ELSEVIER

Journal of Chromatography A, 838 (1999) 11–18

JOURNAL OF CHROMATOGRAPHY A

Buffer properties of biopolymer solutions, as related to their behaviour in electrokinetic methodologies

Alexander V. Stoyanov[a], Pier Giorgio Righetti[b,*]

[a]*Institute of Gene Biology, Russian Academy of Sciences, Vavilova Street 34/5, 117334 Moscow B-334, Russia*
[b]*University of Verona, Department of Industrial Biotechnologies, Strada Le Grazie, Ca' Vignal, 37134 Verona, Italy*

Abstract

The buffer properties of biopolymers (proteins and nucleic acids), with special reference to electrokinetic methodologies, are considered. The action of biopolymers as potential titrants is also analyzed. Since the 'buffering capacity' value (β), that is conventionally used, is a local value, we propose to use the concept of 'buffer reserve'. The latter is obtained by integrating the β value over the necessary pH interval, and thus coincides with the concentration of strong titrant needed to induce the appropriate pH shift. With this parameter any buffer system may be characterized and its value will be a function of the ΔpH shift. The theoretical calculations performed for two real amphoteric substances show that the resistivity limit of such two buffers with the same initial β level may be quite different even in case of rather small pH shifts (several tenths of pH unit).

Keywords: Buffering capacity; Buffer composition; Proteins; Nucleic acids

1. Introduction

The 'buffer value', as introduced by Van Slyke [1], is an important characteristic of any buffer system and a very useful tool for choosing experimental conditions in different separation techniques. In our recent work we have analyzed the buffer properties of some simple protolytes [2–5] and proposed the concept of 'normalized buffering power to conductivity ratio' in order to give a simple algorithm for selecting appropriate amphoteric buffers in capillary zone electrophoresis [3]. In the present work we want to draw the readers' attention to the fact that the 'buffering capacity' is a local value, which typically reaches a maximum at pH=pK and in general rapidly worsens, for mono-protic species, both above and below the pK, in a bell-shaped curve [7]. In cases in which it is feared that the sample ions might compete with the buffering power of the selected buffer and locally alter the pH, it is desirable to know what is the 'buffer reserve', i.e. the maximum amount of added titrant resulting in an acceptable pH shift. The aim of the present report is to evaluate the 'buffer reserve', especially of amphoteric buffers, and how their ΔpK value can affect their performance. In addition, some characteristic features of biopolymers (proteins and nucleic acids) will be evaluated and their action as potential titrants in a given buffer solution will be assessed.

2. Theory

2.1. Buffer capacity and buffer reserve

According to Van Slyke [1], the buffer capacity β is defined as the derivative:

*Corresponding author.

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00090-4

$$\beta = \frac{dC_b}{d(pH)} \quad (1)$$

where C_b represents the concentration of a strong-base titrant required to induce a pH increase. Since β is a local value (i.e. it is referred to a precise value of the pH scale), when a pH interval is explored instead, one ought to take into account the β variance with pH. Thus, in order to obtain the amount of C, that will induce a pH shift of $\Delta pH = pH_2 - pH_1$, one has to integrate over this pH interval:

$$C = \int_{pH_1}^{pH_2} \beta(pH)\, d(pH) \quad (2)$$

or, in other form,

$$C = -\frac{1}{2.303} \int_{(H)_1}^{(H)_2} \frac{\beta(H)}{(H)} \cdot d(H) \quad (3)$$

Here we used

$$\frac{d}{d(pH)} = -2.303(H) \cdot \frac{d}{d(H)}$$

In many practical applications we are restricted often by the demand that the pH of the system is to be controlled and not to exceed some defined limit $(\Delta pH)_{max}$. Eq. (2) allows us to calculate the concentration of strong titrant that will cause this change of pH. To this purpose, we introduce here the value of 'buffer reserve', which represents the limit titrant concentration needed to keep our restriction on pH shift[1]:

$$U(\Delta pH_{max}) = C_{max} = \int_{pH_1}^{pH_1 + \Delta pH_{max}} \beta(pH)\, d(pH) \quad (4)$$

Thus, the buffer reserve is a function of a limit on ΔpH, and via its use any concrete buffer system may be characterized.

