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Polyacrylamide Gel Electrophoresis

See **II / ELECTROPHORESIS / One-dimensional Polyacrylamide Gel Electrophoresis; II / ELECTROPHORESIS / One-dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; II / ELECTROPHORESIS / Two-dimentional Polyacrylamide Gel Electrophoresis**

Porosity Gradient Gels

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Introduction

The high resolving power of polyacrylamide (PA) gels for proteins, peptides and nucleic acids can be improved by using gradient gels instead of homogeneous (i.e. single concentration) gels. However, a more specific separation of polynucleotides in PA gels affords separation by incorporating a 40–80% denaturant gradient (7 mol L^{-1} urea, 40% (v/v) formamide) into a homogeneous PA gel (of e.g. 6.5% (w/v) total polymer concentration) or applying a temperature gradient to a homogeneous PA gel.

In PA gradient gels the average pore radius decreases with increasing gel concentrations, i.e. in the direction of the migrating protein (polynucleotide) bands. This results in a sharpening of the bands because the molecules at the front of the moving band are slower than those at the rear. Because of this effect, gradient gels need not be covered by a stacking gel, as in disc gel electrophoresis. In porosity gradient gels with a steep increase of polymer concentration (e.g. from 4 to 30% T (w/v) where $\%T$ = g acrylamide + g Bis = N,N'-methylenebisacrylamide (Bis) per 100 mL) proteins of a large size range (approximately 10^4–10^6 Da) can be separated. In shallow gradients ($> 4\%$ T to $< 30\%$ T), the separable size range of proteins is limited but they still provide an improved band sharpening.

There are two modes to run porosity gradient gels: a fixed-time mode, where electrophoresis is terminated after a certain time, and a time-dependent mode, which means that a number of consecutive electrophoretic mobilities are registered. Fixed-time electrophoresis is performed if protein (polynucleotide) patterns are to be screened, such as in population genetics or when determining the molecular mass of sodium dodecyl sulfate (SDS) denatured proteins. Molecular size properties of nondenatured proteins, however, cannot be elucidated that way, but afford time-dependent investigation of protein mobilities. On the other hand, time-dependent PA gradient gel electrophoresis not only offers the possibility to estimate the molecular mass of native proteins and enzymes but also allows determination of their Stokes radius, frictional coefficient, free electrophoretic

mobility and nett charge. A number of different (iso)enzyme systems have been classified in this way and comparisons between related species used to study the evolution of enzyme systems.

Porosity gradient gels can be easily prepared using one of the different devices on the market. Ready-to-use pore gradient gels are commercially available (Amersham Pharmacia Biotech, Freiburg, Germany; Gradipore, 200 Harris Street, Pyrmont NSW 2009, Sydney, Australia). Porosity gradient gels can be prepared in casting glass cassettes either without any further support or by adhering them to a silanized glass plate or a reactive polyester film. The latter two methods are employed when ultra-thin gels are to be used horizontally. Glass cassette cast PA gradient gels without any further support are used vertically.

Porosity of Polyacrylamide Gradient Gels

In 1962 Ornstein and Davis were the first to suggest a formula to estimate roughly the average pore diameter of homogeneous PA gels:

$$p_{av}\,(\text{nm}) = 12.67 \times (\%T)^{-1/2} \qquad [1]$$

where p_{av} (nm) is the average pore diameter in nanometres and $\%T$ is the total acrylamide concentration (g acrylamide + g Bis in 100 mL).

Based on the Ogston model which describes dextran gels as assembled from arbitrarily arranged gel rods, Raymond and Nakamichi related the average pore diameter of PA gels to the total polymer concentration (T) as follows:

$$p_{av}\,(\text{nm}) = K \times d \times (100 \times p)^{1/2} \times (\%T)^{-1/2} \qquad [2]$$

where K is the factor resulting from the angle in which the gel rods are linked together (1.5), d (nm) is the diameter of a PA gel rod (0.5), p (g cm^{-1}) is the density of gel rod (1.2). This results in:

$$p_{av}\,(\text{nm}) = 8.216 \times (\%T)^{-1/2} \qquad [3]$$

The largest pore diameter in a PA gel of a certain concentration is, however, much larger than the average pore diameter (**Figure 1**). Moreover, the largest pore diameter deviates increasingly from the average pore diameter with decreasing gel concentration. The pores therefore are statistically distributed, but the standard deviations of the average pore radii and the distribution function (Gaussian or logarithmic distribution) are unknown.

The generally held assumption of a random meshwork of cross-linked individual PA rods could not be confirmed by electron microscope images. They revealed sponge-like structures in the submicron range. Such structures are in accordance with the mode in which gels polymerize. PA molecules first arrange as high molecular aggregates that are in the sol state and not interconnected. Thereafter, cross-linkage to a three-dimensional gel occurs: this is indicated by an abrupt start of gelation.

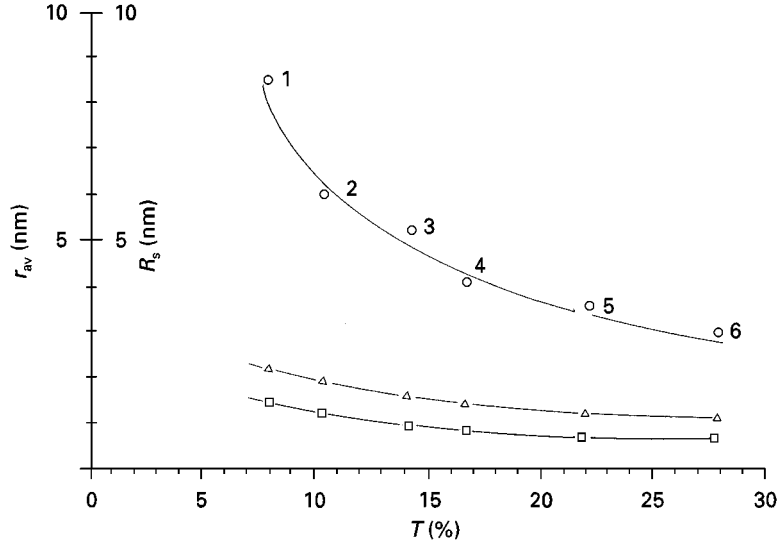


Figure 1 Plot of average pore radius (r_{av} (nm)) against PA gel concentration (T (%)). Triangles, average pore radii calculated as suggested by Ornstein and Davis (1962). Squares, average pore radii calculated as suggested by Raymond and Nakamichi (1962). Circles, maximum pore radii as marked by native proteins of known radius: 1, thyroglobulin; 2, ferritin; 3, catalase; 4, lactate dehydrogenase; 5, bovine serum albumin; 6, ovalbumin. Reproduced with permission from Rothe and Maurer (1986).

Analytical Separation of Native Proteins in a Glass Cassette-Cast Porosity Gradient

Gradient preparation is performed with acrylamide solutions of high and low concentrations, usually by using a two-chamber gradient mixer, although more sophisticated gradient formers have been developed. Linear PA gradients are usually prepared by the technique which was first described by Martin and Ames in 1961 for the preparation of linear sucrose gradients. Glass cassette-cast gels are mostly 82×82 (140) mm or 125×250 mm and a thickness of 3.0, 1.0, 0.8, 0.5 or 0.1 mm.

Preparation of a Batch of Unattached Gradient Gels

Polyacrylamide gradient gels cast in glass cassettes may be prepared individually or simultaneously in batches (**Figure 2**). The latter method saves time and, although the gradients usually deviate slightly from each other, they are well suited to determine protein patterns, e.g. isozyme patterns as in population genetics. Any form of gradient (linear, concave, convex) may be prepared but linearly increasing gradients of total polymer concentration are most commonly used.

The device shown in **Figure 3** can prepare six gradient gels simultaneously. In each gel the PA concentration increases linearly from top to bottom from approximately 5 to 25% *T*. The gels are encased in glass cassettes of internal dimensions $172 \times 82 \times 1.0$ mm. Each cassette is fitted with a slot former and inserted in a gel-casting device. The linear PA gradient is prepared by using a two-chamber gradient mixer, a separate reservoir (for the catalyst solution), a proportioning pump, a 1 mL mixing chamber (and a reservoir filled with sucrose and a pump to lift the gradient into the cassettes). The device shown in Figure 3 is used as follows: The inner chamber (1) and the connecting tube (3) to the outer chamber (2) of the gradient mixer are filled with 57 mL of T_{min} solution. Then the tube (3) to chamber (2) of the mixer is closed. Afterwards 57 mL of the T_{max} solution is pipetted into chamber (2) of the gradient mixer. Now 22.5 µL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) is mixed with the T_{min} and the T_{max} solution, respectively. A separate reservoir (4) is filled with 35 mL of gel buffer containing 50 mg ammonium persulfate. The connection between chamber (1) and (2) of the gradient mixer is opened, after which the stirrer (5) of the gradient mixer and the stirrer (6) of the mixing chamber (7) as well as the peristaltic pump (8) are switched on. Immediately after chamber (1) is empty, the pump (8) is switched off and a sufficient amount of sucrose solution (50% (w/v)) is pumped from the corresponding reservoir (9) with the help of a separate pump (10) underneath the gel cassettes (12) to lift the whole gradient into the cassettes, which are in the gel-casting device (14).

The T_{min} and T_{max} solution contain acrylamide and Bis at the same ratio (acrylamide–Bis = 24 : 1). The T_{min} solution contains 4.205 g acrylamide and

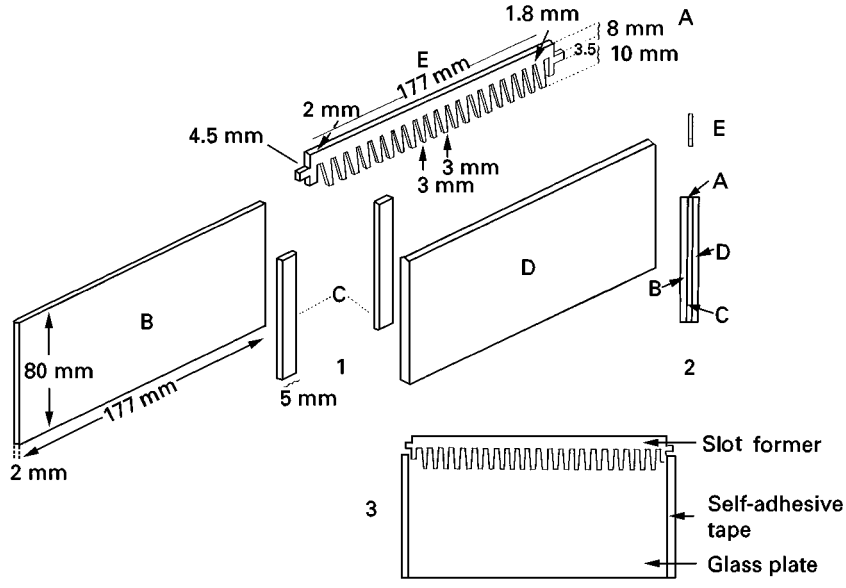
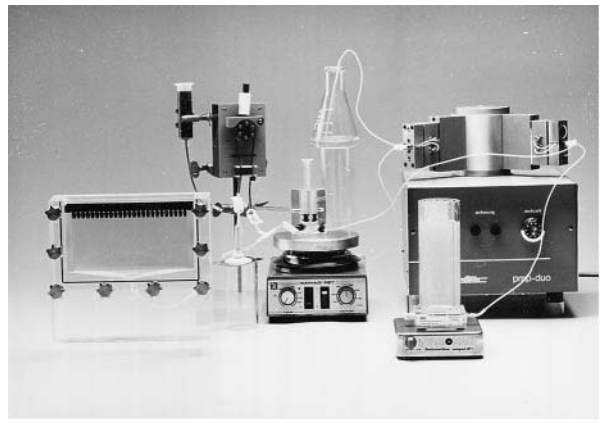


Figure 2 Assembly of a glass cassette to cast a PA (gradient) gel slab. A, Slot former; B, front and D rear glass plate of cassette; C, left and right distance bar. 1, Exploded view of cassette; 2, side view; 3, front view. Procedure according to Pharmacia, Uppsala, Sweden. Reproduced with permission from Rothe (1991).



(A)

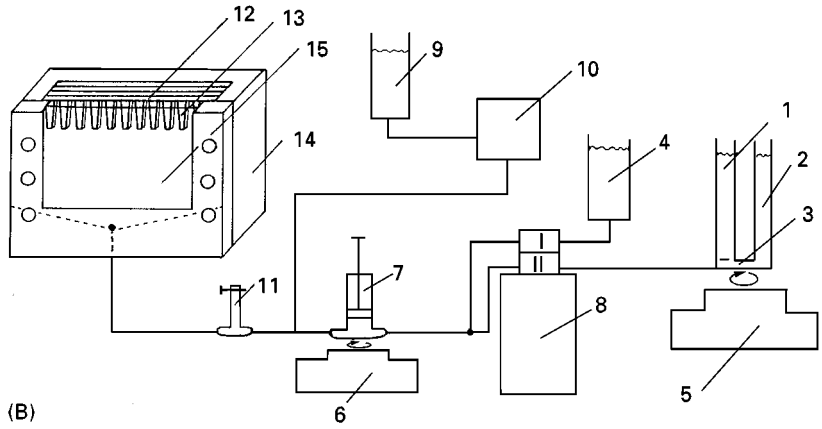


Figure 3 (A) Device for preparing a batch of six PA porosity gradient gels. (B) Scheme for preparing a batch of six porosity gradient gels each encased in a glass cassette without further support. 1 and 2, chambers of the gradient mixer (1 with magnetic bar); 3, connecting tube between both chambers which can be closed by a stopcock (not shown); 4, reservoir to hold the catalyst solution (ammonium persulfate); 5 and 6, stirrers; 7, mixing chamber (modified 1 mL syringe); 8, two-channel pump; 9, reservoir to hold sucrose solution; 10, one-channel pump; 11, air trap; 12, gel cassettes with 13, inserted slot formers; 14, gel-casting apparatus (made of perspex) with 15 removable front plate. Reproduced with permission from Rothe (1994).

0.175 g Bis per 100 mL gel buffer while the T_{max} solution contains 31.54 g acrylamide and 1.314 g Bis in 100 mL gel buffer. The T_{min} and T_{max} solutions are diluted upon gradient formation with catalyst solution by a factor of 1.255 (Figure 3) and the gel solution is pumped to about 5 mm above the slot template. This results in a final concentration range of approximately 5–25% *T*. (Mixing both catalysts into the T_{min} and T_{max} solution is also possible but carries the danger that the gel may solidify before being completely cast in the cassette). Prior to use all solutions are brought to room temperature and degassed. The ammonium persulfate solution should be prepared freshly each time. 90 mmol L^{-1} Tris, 45 mmol L^{-1} boric acid and 2.5 mmol L^{-1} EDTA–Na_2, pH 8.4 is used as gel and electrode buffer. Further buffer systems are given in **Table 1**.

Vertical Electrophoresis

Glass cassette-cast gradient gels are mounted vertically into an electrophoretic apparatus consisting of an upper and lower electrode vessel (**Figure 4**). The upper buffer tank has rubber gaskets into which two or four cassettes can be inserted. The lower electrode vessel is filled with cooled buffer; the upper electrode vessel with the inserted gel cassettes is mounted into the electrophoresis apparatus and filled with electrode buffer. Then the samples (enriched with 10% sucrose) are added to the slots (with a Hamilton syringe). Afterwards the voltage is switched on (≤ 40 V cm^{-1}) for 15 min for the proteins to migrate into the gel. Finally the buffer is circulated from buffer tank to buffer tank at the same voltage. The lower buffer tank is cooled to 5°C during electrophoresis by a cooling coil.

Table 1 Buffer systems used in porosity gradient gel electrophoresis to separate native proteins

Gel buffer	*Electrode buffer*	%T *range*	*Authors*
0.35 mol L^{-1} Tris HCl, pH 8.9	0.06 mol L^{-1}, Tris, 0.40 mol L^{-1} glycine, pH 8.3	3–20	Kopperschläger *et al.* (1969)
0.09 mol L^{-1} Tris, 0.08 mol L^{-1} boric acid, 0.003 mol L^{-1} EDTA–Na_2, pH 8.3	Same as gel buffer	4–26	Anderson *et al.* (1972) Lasky (1978)
0.01 mol L^{-1} Tris, 0.08 mol L^{-1} glycine, pH 8.3	Same as gel buffer	5–30 5–15	Slater (1969)
0.04 mol L^{-1} Veronal–Na, 0.04 mol L^{-1} Tris, 0.01 mol L^{-1} glycine, 0.04 mol L^{-1} ethanolamine, 0.001 mol L^{-1} EDTA–Na_2 pH 9.8	Same as gel buffer	5–30	Lambin and Fine (1979)
0.01 mol L^{-1} Na–phosphate, pH 7.2	Same as gel buffer	5–30	Lambin and Fine (1979)

References as given in Rothe and Maurer (1986). Reproduced with permission from Rothe and Maurer (1986).

