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PG AND RESEARCH DEPARTMENT OF BIOCHEMISTRY
E-NOTES

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SYLLABUS

UNIT –IV **Electrophoretic Techniques**

Electrophoretic Techniques:Polyacrlamide gel electrophoresis, SDS PAGE, 2D Electrophoresis, agarose gel Electrophoresis,isoelectric focusing, phase liquid Electrophoresis.

1 Electrophoresis

1.0 General

Electrophoresis in free solution

Moving boundary electrophoresis: Arne Tiselius (1937) developed the moving boundary technique for the electrophoretic separation of substances, for which, besides his work on adsorption analysis, he received the Nobel prize in 1948. The sample, a mixture of proteins for example, is applied in a U-shaped cell filled with a buffer solution and at the end of which electrodes are immersed. Under the influence of the applied voltage, the compounds will migrate at different velocities towards the anode or the cathode depending on their charges. The changes in the refractive index at the boundary during migration can be detected at both ends on the solution using Schlieren optics.

Tiselius A. Trans Faraday Soc. 33 (1937) 524–531.

Nowadays movingboundary electrophoresis in free solution is mainly used in fundamental research to determine exact electrophoretic mobilities.

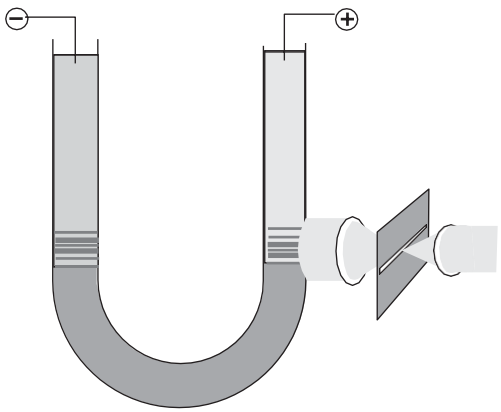

Fig. 3: Moving boundary electrophoresis in a U-shaped cell according to Tiselius. Measurement of the electrophoretic mobility with Schlieren optics.

Electrophoresis in Practice, Fourth Edition. Reiner Westermeier

ISBN: 3-527-31181-5

HannigK. Electrophoresis 3 (1982) 235–243.

This is the only continuous electrophoretic separation method.

Free flow electrophoresis: in this technique developed by Hannig (1982) a continuous stream of buffer flows perpendicular to the electrical field through a buffer film between two cooled glass plates which is 0.5 to 1.0 mm wide. At one end the sample is injected at a defined spot and at the other end, the fractions are collected in an array of tubes.

Wagner H, Kuhn R, Hofstetter S. In: Wagner H, Blasius E. Ed. Praxis der elektrophoretischen Trennmethoden. Springer Verlag, Heidelberg (1989) 223–261.

The varying electrophoretic mobilities perpendicular to the flow lead to differently heavy but constant deviations of the components so that they reach the end of the separation chamber at different though stable positions (see Fig. 4).

Unfortunately electrophoresis in free solution cannot yet be applied on an industrial scale. The upscalingof the instrumentation is limited by the thermal convection which results from the insufficient dissipation of Joule heat from the flowing electrolyte. Loadingcannot be freely increased because highly concentrated samples begin to sediment. Both these limiting factors occur only under gravity. Since 1971, ever since Apollo 14, experiments have been conducted in space to try and develop production in an orbital station.

Besides the separation of soluble substances, this technique is also used for the identification, purification and isolation of cell organelles and membranes or whole cells such as erythrocytes, leukocytes, tissue cells, the causal agent of malaria and other parasites (Hannig 1982, Wagner *et al.* 1989).This method is very effective since even minimal differences in the surface charge of particles and cells can be used for separation.

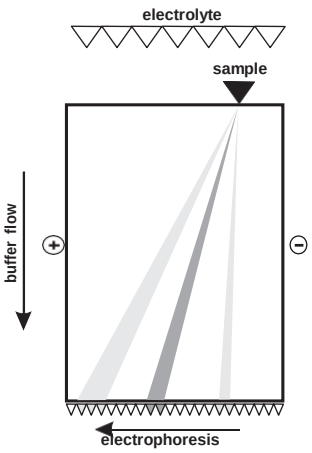

Fig. 4: Schemtic drawing of a continuous free flow electrophoresis system. According to Wagner *et al.* (1989)

Jorgenson JW, Lukacs KD. Anal Chem 53 (1981) 1298–1302

Hjert=n S. J Chromatogr. 270 (1983) 1–6.
Fused silica capillaries are otherwise used in gas chromatography.

Capillary electrophoresis (CE): this technique is being used increasingly for analytical and micropreparative electrophoresis (Jorgenson and Lukacs, 1981; Hjert@n, 1983): as for HPLC, the abbreviation HPCE for High Performance Capillary Electrophoresis is often used. Separation is carried out in a fused silica capillary 20 to 30 cm long and with an internal diameter of 50 to 100 1m. Both ends are immersed in a buffer container into which the electrodes are built (see Fig. 5).

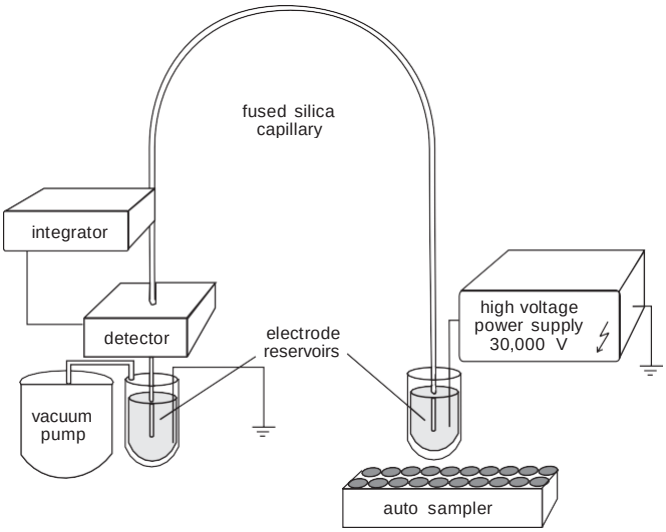

Fig. 5: Example of the instrumentation for capillary electrophoresis.

The amount of chemicals and sample needed is very low. The volume of injected material is usually not more than 2 – 4 nL, nanograms of sample material is required.
Field strengths of up to 1 kV/cm and currents of 10 to 20 mA are used; for this reason a power supply which can yield voltages up to 30 kV is needed. Joule heat can be dissipated very effectively from these thin capillaries with a fan.

CE separations typically take 10 to 20 min. There are many detection methods possible: UV/VIS, fluorescence, conductivity, electrochemistry etc. In most applications the fractions are detected by UV measurement at 280, 260 or in some cases even 185 nm directly in the capillary.

For some substances and applications the limit of detection can go as low as to the attomole level.

In general the results are then further processed by HPLC interpretation software on personal computers.

To prevent adsorption of components on the surface of the capillary and electro-osmotic effects, the inside of the capillary can be coated with linear polyacrylamide or methyl cellulose. Capillary electrophoresis instruments can be used for all three of the separation methods: electrophoresis, isotachophoresis and isoelectric focusing. Even an additional new method, a hybrid of electrophoresis and chromatography, has been developed:

The buffer used depends on the nature of the separation: e.g. 20 to 30 mmol/L sodium phosphate buffer pH 2.6 for electrophoresis of peptides.

Terabe S, Otsuka K, Ichikawa K, Tsuchiya A, Ando T. Anal Chem.64 (1984) 111–113 Terabe S, Chen N, Otsuka K. In Chrambach A, Dunn M, Radola BJ. Eds. Advances in Electrophoresis 7. VCH Weinheim (1994) 87–153.

Micellar electrokinetic chromatography (MEKC) introduced by Terabe *et al.* (1984). It is the only electrophoretic method, which can separate neutral as well as charged compounds. Surfactants are used at concentrations over the the critical micelle concentration. The charged micelles migrate in the opposite direction to the electro-osmotic flow created by the capillary wall. The electro-osmotic counter-flow is faster than the migration of the micelles. During migration, the micelles interact with the sample compounds in a chromatographic manner through both hydrophobic and electrostatic interactions.It has become one of the most widely used CE methods. More details on this method are found in a review by Terabe *et al.* (1994).

However, the investment for such an instrument is by far higher compared to a gel electrophoresis equipment.

One great advantage of capillary electrophoresis lies in its automation. Every step can be controlled by semiautomatic or full automatic instrumentation. An autosampler is a standard part of this equipment.

Another advantage is the possibility of linking with other analytical instruments either before electrophoresis: HPLC/HPCE or after: HPCE/MS.

In contrast to Reversed Phase Chromatography proteins are not damaged during HPCE and, in addition, the resolution is better.

For preparative separations a fraction collector is attached to the UV detector. The identification of the individual substances is done by the relative mobility or the molecular weight, or else the collected fractions are analyzed.

Cohen AS, Karger BL. J Chromatogr. 397 (1987) 409–417.

For molecular weight separations of proteins, peptides,and nucleic acids capillaries filled with linear (non crosslinked) polyacrylamide gel are used (Cohen *et al.* 1987).

See also page 27

The most successful application of capillary electrophoresis is the separation of DNA fragments. Because of the possibility of automation and the repeated use of a high number of capillaries in parallel, this technique is ideal for high throughput DNA sequencing. It had been predicted, that the complete knowledge of the human genome would be available in the year 2005. But the introduction of the new multi-capillary sequencers has speeded up the Human Genome Project considerably.

Electrophoresis in supporting media

The instructions in the second part are limited to electrophoresis in supportingmedia since these techniques only require minimal equipment.

Compact material such as paper, films or gels are used. So as to monitor the progress of the separation and to recognize the end of the run, dyes with a high electrophoretic mobility are applied together with the sample.

For separation of proteins in anodal direction Bromophenol Blue, Xylenecyanol or Orange G are used, in the cathodal direction Bromocresol Green, Pyronine or Methylene Blue.

Detection of the separated zones can either be done directly in the medium by positive staining with Coomassie blue or silver, negative staining with zinc-imidazole, spraying with specific reagents, enzyme substrate coupling reactions, immuno precipitation, autoradiography, fluorography, or indirectly by immunoprinting or blotting methods. A comprehensive survey on enzyme staining methods has been published by Rothe (1994).

Blotting: transfer to immobilizingmembranes followed by stainingor specific ligand binding.

Rothe G. Electrophoresis of enzymes. Springer Verlag, Berlin, (1994).

Paper and thin-layer electrophoresis: These methods have mostly been abandoned in profit of gel electrophoresis, because of improved separation and the higher loading capacity of agarose and polyacrylamide gels. Electrophoretic separations on thin-layer silica gel plates linked to buffer tanks are only carried out for the analysis of polysaccharides of high molecular weight and lipopolysaccharides, which would obstruct the pores of the gels (Scherz, 1990).

Scherz H. Electrophoresis 11 (1990) 18–22.

Cellulose acetate membrane electrophoresis: cellulose acetate membranes have large pores and therefore hardly exert any sieving effect on proteins (Kohn, 1957). This means that these electrophoretic separations are entirely based on charge density.

Kohn J. Nature 180 (1957) 986-988.

The matrix exerts little effect on diffusion so that the separated zones are relatively wide while the resolution and limit of detection are low. On the other hand they are easy to handle and separation and staining are rapid. The cellulose acetate strips are suspended in the tank of a horizontal apparatus, so that both ends dip in the buffer; no cooling is necessary during separation. This technique is widely used for routine clinical analysis and related applications for the analysis of serum or isoenzymes.

Because the resolution and reproducibility of separations in agarose and polyacrylamide gels are better, cellulose acetate membranes are more and more often replaced by gel electrophoresis.

Gel electrophoresis

The gel: The gel matrix should have adjustable and regular pore sizes, be chemically inert and not exhibit electroendosmosis. Vertical cylindrical gel rods or plates as well as horizontal gel slabs are employed, the latter being usually cast on to stable support film to facilitate handling (Fig. 6).

