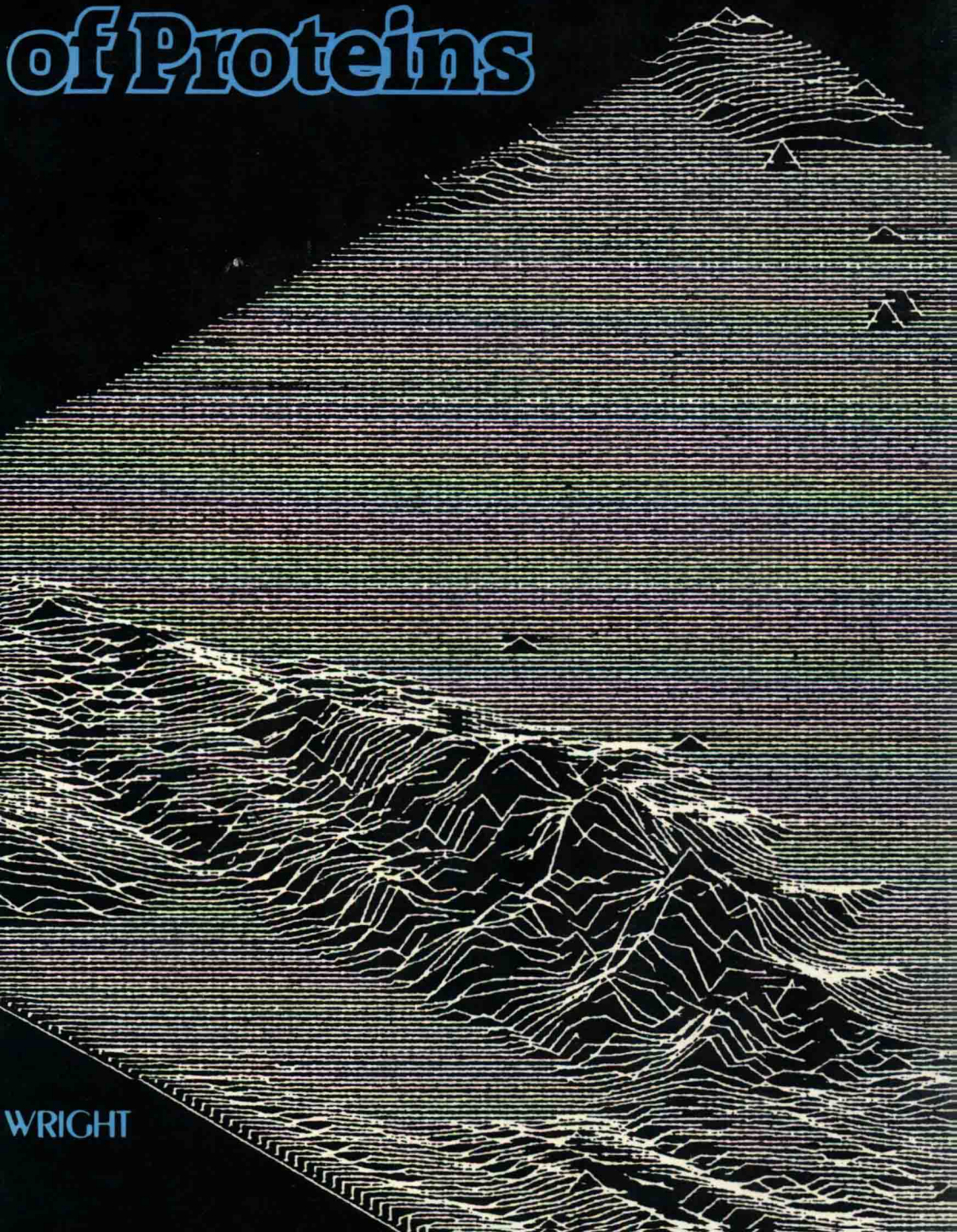
M.J. Dunn

Gel Electrophoresis of Proteins



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Gel Electrophoresis of Proteins