PA gradient gel electrophoresis under nondenaturing conditions has proved to be advantageous compared to electrophoresis in homogeneous gels, e.g. in plant population genetics. **Figure 5** gives an example.

Separation of Native Proteins in an Ultra-Thin Support-Bound Porosity Gradient

To prepare a thin gradient gel of the dimensions $120 \times 250 \times 0.5$ mm fixed to a derivatized clear and flexible polyester foil (e.g. manufactured by Gel Bond, Marine Colloids, Rockland, MN, USA or Serva, Heidelberg, Germany), the gel-forming devices shown in **Figure 6** may be used. (When the cassettes are assembled the slot formers must not touch the opposite glass wall but leave a space of 0.1 mm in between). The following solutions may be used to form a gradient ranging from 3 to 30% *T*:

1. gel buffer: 90 mmol L^{-1} Tris, 80 mmol L^{-1} boric acid, 2.5 mmol L^{-1} EDTA–Na_2, pH 8.4;
2. electrode buffer: 1 in 2 diluted gel buffer;
3. stock acrylamide solution (30% *T*: 28.8 g acrylamide plus 1.2 g Bis plus 50 mL gel buffer, made to 100 mL with distilled water);

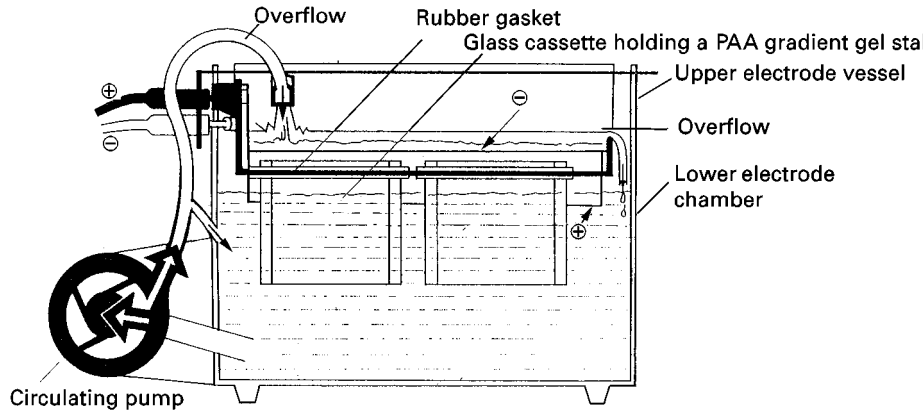


Figure 4 Vertical electrophoretic apparatus in which up to four glass cassette-cast porosity gradient gels can be inserted. Upper electrode vessel with 2 rubber gaskets to hold 2 to 4 glass cassettes, each containing a porosity gradient made of PA; +, −, electrodes. Modified from an instruction leaflet published by Pharmacia, Uppsala, Sweden.

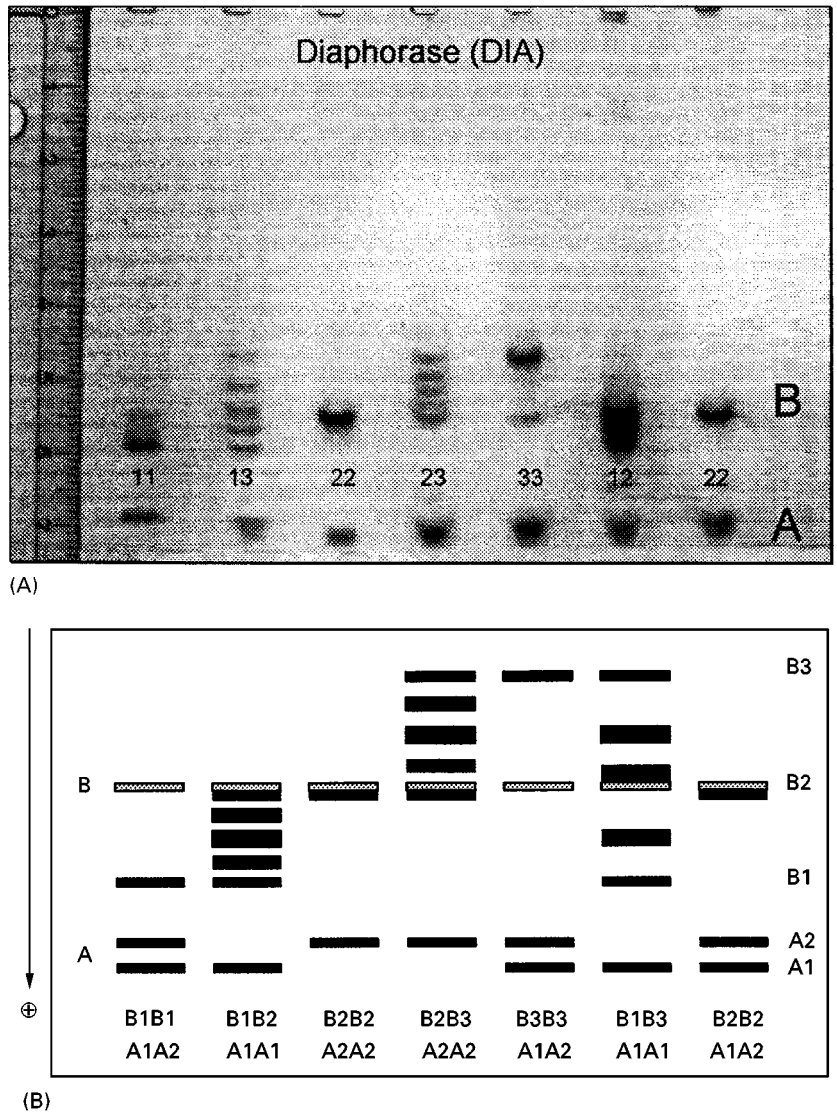


Figure 5 Electrophoresis of plant diaphorase isoenzymes in a 4–20% *T* PA gradient gel of 0.8 mm thickness (length 175 mm, height 75 mm). (A) Zymogram of diaphorase enzymes (numbers indicate genotypes of the tetrameric enzyme at locus B. (B) Schematic representation of genotypes at locus DIA-A and DIA-B. Enzyme source: leaf buds of seven different trees of European beech (*Fagus sylvatica* L.). Conditions of electrophoresis: gel and electrode buffer: 45 mmol L^{-1} Tris, 40 mmol L^{-1} boric acid, 1.25 mmol L^{-1} EDTA–Na_2; pH 8.4; running time 4 h; voltage gradient 40 V cm^{-1}; temperature 5°C.

Enzyme extraction: 1.5 mL Eppendorf tubes containing 150 mg of green bud leaves, 50 mg of quartz sand and 600 μL of extraction medium were cooled from underneath with ice water. A motor-driven grinding cone adapted in the shape of the tube (rotating at 700 rpm) was used to homogenize the material. The extraction medium contained in 100 mL: 1.21 g Tris, 1.43 g Na_2HPO_4, 60 mg L-cysteine, 210 mg ascorbic acid, 14 g sucrose, 40 mg NADP, 15 g polyclar AT (PVPP) and 1 g polyethylene glycol, pH 7.5 (with H_3PO_4). The homogenate was centrifuged for 30 min at 4°C and 10 000 **g** and the clear supernatant used as crude enzyme extract. Samples of 8 μL were applied per lane. Diaphorase isozymes were visualized histochemically (60 mL 25 mmol L^{-1} Tris-HCl, pH 8.5, containing 24 mg NADH, 1.5 mg 2,5 dichlorphenolindophenol-Na × $2H_2O$ (DCPIP) and 1.8 mL MTT (500 mg 100 mL^{-1} aq. bidest. water). Anode at bottom. A, Enzymes of gene locus DIA-A; B, locus DIA-B. In (A) not all genotypes indicated in (B) are shown.

4. dense acrylamide solution (30% *T*: to 6.5 mL stock solution is added, shortly before use, 20 μL TEMED (1 in 10 with H_2O diluted solution) and 5 μL ammonium persulfate solution (40% w/v in distilled water));
5. light acrylamide solution (3% *T*: 1 vol of stock acrylamide solution is diluted with 4.5 vol distilled water and 4.5 vol of gel buffer shortly before use and 40 μL TEMED (1 in 10 with distilled water diluted solution) and 10 μL ammonium persulfate

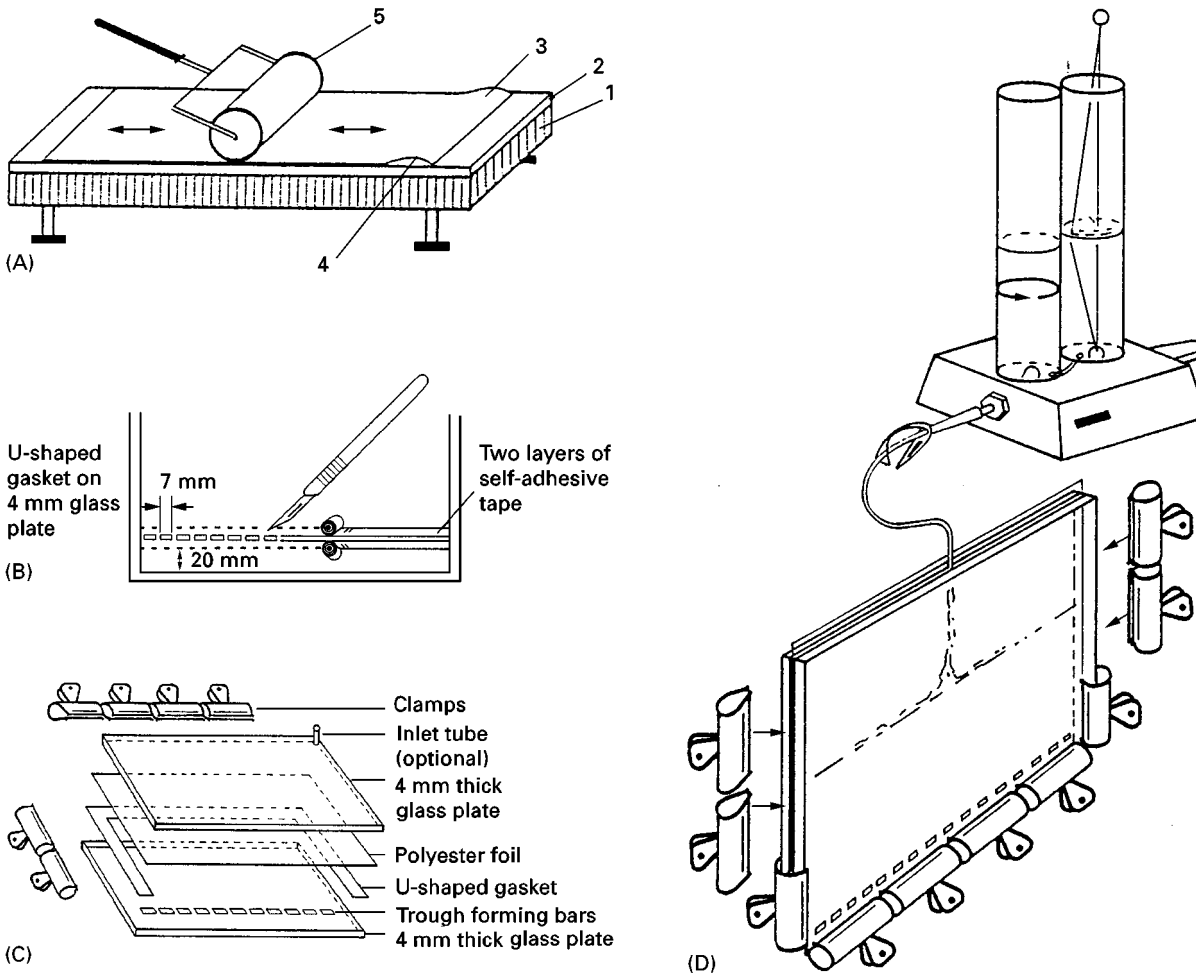


Figure 6 Preparation of an ultra-thin PA gradient gel fixed to a polyester foil. (A) Rolling the polyester foil (reactive side up, e.g. Gel Bond) on to one of the glass plates used to build the casting glass cassette: 1, levelling table; 2, glass plate; 3, hydrophilic side of polyester foil; 4, water layer; 5, rubber roller. (B) Trough template preparation. The bars are prepared from two layers of self-adhesive tape with a scalpel. (C) Assembling the glass cassette to cast the PA gradient. (D) Casting the porosity gradient: two-chamber mixer and glass cassette. Reproduced with permission from Rothe (1991).

solution (40% w/v in distilled water) is added. The gradient is made of 6.5 mL of dense acrylamide solution and 6.5 mL of light acrylamide solution. After gradient formation, 2 mL of light acrylamide solution is overlaid; the slots must be situated in the middle of the 3% T range.

Horizontal Electrophoresis

Before electrophoresis, the gel is taken out of the cassette. A few drops of kerosene are put on the cooling plate of the opened electrophoretic apparatus (**Figure 7**) and the gel, firmly adhering to the polyester foil, is placed on it, carefully avoiding the inclusion of air bubbles. Both ends of the gel are connected with the buffer vessels by paper wicks or a household sponge-like material. A 15–30 min pre-electrophoresis is performed at 1000 V (50 V cm^{-1}). Then the slots are filled with protein solution (or electrode buffer) and the power is turned on again at a voltage of 1000 V for approximately 2 h. Afterwards the gel, fixed on the polyester foil, may be stained for proteins or (iso)enzymes (**Figure 8**).

Determination of the Course and Concentration of a Porosity Gradient Gel

The course and %T range of laboratory-made PA gradient gels can be controlled by densitometry if a coloured dye such as *p*-nitrophenol is added to the denser acrylamide solution prior to gradient formation. After polymerization, the increase in colour

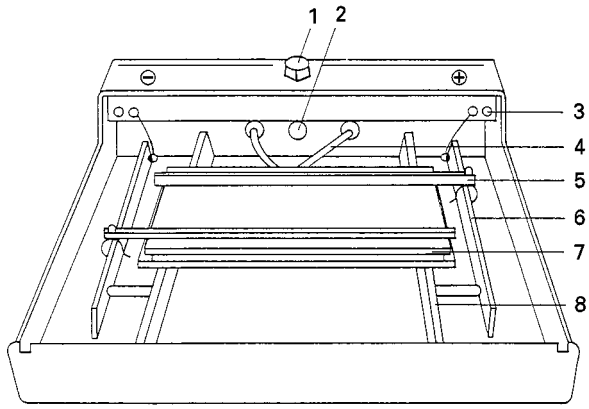


Figure 7 Horizontal electrophoretic apparatus with cooling plate. 1, Cover lock; 2, gassing stud; 3, high voltage connection of the lid; 4, flexible tube to the cooling plate; 7, with a cooling device (not shown); 5, electrode bar (used in isoelectric focusing); 6, electrode ledge; 8, support for cooling plate. For PA gradient gel electrophoresis the electrode bars are replaced by two buffer vessels (not shown) under the cooling plate and connected to the electrode ledge. The gel is connected to the buffer reservoirs by (paper) wicks (not shown). Reproduced with permission from Rothe (1991).

intensity from top to bottom of the gel can be used to measure the course of the gradient and its precise concentration in polyacrylamide. For a 1 mm thick gel 15 mg *p*-nitrophenol may be added to 100 mL of the dense acrylamide solution. After gelation the colour intensity is quantified by densitometry at 405 nm. Whilst the course of the gradient can be seen directly on the densitogram, the %*T* range of the gradient can be calculated with the formula:

$$T(\%) = T_s \times (E_{405} - E_p) \times M_r \times (c \times d \times \varepsilon)^{-1} \quad [4]$$

where T_s (%) is PA concentration of stock solution, E_{405} is absorbance of *p*-nitrophenol, E_p is absorbance of empty cassette at 405 nm, c (g L^{-1}) is concentration of *p*-nitrophenol in stock acrylamide solution ($c = 0.150$), M_r (g L^{-1}) is mol mass of *p*-nitrophenol ($M_r = 139.1$), d (mm) is thickness of gel (e.g. 0.5), E [L (mol mm)$^{-1}$] = molar extinction coefficient of *p*-nitrophenol at 405 nm ($\varepsilon = 1728$) and T (%), as in eqn [1].

