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RECIPE FOR A pH 3-4 IMMOBILIZED GRADIENT FOR ISOELECTRIC FOCUSING

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SUMMARY

A linearized, optimized recipe for a pH 3-4 gradient is described. In this formulation, water is added as a basic Immobiline with $pK = 0.00$ (when the concentration is expressed as the activity coefficient on the molar fraction scale, *i.e.*, 1 *M*) or as a basic Immobiline with $pK = -1.74$ (when the concentration is expressed as the molarity of water in pure water, *i.e.*, 55.56 *M*).

INTRODUCTION

We have described in a series of papers¹⁻⁴ a computer program for the generation and optimization of any narrow or extended pH interval with Immobiline chemicals (immobilized pH gradients, IPG). The program, first adopted for a multi-chamber mixing device¹, was then applied to two chamber mixers² and recipes were given for any possible pH interval, spanning from a minimum of 1.5 to a maximum of 7 pH units, with³ or without⁴ the aid of strong titrants. Recently, we have also given formulations for non-linear, extended pH gradients to be used in the first dimension of two-dimensional maps of complex samples, such as cell lysates and biological fluids⁵.

Acidic pH gradients (below pH 4) had never been taken into consideration, since it was correctly believed that the strong conductivity of free protons would hamper the correct functioning of an IPG matrix⁶. Recently, however, we have demonstrated both by computer simulation⁷ and by experimental verification⁸ the feasibility of focusing in acidic gradients, such as a pH 3-4 interval. This was obtained by resorting to gradients of "conductivity quenchers", incorporated in the matrix, with the dense region located on the anodic side, for smoothing the exponential conductivity profile. These "quenchers" accomplish two functions: they will smooth the voltage gradient across the separation cell and will reduce the cathodic electro-osmotic flow due to the net negative charge acquired by the matrix at pH 3.

We give here an optimized recipe for a linear pH 3-4 gradient, computed by

using the premise that water in an acidic medium (pH 3 and below) behaves as a basic buffer with $pK = 0.00$. As water is present in the system in a large excess, it can be considered, for all practical purposes, as an Immobiline*.

EXPERIMENTAL

Immobilines of pK 3.6, 4.4, 4.6, 6.2 and 7.0 were obtained from LKB (Bromma, Sweden). Immobiline of pK 0.00 (1 M) or $pK -1.74$ (55.56 M) was prepared from tap water purified first on an ion-exchanger bed and then distilled. Immobiline of pK 0.8 was a gift from Dr. E. Boschetti (IBF, Villeneuve La Garenne, France). Computing algorithms were as previously described^{1,2}.

RESULTS

As no recipe was available for a pH 3–4 gradient, we tried to derive one from the nearest formula described, a pH 3.5–5 interval, as reported in ref. 3. By linear interpolation, the new values for a pH 4.0 extreme were derived (see Fig. 1). By extrapolation, a new set of concentrations for Immobilines generating a pH 3.0 starting point was also calculated (Table I, central row, and Fig. 1). When this set of data were analysed by computer, the pH 4.0 formulation turned out to be correct, whereas the pH 3.0 recipe was markedly inaccurate, resulting in pH 3.27 instead of the pre-

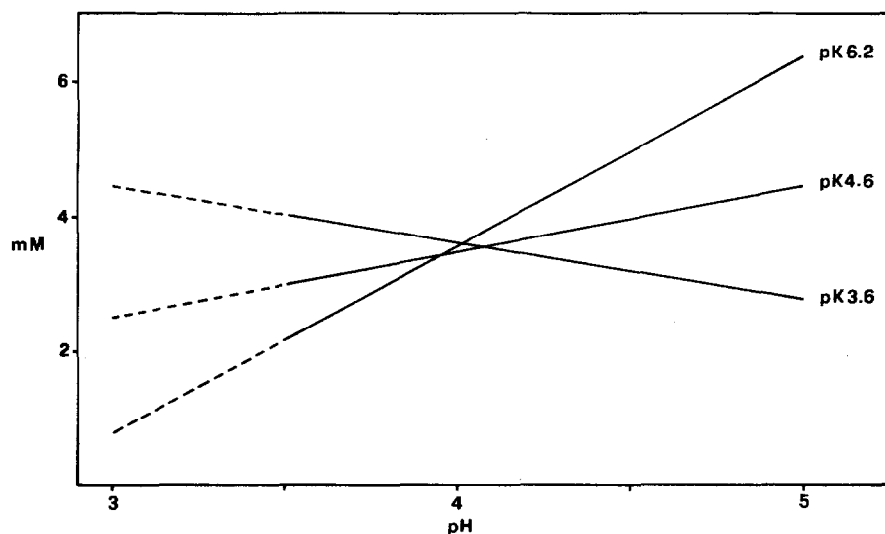


Fig. 1. Algebraic derivation of a pH 3–4 gradient. The molarities of the nearest tabulated recipe (pH 3.5–5) were plotted against pH and new values were derived by interpolation for the pH 4.0 extreme and by extrapolation for pH 3.0.