The use of gel rods has become very rare, because of the complicated handling and difficult pattern comparison. Slab gels for vertical and flatbed systems can be easily polymerized in the laboratory, but prefabricated gels of many different types are available from various suppliers. An overview over the features, benefits and drawbacks of vertical and horizontal slab gel systems is given in Table 1.

The most instructions in the second part are describing horizontal gels on support films since these can be used for all applications and with universally applicable equipment.

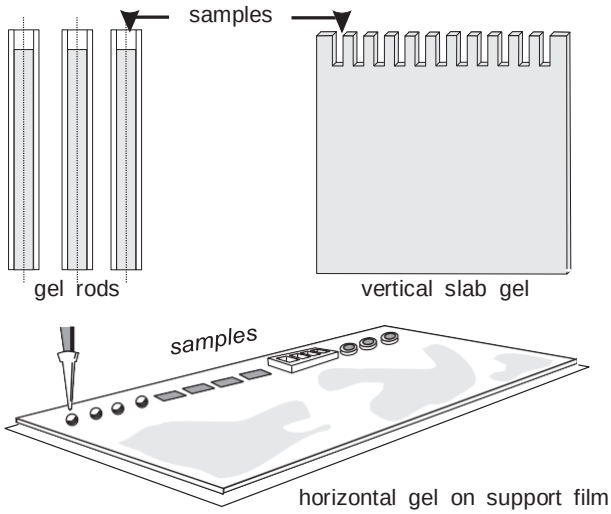

Fig. 6: Gel geometries for electrophoretic separations.

Tab. 1: Comparison of flatbed and vertical gel systems

	Flatbed Systems:	*Vertical Systems:*
Up to 3 mm thick gels can be used in vertical systems.	Gel thickness is limited, because cooling is only possible from one side	Higher protein loading capacity, because thicker gels can be used, which are cooled from both sides
Also, on flatbed systems mostly gels on film supports are employed. But films can be removed.		Blotting is easier because of higher gel thickness
	One gel per instrument is run	Multiple gel runs possible
In IEF, samples very often have to be loaded inside the pH gradient.	Very versatile for different methods, ideal for isoelectric focusing	Limited technical possibilities, not optimal for isoelectric focusing
Very thin gels show higher sensitivity of detection, and are easier and quicker to stain.	Thin layers can easily be used, easy sample application	The thinner the gel, the more complicated is sample application
The buffer strip concept reduces chemical and radioactive liquid waste considerably (Kleine et al. 1992).	Buffer strips (polyacrylamide or filter paper) can be used instead of large volumes of liquid buffers	
On flatbed systems mostly gels on film supports are employed.	Easy to handle and to clean, no glass plates necessary, thus ideal for routine applications	Many pieces to set up and to clean
In a flatbed system the buffers can not leak into each other.	Higher electric safety	

Starch gels were introduced by Smithies (1955) and are prepared from hydrolyzed potato starch which is dissolved by heating and poured to a thickness of 5 to 10 mm. The pore size can be adjusted by the starch concentration of the solution. Because of the low reproducibility and the impractical handling these gels have been largely replaced by polyacrylamide gels.

Smithies O. Biochem J. 61 (1955) 629–641.

Starch is a natural product whose properties can vary greatly.

Agarose gels are mostly used when large pores for the analysis of molecules over 10 nm in diameter are needed. Agarose is a polysaccharide obtained from red seaweed.

By removal of the agaropectin, gels of varying electroendosmosis and degrees of purity can be obtained. They are characterized by their melting point (35 IC to 95 IC) and the degree of electroendosmosis (m_r).

m_r is dependent on the number of polar groups left. The definition is the same like for relative electrophoretic mobility.

The pore size depends on the concentration of agarose: one usually refers to the weight of agarose and the volume of water. The unavoidable losses of water which occur during heating can vary from batch to batch, so in practice, this value cannot be absolutely exact. In general gels with a pore size from 150 nm at 1% (w/v) to 500 nm at 0.16% are used.

For pore diameters up to 800 nm (0.075% agarose): Serwer P. Biochemistry 19 (1980) 3001–3005.

Agarose is dissolved in boiling water and then forms a gel upon cooling. During this process double helixes form which are joined laterally to form relatively thick filaments (Fig. 7).

For DNA separations 1 to 10 mm thick gels are cast on UV-transparent trays, because the bands are usually stained with fluorescent dyes: Ethidium bromide or SYBR Green.

The gels are run under buffer in order to prevent dryingout due to electroendosmosis.

For protein electrophoresis the gels are made by coating horizontal glass plates or support films with a solution of agarose. The thickness of the gel – usually 1 – 2 mm – is determined by the volume of the solution and the surface it covers.

Very even gel thicknesses are obtained by pouringthe solution in prewarmed molds.

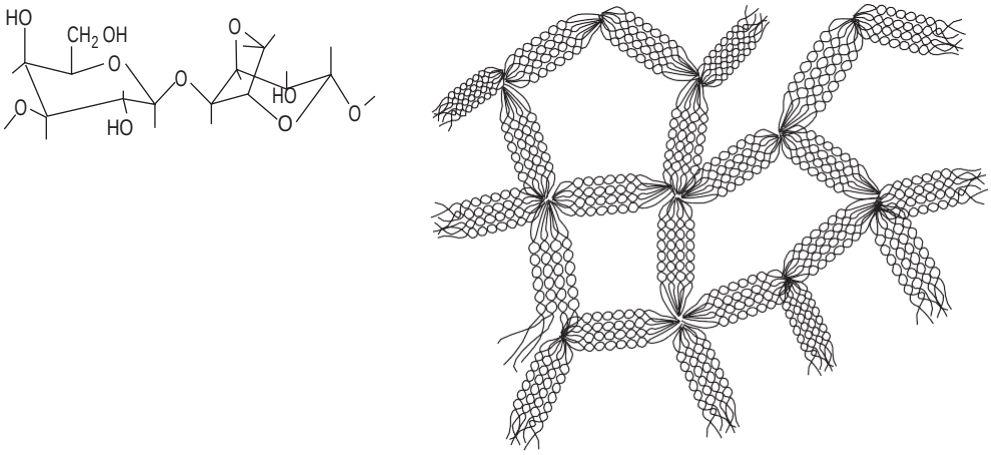

Fig. 7: Chemical structure of agarose and structure of the polymers during gel formation.

Kerenyi L, Gallyas F. Clin Chim Acta 38 (1972) 465–467.

The separated protein bands are mostly detected by Amido Black or Coomassie Brilliant blue staining of the gels after drying them. In order to improve the protein detection limit, the first silver staining technique had been developed for agarose gels to detect oligoclonal IgGs in cerebrospinal fluid (Kerenyi and Gallyas, 1972)

Raymond S. Weintraub L. Science. 130 (1959) 711–711. The reaction is started with ammonium persulphate as catalyst, TEMED provides the tertiary amino groups to release the radicals.

Polyacrylamide gels were first used for electrophoresis by Raymond and Weintraub (1959). They are chemically inert and mechanically stable. By chemical co-polymerization of acrylamide monomers with a cross-linking reagent – usually N,N¢-methylenebisacrylamide (Fig. 8) – a clear transparent gel which exhibits very little electroendosmosis is obtained.

Hjert=n S. Arch Biochem Biophys Suppl 1 (1962) 147.

The pore size can be exactly and reproducibly controlled by the total acrylamide concentration T and the degree of cross-linking C (Hjert@n, 1962):

$$T = \frac{a+b}{V} \times 100\,[\%], \quad C = \frac{b}{a+b} \times 100\,[\%]$$

a is the mass of acrylamide in g,
b the mass of methylenebisacrylamide in g, and
V the volume in mL.

Gels with C > 5 % are brittle and relatively hydrophobic. They are only used in special cases.

When C remains constant and T increases, the pore size decreases. When T remains constant and C increases, the pore size follows a parabolic function: at high and low values of C the pores are large, the minimum being at $C = 5\%$.

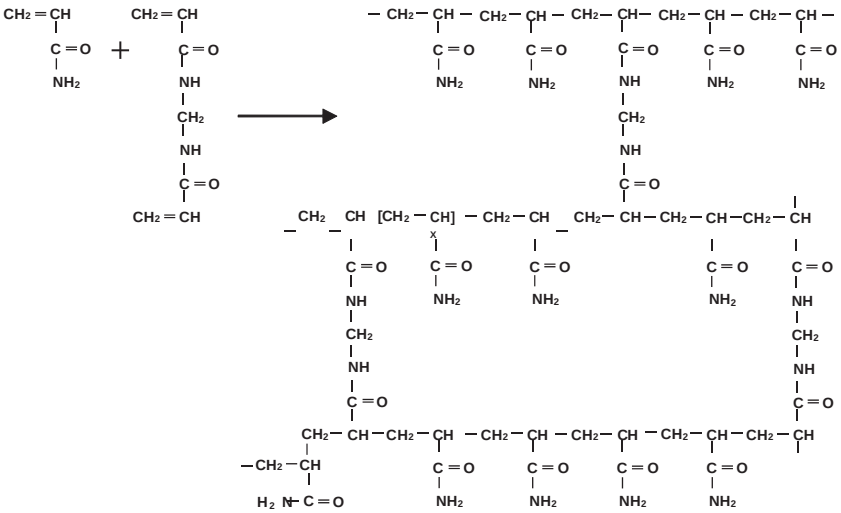

Fig. 8: The polymerization reaction of acrylamide and methylenebisacrylamide.

Besides methylenebisacrylamide a number of other cross-linking reagents exist, they have been listed and compared by Righetti (1983). N,N¢-Bisacryloylcystamine is mentioned here, it possesses a disulfide bond which can be cleaved by thiol reagents. Because of this, it is possible to solubilize the gel matrix after electrophoresis.

Righetti PG. Isoelectric focusing: theory, methodology and applications. Elsevier Biomedical Press, Amsterdam (1983).

Polymerization should take place under an inert atmosphere since oxygen can act as a free radical trap. The polymerization is temperature dependent: to prevent incomplete polymerization the temperature should be maintained above 20 IC.

The monomers are toxic and should be handled with precaution.

To minimize oxygen absorption gels are usually polymerized in vertical casting chambers: cylindrical gels in glass tubes and flat gels in moulds formed by two glass plates sealed together around the edges.

With horizontal castingoxygen intake is increased. That must be compensated by a higher amount of catalyst, often leadingto problems during separation.

For electrophoresis in vertical systems the gel in glass rods or cassettes are placed into the buffer tanks, and are in direct contact with the electrode buffers. Gels for flatbed systems are polymerized on a support and removed from the mould before use.

For sample application wells are formed at the upper edge of the gel during polymerization (see Fig. 6). These are made by insertion of a sample comb between the glass plates. In horizontal gels, sample wells are not always necessary; the samples can be applied directly on the surface with strips of filter paper or silicone rubber.

In homogeneous buffer systems, narrow sample slots on the surface of horizontal gels are also important to obtain good results.

The various gel electrophoresis methods can be divided into those in restrictive and non-restrictive media. Restrictive gels work against diffusion so the zones are more distinctly separated and better resolved than in non-restrictive gels. The limit of detection is thus increased.

In restrictive gels, the molecule size has a major influence on the result of the separation.

1.1 Electrophoresis in non-restrictive gels

For these techniques the frictional resistance of the gel is kept negligibly low so that the electrophoretic mobility depends only on the net charge of the sample molecule. Horizontal agarose gels are used for high molecular weight samples such as proteins or enzymes and polyacrylamide gels for low molecular weight peptides or polyphenols.

1.1.1 Agarose gel electrophoresis

Zone electrophoresis

Agarose gels with concentrations of 0.7 to 1% are often used in clinical laboratories for the analysis of serum proteins. The separation

times are exceedingly low: about 30 min. Agarose gels are also used for the analysis of isoenzymes of diagnostic importance such as lactate dehydrogenase (Fig. 9) and creatine kinase.

Because of their large pore size, agarose gels are especially suited to specific protein detection by immunofixation: after electrophoresis the specific antibody is allowed to diffuse through the gel. The insoluble immunocomplexes formed with the respective antigen result in insoluble precipitates and the non-precipitated proteins can be washed out. In this way only the desired fractions are detected during development.

