H. R. Maurer

Disc Electrophoresis

and Related Techniques of Polyacrylamide Gel Electrophoresis

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by H. Rainer Maurer Dr. rer. nat.

Max-Planck-Institut für Virus-Forschung
Tübingen, Germany

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Preface

The excellent fractionations of proteins, polypeptides and nucleic acids obtained with *disc electrophoresis* are attracting increasing attention in

biochemistry, clinical chemistry, forensic chemistry, food chemistry, toxicology, pharmacy, pharmacology, enzymology, immunology, zoology, microbiology, botany, cytology, molecular biology, etc.

This is demonstrated by the growing number of studies which make use of this primarily *microanalytical method.*

We are indebted to ORNSTEIN and DAVIS who first described the method in 1959 and who have developed its theoretical and practical aspects in subsequent years.

What is the reason for the high resolving power of disc electrophoresis? The advantages of *gels* as electrophoretic matrices compared to liquid, granular or solid media have become generally known since the introduction of starch gel and especially, polyacrylamide gel.

Both gels afford separations in continuous homogeneous systems on the basis of the *molecular sieving effect.* As a synthetic polymerization product, polyacrylamide gel is distinguished by its

chemical stability and inertia, transparency, the possibility of controlling its broadly variable structure (to produce the most diverse pore sizes), the absence of adsorption and electro-osmosis, stability to pH and temperature variations, insolubility in most solvents, analytical purity of its components as well as the good reproducibility obtained in its preparation.

Disc electrophoresis differs from continuous polyacrylamide gel electrophoresis by the introduction of discontinuities in buffer composition, pH and pore size in the gel. These discontinuities should produce the *concentrating (stacking) effect:* the samples first concentrate as thin starting zones in the upper, e. g. acid, section of the gel, then separate in the lower, e. g. alkaline, section; the upper part with large pores through which the material migrates unhindered is called the stacking or spacer gel, while the lower section with small pores is known as the separation or running gel. The method received its name from the discontinuities and – by coincidence – the discoid shape of the zones of separated samples.

The resolving power of the method therefore is based on a *combination of the concentrating and the molecular sieving effects.* These properties as well as the special equipment for the performance of the process lead to several *advantages* compared to conventional zone electrophoretic techniques:

The small sample requirement (1–100 μg), the concentration of dilute solutions of material, simple equipment (use of glass tubes), short running times (30–60 min.), a high resolving power for molecules of the most diverse sizes (molecular weights of $< 10^4$ to $> 10^6$) and shapes, good reproducibility of the separations, simple means of detection of protein and nucleic acids in general, as well as of enzymes, antigens, polysaccharide ions and radioactively labeled polyelectrolytes in particular, and others.

The *application* of the method therefore extends to a

purity analysis of preparations and chromatographic fractions, evaluation of extraction and purification methods, analysis of protein and enzyme patterns, determination of relative protein concentrations, identification of various extracts on the basis of their protein patterns, separation and identification of microgram-quantities of radioactively labeled proteins, detection of antigens, evaluation of chemical reactions of proteins and nucleic acids after chemical or physical treatment, etc.

Disc electrophoresis is not limited to analytical purposes: *preparative* (on the milligram scale) and *ultramicroanalytical* separations (on the nano- and picogram scale) have been reported.

For example, in *applied biochemistry*, the method has proved useful as a sensitive purity test. In many cases, it surpassed ultracentrifuge and conventional chromatographic and electrophoretic techniques in its resolving power; comparable sensitivities were often obtained only with immunoelectrophoretic methods. Its field of application is notable: it extends from polypeptides to ribosomes and viruses.

