, which contains many phosphoserines.

As a conclusion, a systematic investigation of the role of the chemical nature of the buffer would allow us to obtain a better understanding of the specific adsorption phenomenon, and a possible improvement in separation.

4.2. Capillary zone electrophoresis

The capillary electrophoresis (CE) of proteins and DNAs is mainly performed for analytical purposes. Abnormal separations of proteins have been observed, indicating that an interaction between the protein and a component of the BGE takes place [128]. Only very few studies have dealt with the characterisation of biological molecules. This is why the dependence of the electrophoretic mobility on the ionic strength is often neglected, whereas it is of prime importance for any conclusion to be made concerning the possible ion binding.

In CE, evidence of "ion binding" to charged biological molecules is generally observed when:

(i) the electrophoretic mobility varies with the physicochemical environment (ionic strength, concentration of the ion of interest). This is the case for globular proteins and, to some extent, for DNA with inorganic cations;

(ii) the electrophoretic mobility varies with the physicochemical environment and, moreover, peak distortion and splitting are observed in the electropherogram. This holds for DNAs with organic cations.

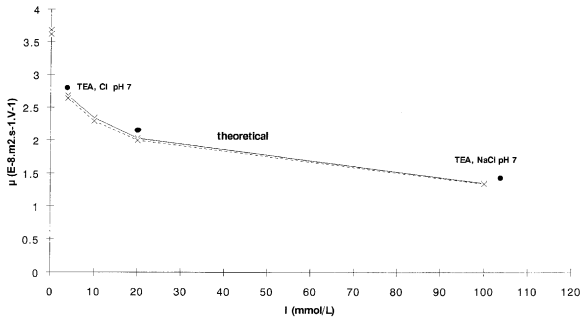

Fig. 2. Mobility of lysozyme (10^{-8} m^2 V^{-1} s^{-1}) versus ionic strength at pH 7.0. (●) Experimental mobility in 5 mmol L^{-1} triethanolamine (TEA) chloride + NaCl and (×) calculated mobility according to Eq. (8) (reprinted with permission from the author and publisher) [72].

4.2.1. Globular proteins

All data on this subject are from our group at the University of Rennes (France).

4.2.1.1. Binding of indifferent anions on a globular protein. The calculated electrophoretic mobility of lysozyme [Eq. (8)] was compared with the experimental value measured in triethanolammonium chloride (TEACl) + NaCl at pH 7.0 (Fig. 2). The two sets of data match well, highlighting the effect of the ionic strength on protein mobility [72]. TEACl and NaCl are indifferent electrolytes towards lysozyme.

4.2.1.2. Oxyanion specific adsorption on a globular protein. Two different cases were studied, as the ion of interest was either a counter-ion or a co-ion of the protein. (i) *Counter-ion*. (a) *Phosphate*. The specific adsorption of phosphate (sodium or potassium salt) was studied on positively charged proteins (lysozyme and lactoferrin) at pH 7.0 versus ionic strength in the range 0–775 mmol L^{-1} [72]. For lysozyme (pI = 10.7) the experimental electrophoretic mobility was positive, but lower than predicted [Eq. (8), Fig. 3]. The difference is due to the specific adsorption of phosphate ions. The phosphate content per protein molecule was determined from the experimental charge number [Z^{eff}, Eq. (8)]

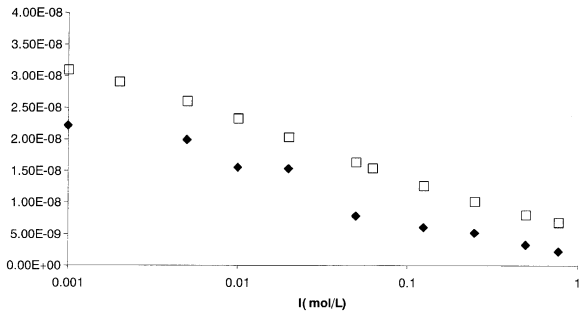

Fig. 3. CZE—Mobility of lysozyme (10^{-8} m^2 V^{-1} s^{-1}) versus ionic strength at pH 7.0. (◆) Experimental mobility in phosphate and (□) calculated mobility according to Eq. (8) (reprinted with permission from the author and publisher) [70].

