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Isoelectric focusing field-flow fractionation

Experimental study of the generation of pH gradient[a]

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ABSTRACT

Isoelectric focusing field-flow fractionation is a method for the separation of ampholytes using, in addition to the electric field and pH gradient, the flow of the carrier liquid through the fractionation channel as the third active separating factor. Flow action permits a decrease in the channel dimension in the direction of the electric field. It makes it possible to decrease the absolute value of the voltage while maintaining a high field strength and results in a lower Joule heat production. Moreover, the laminar flow of the carrier liquid stabilizes the pH gradient against convection. A channel was designed from which ampholyte samples can be taken at twelve different positions. pH value of the samples taken were measured with the aid of a capillary pH electrode. The pH dependence of the applied voltage, flow-rate of ampholyte solutions and concentrations of the solutions of ampholyte and electrode electrolytes were measured at different channel positions. It follows from the measurements performed so far that the pH gradient generation is sufficiently fast and reproducible for use in isoelectric focusing field-flow fractionation.

INTRODUCTION

Isoelectric focusing (IEF), an electromigration method for the separation and characterization of amphoteric compounds [1], has become an important method for protein studies. Gel IEF, a technique with very high resolution [2], has been widely used, but a number of works have recently appeared that assume the initial idea of IEF in solutions [1]. This makes it possible to extend the field of applications to particles and cells which cannot pass through gels, to create continuous and discontinuous preparative systems and to reduce the time required for analyses [3–8].

Despite considerable progress in instrumentation, some problems have remained unsolved. The first is the time required for the analysis which, with rare exceptions, reaches several hours, also the application of high voltages, leading to intense Joule heat production and requiring the use of efficient cooling systems and finally the difficult detection and characterization of the substances being separated.

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The application of focusing field-flow fractionation is a possible solution to these problems [9]. The method, utilizing for the separation, in addition to the electric field and pH gradient, the flow of a liquid through the separation channel as the third active separating factor, was named isoelectric focusing field-flow fractionation (IEF_4) [10]. The shape of the flow velocity profile of the carrier liquid is influenced by the geometry of the fractionation channel [9]. The method was put into practical use by Chmelík *et al.* [10] in a trapezoidal cross-section channel and by Thormann *et al.* [11] in a rectangular cross-section channel. The latter group named this technique electrical hyperlayer field-flow fractionation (EHF_3).

The zone of an amphoteric solute is established owing to the action of the electric field and pH gradient at that position in the channel where the intensity of forces is zero. After dynamic equilibrium between the focusing and dispersive processes has been reached, the narrow focused solute zones, having an approximately Gaussian distribution of concentrations, are located at different positions of the channel depending on their isoelectric points. The direction of the liquid flow inside the fractionation channel is perpendicular to the direction of the effective electric field. The velocity profile formed in the liquid flow causes the migration of focused zones along the channel at different velocities, so that the solutes are longitudinally separated.

The flow acting as the separation factor makes it possible to reduce the channel dimension in the direction of the electric field, which enables absolute voltage values to be decreased with the maintenance of a high field strength. This results in a decrease in the Joule heat production. The reduction in the channel dimensions also decreases the time required for focusing. Further, the laminar flow of the carrier liquid stabilizes the pH gradient against convection [10]. Another advantage is provided by the detection mode permitted by the elution character of the method [11]. Steady-state solute zones need not be mobilized in order to pass them across a sensor, but detectors used in liquid chromatography can be used.

Sufficient speed of the establishment of the pH gradient and its stability are fundamental assumptions for effective separations by IEF_4. A number of theoretical and experimental studies characterizing pH gradients in different techniques have been published (for reviews, see refs. 12 and 13). However, no study has been published concerning the generation of pH gradients in IEF_4. This work was aimed at investigating the dynamics of the pH gradient in the IEF_4 channel and studying the influence of various experimental parameters on the pH gradient.