Edited by

Michael J Dunn

Lecturer Royal Postgraduate Medical School
Member of the Jerry Lewis Muscle Research Centre
Hammersmith Hospital
President of the International and the British Electrophoresis Society

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GEL ELECTROPHORESIS OF PROTEINS

Preface

Methods of gel electrophoresis have been developed to such a state where in many situations they are the techniques of highest resolution available for protein analysis. This book is an attempt to give a comprehensive overview of the major techniques of analytical gel electrophoresis currently available. The first seven chapters give the theoretical basis of the major techniques of one-dimensional and two-dimensional gel electrophoresis, describe details of "state-of-the-art" methodologies and give examples of the ways in which these procedures can be applied to a variety of biochemical, biological and biomedical problems. There follows a chapter describing in detail the highly sensitive detection techniques now available for use in conjunction with the various electrophoretic procedures. The final chapter deals with qualitative and quantitative analysis of gel electrophoretograms, particularly with regard to those generated by high resolution two-dimensional methods. Each chapter has an extensive reference list, forming an excellent introduction to the literature for scientists unfamiliar with electrophoretic techniques who might be contemplating their use in a particular research project. For those already initiated into the mysteries of electrophoresis, it is hoped that the material contained within this volume can answer some questions, stimulate new ones and perhaps stimulate some advances in electrophoretic technology.

I would like to thank the authors for their willingness to contribute to this volume and for their punctuality in submitting manuscripts. I also express my gratitude to Dr P A Edge and the staff of John Wright for their patience and assistance in the completion of this book.

M J Dunn
April 1985

List of Contributors

A H M Burghes
Department of Genetics
The Hospital for Sick Children
555 University Avenue
Ontario
CANADA

M J Dunn
Jerry Lewis Muscle Research Centre
Roval Postgraduate Medical School
Ducane Road
LONDON
W12 OHS

K Gooderham
Bioseparations Research Department
LKB Produkter AB
Box 305
S-161 26 Bromma
SWEDEN

P M H Heegaard and T C Bøg-Hansen
The Protein Laboratory
University of Copenhagen
34 Sigurdsgade
DK-2200 Copenhagen N
DENMARK

C R Merril, M G Harasewych and M G Harrington
Section on Biochemical Genetics
Clinical Neurogenetics Branch
National Institute of Mental Health
Bethesda
Maryland 202051000
USA

P G Righetti, C Gelfi and E Gianazza
Faculty of Pharmacy and Department of
Biomedical Sciences and Technologies
University of Milano
Via Celoria 2
Milano 20133
ITALY

G M Rothe and W D Maurer
Institut für Allgemeine Botanik
Johannes-Gutenberg Universitat Mainz
Saarstrasse 21
6500 Mainz
FRG

C Schafer-Nielsen
The Protein Laboratory
University of Copenhagen
34 Sigurdsgade
DK-2200 Copenhagen N
DENMARK

S P Spragg, R Amess, M I Jones and R Ramasamy
Department of Chemistry
University of Birmingham
BIRMINGHAM
B15 2TT

List of Abbreviations

ACES	N-(2-acetamido)2-amino-ethane sulphonic acid
A/D	analogue/digital
Arg	arginine
Asn	asparagine
Asp	aspartic acid
b-Ala	beta-alanine
ATPase	adenosine 5′-triphosphatase
BAC	N,N′-bisacrylyl-cystamine
BEF	buffer isoelectric focusing
Bis	N,N′-methylene bisacrylamide
C	total g crosslinker per 100 ml
CA	carrier ampholyte
CCD	charge coupled device
CHAPS	3-[(cholamidopropyl)-dimethylammonio]-1-propane sulphonate
CMC	critical micelle concentration
Con A	concanavalin A
CRIE	crossed radio immunoelectrophoresis
CSF	cerebrospinal fluid
Cyt b5	cytochrome b5
Cyt c	cytochrome c
D/A	digital/analogue
DATD	N,N′-diallyltartardiamide
DDA	dodecyl alcohol
DDE	didodecyl ether
DDS	didodecyl sulphate
DHEBA	N,N′-(1,2-dihydroxyethylene) bisacrylamide
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPM	disintegrations per minute
EACA	epsilon amino caproic acid
E. coli	*Escherichia coli*
EDA	ethylene diacrylate
EDTA	ethylenediaminetetraacetic acid
EEO	electroendosmosis
ELISA	enzyme linked immunosorbent assay
FFT	fast Fourier transform
Gly	glycine
Hb	haemoglobin
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethane-sulphonic acid