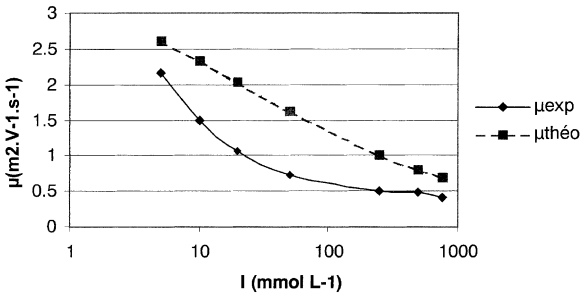

Fig. 4. CZE—Mobility of lysozyme in sodium citrate at pH 7.0 versus ionic strength [126].

of the protein in phosphate medium. Z^{eff} was found to be +5 in the ionic strength range 1–20 mmol L^{-1} and +3 in the range 50–500 mmol L^{-1}, whereas the calculated Z value (primary sequence) was +7 at pH 7.0. Lysozyme binds one phosphate at low ionic strength and between two and three at the highest ionic strength. The ability of lysozyme to adsorb one phosphate per molecule at low ionic strength at pH 8.0 has been reported previously [129]. For lactoferrin (pI = 8–9), the theoretical mobility cannot be calculated as the primary sequence has not been totally resolved. The electrophoretic mobility was constant and negative, highlighting charge reversal due to phosphate specific adsorption. (b) *Borate*. For lysozyme, the mobility at pH 8.0 decreases from $1{\cdot}10^{-8}$ m^2 V^{-1} s^{-1} in 100 mmol L^{-1} boric acid (I = 10 mmol L^{-1} in borate form and Na) to zero in 200 mmol L^{-1} (I = 20 mmol L^{-1} in borate form and Na). The experimental mobilities are lower than the calculated values, indicating specific adsorption. Moreover, an apparent IEP was determined at pH 8 (I = 20 mmol L^{-1} borate) far from pI = 10.7. (c) *Citrate as counter-ion*. The mobility of positively charged lysozyme was studied in citrate (sodium salt) at pH 7.0 in the ionic strength range 5–775 mmol L^{-1} [126] (Fig. 4). The Z^{eff} deduced is constant, about 4, indicating the adsorption of one or two citrate (valency −2.8) per molecule (Fig. 5). (ii) *Co-ion*. (a) *Phosphate as co-ion*. The mobility of a negatively charged protein was studied at pH 7.0 in phosphate (Na or K salt) in the ionic strength range 5–775 mmol L^{-1} [70]. For α-lactalbumin (pI = 4.2–4.6) two forms are

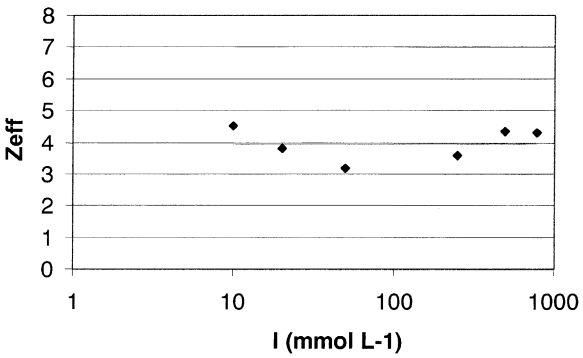

Fig. 5. CZE—Z^{eff} of lysozyme in sodium citrate at pH 7.0 versus ionic strength from μ of Fig. 4. The calculated Z value is +7.1 [126].