Cross-Linkers Other than Bis and Mixed Polyacrylamide Gels

PA is normally cross-linked with Bis to obtain an electrophoretic matrix. The use of *N*,*N*′-(1,2-dihydroxyethylene)bisacrylamide (DHEBA) instead of Bis gives gels that can be solubilized in dilute periodic

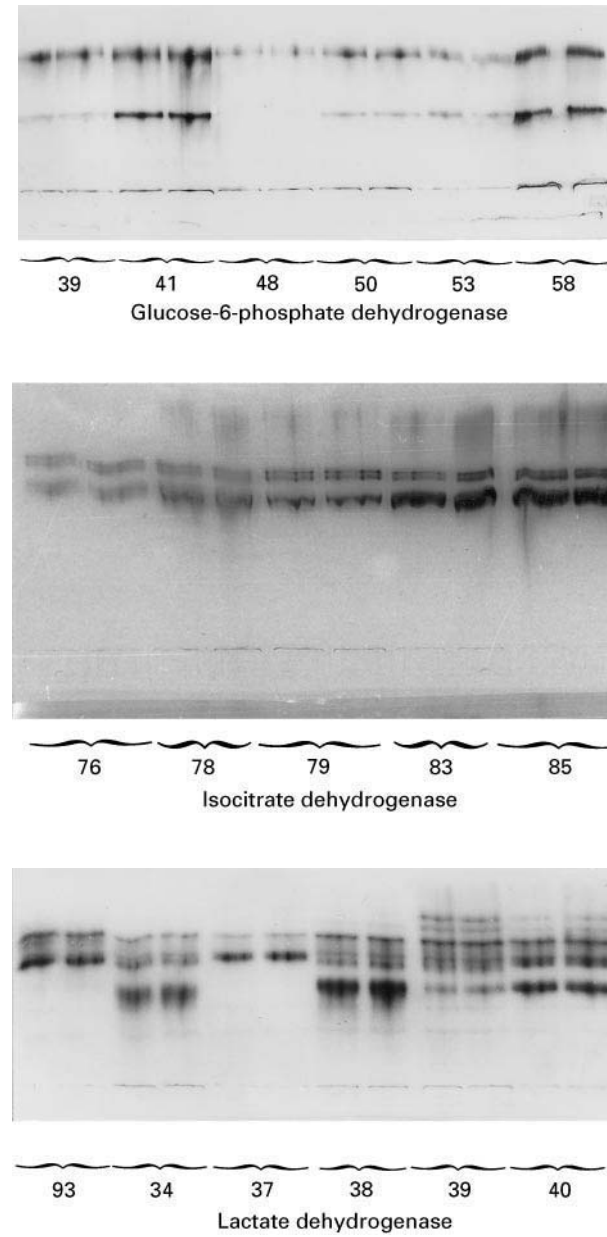


Figure 8 Electrophoresis of plant (iso)enzymes on ultra-thin PA gradient gels fixed on a polyester film. Gel dimensions: 240 × 120 × 0.5 (mm); PA gradient from 4 to 28% *T*. Enzyme source: current-year (1989) needles of Norway spruce (*Picea abies* L., Karst.) sampled from a variety of clones (clone numbers indicated) of the multiple clone variety East Prussian Late Spruce (Hessische Forstliche Versuchsanstalt, Hann. Münden, Germany). Enzyme extraction: 2 g of fresh needles was homogenized in 10 mL of homogenizing medium (0.1 mol L^{-1} Na–phosphate, pH 7.5, containing 5% w/v Polyclar AT and 0.5% w/v Triton X-100. The crude extract was centrifuged for 30 min at 38 000 g and the supernatant concentrated by a factor of 4 using the ultrafiltration system Centrisart I (Sartorius, Göttingen, Germany). Samples of 10 μL were applied per lane. Conditions of electrophoresis: 1000 V for 90 min at 4°C; gel and electrode buffer: 45 mmol L^{-1} Tris, 40 mmol L^{-1} boric acid, 1.25 mmol L^{-1} EDTA–Na_2, pH 8.4. Enzymes were stained histochemically. Anode at top. Reproduced with permission from Rothe (1991).

acid or dilute aqueous solutions of bases to liberate proteins after the electrophoretic separation. Gradient flat gels ($140 \times 120 \times 3$ mm) with an increasing acrylamide concentration but a constant ratio of DHEBA have been used to separate protein mixtures from fruit with radio-labelled amino acids. Following electrophoresis, gel slices containing protein zones are placed in a glass scintillation counting vial fitted with a Teflon-lined plastic cap, 1 mL of 0.025 mol L^{-1} periodic acid is added and the vials are sealed. After incubation for 48 h at 50°C, 10 mL of Mix I scintillation fluid is added, and the vials cooled overnight before counting. PA gels produced with DHEBA may be used with the common alkaline buffer systems except borate buffers, which form negatively charged complexes with the *cis*-1, 2-diol structure of the cross-linker DHEBA.

To improve the retardation of PA gradient gels for low molecular mass proteins, a mixture of acrylamide, Bis and *N*,*N*′,*N*′-triallylcitric triamide has been suggested.

The use of *N*-substituted acrylamido derivatives, such as *N*-acryloyltris(hydroxymethyl)aminomethane (NAT) gives PA gels with larger pores, although the pores are still smaller than those of agarose. Gels of similar pore sizes can be made from allyl-activated agarose and acrylamide or *N*-substituted acrylamido derivatives. The mixed-bed gels of agarose–acrylamide have average pore sizes which are about 30% larger than those of a regular 3.3% Bis cross-linked gel with the same %*T*.

Size Estimation of Native Proteins and Enzymes

The size of native proteins can be deduced from their migration behaviour in homogeneous or gradient gels. Both methods have the advantage that crude tissue or cell extracts can be used as the protein source, provided a specific staining method exists with which they can be located in the gel after electrophoresis. The method with homogeneous gels uses a number of gels of different PA concentration in the range of 4–35% *T* and estimates the relative electrophoretic mobility referred to Bromophenol blue (R_F value) of a set of marker proteins and the sample protein(s). From these values the gel concentration is estimated at which the electrophoretic mobility is zero (or would become zero). This is achieved by plotting the logarithm of the %*T* concentration (log *T*) in which the mobility is measured against the respective R_F value. In the underlying linear function ($\log T = -k \times R_F + \log T_{lim}$), the value of T_{lim} represents the exclusion limit, the %*T* concentration at which protein mobility stops. The T_{lim} values calculated for a number of marker proteins can be correlated to their corresponding Stokes radii (R_S) to obtain a calibration line. A linear function is obtained when R_S is plotted against the reciprocal of T_{lim} ($R_S = a \times 1/T_{lim} + b$). Into this equation the exclusion limit of a sample protein is inserted and this then allows calculation of the corresponding Stokes radius.

Polyacrylamide gradient gel electrophoresis can also be used to estimate the molecular size of nondenatured proteins, provided it is performed in a time-dependent way. The following physicochemical properties of native proteins (enzymes) are obtainable:

1. molecular mass (M_r);
2. hydrodynamic radius (Stokes radius (R_S));
3. frictional coefficient (f/f_o) (molecular eccentricity, considering the molecular shape as a rotational ellipsoid and f/f_o as the quotient of the ratio of the two half axes of the rotational ellipsoid, f = half axis of ellipsoid, f_o = half axis of circle);
4. isomeric nature of multiple protein forms (size isomers or charge isomers);
5. free electrophoretic mobility (and nett negative charge (valence *Z*, charge *Q*)) at the pH value of the electrophoresis.

The mathematical procedures used to calculate these parameters are bound by several preconditions:

1. The PA gradient increases linearly (at a constant ratio of acrylamide to Bis). The gradient range however, can be chosen freely.
2. The electrophoretic pH value and the voltage gradient are chosen in a way that marker and sample proteins migrate sufficiently.
3. The same buffer system has been used as gel and electrode buffer, if net charges are to be obtained.
4. The sizes of the marker and sample proteins fit the pore range of the PA gradient.
5. Marker and sample proteins have migrated on the same gel slab.
6. Parts of the gel slab which have been cut into two or more parts and stained differently are re-equilibrated to the original gel length before protein migrations are measured.
7. Approximately 10 (or more) time-dependent migration distances of marker and sample proteins are accurately measured.

Estimation of the Maximum Migration Distance and Recognition of Size Isomers

With increasing times of electrophoresis under nondenaturing conditions, the migration of proteins in a PA gradient gel gradually decreases (**Figures 9–11**).

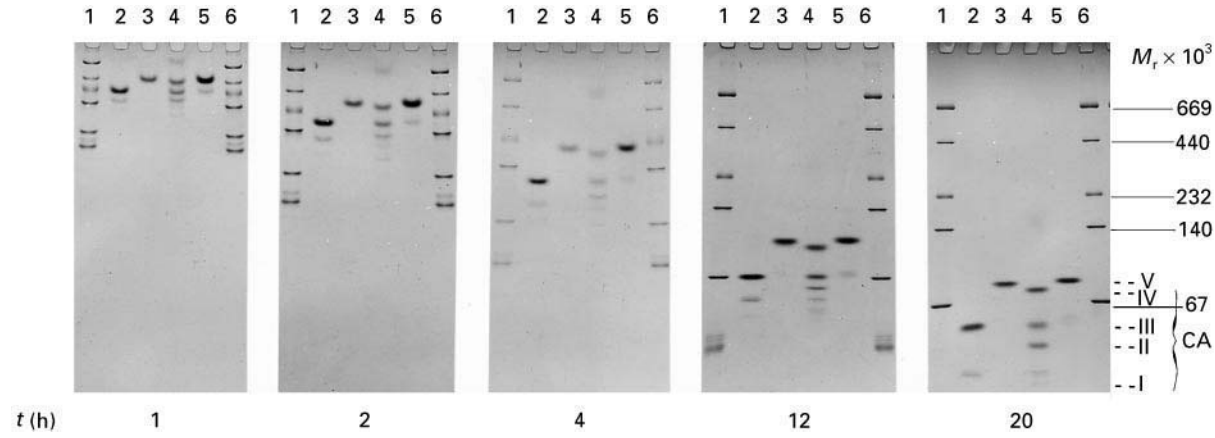


Figure 9 Time-dependent migration patterns of marker proteins and carbonic anhydrase (EC 4.2.1.1) (iso)enzymes from mammalian erythrocytes. Lanes 1 and 6, marker proteins. Lanes 2–5; carbonic anhydrases from (2) bovine, (3) human, (4) rabbit and (5) canine. Mol mass of marker proteins: ovalbumin (45 000), bovine serum albumin (67 000), lactate dehydrogenase (140 000), catalase (232 000), ferritin (440 000) and thyroglobulin (669 000). Linear PA 4–30% T gradient (acrylamide–Bis = 24 : 1), 300 V per 73 mm of gel length, 5°C. Running times: 2, 8 and 16 h. Gel and electrode buffer: 90 mmol L^{-1} Tris, 80 mmol L^{-1} boric acid, 1.25 mmol L^{-1} EDTA–Na_2–H_2O, pH 8.4. Protein staining with Coomassie brilliant blue. Enzyme preparations from Sigma, Munich, Germany. Reproduced with permission from Rothe (1991).

Migration of globular proteins comes to an end when the maximum pore size of a gel region equals their own size. The corresponding migration distance is called the maximum migration distance (D_{max} (mm)). The maximum migration distance can be obtained from a number of time-dependent protein migrations (D (mm)) (**Figures 9** and **10**) which are directly measured on the gel after proteins have been visualized following electrophoretic separation (**Table 2**). To obtain the maximum migration distance of a certain protein, the following mathematical approximation procedure can be applied: the migration distances are double-logarithmized ($\ln(\ln D)$) and plotted versus the reciprocal of the square root of electrophoretic migration time, $1/t^{1/2}$ (t (h)). This results in a straight line (**Figure 11**) whereby the transformed migration values ($\ln(\ln D)$) and the transformed times of electrophoresis ($t^{-1/2}$) are interrelated by the equation:

$$\ln(\ln D) = -a \times t^{-1/2} + b \qquad [5]$$

where a and b are the slope and the intercept of the corresponding straight line. The equation predicts that at very high values of t, $t^{-1/2}$ reaches zero. This means that the maximum migration of a protein (D_{max} (mm)) can be taken from the intercept of the straight line with the ordinate in a plot of $\ln(\ln D)$ versus $t^{-1/2}$ provided protein migrations were larger than 2 mm and a sufficient number of different migration distances are registered. Letting t approximate to infinity means that eqn [5] becomes:

$$\ln(\ln D) = \ln(\ln D_{max}) = b \qquad [6]$$

and:

$$D = D_{max} = \exp(e^b) \qquad [7]$$

A plot of $\ln(\ln D)$ versus $t^{-1/2}$ can also be used to distinguish size isomers from charge isomers. Equally sized but differently charged forms of an enzyme or protein system are recognized by the fact that the straight line of each enzyme form intersects at the same point on the $\ln(\ln D)$ axis as is for example the case with mammalian carbonic anhydrase (cf. **Figure 11**) and mammalian lactate dehydrogenase. On the other hand, migration of charge isomers should result in lines of equal slope. Proteins differing in charge and size, however, give straight lines with both different slopes and intercepts.

Estimation of Stokes Radius and Molecular Mass

The maximum migration distance of globular proteins is related to the maximum gel pore radius at the respective gel concentration (cf. **Figure 1**). Therefore, the maximum migration distances (D_{max}) of proteins can be correlated to their Stokes radius (R_S). A linear relationship is obtained if the logarithm of the maximum migration distance ($\ln D_{max}$) of proteins is plotted versus the logarithm of their Stokes radius ($\ln R_S$):

$$\ln D_{max} = -m \times \ln R_S + b \qquad [8]$$

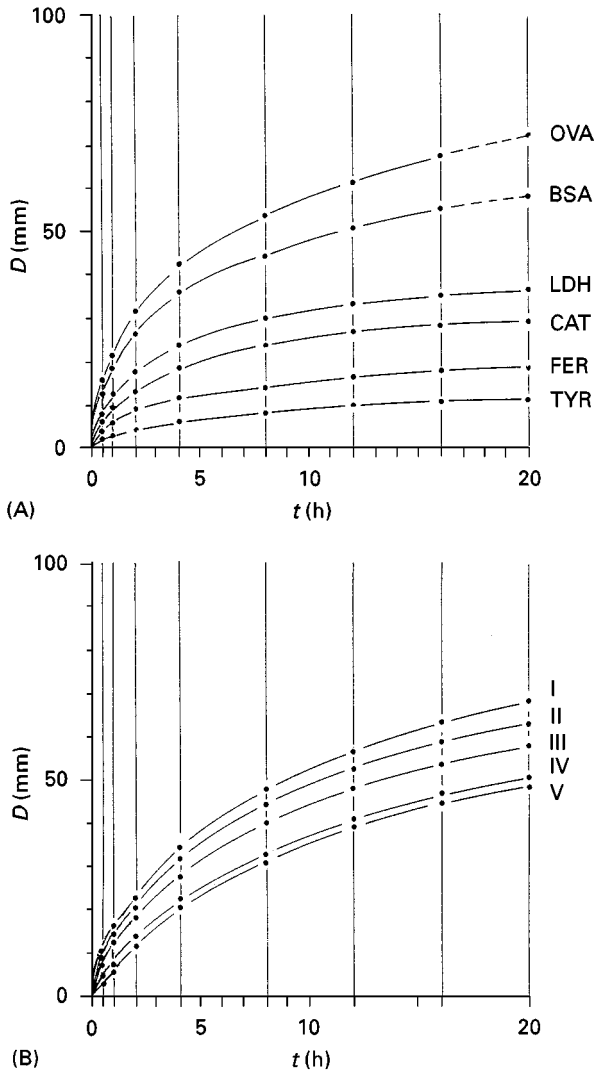


Figure 10 (A) Plot of migration distances (D (mm)) of marker proteins and (B) of five different carbonic anhydrases versus times of electrophoresis (t (h)) in a linear PA gradient gel of 4–30% T. Conditions of electrophoresis are given in Figure 9. Migration distances and times of electrophoresis as listed in Table 2. OVA, Ovalbumin; BSA, bovine serum albumin; LDH, lactate dehydrogenase; CAT, catalase; FER, ferritin; TYR, thyroglobulin. Marker proteins and carbonic anhydrases were migrated on the same gradient gel. Purified enzyme preparations (Sigma, Munich, Germany) comprised carbonic anhydrases from bovine (I–III), rabbit (III, IV), human (V) and canine (V) erythrocytes.
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where $\ln D_{max}$ equals e^b of eqn [7], and m and b represent the slope and intercept of the straight line (**Figure 12**).