EXPERIMENTAL

The IEF_4 channel used in the study of pH gradient generation is illustrated schematically in Fig. 1. The channel is composed of three Perspex parts clamped together with brass bolts. The core is the block in which is the channel proper with dimensions of length 250 mm, height 12 mm and width 1 mm. This block also includes inlet and outlet capillaries which are used for the introduction of ampholyte solution (the capillary situated at the channel head at its half-height). Injection of the sample (the capillary situated in the distance of 2 cm from the channel head at its half-height) and taking of ampholyte samples for pH measurements (four triplets of capillaries situated at one quarter, half and three quarters of the channel length and at its end with individual capillaries of one triplet situated at one quarter, half and three quarters of

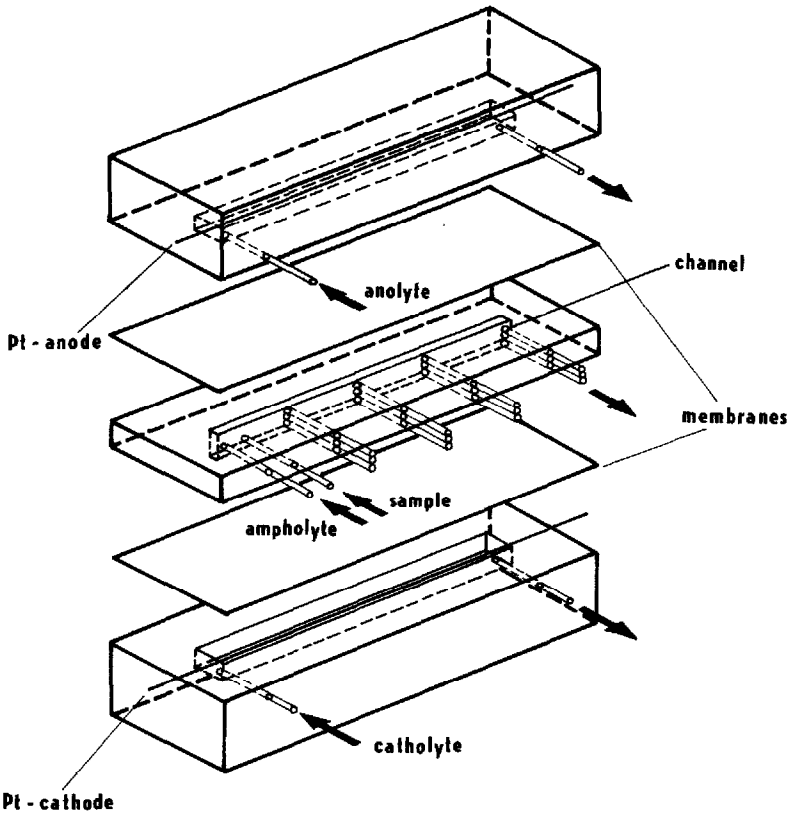

Fig. 1. Schematic illustration of the IEF_4 channel used for the study of pH gradient generation.

the channel height). The remaining two blocks, used for fixing platinum wire capillaries and comprising electrode reservoirs, are separated from the channel interior space with PGCL ultrafiltration membranes (Millipore, Bedford, MA, U.S.A.) with a nominal molecular weight cut-off of 10 000.

The scheme of the experimental arrangement for the measurement of pH gradients is shown in Fig. 2. An M 122 doser (Mikrotechna, Prague, Czechoslovakia) with two injection syringes was used to pump solutions of electrode electrolytes, *i.e.*, solutions of sodium hydroxide and acetic acid, into electrode reservoirs. The flow-rate was 500 μl/min in all instances. An LD 2 linear feeder (Development Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia) was used to pump ampholytes into the channel. Another LD 2 device with three identical syringes was used to suck ampholyte samples from the channel. These linear feeders were adjusted in such a way that the volumes of the ampholyte injected were identical with the total volume taken by all three syringes. The syringe denoted a was used to take ampholyte samples from the anodic side of the channel (*i.e.*, in the acidic range of the pH gradient), syringe b was used to take samples from the alkaline range of the pH gradient in the cathodic side of the channel and syringe c served for taking samples

