A New Approach to Isoelectric Focusing and Fractionation of Proteins in a pH Gradient

Stephen J. Luner and Alexander Kolin

DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF CALIFORNIA, LOS ANGELES

Communicated by Louis B. Slichter, April 13, 1970

Abstract. This paper describes a new method of condensation (focusing) of extended volumes of mixtures of proteins (or other ampholytes) into an isoelectric spectrum of discrete zones located at points of a pH gradient corresponding to the pI value of the individual proteins. In contradistinction to the currently practiced isoelectric focusing in “natural” pH gradients which may require as much as 96 hours for completion, the present method yields clear-cut condensations marking the isoelectric pH within about five minutes and complete fractionation within about 15 minutes. No “Ampholines” or other special buffers are required and establishment of the pH gradient requires only about 10 seconds rather than days as is common in natural pH gradient focusing. The pH gradient is generated by utilization of the temperature dependence of pH. By establishing a temperature gradient between 0° and 50°C in an electrophoretic column, a stable pH gradient extending over 1 pH unit can be maintained in the absence as well as the presence of a current. The pH gradient can be easily controlled by variation of the temperature limits so that high resolving power can be achieved in shallow pH gradients. The pI of the focused fractions is determined by measurement (by a thermistor or resistance thermometer) of the local temperature within each of the isoelectric zones that determine the local pH. The individual zones can be pipetted out. The method is illustrated by simultaneous condensation and evacuation of hemoglobin in two pH gradients traversed by opposite currents and by separation of hemoglobins A and S in 16 minutes in a pH gradient where the current passes in the direction of increasing pH in Tris buffer solutions stabilized by a sucrose density gradient.

Introduction. Electrophoretic mobility has been used for decades as a basis for separation and characterization of proteins. The resolving power of the separations is limited, and the characterization by mobility is not unique because of the dependence of the protein mobilities on the pH and ionic strength of the buffers.

In 19541-2 a new method was proposed for separation and characterization of proteins on the basis of differences in their isoelectric points. This approach led in a subsequent modification3-4 to unique characterization of proteins and to high resolution in preparative separations. Proteins differing by as little as 0.01 pH unit in their pI values could be separated. The protein is distributed in an electrophoretic column in which a pH gradient is maintained with a concomitant density gradient (the latter for stabilization against thermal convection). An
electrical current directed toward increasing pH sweeps a protein which is positively charged in sections where pH < pI toward the isoelectric zone where it is arrested due to loss of charge. Similarly, protein ions which are negatively charged in regions where pH > pI are swept in the direction opposite to the current toward the isoelectric zone where they come to a stop. Thus, eventually all of the protein is condensed in a sharp isoelectric zone whose pH can be measured to ascertain the isoelectric point of the protein. N protein components differing in their pI values give as many sharp isoelectric condensation zones.

In the initial approach, the pH gradient was prepared by appropriate buffer mixture. Rapid isoelectric condensations (or “focusing”) have been obtained in less than 5 min. The weakness of this method of implementing the isoelectric focusing idea was a drift of the pH gradient which made it impossible to reach a stable steady-state pattern with each isoelectric protein zone created precisely at its isoelectric point within the pH gradient. This instability was removed by creating a stable steady-state pH distribution through achievement of an equilibrium between electromigration and diffusion of buffer ions. This process is time consuming (in some cases requiring as much as 96 hr). In addition it requires special costly synthetic ampholytes ("Ampholines") to buffer the fractionation column. The Ampholines must be removed from the collected fractions by dialysis at the end of the process. Their absorption in the ultraviolet may interfere with spectrophotometric evaluation of the fractionation. These drawbacks are offset by an unprecedented resolving power in separation as well as in characterization of proteins which can be separated and characterized within about one-hundredth of a pH unit.