considered for calculations: the apo form and the holo form (+1 calcium per molecule), known to be the stable form in milk (pH 6.6). The experimental μ of α-lactalbumin (in the holo form) was roughly constant and more negative than predicted. Z^{eff} increased continuously from −4 to −11 in the ionic strength range from 5 to 775 mmol L^{-1}, respectively. Accordingly, one to six phosphates were bound per protein, respectively. (b) *Borate as co-ion*. The mobility of β-lactoglobulin (pI = 5.1) was calculated considering the two genetic variants, A and B, of the protein in monomer form at pH 8 in 200 mmol L^{-1} boric acid BGE (I = 20 mmol L^{-1} in borate form and Na). The calculated values were slightly higher than the experimental mobilities at the same ionic strength [72]. The mobility of β-lactoglobulin was calculated considering the two genetic variants, A and B, of the protein in dimer form at pH 7. In a BGE consisting of 200 mmol L^{-1} boric acid+20 mmol L^{-1} NaCl (I = 20 mmol L^{-1} in NaCl) the calculated values were about twice the experimental mobilities. It is clear that binding occurs on β-lactoglobulin either at pH 7.0 or 8.0, but the form of the binding (borate or boric acid) remains unclear. As such specific adsorption was observed with phosphate and citrate, we believe that it is probably the borate form that specifically adsorbs on proteins. Deprotonation of H_3BO_3 occurs at acidic pH, probably via an acid/base reaction with free amino groups of the protein, allowing borate adsorption in a second step. This hypothesis requires further investigation.

4.2.1.3. Conclusions. The following major conclusions can be deduced from these experiments.

(i) Specific adsorption can occur when the small ion is either a counter-ion or a co-ion of the protein. It appears unambiguous that the bond between the ion and the protein might be different in nature. In the case of co-ions, the role played by cations (particularly in metalloproteins such as α-lactalbumin and lactoferrin) should be studied.

(ii) Specific adsorption of phosphate, borate and citrate on proteins was observed. As shown in the following section, borate adsorption also occurs on free uncharged adenosine, negatively charged AMP and, to a smaller extent, on negatively charged DNA. One can expect, even if further investigations have to be performed, that the specific adsorption of oxyanions is a common property of proteins.

A charged protein, in fact, bears both positive and negative charges. Our current opinion is that specific adsorption of the ion of interest occurs on the counter-ion site of the protein via electrostatic attraction and can be stabilised by other weak forces. There are no specific sites for this adsorption, contrary to the "specific" sites of enzymatic studies.

The positively charged site of the protein is taken into consideration whatever the total of negatively charged ions bound from the BGE via electrostatic attraction. Consequently, the balance of positive and negative charges of the protein is modified by ion binding, and its isoelectric point is shifted.

This review also highlights the lack of information concerning the nature of the chemical bond, the adsorption sites and the reversibility of specific adsorption that can be obtained using spectroscopic techniques. Some preliminary experiments have been performed for BGEs containing ethanol in order to show possible hydrogen bonding [130] and these hold promise for a better understanding. Nevertheless, further investigations are needed.

4.2.2. Adenosine, AMP and DNAs

Papers dealing with this subject are due to Righetti's group from the University of Verona (Italy) and Stellwagen's group from the University of Iowa (USA).

All studies were performed in the pH range 7.7–8.9, at which DNAs are negatively charged (due to their phosphate groups). The main experiments concern the binding of either inorganic or organic cations. Few results have involved anions such as borate.

4.2.2.1. Counter-ion. (i) *Inorganic cations*. The binding of inorganic monovalent alkaline cations (Li, Na, K, Rb, Cs) was studied on ds-20 mer DNA in a TAE-based buffer at pH 8.3 and I = 40 mmol L^{-1}. Different electrophoretic mobilities were obtained according to Eq. (20), with μ the experimental mobility in TAE buffer+alkaline cation of interest (in chloride form) and μ^{ref} the experimental mobility in TAE buffer ($3.18{\cdot}10^{-8}$ m^2 V^{-1} s^{-1}). For all alkaline cations, $\Delta\mu$ increased linearly with salt concentration in the range 0–20 mmol L^{-1}, as double layer compression occurs. Small differences were observed between the cations as $\Delta\mu$ decreased slightly from Cs to Li [88]. It was demonstrated that inorganic cations only provide a shielding of the charge via electrostatic interactions. Sodium binding on 20 bp DNA was studied with the aim of identifying the sodium binding site to A-Tract DNA oligomers [87]. Our current opinion is that this behaviour seems to be typical of the binding of an indifferent ion. (ii) *Organic cations*. The Binding of organic cations has been studied extensively as many of the buffers used in biological studies are based on amines. (a) *Histidine* (pI = 7.7). Stellwagen et al. [91] compared the electropherograms of two DNAs (pUC19 and 18-mer), both in 40 mmol L^{-1} TAE buffer at pH 8.0 and in 50 mmol L^{-1} histidine buffer at pH 7.7. Each DNA migrates as a single Gaussian peak in TAE buffer and peak splitting is observed in histidine buffer. This is unmistakable evidence of histidine binding on DNAs. When increasing NaCl (0–50 mmol L^{-1}) is added to the BGE the double peak disappears until a single peak is observed at higher ionic strength. The histidine is bound to DNA through electrostatic interactions. Further experiments [89] have shown that monovalent inorganic cations are more effective at releasing histidine from DNA than divalent cations. Moreover, it was shown that analysis performed in an old histidine buffer, where degradation has occurred with time, produces poorly resolved peaks compared with a fresh buffer, which argues for the effectiveness of histidine

