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Isoelectric Focusing: Fundamental Aspects

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Abstract

The principles, history, types, and applications of isoelectric focusing are presented. Also discussed are the theoretical aspects, electrooptical scanning, methodological parameters, and apparent physical constants. The lack of suitable carrier ampholytes is the primary reason why isoelectric focusing has not reached its full potential.

INTRODUCTION

Principle

Isoelectric focusing is an electrophoretic method which utilizes the migration behavior of amphoteric molecules in a pH gradient to achieve their condensation into narrow isoelectric zones that are stationary in the electric field. The steady-state position of each zone in the pH gradient depends on the isoelectric point (pI) of a particular amphoteric molecule, therefore isoelectric focusing can be used as a separation technique. The method involves mainly two processes which can be carried out either simultaneously or separately. These include (a) the formation of a stable pH gradient which increases from the anode to the cathode, and (b) the electrophoretic migration of the amphoteric molecules under study (e.g., proteins) toward their respective pI positions with subsequent attainment of the steady-state. At present the stable pH gradient is formed by the use of carrier ampholyte mixtures with specific properties which contain components with pI's within a defined pH range.

In order to understand how isoelectric focusing takes place, let us consider a model system where convective remixing of separating components does not occur. The anode (positive charge electrode) is placed in a strong acid (e.g., phosphoric acid) and the cathode in a strong base (e.g., NaOH). The two electrodes are then connected to a dc constant voltage power supply to produce an electric field between them. The negatively charged cathode in the electrolysis cell attracts positive ions and repels negative ions; the opposite occurs at the anode. If an amphoteric compound is present in the system, such as a carrier ampholyte, it will become negatively charged at the cathode and positively charged at the anode. This causes repulsion of the ampholyte from the electrodes. The ampholyte which has the lowest pI (most acidic) will migrate closer to the anode where it will condense in its isoelectric state (zero net charge) at some distance from the anode; the opposite will occur for the ampholyte with the highest pI (most basic) where it will condense close to the cathode. If a mixture of carrier ampholytes is used with intermediate pI values, these will focus at different positions along the electric field so that a pH gradient is formed which is defined by the pH of the ampholytes at the point of focusing. The nature of the pH gradient will depend on the range of isoelectric points, the number of carrier ampholyte species in the system, and their relative concentration and buffering capacity.

The formation of a stable pH gradient with adequate buffering capacity and conductance provides the basis for the isoelectric focusing of amphoteric molecules of interest. This process involves electrophoretic migration in a pH gradient subject to the properties of the pH-mobility curve of each particular species. The addition of an anticonvection medium (e.g., density gradient, gel matrix) and a zone detection system (e.g., UV absorbance, staining) is necessary for the practice of the method either at the preparative or analytical levels.

History

Early "stationary electrolysis" experiments involving isoelectric condensation of dyes (*1*) and glutamic acid (*2*) did not receive any attention probably because the principle of isoelectric focusing was not clearly expressed. Several years passed by until Williams and Waterman (*3*) defined the basic concept of isoelectric condensation and performed experiments with a multichambered apparatus in which adjacent compartments were separated by membranes to avoid remixing of isoelectric components. Sporadic applications of this technique for separation of

biological materials including proteins was reported in the scientific literature (*4–8*). However, the method suffered from the lack of a stable pH gradient of adequate conductance and the use of a severely limited anti-convection system.

The next stage in the development of the isoelectric focusing technique was the introduction of short-lived "artificial" pH gradients by Kolin (*9–12*). These pH gradients–which were produced by mixing two buffer solutions–were not stable in the electric field because the buffer ions migrated electrophoretically. However, Kolin realized the importance of forming a pH gradient with sufficient buffering capacity and uniform high conductivity. He also introduced a density gradient system to overcome convective disturbances. Several other systems utilizing poorly reproducible and unstable pH gradients were also reported in the middle fifties (*13–16*). Svensson (*17, 18*) introduced the concept of carrier ampholytes which were defined as ampholytes with appreciable conductance and buffer capacity in the isoionic form. He realized that for isoelectric focusing to be successful, it is necessary to have several species of ampholytes with isoelectric points distributed throughout the pH range of interest. Svensson also derived the differential equations describing the dynamic equilibrium between diffusion and isoelectric condensation at the steady-state. In addition, the design of a preparative isoelectric focusing column utilizing density gradients for stabilization of protein zones was reported by him (*18*). Thus the pace was set for meaningful isoelectric focusing experiments which were not realized because of the lack of suitable compounds to serve as carrier ampholytes. However, this was remedied by Vesterberg (*19*) who was able to synthesize a mixture of polyaminopolycarboxylic acids which met the requirements for the formation of a natural pH gradient. The first successful modern isoelectric focusing experiment was reported by Vesterberg and Svensson (*20*) which opened the way to the widespread use of the method with the help of the LKB Company which made commercially available Svensson's preparative column and Vesterberg's carrier ampholytes. Shortly after Vesterberg's and Svensson's (*20*) publication, a number of microtechniques utilizing gels as stabilization medium were reported in the literature (*21–32*). The further development of new gel and density gradient microcolumn methods [for reviews, see Catsimpoolas (*33*), (*34*)] contributed to the popularization of the method because of the advantages of rapid analysis, low cost, and widely diversified selection of detection methods.