Besides immunofixingand immunoprinting, immunoblottingalso exists for protein identification: immobilizing membranes, for example nitrocellulose, are used on the surface of which the proteins are adsorbed, see "blotting" on page 67 ff.

Immunoprinting functions in a similar way: after the electrophoretic separation, an agarose gel containing antibodies or a cellulose acetate membrane impregnated with antibodies is placed on the gel. The antigens then diffuse towards the antibodies and the identification of the zone is done in the antibody-containing medium. Immunoprinting is mainly used for gels with small pores.

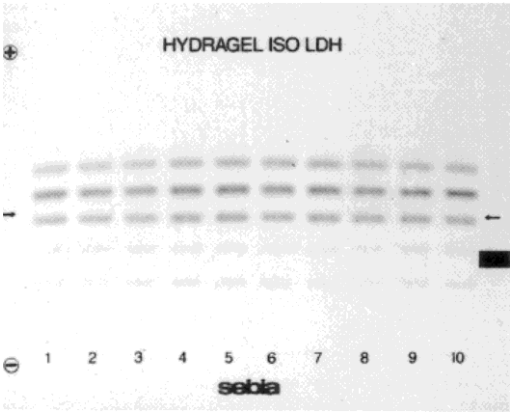

Fig. 9: Agarose electrophoresis of lactate dehydrogenase isoenzymes. Specific staining with the zymogram technique.

Immunoelectrophoresis

The principle of immunoelectrophoresis is the formation of precipitate lines at the equivalence point of the antigen and its corresponding antibody. In this method it is important that the ratio between the quantities of antigen and antibody be correct (antibody titer).

When the antibody is in excess, statistically at most one antigen binds to each antibody while when the antigen is in excess at most one antibody binds to each antigen. Yet at a specific antigen/antibody ratio (equivalence point) huge macromolecules are formed.

They consist of an antigen-antibody-antigen-antibody-... sequence and are immobilized in the gel matrix as an immunoprecipitate. The white precipitate lines are visible in the gel and can be revealed with protein stains. The method is specific and the sensitivity very high because distinct zones are formed. Immunoelectrophoresis can be divided into three principles (Fig. 10):

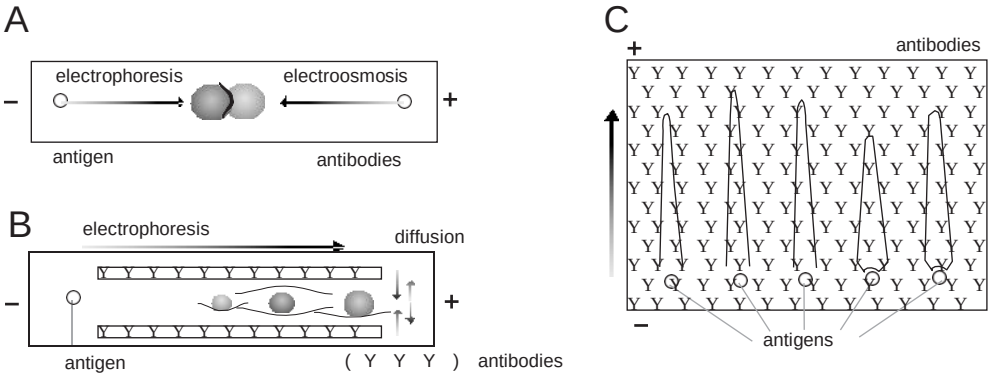

Fig. 10: The three principles of immunoelectrophoresis A, B and C, see text for details.

A. *Counter immunoelectrophoresis* according to Bussard (1959): in an agarose gel exhibiting high electroendosmosis, the buffer is set at a pH about 8.6 so that the antibody does not carry any net charge. The sample and the antibody are placed in their respective wells and move towards each other: the charged antigens migrate electrophoretically and the antibodies are carried by the electro-osmotic flow.

Bussard A. Biochim. Biophys Acta. 34 (1959) 258–260.

B. *Zone electrophoresis/immunodiffusion* according to Grabar and Williams (1953): first a zone electrophoresis is run in an agarose gel, followed by the diffusion of the antigen fraction towards the antibody which is pipetted into troughs cut in the side parallel to the electrophoretic run.

Grabar P, Williams CA. Biochim Biophys Acta. 10 (1953) 193.

C. The *"rocket"* technique according to Laurell (1966) and the related methods: antigens migrate in an agarose gel which contains a definite concentration of antibody. As in method A the antibodies are not charged because of the choice of the buffer. As the sample migrates one antibody will bind to one antigen until the ratio of concentrations corresponds to the equivalence point of the immunocomplex.

Laurell CB. Anal Biochem. 15 (1966) 45–52.

The result is that rocket shaped precipitation lines are formed, the enclosed areas are proportional to the concentration of antigen ion in the sample. A series of modifications to this technique exist, including two-dimensional ones.

Affinity electrophoresis

Bøg-Hansen TC, Hau J. J Chrom Library. 18 B (1981) 219–252.

This is a method related to immunoelectrophoresis which is based on the interactions between various macromolecules for example lectin-glycoprotein, enzyme-substrate and enzyme-inhibitor complexes (Bøg-Hansen and Hau, 1981)

All the techniques known from immunoelectrophoresis can be employed. For example, specific binding lectin collected worldwide from plant seeds are examined with line affinity electrophoresis. In this way carbohydrate changes in glycoproteins during different biological processes can be identified. In Fig. 11 an application of affinity electrophoresis to differentiate between alkaline phosphatase of liver and bone is shown.

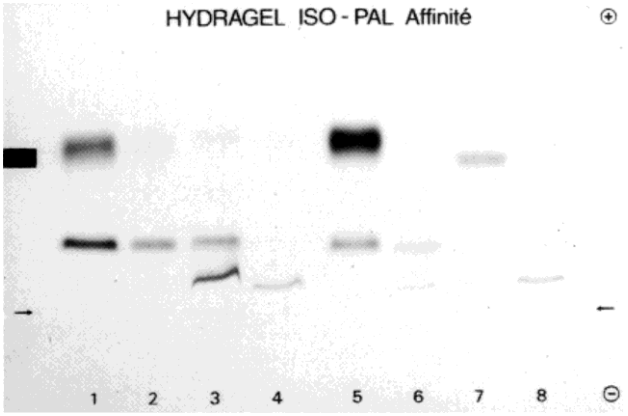

Fig. 11: Affinity electrophoresis of isoenzymes of alkaline phosphatase from the liver and the bones. The wheat germ agglutinin specifically binds the bone fraction which is recognizable as a characteristic band close to the application point. Alkaline phosphatase staining.

1.1.2 Polyacrylamide gel electrophoresis of low molecular weight substances

See method 1

**Accordingto the guide-lines of the SI, the use of the term Dalton for 1.6601 J 10^{-27} kg is no longer recommended. However it is still a current unit in biochemistry.*

Since low molecular weight fractions cannot be chemically fixed in the matrix, horizontal ultra-thin layer polyacrylamide gels on film supports are used. Those are dried at 100 IC immediately after electrophoresis and then sprayed with specific reagents. With this method for example, dyes with molecular weights of approximately 500 Da* can be separated.

1.2 Electrophoresis in restrictive gels

1.2.1 The Ferguson plot

Although during electrophoresis in restrictive gels, electrophoretic mobility depends both on net charge and on molecular radius this method can also be used for the physico-chemical analysis of proteins. The principle was formulated by Ferguson (1964): the samples are separated under identical buffer, time and temperature conditions but with different gel concentrations (g/100 mL for agarose, %*T* for polyacrylamide). The distances traveled will vary: m_r is the relative mobility. A plot of log 10 m_r versus the gel concentration yields a straight line.

Ferguson KA. Metabolism. 13 (1964) 985–995.

The slope (see Fig. 12) is a measure of the molecular size and is called the retardation coefficient K_R.

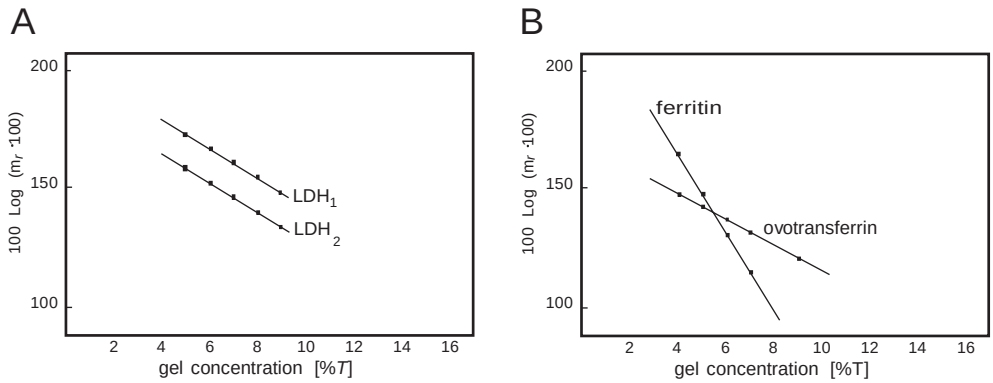

Fig. 12: Ferguson plots: plots of the electrophoretic migrations of proteins versus gel concentrations. (A) Lactate dehydrogenase isoenzymes; (B) Different proteins. See text for further details.

For globular proteins there is a linear relationship between K_R and the molecular radius *r* (Stokes radius), so the molecular size can be calculated from the slope of the plot. Once the free mobility and the molecular radius are known the net charge can also be calculated (Hedrick and Smith, 1968). For protein mixtures the following deductions can be made according to the appearance of the plots:

Hedrick JL, Smith AJ. Arch Biochem Biophys. 126 (1968) 155–163.

- The lines are parallel: The proteins have the same size but different mobilities e.g. iosenzymes. *Fig. 12A!*
- The slopes are different but the lines do not cross: the protein corresponding to the upper curve is smaller and has a higher net charge.

Fig. 12B!

Same net charge, different molecular sizes.

- The lines cross beyond T=2%: the larger protein has the higher charge density and intercepts the y-axis at a higher value.
- Several lines cross at a point where T< 2%: these are obviously the various polymers of one protein.

1.2.2 Agarose gel electrophoresis

Proteins

Jovin TM, Dante ML, Chrambach A. Multiphasic buffer systems output. Natl Techn Inf Serv. Springfield VA USA PB (1970) 196 085–196 091.

Since highly concentrated agarose gels above 1% (1g/100 mL agarose in water) are cloudy and the electro-osmotic flow is high, agarose gels are only used for the separation of very high molecular weight proteins or protein aggregates. Since agarose gels do not contain catalysts which can influence the buffer system, they have also been used to develop a series of multiphasic discontinuous buffer systems (Jovin *et al.* 1970).

Nucleic acids

Maniatis T, Fritsch EF, Sambrook J. Molecular cloning a laboratory manual. Cold SpringLaboratory (1982). Rickwood D, Hames BD. Gel electrophoresis of nucleic acids. IRL Press Ltd. (1982).

Perlman D, Chikarmane H, Halvorson HO. Anal Biochem. 163 (1987) 247–254.

Agarose electrophoresis is the standard method for separation, DNA restriction fragment-analysis and purification of DNA and RNA fragments (Maniatis *et al.* 1982; Rickwood and Hames, 1982). The fragment sizes analysed are in the range between 1,000 and 23,000 bp. Horizontal "submarine" gels are used for these nucleic acid separations: the agarose gel lies directly in the buffer (Fig. 13). This prevents the gel from drying out.

When a narrow pore size gel is required, agarose can be partially substituted by polysaccharides (Perlman *et al.* 1987).

These dyes have to be handled with care, because they are mutagens.

The gels are stained with fluorescent dyes like Ethidium bromide or SYBR Green, and the bands are visible under UV light. Their sensitivites ranges are between 100 pg and 1 ng / band. Because they are intercalating in the helix, the sensitivity is dependent on the size of the DNA fragment and is lower for RNA detection.

For RFLP (restriction fragment length polymorphism) analysis, the separated DNA fragments are transferred onto an immobilizingmembrane followed by hybridization with radiolabelled probes (s. 4 Blotting).

For a permanent record, mostly instant photos had been taken from the gels in a darkroom. Video documentation systems take the images inside a box, print the results on thermopaper, or feed them to a computer.

