Journal of Chromatography, 496 (1989) 121–128
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO 4951

PECULIAR FEATURES OF APPLICATION OF pH GRADIENTS FORMED IN BORATE BUFFER WITH A POLYHYDROXY COMPOUND FOR SEPARATION OF PROTEINS IN A FREE-FLOW ELECTROPHORETIC APPARATUS

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(First received March 24th, 1989; revised manuscript received July 10th, 1989)

SUMMARY

The use of pH gradients in borate buffer with a polyhydroxy compound for separation of proteins in a free-flow electrophoretic apparatus is outlined. The composition of the pH gradients, the free-flow apparatus design and its operation, and the main causes of failures in the protein separation are considered.

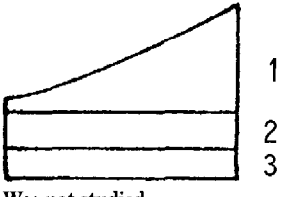
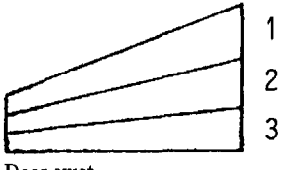
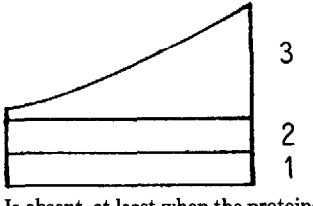
INTRODUCTION

Previously we reported the separation of proteins in the free-flow electrophoretic apparatus using pH gradients formed in borate buffer with a polyhydroxy compound [1–3]. In particular, borate–mannitol pH gradients were described [1,3], as well as the pH gradients obtained by the formation of a concentration gradient of boric acid in solutions of borax and mannitol [2,3]. These pH gradients are destroyed in the electric field, hence they cannot be applied for isoelectric focusing of proteins in the free-flow apparatus. However, the use of pH gradients in borate buffer with a polyhydroxy compound allows separation of proteins better than that obtained with conventional free-flow electrophoresis. A promising field for the application of pH gradients is the separation of proteins when it is necessary to purify a protein from a mixture without separating the latter.

0378-4347/89/$03.50

TABLE I

PROPERTIES OF pH GRADIENTS IN BORATE BUFFER WITH A POLYHYDROXY COMPOUND (PHC)

Property	The pH gradient with a variable ratio of analytical concentrations of borate ions to PHC molecules	The pH gradient with an initially constant ratio of analytical concentrations of borate ions to PHC molecules	The pH gradient in a solution of borax and PHC
Mode of creation of the pH gradient	Formation of the concentration gradient of PHC in borate buffer	Formation of the concentration gradient of PHC in borate buffer	Formation of the concentration gradient of boric acid in borate buffer
Function of PHC	Creates the pH gradient	Creates the pH gradient	Shifts the pH gradient along the pH scale
PHC concentration along the pH gradient	Increases towards the acidic end of the pH gradient	Increases towards the acidic end of the pH gradient	Does not change
Concentration of borate buffer along the pH gradient	Does not change	Changes in the same way as PHC concentration	Increases simultaneously with the rise of the concentration of boric acid
Analytical concentrations of buffer ions along the pH gradient	Do not change	Change in the same way as PHC concentration	Do not change
Ratio of analytical concentrations of PHC to buffer ions along the pH gradient	Is variable	Is constant	Is constant
Presence of the conductivity gradient along the pH gradient	Small decrease of conductivity with increasing PHC concentration	Increase of conductivity with increasing concentration of borate buffer	Is absent
Viscosity of solutions along the pH gradient	Increases with the rise of PHC concentration	Increases with the rise of PHC concentration	Increases (insignificantly) with the rise of the concentration of boric acid
General scheme of formation of the pH gradients (1 = PHC concentration; 2 = concentration of buffer ions; 3 = concentration of boric acid)			
Dependence of the results of protein separation on the direction of migration of their molecules along the pH gradient	Was not studied	Does exist	Is absent, at least when the proteins are introduced near the isoelectric points

The aim of the present work is to generalize the experience gained in using such pH gradients for protein purification in the free-flow apparatus.

