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Micromethods in Molecular Biology

With Contributions by

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With 275 Figures

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Preface

This book is based on practical experience and is therefore written as a practical manual. The fore-runners of the book were the manuals of the first and second EMBO-Courses on "Micromethods in Molecular Biology" which were held in Göttingen in the spring of 1970 and the autumn of 1971. This book may serve as a manual not only for the participants of the third EMBO-Course to be held in Göttingen in autumn 1973, but also for all experimenters who are interested in using micromethods. It must be emphasized from the outset that this book is conceived as a "cook book" and not as a monograph which attempts to revue the literature on micromethods critically.

The methods described here in detail are performed routinely in the authors' laboratories and include all the practical details necessary for the successful application of the micromethods. There are many other sensitive and excellent micromethods which are not included in this book, because the authors feel that in a "cook book" only methods for which they have personal experience and proficiency should be described. Some readers may feel that the title promises more than the present contents of this book; however, if sufficient interest is shown in this volume, it may be possible to remedy such deficiencies in future editions.

In general, micromethods are no more arduous than the equivalent method on the macro scale, and the saving in time is usually considerable. For instance, sometimes a procedure in the macro scale takes hours, and on the micro scale only minutes, yet the amount of information obtained is the same. Thus it is often advantageous to use micro methods even when there is sufficient material available for macro scale analysis.

Many existing macro scale methods can be made a hundred- or thousand-fold more sensitive by simply scaling down the dimensions of the analytical medium in use, but in some cases it may be necessary to change the conditions of separation when adapting a method. However, in changing from the normal to the micro scale, the biggest barrier is often the sceptism of the experimenter about any method which has been reduced to a micro scale. Once this has been overcome it is fascinating to see what possibilities exist for micro methods in one's own field of research.

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V. NEUHOFF

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Chapter 1

Micro-Electrophoresis on Polyacrylamide Gels

Polyacrylamide gels were introduced in 1959 by RAYMOND and WEINTRAUB, as supports for electrophoretic separations. The polyacrylamide gel is produced by polymerising acrylamide, with N,N-methylenebisacrylamide or ethylene diacrylate as the cross-linking component. Catalytic redox systems, which yield free radicals, are used to initate copolymerisation (e.g. ammonium peroxydisulphate and N,N,N′,N′-tetramethylethylene diamine). Electrophoresis on polyacrylamide gels is now in general use as a laboratory technique. Its popularity owes much to the transparency of the gel, its mechanical stability and inertness, its stability over a very wide range of pH and its insolubility in most of the solvents commonly used for electrophoresis. The gels can be prepared reliably and reproducibly from analytically pure starting materials, and possesses the decisive advantage that by varying the proportions of the starting materials, gels of different density and pore diameter can be prepared. Various other substances can also be copolymerised into these gels.

In carrier electrophoresis, the chemical and physical properties of the support influence the mobility of the components to be fractionated and the sharpness of separation. The capacity of the carrier material is limited, and it is inhomogeneous; this gives rise to adsorption, electroosmotic suction, and "wick" effects, which are difficult to control and which influence the fractionation adversely. In contrast, polyacrylamide gels are almost completely homogeneous (compare Fig. 1) and therefore adsorption and electroosmosis do not occur. Wick effects can also be minimized by choice of a suitable gel concentration, buffer system, and current strength. These advantage are particularly valuable when macromolecules are to be fractionated and characterised on such gels.

Continuous electrophoresis on polyacrylamide gels (RAYMOND and WEINTRAUB, 1959), as for all other methods of carrier electrophoresis, is based on a homogeneous buffer system of fixed pH. In contrast, disc electrophoresis, developed by ORNSTEIN (1964) and DAVIES (1964), employs a discontinuous separating system. Disc electrophoresis can be performed with different buffer systems, different pH values, and different pore sizes of the polyacrylamide gel used as carrier, yet it still maintains its amazingly high quality of separation. In practice, the degree of gel discontinuity can be adjusted for each separation problem. The term "disc" also indicates a characteristic which determines the quality of separation: the macromolecules to be fractionated are concentrated from dilute solution into a sharply defined zone. Since discontinuous polyacrylamide gel electrophoresis is usually carried out in a glass tube with an inner diameter of 5–7 mm, the starting zone is