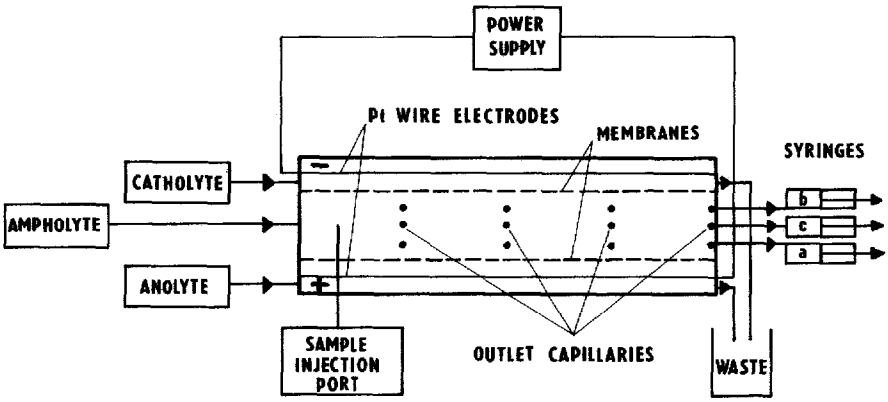

Fig. 2. Scheme of the experimental arrangement for the measurement of pH gradients in the IEF_4 channel.

from the middle of the channel. This arrangement ensured good reproducibility of the measurements. Another LD 2 feeder was used for the continuous injection of amphoteric dyestuffs.

The pH values of ampholyte samples taken with syringes a, b and c were measured with a capillary pH microelectrode (Radelkis, Budapest, Hungary).

The synthetic carrier ampholyte Servalyt 4-9T was a product of Serva (Heidelberg, F.R.G.). Other chemicals were obtained from Lachema (Brno, Czechoslovakia).

RESULTS AND DISCUSSION

Generation of pH gradient in the IEF_4 channel was studied as a function of various experimental parameters. Sampling from twelve different positions in the channel at three flow-rates of ampholyte solution (50, 150 and 500 μl/min), at three different concentrations (0.25, 0.5 and 1%, w/v) and at three applied voltages (25, 50 and 100 V) made it possible to obtain numerous experimental data characterizing the generation of pH gradients. In view of the large number of the data, it is not possible to present all of them here and only some examples have been selected. In spite of the fact that the height of the channel used exceeds several-fold the height of the channels used in earlier work [10,11], it is probable that, with certain restrictions, the results obtained could also be applied to channels of smaller height that are used for analytical purposes.

To illustrate the results, with the following four dependences the data obtained from the first triplet of sampling capillaries situated at one quarter of the channel length and those from the triplet of the sampling capillaries situated at the channel end only are presented in the figures. In the former instances this means from the location where pH gradient is just being generated and in the latter from the location where the steady-state pH gradient has already been reached, *i.e.*, a pH gradient which corresponds to the pH gradient that would be generated in a flowless arrangement at the given voltage and at the given compositions of ampholyte solutions and electrode electrolytes in the course of a sufficiently long time period. pH values from the

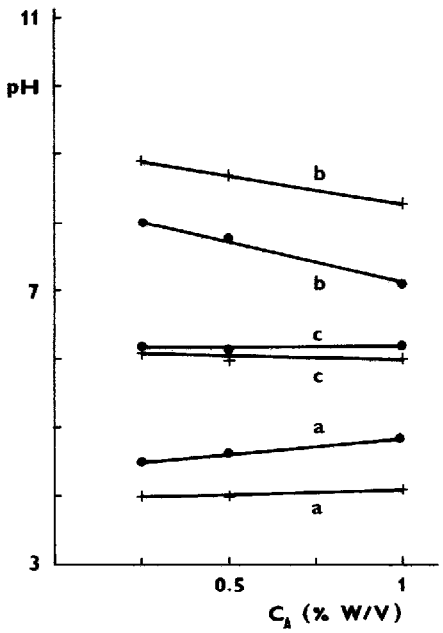

Fig. 3. Dependence of pH gradient on the concentration of ampholyte, c_A. Conditions: applied voltage, E = 100 V; flow-rate of ampholyte solution, v = 150 μl/min; concentration of electrode electrolytes, c_E = 0.05 M. Letters a, b and c denote syringes sucking ampholyte samples from the anodic and cathodic sides and the middle of the channel, respectively. (●) pH values obtained from the capillaries situated at one quarter of the channel length; (+) data from the capillaries situated at the channel end.

capillaries situated at half and three quarters of the channel length lie between these boundary values.