It has been shown that a similar equation correlates the logarithm of the maximum migration distance ($\ln D_{max}$) to the logarithm of the molecular mass ($\ln M_r$):

$$\ln D_{max} = -z \times \ln M_r + c \qquad [9]$$

where $\ln D_{max}$ equals e^b of eqn [7], and z and c represent the slope and intercept of the straight line (**Figure 12**).

Knowing the maximum migration distance of any native globular protein, the calibration line can be used to calculate the molecular mass of the protein by inserting the calculated $\ln D_{max}$ value and the values of the slope (z) and the intercept (c) of the calibration line into the equation $\ln D_{max} = -z \times \ln M_r + c$ (**Table 3**) or inserting the $\ln D_{max}$ value and the values of the slope (m) and the intercept (b) of the calibration line into the equation $\ln D_{max} = -m \times \ln R_S + b$ (**Table 4**).

When using PA gradients of 4–30% T and a buffer of pH 8.4 (45 mmol L^{-1} Tris, 40 mmol L^{-1} boric acid, 1.25 mmol L^{-1} EDTA–Na_2, pH 8.4) a number of markers can be used, ranging from carbonic anhydrase (M_r 30 000, R_S 3.05) to thyroglobulin (M_r 669 000, R_S 8.50; **Table 5**). β-Galactosidase (M_r 116 000, R_S 4.23) and carbonic anhydratase (Sigma, St Louis, MO, USA) are run in the same lane and the other marker proteins are run in a separate one. The marker proteins bovine serum albumin, lactate dehydrogenase, catalase, ferritin and thyroglobulin can be obtained as a freeze-dried mixture (Amersham Pharmacia Biotech, Freiburg, Germany) and dissolved in a solution of pure ovalbumin (Boehringer, Mannheim, Germany). Separation times depend on the voltage gradient and may range from 0.5 to more than 20 h (**Table 2**).

Estimation of Frictional Coefficient

The frictional coefficient (f/f_o) relates the hydrodynamic volume of a protein molecule to its molecular mass. According to Siegel and Monty, the Stokes radius (R_S) of a protein is related to its molecular mass (M_r) by the following equation:

$$R_S\,(\mathrm{m}) = f/f_o \times (3 \times \upsilon \times M_r)^{1/3} \times (4 \times \pi \times N_A)^{-1/3} \qquad [10]$$

where R_S (m) is the Stokes radius, f/f_o is the frictional coefficient (equivalent to the quotient of the half axes of a rotational ellipsoid), υ ($m^3\,g^{-1}$) is the partial specific volume (the reciprocal of the average density of a protein, ($\upsilon = 0.75 \times 10^{-6}$), N_A (mol^{-1}) is Avogadro's number ($N_A = 6.022 \times 10^{23}$), and M_r ($Da = g\,mol^{-1}$) is the molecular mass of a protein. By substituting the actual values one obtains:

$$R_S\,(\mathrm{m}) = f/f_o \times 66.1 \times 10^{-12} \times M_r^{1/3} \qquad [11]$$

The geometric mean radius of a molecular mass equivalent sphere is defined as R_m (m). It is obtained

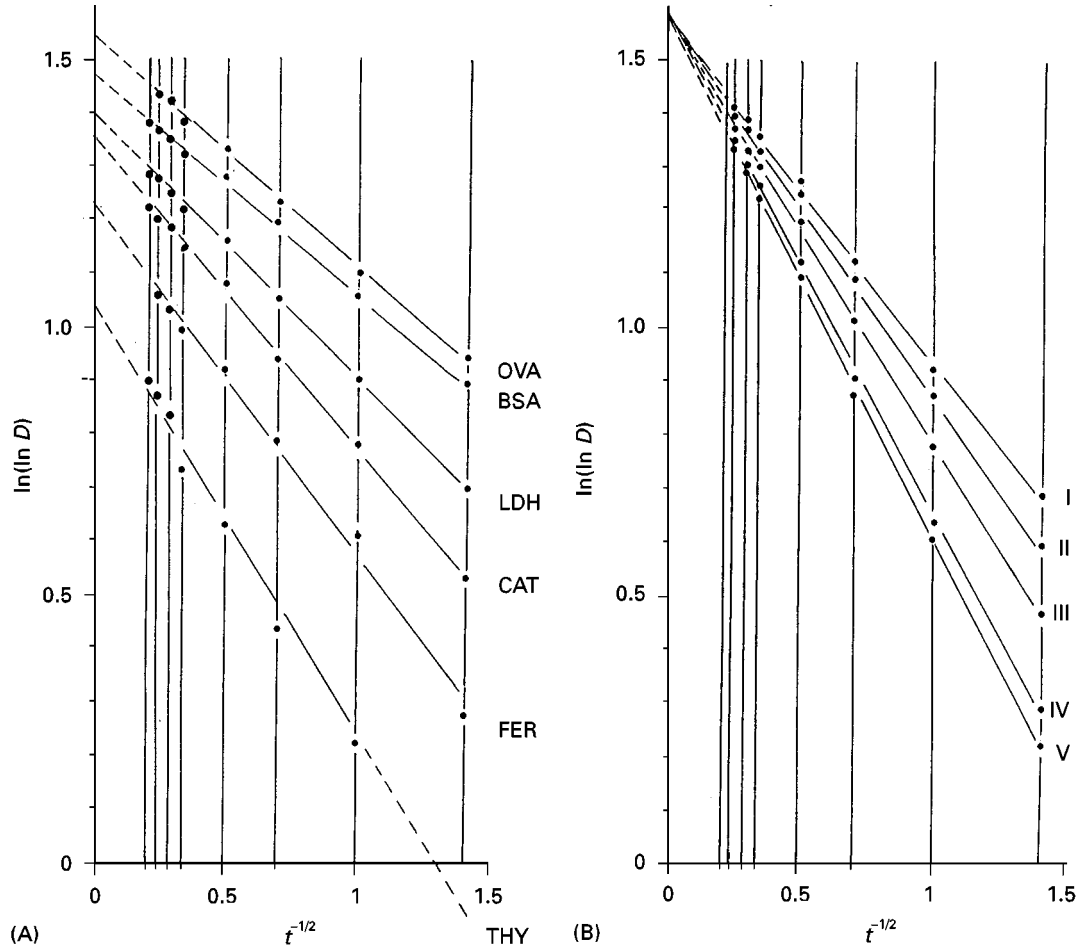

Figure 11 Plot of transformed migration distances (ln(ln D)) against transformed migration times ($t^{-1/2}$) of (A) marker proteins and (B) five carbonic anhydrase variants. Migration distances and times of electrophoresis as listed in Table 2. Abbreviations as in Figure 10. The common point of intersection of the various straight lines marked I–V on the ln(ln D) axis indicates that the investigated enzymes are size isomers. Reproduced with permission from Rothe (1991).

by setting $f/f_o = 1$ in eqn [11] to give eqn [12]:

$$R_S\,(\mathrm{m}) = 66.1 \times 10^{-12} \times M_r^{1/3} \qquad [12]$$

This means that R_S and R_m are interrelated through the frictional coefficient:

$$R_S = f/f_o \times R_m \qquad [13]$$

The frictional coefficient can be obtained from the experimentally obtained M_r and R_S values and eqn [13].

Extremely high frictional ratios are to be expected for molecules with rod-like or fibrous structures, which are characterized by a high axial ratio such as fibrinogen or myosin or by bulky and voluminous globular molecules with normal axial ratios. Examples of the latter are the spider-like immunoglobulin M, the shell-like apoferritin or the branched α-macroglobulin. Usually, native proteins and enzymes do not belong to these groups of proteins.

In eqn [11] the frictional coefficient of native proteins is assumed to be constant. However, when analysing the molecular mass (M_r) and Stokes radius (R_S) of more than 60 native proteins it became apparent that the frictional coefficient increases with increasing protein size (see Further Reading). A more precise equation relating R_S and M_r is the following:

$$R_S\,(\mathrm{m}) = M_r^{0.0225} \times 55.1 \times 10^{-12} \times M_r^{0.0142} \times M_r^{1/3} \qquad [14]$$

According to this expression the frictional coefficient of globular proteins equals $f/f_o = M_r^{0.0225}$ and increases with molecular masses of 10^3 to 9×10^6 from $f/f_o = 1.17$ to $f/f_o = 1.43$ while the factor 66×10^{-12} of the expression of Siegel and Monty ($R_S\,(\mathrm{nm}) = f/f_o \times 66.1 \times 10^{-12} \times M_r^{1/3}$) increases from 61×10^{-12} to 67×10^{-12}.

As an average, the frictional ratio of globular proteins sized 45–100 kDa is $f/f_o = 1.23$, for those in the range of 100–500 kDa $f/f_o = 1.28$ and in the range of

Table 2 Time-dependent migration distances of marker proteins and carbonic anhydrase (iso)enzymes from erythrocytes of four mammalian species in a porosity gradient gel from 4 to 30% *T*

Protein		*Time* t (*h*) *of electrophoresis* ($1/\sqrt{t}$ *given in brackets*)							
		0.5 (1.41421)	*1 (1.00000)*	*2 (0.70711)*	*4 (0.50000)*	*8 (0.35355)*	*12 (0.28868)*	*16 (0.25000)*	*20 (0.22361)*
Ovalbumin	*D* (mm)	13.05	20.25	31.5	44.0	54.0	61.0	67.5	
Bovine serum albumin	*D* (mm)	11.7	17.8	26.5	36.3	43.5	47.5	50.5	53.2
L-lactate dehydrogenase	*D* (mm)	7.5	11.9	17.5	24.5	30.2	33.3	35.5	37.5
Catalase	*D* (mm)	5.5	8.8	13.2	18.8	23.5	26.6	28.5	30.0
Ferritin	*D* (mm)	3.7	6.5	9.0	12.0	14.3	16.6	17.9	18.9
Thyroglobulin	*D* (mm)	1.9	3.5	4.7	6.5	8.0	10.0	10.8	11.6
Bovine I	*D* (mm)	7.3	12.5	21.5	35.5	48.2	56.0	62.5	
Bovine II	*D* (mm)	6.3	11.0	19.0	32.5	45.0	52.3	58.8	68.0
Bovine, rabbit III	*D* (mm)	5.0	8.8	15.5	27.5	40.1	47.5	52.0	58.0
Rabbit IV	*D* (mm)	3.8	6.7	11.8	21.5	33.6	41.0	45.7	50.2
Canine, Human V	*D* (mm)	3.5	6.3	11.2	20.0	32.5	39.8	44.5	48.5

D (mm), Time-dependent migration distances of marker proteins and carbonic anhydrase (EC 4.2.1.1) variants. Gel length (*D* (mm)) and gel concentration (*T* (%)) are interrelated by the equation $T = \alpha D + \beta$ where $\alpha = 0.3528 \pm 0.0054$ and $\beta = 4.1116 \pm 0.2344$; the correlation coefficient is $r = 0.9985$. Reproduced with permission from Rothe (1991).

500–1000 kDa $f/f_o = 1.43$. From these data and the Stokes radius of a globular protein its molecular mass can be estimated:

$$M_r = (1/(f/f_o))^3 \times 3463 \times R_S^3 \qquad [15]$$

with M_r, f/f_o and R_S as in eqn [10].

This can be exemplified by mammalian liver alcohol dehydrogenase (EC 1.1.1.1), which has a molecular mass of 80 kDa and a Stokes radius of 3.5 nm; the average frictional coefficient of globular proteins in that range is $f/f_o = 1.23$. By inserting these values into eqn [15] one obtains: $M_r\,(\mathrm{Da}) = (1/1.23)^3 \times 3463 \times 3.5^3 = 79\,791$.

Determination of Migration Velocities

The migration velocity of a protein migrating in an electrophoretic support medium can be obtained by computing the quotient of the difference in the distance migrated between two consecutive time

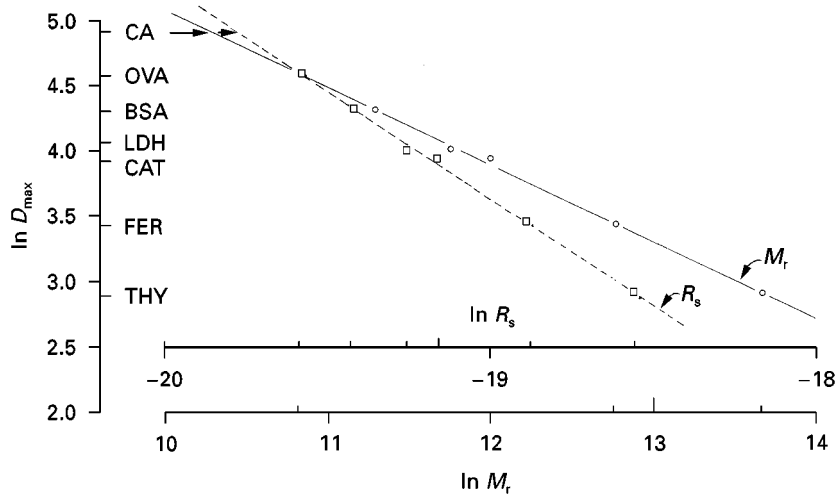

Figure 12 Calibration lines to calculate the molecular mass (*M*) and Stokes radius (R_S) of five carbonic anhydrase isoenzymes. The logarithm of the maximum migration distance (ln D_{max}) correlates linearly to the logarithm of the mol mass (ln M_r) and the logarithm of the Stokes radius (ln R_S), respectively. CA, Carbonic anhydrase (average ln D_{max} of isozymes I–V); OVA, ovalbumin; BSA; bovine serum albumin; LDH, lactate dehydrogenase; CAT, catalase; FER, ferritin; THY, thyroglobulin. The calculated mol masses and Stokes radii are listed in Tables 3 and 4.

Table 3 Calculated molecular mass of marker proteins and mammalian carbonic anhydrase (iso)enzymes and calculation of percentage of deviation of the calculated values from the literature

Protein	*Mol mass* (M_r (*g mol*$^{-1}$))[a]	*ln* M_r	Frictional *coefficient* (f/f_o)	*Calculated mol mass* (M_r)			
				ln M_r	M_r[b]	*Deviation*[c] (%)	ln D_{max}
Ovalbumin	43 000	10.6690	1.18	10.8234	50 181	+ 16.7	4.6563
Bovine serum albumin	67 000	11.1125	1.34	11.2981	80 668	+ 20.4	4.3537
L-lactate dehydrogenase	140 000	11.8494		11.7695	129 249	− 7.7	4.0532
Catalase	232 000	12.3545	1.27	12.0356	168 653	− 27.3	3.8836
Ferritin	440 000	12.9945	1.40	12.7717	352 110	− 20	3.4144
Thyroglobulin	669 000	13.4135		13.6949	886 379	+ 32	2.8259
Carbonic anhydrase							
Bovine I				10.5233	37 171		4.8476
Bovine II				10.4904	35 968		4.8686
Bovine/rabbit III	38 000			10.5373	37 695		4.8387
Rabbit IV				10.5775	39 241		4.8131
Canine/human V	29 700			10.5462	38 032		4.8330
Arithmetic mean				10.5346	37 594		4.8404

[a] Literature values.
[b] The molecular mass of bovine carbonic anhydrase as estimated by sequence analysis was reported to be 28 980 while that of the enzyme from mouse was found to be 29 068.
[c] The molecular sizes calculated are compared with the literature data and the percentage deviation indicated.
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intervals during electrophoresis, and the corresponding time difference:

$$v\ (\text{mm s}^{-1}) = (D_1 - D_0) \times (t_1 - t_0)^{-1}$$
$$v\ (\text{mm s}^{-1}) = (D_2 - D_1) \times (t_2 - t_1)^{-1}$$
$$v\ (\text{mm s}^{-1}) = (D_3 - D_2) \times (t_3 - t_2)^{-1}$$
$$\vdots$$
$$v\ (\text{mm s}^{-1}) = (D_Z - D_{Z-1}) \times (t_Z - t_{Z-1})^{-1}$$

Eqn [16] summarizes this procedure:

$$v\ (\text{mm s}^{-1}) = (D_n - D_m) \times (t_n - t_m)^{-1} = dD \times dt^{-1} \quad [16]$$

where D_n (mm) equals the migration distance of a protein at a time t_n (s) and D_m (mm) equals its migration distance at a time t_m (s) where $t_n > t_m$ (**Figure 13**).

