METHODS OF BIOCHEMICAL ANALYSIS

DAVID GLICK

VOLUME 19

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The current volume is another in this series which is designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation

of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details, a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

DAVID GLICK

CONTENTS

Isoelectric Focusing in pH Gradients —A Technique for Fractionation and Characterization of Ampholytes

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I. INTRODUCTION

1. Main Applications in Isoelectric Focusing

Isoelectric focusing has rapidly become an important tool for many researchers, mainly in biochemistry and related sciences. The two main applications of isoelectric focusing are:

1. Analytical or preparative separation of ampholytes, especially proteins, according to isoelectric point.

2. Characterization of ampholytes, especially proteins, by determining isoelectric point. This can be done simply and exactly in a single experiment.

The technique is characterized by very high analytical resolution and by simplicity of apparatus and method. Electrofocusing has been applied to many types of proteins. Proteins with as small a difference as 0.02 pH units at the isoelectric point have been successfully separated.

The capabilities of measuring isoelectric points are outstanding when compared with the previously available method of free electrophoresis.

Limiting aspects of the electrofocusing method are:

1. The technique requires salt-free solutions. This can cause precipitation of sample proteins. However, the carrier ampholytes—*Ampholine* is the trade name—a necessity in electrofocusing, contribute to the ionic properties of the solution in such a way that the lack of salts is counteracted.

2. The components of a sample are compelled to remain at their isoelectric point. This feature means that the technique should not be used with proteins which become insoluble or denatured at the isoelectric point.

2. Simplified Basic Description

Every protein or other ampholyte has a so-called isoelectric point, pI, which is a pH value at which it may be dissolved to yield a net charge of zero. If the protein is added to a solution with a higher pH (more basic) than the pI, it loses protons and becomes negatively charged. Conversely, if the protein is in surroundings with a lower pH (more acidic) than the pI, it will capture protons and become positively charged.

In an electric field, positively charged particles migrate towards the cathode (the negative pole) and negatively charged particles towards the anode (the positive pole).

In electrophoresis a constant pH, achieved by a buffer, is usually used. If, in contrast to this, one arranges a buffer with a varying pH, that is, increasing from one end to the other, a pH-gradient is obtained. Such a gradient has always the lowest pH value at the anode and the highest pH at the cathode.

When a sample of proteins with isoelectric points within the pH range of the gradient is added to the pH-gradient, the protein molecules will obtain different charges. The charge of every individual protein will be determined by the pI for that protein and the pH corresponding to the position where the protein is located. Applying a voltage across such a system results in each protein molecule migrating toward the pH value in the system where it is isoelectric, that is, where its net charge is zero. The proteins are thus exactly focused at the point where the pH is equal to the pI.

For a long time the obstacle to the development of the electrofocusing technique was the difficulty in obtaining stable pH-gradients. However, Svensson (1,2,3,4,5,6) and Vesterberg (7,8) found that when a mixture of low molecular weight carrier ampholytes, for example, Ampholine chemicals, are exposed to a voltage in a convection-free water solution, a pH-gradient is formed, created by the current itself. When large molecular weight ampholytes such as proteins are inserted as a sample

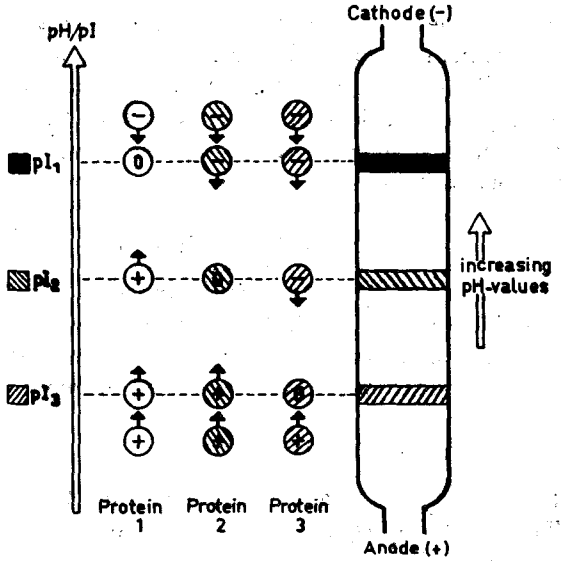

Figure 1. Three proteins, marked pI_1, pI_2, and pI_3, schematically electrofocused in a column. Each of the three protein components is negatively charged above its respective point and positively charged beneath. For that reason it will move in the electric field until the position in the column is reached where the pH of the pH-gradient is the same as the isoelectric point for that particular protein.

in such a pH-gradient, they will concentrate in narrow zones. The focusing will occur at the isoelectric point of the protein. The isoelectric point is relative and characteristic for each protein.