His	histidine
Hp	haptoglobin
HRP	horseradish peroxidase
HSA	human seum albumin
IEF	isoelectric focusing
IPG	immobilised pH gradients
ITP	isotachophoresis
K_d	dissociation constant
K_R	retardation coefficient
LPS	lipopolysaccharide
Lys	lysine
MDPF	2-methoxy-2,4-diphenyl-3(2H)-furanone
MES	morpholinoethane sulphonic acid
mol M	molecular mass
MTT	methyl thiazolyl tetrazolium
N_A	Avogadro's number, 6.022×10^3
NAA	neutron activation analysis
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of NAD
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of NADP
NEPHGE	non-equilibrium pH gradient electrophoresis
PAA	polyacrylamide
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
PAGGE	polyacrylamide gradient gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethyleneglycol
pI	isoelectric point
PM	photomultiplier
PMS	phenazine methasulphate
PMSF	phenylmethanesulphonyl fluoride
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
R_S	Stokes' radius
SB	sulphobetaine
SDS	sodium dodecyl sulphate
SNEP	snow electrophoresis
T	total g acrylamide + g Bis per 100 ml
TACT	N,N′,N′-triallylcitric triamide
TCA	trichloroacetic acid
TEMED	N,N,N′,N′-tetramethylethylenediamine
TEP	telescope electrophoresis

Tris	Tris hydroxymethyl aminomethane
Vh	volt hours
VT	vidicon camera
Wh	watt hours
2-D	two-dimensional

Contents

Chapter 1

Steady-state Gel Electrophoresis Systems

by
C. Schafer-Nielsen

1.1 Introduction

The term steady-state electrophoresis will in the present outline be used as a reference to electrophoresis characterised by a steady state, in which the electrolyte phases remain of constant ionic composition and in which there is no net transport of ion constituents by diffusion processes. The term is used in connection with isotachophoresis systems, moving boundary systems and isoelectric focusing systems.

Most workers in the field of protein analysis are acquainted with isotachophoresis through the daily use of discontinuous buffer systems in SDS-electrophoresis (sodium dodecyl sulphate). Here a sharp boundary between the electrolyte phases is employed for concentration of sample proteins in the early part of the run. Moving boundary systems are for the time being less widely used, but were in fact among the first systems applied in controlled electrophoresis of proteins and colloids by Tiselius in the first half of the century. A notable example of moving boundary electrophoresis is found in the early stage of isoelectric focusing with polyampholytes, i.e. before the final separation of ampholytes into discrete zones occupied by only one ampholyte species.

The aim of this article is to outline the various types of electrophoresis systems encountered in daily laboratory work and to provide the reader with an insight in the quantitative basis for calculation of their ionic composition. The reluctance with which the non-expert sets out to design electrophoresis systems is justified by the barrier presented by the somewhat

complicated electrophoresis theory. However, a deeper understanding of the quantitative relations involved in the calculation of electrolyte phases for steady-state electrophoresis systems is not a pre-requisite for the worker who wants to design an electrophoresis system for his own needs. In fact, a qualitative understanding combined with simple calculation equipment is sufficient for this purpose in the majority of cases, especially where the task is to design a discontinuous electrophoresis system involving a few buffer constituents. At a later stage, if necessary, one can activate a computer program and obtain more precise knowledge of the quantitative composition of the systems. As will be demonstrated below, the main modest requirement for quantitative work often turns out to be programmable pocket calculator and a table of the pK and mobility values of the system constituents.