Table 4 Calculated Stokes radius of marker proteins and mammalian carbonic anhydrase (iso)enzymes and calculation of percentage of deviation of calculated values from the literature

Protein	*Stokes radius* (R_S (*nm*))[a]	ln R_S	*Calculated Stokes radius* (R_S)			ln D_{max}
			ln R_S	R_S (*nm*)	*Percentage deviation*[b]	
Ovalbumin	3.05	− 19.6081	− 19.5992	3.08	+ 0.9	4.6563
Bovine serum albumin	3.55	− 19.4563	− 19.4285	3.65	+ 2.8	4.3537
L-Lactate dehydrogenase	4.20	− 19.2881	− 19.2590	4.32	+ 2.9	4.0532
Catalase	5.25	− 19.0650	− 19.1634	4.76	− 9.3	3.8836
Ferritin	6.10	− 18.9150	− 18.8987	6.20	+ 1.6	3.4144
Thyroglobulin	8.50	− 18.5832	− 18.5668	8.64	+ 1.7	2.8259
Carbonic anhydrase						
Bovine I			− 19.7070	2.76		4.8476
Bovine II			− 19.7189	2.73		4.8686
Bovine/rabbit III			− 19.7020	2.78		4.8387
Rabbit IV			− 19.6876	2.82		4.8131
Canine/human V			− 19.6988	2.78		4.8330
Arithmetic mean			− 19.7030	2.77		4.8404

[a] Literature values.
[b] The molecular sizes calculated are compared with the literature data and the percentage deviation indicated.
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Table 5 Marker proteins that can be used to estimate the native molecular size of proteins

Marker protein	M_r	R_S
Carbonic anhydrase	30 000	2.43
Ovalbumin	45 000	3.05
Bovine serum albumin	67 000	3.55
β-Galactosidase	116 000	4.23
Lactate dehydrogenase	140 000	4.20
Catalase	232 000	5.25
Ferritin	440 000	6.10
Thyroglobulin	669 000	8.50

M_r (Da), Molecular mass; R_S (nm), Stokes' radius of proteins. These markers can be taken when using PA gradients of 4–30% *T* and a buffer of pH 8.4 (45 mmol L^{-1} Tris, 40 mmol L^{-1} boric acid, 1.25 mmol L^{-1} EDTA–Na_2, pH 8.4).

Correlating Migration Velocities and Migration Distances

The migration velocities may be plotted against the corresponding migration distances at the end of each time interval to correlate migration velocities and migration distances (Figure 13). The function by which v and D are interrelated is best described by the following exponential equation:

$$v\ (\text{mm s}^{-1}) = \varepsilon(D_{max} - D)^{\delta} \qquad [17]$$

where ε, D_{max} and δ are constants, D (mm) is the independent variable and v (mm s^{-1}) the dependent variable. D_{max} represents the maximum migration distance which a protein can cover, i.e. the migration distance at which the migration velocity becomes zero. If this point is reached then $D_{max} = D$ and:

$$v\ (\text{mm s}^{-1}) = \varepsilon(D - D)^{\delta} = 0 \qquad [18]$$

Eqn [17] can be used to relate the apparent migration velocity (v) of a protein to the PA concentration (T (%)) that corresponds to the migration distance travelled during a given period of electrophoresis. When using a linear gel gradient, the PA concentration and the gel length are interrelated by eqn [19]:

$$D = \alpha^{-1}\ (T - \beta) \qquad [19]$$

whilst T_{max} (%), the stacking gel concentration, is related to the maximum distance D_{max} (mm) by eqn [20]:

$$D_{max} = \alpha^{-1}\ (T_{max} - \beta) \qquad [20]$$

Substituting eqns [19] and [20] into eqn [17] yields the formula:

$$v\ (\text{mm s}^{-1}) = \varepsilon[((T_{max} - \beta) \times \alpha^{-1}) - ((T - \beta) \times \alpha^{-1})]^{\delta} \qquad [21]$$

which can be arranged to:

$$v\ (\text{mm s}^{-1}) = \varepsilon \times \alpha^{-\delta} \times (T_{max} - T)^{\delta} \qquad [22]$$

and:

$$v\ (\text{mm s}^{-1}) = h \times (T_{max} - T)^{\delta} \qquad [23]$$

where $h = \varepsilon \times \alpha^{-\delta}$.

This derivation shows that, indeed, the apparent migration velocity of a protein (v) is related by the same function to the distance (D) as to the PA concentration (T) it has reached in a linear pore gradient, although the constants (ε and D_{max}, respectively, h and T_{max}) are different. The exponent δ in both equations, however, is the same.

Eqn [23] predicts that zero protein mobility ($v = 0$) results if the apparent gel concentration (T (%)) is equal to the stacking gel concentration (T_{max} (%)), i.e. if $T = T_{max}$. The apparent free electrophoretic mobility of a protein unhindered by the PA matrix (μ (mm s^{-1})), can be calculated by simply extrapolating its apparent mobility to zero T (%):

$$\mu\ (\text{mm s}^{-1}) = h \times (T_{max} - 0)^{\delta} \qquad [24]$$

thus:

$$\mu\ (\text{mm s}^{-1}) = h \times T_{max}^{\delta} \qquad [25]$$

This expression may be used to divide eqn [23] to yield eqns [26] and [27]:

$$v \times \mu^{-1} = (h \times (T_{max} - T)^{\delta}) \times (h \times T_{max}^{\delta})^{-1} \qquad [26]$$

which can be rewritten as:

$$v = \mu[1 - (T \times T_{max}^{-1})]^{\delta} \qquad [27]$$

The value of the quotient $(T_{max} - T) \times T_{max}^{-1}$ ranges from one ($T = 0$) to zero ($T = T_{max}$) and thus the value of v extends from the apparent free electrophoretic mobility (μ) to zero.

This means that, in a linear PA gradient, the apparent migration velocity (v) of a protein (migrating under a constant electrical field strength) is equal to its apparent free mobility (μ) times a retardation factor ($[1 - (T \times T_{max})^{-1}]^{\delta}$ which depends on the PA concentration (T) that the protein has just reached and its exclusion limit (T_{max}). This factor always takes

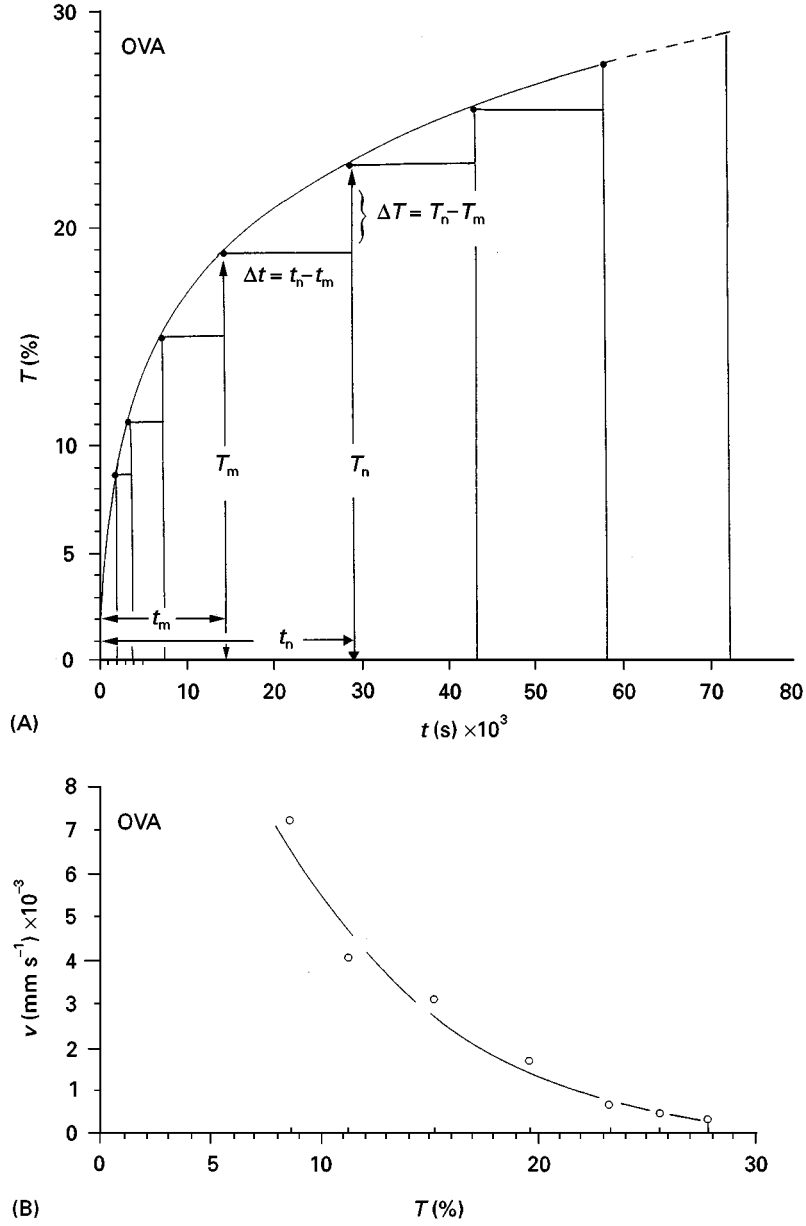

Figure 13 (A) Estimation of the migration velocity of a protein (OVA, ovalbumin) in a linear PA gradient gel. T_n, migration distance at a longer time of electrophoresis (t_n); T_m, migration distance at a shorter time of electrophoresis (t_m). (B) Plot of the resulting migration velocities (v (mm s^{-1}) versus the corresponding gel concentrations (T (%)) at the end of each time interval.

values between zero and one and increases exponentially with increasing gel concentrations.

In order to solve eqn [23] (v (mm s^{-1}) $= h \times (T_{max} - T)^{\delta}$), the following sequence of calculations is recommended:

1. determination of the maximum migration distance of the protein under investigation from a plot of ln (ln D) vs. $t^{-1/2}$ (eqn [5])
2. computation of the maximum gel concentration (T_{max}) by use of eqn [20] ($D_{max} = \alpha^{-1}(T_{max} - \beta)$);
3. calculation of the gel concentration equivalent to the migration distances with eqn [19] ($D = \alpha^{-1}(T - \beta)$), (the values of the constants α and β may be obtained from a gel scan at 405 nm if *p*-nitrophenol has been mixed into the more concentrated of the two solutions used to prepare the gradient gel);
4. then the values of ($T_{max} - T$) are calculated
5. finally the constants h and δ in eqn [23] are calculated by plotting ln v vs. ln ($T_{max} - T$) and performing a linear regression analysis with these data,

i.e. taking the logarithmized version of eqn [23]:

$$\ln \nu = \delta \times \ln(T_{max} - T) + \ln h \qquad [28]$$

Calculation of the Free Electrophoretic Mobility

The free electrophoretic mobility (U ($m^2\ V^{-1}\ s^{-1}$)) of a protein results from its apparent free electrophotetic mobility unhindered by the gel matrix (μ ($m\ s^{-1}$)) and the electric field strength E ($V\ m^{-1}$) acting on it:

$$U = \mu \times E^{-1}\ (m\ s^{-1}\ (V\ m^{-1})^{-1} = m^2\ V^{-1}\ s^{-1}) \qquad [29]$$

The apparent free electrophoretic mobility can be obtained by applying eqn [25] (μ ($mm\ s^{-1}$) = $h \times T_{max}^{\delta}$). The free electrophoretic mobilities of various marker proteins and five different mammalian carbonic anhydrases calculated by these procedures are listed in **Table 6**.

Computation of the Nett Charge

Estimation of the number of unit charges (Z) in a nondenatured protein requires prior knowledge of its Stokes radius (R_S) and its apparent free electrophoretic mobility (μ) or its free electrophoretic mobility (U). In addition to this, the ionic strength (I) and viscosity (η) of the buffer system used to estimate Z and R_S must be known. Time-dependent gradient gel electrophoresis can be used to determine the Stokes radius of a protein and its free electrophoretic mobility.

At a first approximation, the free electrophoretic mobility, unhindered by a gel matrix (U ($m^2\ V^{-1}\ s^{-1}$)), can be described by eqn [30]:

$$U = (Z \times \varepsilon) \times (6 \times \pi \times \eta \times R_S)^{-1}$$

$$(C\ (Pa\ s\ m)^{-1} = m^2\ (V\ s^{-1}) \qquad [30]$$

where Z is the number of unit charges (1); ε is the unit charge (protonic charge) = 1.602×10^{-19} (C); $\pi = 3.14\ldots$; η is the dynamic viscosity of the medium (Pa s); R_S is the Stokes radius (m) and the following coherences 1 C = 1 A s, 1 Pa = 1 N m^{-2}, 1 V A = 1 W and 1 W s = 1 N m.

Since migration of proteins is studied in buffered solutions, there are also positive and negative buffer ions present, in addition to the protein ions. The small ions of sign opposite to that of the protein, also called counterions, are present in excess and to be found in the vicinity of the protein molecules. The electric field which drives the protein molecules also acts on the counterions, but in the opposite direction and since the migrating counterions drag solvent along with them and the solvent in turn acts on the protein, the nett effect is a secondary force on the protein opposite in direction to the primary force. The migration velocity of the protein molecules towards the electric field may therefore be reduced well below that predicted by eqn [30], an effect known as the electrophoretic effect. This is why eqn [30] must be corrected by a retardation factor (F),

Table 6 Free electrophoretic mobility (U) and net negative charge (valence, Z; charge, Q) of several marker proteins and carbonic anhydrase (iso)enzymes from mammalia at pH 8.4

Protein	U (m^2 (V s)$^{-1} \times 10^{-9}$) $I = 0.529 \times 10^{3a}$ ($mol\,m^{-3}$)	*Negative charge*		
		$I = 0.1 \times 10^{3b}$ ($mol\,m^{-3}$)	Z	Q ($C\ molecule^{-1}$) $\times 10^{-19}$
Ovalbumin	3.45	5.99	13.06	20.92
Bovine serum albumin	4.40	7.85	22.42	35.92
Lactate dehydrogenase	3.27	6.00	22.63	35.25
Catalase	2.60	4.94	21.43	34.33
Ferritin	3.28	6.38	43.81	70.18
Thyroglobulin	2.78	5.62	68.46	109.67
CA I	1.58	2.69	4.93	7.90
CA II	1.17	1.99	3.58	5.74
CA III	1.05	1.79	3.32	5.32
CA IV	0.851	1.46	2.75	4.41
CA V	0.734	1.25	2.31	3.70

[a] Ionic strength of electrophoretic buffer system.

[b] Free electrophoretic mobility at ionic strength 0.1×10^3 (m^2 (V s)$^{-1}$).

CA, Carbonic anhydrase (iso)enzymes from mammalian erythrocytes: (bovine, I, II), bovine, rabbit (III), rabbit (IV) and canine, human (V). Conditions of electrophoresis: linear polyacrylamide gradient from 4 to 27% T (acrylamide–Bis = 24 : 1); gel length 73 mm; buffer system 90 mmol L^{-1} Tris; 80 mmol L^{-1} boric acid; 1.25 mmol L^{-1} EDTA–Na_2, pH 8.4 (I = 529 (mol m^{-3}); field strength: 41 V cm^{-1}; 4°C.