An investigator making a decision to use pH gradients in borate buffer for protein separation must be able to answer the following questions: (1) What pH gradient is necessary? (2) What must be done to achieve success in protein purification in the free-flow apparatus using the selected pH gradient?

It may seem surprising that the same thesis underlies the answer to both questions: all efforts must be directed to purifying the protein of interest from the mixture prior to the decay of the pH gradient.

The main requirements to the pH gradient are as follows. It must be resistant to action of the electric field of the free-flow apparatus. The resistance of the pH gradient is dependent on its chemical composition (e.g. on the presence of the initially constant ratio of concentrations of borate ions and polyhydroxy compounds along the pH gradient) and on the apparatus design (in particular, on the properties of the membranes between the electrode compartments and separation chamber). The pH gradient must be in the region of the pH scale where electrophoretic mobility of the protein to be purified is clearly distinct from those of mixtures. This region is often (but not always) found in the vicinity of the isoelectric point of the protein of interest. The slope of the pH gradient must be neither very smooth nor very sharp. In the first case the situation will resemble conventional free-flow electrophoresis, and that can cause a deterioration of the separation quality [4]. In the second case the situation is opposite: the pH gradient becomes similar to the step pH gradient [5] and one should not expect a fine protein separation.

COMPOSITION OF pH GRADIENTS

In routine work it is desirable that the pH gradients are easily formed and that the reagents necessary for gradient formation are inexpensive and readily available. The pH gradients that are simplest in composition and in their mode of formation in borate buffer are those generated in the presence of a single polyhydroxy compound. These gradients will be described in this paper.

Any available polyhydroxy compound that does not affect the properties of proteins in solutions and is capable of changing the pH of borate buffers over a wide pH range is considered to be suitable for pH gradient formation. We chose D-mannitol [1,2]. The concentration of the compound is constant in pH gradients obtained by formation of a concentration gradient of boric acid in borax solution. In the borate–polyol pH gradients the concentration of the polyhydroxy compound changes (see Table I).

We obtained good results, in terms of the quality of the protein separation, pH gradient stability and operating conditions of the apparatus with a vertical planar separation chamber (dimensions: $38 \times 5 \times 0.06$ cm, 25-cm-long electrodes, a cooling plate), in the pH gradients generated in the 2.5 m*M* borax

solution. In the pH gradients formed by the concentration gradient of boric acid in such a solution, buffering capacity and conductivity are on a par with those known for the pH gradients in 1% ampholine solutions [6,7]. In the borate–polyol gradients with the initially constant ratio of analytical concentrations of components, these values decrease from the acidic towards the alkaline end of the pH gradient, in a similar manner to the change of borax concentration. The 2.5 m*M* borax solution can be recommended as a base to form the pH gradients (the procedure for their production was described previously [1,2]. In principle, the borax concentration can be varied. But it must be borne in mind that in case of pH gradients generated by the concentration gradient of boric acid, this would necessitate the formation of a new concentration gradient of acid to obtain the same pH gradient. An increase in the borax concentration in solution would require an appropriate increase in the concentration of boric acid (see below, eqn. 5). The necessary concentration of boric acid may appear to exceed its solubility limit, i.e. the required pH gradient would not be obtained. On the other hand, as the borax concentration decreases the buffering capacity diminishes. In case of the borate–polyol pH gradients the decrease in the borax concentration is undesirable since this would lead to a lowering of the buffering capacity in solution.

The range and shape of the pH gradient in a solution of borax and the polyhydroxy compound are determined by the concentration gradient of boric acid and also by the borax concentration. The pH value of the buffer solution is described by the Henderson–Hasselbalch equation. When the buffer solution consists of a weak acid HA and its sodium salt NaA, the equation is as follows [8]:

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\mathrm{A}^-]}{[\mathrm{HA}]} \tag{1}$$

where $\mathrm{p}K_a = -\log K_a$ (K_a is the dissociation constant of the weak acid $\mathrm{HA} \rightleftarrows \mathrm{H}^+ + \mathrm{A}^-$); [HA] and [$\mathrm{A}^-$] are equilibrium concentrations of the acid and its anion, respectively.