1.2 Historical Developments

The formation of sharp migrating boundaries between different electrolyte phases suspended in an electric field was observed independently by several workers in the nineteenth century. A quantitative theory accounting for the steady-state composition of the electrolyte phases was worked out by Friedrich Kohlrausch (1897). He realised that the ionic constituents were migrating independently with specific mobilities. In the first half of this century the phenomenon was extensively employed as a means for determination of ionic conductances and mobilities. However, although early experiments (Picton and Linder 1892) had been carried out on haemoglobin, the potential of the method, and indeed of electrophoresis in general, as a tool in biochemical investigations was not realised until Tiselius in the late 1920s carried out his studies on electrophoretic separation of proteins and colloids (Tiselius 1930). With the advent of the Tiselius apparatus, boundaries formed by electrophoretically migrating proteins in buffer solutions could be recorded optically and by light absorption and it became clear that the principle offered unique possibilities for the study of heterogenous biological fluids. The separation technique preceeded the application of useful chromatographic separations of proteins by more than a decade, and it was for a while the only useful method by which one could monitor the overall protein composition of, for example, blood serum. Tiselius himself did not formulate a quantitative theory for the moving boundary systems. A useful theory confined to strong electrolytes was published in 1945 by Dole and the theory was supported experimentally in an accompanying paper by Longsworth (1945), who carried out measurements in electrophoresis cells containing up to six monovalent ion species forming five independent boundaries. The moving boundary theory was extended to comprise weak electrolytes by Svensson (1948) and Alberty

(1950) and was later unified by the author in collaboration with P J Svendsen (Schafer-Nielsen and Svendsen 1981) to comprise any *n*-component steady-state electrophoresis system characterised by electrolyte phases of constant and self-adjusting composition.

The analytical device of Tiselius in which the sample ions are subjected to electrophoresis in free buffer, i.e. without an anticonvection medium, was supplemented in the late forties by the introduction of paper electrophoresis (Durrum 1950). The use of porous paper as an anti-convection medium led to a drastic reduction in the cost of equipment and at the same time a higher resolution was obtained, at least for some purposes. The paper strips were soon followed by gel based anti-convection media, e.g. starch (Smithies 1955), all of which are characterised by a low content although not complete lack of fixed net charges. The presence of fixed charges was found to interfere with electrophoresis, partly because of ionic binding of charged sample molecules and partly because fixed net charges give rise to electroendosmosis, i.e. to electrophoretic migration of water (Vanderhoff and Micale 1979). This intriguing phenomenon was a nuisance in steady-state electrophoresis systems, especially where local changes in the field gradient are accompanied by local deformation of the anticonvection medium due to changes in the electroendosmotic flow of water.

A major breakthrough in analytical electrophoresis followed from the advent of polyacrylamide gel (Raymond and Weintraub 1959), a hydrophilic non-ionic polymer with controllable protein permeability. In these gels one could obtain hitherto unprecedented resolution during electrophoresis of proteins, because of the low adsorption of the sample molecules and because the lack of electroendosmosis permitted the use of discontinuous steady-state electrophoresis systems. The first to employ the polyacrylamide gels in this context were Ornstein (1964) and Davis (1964) who introduced the technique under the name still used today: 'disc electrophoresis'. The word 'disc' refers both to the discontinuous electrolyte phases and to the discoid shape of the boundaries obtained in cylindrical electrophoresis tubes. Ornstein presented in 1964 a theoretical outline of the ionic composition of disc electrophoresis systems involving weak monovalent electrolytes and the theory was later expanded by others to comprise polyvalent electrolytes (Routs 1971, Jovin 1973, Everaerts 1976) and ampholytes (Schafer-Nielsen *et al* 1980).

With polyacrylamide as a nearly ideal anticonvection medium and disc electrophoresis as a perfect means for *in situ* concentration of applied sample proteins, the resolving power of electrophoresis was increased to an extent where up to about 20 protein species could be distinguished in a given run compared to the five to seven peaks obtainable with the Tiselius technique. However, two more refinements established analytical electrophoresis as an indispensable tool in biochemical work with protein mixtures.