Let us consider some simple examples. When we have two different buffers and the value of their buffering capacity is close or even the same, their 'buffer reserve', also for a moderate pH variation, may be considerably different (or, in other terms, the same amount of titrant will result in different pH shifts). In Fig. 1A the behavior of the buffer power function in the vicinity of the isoelectric point for two different model biprotic protolytes is shown. If these two substances are ampholytes, the pH of their solutions should be close to their isoelectric points. Let us suppose also that we are at moderate pH, so that the water ions contribution to the buffer power may be omitted. We see that the β-function for an amphotere with $\Delta pK = 1$ is quasi constant in an interval covering ca. one pH unit (curve I), whereas, for $\Delta pK = 0$, we can see an appreciable decrease (curve II). In order to obtain the same β value at the isoelectric point, the protolyte concentration for curve II had to be taken lower ($C_{II}/C_I = 0.73$). One even more striking example is given in Fig. 1B. Here we have two β-functions with derivatives of opposite sign: $\Delta pK = 3$ (curve I) and $\Delta pK = 0$ (curve II) (with $C_{II}/C_I = 0.119$). Paradoxically, here we will have an increase in buffer capacity with titration for buffer I and a quasi specular decrease for buffer II, that obviously will produce considerable differences in 'buffer reserve'.

One should additionally take into account that the high buffer capacity values of amphoteric buffer systems having their isoelectric point (pI) at pH extremes are always connected with fast β decrements with titration (high negative derivative), that can be explained by a relatively high contribution of water ions. Let us consider some examples with two real substances. Iminodiacetic acid (IDA) has two acidic ionogenic groups: $pK_1 = 1.73$, $pK_2 = 2.73$ (pI 2.23) [6], and it possesses a very high buffer power [for a 10 mM solution (pH = 2.56), the calculated β value is $\beta = 14.4 \cdot 10^{-3}$ equiv. l^{-1} pH^{-1}]. A solution of glutamic acid ($pK_1 = 2.162$, $pK_2 = 4.324$, pI 3.243) at the same concentration will provide only $\beta = 4.33 \cdot 10^{-3}$ equiv. l^{-1} pH^{-1}. In order to obtain the same level of buffer power one should perform a four fold concentration increase ($C = 40.05$ mM), but the solution pH will shift to higher values, precisely pH 3.28 instead of the pH 3.24 reported for a 10 mM solution. Now we have the same buffer capacity, but the former is characterized by a high negative derivative. On the contrary, the latter has a small derivative value, which increases as one performs the

[1]Generally, the information we need may be obtained directly by solving the electroneutrality equation. Eq. (4) is more suitable for approximate calculations.