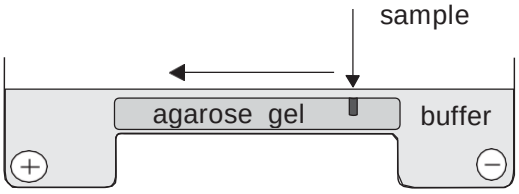

Fig. 13: The "submarine" technique for the separation of nucleic acids.

Pulsed field gel electrophoresis

For chromosome separation, pulsed field electrophoresis (PFG) according to Schwartz and Cantor (1984) is used; it is a modified submarine technique.

Schwartz DC, Cantor CR. Cell. 37 (1984) 67–75.

High molecular weight DNA molecules over 20 kb align themselves lengthwise during conventional electrophoresis and migrate with the same mobility so that no separation is achieved.

kb kilobases

In PFG the molecules must change their orientation with changes in the electric field, their helical structure is first stretched and then compressed. The "viscoelastic relaxation time" is dependent on the molecular weight. In addition, small molecules need less time to reorient themselves than large ones. This means that after renewed stretching and reorientation, larger molecules have – for a defined pulse – less time left for actual electrophoretic migration. The resulting electrophoretic mobility thus depends on the pulse time or on the duration of the electric field: a separation according to the molecular weight up to the magnitude of 10 megabases is obtained.

For shorter DNA fragments the resolution with PFG is also better than with conventional submarine electrophoresis.

For the analysis of chromosomes, the sample preparation including cell disintegration, is done in agarose blocks which are placed in

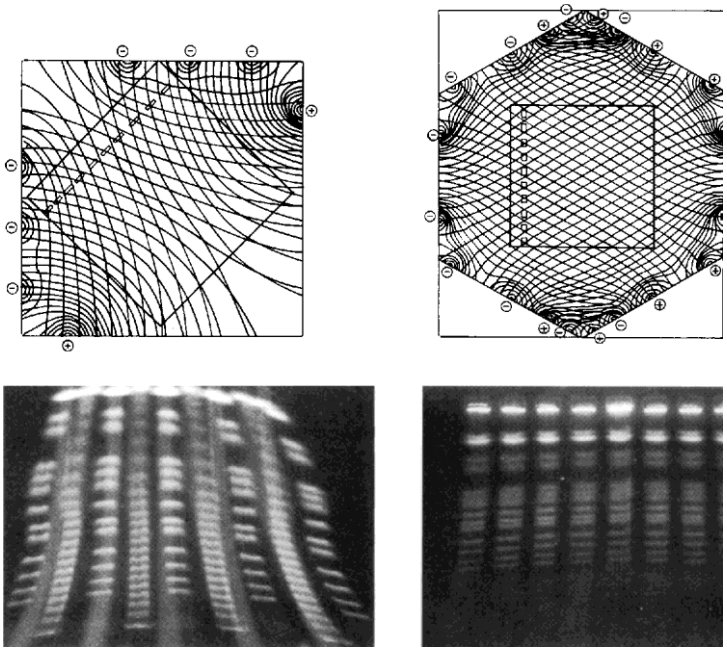

Fig. 14: Field lines and separation results for two types of PFG electrophoresis: *left* orthogonal doubly inhomogeneous fields and *right* homogeneous fields for hexagonally arranged point electrodes.

the pre-formed sample pockets. These molecules would be broken by the shear forces. 1.0 to 1.5% agarose gels are used for the separation.

There are in addition other field geometries:
Field Inversion (FI) electrophoresis: the electric field is pulsed back and forth in one direction.
Transverse Alternating Field electrophoresis (TAFE): The gel is mounted vertically in an aquarium-like tank and the field is pulsed back and forth between electrode pairs mounted on the top and the bottom of both sides of the gel.

The electric fields should have an angle of at least 110I relative to the sample. This is obtained for example by an inhomogeneous field with point electrodes mounted on orthogonal rails or in hexagonal configuration. The pulse time is of 1 s to 90 min for these techniques, depending on the length of the DNA molecules to be separated. Large molecules are better separated when the pulse time is long, small molecules needs short pulse times. The separations can last several days.

Fig. 14 shows the field lines for an orthogonal configuration with an inhomogeneous field and for an hexagonal configuration with a homogeneous field as well as the corresponding separations.

Pulsed field gel electrophoresis is mainly employed for research, but it has also found its place in routine analysis for bacterial taxonomy.

1.2.3 Polyacrylamide gel electrophoresis of nucleic acids

DNA sequencing

Sanger F, Coulson AR. J Mol Biol. 94 (1975) 441–448. Maxam AM, Gilbert W. Proc Natl AcadSci USA. 74 (1977) 560–564.

In the DNA sequencing methods according to Sanger and Coulson (1975) or Maxam and Gilbert (1977), the last step is electrophoresis in a polyacrylamide gel under denaturing conditions. The four reactions – containing variously long fragments of the DNA strand to be analyzed, each terminating with a specific base – are separated one beside the other. Determination of the order of the bands in these four lanes from the bottom to the top of the gel yields the base sequence, that is, the genetic information.

Smilingeffect: When the temperature in the middle of the gel is higher than at the edges the DNA fragments migrate faster.

Tris-borate EDTA (TBE) buffer is used. To completely denature the molecule, the process is usually carried out at a temperature over 50 IC and in the presence of urea. Irregular heat distribution results in the *"smiling"* effect, when the bands are turned up at the ends. For this reason, it has proved effective to prewarm the gels with thermoplates independent from the electric field.

In practice, vertical gel slabs are used, which are – in most cases – heated by the electric field. An aluminum plate behind one of the glass plates distributes the heat evenly.

Manual sequencing: in the manual technique the bands are mostly revealed by autoradiography. Nucleotides or primers labelled with ^{32}P or ^{35}S are used. The gels are usually thinner than 0.4 mm since they must be dried for autoradiography.

Alternative nonradioactive detection methods have been developed:

This requires biotinylated or fluorescent primers, nucleotides or probes.

- Chromogenic or chemiluminescent detection on a membrane after the separated DNA fragments have been transferred from the gel.

- Silver staining of the gel.

This requires cycle sequencing.

The use of wedge shaped gels has proved useful: they generate a field strength gradient which induces a compression of the band pattern in the low molecular weight area and enables the analysis of substantially more bases in one gel.

The samples are introduced in sample wells (formed in the gel by a sample comb during polymerization) with microcapillaries or syringes with an extra thin needle. A typical sequencing autoradiogram is shown in Fig. 15.

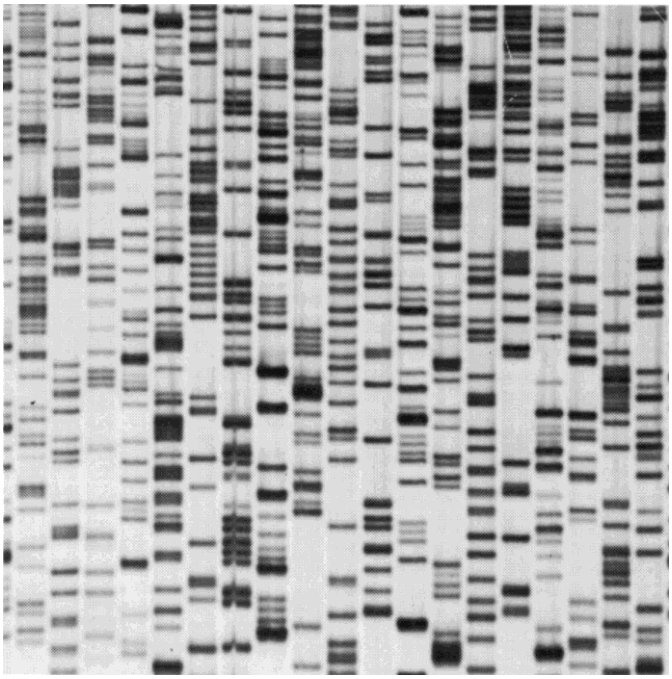

Fig. 15: Autoradiogram of DNA sequencing.

Automated sequencing: samples with fluorescent tags are used. There are two principles:

Also here almost exclusively vertical slab gels are employed.

1. *Single track system:* for the four necessary reactions – with the base endings A, C, G, T – four different fluorescent markers are used. For separation, the four reagents are applied on the gel and the zones which migrate in one track are measured with selective photodetectors.

Ansorge W, Sproat BS, Stegemann J, Schwager C. J Biochem Biophys Methods. 13 (1986) 315–323.

Since the introduction of the Cy5 label, a red laser can be employed.

2. *Four track system:* This principle is based on the traditional Sanger method (Sanger and Coulson 1975). Only one dye is used, for example fluoresceine, which is used to mark the primer. The samples are separated in four tracks per clone. A fixed laser beam constantly scans the whole width of the gel in the lower fifth of the separation distance. At this height, a photovoltaic cell is fixed to the glass plate behind each band. When the migrating bands reach that spot, the fluorescent DNA fragments will be excited and emit a light signal (Ansorge *et al.* 1986). Since a single photo cell corresponds to each band, the migrating bands will be registered one after the other by the computer, giving the sequence. In one track systems, the rawdata must be processed so that the mobility shifts due to the different markers are compensated. In four track systems, the sequence can be recognized directly from the rawdata (see Fig. 16).

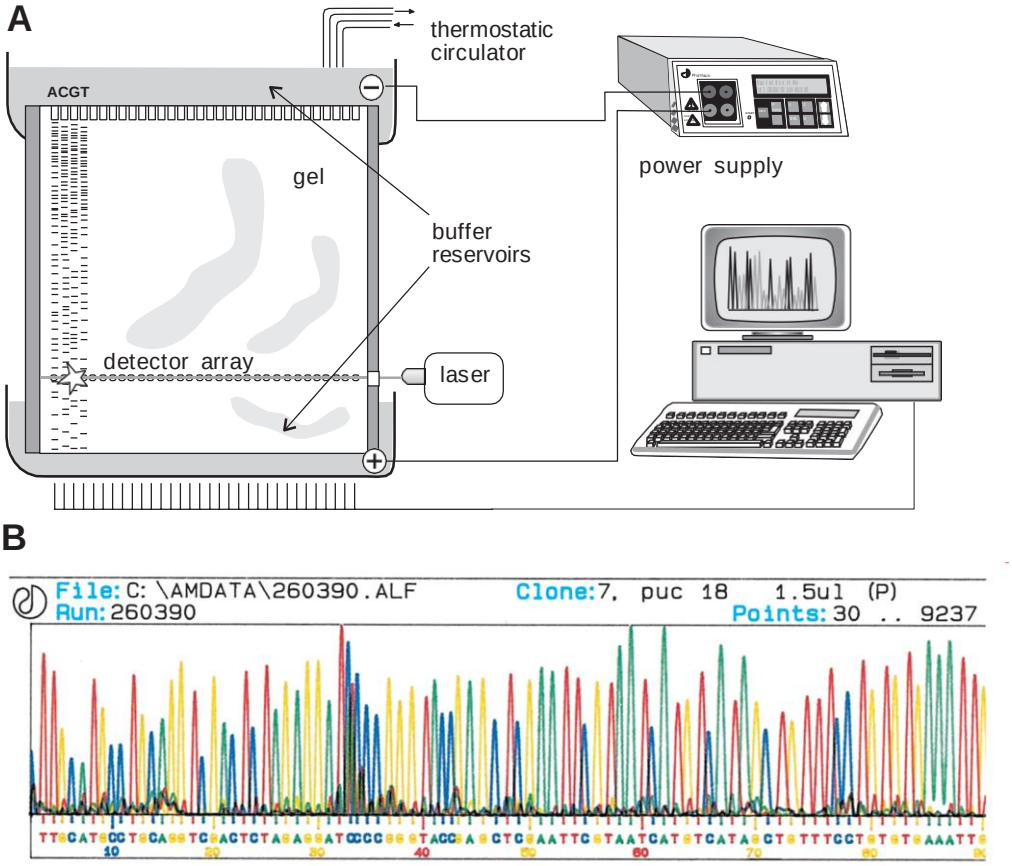

Fig. 16: (A) Instrumentation for automated DNA sequencing with a four track system; (B) Typical trace after treatment of the crude data by a computer.