binding to DNAs [131]. Using Eq. (11), according to the added KCl, the number of histidines per DNA molecules was estimated to be from 0 to 1.9 for 18-mer DNA and from 1.9 to 2.6 for linear UPC19 DNA, with 60–10 mmol L^{-1} KCl added, respectively. In our opinion, considering that, at pH 7.7, histidine is in zwitterionic form, most of the charges are due to α-amino groups, consequently electrostatic interactions are probably not "located" on the imidazole residues. (b) *Other amines*. Complementary studies [88,89] with more bulky amine buffers {HEPES [*N*-(2-hydroxylethyl)-piperazine-*N*′-(2-ethanesulfonic acid)], MOPS [3-(*N*-morpholino)propanesulfonic acid], BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid], TES [*N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid], Tris [tris(hydroxymethyl)aminoethane], and Tricine [*N*-tris(hydroxymethyl)methylglycine]} show behaviour similar to histidine (Fig. 6), allowing us to come to the conclusion that the most commonly used amine-based buffers interact with DNAs.

4.2.2.2. Co-ion. The only co-ion studied was borate, as it is a buffer ion often used in biological studies [86,89,90]. (i) *Free adenosine*. Adenosine is uncharged at pH 8.25 and migrates with the EOF in Tris–acetate (TA) buffer (∼108 min), whereas in TB buffer the migration time is about 16 min (under similar conditions), highlighting the strong binding of borate [86]. (ii) *DNA*. Stellwagen et al. [89] reported that, in 45 mmol L^{-1} tris–borate–EDTA (TBE) buffer, mixtures of large and small DNA molecules (linear pUC19 and double-stranded 20 mer) migrate as a single component. On increasing the borate concentration in the TBE buffer from 90 to 270 mmol L^{-1}, a gradual dissociation of the combination of the two DNAs was observed (Fig. 7). Moreover, peak distortion was observed. This interaction between DNAs and borate was attributed to the progressive saturation of the borate binding sites of each DNA. D'Acunto et al. [86] measured a similar mobility ($\mu = 3.68 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}) for a ds 24-mer DNA in TA and TB buffers of $I = 45$ mmol L^{-1} at pH 8.25, indicating a similar behaviour of acetate and borate towards DNA. However, at 70 mmol L^{-1} ionic strength and high borate concentration (200 mmol L^{-1}) the experimental mobility of the DNA was strongly dependent on the pH and was reduced by about 20% at pH 8.9 compared to pH 7.7. This indicates that some borate binding could occur on DNA at high pH when a significant part of the buffer is in borate form ($pK_a = 9.3$) [86]. (iii) *AMP*. Similar experiments performed with low-molecular-mass AMP show a higher mobility in TB than in TA buffer. Moreover, in TB buffer, fronting peaks were observed for AMP, whereas Gaussian peaks were obtained in TA buffer. The peak distortion was studied according to the borate concentration (Fig. 8). The peak shape changed from fronting to tailing when the borate concentration decreased, emphasising increased borate binding to AMP [86]. The data suggest that borate binding mainly occurs on free nucleosides. From this the authors assumed that the only possible way to bind borate

