Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 21
Sofia ● 2015

Abnormal Maximum in the Current during Isoelectric Focusing and Certain Possibilities to Control and Utilize It

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Studying the influence of the electrochemical reactions occurring at the electrodes on the attainment of steady state we found that an additional process occurs at the electrodes, causing an abnormal increase of the current. Since the magnitude of the current determines the progress of IEF, knowledge gained from studies on its nature and generation reveals a possibility for this entity to be controlled. We observed that the addition of gelatin into the electrode solutions suppresses the magnitude of the current flowing through the system, which allows the IEF system to approach steady state for a shorter time.

Key words: isoelectric focusing, abnormal current, control of the electric current.

Introduction

Svensson formulated the basic concept of isoelectric focusing, according to which at steady state a dynamic equilibrium between the thermal diffusion and electrophoretic migration in the direction of the isoelectric state is attained, where the amphoteric molecules should remain indefinitely [16, 17, 18]. One of the important considerations in Svensson's theory concerns the electrochemical reactions which take place at the electrodes. The significant feature is that under the influence of the electric field water decomposition commences and the anode naturally becomes acidic, while the cathode naturally becomes alkaline. According to this concept, the continuous production of water ions causes a very steep micro pH gradient only in the near vicinity of the electrodes, which does not spread over the whole carrier matrix. Thus the large part of the gel remains neutral. However, a number of studies have shown that the steady state postulated by the theory is never attained [1, 9]. Furthermore, during IEF the measured pH gradient, created only by the electrode solutions, has a rather different profile along the carrier than that of the carrier ampholytes. These pH gradients, termed "primary", are characterized by a specific dynamics that provokes corresponding changes of the ampholyte pH gradient [7, 8]. These findings prompted us to study more closely the

"background" processes occurring in the electrode solutions, which have a bearing to the current flowing through the electrophoretic system.

Electrode Solutions used in IEF

Non-volatile solutions of acids and bases are used routinely in IEF, as summarized by Righetti [14]. This author does not recommend water as electrode solution. However, in another paper [15] the same author offers a list of electrode solutions commonly used for electrophoresis in Immobiline gels and specifies that water is applicable only when samples with high salt concentration are analyzed. A number of other authors have also employed water as electrode solutions [3, 4, 5, 6, 10, 12].

Water Decomposition and Transport phenomena during IEF

In a recent paper [8] we discussed the kinetics of the electrochemical reactions transferring electrons during IEF. Routinely, in the isoelectric focusing numerous acids and bases are employed as electrode solutions and a porous barrier of polyacrylamide gel separates them. There are many IEF techniques, but in the popular implementation of this method a thin platinum wire is laid on paper strips, soaked with electrode solutions that are in contact with the carrier gel, or the electrodes are immersed into appropriate electrode reservoirs. In all cases the products of water electrolysis are liberated into the electrode solutions, thus influencing their physicochemical properties during the process, by alteration of their pH.

Studying the process of water electrolysis it was found that there is a non symmetrical acidification and alkalization of both electrode solutions [8]. When an electric field is applied a two-way process begins, whereby the migration of the produced water ions is accompanied by diffusion of charge-compensating particles originating from the electrode solutions, and/or some constituents of the carrier matrix [7]. This process determines the conductivity of the system, and thus the magnitude of the current flowing through the system. As we reported earlier [8], the electrode current represents the reaction rate at which water is discharged on the platinum wire, which can be calculated applying the equation

$$i \sim k \frac{\Delta \mathrm{pH}}{\sqrt{t}}, \qquad (1)$$

where the rate constant k includes the number of transferred electrons, Faraday's constant, the electrode surface and diffusion coefficient of the respective particle, $\sqrt{t}$ is the duration of the electrophoresis and ΔpH is the difference between the initial pH of a definite electrode solution and the pH measured after a definite interval of time during the IEF process.

Steady state Condition

Irrespectively of the type of electrode solutions employed, when electrophoresis is carried out in power mode the current gradually decreases, tending to reach a minimal value. We observed the same correlation for the yield of water ions, which decreases during the process, following a non-linear relationship similar to that of the current. As we concluded in a preceding paper [8], as long as the electrode solutions are a continuous source of hydrogen and hydroxide ions the electrophoretic system will go through a sequence of transient states, gradually approaching steady state ($i = 0$), when the equal-

ity $i_{anode} = i_{cathode}$ should be valid. Thus studying the pH alterations when water alone is used as electrode solutions we formulated a criterion for the attainment of steady state, based on the proximity of the sum $pH_{anode} + pH_{cathode}$ to 14.