Recently, Catsimpoolas and co-workers (*35–42*) reported the development of the "transient state isoelectric focusing" (TRANSIF) method

based on a new kinetic theory and the use of an analytical instrumentation system utilizing continuous *in situ* electrooptical monitoring of the separation path (*43*). This new technique made possible the direct on-line measurement of methodological parameters and apparent physical constants, and the evaluation of the "steady-state" of isoelectric focusing. It was soon realized that TRANSIF offered the only feasible approach to the exact evaluation of an isoelectric separation because of instabilities in the pH gradient and the presence of nonuniform conductance along the separation field.

Types of Isoelectric Focusing

There are several ways to classify isoelectric focusing experiments in some useful operational fashion (*33*, *34*). The distinction between preparative and analytical isoelectric focusing pertains to the end result of either collecting the separated material at the conclusion of the experiment, or being interested only in the analytical aspects without consideration for the fate of the sample. Usually preparative isoelectric focusing is carried out in large columns (100 to 400 ml volume) using a sucrose density gradient as supporting medium. Alternatively, blocks of granular gels (e.g., Sephadex) or zigzag horizontal electrolysis cells can be used. Proteins are isolated in milligram or even gram quantities. On the other hand, analytical techniques can be performed with microgram or even nanogram amounts of material in small columns (1 to 10 ml) of density gradient or gel, and in flat gel slabs.

In regard to the kind of supporting medium, or the absence of it, we can distinguish several types of isoelectric focusing experiments. These include *density gradient*, *gel*, *zone convection*, and *free solution* isoelectric focusing. The density gradient is formed using two different concentrations of sucrose or other neutral substances such as Ficoll and ethylene glycol with special devices, or by layering fractions of different density. Gel isoelectric focusing is carried out either in homogeneous (continuous phase) or granular (beads) polyacrylamide, agarose, or cross-linked dextran gels. Zone convection and free solution isoelectric focusing requires specially constructed apparatus.

The application of specific detection methods involving antigen–antibody precipitin reactions in gels after isoelectric separation gave rise to the technique of *immunoisoelectrofocusing*. Another detection method utilizing UV absorption optics for *in situ* continuous electrooptical

monitoring of the separation field has been called *scanning* isoelectric focusing. Finally, if the kinetic aspects of pH gradient electrophoresis focusing and isoelectric diffusion can be measured by electrooptical methods, the technique is called *transient state* isoelectric focusing.

Applications

The main applications of isoelectric focusing to-date have been in the field of protein and peptide separation and characterization at both the preparative and analytical levels. Proteins from various sources (e.g., animal, plant, and microbial origin) having different functions, such as enzymes, hormones, immunoglobulins, and toxins, have been isolated in homogeneous form by isoelectric focusing. Usually the method is applied to the final step of purification preceded by other fractionation techniques such as ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. Often a protein that has been found homogeneous by other methods (e.g., ultracentrifugation, chromatography, electrophoresis) can be resolved into several components by isoelectric focusing. This occurs because of the high resolving power of the technique being able to separate proteins differing by only a few hundredths of a pH unit in their isoelectric points. The observed microheterogeneity may be due to: (a) minute differences in the primary structure (asparagine and glutamine residues are part of the primary structure); (b) conformational isomers; (c) denaturation; (d) presence of variable moieties such as carbohydrates, lipids, and metals which alter the isoelectric point; and (e) strong complexing with ionic and nonionic compounds including the carrier ampholytes themselves.

Direct determination of the isoelectric point of a protein is another great advantage of the isoelectric focusing technique, and this extra feature is utilized in most applications involving separation of a mixture of proteins. Measurement of the isoelectric point is one of the physicochemical parameters required for the characterization of an unknown protein, or establishing the identity of two similar proteins.

In addition to the above applications, isoelectric focusing can be used in two-dimensional separations in gels in combination either with other electrophoretic techniques or with immunodiffusion. Such methods are very useful in resolving protein components in a complex mixture and have very important applications in fields such as clinical chemistry, biochemical genetics, and plant taxonomy.

THEORETICAL ASPECTS

The basic theory of isoelectric focusing dealing with the "steady-state" was developed by Svensson (*17*, *18*) and with the "transient-state" (kinetic) by Weiss, Catsimpoolas, and Rodbard (*40*). Knowledge of the basic assumptions, physical parameters, and equations involved in the present state of the theory of isoelectric focusing is important in the interpretation of results obtained with the technique and in the further advancement of the method both from the theoretical and methodological point of view.