Borax in aqueous solution dissociates in the following way [9]:

$$\mathrm{Na_2B_4O_7} + 7\mathrm{H_2O} \rightleftarrows 2\mathrm{H_3BO_3} + 2\mathrm{B(OH)_4^-} + 2\mathrm{Na^+} \tag{2}$$

Thus, borax solution is a buffer that includes boric acid $\mathrm{H_3BO_3}$ and its anion $\mathrm{B(OH)_4^-}$. The pH of such a solution is described by the equation

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\mathrm{B(OH)_4^-}]}{[\mathrm{H_3BO_3}]} \tag{3}$$

where $\mathrm{p}K_a = 9.24$ at 25°C (K_a is the dissociation constant of boric acid). For our calculations, replacement of the equilibrium concentrations [$\mathrm{H_3BO_3}$] and [$\mathrm{B(OH)_4^-}$] with the analytical concentrations $C_{\mathrm{H_3BO_3}}$ and $C_{\mathrm{B(OH)_4^-}}$ is permis-

sible. Assuming the borax concentration C_b and taking into account that dissociation of a borax molecule yields two molecules of boric acid and two borate ions, i.e. $C_{H_3BO_3} = C_{B(OH)_4^-} = 2C_b$, eqn. 3 becomes

$$pH = pK_a + \log\frac{2C_b}{2C_b} \tag{4}$$

If such a solution is supplemented additionally with boric acid at a concentration of C_a, the pH will be described by the equation

$$pH = pK_a + \log\frac{2C_b}{2C_b + C_a} \tag{5}$$

Eqn. 5 shows how the concentration of boric acid should be varied to achieve a particular change in the pH of the borax solution. Using this equation, we calculated the concentration gradients of boric acid to form the pH gradient. The calculations are simple and do not need special mention. In our experiments [2] a linear pH gradient covering 1 pH unit appeared to be sufficient for successful separation of proteins. In the 2.5 m*M* borax solution it is formed by the logarithmic concentration gradient of boric acid in the range 0–45 m*M*. A pH gradient covering 2 pH units is generated in 2.5 m*M* borax solution by the formation of the concentration gradient of boric acid in the range 0–495 m*M*. It should be noted that, if the borax concentration is fixed, the particular concentration gradient of boric acid generates a pH gradient that is unique in its range, shape and location on the pH scale. The introduction of the polyhydroxy compound into the borax solution induces a shift of the pH gradient to a more acidic region without any change in its shape and range, as if the pK_a in eqn. 3 changed from 9.24 by the pH value of the starting borax solution in the presence of the polyhydroxy compound.

The range and shape of the borate–polyol pH gradients are mainly determined by range and shape of the concentration gradient of the polyhydroxy compound. The shift within the pH scale is dependent on the overall concentration of the polyhydroxy compound: the greater the concentration the more acidic the region where the pH gradient is generated.

Salts, such as NaCl, enhance changes of the pH gradients in the electric field, therefore their presence in solutions is undesirable.

WORK WITH pH GRADIENTS IN THE FREE-FLOW APPARATUS

The pH gradients in borate buffer with a polyhydroxy compound represent a preliminary created set of solutions with gradually varying pH values. Therefore the separation chamber of the free-flow apparatus should be equipped with a multi-channel inlet to introduce the pH gradient solutions. When using the apparatus with a planar separation chamber the best results are achieved with the arrangement vertical (inlet in the upper part). A multi-channel peristaltic

pump to pump solutions through the chamber is better installed below the chamber. In this case the solutions are introduced via all the channels more evenly than when the pump is placed at the inlet. The multi-channel pump should ensure stable laminar flow of the liquid in the separation chamber during the running time.

Dialysis and cation-exchange membranes are suitable for separating the electrodes from the separation chamber, but anion-exchange membranes are not. A correct choice of membrane allows one to decrease drastically changes of the pH gradient, and hence to improve the conditions of protein separation. It is possible that membranes with amphoteric properties may prove suitable when the pH gradients under study are used in the free-flow apparatus.