Alteration in the Electrode Current during IEF

Studying the processes occurring on both electrodes we observed that during the early stage of the experiment the current changes abnormally reaching unusually high values (**Fig. 1**). This side effect cannot be predicted by the theory [8]. After a relatively short interval of time the current decreases to a definite value from which asymptotically tends to zero, while the system is driving slowly toward a steady state. Analyzing the relationship between the electrode current and the pH of the electrode solutions we reached the conclusion that the attainment of steady state requires an indefinitely long isoelectric focusing process, the duration of which is proportional to the applied voltage [8]. A similar conclusion was reached earlier by Bier and Palusinski et al. [2, 11].

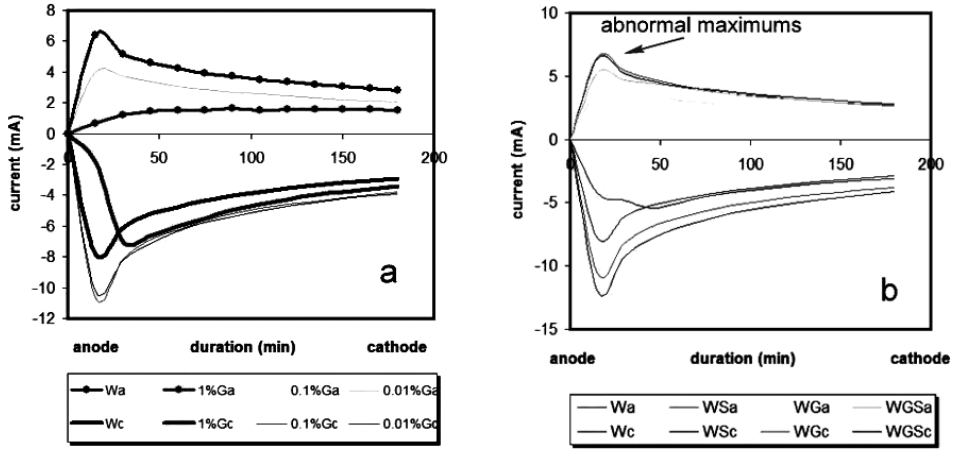

Fig. 1. a) Comparison of the electrode current when different electrode solutions are applied: W_a, W_c – distilled water alone; G_a, G_c – 1%, 0.1% and 0.01% (m/V) solution of gelatin. The anode and cathode currents are denoted by the indexes ($_a$) and ($_c$) respectively. PAG was prepared in the absence of carrier ampholytes. The extrapolation of the curves to current zero shows that the duration of electrophoresis needed to reach steady state should be different. The presence of 1% (m/V) gelatin brings about considerable decrease of both electrode currents;
b) Comparison of the electrode current when different electrode solutions are applied: W_a, W_c – distilled water alone; WS_a, WS_c – 0.1 M solution of Na_2SO_4; WG_a, WG_c – 0.1% (m/V) solution of gelatin; WGS_a, WGS_c – the solution contains 0.1 M Na_2SO_4 and 0.1% (m/V) gelatin. The anode and cathode currents are denoted by the indexes ($_a$) and ($_c$). PAG was prepared in the absence of carrier ampholytes

Therefore we can suggest that any substance present in the electrode solutions, which can suppress the electrolysis of water, will be suitable to bring the system nearer to steady state for a shorter time, which is the essence of the present study.

pH gradient Drifts

In a recent paper we discussed widely the reasons for the pH gradient drift during the IEF process. The considerations presented there enabled us to be the first to arrive at

the conclusion, that the gradient drift can be explained and predicted with a very good approximation by the alteration of both most important electrical parameters of the system: electrode potential and current [8]. The analysis of this phenomenon showed that during the early phase of the electrophoretic process the acidity of the electrode solutions changes asymmetrically and the system is strongly diverted from its steady state ($i_{anode} \neq i_{cathode}$). With time the change of the reaction rate of the electrolytic processes shows a clearly defined tendency for equalization of the electrode currents ($i_{anode} = i_{cathode}$), which is the reason for the observed decrease of the pH changes, occurring in the electrode solutions. Concomitantly with the processes of electron transfer, corresponding changes in mass transport take place in the system, which is the reason for the observed gradient drift.