It is obvious from Fig. 3 that under the given experimental conditions the difference between the pH values in the anodic and cathodic sides of the channel decreases with increasing ampholyte concentration. The influence of the increase in the concentration of H^+ ions in the anolyte and of OH^- ions in catholyte appears as a decrease or increase in the pH of the anodic and cathodic sides of pH gradients (see Fig. 4). An increase in the applied voltage results in an acceleration of the migration of compounds that show electrophoretic mobility and a faster generation of the pH gradient, which under the experimental conditions used (Fig. 5) also appeared as an increase in the difference between the pH values in the cathodic and anodic sides of the channel. It follows from the results obtained that the steady-state pH gradient is not generated in the channel at lower values of the applied voltage and at higher flow-rates.

The influence of the flow-rate of the ampholytes on the generation of the pH gradient is illustrated in Fig. 6. This influence is evident in those parts of the channel where, under the given conditions, the steady-state gradient is not generated. This is illustrated in Fig. 6 by the pH values obtained from the capillaries situated in the first quarter of the channel. As long as under the given conditions (applied voltage and composition of solutions) the steady-state pH value is generated at a certain position in the channel at the highest flow-rate used, it is evident that the pH at this position in the

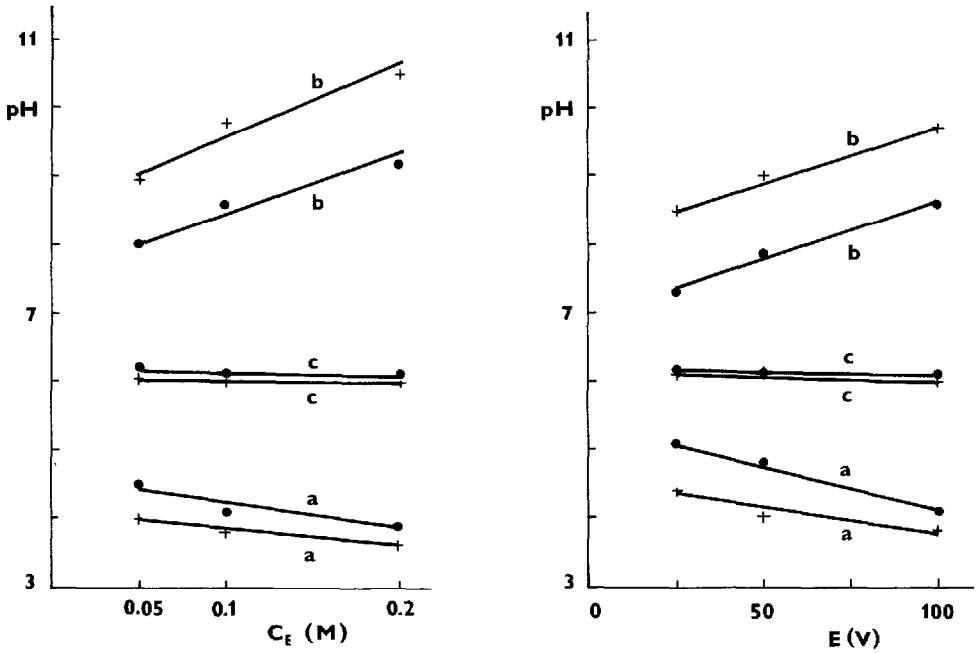

Fig. 4. Dependence of pH gradient on the concentration of electrode electrolytes, c_E. Conditions: E = 100 V; v = 150 μl/min; c_A = 0.25%. Other symbols as in Fig. 3.