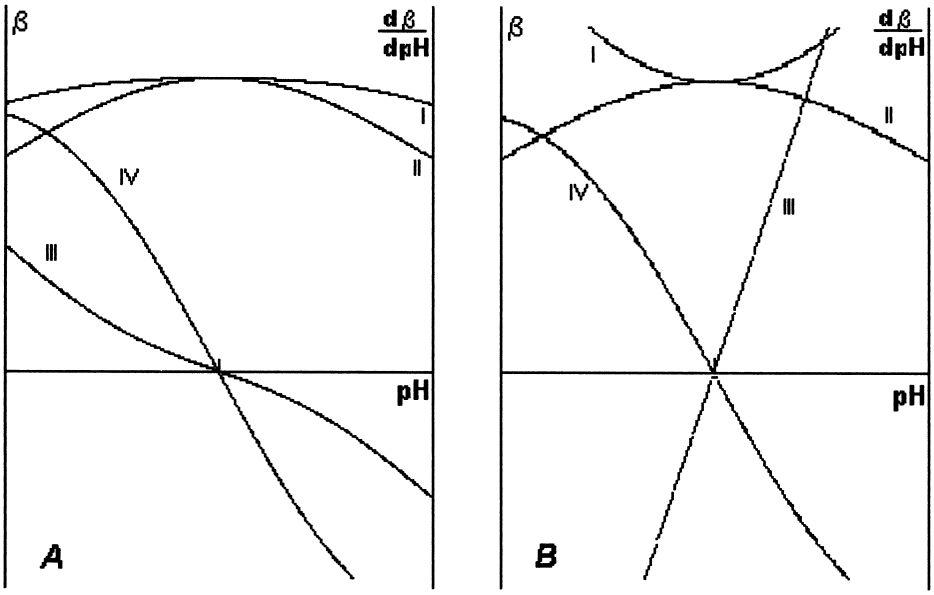

Fig. 1. Different behavior of the buffer power function in the vicinity of the isoelectric point for model biprotic protolytes. (A) Curves I and II represent the buffer capacity courses for $\Delta pK=1$ and 0, respectively (in order to obtain the same β value at the isoelectric point, the protolyte concentration for curve II is taken lower ($C_{II}/C_{I}=0.73$). (B) Example of two β-functions with derivatives of opposite sign: $\Delta pK=3$ (curve I); $\Delta pK=0$ (curve II) ($C_{II}/C_{I}=0.119$). Curves III and IV are the derivatives of the curves I and II, respectively. The total pH interval is one unit.

titration procedure (Fig. 2). If one calculates the 'buffer reserve' for these two systems, supposing $\Delta pH_{max}=0.3$, one will obtain: U^{β}_{IDA} ($\Delta=0.3$)=3.64 m*M*, and U^{β}_{Glu} ($\Delta=0.3$)=4.5 m*M*.

3. Results

3.1. Buffer properties of solutions of proteins and nucleic acids

The buffer power of a biopolymer may be approximated as an additive sum of the ionogenic groups of their monomer units[2]. Each one represents a function $(H)K_d/((H)+K_d)^2$, which decreases rather slowly as the pH moves away from that corresponding to the maximum (Fig. 3A and C). In the case of proteins, the main contribution to the buffering capacity is due to the ionogenic groups of the amino acid side chains. In Fig. 3A the separate contribution of amino acids is shown from left to the right: Asp (p*K* 3.9), Glu (p*K* 4.32), His (p*K* 6.04), Cys (p*K* 8.6), Tyr (p*K* 10.12), Lys (p*K* 10.79), Arg (p*K* 12.48). Here we used the data of Hirokawa [10]. Although the p*K* values of the various ionogenic groups are distributed rather uniformly within the pH 4–11 interval[3], one can appreciate that in general for proteins there exist a minimum in the pH 7–9 range, since Cys usually does not take part in dissociation (and its relative concentration is also very low). So one can correctly assume a local minimum in this range (see Fig. 3B), although the β value remains still appreciable. In fact, in the case of proteins, there are no pH ranges where β approaches zero (in contrast with small amphoteric compounds). One should also take

[2]The expression for buffering capacity depends on the dissociation scheme we used [8,9]. In the framework of 'parallel schemes', the assumption of independent dissociation leads to the approximation used in the present paper. Nevertheless one should remember, that some groups within monomer may form a subsystem with sequential dissociation type.

[3]As a first approximation we may assume that the p*K* values of monomeric units remain the same, or more correctly, that the model of parallel independent dissociation is valid [8]. In reality more diffused pattern should be observed.