Automated sequencing has many advantages over the manual technique:

- Since fluorescent markers are used, the use of radioactivity in the laboratory can be avoided.
- Neither extensive treatment of the gel after separation nor time-consuming autoradiography are necessary.
- The laborious reading of the bands becomes unnecessary.
- The sequences are directly fed into the computer.
- The reactions labelled with the fluorescent label can easily be kept for a long time, so that the separation can be repeated later in case of doubt.
- The high sensitivity of fluorescent labelling also allows the sequencing of cosmids and lambda DNA as well as the products of the polymerase chain reaction PCR[Q]*). In addition restriction analyses can be carried out.

No need for isotope laboratory.

**) The PCR process is covered by U.S. patents 4,683,195 and 4,683,302 owned by Hoffman-La Roche Inc. Use of the PCR process requires a license.*

This "on-line electrophoresis" setup can also be employed for various DNA typing methods.

For high throughput genome sequencing, multi-capillary instruments have replaced the slabgel technique. The capillaries are usually filled with linear – non-crosslinked – polyacrylamide. The entire procedure, including sample application is automated.

Those are the biggest and most expensive electrophoresis instruments existing.

DNA typing

Many newtechniques and applications have recently been developed in this field. Because those are almost exclusively based on PCR[Q] technology, the size range of the DNA fragments to be analysed lies between 50 and 1,500 bp. In this range the sensitivity and resolution of agarose electrophoresis with Ethidium bromide staining is coming to its limits, because the gel pores are too large for proper sieving and the intercalating fluorescent dyes are much less sensitive than for larger fragments.

Amplification of fragments larger than 1,500 bp is possible, however, with a lot of problems with reproduciblity.

PAGE and silver staining:

The use polyacrylamide gels leads to much sharper bands and higher resolution; with subsequent silver staining a sensitivity of 15 pg per band can be achieved (Bassam *et al.* 1991). Vertical and horizontal slab gels can be used. Whereas in agarose electrophoresis the mobilities of DNA fragments are solely proportional to their sizes, the band positions in polyacrylamide gels are partly influenced by the base sequence as well. A and T rich fragments migrate slower than others.

Bassam BJ, Caetano-Annol-l=s G, Gresshoff PM. Anal Biochem. 196 (1991) 80–83. Silver stainingof DNA is much easier than of proteins, because fixation is very easy.

Silver stained DNA bands can be directly reamplified after scratching them out of the gel without intermediate purification. About 20 % of the DNA molecules of a band remain undestroyed by the silver staining procedure. They are locked inside the stained band, thus DNA fragments do not contaminate the gel surface during staining.

Reamplification of DNA works only, when silver staining techniques specially designed for DNA detection are employed.

Horizontal (flatbed) electrophoresis

GörgA, Postel W, Westermeier R, Gianazza E, Righetti PG, J Biochem Biophys Methods. 3 (1980) 273–284.

Flatbed polyacrylamide systems have a number of advantages over the vertical ones when ultrathin gels polymerized on support films are used (Görg *et al.* 1980): simple handling, easy use of ready-made gels and buffer strips instead of large buffer volumes; good cooling efficiency and temperature control; possibility of washing, drying and rehydrating the gels; possibility of automation.

Amplified ribosomal DNA restriction analysis (ARDRA)

This method is derived from ribotypingand is mainly employed fro the identification of bacteria species.

Fragments of ribosomal DNA with polymorphic restriction sites of an organism are amplified with a primer pair and subsequently digested with a restriction enzyme. After gel electrophoresis and silver staining, species specific pattern are obtained.

Random amplified polymorphic DNA (RAPD)

Welsh J, McClelland M. Nucleic Acids Res. 18 (1990) 7213–7218.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. Nucleic Acids Res. 18 (1990) 6531–6535.

BergDE, Akopyants NS, Kersulyte D. Meth Molec Cell Biol. 5 (1994) 13–24.

Caetano-Annoll=s G, Bassam BJ, Gresshoff PM. Bio/Technology 9 (1991) 553–557.

Even one additional band detected can make a bigdifference in the evaluation. With optimized separation and detection strain-specific pattern are achieved. See Method 12.

This method is applied for rapid detections of DNA polymorphisms of a wide variety of organisms: bacteria, fungi, plants, and animals. One single short oligonucleotide primer (10mer) of arbitrary sequence is used to amplify fragments of the genomic DNA (Welsh and McClelland, 1990; Williams *et al.* 1990). The low stringency annealing conditions lead to an amplification of a set of multiple DNA fragments of different sizes. Berg *et al.* (1984) have found a series of primers, which allow a very good differentiation of microorganisms. When optimized and uniform PCR conditions are employed, specific and reproducible band patterns are achieved.

A modification using 5mer primers is called DNA amplification fingerprinting (DAF) and has been introduced by Caetano-Annoll@s *et al.* (1991).

RAPD samples can be run on agarose gels with Ethidium bromide staining or on polyacrylamide gels with subsequent silver staining. As the resolution and sensitivity of the latter method is much higher, more variety differences can be detected. Figure 17 shows the RAPD patterns of different fungus varieties separated in a horizontal polyacrylamide gel and silver stained. The primers are based on those published by Berg *et al.* (1984).

Mutation detection methods

Landegren U, Ed. Laboratory protocols for mutation Detection. Oxford University Press (1996).

A comprehensive description of mutation detection methods can be found in the book “Laboratory Protocols for Mutation Detection”, edited by Ulf Landegren (1996).

The most certain and sensitive method for the detection of mutations is the DNA sequence analysis. However, this method is too costly and time-consuming for screening purposes.

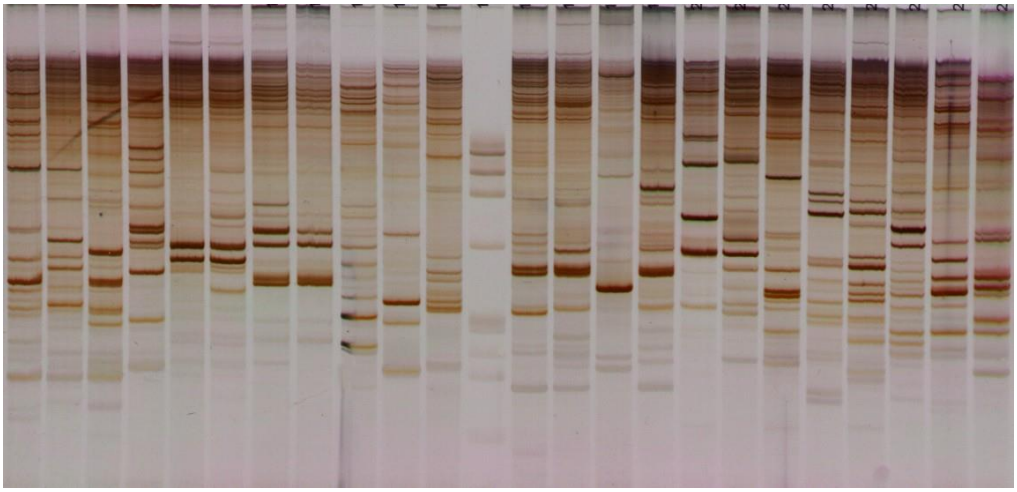

Fig. 17: RAPD electrophoresis of fungi varieties in a horizontal polyacrylamide gel. Silver staining. By kind permission of Birgit J:ger and Dr. Hans-Volker Tichy, T=V S>dwest GmbH – Biological Safety Division, Freiburg im Breisgau.

Single strand conformation polymorphism (SSCP)

The principle: Variations in the sequence as small as one base exchange alter the secondary structure of ssDNA, e.g. by different intramolecular base pairing. The changes in the sequence cause differences in the electrophoretic mobility, which are observed as band shifts (Orita *et al.* 1989).

Orita M, Iwahana H, Kanazewa H, Hayashi K, Sekiya T. Proc Natl Acad Sci USA. 86 (1989) 2766–2770.

The mechanism of SSCP is described as: Differential transient interactions of the bent and curved molecules with the gel fibers during electrophoresis, causing the various sequence isomers to migrate with different mobilities.

Single strands migrate much slower than the corresponding double strands.

Before screening, the mutants have to be defined by direct sequencing. The sequences fo the appropriate primer pair have to be found. The PCR products are denatured by heating with formamide or sodium hydroxid, and loaded onto a non-denaturing polyacrylamide gel for electrophoresis. Silver staining has to be employed for detection of the DNA fragments.

SSCP analysis is not a replacement but an addition to sequencing, when 100 % of defined mutations have to be detected. Intercalatingdyes do not work here.

A high number of samples can be screened with a considerably lower effort than direct sequencing in a relatively short time, namely within a few hours.

However, the band shifts do not show up automatically for all mutations and under all conditions. Unfortunately, there is not a single and unique separation condition, which can be applied to the separations of all exons. The parameters influencing the result have been reviewed by Hayashi and Yandell (1993) and will be further discussed in Method 13 in part II.

Hayashi K, Yandell DW. Hum Mutat. 2 (1993) 338–346.

For this method, good cooling and temperature control system is very important.

Rehbein H, Mackie IM, Pryde S, Gonzales-Sotelo C, Perez-Martin R, Quintero J, Rey-Mendez M. Inf. Fischwirtsch. 42 (1995) 209–212.

SSCP of the mitochondrial cytochrome b gene is also employed for differentiation of animal species. Rehbein *et al.* (1995) have used the method for the identification of the species in canned tuna.

Heteroduplex and DSCP

Keen JD, Lester D, Inglehearn C, Curtis A, Bhattacharya. Trends Genet. 7 (1991) 5.

White MB, Carvalho M, Derse D, O'Brien SJ, Dean M. Genomics 12 (1992) 301–306.

Barros F, Carracedo A, Victoria ML, Rodriguez-Calvo MS. Electrophoresis 12 (1991) 1041–1045.

Dockhorn-Dworniczak B, Aulekla-Acholz C, Dworniczak B. Pharmacia LKB Offprint A37 (1990).

Single base substitutions can also be detected by heating the mixtures amplified wild type and mutant DNA and run the resulting heteroduplexes on a native polyacrylamide gel electrophoresis (Keen *et al.* 1991; White *et al.* 1992). The mobilities of heteroduplexes lie between the mobilities of the corresponding homoduplexes and single strands. Different mutations cause different mobility shifts of heteroduplexes. The bands can be detected with Ethidiumbromide or with silver staining.

Sometimes the technique is also called DSCP (double strand conformation polymorphism) (Barros *et al.* 1992). But it should not be forgotten, that also homoduplexes can show band shifts in native gels due to the influence of the contents of A and T.

For DNA diagnosis, DNA point mutations can quickly be revealed with the Primer Mismatch process in combination with electrophoresis of the amplification products in horizontal polyacrylamide gels (Dockhorn-Dworniczak *et al.*, 1990).

Denaturing gradient gel electrophoresis (DGGE) and constant denaturing gel electrophoresis (CDGE)

Fischer SG, Lerman LS. Proc Natl Acad Sci. 60 (1983) 1579-1583.

With DGGE single base exchanges in segments of DNA can be detected with almost 100 % efficiency. The principle of DGGE is based on the different electrophoretic mobilities of partially denatured molecules caused by differences in DNA melting (Fischer and Lerman, 1983).

Typically the 100 % denaturant solution contains 6 to 7 mol/L urea and 20 to 40 % formamide. The gels are run at temperatures between 40 PC and 60 PC.

With a denaturant gradient perpendicular to the electrophoresis direction, the region of a point mutation can be identified. Denaturant gradients parallel to the electrophoresis runs are better for screening applications.

The practical aspects and the gradient casting technique are described in method 14.

Constant denaturing gel electrophoresis (CDGE) is employed for screening, when the denaturant concentration of differential melting of a DNA segment has been detected with DGGE. Figure 18 is a schematic representation of perpendicular and parallel DGGE. As DGGE is not very easy to perform, it is only employed, when the techniques other than sequence analysis fail in detecting a mutation.