The first of these was due to Svensson (1961), who, following on from

results obtained by earlier workers (Williams and Waterman 1930, Kolin 1954, 1955), initiated studies around 1960 on the possibility for controlled use of ampholytes for electrophoretic generation of self-stabilising pH gradients. The basic consideration was, that if an aqueous solution of different ampholytes is subjected to an electric field in a closed system, the ampholytes will separate into discrete zones each occupied by one ampholyte species inducing a pH value close to its isoelectric point. In this way a stable pH gradient can be formed by electrophoresis of a mixture of ampholytes with different isoelectric points. Since proteins themselves are ampholytes, they can be separated by this process, and adequate spacing of the individual proteins can be obtained by addition of ampholytes with isoelectric points intermediate to those of the proteins. In his theoretical treatise on the principle (Svensson 1961) and the accompanying papers (Svensson 1962, Vesterberg 1966), Svensson pointed out that the number of known low molecular weight ampholytes with suitable isoelectric points is on the short side of what is needed for adequate spacing in isoelectric fractionation of complex protein mixtures. He further found that few of these ampholytes possess reasonable buffering capacity at their isoelectric points. The low buffering capacity is because most of the compounds (mainly amino acids and their derivatives) possess pK values two or more units from the isoelectric points. Svensson in addition pointed out that the low buffering capacity was accompanied by a low molar conductivity at the isoelectric points. The use of such ampholytes would thus lead to poorly defined pH gradients and the field strength required for their electrophoretic separation would be very high. The requirement then was for mixtures containing a large number of low molecular weight ampholytes with different isoelectric points and with closely spaced pK values. The riddle was solved when Vesterberg (1969) managed to work out a synthesis of highly diverse polyampholytes through a reaction of acrylic acid with oligoamines, the latter formed from polymerisation of ethylenediamine with dichloroethane. The product was made commercially available by LKB under the trade name 'Ampholine' and the type of electrophoresis became known as 'isoelectric focusing'.

The other major development was the introduction of sodium dodecyl sulphate (SDS) as the solubility agent for electrophoresis of proteins in polyacrylamide gels (Maizel 1969). This anionic detergent was known to be an extremely efficient solubiliser and denaturant of proteins. The efficiency is due to mutual electrostatic repulsion of the proteins following binding of large numbers of the negatively charged detergent molecules on their surface. In excess of the detergent, the amount bound to a given amount of protein differs little from one protein species to another, and the amount of sulphate groups in the protein–detergent complex is large compared to the number of charges of the native protein. The electrostatic repulsion of the sulphate groups is sufficient not only to dissociate protein aggregates, but also to change the tertiary structure of the individual protein molecules,

so that they tend to assume a rod-shaped structure. Proteins treated in this way thus tend to have the same shape and charge density, and therefore the same electrophoretic mobility in a homogenous medium. In order to achieve an electrophoretic separation of SDS-treated proteins, it is necessary to perform the electrophoresis in an inhomogenous medium where separation parameters other than shape and charge can be exploited. Polyacrylamide gels provide an answer to this problem. With this material, it is possible to obtain a matrix in which the electrophoretic migration of macromolecules is restricted by partition between the polymer network and the aqueous phase. When SDS-treated proteins are subjected to electrophoresis in polyacrylamide, the migration velocity obtained shows an inverse linear correlation with the logarithm of the molecular weight. Although the exact mechanism of action is still open to theoretical interpretation (Bode 1980), it seems safe to conclude that the separation is due to the higher degree of interaction with the polymer network experienced by large as compared with small molecules, i.e. to the inhomogeneity obtained by the coexistence of a hydrated polymer and a free solvent. Molecular sieving of this type is observed in other types of gel as well, but due to its inertness and the unique possibility for controlled regulation of the sieving characteristics, polyacrylamide is still the material of choice.