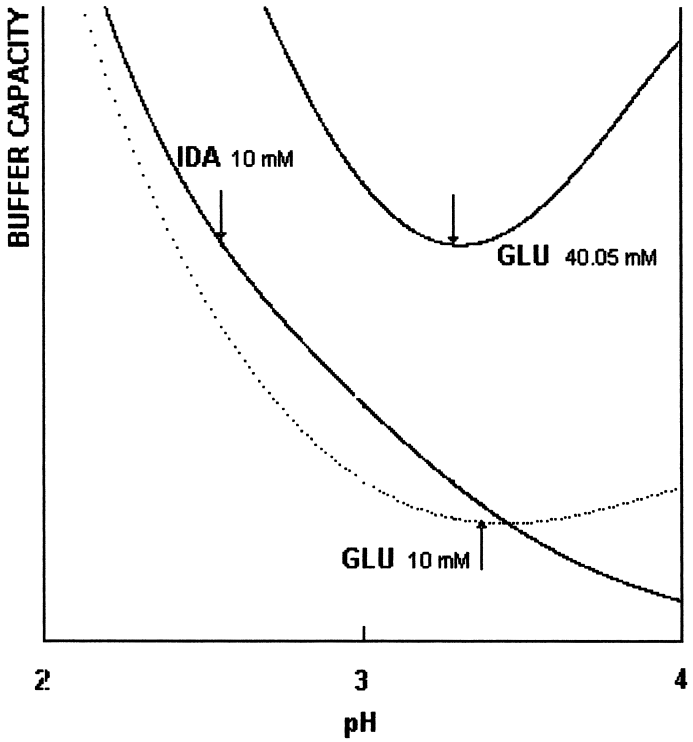

Fig. 2. Buffer capacity of iminodiacetic acid (10 m*M*) and glutamic acid (40.5 m*M*) solutions at and in the proximity of their respective isoelectric points. These two buffers have the same buffer capacity at the given molarities, but the former is characterized by a high negative derivative. On the contrary, the latter has a small derivative value, and, additionally, its β value increases as the ampholyte is titrated on both sides of the p*I* value. The dotted line shows the β-function of a 10 m*M* solution of glutamic acid. The vertical arrows indicate the appropriate pH (approximating the p*I*) values of the pure ampholyte solutions at the given molarities.

into account the fact that the frequency rate of ionogenic groups in proteins may be taken as 0.1, which has a consequence the well-known fact that protein solutions, in general, provide relatively moderate buffering power with respect to mass concentration.

A quite different situation is observed in the case of nucleic acids. First of all they possess a considerably higher density of ionogenic groups per monomer (and, also, per mass unit). Fig. 3C shows the separate contribution of each ionogenic group, from left to right: phosphate (p*K* 1.0), G (pK_1 2.4), A (p*K* 3.8), C (p*K* 4.5), G_2 (pK_2 9.4), T (p*K* 10.0) (the latter two p*K* values referring to the dissociation of enolate ions in the keto–enolic tautomerism). The bases incorporated are weak (p*K* 2.4; p*K* 3.8; p*K* 4.5), so that the isoelectric points of nucleic acids are always in the acidic region, where the buffering power is very high. For both RNAs and DNAs this is due to the presence of basic groups in the G, A and C nucleotides as well as to the contribution of the prime phosphate (see Fig. 3D).

3.2. Biopolymers as titrating agents

In the previous section we considered the buffer properties of solutions of biopolymers, but for many practical purposes another aspect is also quite important, namely the influence of a biopolymer sample when added to a buffer. So, one could explore the behavior of the macromolecular sample as a ‘titrant’. It is reasonable to approximate the action of many dissociating groups in biopolymers as adding equivalent amounts of strong titrant. The latter, obviously, coincides with the total amount of charges that will