* The concept of water as an Immobiline is introduced here just as a provocative idea and for computational purposes. We all agree that water is not, in fact, an Immobiline, especially at such extreme pH gradients where an electrochemical water pump (electroendosmosis) is active.

TABLE I
 RECIPE FOR A pH 3-4 GRADIENT BY ALGEBRAIC DERIVATION

All values in mM.

<i>Immobiline</i>	<i>Chamber 1*</i> (pH 3.0)	<i>Chamber 2**</i> (pH 4.0)
pK 3.6	4.45	3.60
pK 4.6	2.50	3.42
pK 6.2	0.75	3.50

* By extrapolation.

** By interpolation.

dicted value. This was unexpected, as over such a short distance of extrapolation (only 0.5 pH unit) an error of this order of magnitude (50% of the pH interval) has never been found when extrapolating any other pH range (maximum 5-10% deviation; results not shown).

It occurred to us that at pH 3.0 (and below) the water present in the system would start to act as a buffering base, thus rendering more alkaline the pH extreme given in Table I, calculated in the absence of water. We therefore recalculated a new, optimized and linearized recipe for a pH 3-4 interval, which is shown in Table II.

Note in this formulation the presence of a new Immobiline, namely water, with pK = 0.00. The strong titrant pK 0.8 Immobiline was added only to generate a linear pH 3-4 gradient. An anodic extreme of pH 3.0 can be obtained by replacing the pK 0.8 acid with the pK 3.6 Immobiline, but the deviation from linearity in the middle of the pH gradient will be large (20% instead of the mere 1-2% of the pH interval as given in the optimized formulation). The physico-chemical parameters of the formulation for the pH 3-4 gradient are given in Fig. 2.

To test our hypothesis of water as an Immobiline we simulated different kinds of pH gradients that can be generated by subtracting one or more components from the pH 3.0 formulation given in Table II. If water is ignored, the pH 3.0 recipe gives

TABLE II
 RECIPE FOR AN OPTIMIZED pH 3-4 GRADIENT

All values in mM.

<i>Immobiline</i>	<i>Chamber 1</i> (pH 3.0)	<i>Chamber 2</i> (pH 4.0)
pK 0.00 (water)*	1000	1000
pK 0.8	1.01	0.00
pK 3.6	3.85	3.49
pK 4.4	2.49	3.42
pK 7.0	0.90	3.42

* Water is considered as a basic Immobiline with pK = 0.00.

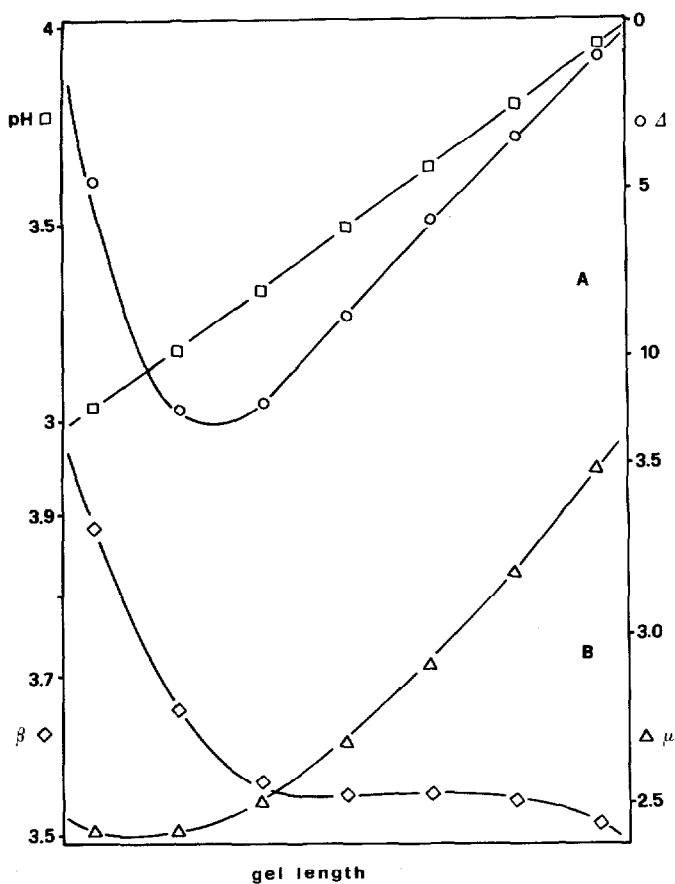


Fig. 2. Physico-chemical parameters of the optimized pH 3-4 recipe given in Table II. (A) Plot of pH (left-hand scale) and its deviation from linearity (Δ , right-hand scale, in thousandths of a pH unit). (B) Corresponding buffering power (β , left-hand scale) and ionic strength (μ , right-hand scale). β is expressed in mequiv. $l^{-1} pH^{-1}$ and μ in mequiv. l^{-1} .