The present development was prompted by a desire to achieve isoelectric focusing and fractionation within a short time span with a high and easily adjustable resolving power without the use of special buffering materials such as the Ampholines. The initial implementation of the new idea is a simple U-tube apparatus filled with a common buffer such as Tris adjusted to a pH of intermediate value within the range between the highest and lowest pI of the components to be fractionated. A pH gradient is then generated by purely physical means within a few seconds so that isoelectric focusing can begin almost immediately leading to separations within several minutes and within a pH range of about one pH unit.

The thermally engendered pH gradient column: The generation of the pH gradient in the present method is based on the following considerations. In a Tris-HCl buffer the pH is established according to the equilibrium relation:

$$K = \frac{[H^+](\text{Tris})}{(\text{Tris} H^+)}.$$  

(1)

The variation in hydrogen ion activity of a buffer solution with temperature can be generally expressed logarithmically as:

$$\frac{\partial \rho_{H^+}}{\partial T} \approx - \frac{\partial \log K}{\partial T} - (2z + 1) \frac{\partial \log \gamma}{\partial T},$$  

(2)

where \(\gamma\) is the activity coefficient of an average ion of valence \(z = 1\). The first
of these two terms can be obtained from the gas constant \( (R) \) and the molar heat of dissociation of an acid \( \Delta H^\circ \):

\[
\frac{\partial \ln K}{\partial T} = \frac{\Delta H^\circ}{RT^2}.
\]

For the case of Tris, the second term is less than 10% of the value of the first term. Thus, if we start with a buffer of a given pH at room temperature and cool its bottom portion to close to \( 0^\circ \)C and raise the temperature of its top section to, say, \( 50^\circ \)C, we shift the pH values of the top and bottom sections of the buffer column in opposite directions and create a vertical pH gradient within the buffer column if leakage of heat through the side walls of the column is avoided. The time required for establishment of the pH gradient depends on the rate of heat flow in the system and has been found to be less than 1 min in the present apparatus.

The pH gradient is stable against redistribution of the ion concentration by the current compared to the artificial pH gradients originally used. This is because the concentration ratio of ions present in more than negligible amounts, \( \text{viz.}, \text{Tris H}^+ \) and \( \text{Cl}^- \), is practically constant (1:1) everywhere. The apparatus resembles the original U tube + "M layer" scheme used in the first pH gradient separator. In the present instrument (Fig. 1), the U tube is, however, surrounded with two jackets \( J_1 \) and \( J_2 \) through which water of \( 0^\circ \) and \( 50^\circ \)C, respectively, is circulated. The jackets are separated by a horizontal block of lucite 2 cm in thickness, \( L \). The U tube section below \( L \) is filled with a heavy 0.025 \( M \) Tris-HCl buffer solution \( B_1 \), containing 1 \( M \) sucrose. There is no sucrose in the upper buffer, \( B_2 \), which is otherwise of exactly the same composition as \( B_1 \).

![Fig. 1.—Apparatus for isoelectric focusing and fractionation in a thermal pH gradient. The clear sections represent a framework milled from a 3-inch lucite block. \( J_1, J_2 \): Water jackets through which water of 0° and 50°C respectively is circulated. The glass U tube passes through the water jackets and the channels drilled for it through the solid lucite partition \( L \). \( C_1, C_2 \): Electrode compartments containing the electrodes \( + \) and \(-\). \( B_1 \): Sucrose-buffer solution of maximum density; \( B_2 \): sucrose-free buffer, \( M_L, M_K \): solutions of intermediate density containing the ampholyte mixture. The arrow indicates the direction of the thermally generated pH gradient.](image-url)
The solution in the intermediate layers \( M \) (labeled \( M_L \) in the left leg and \( M_R \) in the right leg of the U tube) are of intermediate density (\( M/2 \) sucrose). The solution in the “\( M \) layers” is made up by dissolving in it sucrose and the protein mixture to be fractionated (the latter at great dilution) and adjusting with Tris to the same pH as solutions \( B_1 \) and \( B_2 \). The upper and lower boundaries of the \( M \) layer are stirred to create a uniform density gradient to stabilize the interior of the \( M \) layer against thermal convection. Actually only the left \( M \) layer (\( M_L \)) is used for fractionation. The right layer (\( M_R \)) has been used in this study only for demonstration of the protein evacuation effect which is the opposite of the focusing effect. \( M_R \) is to be omitted in the practical use of the method.