This process is illustrated in Figure 1, which schematically shows how three proteins, marked (pI_1), (pI_2) and (pI_3), are focused in a column. Diffusion counteracts the electrofocusing. As the diffusion coefficient is inversely proportional to the molecular weight, it is understandable that better focusing is obtained with higher molecular weight proteins.

3. Principle

The situation can be summarized as follows: Isoelectric analysis, focusing, and fractionation are obtained by imposing a d.c. potential on an electrolyte system in which the pH steadily increases from the anode to the cathode. Provided that the pH-gradient is sufficiently stable for the duration of an experiment, ampholytes, such as proteins and peptides, present in the electrolyte system will be repelled by both electrodes.

Each ampholyte species will collect at the place in the gradient where the pH of the gradient is equal to the isoelectric point of the species. This collecting or focusing is caused by the electric field; thus the name isoelectric focusing. A necessary prerequisite is that the electrolyte system is stabilized against uncontrolled convection and against remixing of focused ampholytes.

4. The pH-Gradient

For the practical use of electrofocusing it must be possible to arrange a pH-gradient with suitable properties. The gradient should comprise the desired pH range and other parameters of importance as slope, and conductivity. The pH-gradient may be achieved in two different ways.

A. NATURAL PH-GRADIENTS

Natural pH-gradients are located and maintained by the electric current itself. The following is a simplified explanation of the natural pH-gradient.

Suppose that a large variety of low molecular weight ampholytes are dissolved in the electrolyte system. A mixture of peptides can be used as an example. This mixture represents a variety of isoelectric points distributed over the pH interval corresponding to the desired pH-gradient. When the electric current is turned on, the different ampholytes start to move, the movement depending on the charge of each individual ampholyte. The ampholyte which is most acidic (i.e., has lowest isoelectric point, pI) will migrate to the anode. There it will collect in its isoelectric (zero net charge) stage and, due to a certain buffering capacity, give the surrounding solution a pH corresponding to the pI. The ampholyte with the acidity closest to the most acidic one (i.e., with second lowest pI) also migrates towards the anode. However, it cannot pass through the region already occupied by the first ampholyte because its own isoelectric point would be passed and its molecules would then become oppositely charged. The second ampholyte will form a layer close behind the region of the first ampholyte and there define a pH equal to its pI. The third ampholyte has a pI slightly higher than those of its predecessors. It migrates close to the second one and remains there. The fourth, fifth and sixth arrive and queue up in the correct order. Finally the whole interval between the electrodes is occupied by a series of ampholytes with pI ascending from anode to cathode, thus creating a pH-gradient defined in each point by carrier ampholytes which are in their isoelectric states. Each site along the gradient has its pH determined by the pH of the ampholyte at that point The profile of the gradient is determined by

the number of the ampholytes, their buffering capacity, relative amounts and isoelectric points. The pH-gradient reaches a stable equilibrium if the system is stabilized against convection and other movements of the liquid which cause remixing. This is usually achieved by means of a density gradient technique or gel stabilization, as will be described later on.

B. ARTIFICAL pH-GRADIENTS

Artificial pH-gradients can be obtained by arranging buffer solutions of different pH in series so that they can diffuse into each other. This pH-gradient is not in equilibrium, but changes continuously during an experiment due to diffusion and electrophoretic migration. Such a pH-gradient has been named *artificial* (9,10). However, this article discusses mainly those systems with natural pH-gradients.

II. HISTORY

"Isoelectric focusing" and "electrofocusing" are names that have been accepted, and in current use, since 1967. Before that time the technique was called isoelectric separation, isoelectric fractionation, isoelectric condensation, isoelectric analysis and focusing electrophoresis, as well as stationary electrolysis. Terms such as "density gradient electrofocusing" or "gel electrofocusing" indicate the medium in which the experiments are carried out.

1. Early Achievements

The idea of isoelectric fractionation of ampholytes has been known for a long time and was described in many articles before a technique became available for practical work. The difficulties which delayed development and application were technical. In particular, it was difficult to achieve a stable pH-gradient and prevent convection in the electrolyte system.