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the quantity of which depends on the composition and strength of the small ions of the buffer used. Henry proposed a method for computing this factor using the formula:

$$F = (X_1(\kappa \times R_S)) \times (1 + (\kappa \times R_S))^{-1} \qquad [31]$$

where X_1 is a function of $\kappa \times R_S$. Introducing this factor into eqn [30] yields eqn [32]:

$$U = (Z \times \varepsilon) \times (6 \times \pi \times \eta \times R_S)^{-1} \times (X_1(\kappa \times R_S)) \times (1 + (\kappa \times R_S))^{-1} \, (m^2 \, (V \, s^{-1})) \qquad [32]$$

The function $X_1(\kappa \times R_S)$ is complicated but always gives values between 1.0 and 1.5, as shown in **Figure 14**. According to Henry, three different equations must be used to compute the values of the function X_1. If $\kappa \times R_S > 24$ then the first of the three equations indicated in Figure 14 must be used. When $\kappa \times R_S \leqslant 5$ the last of the three equations in **Figure 14** is applied. In the range between the two border values 5 and 24, a linear equation is taken, which is also given in Figure 14. It is somewhat difficult to calculate the X_1 values when $\kappa \times R_S \leqslant 5$. Therefore, **Table 7** provides a number of values in the range of $\kappa \times R_S = 0.01$–5. Kappa (κ (m^{-1})) represents the reciprocal of the radius of the ion cloud, i.e. the radius of the cloud of counterions surrounding the protein. Depending on the ionic composition, ionic strength and temperature of the solution, κ acquires values ranging from zero to infinity, and at increasing ionic strengths the value of κ increases whilst the radius of the ionic cloud decreases and vice versa. In a salt-free solution, $\kappa = 0$ so that the electrophoretic mobility U is not influenced at all, whilst conversely it decreases permanently in solutions with increasing salt concentrations. The value of kappa can be obtained from the equation:

$$\kappa = [(2N_A \times \varepsilon^2) \times (D_0 \times D \times k \times T)^{-1}]^{1/2} \times I^{1/2}(m^{-1}) \qquad [33]$$

where $N_A = 6.025 \times 10^{23}$ (mol^{-1}); ε is the unit charge (protonic charge) $= 1.602 \times 10^{-19}$ (C); D_0 represents

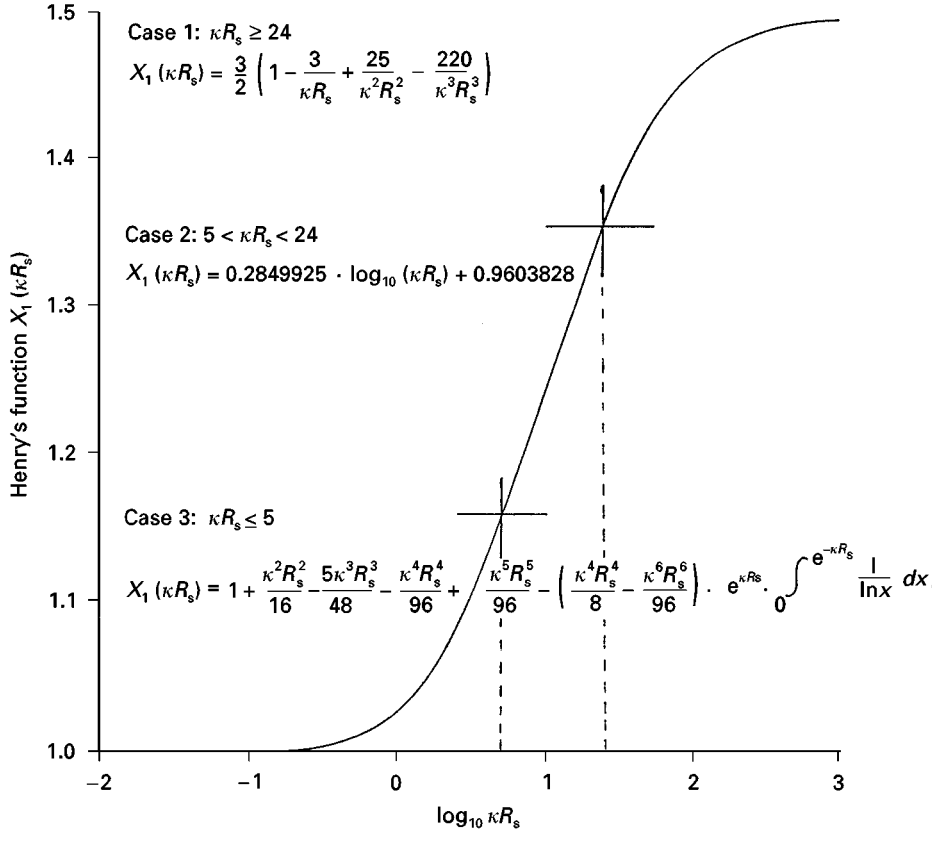

Figure 14 Graphical representation of Henry's function X_1 (κR_S). Depending on the value of κR_S three different equations must be used to compute the values of X_1. If $\kappa R_S > 24$ (case 1), the first of the three equations given is used. The second equation (case 2) comes into use if $5 \leq \kappa R_S \leq 24$ while the third equation (case 3) is applied if $\kappa R_S \leqslant 5$. In the latter case, Table 6 provides a number of values. Reproduced with permission from Rothe (1991).

Table 7 Values of Henry's function ($X_1(\kappa \times R_S)$) if $\kappa \times R_S < 5$ (cf. Figure 14)[a]

$\kappa \times R_S$	$\log_{10}(\kappa \times R_S)$	X_1 according to Overbeek's modification of Henry's equation	$\kappa \times R_S$	$\log_{10}(\kappa \times R_S)$	X_1 according to Overbeek's modification of Henry's equation
0.01	− 2	1.0000062	1.95	0.2900346	1.0632127
0.05	− 1.30103	1.0001452	2.00	0.30103	1.0651048
0.10	− 1	1.0005451	2.05	0.3117539	1.0669887
0.15	− 0.8239087	1.0011577	2.10	0.3222193	1.0688642
0.20	− 0.69897	1.001951	2.15	0.3324385	1.0707308
0.25	− 0.60206	1.0028994	2.20	0.3424227	1.0725882
0.30	− 0.5228787	1.003982	2.25	0.3521825	1.0744361
0.35	− 0.455932	1.005181	2.30	0.3617278	1.0762744
0.40	− 0.39794	1.0064817	2.35	0.3710679	1.0781027
0.45	− 0.3467875	1.0078712	2.40	0.3802112	1.0799208
0.50	− 0.30103	1.0093387	2.45	0.3891661	1.0817286
0.55	− 0.2596373	1.0108744	2.50	0.39794	1.0835259
0.60	− 0.2218487	1.0124701	2.55	0.4065402	1.0853126
0.65	− 0.1870866	1.0141185	2.60	0.4149733	1.0870886
0.70	− 0.154902	1.0158129	2.65	0.4232459	1.0888537
0.75	− 0.1249387	1.0175476	2.70	0.4313638	1.0906078
0.80	− 0.09691	1.0193175	2.75	0.4393327	1.0923509
0.85	− 0.0705811	1.0211181	2.80	0.447158	1.094083
0.90	− 0.0457575	1.0229452	2.85	0.4548449	1.0958039
0.95	− 0.0222764	1.0247952	2.90	0.462398	1.0975136
1.00	0.0	1.0266648	2.95	0.469822	1.0992121
1.05	0.0211893	1.028551	3.00	0.4771213	1.1008994
1.10	0.0413927	1.0304511	3.05	0.4842998	1.1025754
1.15	0.0606978	1.0323626	3.10	0.4913617	1.1042402
1.20	0.0791812	1.0342836	3.15	0.4983106	1.1058938
1.25	0.09691	1.0362118	3.20	0.50515	1.1075361
1.30	0.1139434	1.0381455	3.25	0.5118834	1.1091672
1.35	0.1303338	1.0400832	3.30	0.5185139	1.1107871
1.40	0.146128	1.0420233	3.35	0.5250448	1.1123958
1.45	0.161368	1.0439644	3.40	0.5314789	1.1139934
1.50	0.1760913	1.0459054	3.45	0.5378191	1.11558
1.55	0.1903317	1.0478451	3.50	0.544068	1.1171554
1.60	0.20412	1.0497825	3.55	0.5502284	1.1187199
1.65	0.2174839	1.0517167	3.60	0.5563025	1.1202734
1.70	0.2304489	1.0536469	3.65	0.5622929	1.1218159
1.75	0.243038	1.0555723	3.70	0.5682017	1.1233477
1.80	0.2552725	1.0574921	3.75	0.5740313	1.1248686
1.85	0.2671717	1.0594059	3.80	0.5797836	1.1263788
1.90	0.2787536	1.0613129	3.85	0.5854607	1.1278783
3.90	0.5910646	1.1293672	4.45	0.64836	1.1450647
3.95	0.5965971	1.1308456	4.50	0.6532125	1.1464318
4.00	0.60206	1.1323134	4.55	0.6580114	1.1477892
4.05	0.607455	1.1337709	4.60	0.6627578	1.1491371
4.10	0.6127839	1.135218	4.65	0.667453	1.1504754
4.15	0.6180481	1.1366549	4.70	0.6720979	1.1518043
4.20	0.6232493	1.1380816	4.75	0.6766936	1.1531238
4.25	0.6283889	1.1394981	4.80	0.6812412	1.1544341
4.30	0.6334685	1.1409047	4.85	0.6857417	1.1557352
4.35	0.6384893	1.1423012	4.90	0.6901961	1.1570272
4.40	0.6434527	1.1436879	4.95	0.6946052	1.1583101
			5.00	0.69897	1.159584

[a]Values were calculated using eqn [3] of Figure 14 (cf. Overbeek JTG (1950) *Advances in Colloid Science*, 3: 97–135). Tolerance of values: 10^{-6}, calculation of integral: 7 digits. Data from Rothe (1991).

the dielectric constant of vacuum $= 8.8542 \times 10^{-12}$ ($C\,V^{-1}\,m^{-1} = C^2\,N^{-1}\,m^{-2}$); D is the temperature-dependent dielectric constant of water (without dimension, cf. **Table 8**), k is Boltzmann's constant $= 1.3805 \times 10^{-23}$ ($J\,K^{-1} = N\,m\,K^{-1}$); T is absolute temperature (K) and I is the ionic strength (mol m^{-3}) of the buffer that was used for electrophoresis.

Table 8 Dielectric constant (D) of water depending on the temperature t (°C)

t (°C)	D	t (°C)	D
0	87.90	18	80.93
5	85.90	20	80.18
10	83.95	25	78.36
15	82.04	30	76.58

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By substituting these values into the equation one obtains:

$$\kappa = [(2 \times 6.025 \times 10^{23} \times (1.602 \times 10^{-19})^2) \times (8.8542 \times 10^{-12} \times 1.3805 \times 10^{-23})^{-1}]^{1/2} \,(\mathrm{K\,m\,(mol)^{-1}})^{1/2} \times (I\,(D\,T)^{-1})^{1/2} \,(\mathrm{mol\,m^{-3}\,K^{-1}})^{1/2} \quad [34]$$

thus:

$$\kappa = 1.590608013 \times 10^{10} \,(\mathrm{K\,m\,mol^{-1}})^{1/2} \times (I\,(D\,T)^{-1})^{1/2} \,(\mathrm{mol\,m^{-3}\,K^{-1}})^{1/2} \quad [35]$$

At a temperature of 5°C (278 K), the dielectric constant of water is 85.90 (cf. **Table 8**). Inserting both values into eqn [35] yields eqn [36]:

$$\kappa = 1.02930525 \times 10^{8} \,(\mathrm{m\,mol^{-1}})^{1/2} \times \sqrt{I} (\mathrm{mol\,m^{-3}})^{1/2} \quad [36]$$

The ionic strength I (mol m^{-3}) is calculated using the formula:

$$I = 1/2 \sum c_i Z_i^2 \,(\mathrm{mol\,m^{-3}}) \quad [37]$$

where $\sum c_i$ (mol m^{-3}) represents the concentrations of the ionic species of the buffer times their squared charges (Z_i).

Taking , for example, a 90 mmol L^{-1} Tris, 80 mmol L^{-1} boric acid, 1.25 mmol L^{-1} EDTA-Na_2 buffer of pH 8.0, the ionic strength of this buffer is:

$$I = 1/2 \sum c_i Z_i^2 \,(\mathrm{mol\,dm^{-3}}) \quad [38]$$

thus:

$$I = 1/2[(0.09 \times 1^2) + (3 \times 0.08 \times 1^2) + (0.08 \times (-3)^2) + (2 \times 0.00125 \times 1^2) + (0.00125 \times (-2)^2)] \quad [39]$$

which becomes:

$$I = 0.52875 \,(\mathrm{mol\,dm^{-3}}) \quad [40]$$

or:

$$I = 0.52875 \times 10^3 \,(\mathrm{mol\,m^{-3}}) \quad [41]$$

Substituting this value into eqn [36] gives:

$$\kappa = 1.02930525 \times 10^{8} \,(\mathrm{m\,mol^{-1}})^{1/2} \times (528.75)^{1/2} \,(\mathrm{mol\,m^{-3}})^{1/2} \quad [42]$$

which rearranges to:

$$\kappa = 2.366842604 \times 10^{9} \,(\mathrm{m^{-1}}) \quad [43]$$

Taking ferritin as an example, with a Stokes radius of 6.20×10^{-9} (m), then $\kappa \times R_S = 14.67$. The log of $\kappa \times R_S$ equals 1.167 and using this value one obtains from the equation in case 2 shown in **Figure 14**, a value of 1.293 for the function X_1 ($\kappa \times R_S$). Thus, inserting these values into eqn [31], it follows that:

$$F = (X_1(\kappa \times R_S)) \times (1 + (\kappa \times R_S))^{-1} = 1.293 \times (1 + 14.67)^{-1} = 0.08251 \quad [44]$$

To calculate the number of nett charges in ferritin, eqn [32] must be solved for Z:

$$Z = ((U \times 6 \times \pi \times \eta \times R_S) \times \varepsilon^{-1}) \times ((1 + (\kappa \times R_S)) \times (X_1(\kappa \times R_S))^{-1}) \quad [45]$$

From gradient gel electrophoresis results, the free electrophoretic mobility of ferritin was calculated as $U = 3.28 \times 10^{-9}$ (m^2 V^{-1} s^{-1}). Substituting this value, that of factor F and the value for the temperature-dependent dynamic viscosity (η (N s m^{-2})) of water as taken from **Table 9** into eqn [45], the number of unit charges that ferritin acquires under the electrophoretic conditions indicated above can be computed as:

$$Z = (3.28 \times 10^{-9} \times 6 \times \pi \times 1.519 \times 10^{-3} \times 6.20 \times 10^{-9}) \times (1.602 \times 10^{-19})^{-1} \times ((1 \times 0.08251)^{-1}) \quad [46]$$

which works out to:

$$Z = 44.05 \quad [47]$$

The actual charge on the molecule is given by $Z \times \varepsilon$ [C molecule^{-1}] $= 44.05 \times 1.602 \times 10^{-19} = 7.057 \times 10^{-18}$ (**Table 6**).

Table 9 Dynamic viscosity (η (N s m^{-2})) of water depending on the temperature (t (°C))

t (°C)	($N\,s\,m^{-2}$) 10^{-3}	t (°C)	($N\,s\,m^{-2}$) 10^{-3}
0	1.787	16	1.109
1	1.728	17	1.081
2	1.671	18	1.053
3	1.618	19	1.027
4	1.567	20	1.002
5	1.519	21	0.9779
6	1.472	22	0.9548
7	1.428	23	0.9325
8	1.386	24	0.9111
9	1.346	25	0.8904
10	1.307	26	0.8705
11	1.271	27	0.8513
12	1.235	28	0.8327
13	1.202	29	0.8148
14	1.169	30	0.7975
15	1.139		

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Evaluation of the Free Electrophoretic Mobility at an Ionic Strength of 0.1 mol L^{-1}

For reasons of comparability, the free electrophoretic mobility obtained for a given set of experimental conditions may be corrected to an effective mobility at an ionic strength of 0.1 mol L^{-1}. This can be achieved by substituting the relevant values into Abramson's equation:

$$U_{0.1} = (U_{>0.1}(\kappa_{>0.1} \times R_S + 2.4)) \times (\kappa_{0.1} \times R_S + 2.4)^{-1} \quad [48]$$

where $U_{0.1}$ and $U_{>0.1}$ (m^2 V^{-1} s^{-1}) represent the free electrophoretic mobilities at an ionic strength of 0.1 (mol m^{-3}) and > 0.1 (mol m^{-3}) respectively; $\kappa_{>0.1}$ and $\kappa_{0.1}$ represent the reciprocal of the effective thickness of the ionic cloud at ionic strength of 0.1 (mol m^{-3}) and > 0.1 (mol m^{-3}) respectively and R_S (m) is the Stokes radius of the protein.