Materials and Methods

Materials

Polyacrylamide gel slabs were prepared as previously described [8]. 2.2 ml of carrier ampholytes (CA) "Ampholyte high-resolution 3-10", Fluka & Riedel (catalogue No. 39878) per 60 ml gel were introduced, followed by 20 mg ammonium persulfate and 0.06 ml TEMED. Solutions of 0.1 M phosphoric acid (Merck, Germany) and sodium hydroxide (Reanal, Hungary) or distilled water alone (pH=6.75) were used as electrode solutions. In some instances 0.01%, 0.1% or 1% (m/v) gelatin (for electrophoresis, type A, G8150, Sigma) or Triton X-100 (CAS number 9002-93-1, laboratory grade, Sigma Chemical) and/or 0.1 M Na_2SO_4 were added to the electrode solutions. The total volume of each electrode solution was 100 ml. Each electrode solution was bubbled with argon prior to use for about 15 minutes. 5 µl of 5% (m/v) solution of Protein Test Mixture 9 ("wide-range" pI-Marker Proteins), purchased from Serva (catalogue No. 39206) were used as a protein standard. To avoid the interaction between the electrode solutions and the gel with atmospheric carbon dioxide, electrophoresis and measurements were performed under argon.

Equipment and Isoelectrophoretic conditions

We should stress that we selected the conditions for IEF so as to be able to register the changes of pH of the electrode solutions, and to ensure a satisfactory separation of the standard protein mixture employed by us. The choice of a rather larger volume of the electrode solutions is due to the technical characteristics of the electrophoresis bath used by us, as well as to ensure a reliable and easy measurement of pH without interrupting the IEF process.

Electrophoresis was carried out using a Pharmacia ECPS 3000/150 Power Supply (Sweden) and an LKB 2117 Multiphor (Sweden) apparatus cooled by running water. The gel was maintained at a temperature of about 10 °C. Platinum electrodes (thin platinum wire – 0.3 mm in diameter, 26 cm length) hanging on a plastic plate (LKB, Sweden) were immersed to the bottom of both electrode solution reservoirs, where the electrode strips were soaked in the corresponding electrode solution. The strips were connected to the gel ends by Whatman 3MM chromatographic paper. The power supply was set to the limiting values of 800 V, 20 mA and 15 W. The duration of the process was read from the moment when the apparatus was switched on only when the purpose of the electrophoretic run was to calculate the alteration of the electrode current during the process. Routinely the duration was read from the moment when the voltage reached the limiting value of 800 V. A pH-meter for water with low conductivity

780 pH meter Metrohm (Switzerland) equipped with Aquatrode plus combined LL pH glass electrode was used.

Measuring Procedure

To follow up the pH changes occurring in the electrode solutions composed of water alone, or containing gelatin or Triton X-100 and in the absence or in the presence of sodium sulfate, we performed a series of experiments as follows: at regular 15 min intervals during the IEF process 6 ml samples were withdrawn from the electrode solutions and were transferred to tightly capped vials. The pH was measured and the samples were returned to the electrode reservoirs. The temperature of the electrode solutions was 22 °C. The electrode current was calculated using equation (1).

Staining and destaining was performed according to the method described by Righetti and Drysdale [13].

Results and Discussion

Physicochemical Influence on the Electrode Current

Studying the electrochemical reactions occurring on the electrode surface and taking into consideration their relationship with the electrode current we reached the conclusion that under the conditions of IEF the abnormal maximum, which is always registered, is analogous to the same phenomenon observed in polarography. In polarography the abnormal jump of the current is suppressed by addition of small amounts of certain substances like the non-ionic detergent Triton X-100 or gelatin. In this paper we turned our attention to the analogous side effect observed by us, which is registered for both electrode currents in electrophoresis. We tried to resolve this problem adapting the knowledge gained from polarographic studies in an attempt to find a way for effective suppression of the electrolysis of water, which in its turn will bring about a decrease of current.

Influence of Gelatin added to the Electrode Solutions on the Electrode Current

We studied the influence of gelatin contained in the electrode solutions on the current flowing through the electrophoretic system. The obtained data presented in **Fig. 1a** show that the decrease of the anode current is proportional to the concentration of the gelatin dissolved in electrode solutions of distilled water. Furthermore, the decrease of the anode current leads to a corresponding increase of the cathode current when the concentration of gelatin is in the range of 0.01-0.1%. However, when the highest feasible 1% concentration of gelatin was employed there was a considerable lowering of both currents.