Fig. 5. Dependence of pH gradient on the applied voltage E. Conditions: v = 150 μl/min; c_A = 0.25%; c_E = 0.1 M. Other symbols as in Fig. 3.

channel will not be dependent on the flow-rate at lower values. This situation is illustrated in Fig. 6 by the pH values obtained from the capillaries situated at the channel half-height and by those obtained from the capillary situated at the end of the channel in the anodic side of the channel. On the other hand, the situation in the cathodic side of the channel is more complicated, as certain changes in pH values appear, depending on the flow-rate.

It follows from the results of the measurements that at an applied voltage of 100 V the pH gradient corresponding to the steady-state pH gradient at a flow-rate of 50 μl/min is reached between a quarter and a third of the channel length. It is also evident that the steady-state pH values are reached faster in the acidic than the alkaline region of the pH gradients. At a flow-rate of 150 μl/min, pH values corresponding to the steady-state values are reached in the last quarter of the channel. At a flow-rate of 500 μl/min the pH values corresponding to the steady-state values in acidic and neutral regions of the pH gradient only are reached.

Measurements of the long-term stability of the pH values of the samples taken from a certain triplet of capillaries were also performed. The highest stability was obtained in the case of the capillaries situated at the end of the channel and at three quarters of the channel length, where no changes were observed at a flow-rate of 150 μl/min. The greatest changes were observed with the capillaries situated at one quarter

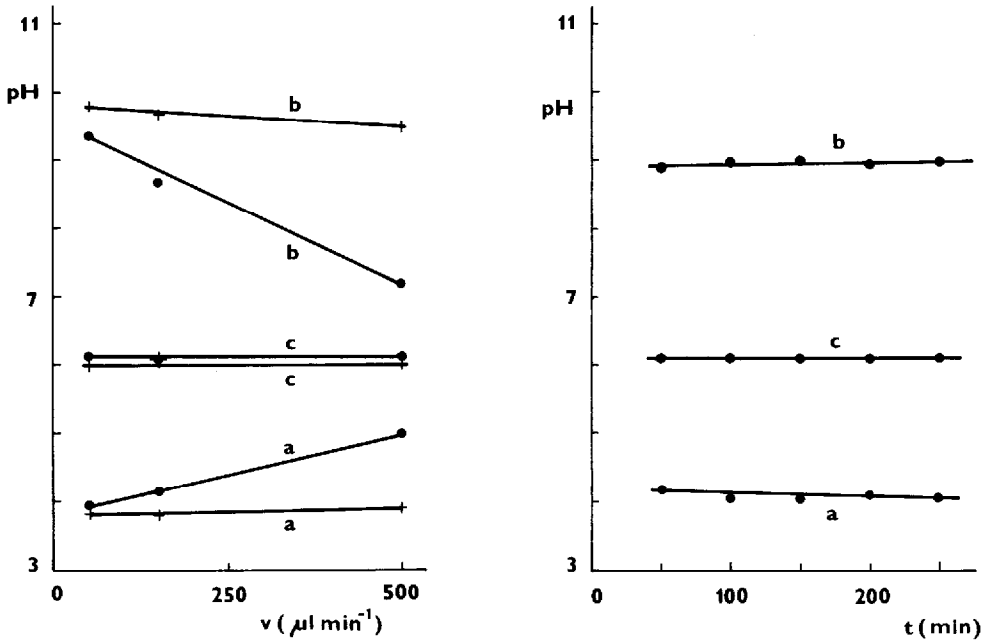

Fig. 6. Dependence of pH gradient on the flow-rate of the solution of ampholytes, v. Conditions: E = 100 V; c_A = 0.25%; c_E = 0.1 M. Other symbols as in Fig. 3.

Fig. 7. Stability of pH gradient studied with the aid of ampholyte samples taken from the channel half-length. Conditions: E = 100 V; v = 150 μl/min; c_A = 0.25%; c_E = 0.1 M. Letters a, b and c as in Fig. 3.

of the channel length in the cathodic side of the channel (±0.2 pH unit) and smaller changes in the anodic side of the channel (±0.1 pH unit). In the case of the capillaries situated at the channel half-length, no changes were observed if the samples were taken from the middle of the channel, whereas certain changes were observed if the samples were taken from the anodic and cathodic sides of the channel (±0.05 pH unit). As follows from Fig. 7, the stability of the pH gradient studied at the channel half-length for 250 min is very good. This is supported by the findings that the laminar flow of the carrier liquid favourably affects the stability of the pH gradient [14].