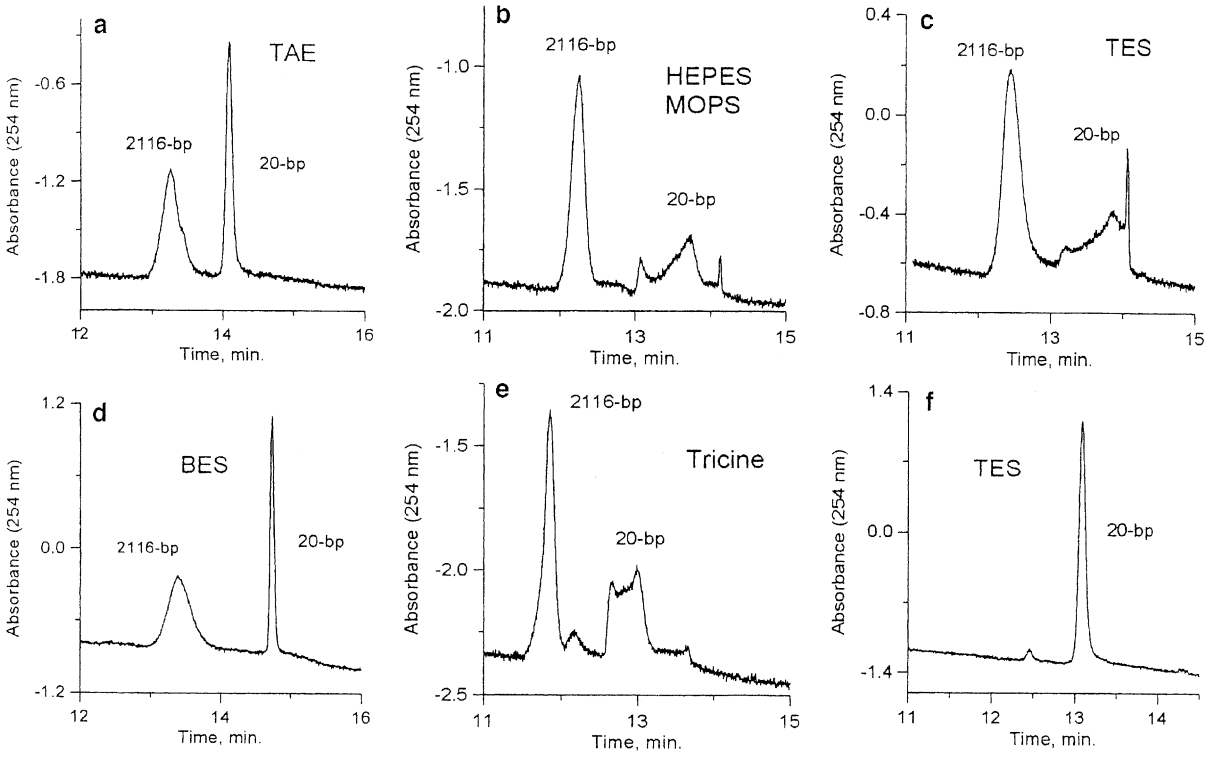

Fig. 6. CZE—Electropherograms of the 2116 and 20 bp fragments in (a) TAE, (b) HEPES, (c) MOPS, (d) TES, (e) BES and (f) Tricine. Only the 20 bp oligomer for TES. Capillary diameter 75 μm, $E = 150$ V/cm (reprinted with permission from the author and publisher) [89].