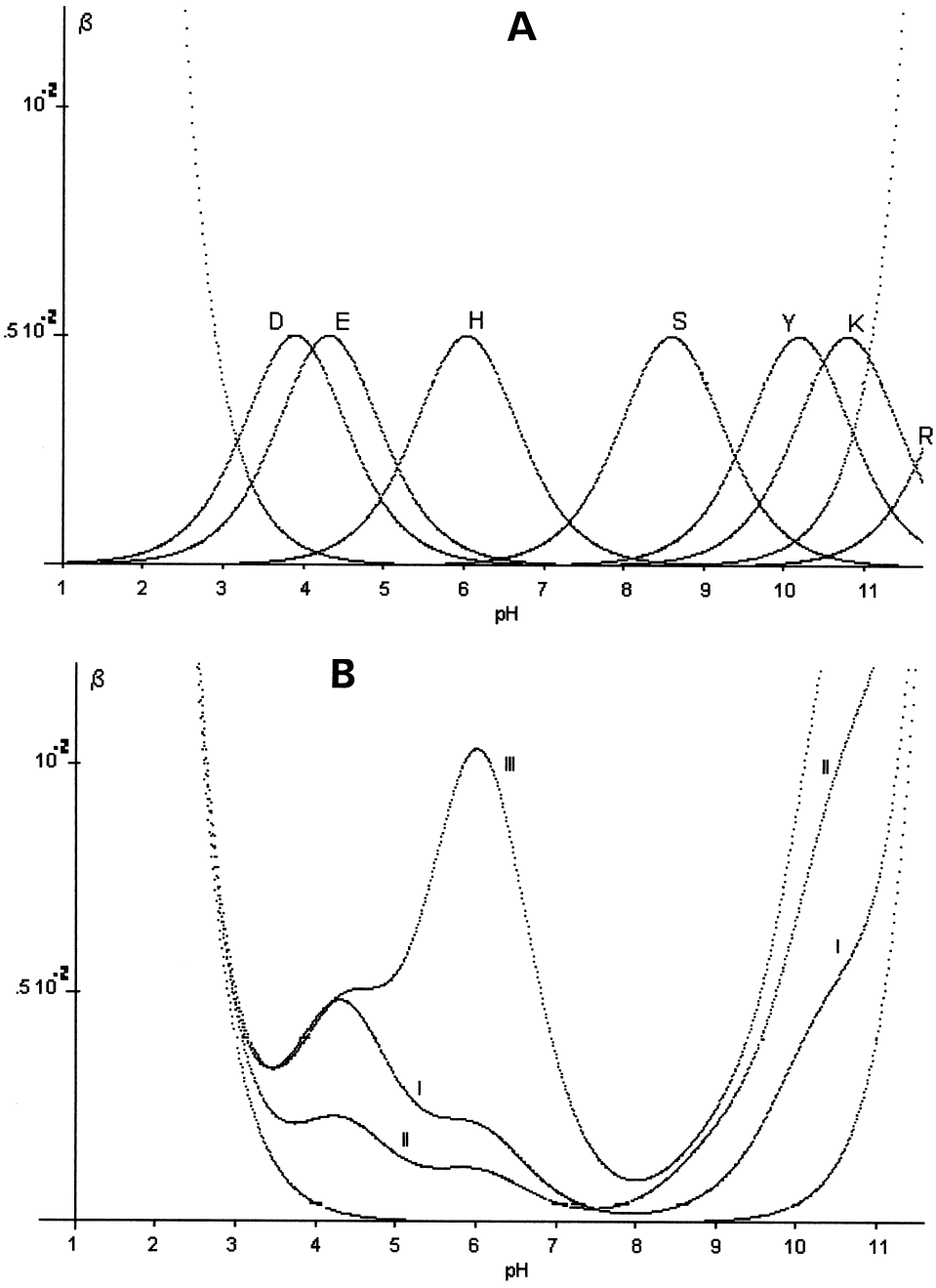

Fig. 3. Buffering power of proteins and nucleic acids in a wide pH interval. (A and C) Contribution of the ionogenic monomeric units [amino acids (A) and nucleotides (C)]. Calculated buffering capacity of three different proteins: albumin 10^{-4} m*M* (curve I); lysozyme 10^{-3} m*M* (curve II) and haemoglobin (Hb) α-chain 10^{-3} m*M* (curve III). Note the huge peak of buffering power of the α-chains of Hb around pH 6, due to its unusually large content of His. (D) Overall β power of a solution of an 18-mer II oligonucleotide (5′-TCTGAAAGTGCTCTACTG-3′) in the pH 1 to 11 interval. The concentration of the monomers is taken as 5 m*M*. The buffer capacity is plotted in relative units (1/4 log 10). For further explanations see the text.