In 1920 two Japanese chemists, Ikeda and Suzuki (11), were granted a patent on a method of producing glutamic acid from a hydrolysate of plant proteins. The mixture of amino acids in the hydrolysate was electrolysed in a rectangular three-chamber apparatus, the chambers of which were separated by membrane walls. After the inorganic salts had collected close to the electrodes it was found that the amino acids had become coarsely separated in groups. The most acidic, glutamic acid, had become enriched close to the anode. The other amino acids consequently tended to arrange themselves according to their isoelectric points. Because of electro-osmosis and other convective forces, the separated amino acids could not, in the boxlike apparatus of Ikeda and Suzuki, develop

into a stable and well-defined pH-gradient. The inventors, however, were able to isolate glutamic acid in the form of glutamate bound to the metal of the anode and thus to separate it from the other acids.

A. MULTICHAMBER DEVICES

Numerous multichamber devices in which the chambers were separated by membranes to prevent convection have since been built and described. By increasing the number of chambers it became possible to obtain a finer separation, as well as to isolate other fractions than the ones located closest to the electrodes. William and Waterman (12) developed a theory for their 14-chamber apparatus and showed "that if the substance sought is an ampholyte, it may be concluded that the pH of that portion of the solution which contains the substance in maximum concentration approximates the isoelectric point of the substance, though, at any pH greater or less than that of the isoelectric point, the substance will be ionized more strongly as a base than as an acid, or vice versa, and will therefore migrate toward the region at which ionization as acid and as base are equal, namely the region of its isoelectric point." Their apparatus is sketched in Figure 2.

The principle was later used for separation work, mostly for ampholytes of low molecular weight. Williams (13) applied it to vitamins and to growth factors. Du Vigneand *et al.* (14) used multichambers to isolate vasopressin and oxytocin hormones from pituitary gland extract.

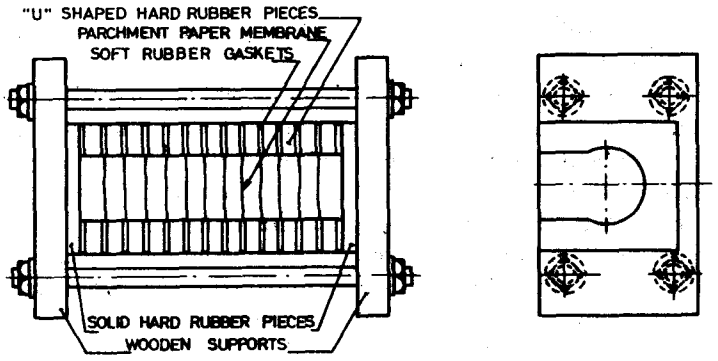

Figure 2. The multichamber apparatus described by Williams and Waterman in 1929 (12). They described their electrofocusing experiments as electrodialyzing in a multiple compartment cell. After electrolysis the contents of the compartments were separately removed and their pH determined. The solutions were assayed for the constituents.

Tiselius (15) described the focusing of ampholytes at their respective isoelectric points as a dynamic equilibrium between electrophoretic migration and diffusion. He used the method on a mixture of egg albumin and hemoglobin. Separation was poorer than by electrophoresis alone. Synge (16) and Sanger and Tuppy (17) used isoelectric focusing for a rough separation of amino acids. This apparatus had four chambers.

Separation of proteins and polypeptides in such multichambers has been more successful than separation of amino acids. The reason for this is that the former molecules retain enough charge in the neighborhood of their pI to become separated. Many amino acids, however, are almost without charge near their pI. This means they are unable to migrate by electrophoresis to a sharply defined pI. Another limiting factor was the difficulty of achieving uniform field strength between the electrodes while using the very high voltages required to give even moderate resolution.

B. ARTIFICAL pH-GRADIENTS

Isoelectric focusing made great progress through Kolin's work (18,19, 20,10). He pointed out that uniform field strength depended on the use of electrolytes with high and uniform conductivity. He also pointed out the necessity of having a pH-gradient of sufficient buffering ability.

Kolin (10) separated proteins. He used columns stabilized with a sucrose density gradient. This stabilized against remixing and convection. The method has considerable advantages over the use of a capillary system, avoiding electro-osmosis and protein loss by adsorption. Kolin allowed two buffer solutions of different pH to diffuse into each other. The pH-gradient arose as the buffers diffused into the intermediate liquid zone. Such gradients were later called "artificial pH-gradients" by Svensson (9). Since buffers are electrolytes, such a pH-gradient is unstable in an electric field. This is due to the electrolytic migration of buffer ions. Thus if substances are to be separated by means of this type of gradient, they must move more rapidly than the buffer ions. Kolin (10) achieved fractionations of considerable sharpness. However, the zones lay very close together. Thus it was difficult to fractionate them separately.