Isoelectric and Isoionic Points

The isoelectric point of a protein is defined as the pH at which an applied continuous electric field (dc) has no effect on the electrophoretic migration of the molecule in respect to the solvent (*44*). In the absence of complexing ions other than protons, the isoelectric point of an amphoteric molecule is virtually identical to its isoionic (isoprotic) point (*44–47*). Knowledge of the isoionic (isoprotic) point of a protein is very valuable because it is characteristic of its intrinsic acidity. The isoionic point of a protein solution is defined as that pH which does not change when a small amount of pure protein is added to the solution. In this regard the isoelectric point of a protein determined by isoelectric focusing should be very close to its isoionic point if the following requirements are met: (a) the carrier ampholytes completely dominate the buffering of the isoelectric zone site; that is, increasing amounts of focused protein has no effect on the pH; (b) the carrier ampholytes do not complex with the protein; and (c) ions that can form complexes with the proteins are removed either by migration toward the electrodes or by strong binding to the carrier ampholytes.

Direct comparison of isoelectric points measured by isoelectric focusing with those determined by other electrophoretic methods often shows that higher pI values are obtained with the former technique (*20*). This phenomena occurs because the buffer ions used in electrophoresis can form complexes with the protein thus altering its isoelectric point. Generally, the lower the ionic strength of the buffer, the higher are the pI values. Extrapolation of the pI values determined by electrophoresis to zero ionic strength should produce isoelectric points approaching those obtained by isolectric focusing.

Dissociation Theory

This theory is given here essentially as presented by Rilbe (*48*).

Dissociation of Biprotic Ampholytes

If we consider an ampholyte consisting of its zwitterionic form HA, its anion A^-, and its cation H_2A^+, the two mutual chemical equilibria of the three subspecies are:

$$HA + H^+ \rightleftarrows H_2A^+ \tag{1}$$

$$HA \rightleftarrows A^- + H^+ \tag{2}$$

giving rise to the mass-action equations

$$K_1 = \frac{[HA][H^+]}{[H_2A^+]} \tag{3}$$

$$K_2 = \frac{[A^-][H^+]}{[HA]} \tag{4}$$

where K_1 and K_2 are the thermodynamic equation constants and [ ] denotes activity. Introducing the simpler notation:

h = $[H^+]$
pH = $-\log h$
C_+ = concentration of cation H_2A^+
C_- = concentration of anion A^-
C_0 = concentration of molecular and zwitterionic ampholyte
C = $C_+ + C_0 + C_-$ = total concentration of ampholyte
f_+ = activity coefficient of cation
f_- = activity coefficient of anion

We can write instead of Eqs. (3) and (4):

$$hC_0 = f + K_1C_+ \tag{5}$$

$$hC_- = C_0(K_2/f_-) \tag{6}$$

Activity coefficient for the uncharged species is assumed to be unitary. For simplification, the stoichiometric dissociation constants K'_1 and K'_2 are introduced, such that

$$f_+K_1 = K'_1 \tag{7}$$

$$K_2/f_- = K'_2 \tag{8}$$

Equations (5) and (6) become

$$C_+ = hC_0/K'_1 \tag{9}$$

$$C_- = K'_2C_0/h \tag{10}$$

by adding them together with C_0 to obtain the total concentration, one arrives at a set of equations describing the concentration of the three subspecies:

$$C_+ = h^2C/(h^2 + hK'_1 + K'_1K'_2) \tag{11}$$

$$C_0 = hK'_1C/(h^2 + hK'_1 + K'_1K'_2) \tag{12}$$

$$C_- = K'_1K'_2C/(h^2 + hK'_1 + K'_1K'_2) \tag{13}$$

Mean Valence and Isoprotic Point

The mean valence due to proton binding is defined as

$$z = \frac{C_+ - C_-}{C} = \frac{h^2 - K'_1K'_2}{h^2 + hK'_1 + K'_1K'_2} \tag{14}$$

This is zero at hydrogen ion activity h_i, satisfying the equation

$$h_i^2 = K'_1K'_2 \tag{15}$$

Since $p = -\log$, one obtains the isoprotic pH:

$$(\mathrm{pH})_i = (\mathrm{p}K'_1 + \mathrm{p}K'_2)/2 \tag{16}$$

Buffer Capacity of Isoprotic Ampholytes

The specific buffer capacity B of a weak protolyte is defined by the expression

$$B = (1/m)[dn/d(\mathrm{pH})] \tag{17}$$

where m is the amount of weak protolyte and n the amount of alkali. For a monovalent weak acid:

$$B = \alpha(1 - \alpha) \ln 10 \tag{18}$$

where α is the degree of dissociation. A maximum is reached

$$B = (\ln 10)/4 \tag{19}$$

for $\alpha = 0.5$ corresponding to $\mathrm{pH} = \mathrm{p}K'$ for a weak acid. The specific buffer capacity for a protolyte with an arbitrary number of protolytic groups can be shown to be identical with the derivative

$$B = -dz/d(\text{pH}) \tag{20}$$

where z is the mean valence (Eq. 14). Thus the buffer capacity becomes

$$B = \frac{1}{C}\frac{d(C_- - C_+)}{d(\text{pH})} \tag{21}$$

or

$$B = \frac{d}{d(\text{pH})}\left[\frac{K'_1K'_2 - h^2}{h^2 + hK'_1 + K'_1K'_2}\right] \tag{22}$$