Its usefulness in *practical clinical chemistry* is now being recognized, primarily as a unique means to diagnose the various hyperlipemia syndromes, and also as a rapid means to diagnose hemoglobinopathy, isozyme-indicated pathology and to screen for gammopathy in serum, cerebrospinal fluid and urine. The usefulness, in routine clinical chemistry, of its full resolving capability in the interpretation of complex abnormal protein patterns, remains to be established because the available data of statistically significant tests are not yet sufficient. In *clinical research*, however (serology, hematology, immunology, enzyme pathology, etc.) its high resolving power undoubtedly represents a valuable and in many cases, indispensable, tool; this is indicated for example, by the studies of dys- and paraproteinemias. The concentrating effect of disc electrophoresis permits the direct analysis of dilute protein solutions, for example, of cerebrospinal fluid and urine: The denaturing influences encountered during the concentration of dilute protein solutions by customary methods are thus avoided. On the whole, however, the analytical possibilities offered by discontinuous polyacrylamide gel electrophoresis have not yet been fully exhausted and explored.

The present monograph offers a summary of methodic experiences with disc electrophoresis and related techniques of polyacrylamide gel electrophoresis as far as this is possible. Moreover, it presents examples of research results obtained with this method; these should only serve as stimuli and

demonstrate the diverse applicabilities of the technique. Such a "catch-all" review cannot be considered complete in view of our present-day rapid development of clinical chemistry and biochemistry. The author therefore will gratefully accept any important additions, informations, practical experiences, corrections and suggested modifications. The theoretical fundamentals have been kept brief; references are made to more detailed presentations. In the examples of application in clinical chemistry and biochemistry, it was necessary to limit the selection. Moreover, some overlapping in the description of biochemical and clinical-chemical applications was unavoidable.

The text occassionally refers to *manufacturing firms* in order to facilitate the search for sources of supplies for the reader. A list of firms appears on p. 211. It was not always possible to list comparable products of several firms. Mention of an instrument or a product therefore does not represent a quality rating.

Since the first German edition has appeared in early 1968, a great number of papers have been published dealing with the method. It is impossible to cite all of them, particularly in view of the existence of highly useful information sources such as the Canalco Disc Electrophoresis Information Center (firm 5), several handbook chapters and reviews to which the reader is explicitly referred.

Besides the early account of a conference on gel electrophoresis [W 14], which was held by the New York Academy of Sciences in late 1963 and which covered the basic principles and first applications of the methods, the excellent monograph by Gordon [G 23] and the highly informative and useful expert chapters in a volume edited by Smith [S 53] merit particular attention.

However it was felt that each user of the book would be predominantly interested in a comprehensive survey on most methodic developments hitherto known. Such a review might enable him to select the most suitable technique to solve his particular problems. This book is primarily intended to assist him in this search, to provide him with a useful guideline for laboratory practice and to save him a time-consuming literature study and technical errors.

H. R. M.

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1. THEORETICAL BACKGROUND

1.1. The Polyacrylamide Gel

1.1.1. Formation and Structure of the Gel

Polyacrylamide gel is the *polymerization and cross-linking product* of the monomer *acrylamide*, $CH_2=CH-CO-NH_2$, and a cross-linking comonomer, usually *N,N'-methylene-bis-acrylamide(Bis)*, $CH_2=CH-CO-NH-CH_2-NH-CO-CH=CH_2$ [B 39, O 12, R 16]. The three-dimensional network of the gel is formed by cross-linking of polyacrylamide chains growing side-by-side by the mechanism of vinyl polymerization (copolymerization in solution). This leads to the development of numerous, random polymer gel coils (Fig. 1 A) in which the polyacrylamide chains assume a state of maximum entropy, i. e. the most irregular shape. The growing coils move together (Fig. 1 B) and are cross-linked by main valencies (Fig. 1 C), where bifunctional compounds, such as N,N'-methylene-bis-acrylamide, are built into the polymer chains as cross-linking agents and can react with free functional groups at terminals of other chains. The chemical structure of the gel is shown in Fig. 2. (For other cross-linking comonomers see page 60.)

The concentration of monomer and comonomer in the gelating solution and the *degree of polymerization* (chain length) and *cross-linking* (i. e. the quantity of built-in cross-linker) determine the density, viscosity, elasticity and mechanical strength of the gel [O 9, O 12].