Unfortunately, when electrode solutions similar to the widely used phosphoric acid and sodium hydroxide are employed, because of their masking role it is not possible to follow up the changes in pH during electrophoresis. However, it is beyond doubt that as a result of the ongoing electrolysis of water there is continuous release of hydrogen and hydroxide ions into the electrode solutions [7, 8]. Considering that the presence of an acid and/or a base increases the intensity of water electrolysis, as a substitute of the routinely used electrode solutions, we employed water solutions of the non-hydrolyzing salt sodium sulfate, which only brings about increased water electrolysis and higher conductivity, without hampering the measurement of pH. The obtained results showed that when sodium sulfate is introduced into the electrode solutions the anode current increases become higher than those calculated when distilled water alone was used. However, at the cathode this effect is very small (**Fig. 1b**). Indeed, when the anodal cur-

rent increases the current at the opposite electrode compensatory decreases during the early stage of the experiment.

When in addition to the salt gelatin is introduced in the electrode solutions to a final concentration of 0.01% or 0.1%, the abnormal maximum, as well as the anode current as a whole, slightly decreases, but the registered decrease becomes greater at high gelatin concentration of 1%. These experimental facts gave us good reason to conclude, that the presence of gelatin in electrode solutions composed of phosphoric acid and sodium hydroxide will ensure the attainment of the maximal preset voltage for a shorter run time, i.e. the electrophoretic system will approach steady state faster. Furthermore, our data clearly show that there is a direct relationship between the concentration of gelatin and the decrease of the electrode current, which leads to faster equalization of anode and cathode currents, i.e. to steady state, where $i = 0$.

We can now propose that the current flowing through both electrodes can be decreased efficiently by adding 1% gelatin to the electrode solutions, so that steady state is attained for a shorter run time.

Influence of Triton X-100 on the Electrode Current

On the basis of the experience in polarography next we studied the influence of 0.01%, 0.1% or 1% concentrations of Triton X-100 introduced in the electrode solutions on the electrode current. Contrary to the results obtained with gelatin containing electrode solutions, in the case of Triton X-100 we found a reciprocal relationship. The data presented in **Fig. 2** show that there is a directly proportional relationship between the concentration of Triton X-100 and the magnitude of both electrode currents, which means that the electrophoretic system will be diverted significantly away from the steady state. This result can be exploited in some analytical cases, since it can be employed as a tool to manage the electrode currents according to our needs.

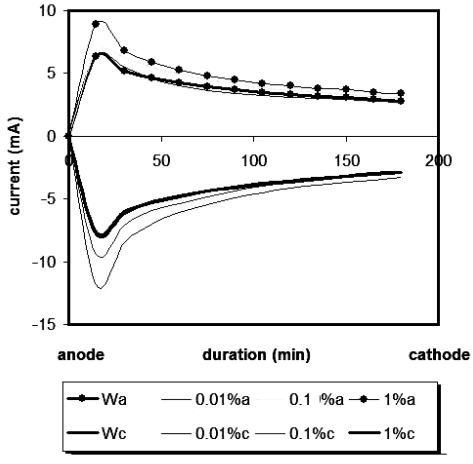

Fig. 2. Comparison of the electrode current when different electrode solutions are applied: W_a, W_c – distilled water alone; 0.01%, 0.1% and 1% solutions of Triton X-100. The anode and cathode currents are denoted by the indexes ($_a$) and ($_c$) respectively. PAG was prepared as in Fig. 1

IEF in the presence of Gelatin or Triton X-100 in the Electrode Solutions

To further verify the influence of gelatin added to the electrode solutions consisting of phosphoric acid and sodium hydroxide, we carried out IEF of a standard protein mixture. For the purposes of comparison we used two types of electrode solutions – the first contained 1% gelatin and in the second gelatin was omitted. It was established that the maximal preset voltage is reached 30 min faster when gelatin is introduced into the electrode solutions, as compared to the case when gelatin is absent. Electrophoresis was continued for two hours after the maximal preset voltage was reached when the process was interrupted and the electrophoregrams were compared. As can be seen in **Fig. 3** the focused protein bands have a very similar separation concerning the number of separated bands, however, their position along the gel is different. This result apparently reflects the pH gradient drift during electrophoresis, which is influenced by the electrochemical processes occurring at the electrodes [8].

Obviously, the presence of gelatin in the electrode solutions brings about suppression of the electrolysis of water, which results in a reduction of the amounts of hydrogen and hydroxide ions liberated in the electrode solutions. At this stage of our investigations we cannot rule out the possibility for a certain buffering action of gelatin, which can bring about a smaller alteration of the pH of the electrode solutions, as compared to the case when gelatin is absent. Moreover, this fact is closely related to the changes of the electrode potentials during the IEF process, which are a function of pH electrode solutions [8].