After differentiation and insertion of the isoprotic condition (Eq. 15), we obtain

$$B_i = \ln 10/(1 + \sqrt{K'_1/4K'_2}) \tag{23}$$

Division of Eq. (23) by Eq. (19) gives the relative buffer capacity b_i in the isoprotic state, i.e., the capacity in units of the maximum capacity of a monovalent weak protolyte:

$$b_i = 4/(1 + \sqrt{K'_1/4K'_2}) \tag{24}$$

Because a bivalent protolyte cannot exert a buffer action better than twice that of a monovalent one, the maximum value of b_i must be 2. This leads to the conditions:

$$K'_1 \geqq 4K'_2 \qquad \text{and} \qquad \Delta pK' \geqq \log 4 \tag{25}$$

Conductivity of Isoprotic Ampholytes

The degree of ionization of an isoprotic ampholyte can be defined as

$$\alpha = \frac{C_+ + C_-}{C} = \frac{h^2 + K'_1K'_2}{h^2 + hK'_1 + K'_1K'_2} \tag{26}$$

It is unity at very low as well as at very high pH values. It also has a minimum at the isoprotic point which can be found by insertion of Eq. (15) into Eq. (26):

$$\alpha_i = 1/(1 + \sqrt{K'_1/4K'_2}) \tag{27}$$

Comparison of Eq. (24) and (27) produces

$$b_i = 4\alpha_i \tag{28}$$

which shows that a high degree of ionization (good conductivity) is accompanied by good buffering capacity and vice versa.

Carrier Ampholytes

The buffer capacity of carrier ampholytes at the isoprotic state (Eq. 24) is very important because they should exhibit a buffer action stronger than that of the proteins and therefore dictate the pH gradient. In addition it is necessary that the carrier ampholytes have appreciable conductivity in the isoelectric state and in the pH region around the neutral point in order to avoid local overheating and absorbance of the applied voltage in areas of low conductivity. This condition (i.e., local low conductivity) may reduce the field strength in conducting zones and in excess may abolish electrolytic transport and therefore focusing. Since the conductivity contribution of an isoprotic ampholyte is proportional to α_i (degree of ionization, Eq. 27), it is evident that ampholytes with a low pK' difference have adequate conductivity in the isoprotic state. Thus histidine ($\Delta pK' = 3.0$), glutamic acid ($\Delta pK' = 2.1$), and lysine ($\Delta pK' = 1.6$) can be considered useful carrier ampholytes whereas glycine ($\Delta pK' = 7.4$, corresponding to a degree of ionization of 0.00038 in the isoprotic state) is useless.

In order to be useful for isoelectric focusing experiments, the carrier ampholytes should contain a large number of isoelectric species differing less than 0.1 pH unit and should preferably be able to cover the range of pH 2.5 to 11.0. Such species should produce overlapping isoelectric distributions and therefore an approximately linear and smooth pH gradient. Other desirable properties of the ampholytes include good solubility in water, absence of hydrophobic groups, and low UV absorbance, especially at 280 nm. Vesterberg's (*19*) synthetic procedure was designed to produce a large number of isomers and homologs by coupling residues containing a carboxylic group to suitable amines. The resulting polyaminopolycarboxylic acids fulfill satisfactorily some of the requirements as expressed above and have found wide use in the isoelectric separation of proteins. However, these carrier ampholytes are far from ideal because (a) they produce a nonuniform conductance course, (b) their concentration differs throughout the pH gradient, (c) the pH gradient is not strictly linear, and (d) they may bind to certain proteins. These synthetic ampholytes are commercially available from LKB and cover the following pH ranges: 3.5–10.0, 2.5–4.0, 3.5–5.0, 4.0–6.0, 5.0–7.0, 5.0–8.0, 6.0–8.0, 7.0–9.0, 8.0–9.5, and 9.0–11.0.

The Steady State

Svensson (*17*) described the concentration distribution of an electrolyte

at the isoelectric point as an "equilibrium" between mass transport and diffusional flow.

$$CME = D(dC/dx) \tag{29}$$

where C is the protein concentration, M is its mobility, E is the field strength, D is its diffusion coefficient, and x is the coordinate along the direction of current. The mobility M can be regarded as a linear function of x because of the narrowness of the zone at the pI. With the introduction of the proportionality factor p such that

$$M = -px \tag{30}$$

Eq. (29) can be written

$$dC/C = (Ep/D)xdx \tag{31}$$

If E, p, and D are treated as constants, this equation is integrated to give

$$C = C(0) \exp(-pEx^2/2D) \tag{32}$$

which expresses a Gaussian concentration distribution with a standard deviation (σ):

$$\sigma = (D/pE)^{1/2} \tag{33}$$

The proportionality factor p may be written as a derivative:

$$p = dM/dx = [-dM/d(\text{pH})][d(\text{pH})/dx] \tag{34}$$

The Transient State

Continuous analytical scanning isoelectric focusing techniques (*43*) have made possible the accurate estimation of the first and second moments of the concentration profile repetitively throughout the course of the isoelectric focusing experiment. This suggested the possibility of measuring the parameters D, p, and $dM/d(\text{pH})$ through a mathematical analysis of the kinetics of isoelectric focusing. The method of TRANSIF (*49*) was thus born, promising to provide considerably more information than the analysis of the steady-state distribution alone. Weiss, Catsimpoolas, and Rodbard (*40*) presented a restricted theory of the kinetics of the new method. The principal assumption is that the mobility of the protein is a linear function of position at all times. The TRANSIF method is assumed to consist of three stages:

(1) Focusing, in which the system is allowed to approach the steady-state distribution for a time t_1.