Many other publications report the production and use of pH-gradients. Hoch and Barr (21) and McDonald and Williamson (22) used such pH-gradients in electrophoresis using paper strips. They had difficulties with electro-osmosis and evaporation. Maher *et al.* (23) also used a similar method, with limited success. Tuttle (24) described successful analytical separations of hemoglobin.

There was one shortcoming in all these methods—poor reproducibility of the pH-gradient. Kolin (10) considered in 1958 that the great problem

to be solved was how to stabilize the pH-gradient against changes due to the passage of current.

2. Recent Developments

A. NATURAL pH-GRADIENTS

Svensson's pioneering work on natural pH-gradients made the development of electrofocusing possible. He performed brilliant theoretical and practical work on these problems in the early 1960's. This work provided the necessary foundation for subsequent successful developments. In a series of fundamental reports, Svensson unravelled the problems of natural pH-gradients (1,2,3). He defined the theoretical requirements of electrolytes for isoelectric focusing, taking into account the requirements defined by Kolin (10). The electrolytes must be ampholytes with certain properties. They must retain good conductivity at the isoelectric point, and have sufficient buffering power there. Svensson showed that the two pK values of the ampholyte, lying on either side of the pI, should be close together, not more than one and a half pH units apart. Svensson and his collaborators also checked the catalogs of chemical supply houses in an endeavor to find substances which would meet the criteria he had drawn up. However he found nothing suitable, least of all in the pH range 5–8. Svensson then made experiments with peptide mixtures from the hydrolysis of hemoglobin (3). These peptide mixtures enabled him to confirm his theoretical arguments. He also established that the method allowed separation with a promising resolution. Svensson used sucrose density gradients as a stabilizing medium against convection in his experiments.

Peptides had obvious disadvantages as carrier ampholytes. However, this fact encouraged Vesterberg to conduct a systematic search for a synthetic method of producing carrier ampholytes with the desired properties. He succeeded in synthetizing a series of aliphatic aminocarboxyl acids which satisfied the requirements defined by Svensson (7,25,26). With this development he found the key to the isoelectric focusing technique as a practical research tool. Vesterberg's synthesis has also made possible the industrial manufacture of the necessary carrier ampholytes. These are now being produced under the name of "Ampholine" chemicals.*

B. GEL ELECTROFOCUSING

Recently isoelectric focusing has also been applied in gels with great success. Wrigley (27,28,29) reported highly resolved separations with

* Marketed by LKB-Produkter AB, Fack S-16125 BROMMA I, Sweden.

polyacrylamide as the stabilizing medium. He employed conventional disc electrophoresis apparatus for his experiments. Later, Riley *et al.* (30) also successfully used the focusing method in gels. Fawcett (31,32) reported isoelectric focusing with polyacrylamide as the stabilizer against convection. Dale and Latner (33,34,35) and Leaback and Rutter (36,37, 38) performed gel isoelectric focusing on thin layers of polyacrylamide in a specially constructed apparatus. Awdeh, Williamson and Askonas (39) have published a description of very simple special gel electrofocusing equipment and have also reported high analytical resolution.

In all electrofocusing, some method must be used to stabilize the liquid against convection currents. The usual methods employ sucrose (density gradient) or polyacrylamide (gel). These substances are present in much larger quantities than the actual substance being tested. This always involves the risk of disturbances. Sucrose must be removed by dialysis at the end of the fractionation. It is also difficult to separate the protein from the polyacrylamide.

C. ZONE CONVECTION ELECTROFOCUSING

A great advance in the equipment for isoelectric focusing has recently been made by Valmet (40). He has described a new principle for electrofocusing called *zone convection electrofocusing*. In this technique the stabilizing against convection does not call for any capillary system, density gradient or membranes.

One form of Valmet's apparatus consists of a trough and a lid which are both corrugated. The corrugations mate when the lid is lowered onto the trough, leaving a continuous gap. The solution of carrier ampholytes fills the gap, forming a horizontal convoluted layer. When a voltage is applied to the ends of the layer, a current passes. Because cooling is applied from the outside, a temperature gradient arises between the middle of the layer of ampholytes and the walls containing it, that is, the lid above it and the trough beneath it.

A density gradient is created at first. This is due to "thermal diffusion"—dissolved molecules of proteins and ampholytes tend to migrate to the cooler walls. This means that a self-stabilizing density gradient is located in each fold early in the experiment. After a time, each protein has migrated towards its isoelectric point, and perhaps reached it. Focused in this way, the proteins thus contribute to the density gradient and reinforce it. This self-stabilizing system has turned out very fruitful for both analytical and preparative focusing. Valmet's technique has the following major advantages:

1. There are no foreign additives, such as sugar or gel, used to stabilize against convection.