The dynamics of the generation of the pH gradient in the IEF_4 channel can be deduced from the results illustrated in Fig. 8, showing the pH values measured at different positions in the channel. The pH values measured at a flow-rate of 50 μl/min are plotted at relative positions corresponding to the positions of the sampling capillaries in the channel. In order to characterize pH gradient at the beginning of the channel, *i.e.*, before the sampling capillaries situated at one quarter of the channel length, the data from the sampling capillaries obtained at a flow-rate of 150 and 500 μl/min, respectively, were transformed.

The transformation was performed in such a way that the position of the given triplet of sampling capillaries was divided by the ratio of the flow-rate used to a flow-rate of 50 μl/min. This means that, *e.g.*, at a flow-rate used of 150 μl/min, the positions of the sampling capillaries were divided by three and the measured pH values

were plotted for these calculated positions. This transformation substantially means plotting pH values for those positions of the channel which would be reached by ampholyte solutions at a flow-rate of 50 μl/min within the same time during which the sampling capillaries are reached at a higher flow-rate. The data obtained at a flow-rate of 50 μl/min can easily be transformed into the state occurring at higher flow-rates and thereby an idea of the course of the pH gradient under these conditions can be acquired. Although the agreement of the data obtained in this way is not perfect (see Fig. 8), these results give an idea of the speed of the generation of the pH gradient under the conditions of the flow along the IEF_4 channel, for the scale of relative distances can be transformed into the time scale by dividing the absolute channel lengths by the linear flow-rate.

The results confirm the finding that the generation of pH gradients is relatively fast [15], but it is nevertheless necessary to be aware that the generation of a pH gradient in solutions of carrier ampholytes is a much faster process than reaching the steady-state distribution of various components of a mixture of carrier ampholytes and conductivity [13]. The generation of a pH gradient is thus one of the conditions required for successful separation. Reaching the steady-state distribution of carrier ampholytes gives rise to an increase in buffering capacity, *i.e.*, an increase in the stability of the pH gradient and thereby the ability of carrier ampholytes to dictate the pH characteristics of the gradient even in the presence of further ampholytes whose zones in such a pH gradient are narrower than those in a pH gradient with low buffering capacity.