a gradient starting at pH 1.56 (Fig. 3). If the pK 0.8 titrant is subtracted, the pH gradient will start at pH 3.27 (just as predicted by computer for the original recipe of Table I obtained by extrapolation in Fig. 1). If both water and pK 0.8 titrant are omitted, the pH gradient will apparently start at pH 3.0; in reality, because water is present in all formulations, the real pH of such a mixture will still be 3.27 (as also measured with a pH meter). Note that, owing to the very low pK of water (0.00), its contribution to the buffering power of the pH 4.0 formulation (and, of course, of any other pH value above that) will be negligible; in fact, by re-simulating the pH extreme in the absence of water, a pH of 3.96 is obtained.

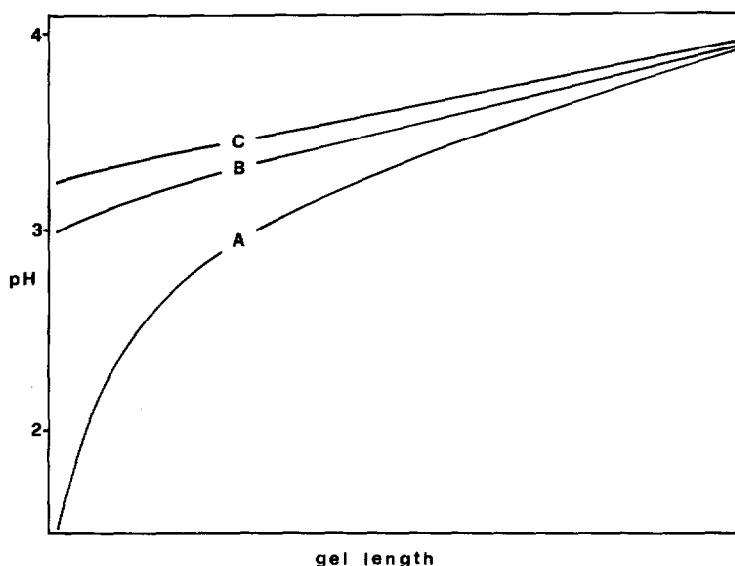


Fig. 3. Simulated pH gradients in the absence of some components from the recipe for the pH 3-4 gradient in Table II. (A) pH gradient in the absence of the Immobiline water (1 *M*); (B) pH gradient obtained by the simultaneous subtraction of water and Immobiline of *pK* 0.8; (C) pH gradient without Immobiline of *pK* 0.8 in the formulation.

DISCUSSION

According to the Brönsted-Lowry theory, water can be considered, at acidic pH, as a buffering base with $pK = 0.00$; at alkaline pH, the conjugated acid will have a pK of 14.0. The isoionic point of water will thus be pH 7.0. Based on these considerations, any Immobiline formulation given for pH ranges at pH 11 and above will have to contain a new acidic Immobiline, water, in the recipe, with $pK = 14.0$. In a previous paper⁷ dealing with acidic IPG ranges, while the concept of water as a basic Immobiline was not specifically dealt with, its buffering capacity was taken into consideration in the calculation of the β power, so that similar results were obtained even though no optimized recipe for a pH 3-4 gradient was given.

It should be noted that Table II contains a constant amount of the Immobiline water (1 *M*) independent of the pH of the solution. This might appear paradoxical, as the concentration of water in pure water is 55.56 *M*. The discrepancy can be resolved by considering not just the molarity of water, but also its activity. In fact, both the water activity and the activity coefficient of pure water are unity on the molar fraction scale. In the Immobiline solution the water molar fraction still remains practically unity and the Immobiline molarities and their molar fractions remain practically identical, differing, in the case of our recipe, by slightly less than 1%. The problem can also be resolved in a completely symmetrical fashion. According to the original Brönsted-Lowry theory, water should be considered, at acidic pH, as a buffering base with $pK = -1.74$ and thus, at alkaline pH, as a conjugated acid with $pK = 15.74$. In both instances, of course, in the equation of the equilibrium constant

referring to the dissociation of water at 25°C one should use, for its concentration, not just the activity coefficient (1 *M*) but the true molarity of water in pure water (55.56 *M*). Note that in our formulation in Table II, by replacing the original set of values for the Immobiline water ($pK = 0.00$; molarity = 1 *M*) with the new set described above ($pK = -1.74$; molarity = 55.56 *M*), identical results are obtained.

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