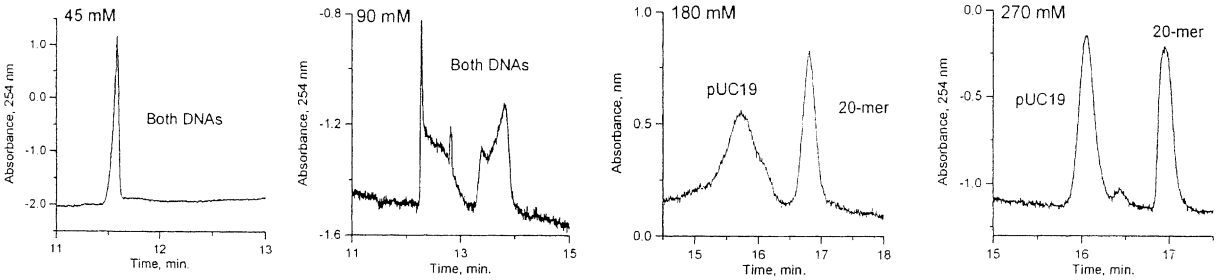

Fig. 7. CZE—electropherograms obtained for a mixture of the 2116 and 20 bp fragments in TBE with various borate concentrations (concentration in m*M*). Capillary 75 μm, $E = 150$ V/cm (reprinted with permission from the author and publisher) [89].

to a DNA double filament is via bridging the only free OH group located at the free 3′ extremity of each filament [86].

4.2.2.3. Conclusion. At this time we cannot draw conclusions concerning the possibility of the specific adsorption of amine buffers on DNAs. The question asked by Stellawagen and Righetti [89] in the title of a recent paper sums up the situation: "DNA and buffers: are there any non-interacting neutral pH buffers?" Until now the answer seems to be "no". This will be taken into account not only for analysis, but also for in vitro experiments using DNAs.

4.3. Ultrafiltration of globular proteins

In ultrafiltration, as shown in the CDE model [116], only repulsive electrostatic interactions are taken into account. The electrophoretic mobility of the protein can be considered as a key parameter for the ultrafiltration of a charged protein.