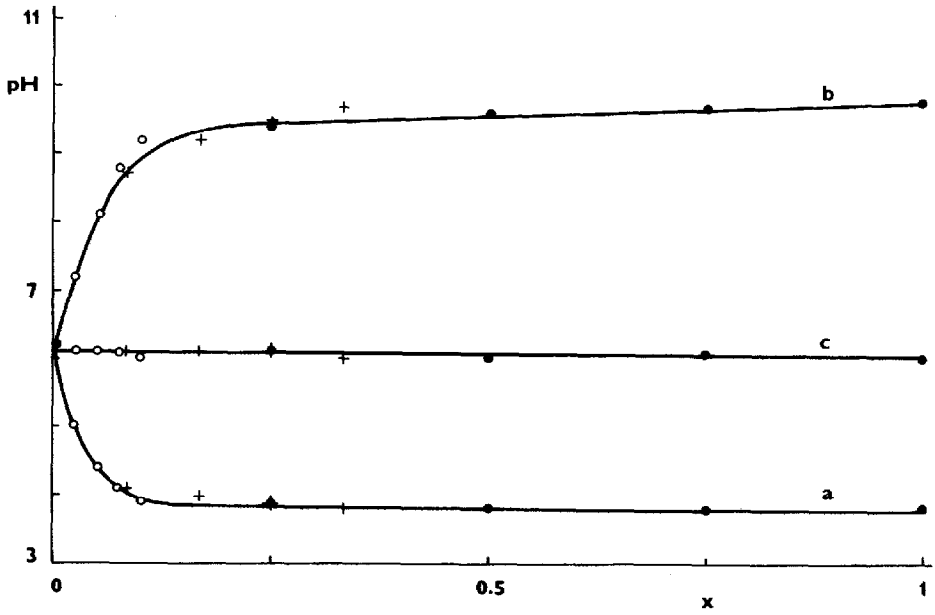

Fig. 8. Dependence of pH gradient on the relative position in the IEF_4 channel. Conditions: E = 100 V; c_A = 0.25%; c_E = 0.1 M. Relative positions x = 0 and x = 1 correspond to the beginning and end of the channel, respectively. Letters a, b and c as in Fig. 3. (●) pH values at a flow-rate of 50 μl/min; (+) and (○) pH values obtained at flow-rates of 150 and 500 μl/min, respectively, plotted on transformed positions.

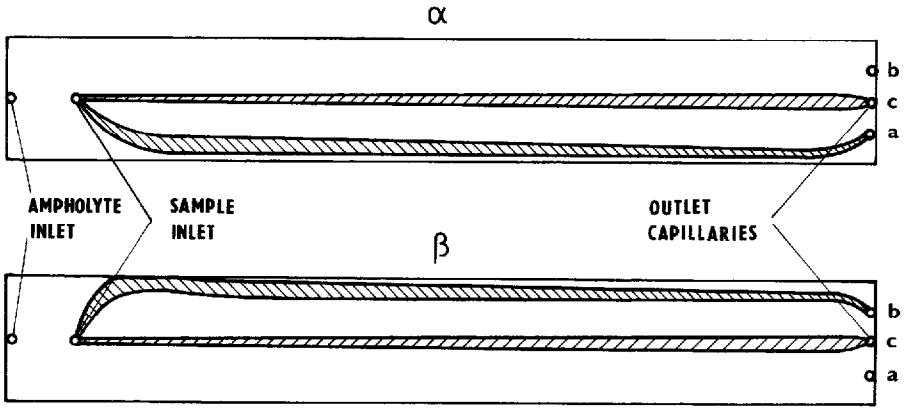

Fig. 9. Schematic illustration of the behaviour of continuously injected amphoteric dyestuffs in the IEF_4 channel. Conditions: flow-rate of the solution of dyestuffs, v_D = 30 μl/min; flow-rate of ampholyte, v = 150 μl/min; c_A = 0.25%; concentration of dyestuffs, c_D = 0.1 *M*. The hatched regions in the middle of both panels denote the flow of the dyestuff without the action of the applied electric field. The other hatched regions denote the flow of the dyestuff at an applied electric field of 100 V. Letters a, b and c as in Fig. 3. (α) Methyl red, electrode electrolytes used at a concentration c_E = 0.1 *M*; (β) 4-(4′-hydroxyphenylazo)-1-naphthylethylenediamine, electrode electrolyte used at a concentration c_E = 0.2 *M*.

The behaviour of low-molecular-weight ampholytes was studied with the aid of continuous injection of amphoteric dyestuffs into the channel. With both of the dyestuffs selected, methyl red for the acidic region and 4-(4′-hydroxyphenylazo)-1-naphthylethylenediamine for the alkaline region, the transformation from yellow to red occurs in the pH range between the neutral medium of the solution of ampholytes (pH 6.2) and their isoelectric points. This change in colour aids the visual investigation of the processes in the channel. As is obvious from the schematic illustration in Fig. 9, only a small part of the channel is required for transferring dyestuffs from the site of their injection to the positions corresponding to their isoelectric points. In the next part of the channel the zone of dyestuffs becomes narrower, which corresponds to gradual focusing of the dyestuff and obviously also to an increase in the buffering capacity of the pH gradient.

In addition to this visual investigation of the focusing of low-molecular-weight amphoteric dyestuffs, a more detailed study of the focusing of methyl red was performed by using Hewlett-Packard HP 1040 A diode-array detector. The detector was situated between a sampling capillary and a suction syringe. It was found that without the applied electric field, methyl red was only detected in samples taken from the middle of the channel (capillaries c). With an applied electric field of 100 V methyl red was only detected in samples taken from the anodic side of the channel (capillaries a).