(2) Defocusing, in which the electrical field is abolished for a time t_2. This is assumed to be a pure diffusion process.

(3) Refocusing for a time t_3, in which the field is reapplied and the distribution again approaches the steady-state.

The following assumptions have been adopted to permit a first theoretical approximation:

1. A linear pH gradient is established prior to application of the sample protein. Alternatively, we may assume that the pH gradient is formed very rapidly compared with the kinetics of focusing of the macromolecule of interest.

2. The pH mobility curve of the protein is assumed to be linear. This assumption is valid only for a limited region near the isoelectric point. On the basis of these two assumptions, $p = dM/dx$ is constant. This single assumption could be used in lieu of the above.

3. The electrical field strength (E) is assumed to be uniform throughout the entire separation path. In lieu of assumptions 1–3, we could simply assume that $pE = dv/dx$ is constant, where v is velocity.

4. Diffusion and mobility coefficients are assumed to be independent of concentration.

5. Diffusion coefficients are assumed to be independent of pH (at least in the region near the isoelectric point).

6. It is assumed that there are no physical/chemical interactions between the protein and other chemical species present (e.g., ampholytes), and no self-association or protein–protein interactions).

7. Band spreading is governed only by diffusion or by a diffusion–like process. Thus electrostatic effects are ignored and it is assumed that the protein is perfectly homogeneous with respect to pI, charge, mobility, radius, and diffusion coefficient.

8. No perturbing phenomena such as electroendosmosis, convective disturbances, or precipitation at the isoelectric point are present.

9. If a gel or density gradient is used as a supporting medium, their effects on diffusion coefficients and on mobility are neglibible (or at least constant throughout the gel), and there is no effect on the uniformity of the electrical field. Thus the effect of the viscosity gradient which is superimposed on the density gradient in sucrose–gradient columns is ignored. Likewise, the molecular sieving effects which are present when polyacrylamide gels are used as a supportive medium are ignored.

10. The effect of the boundary condition that there can be no flux

of the species of interest through the ends of the gel column, or that there is an abrupt discontinuity of pH at the ends of the column, is ignored. These effects should become insignificant shortly after the start of the experiment.

The above assumptions may be relaxed later to provide a more generalized and practical theory. For experimental purposes it is sufficient and convenient to find $\mu_1(\tau)$ and $\sigma^2(\tau)$, i.e., the mean and square of the standard deviation of peak width. These equations are:

1. Focusing: $0 \leqq \tau \leqq \tau_1$

$$\mu_1(\tau) = \mu_1(0)e^{-\tau} + y_0(1 - e^{-\tau}) \tag{35}$$

$$\sigma^2(\tau) = \sigma^2(0)e^{-2\tau} + \alpha(1 - e^{-2\tau}) \tag{36}$$

2. Defocusing: $\tau_1 \leqq \tau \leqq \tau_1 + \tau_2$

$$\mu_1(\tau) = \mu(0)e^{-\tau_1} + y_0(1 - e^{-\tau_1}) = \text{constant} \tag{37}$$

$$\sigma^2(\tau) = \sigma^2(0)e^{-2\tau_1} + \alpha(1 - e^{-2\tau_1}) + 2\alpha(\tau - \tau_1) \tag{38}$$

3. Refocusing: $\tau_1 + \tau_2 \leqq \tau$

$$\begin{aligned}\mu_1(\tau) = {} & \{\mu_1(0)e^{-\tau_1} + y_0(1 - e^{-\tau_1})\} \exp\{-(\tau - \tau_1 - \tau_2)\} \\ & + y_0(1 - \exp\{-(\tau - \tau_1 - \tau_2)\})\end{aligned} \tag{39}$$

$$\begin{aligned}\sigma^2(\tau) = {} & \{\sigma^2(0)e^{-2\tau_1} + \alpha(1 - e^{-2\tau_1}) + 2\alpha\tau_2\} \\ & \times \exp\{-2(\tau - \tau_1 - \tau_2)\} \\ & + \alpha(1 - \exp\{-2(\tau - \tau_1 - \tau_2)\})\end{aligned} \tag{40}$$

where L is the column length
x_0 is the position of the isoelectric point
$\alpha = D/(L^2pE)$
$\tau = pEt$
$y_0 = x_0/L$

A computer simulation study derived from theory of the time course of the centroid (μ) and σ^2 in TRANSIF is shown in Fig. 1.