The ends of the U tube are submerged in buffer solution which fills the compartments \( C_1 \) and \( C_2 \). \( C_2 \) harbors the Pt cathode while the Pt anode is in compartment \( C_1 \). It is essential to prevent electrolysis products from traveling from the electrodes to the vicinity of the \( M \) layer. Appropriate protection is achieved either by use of large electrode compartments or by connecting \( C_1 \) and \( C_2 \) by salt bridges to large adjacent electrode vessels.

A voltage of about 300 volts maintains a current on the order of 9 ma through the apparatus under normal operating conditions. The 0° and 50°C solutions circulated through the jackets \( J_1 \) and \( J_2 \) are derived from an ice-water bath and a Precision Thelco constant temperature bath, respectively.

**Experimental Procedure.** At the beginning of a run, the solutions are shown to be at the same pH (within 0.01 pH unit) at room temperature (22°C) prior to generation of the temperature gradient. After establishment of the temperature difference between \( J_1 \) and \( J_2 \), the pH of \( B_2 \) exceeds that of \( B_1 \) by 0.5 to 1 pH unit depending on the temperature difference and the choice of buffer. If isoelectric focusing of a protein, say, hemoglobin, is to be demonstrated, the original buffer pH is chosen so that there will be a point within the thermally established pH gradient at which hemoglobin will be isoelectric at the temperature of the isoelectric zone. For hemoglobin, we used a 0.025 M Tris-HCl buffer of pH 7.40 at 22°C. This buffer gave a pH gradient between pH_1 = 7.78 and pH_2 = 6.78 in the 7°-45°C temperature range. Other possible buffers with high \( \frac{\partial \text{pH}}{\partial T} \) values over various pH ranges can be selected from data given in references 6 and 9.

Human hemoglobins in the oxyhemoglobin form have been chosen to illustrate the mode of operation of the method. Hemoglobin solutions were prepared after Drabkin’s method by lysing washed erythrocytes with 1.5 vol of water, shaking with 0.5 vol of toluene, and then centrifuging. The aqueous phase containing the hemoglobin was pipetted out and dialysed against water. The \( M \) layer solution was prepared by adding 0.1 ml of the dialysed hemoglobin solution to 40 ml of \( M/2 \) sucrose solution and adjusting the pH with Tris to that of the buffer solutions.

About 5 min after application of the voltage, one sees a clear-cut condensation zone of hemoglobin in the \( M_L \) layer as shown in Figure 2B as compared to the initial hemoglobin distribution seen in Figure 2A. Simultaneously a clear evacuation zone free of protein develops in the \( M_R \) layer seen in Figure 2B. In the latter region, hemoglobin migrates toward the upper and lower edges of the \( M_R \) layer where its migration velocity is retarded due to a low potential gradient in the more highly conductive buffer columns \( B_1 \) and \( B_2 \). The dark band near the bottom of the \( M_R \) layer has been formed by this mechanism. Reversing the current restores the original condition of the \( M \) layer and one can, in fact, obtain a focusing of the hemoglobin in the \( M_R \) and a defocusing of the focused hemoglobin zone in the \( M_L \) layer followed by formation of an evacuation zone at the location of the original condensation zone. The \( C \) and \( E \) zones are not at the same level because of differences in the conductivity distributions in the two \( M \) layers whose boun-
FIG. 2.—(a) The sample-containing belts \( M_L \) and \( M_R \) containing a hemoglobin A solution prior to passage of current. (b) Evidence of hemoglobin condensation \((C)\) inside the \( M_L \) column and evacuation from a narrow zone \((E)\) in the \( M_R \) column after passage of 9 ma for approximately 5 min. The dark zone at the bottom of the \( M_R \) column is formed by conductivity gradient focusing of the descending protein ions. (Buffer pH 7.40 at 22°C.)