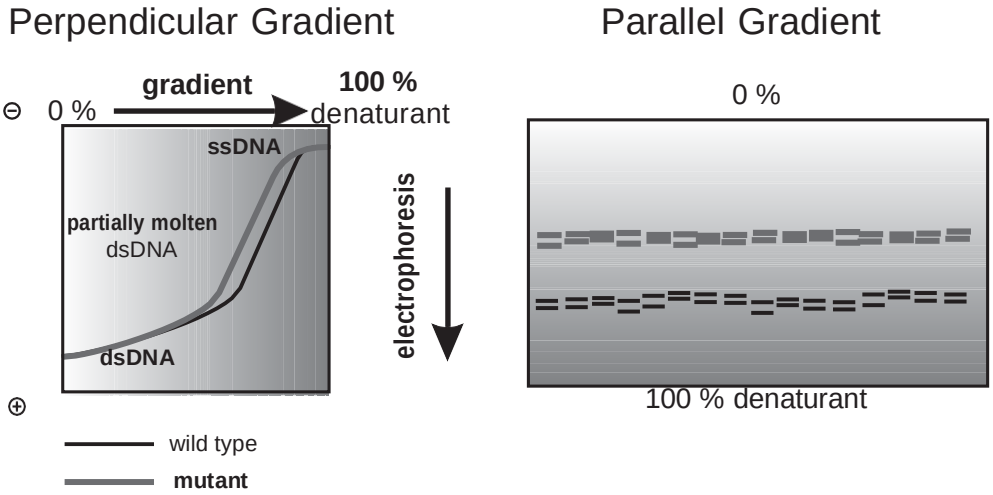

Fig. 18: Schematic representation of typical results of a perpendicular and a parallel DGGE.

Temperature gradient gel electrophoresis (TGGE)

Temperature gradient gel electrophoresis resolves homo- and heteroduplexes according to their thermal stabilities (Riesner *et al.* 1989). In this technique, denaturing gels are run on a plate with a cold (15 IC) side at the cathode and a hot side (60 IC) at the anode. The method is well suitable for screening purposes. Suttorp *et al.* (1996) have described how to change a standard horizontal electrophoretic chamber into a TGGE device.

Riesner D, Steger G, Wiese U, Wulfert M, Heibey M, Henco K. Electrophoresis 10 (1989) 377–389.
Suttorp M, von Neuhoff N, Tiemann M, Dreger P, Schaub J, Löffer H, Parwaresch R, Schmitz N. Electrophoresis 17 (1996) 672–677.

Single Nucleotide Polymorphism (SNP) analysis

According to estimations, one single nucleotide (SNP) occurs approximately in every 100–300 bases of the human genome. Single nucleotide polymorphisms are present in both the coding and non-coding regions. The SNPs found in the coding regions of the genome are interesting for clinical research, because they may be indicators for the different responses of different patients to drug treatment and other factors. High-throughput systems are preferably employed, like the multi-capillary electrophoresis instruments used for DNA sequencing.

Denaturing PAGE of microsatellites

Denaturing gels provide a very high resolving power, thus they are very useful for separating of microsatellites with very short repeats down to 2 bp. Because the Taq-polymerase used in PCR adds an addi-

It is not always necessary to apply completely denaturing conditons on the gel: 7 mol/L urea in the gel and 25 PC separation temperature are often sufficient.

tional A to the 3¢-end of a part of the single strands, double bands are frequently seen after silver staining of denaturing gels.

Instructions for denaturingelectrophoresis are found in method 14.

When labelling techniques like radioactivity or fluorescence are employed, only one of the primer pair is marked to avoid visualization of the duplets.

Native PAGE of mini- and microsatellites

Variable number of tandem repeats (VNTR) and short tandem repeats (STR) analysis are used in forensic laboratories: They are performed in denaturing and in non-denaturing gels. In both cases, the assignment of alleles with well-defined (sequenced) allelic ladders of the respective VNTR or STR locus, which are run in the same gel, proved to be the most reliable method (Puers *et al*. 1993; Möller *et al*. 1994).

Möller A, Wiegand P, Grüschow C, Seuchter SA, Baur MP, Brinkmann B. Int J LegMed 106 (1994) 183–189.

Puers C, Hammond HA, Jin L, Caskey CT, Schumm JW. Am J Hum Genet 53 (1993) 953–958.

However, additionally to the regular types with length variations, there are sequence variants existing in some STR loci, which can only be identified by sequencing the fragments or running them on non-denaturing polyacrylamide gels. In order to achieve adequate resolution in native gels, long separation distances or special gel media and buffer systems have to be used (Schickle, 1996).

Schickle HP. GIT Labormedizin. 19 (1996) 228–231.

With new special gel media fragments differing by 2 bp (in the size range of 100 to 200 bp) can be resolved in a 10 cm long gel under native conditions.

Differential Display PCR Electrophoresis

This is a method to screen the total amount of cDNAs coming from the messenger RNA-pool of specific cell lines. The purpose is to display only the active genes besides the total amount of ca. 1 Million genes of a cell.

Not only cell regulation and differentiation can be monitored, but also miscontrolled cells can be visualized in cancer research.

The method “DDRT” (Differential display reverse transcription) has been introduced by Liang and Pardee (1992) and improved by Bauer *et al*. (1993). The mRNA from the original cell and the stimulated cell are processed in parallel. Extracted mRNA is reverse transcribed with oligo-dT-NN anchor primers. The resulting 12 cDNA pools are amplified with the respective oligo-dT primer and a set of arbitrary 10mer primers. After high resolution electrophoresis of the amplification products, those additional bands, which have been expressed by the stimulated cell, are cut out and reamplified for cloning and sequencing (see Fig. 19). The original technique employes autoradiography for the detection of the bands.

Liang, P, Pardee AB. Science 257 (1992) 967–971.

Bauer D, Müller H, Reich J, Riedel H, Ahrenkiel V, Warthoe P, Strauss M. Nucleic Acid Res. 21 (1993) 4272–4280.

The additional bands are identified on the developed X-ray film; after cuttinga hole in this position the film is matched with the gel again, the band is scratched out.

Lohmann *et al.* (1995) have taken a big step foreward with their "REN" (rapid, efficient, nonradioactive) technique: they use horizontal film-supported gels and cut out the silver stained DNA bands for reamplification. In this way, the method can be performed much faster, cheaper, and with a higher success of finding a gene, which has been expressed as a response of the cell.

Lohmann J, Schickle HP, Bosch TCG. BioTechniques 18 (1995) 200–202.
Urea and native gels can be employed. Sometimes it is necessary to use long gels, because the bands are spread over a wide range basepair-lengths.

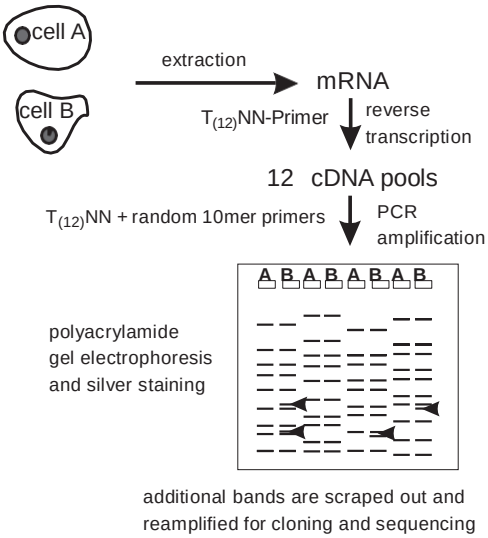

Fig. 19: The steps in a DDRT experiment.

Practical hints for improved reamplification can be found in the paper by Böckelmann *et al.* (1999).

Böckelmann R, Bonnekoh B, Gollnick H. Skin Pharmacol Appl Skin Physiol.12 (1999) 54–63.

Two-dimensional DNA electrophoresis

Complex DNA samples can also be displayed with a two-dimensional electrophoresis: First the DNA is digested with a rare cutting restriction enzyme and the fragments are separated in an agarose gel; then the agarose gel is soaked in a mixture of selected restriction enzyme and applied on a polyacrylamide gel. Schickle *et al.* (1999) have converted the time consuming "handcraft" procedure into a faster technique, which is based on ready-made gels. The resulting tiny spots are visualized with autoradiography.

Schickle HP, Lamb B, Hanash SM. Electrophoresis 20 (1999) 1233–1238.

RNA and viroids

Bi-directional electrophoresis (Schumacher *et al.* 1986) is used for viroid tests: the plant extract (RNA fragment + viroid) is first separated under native conditions at 15 IC. After a certain separation time, the gel is cut behind a zone marked with a dye such as Bromophenol Blue or xylenecyanol.

Schumacher J, Meyer N, Riesner D, Weidemann HL. J Phytophathol. 115 (1986) 332–343.

The gel contains 4 mol/L urea. The molecules are denatured, that is unfolded by the combination of urea and elevated temperature. For practical reasons this method is only carried out in horizontal systems.

An electrophoretic separation under denaturing conditions is carried out. The viroid forms a ring which cannot migrate. The RNA fragments which migrate more slowly during the first native separation, do not lose their mobility at 50 IC and migrate out of the gel. If a viroid is present, only one band is found when the gel is stained. The position of the viroid in the gel depends on its kind. Several new viroids have been discovered in this way.

1.2.4 Polyacrylamide gel electrophoresis of proteins

For analytical PAGE of proteins, the trend is to go from cylindrical gels to flat and thinner ones. Because of the development of more sensitive staining methods such as silver staining for example, very small quantities of concentrated sample solution can be applied for the detection of trace amounts of proteins.
The advantages of thinner gels are:

- faster separation
- better defined bands
- faster staining
- better staining efficiency, higher sensitivity

Disc electrophoresis

Ornstein L. Ann NY Acad Sci. 121 (1964) 321–349.
Davis BJ. Ann NY Acad Sci. 121 (1964) 404–427.
See also page 45: Isotachophoresis

Discontinuous electrophoresis according to Ornstein (1964) and Davis (1964) solves two problems of protein electrophoresis: it prevents aggregation and precipitation of proteins during the entry from liquid sample into the gel matrix, and promotes the formation of well defined bands. The discontinuity is based on four parameters (see Fig. 20):

- the gel structure
- the pH value of the buffer
- the ionic strength of the buffer
- the nature of the ions in the gel and in the electrode buffer

The gel is divided into two areas: resolving and stacking gel. The resolving gel with small pores contains 0.375 mol/L Tris-HCL buffer pH 8.8, the stacking gel with large pores contains 0.125 mol/L Tris-HCL pH 6.8.

Glycine is used because it is very hydrophilic and does not bind to proteins.

The electrode buffer contains only glycine, the gel only Cl^- ions. Glycine has a pI 6.7, it has almost no net charge at pH 6.8: the pH of the stacking gel. Thus glycine has a low mobility.

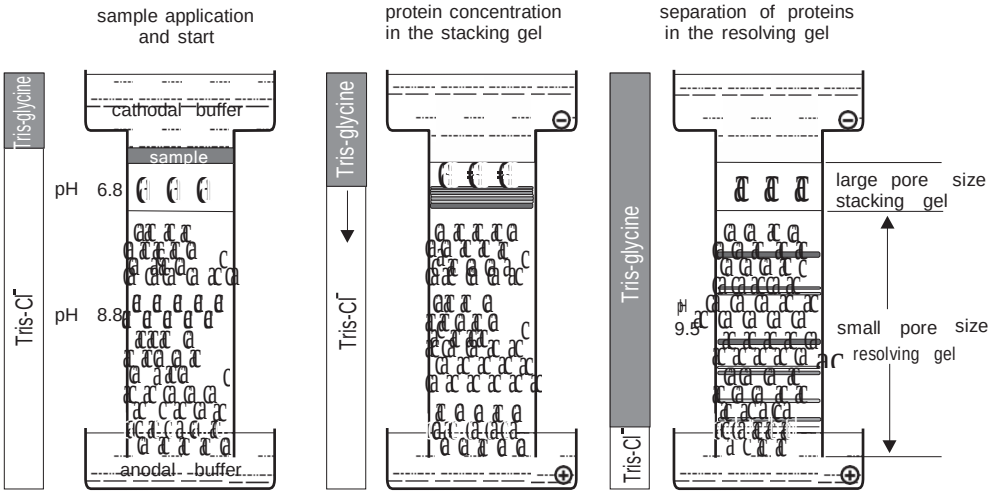

Fig. 20: Schematic diagram of the principles of disc electrophoresis according to Ornstein (1964). The buffer system shown is also employed for discontinuous SDS electrophoresis.