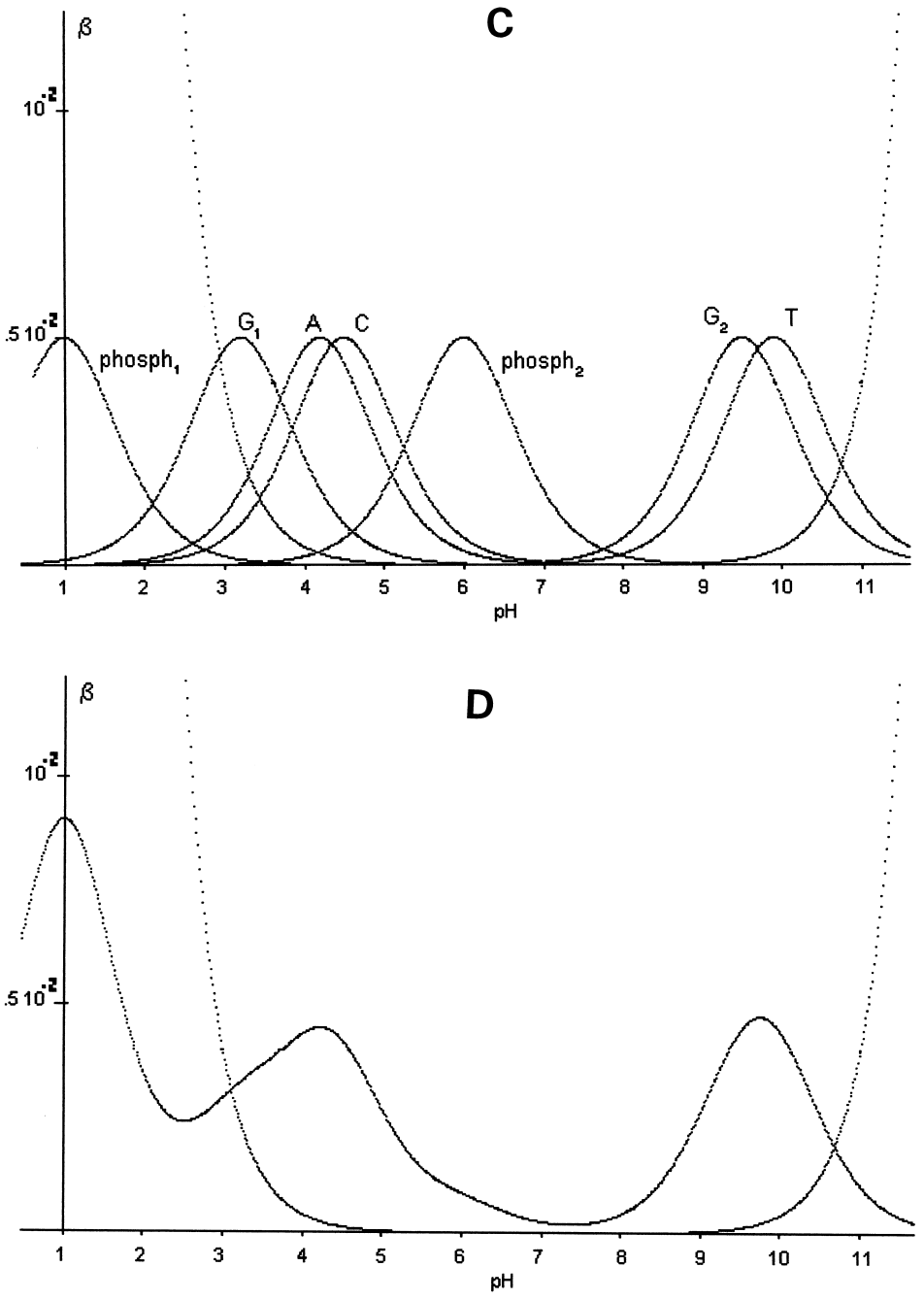

Fig. 3. (*continued*)

be imparted to the biopolymer by being conditioned by the prevailing pH of the given buffer solution.