The SDS–polyacrylamide gel electrophoresis was eventually refined by Laemmli (1970) through a combination with the disc electrophoresis principle of Ornstein and Davis. The resolving power was hereby increased to about 50 peptide species in a single analytical run and, because of the solubilising ability of dodecyl sulphate, almost any protein sample can be prepared for the purpose.

The next step forward in analytical electrophoresis came when O'Farrell (1975) designed a generally applicable combination of isoelectric focusing and SDS–polyacrylamide gel electrophoresis in which radio-labelled proteins could be separated analytically and detected by autoradiography with a resolving power of several hundred different peptides in a single sample.

Recent developments in analytical electrophoresis have mainly been centred on refinements and the extended use of the established techniques already mentioned. Thus Rosengren *et al* (1977) have introduced an elegant technique for obtaining protein titration curves by electrophoresis into isoelectrically separated ampholytes, and the technique has been extensively exploited by Righetti and Gianazza (1980) who have also been involved in the refinement of isoelectric focusing in polyacrylamide gels with pH gradients generated by acids and bases that are covalently coupled to the gel matrix (Gasparic *et al* 1975). This promising development was introduced by Righetti *et al* (1982) in collaboration with the LKB laboratories who have marketed the reactive electrolytes under the trade name 'Immobilines'. With immobilised pH gradients, the resolving power in isoelectric focusing of proteins approaches the highest conceivable level where proteins with isoelectric points differing by as little as 0.001 pH unit can be separated.

1.3 Fundamental Steady-state Electrophoresis Systems

As mentioned above, the steady-state electrophoresis systems usually involve boundaries that separate electrolyte phases with different ionic composition. The electrolyte phases are usually different in the qualitative sense, i.e. one or more ion species is present on only one side of the boundary. It is entirely possible, however, to design systems in which the same ions are present on both sides of the boundary with only their concentration ratios being different to those of the adjacent electrolyte phases. Figure 1.1 summarises the four types of electrophoresis systems most commonly employed in gel electrophoresis systems. In this figure, open circles represent ion constituents with similar sign of charge (positive or negative), whereas the closed circles represent the counter ions. Possible steady-state boundaries between different electrolyte phases are represented by the broken lines. In the zone electrophoresis system, the sample molecules (large open circles) migrate in a background electrolyte (the electrophoresis buffer) which is of uniform composition throughout the system.

In zone electrophoresis, the sample molecules are unrestricted from diffusion, and accordingly no sharp boundaries exist in such systems. The lack of stabilising boundaries excludes zone electrophoresis systems from the family of steady-state electrophoresis systems as defined in this outline.

The moving boundary systems (figure 1.1) are characterised by boundaries between electrolyte phases of different ionic composition in which at least one of the ion constituents that migrates in the same direction as a given boundary is present in the electrolyte phases on both sides of that

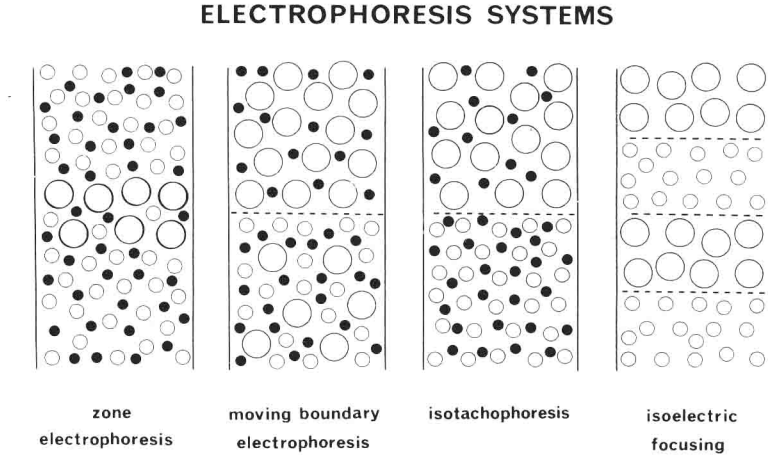

Figure 1.1 Schematic representation of the distribution of ion constituents in various types of electrophoresis systems. Open and closed circles represent ions carrying opposite net charge.