Boundaries are stirred by hand. The Joule heat developed in the electrolyte column affects the temperature distribution and, hence, the precise location of the isoelectric zones in the two \( M \) layers.

The stability of the thermally engendered temperature gradient is exhibited by the stationary nature of the protein condensation zones. A further demonstration of stability was provided by pH determination at room temperature of portions of the buffer columns removed from the \( M \) layers and adjacent regions after completion of electrophoresis. The measurements revealed no observable change from the initial uniform pH values.

A unique convenience of the present method is the possibility of pH determination within the isoelectrically focused protein zones with a thermometric microprobe. This probe can be a thermistor or a fine insulated wire serving as a resistance thermometer. Thus, the pH determination amounts to a temperature measurement which could yield the pH in the zone to better than 0.01 pH unit from the known pH versus temperature curve. This creates the possibility of characterization of proteins available in small amounts through experiments performed on a microscale.

Figure 3 shows an example of a fractionation of two proteins, hemoglobin A and hemoglobin S, whose isoelectric points are 0.2 pH units apart as determined by temperature measurements which yielded pH values of 7.12 at 33°C for hemoglobin A and 7.33 at 25°C for hemoglobin S. Their wide separation in a deliberately steep pH gradient suggests that much smaller pH differences could be easily resolved.

A particular advantage of this method is the possibility of varying the range and slope of the pH gradient simply by varying the temperatures in the water jackets \( J_1 \) and \( J_2 \). There is actually no lower limit to the shal-
lowness of the pH gradient which the experimenter could produce to enhance the resolv-
ing power.

In order to separate a given pair of ampholytes it is not necessary for the pH gradient to contain points of a pH equaling their pI values; it is merely necessary for it to contain a point of a pH intermediate between the two pI values. In this case the two proteins will migrate in opposite directions toward the upper and lower boundaries of the $M_L$ layer where they will be condensed into sharp zones by conductivity gradient focusing.2,8

Conclusions. The thermal pH gradient electrophoresis has thus the following advantages: A pH gradient encompassing about 1 pH unit down to any desired smaller width can be generated and stably maintained as well as adjusted by a superimposed temperature gradient. Ampholytes, such as proteins, can be rapidly focused (within about 15 min) in such a pH gradient and the pH can be determined with a thermal microprobe at the site of the condensation zone to characterize the protein. A mixture of proteins can be resolved into an isoelectric spectrum of distinct zones which can be separated from each other by pipetting. No special buffering substances, such as Ampholines, are required which may be difficult to remove as contaminants at the end of the separation and would affect the absorption spectrum of the collected fractions. Common buffer solutions can be used.

Two improvements in apparatus and procedure were suggested by the initial experiments with hemoglobins. To rapidly achieve a steady equilibrium temperature in the compartments $C_1$ and $C_2$ they should be in more efficient thermal contact with the upper water jacket, while they should be better insulated from the surrounding air. Secondly a mechanical density gradient generator is suggested for the introduction of $M$ layers of accurately reproducible density gradient and conductivity.

The demonstrated effects suggest that this method may be implemented in a variety of instrumental arrangements for zonal as well as continuous flow separations.

Support of this work by Cancer Detection Services, Inc., is gratefully acknowledged. We are also indebted to Dr. Patricia N. Konrad of the Department of Pediatrics, and Dr. Robert S. Sparks of the Department of Medicine for blood samples, and to Mr. Gordon W. Culp for his kind assistance in preparing the drawing. Thanks are due to Mr. A. Ken Meadors of the Life Sciences Instrumentation Facility for the fabrication of the apparatus.

2 Kolin, A., these PROCEEDINGS, 41, 101 (1953).