For the experimental conditions given earlier:

$$\kappa_{0.1} = 1.02930525 \times 10^8\ (\mathrm{m\,mol^{-1}})^{1/2} \times (0.1 \times 10^3)^{1/2}\ (\mathrm{mol\,m^{-3}})^{1/2} \quad [49]$$

thus:

$$\kappa = 1.02930525 \times 10^9\ (\mathrm{m^{-1}}) \quad [50]$$

Taking ferritin as an example, for which U (m^2 V^{-1} s^{-1}) $= 3.28 \times 10^{-9}$ (at $I = 0.529 \times 10^3$ (mol m^{-3}), if $\kappa_{>0.1} = 2.366842604 \times 10^9$ and $R_S = 6.20 \times 10^{-9}$ (m) are determined and substituted into eqn [48], it follows that:

$$U_{0.1} = ((3.28 \times 10^{-9}) \times (2.366842604 \times 10^9 \times 6.20 \times 10^{-9} + 2.4)) \times (1.02930525 \times 10^9 \times 6.20 \times 10^{-9} + 2.4)^{-1} \quad [51]$$

thus:

$$U_{0.1} = 6.377 \times 10^{-9}\ (\mathrm{m^2\,V^{-1}\,s^{-1}}) \quad [52]$$

The free electrophoretic mobilities at $I = 0.52875 \times 10^3$ (mol m^{-3}) and at $I = 0.1 \times 10^3$ (mol m^{-3}) of several marker proteins and some carbonic anhydrase isozymes are listed in **Table 10**.

Table 10 Free electrophoretic mobility of ferritin in buffered solution

Experimental conditions	*Free mobility* U (m^2 V^{-1} s^{-1}) $\times 10^{-9}$	*Reference*
Moving boundary method $I = 0.1$ (mol L^{-1}), 0 (°C), pH 8.6	− 6.1	Mazur *et al.* (1950)
Agarose gel electrophoresis $I = 0.05$ (mol L^{-1}), + 20 (°C), pH 6.8	− 10.5	Gosh *et al.* (1974)
Disc electrophoresis $C = 2\%$; 0 (°C), pH 8.88		Rodbard *et al.* (1971)
$I = 0.0034$ (mol L^{-1})	− 10.97	
$I = 0.10$ (mol L^{-1})	− 5.67	
PA gradient gel electrophoresis 5–30 T (%), acrylamide–Bis = 24 : 1; + 4 (°C), pH 8.4[a]		Rothe (1991)
$I = 0.529$ (mol L^{-1})	− 3.28	
$I = 0.10$ (mol L^{-1})	− 6.38	

[a] Electrophoretic conditions; 90 mmol L^{-1} Tris, 80 mmol L^{-1} boric acid, 1.25 mmol L^{-1} EDTA–Na_2, pH 8.4; separation distance 73 mm, voltage gradient 41.1 (V cm^{-1})
References are given in Rothe (1991).

The result of calculating the net protonic charge of a protein of course remains unaffected whether the ionic strength of the experiment or that of a buffer strength of 0.1 mol L^{-1} is used.

Comprehensive Equation Describing Electrophoretic Mobility of Proteins Migrating in a Linear PA Gradient Gel

As explained above, the velocity with which a protein migrates in a linear PA gradient gel depends on its apparent free electrophoretic mobility times a retardation factor (eqn [27]):

$$v = \mu \times [1 - (T \times T_{max}^{-1})]^{\delta} \ (m\ s^{-1}) \qquad [27]$$

where v is the migration velocity ($m\ s^{-1}$), T (%) is the PA concentration which the migrating protein of mobility v has reached and T_{max} (%) represents the exclusion limit of the migrating protein.

Since $U = \mu \times E^{-1}$ ($m^2\ V^{-1}\ s^{-1}$), it follows that:

$$v \times E^{-1} = U \times [1 - (T \times T_{max}^{-1})]^{\delta} \ (m\ V^{-1}\ s^{-1}) \quad [53]$$

U is defined by eqn [32] as equivalent to:

$$U = (Z \times \varepsilon) \times (6 \times \pi \times \eta \times R_S)^{-1} \times (X_1(\kappa \times R_S)) \times (1 + (\kappa \times R_S))^{-1} \ (m^2\ (V\ s)^{-1})$$

with the definitions given above.

Using all this information, a complete description of the electrophoretic mobility of proteins migrating in a linear PA gradient gel can then be given by the equation:

$$v \times E^{-1} = (Z \times \varepsilon) \times (6 \times \pi \times \eta \times R_S)^{-1} \times (X_1(\kappa \times R_S)) \times (1 + (\kappa \times R_S))^{-1} \times [1 - (T \times T_{max}^{-1})]^{\delta} \ (m^2\ (V\ s)^{-1}) \qquad [54]$$

with the definitions as given above.

Sodium Dodecyl sulfate Porosity Gradient Gel Electrophoresis

Polyacrylamide gradient gels also offer greater possibilities for the electrophoretic separation of proteins in the presence of SDS. Porosity gradient gels have a much higher resolving capacity, for example the two chains of haemoglobin of $M_r = 15\ 126$ and 15 866 Da, respectively, can be clearly separated in a 3–30% T gradient gel. An 8% T continuous PA–SDS gel does not exhibit this resolving capacity. In SDS porosity gradient gel electrophoresis the use of continuous buffer systems is recommended (**Table 11**) since partial deloading of SDS–protein complexes has been observed when the gel contained SDS but not the electrode buffer. This results in a confusing multitude of bands.

Estimation of Molecular Mass of Denatured Proteins and Small Peptides

When SDS electrophoresis is performed in a linear PA gradient gel of 3–30% T, a linear relationship can be set up between the logarithm of the mol mass ($\log M_r$) and the log of the PA concentration ($\log T$) reached by proteins after a certain time of electrophoresis. The validity of the corresponding relationship $\log M_r = -a \times \log T + b$ has been confirmed with some 40 proteins between 14 and 950 kDa. In PA gradient gels in the presence of SDS the molar mass of both unreduced and 2-mercaptoethanol-reduced proteins as well as the molar mass of glycoproteins can be determined with the same accuracy ($\pm$ 5%, **Table 12**). Ribonuclease and lysozyme binding normal amounts of SDS migrate anomalously in homogeneous SDS gels but not in SDS PA gradient gels. Papain and pepsin, which also bind only traces of SDS, migrate regularly in SDS PA gradient gels.

The migration distance of proteins in linear SDS PA gradient gels and their respective mol mass can also be correlated by the equation:

$$\log M_r = -a \times \sqrt{D} + b \qquad [55]$$

where D (mm) is the migration distance. This relationship can be applied to SDS-complexed and reduced and to SDS-complexed nonreduced proteins, to glycoproteins and to carbohydrate-free proteins (**Figure 15**). The relationship is not affected by the buffer system, the concentration of the cross-linker within 1–8% C or the concentration range of the gradient within 3–30% T at the commonly used gel length of 8–15 cm. The value of the constants a and b, on the other hand, are changed when the experimental parameters are altered. If SDS electrophoresis is performed in a linear gradient gel of approximately 6–27% T, the relationship $\log M_r = -a \times \sqrt{D} + b$ is practically independent of the time of electrophoresis. This means that the molecular mass estimation can be made when the best resolution of a set of proteins has been obtained. It is not necessary to wait until the proteins have reached their exclusion pore size. On the contrary, under prolonged electrophoresis protein–SDS complexes can reach a pore size where the complexing SDS is stripped off the protein molecules which leads to erroneous banding patterns. This is particularly

Table 11 Gel and buffer systems used in SDS PA gradient gel electrophoresis to separate denatured proteins

PA range (%*T*) (*acrylamide–Bis*)	*Gel shape* (*dimensions* (mm))	*Buffer systems*		*Current or voltage per gel*	*Running time (h)*	*Correlation* (M_r *range* (kDa))	*Notes*	*Authors*
		Gel buffer	*Electrode buffer*					
3–30 (30 : 0.8)	Column (150 × 6)	0.1 mol L^{-1} Na–phosphate, 0.1% SDS, 5–15% (v/v) glycerol, pH 7.0	0.1 mol L^{-1} Na–phosphate, 0.1% SDS, pH 7.0	4 mA	24	— (12–125)	*a*	Exposito and Obijeski (1976)
3–30 (9.62 : 0.38)	Slab gel (length: 80)	10.75 g Tris, 5.04 g boric acid 0.93 g EDTA–Na_2, pH 7.2	0.01 mol L^{-1} Na-phosphate, 1% SDS, 1% 2-mercaptoethanol, pH 7.2	40 V	16	log M_r vs. log T (13–950)	*b*	Lambin *et al.* (1976), Lambin (1978)
1.5–40 (12.57 : 1)	Microcolumn i.d. 0.43, length 15	0.1 mol L^{-1} Na–phosphate, pH 7.2, 0.1% SDS or 0.35 mol L^{-1} Tris-sulfate, 0.1% SDS, pH 8.5, or 0.05 mol L^{-1} Tris–glycine, 0.1% SDS, pH 8.4, or 0.065 mol L^{-1} Tris–borate, 0.1% SDS, pH 9.3	29 g glycine plus Tris to pH 8.4, 1 g SDS, H_2O to 1000 mL	60 V	2	log M_r vs. R_F (13–300)	*c*	Rüchel *et al.* (1974)
1.5–40 (12.57 : 1)	Microcolumn (i.d. 0.43, length 15)	4 g Tris and H_2SO_4 to pH 8.4, H_2O to 10 mL	29 g glycine plus Tris to pH 8.4, 1 g SDS, H_2O to 1000 mL	60 V	0.33	log M_r vs. R_F (13–300)	*c*	Rüchel *et al.* (1974)
3–30 (28 : 1)	Slab gel (width 80, length 80, thickness 1)	0.04 mol L^{-1} Tris, 0.02 mol L^{-1} Na–acetate, 0.02 mol L^{-1} Na–EDTA, pH 7.4, 0.2% SDS	Same as gel buffer	150 V	0.5–8	log M_r vs. $\sqrt{D}$ (13–950)	*d*	Rothe (1982)

[a]Gels were stored at room temperature before use in a solution which contained 0.1 mol L^{-1} Na–phosphate, 0.01% SDS, 15% glycerol, 2 mmol L^{-1} EDTA–Na_2 and 0.01% NaN_3. Samples were dissolved at 100°C for 3 min in 0.01 phosphate buffer, pH 7, containing 2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.005% Bromophenol blue. On each column 20–100 μg protein was loaded.

[b] *T*(%) g acrylamide plus g Bis per 100 mL solvent. Protein samples (0.5 mg mL^{-1}) were incubated in 0.01 mol L^{-1} phosphate buffer, containing 1% SDS, pH 7.2 for 3 min in a 100°C bath; for cleavage of disulfide bridges 1% 2-mercaptoethanol was added. The % *T* concentration reached by each protein after electrophoresis was determined and log *T* plotted versus log *mol mass*.

[c]Resolution was found to be better in discontinuous than in continuous buffer systems. Samples (1 mg protein mL^{-1}) were treated for 2 min at 100°C with 1% SDS and 1% 2-mercaptoethanol in 0.035 mol L^{-1} Tris–sulfate, pH 8.6, 0.35 mol L^{-1} Tris–sulfate, pH 8.6 or 0.1 mol L^{-1} phosphate. Complete removal of SDS from proteins can be achieved with SDS-free electrode buffers. The activity of β-galactosidase denatured with SDS and separated on an SDS-free PA gradient gel could be restored to 10%.

[d]$\sqrt{D}$, square root of migration distance (*D* (mm)). Re-evaluation of the data from Lambin (1978), Lasky (1978) and Poduslo and Rodbard (1980) confirmed the validity of the log $M_r - \sqrt{D}$ relationship, found when evaluating time-dependent SDS-porosity gradient gel electrophoresis using marker proteins in the range of 10–330 kDa.

References as given in Rothe and Maurer (1986). Reproduced with permission from Rothe and Maurer (1986).

true when in an alkaline buffer system the upper electrode buffer contains no SDS.

In SDS electrophoresis with linear PA gradients ranging from 3 to 30% *T*, polypeptides in the range of 1.4–10 kDa cannot be resolved. Separation is possible, however, in 10–18% *T* gels in the presence of 0.1% SDS and 7 mol L^{-1} urea (cf. **Tables 13** and **14**).

Table 12 Separation characteristics of some proteins in SDS PA gel electrophoresis and deviation of calculated mol masses from those given in the literature

No.	*Protein*	M_r (*Da*) (*literature value*)	3–30% *T*, $C = 8.4\%$[a]				3–30% *T*, $C = 3.8\%$[b]			
			D (mm)	M_r[c] ± %	T (%)	M_r[d] ± %	D (mm)	M_r[c] ± %	T (%)	M_r[d] ± %
1	Prealbumin	13745	51.5	− 0.4	20.7	− 4.0	56	− 1.7	22.4	− 6.8
2	Lysozyme	14314	53.5	− 16.5	21.4	− 20.4	51.5	+ 16.9	20.8	+ 13.6
3	Ribonuclease B	14700	52	− 10.0	20.9	− 14.0	55.5	− 6.0	22.2	− 10.3
4	Haemoglobin	15500	51	− 8.6	20.5	− 11.2	55	− 8.7	22	− 12.3
5	Avidin	16000	49.5	− 1.7	20	− 4.2	51	+ 7.1	20.6	+ 4.8
6	Soybean trypsin inhibitor	20095	47	− 6.6	19.2	− 9.2	50	+ 10.4	20.3	− 12.7
7	Papain	23426	44.5	− 4.0	18.3	− 4.8	48	+ 15.1	19.6	− 16.5
8	α-chain of IgG	23500								
9	Chymotrypsinogen A	25666	43.5	− 5.6	18	− 7.0	44	+ 4.8	18.2	− 4.8
10	Carbonic anhydrase B	28739	41	+ 1.8	17.1	+ 2.2	42	+ 5.5	17.5	− 4.7
11	Carboxypeptidase A	34409	40	− 8.1	16.7	− 6.3	39.5	− 9.5	16.7	− 9.0
12	Pepsin	34700	37.5	+ 11.0	15.9	+ 12.5	37.5	+ 0.4	16	+ 1.8
13	Glycerol-3-phosphate dehydrogenase	35700	37.5	+ 7.9	15.9	+ 9.4	36	+ 6.4	15.5	+ 7.9
14	Lactate dehydrogenase	36180	37.5	+ 6.4	15.9	+ 7.9	37	− 1.0	15.8	+ 1.0
15	Aldolase	38994	36	+ 11.5	15.4	+ 13.1	35	+ 3.2	15.1	+ 6.0
16	Alcohol dehydrogenase	39805	35.5	+ 13.8	15.2	+ 16.4	34.5	+ 4.2	14.9	+ 7.7
17	α_1-Acid glycoprotein	40000	35	+ 18.0	15	+ 21.7	32	+ 20.6	14.1	+ 23.9
18	Ovalbumin	43000	35.5	+ 5.4	15.2	+ 7.8	35.5	− 9.1	15.3	− 7.2
19	Fibrinogen γ chain	47000								
20	Glutamate oxalacetate transaminase	50000	32.5	+ 16.7	14.2	+ 19.2	29	+ 16.6	13	+ 21.9
21	Heavy chain IgG	50000								
22	Fibrinogen β chain	56000								
23	Catalase	57500	32	+ 5.9	14	+ 9.1	29	+ 1.4	13	+ 6.0
24	Fibrinogen α chain	63500								
25	Albumin monomer	66290	31.5	+ 10.7	13.8	+ 14.9	28	+ 8.2	12.7	− 12.3
26	Heavy chain IgM	72000								
27	Transferrin	76000	30.5	− 8.6	13.5	− 6.0	24	+ 7.6	11.3	+ 12.4
28	Plasminogen	81000	29	− 1.8	13	+ 0.7	23	+ 8.5	11	+ 12.2
29	Phosphorylase b	96800	25.5	+ 14.2	11.8	+ 17.1	21	+ 5.3	10.3	+ 8.9
30	Ceruloplasmin	124000	23.5	+ 8.8	11.1	+ 11.6	17	+ 13.2	8.9	+ 16.2
31	Albumin, dimer	132580	24	− 3.3	11.2	+ 1.4	17.5	+ 1.6	9	+ 6.2
32	Immunoglobulin G	150000	21.5	+ 10.6	10.4	+ 13.4	15	+ 11.4	8.2	+ 13.4
33	Immunoglobulin A	160000	22	+ 1.6	10.6	+ 0.1	14	+ 14.5	7.8	+ 17.2
34	Reduced α_2-macroglobulin	190000								
35	Albumin, trimer	198870	19.75	+ 0.8	9.8	+ 2.6	12	+ 11.8	7.1	+ 12.6
36	Immunoglobulin A	320000	16	− 3.1	8.5	− 3.5	9	− 4.0	6.1	− 8.4
37	Thyroglobulin	330000	16.5	− 11.6	8.7	− 12.4	11	− 25.4	6.8	− 26.6
38	Fibrinogen	340000	15	+ 3.3	8.2	+ 0.4	8.5	− 4.2	5.9	− 8.8
39	α_2-Macroglobulin	380000	15.5	− 13.2	8.3	− 13.1	8	− 9.0	5.8	− 16.0
40	Immunoglobulin A, trimer	480000	13	− 4.8	7.5	− 9.5	6.5	− 12.4	5.2	− 20.6
41	Immunoglobulin M	950000	8.75	− 9.1	6	− 20.1				
	Average % deviation			± 7.8		± 9.6		± 8.7		± 11.2
	Lambin (1978)					± 5.9				± 7.4

[a] The gel buffer contained no 2-mercaptoethanol (gel length 78.5 mm).
[b] Gel buffer with 2-mercaptoethanol (gel length 81 mm), gel and electrode buffer as well as conditions of electrophoresis as given in Table 8. M_r (Da), mol mass; *D* (mm), migration distance; *T* (%), g acrylamide plus g Bis per 100 mL, as reached by a protein.
[c] M_r ± %, %-deviation of calculated mol mass from the literature value using the relationship $\log M_r = a \times \sqrt{D} + b$.
[d] M_r ± %, %-deviation of calculated mol mass from the literature value using the relationship $\log M_r = a \times \sqrt{T} + b$.
Reproduced with permission from Rothe and Maurer (1986).