The spectra of methyl red recorded both without and with the applied electric field are shown in Fig. 10. Spectrum a (without the applied electric field) corresponds to the yellow form of methyl red in the neutral pH range; the pH of the ampholyte solution was determined to be 6.2. Spectrum b (with an applied electric field of 100 V) corresponds to the red form of methyl red in the acidic pH range. The determined pH

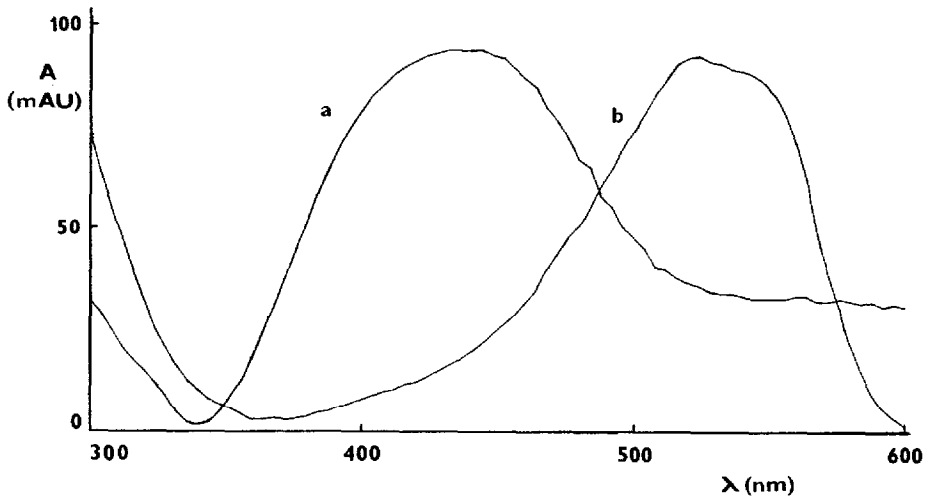

Fig. 10. Spectra of methyl red obtained in the investigation of the behaviour of continuously injected dyestuffs in the IEF_4 channel. Conditions: flow-rate of solution of methyl red, v_D = 30 μl/min; concentration of methyl red, c_D = 0.01 *M*; flow-rate of ampholyte solution, v = 150 μl/min; concentration of ampholytes, c_A = 0.25%; concentration of electrode solutions, c_E = 0.1 *M*. Capillaries a and c as in Fig. 3. (a) Spectrum of methyl red obtained from capillary c without the applied electric field; (b) spectrum of methyl red obtained from capillary a at an applied electric field of 100 V.

values of ampholyte solutions from capillaries a ranged from 3.9 to 3.8, which corresponds to the isoelectric point of methyl red.

Methyl red was not detected in any sample taken from each of the twelve capillaries if the pH of anolyte was increased from 3.0 to 5.0, *i.e.*, the pH of the anolyte was higher than the isoelectric point of methyl red. In this instance methyl red did not focus inside the channel but passed through the membrane into the electrode (anode) reservoir. These results confirm that the zone of methyl red is formed by isoelectric focusing if the pH gradient generated inside the channel contains the pH value corresponding to the isoelectric point of methyl red.

The results obtained only represent the first step in characterizing pH gradients in IEF_4 channels. With respect to the procedure used for taking samples for the pH measurements, these are of only a qualitative character. It will be possible to obtain more detailed information by placing the pH electrodes (*e.g.*, antimony or bismuth) directly in the channel or by studying changes in the potential gradient at different channel positions with the aid of an electrode array detector.

On the basis of the experience obtained so far, the conditions can be found under which a pH gradient is generated in the IEF_4 channel such that amphoteric compounds can be separated successfully. This is confirmed by results published recently on the fractionation of proteins [10,11]. Further research aimed at using IEF_4 must, in addition to a search for suitable experimental conditions, also include optimization of the separation channel with respect to both its design and the materials used [11,16]. The solution to these problems could lead to a reduction in the time required for the analysis to a few minutes for channels of rectangular cross-section [11] or to tens of

minutes for channels of trapezoidal cross-section [10], which would make IEF_4 suitable for various applications.

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