At first, the proteins are separated according to the *principle of isotachophoresis* and form stacks in the order of their mobility ("stacking effect"). The individual zones become concentrated. Because of the large pores in the stacking gel, the mobilities are dependent on the net charge, not on the size of the molecule.

Because of the relatively slow migration velocity of glycine, the samples enter the gel slowly without sudden concentrating. The stackingeffect is described in chapter 2 Isotachophoresis.

The protein stack migrates – slowly and at constant speed – towards the anode, till it reaches the border to the resolving gel. The frictional resistance suddenly increases for the proteins, they migrate slower, and the zones become higher concentrated. The low molecular weight glycine is not affected by this, passes the proteins, and becomes higher charged in the resolving zone; the new Cl^- / $glycine^-$ front moves ahead of the proteins.

2nd zone sharpeningeffect!

Several events now occur simultaneously:

- The proteins are in a homogeneous buffer medium, destack and start to separate according to the principles of zone electrophoresis.
- Their mobility now depends on their charge as well as on their size. The ranking of the protein ions changes.
- The pH value rises to 9.5 and because of this, the net charge of the proteins and increases.

A discontinuity now only exist at the front.

The separation becomes faster.

Maurer RH. Disk-Electrophorese – Theorie und Praxis der diskontinuierlichen Polyacrylamid-Electrophorese. W de Gruyter, Berlin (1968).
For SDS electrophoresis see page 35 ff.

Disc electrophoresis affords high resolution and good band definition. In the example cited above, proteins with pIs higher than pH 6.8 migrate in the direction of the cathode and are lost. Another buffer system must be chosen to separate these proteins. A selection can be found in the works of Maurer (1968) and Jovin (1970). Alternatively SDS can be added to the gel and running buffer to in order to have all proteins negatively charged.

The stacking gels is only cast onto the resolving gel just before electrophoresis because, when the complete gel is left standing for a long time the ions diffuse towards one another.

Gradient gel electrophoresis

Rothe GM, Purkhanbaba M. Electrophoresis 3 (1982) 33–42.

By continuously changing the acrylamide concentration in the polymerization solution, a pore gradient gel is obtained. Gradient gels have a zone sharpening effect and can be used to determine the molecular diameter of proteins in their native state (Rothe and Purkhanbaba, 1982).

The determination of molecular weights in this manner can be problematic, since different proteins have different tertiary structures. Structural proteins cannot be compared with globular proteins.

When the acrylamide concentration and cross-linking are high enough in the small pore area, the protein molecules can be driven to an end point, where they are trapped in the tight gel matrix. Since the speed of migration of the individual protein molecules depends on their charge, the electrophoresis must be carried out long enough so that the molecule with the lowest net charge also reaches its end point.

When several gels are cast simultaneously, the solutions are injected from the bottom. In this case, the solutions in the mixingchamber and the reservoir are interchanged (see page 238).

There are various ways of making gels with linear or exponential porosity gradients. All are based on the same principle: two monomer solutions with different acrylamide concentrations are prepared. During casting, the concentrated solution is continuously mixed with the diluted solution, so that the concentration in the casting mold decreases from bottom to top (Fig. 21). For single gels the solution is poured into the top of the cassette.

The density of the highly concentrated solution is increased with glycerol or sucrose so that the layers in the molds do not mix. In principle a concentration gradient is poured. The mixing of the less dense dilute solution with the high densitiy solution takes place in the mixing chamber using a magnetic stirrer bar.

Exponential gradients are formed when the mixing chamber is sealed. The volume in the mixingchamber stays constant, the same quantity of dilute solution flows in as solution out of the mixing chamber (see Fig. 21).

If the mixing chamber is left open at the top, the principle of communicating vases is valid: so that the height of both fluids stays equal, half as much of the dilute solution flows in as of solution flowing out of the mixing chamber. A linear gradient is thus formed. A compensating stick in the reservoir compensates the volume of the stirrer bar and the difference in the densities of both solutions (see page 219 and 236 for porosity gradients, pages 265 and 281 for pH gradients, and pages 269 and 321 ff for additive gradients).

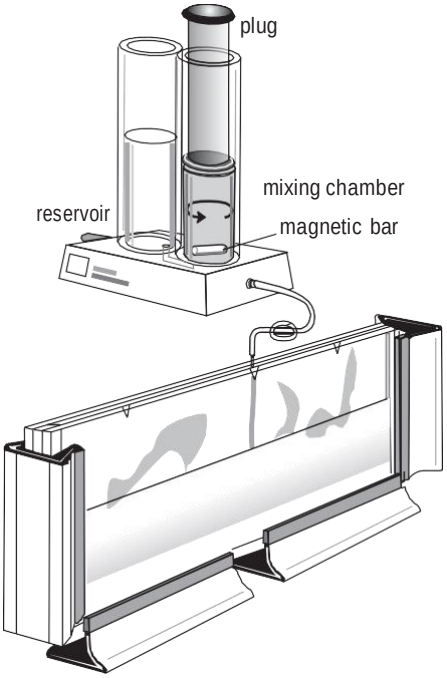

Fig. 21: Casting of an exponential gradient gel with a gradient maker. The stirrer bar is rotated with a magnetic stirrer (not shown).

SDS electrophoresis

SDS electrophoresis – SDS being the abbreviation for *sodium dodecyl sulphate* – which was introduced by Shapiro *et al.* (1967) separates exclusively according to molecular weight. By loading with the anionic detergent SDS, the charge of the proteins is so well masked that anionic micelles with a constant net charge per mass unit result: 1.4 g SDS per g protein.

Shapiro AL, Viñuela E, Maizel JV. Biochem Biophys Res Commun. 28 (1967) 815–822.

In addition, the tertiary and secondary structures are cancelled because of the disruption of the hydrogen bonds and unfolding of the molecules.

Thus there is no influence of the shape of the protein on the runningcondition.

Disulfide bonds between cysteine residues can only be cleaved by a reducing thiol agent such as 2-mercaptoethanol or dithiothreitol. The SH groups are often protected by a subsequent alkylation with iodoacetamide, iodoacetic acid or vinylpyridine (Lane, 1978).

Lane LC. Anal Biochem. 86 (1978) 655–664.

The unfolded amino acid chains, bound to SDS, form ellipsoids with identical central axes. During electrophoresis in restrictive polyacrylamide gels containing 0.1% SDS there is a linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide micelle.

This linear relationship is only valid for a certain interval, which is determined by the ratio of the molecular size to the pore diameter.

Marker proteins for various molecular weight intervals are available.

Gels with a pore gradient show a wider separation range and a larger linear relationship than gels with a constant pore size. In addition, sharper bands result since a gradient gel minimizes diffusion (Fig. 22). The molecular weight of the proteins can be estimated with a calibration curve using marker proteins (Fig. 23).

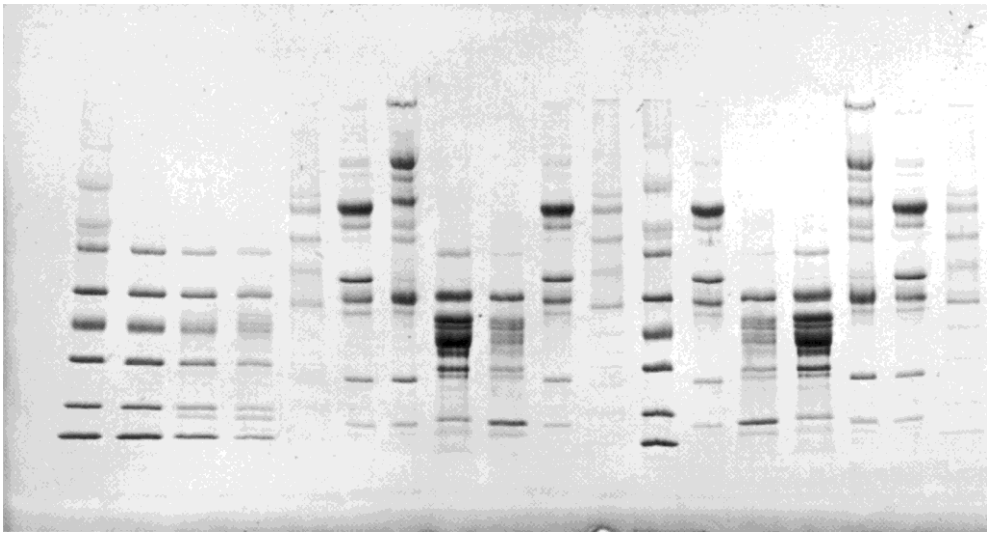

Fig. 22: Separation of proteins in a linear pore gradient gel T = 8% to 18% by SDS electrophoresis. Staining with Coomassie Brilliant Blue (Cathode on top).

For example when it is not reduced, albumin shows a molecular weight of 54 kDa instead of 68 kDa since the polypeptide chain is only partially unfolded.

For separation of physiological fluids or analysis of urine proteins for example, the reduction step is left out to prevent the breakdown of the immunoglobulins into subunits. In these cases the incomplete unfolding of certain proteins must be taken into account and therefore the molecular weight cannot be determined exactly.

There are a number of practical advantages to SDS electrophoresis:

Even very hydrophobic and denatured proteins.
- SDS solubilizes almost all proteins.

This ensures rapid separations.
- Since SDS-protein complexes are highly charged, they possess a high electrophoretic mobility.

Towards the anode.
- Since the fractions are uniformly negatively charged, they all migrate in one direction.

This limits diffusion.
- The polypeptides are unfolded and stretched by the treatment with SDS and the separation is carried out in strongly restrictive gels.

Sharp zones.
- This affords high resolution.

No strongacids are necessary.
- The bands are easy to fix.

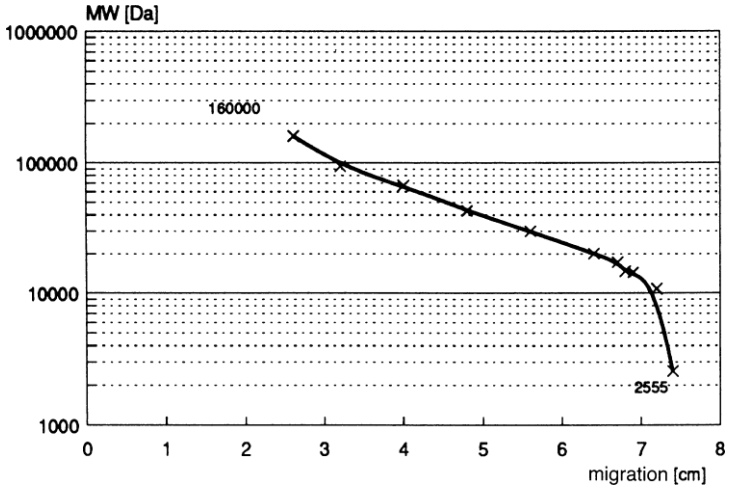

Fig. 23: Semi-logarithmic representation of a molecular weight curve. The molecular weights of the marker proteins are represented as a function of their migration. (SDS linear pore gradient gel according to Fig. 22).

- The separation is based on one physico-chemical parameter, the molecular weight. *It is an easy method for molecular weight determination.*
- Charge microheterogeneities of isoenzymes are cancelled out. *There is one band for one enzyme.*
- Proteins separated with SDS bind dyes better. *The detection limit increases ten-fold compared to native PAGE.*
- After electrophoretic transfer on an immobilizing membrane, the SDS can be removed from the proteins without eluting the proteins themselves. *See chapter 4: Blotting.*

SDS electrophoresis can be carried out in a continuous phosphate buffer system (Weber and Osborn, 1968) or in a discontinuous system (see page 34): *Weber K, Osborn M. J Biol Chem. 244 (1968) 4406–4412.*

Lämmli (1970) has directly adopted the disc electrophoresis method according to Ornstein (1964) and Davis (1964), for proteins charged with SDS, though the discontinuities in pH value and ionic strength are in most cases not necessary. *Lämmli UK. Nature 227 (1970) 680–685.*

- Because the protein-SDS micelles have very high negative charges, the mobility of glycine is lower than that of the proteins in the stacking gel at the beginning of electrophoresis, even at pH 8.8; it does not bind SDS. *However, the discontinuity of the anions and the different gel porosities are very important.*
- During stacking no field strength gradient results, since there are no charge differences within the sample: so no low ionic strength is necessary.