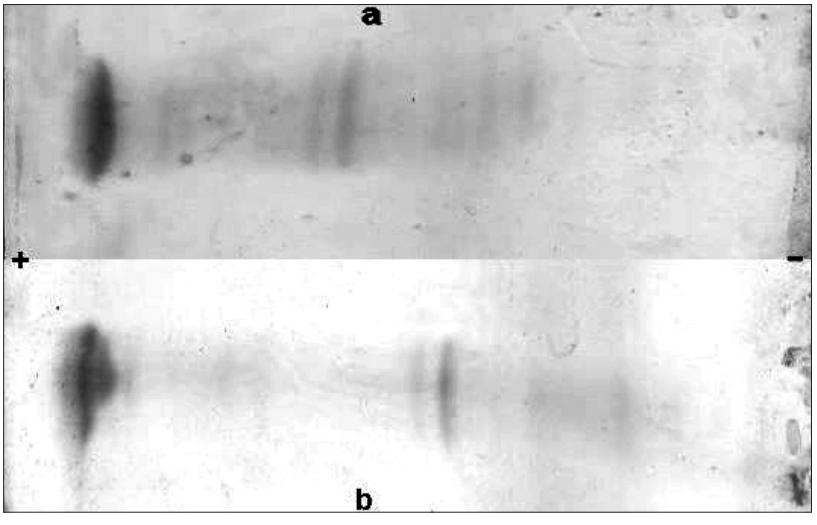

Fig. 3. IEF of Protein standard mixture. Two types of electrode solutions were employed: 0.1 M phosphoric acid and sodium hydroxide (a), and 0.1 M phosphoric acid and sodium hydroxide in the presence of 1% gelatin (b). PAG was prepared in the presence of carrier ampholytes. The maximal preset voltage is reached 30 min faster when gelatin is introduced into the electrode solutions, and then the ectrophoresis was continued for two hours at 800 V. The focused protein bands have an almost identical separation and sharpness, however, their position along the gel differs, which is caused by the pH gradient drift. Running conditions: 800 V, 20 mA, 15 W. The letters denoting the electrophoregrams correspond to the electrode solutions employed

The same electrophoretic system was studied, where gelatin was replaced with 0.01%, 0.1% or 1% concentrations of Triton X-100. We found that the time needed to reach the preset limiting voltage is proportional to the concentration of Triton X-100. Carrying out IEF in the presence of 1% Triton X-100 we observed a considerable prolongation of the time for which the voltage reaches the preset limiting value, approximately 90 min. At the same time the magnitude of the current was very high throughout the process. In addition we observed that the electrophoresis was accompanied by a considerable transport of water toward the anode, which caused a swelling of the carrier gel. Under these conditions the anodal proteins precipitate, thus compromising the electrophoretic separation.

The statistical treatment of the results on the time taken by the electrophoretic system to attain the preset maximum voltage of 800 V shows that in the presence of gelatin in the electrode solutions the decrease is 30 ± 4 min, as compared to electrophoretic runs performed in the absence of gelatin. This 10% decrease of the duration of electrophoresis apparently reflects the contribution of the electrolysis processes, occurring at the electrodes, to the deviation of the system from its steady state.

The comparative analysis of the electrophoregrams obtained in the presence and in the absence of gelatin shows that no additional protein bands can be found. Therefore, the inclusion of gelatin into the electrode solutions only influences the reaction rate of water electrolysis and reduces selectively the quantity of the ions H^+ and HO^- produced during the IEF process.

We found that the addition of Triton X-100 to the electrode solutions brings about the considerable increase of 90 ± 7 min of the time taken by the system to reach 800 V. Presently we cannot assess the significance of this result for the practice of IEF, however, we believe that it has a potential application.

Concluding Remarks

Contemporary IEF applications are typically characterized by a plethora of physical phenomena. This, in addition to their ever-increasing scope and complexities, makes their realistic modeling a daunting task. Central to modeling electrophoresis is the understanding of both transport and stoichiometric behavior of analytes in the electrolyte system in the presence of numerous driving forces. This work is a continuation of our efforts towards developing a comprehensive, and at the same time, a sufficiently simple model for realistic IEF applications and techniques, supplementing certain substances into the electrode solutions. The core of the present work, however, is to stress again the importance of the electrode solutions for the entire IEF process, which so far appears to be overlooked. In this paper we offer a simple modification of the electrophoretic method, allowing to predict and control the electrode current. We believe that the results reported in the present paper are a further contribution toward the elucidation of the role of the electrode solutions in the isoelectrophoretic process.

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