Gel density or *gel concentration* may be defined by two numerals T and C, where the first numeral T denotes the total percentage concentration of both monomeres (acrylamide and Bis) and the second numeral C the percentage concentration of the cross-linker relative to the total concentration T. HJERTÉN [H 23] introduced the following equations to calculate the gel composition:

$$T = \frac{a + b}{m} \cdot 100\ [\%] \quad (1)$$

$$C = \frac{b}{a + b} \cdot 100\ [\%] \quad (2)$$

a = acrylamide (g)
b = N,N'-methylenebisacrylamide (g)
m = volume of buffer (ml)

The *weight ratios of acrylamide to Bis* are highly critical [O 12]: If they are smaller than 10, the gels become brittle, rigid and opaque; if they exceed 100, 5 % gels (relative to acrylamide) are pasty and easily break down.

Elastic and completely transparent gels are obtained with ratios of about 30 in which the acrylamide concentration must be higher than 3 %. DAVIS [D 7] investigated the concentration ranges of acrylamide between 1.5

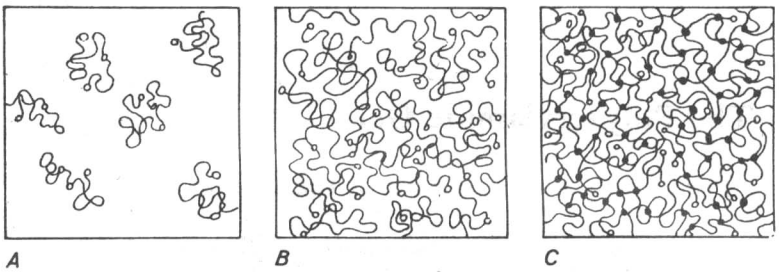

Fig. 1 – Schematic representation of the formation of polyacrylamide gel from random gel coils. Transition from a dilute polymer solution (A) through the concentrated solution (B) to the gel (C). –o– cross-linking agent; –●– tie-points. From VOLLMERT [V7].

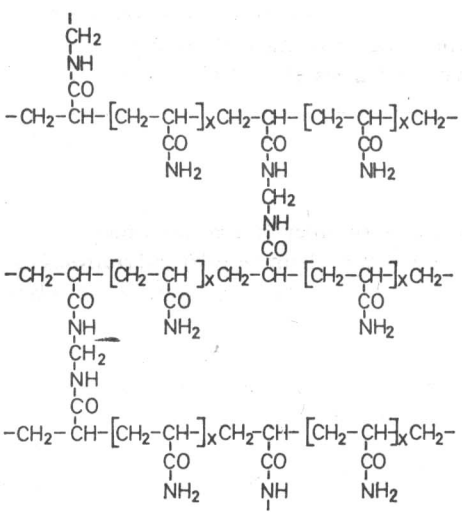

Fig. 2. – Structure of polyacrylamide gel. Monomer: acrylamide; comonomer (cross-linking agent): N,N'-methylene-bis-acrylamide. From OTT [O 12].

and 60 % and of Bis between 0 and 0.625 %. He found that no further gelation takes place at concentrations of less than 2 % acrylamide and 0.5 % Bis; furthermore, he noted that an increase of the acrylamide concentration should be generally accompanied by a decrease of the Bis concentration in

order to produce elastic gels. RICHARDS et al. [R 34] proposed the following "compromise" formula for this requirement:

$$C = 6.5 - 0.3\,T \tag{3}$$

This formula can be used to calculate the composition of manageable gels within the range of 5–20 %; the value of C is not critical and may be varied by about ± 1 % in most cases. Taking optimal resolution of human serum proteins as a basis, BRACKENRIDGE and BACHELARD [B 53] calculated the optimal cross-linkage for a given gel percentage using the least squares regression equation:

$$B = 0.201 - 0.0112\,T \tag{4}$$

where B is the Bis concentration in % and T as above.