The overlayeringof the resolving gel with butanol for example can thus be avoided and especially the laborious removal of the overlay before pouringthe stacking gel.

This means that SDS disc electrophoresis gels can be cast in one step: Glycerol is added to the resolving gel and then the stacking gel, which contains the same buffer but no glycerol, is directly cast on top of it. In addition, the run time is shorter since the separation starts more quickly.

For ready-made gels with longer shelf lives, another buffer system with pH values around 7 should be chosen.

Since there are no diffusion problems between the stacking and the resolving gel buffers with these gels, they can be stored longer than conventional disc gels. Yet their shelflife is limited by the high pH value of the gel buffer, since, after about 10 days, the polyacrylamide matrix starts to hydrolyse.

Since Tricine is much more expensive than glycine, it is only used at the cathode, the anode contains Tris-acetate.
Kleine B, Löffler G, Kaufmann H, Scheipers P, Schickle HP, Westermeier R, Bessler WG. Electrophoresis 13 (1992) 73–75.

Long shelflife gels: Tris-acetate buffer with a pH of 6.7 has proven to have the best storage stability and separation capacity. Tricine is used instead of glycine as the terminating ion. The principle of this buffer system with polyacrylamide electrode buffer strips in a ready-made SDS gel can be seen in Fig. 24. These buffer strips simplify electrophoresis considerably and reduced chemical and radioactive liquid waste Kleine *et al.* (1992).

Schägger H, von Jagow G. Anal Biochem. 166 (1987) 368–379. The major effect is caused by the use of tricine instead of glycine. Also the "long shelflife gels" show a markedly better separation of small peptides.

Low molecular weight peptides: the resolution of peptides below 14 kDa is not sufficient in conventional Tris-glycine-HCl systems. This problem has been solved by the development of a new gel and buffer system by Schägger and von Jagow (1987). In this method an additional spacer gel is introduced, the molarity of the buffer is increased and tricine used as terminating ion instead of glycine. This method yields linear resolution from 100 to 1 kDa.

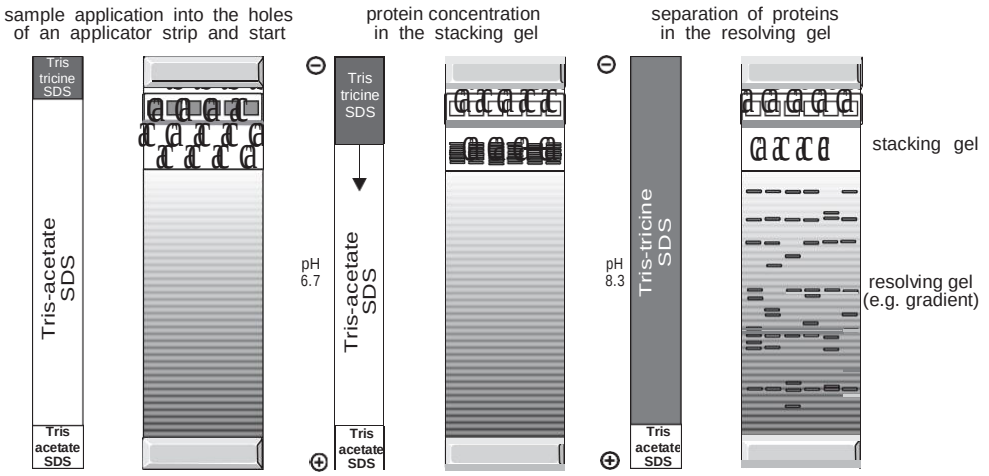

Fig. 24: Principle of the buffer systems of ready-made gels for discontinuous SDS-electrophoresis. Horizontal gels with Tris-Tricine buffer strips.

Glycoproteins migrate too slowly in SDS electrophoresis, since the sugar moiety does not bind SDS. When a Tris-borate-EDTA buffer is used, the sugar moieties are also negatively charged, so the speed of migration increases (Poduslo, 1981).

Poduslo JF. Anal Biochem. 114 (1981) 131–139. The use of gradient gels is also beneficial for better MW estimations.

Blue Native Electrophoresis

Membrane proteins: When membrane proteins are solubilized with nonionic detergents, these detergents would interfere with the SDS. Schägger and von Jagow (1991) have developed "Blue Native electrophoresis" of membrane protein complexes to solve this problem:

Schägger H, von Jagow G. Anal Biochem. 199 (1991) 223–231.

In a vertical chamber Coomassie Blue G-250 is added to the cathodal buffer in a native polyacrylamide gel electrophoresis.

During the run the dye competes with the nonionic detergent and binds to the membrane proteins and complexes and charges them negatively analogous to SDS. All these protein-dye complexes migrate towards the anode, also basic proteins. They are soluble in detergent-free solution, and – as the negatively charged protein surfaces repel each other – aggregation between proteins is minimized.

The membrane proteins and complexes can be isolated in enzymatically active form. The gels do not need to be stained, because the proteins and complexes migrate as blue bands.

The separation lanes of individual samples can be cut out from the polyacrylamide slab and applied directly on SDS polyacrylamide gel for a separation into a second dimension. In presence of SDS the complexes are dissolved and the partners of the respective complexes are displayed in the gel. The procedure has been described in detail (H. Schägger, 1994).

Schägger H. Chapter 4: Native electrophoresis. In: A Practical guide to membrane protein Purification (Von Jagow G, Schägger H, eds.) Academic Press, New York (1994) 81–103.

Cationic detergent electrophoresis

Strongly acidic proteins do not bind SDS and very basic nucleoproteins behave abnormally in SDS gels. The alternative is to use cationic detergents, for instance cetyltrimethylammonium bromide (CTAB), in an acidic medium at pH 3 to 5 (Eley *et al.* 1979). This allows a separation according to the molecular weight in the direction of the cathode. This cationic detergent also causes less damage to the protein than SDS, so CTAB electrophoresis can be used as a form of native electrophoresis (Atin *et al.* 1985). See also next page.

Eley MH, Burns PC, Kannapell CC, Campbell PS. Anal Biochem. 92 (1979) 411–419.

Atin DT, Shapira R, Kinkade JM. Anal Biochem. 145 (1985) 170–176.

Rehydrated polyacrylamide gels:

In washed gels the SDS Tris-HCl / Tris-glycine buffer system shows poor results. However good results are obtained with the Tris-acetate / Tris-tricine system.

The performance of SDS buffer systems are obviously highly influenced by catalysts and / or monomers of acrylamide.

In this method, the gel is rehydrated in Tris-acetate pH 8.0 using a horizontal tray. If, for highly concentrated protein samples, a discontinuity in pH and molarity between stacking and resolving gel is required, the stacking zone can be selectively equilibrated in a higher diluted Tris-acetate buffer pH 5.6 using a vertical chamber (see page 228).

This procedure of washing, drying, rehydration and equilibration can only be performed with gels polymerized on carrier films, which are used in horizontal systems.

The ionic catalysts APS and TEMED would destabilize these buffer systems, see method 4, pages 175 and following.
Hsam SLK, Schickle HP, Westermeier R, Zeller FJ. Brauwissenschaft 3 (1993) 86–94. Rehbein H. Electrophoresis 16 (1995) 820–822.

Native electrophoresis in amphoteric buffers: the polymerization catalysts can be washed out of the polyacrylamide gels on support films used in horizontal systems with deionized water. By equilibration with amphoteric buffers such as HEPES, MES or MOPS for example, there is a wide spectrum for electrophoresis under native conditions. This method proved to be particularly useful for acidic electrophoresis of basic hydrophobic barley hordeins (Hsam *et al.* 1993) and basic fish sarcoplasmic proteins (Rehbein, 1995).

Two-dimensional electrophoresis techniques

Several aims are pursued by the combination of two different electrophoretic methods:

- Proteins separated by electrophoresis are then identified by crossed immunoelectrophoresis.
- A complex protein mixture is first separated by zone electrophoresis and then further purified by IEF, or vice versa (Altland and Hackler, 1984).

Altland K, Hackler R. In: Neuhoff V, Ed. Electrophoresis' 84. VerlagChemie, Weinheim (1984) 362–378.

Langen H, Takács B, Evers S, Berndt P, Lahm H-W, Wipf B, Gray C, Fountoulakis M. Electrophoresis 21 (2000) 411–429.

- Hydrophobic proteins, such as membrane-bound proteins, are separated first in an acidic gel at pH 2.1 in presence of the cationic detergent 16-BAC, followed by an SDS electrophoresis (Langen *et al.* 2000). As the separation patterns in 16-BAC and in SDS differ substantially, a decent resolution is obtained.
- Membrane protein complexes are first separated by Blue native electrophoresis (see above) and then separated with SDS PAGE for the display and identification of complex partners.
- Highly heterogeneous mixtures of proteins such as cell lysates or tissue extracts should be completely fractionated into individual proteins so as to obtain an overall picture of the protein composition and to enable location of individual proteins.

The highest resolution is obtained by first separating accordingto the isoelectric points, the second dimension accordingto the molecule mass.
A flat-bed gel can also be cut into strips after the first separation and transferred onto the second gel.

For these techniques, the first-dimensional runs are carried out in individual gel rods or strips and loaded onto the second-dimensional gels.

O'Farrell PH. J Biol Chem. 250 (1975) 4007–4021.

High resolution 2-D electrophoresis: This method had been introduced by O'Farrell (1975). The sample is denatured with a lysis buffer, the first dimension is isoelectric focusing in presence of 8 or 9 molar urea and a non-ionic detergent. SDS electrophoresis is run as the second dimension. In Fig. 25 the principle of the traditional 2-D electrophoresis methodology is shown.

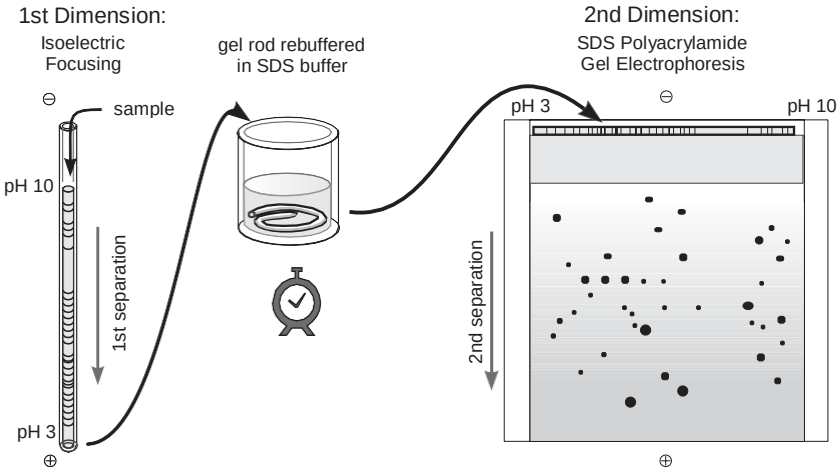

Fig. 25: The principle of the classical high-resolution 2-D electrophoresis according to O'Farrell (1975).

The method has recently become highly interesting, because the protein spots obtained can be further analysed with new methods of mass spectrometry. Protein spots are then identified with the help of genomic databases. This approach is used for "Proteome analysis" (Wasinger *et al.* 1995), which will be described in more detail in the dedicated chapter 6 (page 91ff) and in the book "Proteomics in Practice" (Westermeier and Naven, 2002).

Formerly, the identification of protein spots was very complicated and time consuming: genomic data were not available.

Westermeier R, Naven T. Proteomics in Practice. A laboratory manual of proteome analysis. WILEY-VCH, Weinheim (2002).