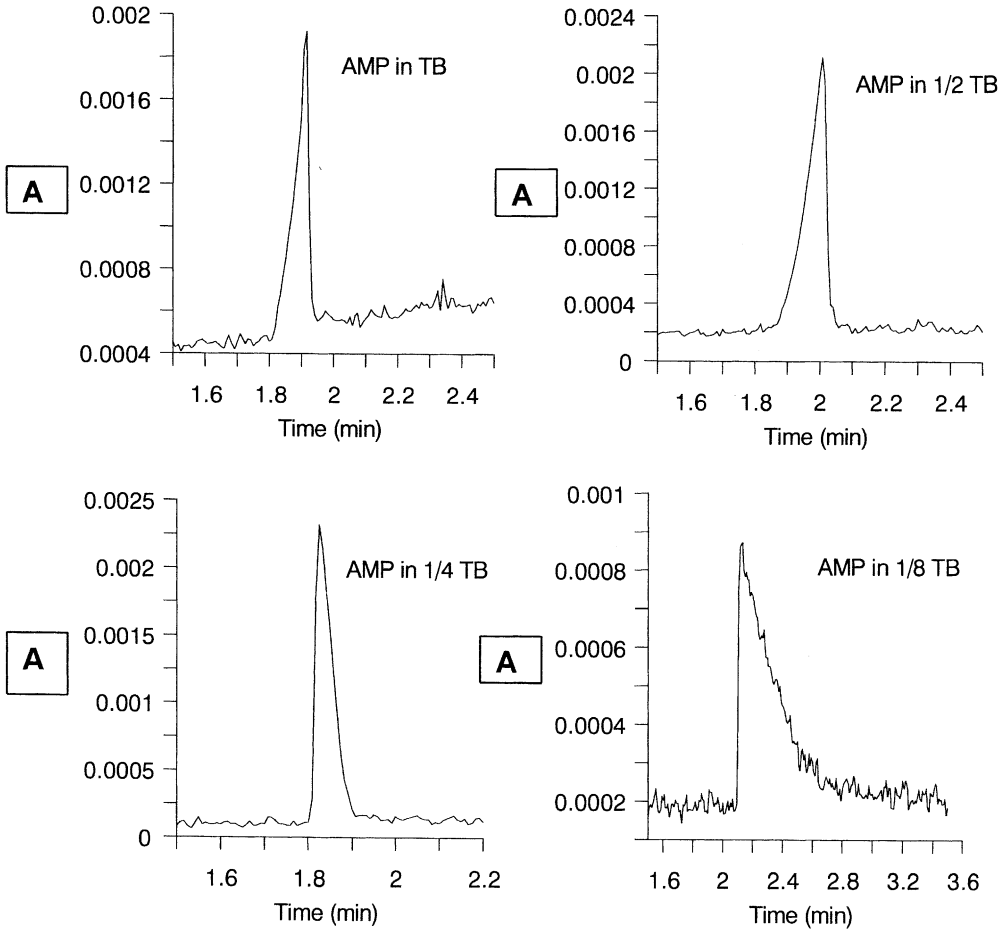

Fig. 8. CZE analysis of AMP in TB of different molarities. Starting borate buffer (upper left panel): 40 mmol L^{-1} Tris, 40 mmol L^{-1} boric acid, pH 8.25. Note the peak distortion (fronting or tailing) versus the borate concentration (reprinted with permission from the author and publisher) [86].

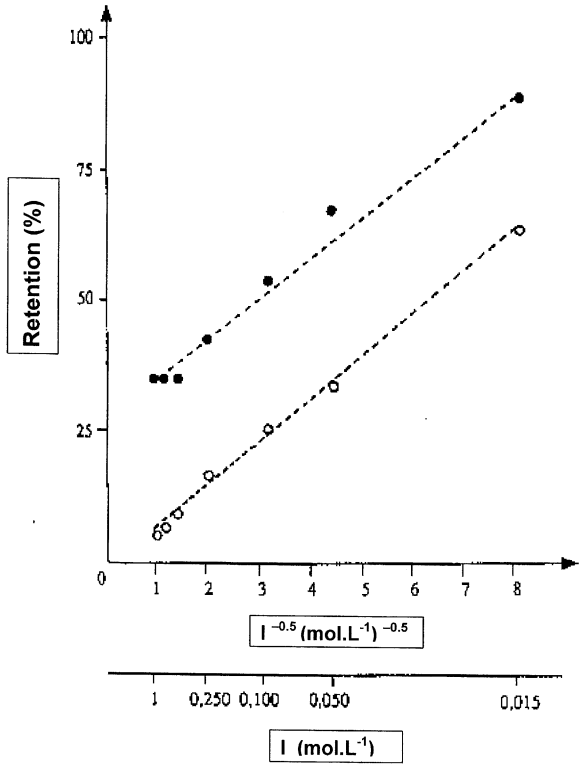

Fig. 9. Retention of BSA (●) and lysozyme (○) in a single solution with a zirconia membrane (M1, 150 kD, Orelis) versus $I^{-0.5}$. UF conditions: 4 m s^{-1}, 2 bar, 12 °C (BSA), 20 °C (lysozyme), batch mode (reprinted with permission from the author and the publisher) [133].

4.3.1. Effect of ionic strength on retention

For a protein, a high ionic strength ($I > 100$ mmol L^{-1}) is needed for cancellation of the electrostatic interactions between the protein and the membrane, either when the protein and membrane are co-ions or counter-ions in their initial state [132,133]. On varying the ionic strength, the retention of the charged solute decreases linearly with the reciprocal of the square root of the ionic strength ($I^{-1/2}$). A semi-empirical model called the "Ionic Strength Control of Retention Model" (ISCR) has been proposed in UF that relates the retention of a charged solute and the ionic strength through $I^{-1/2}$ [133,134] (Fig. 9). Such a decrease of retention with increasing ionic strength is typical of an indifferent electrolyte.

4.3.2. Effect of specific ion binding on retention

4.3.2.1. Lysozyme/lactoferrin mixture. Chaufer et al. [135] compared the separation of two proteins [lysozyme and lactoferrin (50:50, w/w)] with the same membrane versus the physico-chemical environment. The main objective was to attain a high transmission of lysozyme in the permeate and to retain lactoferrin in the retentate. UF of the mixture was performed either at pH 7.0 in NaCl, acting as an indifferent electrolyte, or at pH 9.0 in phosphate (in sodium or potassium form), acting as a specifically adsorbed ion on the two proteins.