The centroid approaches the isoelectric point by an exponential decay during focusing and refocusing. With ideal initial pulse loading, the bandwidth (σ^2) increases during focusing, asymptotically approaching the steady-state value.

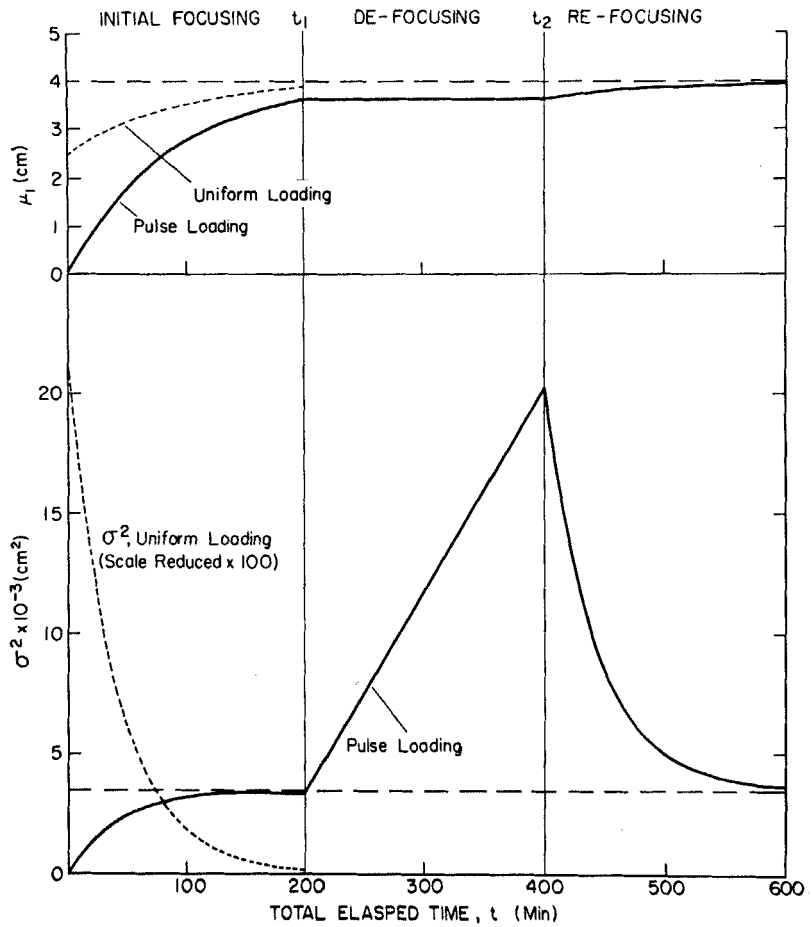

FIG. 1. Time course of the centroid, μ, and σ^2. Both the uniform distribution and "pulse loading" cases are shown for the initial focusing stage.
Parameters: $L=5$ cm; $X_0=4$ cm; $D=7\times10^{-7}$ cm^2/sec; $pE=20\times10^{-5}=2\times10^{-4}$ sec^{-1}; $E=20$ V/cm; $P=10^{-5}$ cm/(sec) (V).

ELECTROOPTICAL SCANNING

Electrooptical Scanning in Isoelectric Focusing: Advantages and Limitations

Biomolecules separated by isoelectric focusing have been detected and analyzed by the following methods: (a) optical and electrooptical *in situ* methods (e.g., visible and UV light absorption); (b) nonspecific dye staining methods (e.g., Coomassie Blue staining of proteins); (c) specific dye staining methods (e.g., for glycoproteins and lipoproteins); (d) immunochemical methods (utilizing antigen–antibody reactions); (e) enzymatic activity methods; (f) specific biological activity methods (bioassay); (g) radioactivity labeling methods; and (h) fluorescent labeling methods.

From all the above techniques, only optical and especially electrooptical methods allow for the continuous monitoring of a particular separation as a function of time without interruption of the electric field. In addition, electrooptical scanning methods lend themselves to precise mathematical peak analysis for the evaluation of methodological parameters and the measurement of physical constants. In particular, reference to the isoelectric focusing and isotachophoresis techniques which require the attainment of a "steady-state," continuous monitoring of the separation field is especially advantageous for evaluation of the minimal focusing time (t_{MF}) in isoelectric focusing of the "constancy of zone length" in isotachophoresis.

To date, the main limitations of the electrooptical detection method are the nonspecificity of detection and reduced sensitivity in comparison to some other detection techniques (e.g., immunological and enzymatic). However, proteins in amounts ranging from 10 to 100 μg can be quantitatively detected by the UV scanning method (at 280 nm). The sensitivity can be increased tenfold if the absorbance at 220 nm is recorded, but this precludes the use of a polyacrylamide gel as a supporting medium. The absorbance at 220 nm is largely due to the peptide bonds and is relatively little affected by the content of aromatic amino acids such as tryptophan and phenylalanine. Therefore, this particular wavelength could be preferentially used for separation in sucrose density gradient. The sensitivity of UV detection at 280 nm can be improved—within 1 to 2 orders of magnitude—by an apparatus which will be utilizing a double-wavelength ratio-recording system to significantly reduce baseline noise and decrease the lower limits of detection.