Separation of Urinary Proteins and Diagnosis of Proteinurias

Diagnosis of pathological urinary profiles and estimation of the molecular size of the corresponding proteins is possible by SDS PA gradient gel electrophoresis under nonreducing conditions. Protein patterns may be estimated in micro-sized ($43 \times 50 \times 0.45$ mm) SDS gradient gels of 8–25% *T* fixed to a plastic backing (GelBond™) as they are

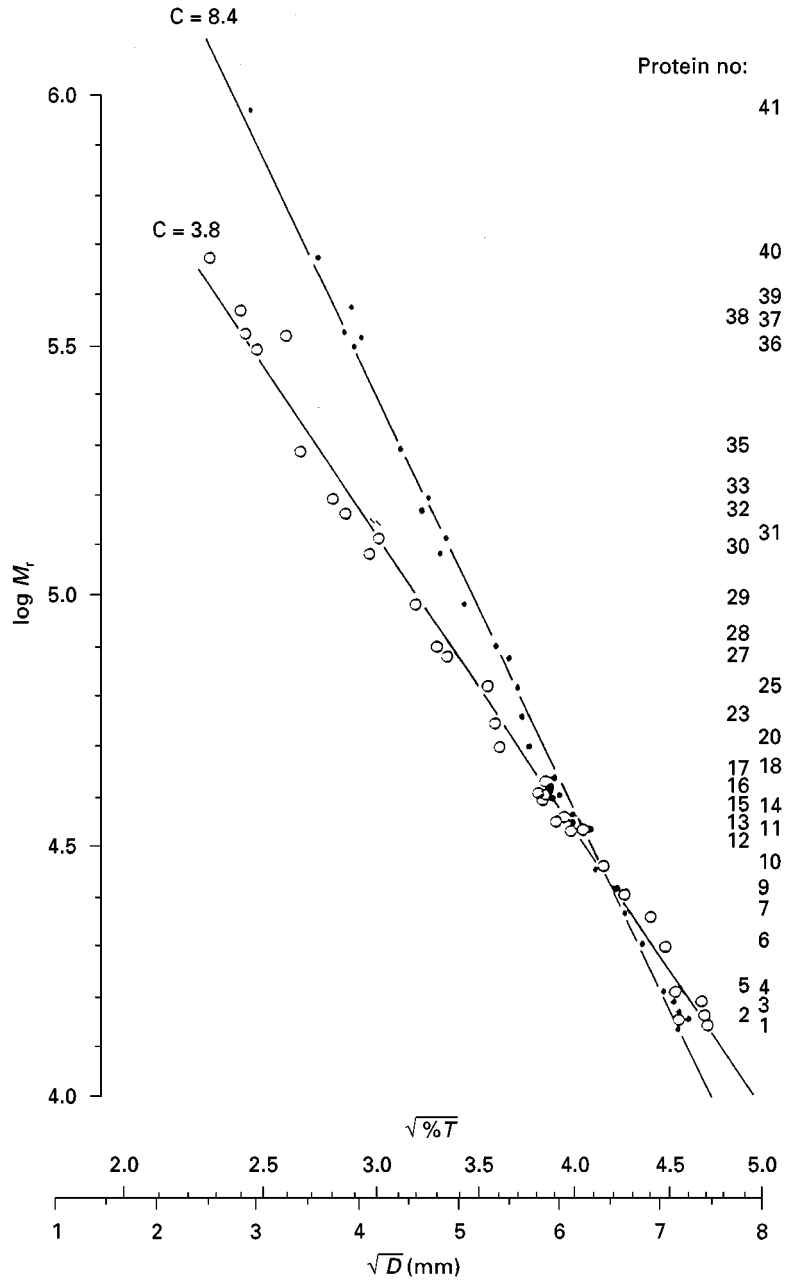

Figure 15 Migration distances of denatured proteins and protein subunits obtained by SDS PA gradient gel electrophoresis. The logarithm of the molecular mass of proteins (log M_r) is linearly correlated to the square root of the PA concentration ($\sqrt{\%\ T}$) which they reached upon electrophoresis. Also, log M_r is linearly correlated to the square root of the migration distance ($\sqrt{D}$ (D (mm)) which proteins reached upon electrophoresis. Reproduced with permission from Rothe (1994).

commercially available together with a suitable horizontal electrophoretic apparatus (Phast system) and an automated silver staining device (Amersham Pharmacia Biotech). The method has the advantage that urine samples need not be concentrated or desalted before electrophoresis. Samples may be stored frozen at $-20^{\circ}C$ after addition of sodium azide and after particulate removal by centrifugation. Samples with protein concentrations above 0.30 mg mL^{-1} must be diluted. Proteins must not be reduced (e.g. with 2-mercaptoethanol) since under SDS and nonreducing conditions the quarternary structure of all major serum proteins excreted in urine is unaffected, except haemoglobin which is split into its monomers and dimers. **Figure 16** shows some selected protein patterns of renal malfunctions.

Table 13 Gel and buffer system used to separate small peptides in SDS PA gradient gel electrophoresis

Acrylamide (g 100 mL^{-1})	*Bis* (g 100 mL^{-1})	*Gel buffer* (*pH*)	*Electrode buffer* (*pH*)	*Correlation* (M_r (Da) *range*)	*Authors*
10–18	0.5–0.9	Stacking gel: 5% acrylamide, 0.13% Bis, 0.067 mol L^{-1} Tris–HCl, pH 6.8, 0.1% SDS, 0.067% ammonium persulfate and 0.067% TEMED; separation gel: 0.45 mol L^{-1} Tris–HCl, pH 6.9, 0.1% SDS, 0.05% ammonium persulfate, 0.05% TEMED, 7 mol L^{-1} urea	0.05 mol L^{-1} Tris, 0.38 mol L^{-1} glycine, 0.1% SDS, pH 8.5	log M_r vs. D (1400–17 000)	Hashimoto *et al.* (1983), Laemmli (1970)

M_r (Da), mol mass; D (mm), migration distance; TEMED, *N,N,N′,N′*-tetramethylethylenediamine. The buffer solution containing 10% acrylamide (0.5% Bis) contains no sucrose while the buffer solution containing 18% acrylamide (0.9% Bis) contains 10% (w/v) of sucrose. The PAA concentration and the sucrose concentration increase linearly from top to bottom. The system can also be used to separate lipopolysaccharides and phospholipids. The addition of iodoacetamide to samples prior to electrophoresis eliminated artifacts currently observed in silver staining of protein bands. Log M_r correlates linearly with migration distance (D (mm)) in the mon mass range of 1.4 (kDa) to 17 (kDa). Flat gels of the dimensions 150 × 140 (height) × 1 (mm) were used. Gels were run for at least 15 h at 120 V. Samples were heated for 2 min at 100°C in a sample buffer containing 10% sucrose, 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10 mM dithiothreitol and 0.0025% Bromophenol blue (if necessary they were treated with iodoacetamide). Reproduced with permission from Rothe and Maurer (1986).

Table 14 Mol masses of polypeptides and peptides employed for urea–SDS gel electrophoresis

Protein	*Mol mass* (*Da*)	
	Literature value[a]	*Computed*[b]
Ovalbumin	46 000	
Carboxypeptidase A	34 500	*c*
Myoglobin	17 200	
Myoglobin I + II	14 900	
Cytochrome *c*	12 300	*c*
Myoglobin I	8270	
Cytochrome *c* I	7760	
Myoglobin II	6420	
Bovine trypsin inhibitor	6160	*c*
Adrenocorticotrophic hormone	4550	6500
Insulin	5700	
Insulin B chain	3400	
Insulin A chain	2300	
Glucagon	3460	1800
Cytochrome *c* II	2780	
Myoglobin III	2550	
Cytochrome *c* III	1810	
Bacitracin	1400	
Polymyxin B	1225	2200

[a] Values as cited by Swank and Munkres (1971).
[b] Values calculated by Swank and Munkres (1971) using least-squares regression analysis and assuming a linear correlation between log M_r (M_r, mol mass (Da)) and migration distance D (mm).
[c] The mol masses of these proteins also deviate considerably if a straight line in a log M_r vs. D plot is drawn through the points of carboxypeptidase A and bacitracin.
References as given in Rothe and Maurer (1986). Reproduced with permission from Rothe and Maurer (1986).

Diagnosis of the following proteinurias is possible:

1. Proteinuria in the normal range of total protein
2. Orthostatic (postural) proteinuria
3. Post-renal proteinurias
   (a) Post-renal haematuria
   (b) Local excretion of proteins
4. Bence–Jones proteinuria
5. Lower and upper urinary tract infections: cystitis and pyelonephritis
6. Diabetes mellitus

Concluding Remarks

Gradient gel electrophoresis has may advantages over conventional gel electrophoresis. Gradient range and course can be adapted to any individual separation problem, and protein bands are much sharper than in Cellogel or starch gel electrophoresis. So far, for example, more than 20 enzyme bands of an enzyme system such as plant acid phosphatase have been clearly resolved and genetically interpreted, and crude enzyme extracts can be used as the enzyme source, provided a specific detection (staining) system is available. The disadvantages are few compared to conventional gel electrophoresis, such as availability of gradient gel, a load of two to three times more enzyme activity per cm^2 of gel cross-section as compared to starch gels, and the exclusive migration of proteins towards the cathode (anode), whereas in Cellogel and starch gel electrophoresis both cathodically and anodically migrating proteins can be detected within the matrix.

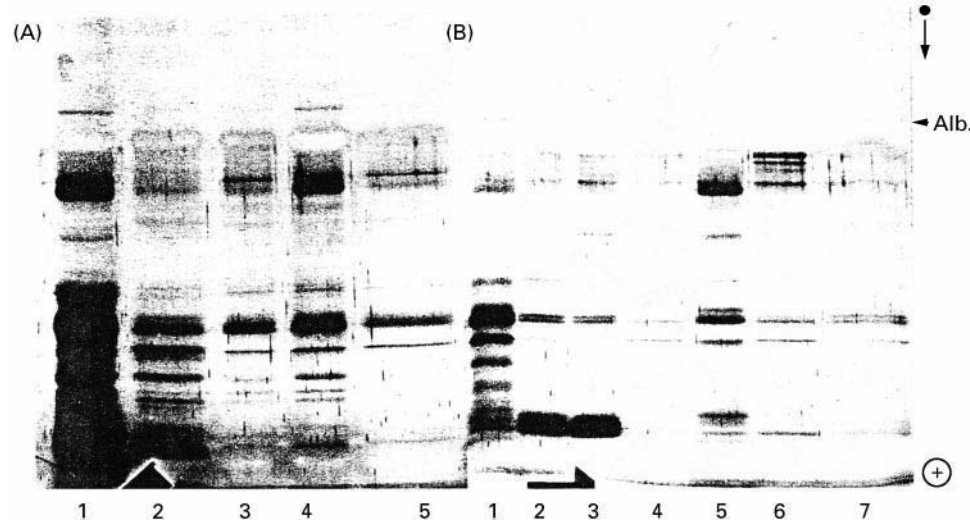

Figure 16 Separation of urinary proteins by macro SDS PA gradient gel electrophoresis. Gel: 4–20% *T*. Running conditions: 3 h at 350 V, 50 mA. Samples: up to 50 μL urine. (A) and (B) show urine samples from paediatric patients with pyelonephritis at various stages of follow-up. Series A: 1, acute phase: 2, and 3, urine taken at weekly intervals; 4, reinfection (acute phase); 5, 1 week follow-up. Series B: 1, acute phase; 2–4, follow-up at weekly intervals (note blood contamination in 2 and 3, α- and β-globin chains at 16 kDa), 5, reinfection, 6, and 7, weekly follow-up. Black dot and vertical arrow on the right side of the gel represent the application point and migration direction, respectively. + Gel polarity. Alb., albumin. Reproduced with permission from Bianchi-Bosisio *et al.* (1991).

In addition to pure PA gels, matrices of mixed polymers can be used for porosity gradients. However, this possibility has been rarely used, although it could extend the separation possibilities.

PA gradients are widely used to determine the molecular mass of SDS-denatured proteins, because this method offers a larger separation range and a much better resolution of protein bands. However, native, time-dependent PA gradient gel electrophoresis has much more possibilities to offer, such as differentiation between size and charge isomers, determination of the molecular size of native proteins and (iso)enzymes (M_r, R_S), estimation of the molecular excentricity (f/f_o), and calculation of the net negative charge at a given pH value. Using these possibilities the evolution of homologous proteins in related animal and plant species can be studied as well as the net charge of isozymes from different cells compartments.

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Proteins, Detection of

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Introduction

After polyacrylamide gel electrophoresis, it is essential that separated protein zones be detected for subsequent analysis, whether this is to be done by simple visual inspection or by quantitative computerized densitometry. In the early days of electrophoresis, methods for the detection of separated zones (ultraviolet absorption, Schlieren optics) were limited and insensitive. The subsequent development of organic dyes able to react with proteins made stains such as Bromophenol Blue and Amido Black 10B popular. In particular, Coomassie Brilliant Blue was for many years the method of choice for protein detection following gel electrophoresis owing to its relatively high sensitivity. However, the need for increased sensitivity resulted in the development of a group of staining methods based on the use of silver (approximately 0.1 ng of protein per band). Recently, there has been a renewed interest in the use of fluorescent methods of protein detection as they provide high sensitivity equivalent to silver staining combined with excellent linearity and extended dynamic range. Detection methods based on the use of radiolabelling also provide high sensitivity but cannot be applied in all situations. Finally, methods are available for the detection of groups of proteins with specific post-translational modifications, for example glycoproteins, phosphoproteins and lipoproteins.

Fixation

After electrophoresis is complete, the gel is removed from the apparatus for localization of the separated zones. Procedures have been described for the direct visualization of unfixed proteins within gels. However, for the majority of protein detection methods it is necessary to precipitate and immobilize (i.e. 'fix') the separated proteins within the gel and to remove any nonprotein components which might interfere with subsequent staining. Gels that are to be used for visualization of enzymatic activity of the separated proteins must not be fixed. The best general purpose fixative is 20% w/v trichloroacetic acid (TCA) as it gives effective precipitation of most proteins. Acid methanol (or ethanol), typically a solution containing 10% v/v acetic acid, 45% v/v methanol, and 45% deionized water, is often used for gel fixation, but it should be noted that this can be ineffective for small proteins, basic proteins and glycoproteins. Aqueous solutions of reagents such as 5% w/v formaldehyde or 2% w/v glutaraldehyde can be used to cross-link proteins covalently to the gel matrix, but this is not a commonly used approach.

Coomassie Brilliant Blue

The most popular general protein-staining procedures following gel electrophoresis are based on the use of the non-polar, sulfated triphenylmethane Coomassie stains, developed for the textile industry, Coomassie Brilliant Blue (CBB) R-250 is most often used and requires an acidic medium for electrostatic interaction between the dye molecules and the amino groups of proteins. Staining is usually carried out using 0.1% w/v CBB R-250 in the same acid methanol solution used for fixation (10% acetic acid, 45% methanol). Depending on gel thickness and polyacrylamide concentration, staining can take from 30 min to several hours. In practice, it is often convenient to stain the gel overnight and then destain it by several changes in the same acid methanol solution until intense blue protein zones can be seen against a clear background. This method is able to detect a minimum of around 100 ng protein per band (**Figure 1**), so that for complex mixtures containing several hundred components, it is necessary to load relatively high amounts of total protein ($> 50\ \mu g$).