Fig. 4 provides an equivalent representation of an 18-mer oligonucleotide as a strong acid (mol equiv./mol), when we use a buffer with different pH. The abscissa represents the difference between the pH of

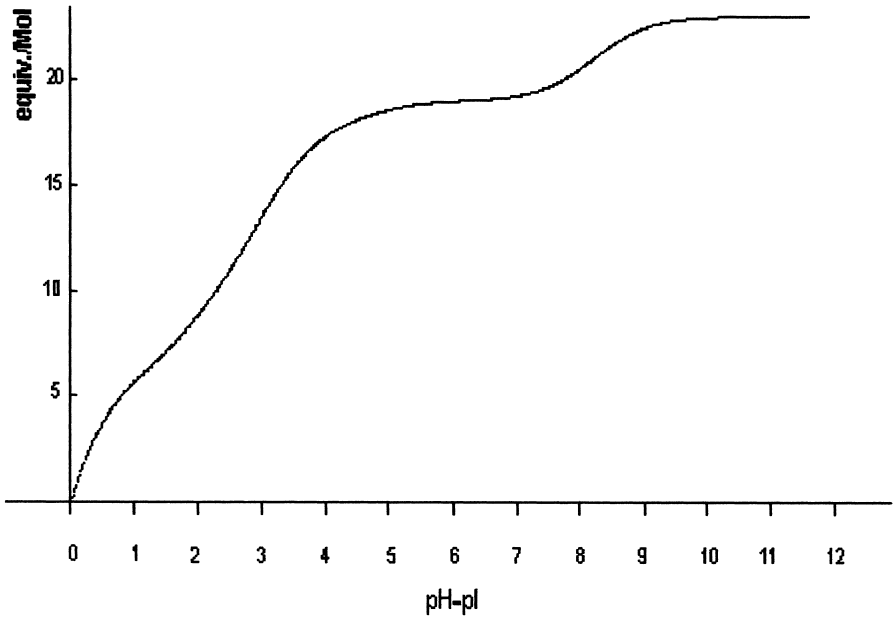

Fig. 4. Equivalent representation of the 18-mer oligonucleotide (having the sequence given in the legend to Fig. 3) as a strong acid (mol equiv./mol), when one uses a buffer with a different pH. The calculated p*I* for the 18-mer oligonucleotide is 1.28.

the buffer, selected for the experiment, and the p*I* value (the calculated p*I* of this oligonucleotide being 1.28). There is a small difference in 'titration' properties in the pH 6–10 range, but in acidic buffers (around pH 3) the sample ion acts as an equivalent amount of acid (taken three times lower in this simulation).

4. Discussion

Ideally, one would prefer to use a buffer with a very high buffering power and to dissolve in it a sample at negligible concentration, so as not to induce any alteration in the local pH value. If the sample influence on the buffer pH becomes non-negligible, an opportunity is now given to calculate theoretically this value, starting from the known chemical composition of both buffer and sample. All that is required is to solve the electroneutrality equation, although some technical problems may arise. At the same time, the information about the buffering capacity of a buffer (as given in Eq. (1)), calculated or taken from Tables, allows us to prepare a buffer at the suitable concentration which will ensure adequate control of the desired pH value upon sample addition, provided the expected pH shift is sufficiently small. In the opposite case, in which one should take into account potential β changes along the pH scale, as induced by sample ions, one should then operate on the principle of 'buffer reserve'. The latter may be easily calculated for simple amphoteric substances (or, at least, be evaluated with the help of the first derivative). In general, the problem of titrating a solution that exhibits a non-constant buffering power and the additional problem encountered when the titrant contains groups having different degrees of dissociation, are not so complicated by themselves, but may prove to be difficult to be understood with the help of simple analogies. Additional general considerations on practical experimental aspects connected with the β value of buffers and macroions in electrophoretic separations are given below.