Principle of in Situ Visible or UV Scanning

Electrophoretic separation is performed in a quartz cell which moves perpendicularly to a thin (25 to 50 μ slit-width) light beam. When the beam encounters separated zones of the sample, it is absorbed proportionally to the amount of material present in the zone and its extinction coefficient at a particular wavelength. A photomultiplier placed behind the quartz column detects the light variation and produces a current which can be converted electronically to an analog voltage related linearly to the zone profile absorbance. The analog voltage can be either recorded directly on a strip-chart recorder or preferentially can be digitized and processed by a computer. The separation path is scanned continuously during the electrophoretic run, resulting in several electropherograms (scans) as a function of time. The sample can be scanned at any preferred wavelength in the 200 to 800 nm range, or the scanner can be stopped and one particular zone can be scanned as a function of wavelength. The electric field is applied at all times. However, the current can be interrupted and the diffusion of the zone can be followed by the broadening of the concentration distribution with time. Thus both electrophoretic and diffusional mass transport phenomena can be evaluated. This is a very important feature in the digital measurement of physical constants of biomolecules and methodological parameters as will be discussed below.

Coupling of the scanning instrument to a digital data acquisition and processing system allows direct measurement of the zeroeth (m_0), reduced first (m'_1), and second (m_2) statistical moments of the zone concentration profile which correspond, respectively, to the area, the position ($\bar{x}$), and the variance (σ^2) of the peak. The experimental values of m_0, σ^2, and $\sigma\,(=\sqrt{\sigma^2})$ in conjunction with $\bar{x}$ values of pI markers are the only parameters required to be measured in order to obtain valuable quantitative information in TRANSIF experiments. These techniques have been described in detail by Catsimpoolas and co-workers (*35–43*).

METHODOLOGICAL PARAMETERS

Determination of Minimal Focusing Time

In the simple case of a single protein uniformly distributed in the column before the electric field is applied, the minimal time required to obtain complete focusing can be determined by following the position ($\bar{x}$) of the discernible peaks migrating from the two ends of the path (positive

and negative) toward the isoelectric point position where they merge into one peak. At the steady-state and in the absence of significant pH gradient instability, the peak position in pI should remain constant with time. Ideally, the peak area (zeroeth moment) and the variance (second moment about the mean) should also remain constant when the steady-state is reached. The latter two parameters can be employed in evaluating "steady-state" conditions (and therefore minimal focusing time) of a mixture of proteins. It should be emphasized that depending on their individual pH–mobility relationship, proteins in a mixture may approach the "steady-state" at different times. Other factors that affect the minimal focusing time of individual proteins are: (a) sieving effects in gels, (b) nonuniform electric field strength, and (c) presence of a viscosity gradient (e.g., sucrose density gradient). The minimal focusing time may be also generally affected by: (a) ampholyte concentration, (b) electric field strength, (c) pH range of carrier ampholytes, (d) temperature, and (e) presence of additives, e.g., urea.

Segmental pH Gradient

This parameter, $\Delta(\text{pH})/\Delta x$ (cm^{-1}), is measured using two pI markers of closely spaced isoelectric points from

$$\frac{\Delta(\text{pH})}{\Delta x} = \frac{\text{pI}_\text{A} - \text{pI}_\text{B}}{\bar{x}_\text{A} - \bar{x}_\text{B}} \tag{41}$$

where pI is the isoelectric point, $\bar{x}$ is the peak position, and subscripts A and B denote two pI markers. In using the above equation, it is assumed that species A and B have reached their isoelectric point, and that $\Delta(\text{pH})/\Delta x$ is constant between pI_A and pI_B where $\bar{x}_\text{A} - \bar{x}_\text{B}$ represents a small segment of the separation path.

Resolution

Arbitrarily assigning resolution of unity to a just resolved double-zone, the resolution R_s can be expressed as

$$R_s = \frac{\Delta\bar{x}}{1.5(\sigma_\text{A} + \sigma_\text{B})} \tag{42}$$

where $\Delta\bar{x}$ is the peak separation of two zones A and B with standard deviations of σ_A and σ_B. Again, $\Delta\bar{x}$, σ_A, and σ_B can be measured directly at any stage of fractionation.

Resolving Power

In isoelectric focusing the resolving power has been defined by Vesterberg and Svensson to be

$$\Delta \mathrm{pI} = 3[d(\mathrm{pH})/dx]\sigma \tag{43}$$

Since $\Delta(\mathrm{pH})/\Delta x$ and σ can be obtained digitally in TRANSIF, the resolving power can be estimated directly.

APPARENT PHYSICAL CONSTANTS

Apparent Isoelectric Point

If an "unknown" protein (U) is included in the segmental pH gradient as described above, its apparent isoelectric point can be calculated by

$$\mathrm{pI_U} = \mathrm{pI_A} + \left(\frac{\Delta(\mathrm{pH})}{\Delta x}\right)(\bar{x}_\mathrm{A} - \bar{x}_\mathrm{U}) \tag{44}$$

All three species A, B, and U should be at pH equilibrium, i.e., at the steady-state.