In the experiments previously described [1,2], the anode buffer differed from the cathode buffer in composition, and separate centrifugal pumps for circulation of the solutions through the electrode compartments were needed, as were separate buffer vessels. This is not an essential condition. A borax solution without a polyhydroxy compound can be used as a common buffer for both electrodes, irrespective of the value of the pH at which the pH gradient was formed. In this instance only one pump and one buffer vessel are required; the concentration of borax in the electrode solution should not be lower than that in the pH gradient. When the borax solution is used as a common electrode buffer, it is important to ensure an electric break in the fluid flow.

The most pronounced changes of the pH gradients in the free-flow apparatus develop near the membranes separating the chamber from the electrodes. Therefore, we recommend that the pH gradient should be admitted into the chamber via the middle entrance channels, and that the solutions of the acidic and alkaline ends of the pH gradient should be introduced into the regions adjacent to the membranes (ca. 25% of the chamber width on each side) via the lateral channels. It is important that the flow-rates of all the solutions (except for that of the sample) are the same for each channel. If the viscosities of the solutions are fairly high and not equal, as is observed in the case of borate–polyol pH gradients in the acidic pH region, uniform flow-rates will not be achieved and hence hydrodynamic distortions of the gradients are possible, leading to poorer protein separation.

Since the pH gradients under study change in the electric field, the operating conditions of the apparatus must be chosen so that the protein separation occurs prior to the decay of the pH gradient. One should not attempt to separate proteins at extremely high field strengths and residence times, since this can cause pronounced decay of the pH gradients. In our experiments [1,2] a satisfactory separation of proteins was achieved at an average voltage drop of 100–300 V/cm and residence time of 40–70 s in the chamber. However, for any other apparatus (e.g. with other dimensions of the separation chamber) the conditions will be expected to be different, and the values presented are to be considered as tentative ones. It is desirable that, during fraction collection and

stabilization of voltage across the electrodes, the current in the apparatus should not change. If the current is stabilized, changes of voltage do not produce any essential influence on the separation quality.

The sample should be injected into the separation chamber by a separate pump at a rate two or three times lower than the flow-rate in the chamber, so that the sample jet does not touch the chamber walls. It is desirable that the sample is injected via one of the middle channels to get it into the centre of the chamber where changes of the pH gradient during the experiment are insignificant. It is clear that the pH of the sample must not differ significantly from those of adjacent gradient solutions. A small excess of the conductivity over those of gradient solutions is permissible (in our experiments [1,2] this was no more than two-fold; differences in conductivities disappeared in the separation chamber). The protein concentration should not be so high that the protein would precipitate near the isoelectric point. Keeping to the conventions mentioned, the protein concentration in the sample does not significantly affect the results of the separation.

To achieve a successful separation of proteins, it is necessary to choose the point of injection of the sample into the pH gradient. In the case of pH gradients generated by the concentration gradient of boric acid in solutions of borax and polyhydroxy compound, the sample can be injected close to the isoelectric point of the protein of interest. When the borate–polyol pH gradients with the initially constant ratio of analytical concentrations of borate ions to molecules of polyhydroxy compound are used, the sample should be injected so that the main part of proteins can migrate from the cathode towards the anode.

MOST PROBABLE CAUSES OF FAILURE OF PROTEIN SEPARATION

Lack of separation is probably due to the following reasons.

(1) The operating conditions of the apparatus, in particular the field strength and residence time, are chosen incorrectly. Two variants are possible: the values of the field strength and (or) residence time are too low and the proteins have insufficient time to separate during the experiment; or they are too high, which induces gradient changes leading to deterioration of the separation pattern. Besides, separation cannot be achieved if the sample injection rate is not low enough compared with the flow-rate in the separation chamber. The operating conditions of the particular apparatus can be optimized by preliminary separation of a known sample, e.g. bovine haemoglobin or human serum albumin.

(2) The point of sample injection into the pH gradient is not chosen correctly: in this case one should attempt to achieve the separation by varying the point of injection, having ascertained that the operating conditions of the apparatus had been chosen correctly.

(3) The electrokinetic properties of the proteins (in particular, charge densities and their dependence on pH values) are similar to or slightly different from each other: in this case it is impossible to separate proteins in the free-flow apparatus under the relatively short-term influence of the electric field.

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