First of all one should remember that the β value of the sample (titrant), in the vicinity of the buffer pH, has no direct relation with the pH shift induced by it. It is important to know the total amount of charges that will be imparted to the biopolymer and it is reasonable to approximate the action of many

dissociating groups in a biopolymer as adding equivalent amounts of strong titrant. Moreover, the high β value of the sample in titration point is connected with fast charge alteration and, obviously, may lead to smaller values of the introduced charge that will result, finally, in a decrease of the pH shift.

A choice of the appropriate buffer in electrophoretic separations should be made by taking into account the opportunity of obtaining a higher resolving power, that usually depends on a number of other factors beside the good buffering capacity. But for biopolymers (in contrast to strong titrants), it is important to know not only the properties of a buffer by itself, but with respect to the concrete sample. With an appropriate buffer selection, one can operate at a higher loading capacity (or at a lower buffer concentration). As mentioned above, the β-function value of the sample is not a matter of great importance and comes to play any role only when the pH shift becomes considerable. The importance of buffer capacity in other electrophoretic techniques, such as capillary electrophoresis, has been also highlighted by Reijenga et al. [11].

A particular case, however, is represented by conventional isoelectric focusing (IEF) in soluble, amphoteric buffers [12]. Here one deals with the effect of sample concentration precisely at the isoelectric point and, thus, it is to be expected that the higher buffering capacity of the sample could result in a flattening of the gradient profile and peak spreading, although one should keep in mind the fact that a high titration curve slope promotes a good sample condensation at the pI. This phenomenon (pH gradient distortion as a function of sample load) should not occur, instead, in IEF in immobilized pH gradients [13], since in the latter case the buffering and titrant ions are grafted to the gel matrix and, additionally, the buffer power is evenly distributed along the pH gradient and not localized in discrete zones, as in the case of conventional IEF.

Acknowledgements

P.G.R. is supported by grants from Agenzia Spaziale Italiana (No. ARS-98-179) and from MURST (Coordinated Project 40%, Biologia Strutturale).

References

[1] D.D. Van Slyke, J. Biol. Chem. 52 (1922) 525–536.
[2] A.V. Stoyanov, C. Gelfi, P.G. Righetti, Electrophoresis 18 (1997) 717–723.
[3] A.V. Stoyanov, P.G. Righetti, J. Chromatogr. A 790 (1997) 169–176.
[4] A.V. Stoyanov, P.G. Righetti, J. Chromatogr. A 799 (1998) 275–282.
[5] A.V. Stoyanov, P.G. Righetti, Electrophoresis 19 (1998) 1674–1676.
[6] A. Bossi, P.G. Righetti, Electrophoresis 18 (1997) 2012–2018.
[7] H. Svensson, Acta Chem. Scand. 16 (1962) 456–466.
[8] A.V. Stoyanov, P.G. Righetti, Electrophoresis 18 (1997) 1944–1950.
[9] A.V. Stoyanov, P.G. Righetti, Electrophoresis 19 (1998) 187–191.
[10] T. Hirokawa, M. Nishino, N. Aoki, Y. Kiso, Y. Sawamoto, T. Yagi, J. Akiyama, J. Chromatogr. 273 (1983) DI-D106.
[11] J.C. Reijenga, T.P.E.M. Verheggen, J.H.P.A. Martens, F.M. Everaerts, J. Chromatogr. A 744 (1996) 147–153.
[12] P.G. Righetti, Isoelectric Focusing: Theory, Methodology and Applications, Elsevier, Amsterdam, 1983.
[13] P.G. Righetti (Ed.), Immobilized pH Gradients: Theory and Methodology, Elsevier, Amsterdam, 1990, pp. 87–90.