Diffusion in Polyacrylamide Gels: Determination of the Retardation Coefficient (C_R)

The apparent diffusion coefficient in polyacrylamide gels is related to the gel concentration by

$$\log D = \log(D_0) - C_R T \tag{45}$$

where D_0 is the free diffusion coefficient, T is the gel concentration, D the apparent diffusion coefficient at any gel concentration T, and C_R is the retardation coefficient obtained from diffusion data. C_R can be measured during the defocusing stage of TRANSIF experiments from the slope of the plot log D vs T. Thus TRANSIF in polyacrylamide gels can provide a measure of molecular size. It is therefore possible that the effective molecular radius $\bar{R}$ and MW could be estimated by the present method from plots of C_R vs $\bar{R}$, or vs MW in analogy to the Rodbard-Chrambach plots (*50*).

Measurement of D and pE

As mentioned above, a TRANSIF experiment is characterized by three stages; namely, focusing, defocusing, and refocusing. In the focusing

stage the sample is subjected to electrophoresis on a pH gradient for time t_1 until a nearly steady-state distribution of the focused zone is achieved. In the defocusing stage, the electrical field is removed for time t_2, allowing the zone to spread by diffusion. In the refocusing stage, the electrical field is reapplied for time t_3 and the distribution reapproaches the steady-state. The advantages of performing kinetic analysis of zone focusing during the refocusing period are: (a) focusing is carried out by starting with a (near) Gaussian distribution; (b) the zone is restricted to a narrow region of the pH (and mobility) spectrum near the isoelectric point; and (c) data are collected under conditions of nearly linear pH gradient (d(pH)/dx) and linear pH–mobility curve [dM/d(pH)]. Thus, if the parameters d(pH)/dx and dM/d(pH) are constant, the experimentally measurable parameter p will also remain constant throughout the refocusing experiment, since

$$p = [dM/d(\text{pH})][d(\text{pH}/dx] \tag{46}$$

The parameter i is related to the standard deviation of the concentration distribution of a focused zone at the steady-state by

$$\sigma = \sqrt{D/pE} \tag{47}$$

where

$$E = i/qK \tag{48}$$

where i is the current, q is the cross-sectional area, k is the conductance, and E is electric field strength.

For experimental purposes, the kinetics of defocusing and refocusing can be evaluated by following the changes of σ^2, which is the square of the standard deviation of peak width, vs elapsed time. The equations describing the behavior of σ^2 during these two stages of the experiment have been derived from theory (*2*) to be:

1. *Defocusing*

$$\sigma^2(t_2) = \sigma^2(t_1) + 2D(t_2 - t_1) \tag{49}$$

2. *Refocusing*

$$\sigma^2(t) = (D/pE) + 2Dt_2 \exp(-2pEt_3) \tag{50}$$

Experimentally, a plot of σ^2 vs $2t_2$ should permit estimation of the apparent diffusion coefficient D (as the slope of the line) during the defocusing stage. Also a plot of $\log[(\sigma_R{}^2 - \sigma_F{}^2)/\sigma_D{}^2]$ vs $2t_3$ during the refocusing stage can be used to determine the parameter pE as the slope of the linear plot. If d(pH)/dx and E are known, the physical constant dM/d(pH) can be estimated from Eq. (46).

CONCLUDING REMARKS

Although isoelectric focusing has been successfully applied to the separation of amphoteric molecules (*51*), especially proteins, as evidenced by more than 1500 published articles to-date, the full potential of the technique has not yet been exploited. This is primarily due to the lack of suitable carrier ampholytes, which should provide a uniform conductance and concentration distribution course throughout the separation path, and a stable linear pH gradient. The commercially available carrier ampholytes fall short in all three of the above desirable properties. The success of steady-state isoelectric focusing—as commonly practiced today—lies primarily in the relative reproducibility of the isoelectric point and the ability to separate different isoelectric species with very high resolution. However, with the development of the TRANSIF technique, it should be possible to utilize the method not only for the separation but also for the physicochemical characterization of amphoteric molecules. To be specific, one should be able to use TRANSIF in obtaining the diffusion coefficient, the $dM/d(\text{pH})$ coefficient, the isoelectric point, and the pH–mobility curve of a protein with a good degree of confidence in the results. The kinetic theory and available instrumentation allow us to do the necessary measurements, but the compiling of correction factors due to nonideal effects, stemming primarily from the present imperfection of carrier ampholytes, renders the method impractical. Some of the corrections that have to be made involve the effect of ampholyte concentration and zone conductance on the measurable physical constants. These should have been relatively easy to carry out if there was assurance that these corrections apply uniformly throughout the column. Other corrections involve "zone load," viscosity, and temperature effects.

Despite the present shortcomings, isoelectric focusing has become an established separation technique with a very promising future both at the preparative and analytical levels. Dynamic development of new methodology and instrumentation coupled with a selective extension of the kinetic theory and the much needed synthesis of "second generation" ampholytes will undoubtedly suggest new avenues of application.

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