1.1.2. Polymerization: Catalysts and Processes

Usually certain *catalyst-redox systems* which furnish free radicals are used for the polymerization of polyacrylamide gels for electrophoresis [L 38], for example:

Ammonium persulfate –	N,N,N',N'-tetramethylethylenediamine (TEMED)
Ammonium persulfate –	3-dimethylaminopropionitrile (DMPN)
Ammonium persulfate –	DMPN-sodium sulfite [S 66 a]
Hydrogen peroxide	– ferrous sulfate - ascorbic acid [J 17]
Riboflavin	– TEMED (as a photocatalytic system).

Even small concentrations of tertiary aliphatic amines, such as TEMED, accelerate the polymerization, while DMPN and triethanolamine appear to be less active [D 7]. Ammonium persulfate has the following advantages: it can be prepared in high purity, is relatively stable at 0° C and has little tendency to develop molecular oxygen. Suitable catalytic systems must not change the selected buffer conditions, electrical conductivity and viscosity of the gels.

The *chemical polymerization* is initiated from free monomer radicals which in turn are produced by the base catalyzed formation of free oxygen radicals from *persulfate*. Since the free bases of TEMED or DMPN are needed, polymerization may be delayed or prevented at lower pH (see also page 55). However, reproducible gels are only obtained if the polymerization is not retarded. Molecular oxygen, cooling and impurities (e. g. metals) can inhibit or even prevent chemical polymerization [0 12]. Consequently, it is usually advisable to select and standardize the conditions such that the solutions will gelate within about one hour.

The initial *rate of polymerization* was found by UV absorbance measurement to be proportional to the square root of the concentration of *ammonium persulfate* [W 4a]. At pH 8.8 a 7.5% solution of acrylamide shows a lag period of several

minutes, then an abrupt increase of the reaction rate which declines as rapidly and levels off after about 30 min. At pH 4.3 the initial rate of polymerization is much slower, hence about 90 min. are required for completion of polymerization. Addition of 5 mM sodium sulfite reduces the lag period to about 2 min.

Polymerization of highly concentrated or large diameter gels produces considerable heat which may lead to cavities in the gel from dissolved gases and to separation of the gel from the wall. The effect can be diminished by decreasing the temperature of the gel solution to about 6° C prior to polymerization [P 15].

In contrast to the chemical polymerization by persulfate the *photochemical polymerization* with *riboflavin* requires traces of oxygen to take place [G 23]. Photodecomposition of riboflavin, with the consequence of reoxidation of leucoflavin and production of free radicals, can only occur if molecular oxygen is initially present. Excess of oxygen, however, may limit chain growth and should be avoided.

Possible artifacts arising from the interaction of sample proteins with the catalysts persulfate and riboflavin are discussed on pages 59 and 60.

NEEDLES [N19a] has discussed the effect of several solution additives (e. g. Tris, TEMED, HCl, Glycine) on *photopolymerization* and has suggested that polymerization accelerators (like TEMED) are generally mild reducing agents (hydrogen donators) which may complex with riboflavin and/or monomers through donor-acceptor interactions.

The starting monomers can also be dissolved in glycerol, ethylene glycol, concentrated urea or saccharose solutions instead of water without a notable inhibition of gelating power; however, a reduction of the gelation time must be anticipated with an increasing viscosity of the solvent [R 8]. Urea can be incorporated in polyacrylamide gel in a concentration of 8 M at acid [E 1] and alkaline [C 47] pH values.

1.1.3. The Molecular Sieving Effect in Polyacrylamide Gel

By W. THORUN and H. R. MAURER

1.1.3.1. Introduction

In conventional electrophoresis using liquid media, electrically charged particles are separated mainly according to their net charge. In paper electrophoresis, for example, the paper merely serves as a supporting and anticonvection medium which exerts little influence on the separation process itself. *Gels*, however, are distinguished from liquid media by high viscosities and high frictional resistances. These supporting media not only prevent convection and minimize diffusion, but also actively participate in the separation process by interacting with the migrating particles. This interaction depends on the particle size. Consequently, in gels the particles are separated according to both charge *and* size. The property of gels to distinguish molecular