At pH 7.0 in NaCl, both proteins are positively charged. The retention of both decreases with increasing ionic strength in the range 1–150 mmol L^{-1}, as the mobility of both proteins also decreases. Moreover, the selectivity S of the separation (defined as the transmission of lysozyme to the transmission of lactoferrin ratio) was shown to be independent of the ionic strength (Fig. 10). The electrostatic exclusion mechanism occurs for both proteins and is not sufficiently selective to enhance the membrane selectivity. Consequently, the instantaneous purity of the lysozyme was 95% in the permeate compared with 50% in the feed.

To enhance the selectivity of the separation, according to the CDE model, lysozyme has to be uncharged, whereas lactoferrin has to be strongly charged for a predominant electrostatic exclusion mechanism by the charged-fouled membrane. The specific adsorption of phosphate appears to be the tool to achieve this goal. In phosphate at pH 9, lactoferrin was negatively charged over the whole range of ionic strength, whereas the sign for lysozyme was successively positive, neutral and negative. Accordingly, enhanced selectivity is expected when lysozyme is neutral in the ionic strength range 10–20 mmol L^{-1}. Enhanced selectivity was obtained close to this ionic strength range (50 mmol L^{-1}, the small shift is due to the variation of membrane fouling with ionic strength during UF), which indicates that the instantaneous purity of the lysozyme was >99% in the permeate (Fig. 10).

4.3.2.2. Whey proteins. Lucas et al. [136] compared the ultrafiltration of whey proteins in different physico-chemical environments at pH 7.0 using the same membrane. The main objective was the selective transmission of α-lactalbumin in the permeate and the retention of β-lactoglobulin in the retentate.

The performance of UF in phosphate, acting as a specifically adsorbed ion on both proteins, was compared with that observed in NaCl, acting as an indifferent electrolyte. The

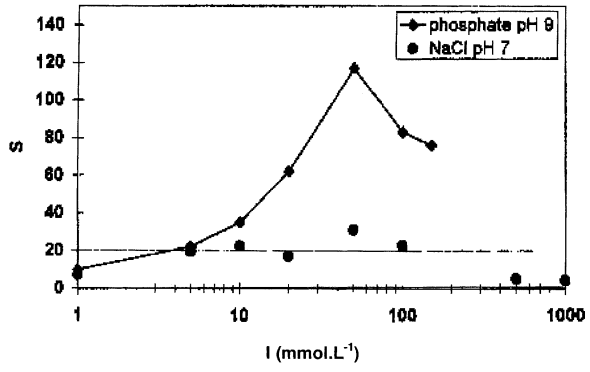

Fig. 10. Influence of the physico-chemical environment on the selectivity (S = transmission of lysozyme/transmission of lactoferrin) of a Kerasep (300 kD) PP membrane for a mixture of lysozyme and lactoferrin (1:1 g L^{-1}) versus the ionic strength of NaCl (●) at pH 7 and phosphate (◆) at pH 9. UF conditions: 1 m s^{-1}, 1.5 bar, 12 °C, batch mode (reprinted with permission from the author and the publisher) [69].

two proteins remained negatively charged in phosphate as in NaCl, and the selectivity of UF remained unchanged for both environments.

The performance of UF in calcium (in chloride form), known to be a specifically adsorbed ion on α-lactalbumin, was compared with that observed in NaCl, acting as an indifferent electrolyte. In $CaCl_2$, enhanced transmission of α-lactalbumin was observed as its charge (mobility) decreased. Unfortunately, the selectivity remains unchanged, because the adsorption of calcium occurs simultaneously on both proteins, with a sufficiently non-selective decrease of the negative charge.

4.3.2.3. Conclusion. From the literature data it appears that the specific adsorption of an ion can, to some extent, be an effective tool to enhance separation by ultrafiltration. When specific adsorption leads only to a decrease in the mobility of the two proteins to be separated, the selectivity of ultrafiltration is not enhanced. However, if specific adsorption allows us to obtain a mixture with one uncharged protein and a charged protein, then selectivity will be enhanced. Unfortunately, as the specific adsorption of ions such as phosphate and calcium is effective on various proteins, this tool needs a preliminary characterisation of the protein of interest and also of the other components of the mixture.

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