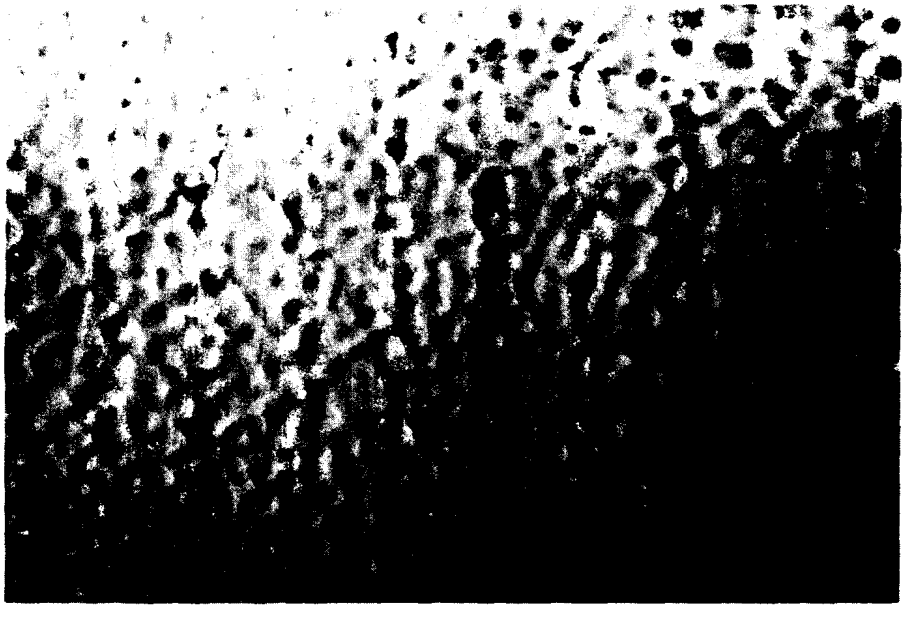

Fig. 1. Electronmicrograph of a 30 % polyacrylamide gel. Freeze-etching and carbon-platinum shadowing. (Preparation of RÜCHEL and AMELUNXEN.) Magnification 53700 ×

actually in the form of a disc in which the mixture of molecules to be separated is highly concentrated; this is difficult to attain if fractionation by continuous electrophoresis is used.

The physical theory for the method of discontinuous electrophoresis, with numerous examples of its use, and practical advice on disc electrophoresis as normally performed, has been amply described by MAURER (1971). In this chapter, therefore, only micro-disc electrophoresis will be described, the application of which is always recommended if only very small quantities of the substances are available for investigation. For example, micro-electrophoresis can be used after a lengthy purification procedure in which only minimal quantities of, for example, a pure enzyme solution, are finally available for analysis. The introduction of micromethods is becoming more and more necessary in neurochemistry, since it is already evident that different anatomical regions of the brain differ in their metabolism; it is therefore necessary to use methods which enable changes in metabolism to be assessed in well defined anatomical regions and also in isolated nerve cells.

The first application on the microscale of polyacrylamide gel electrophoresis was carried out in 1964 when PUN and LOMBROZO fractionated brain proteins. In 1965, GROSSBACH used the 5 µl Drummond microcap for this technique, which was further refined in 1966 by HYDÉN, BJURSTAM and MCEWEN, MCEWEN and HYDÉN, who used 2 µl capillaries for the fractionation of brain proteins. In 1968, NEUHOFF introduced a gel mixture which had been specially developed for the

micro fractionation of water-soluble brain proteins; these gels were later found to be suitable for many different fractionation problems. HYDÉN and LANGE (1972) used the micro-disc electrophoresis for the analysis of the changes in proteins in different brain areas as a function of intermittent training. GRIFFITH and LAVELLE (1971) have analysed changes in the developmental proteins in facial nerve nuclear regions by this method. ANSORG, DAMES and NEUHOFF (1971) have used micro-disc electrophoresis to study the effect of different extraction procedures on the pattern of brain proteins, and ALTHAUS *et al.* (1972) have used the method for the analysis of the effect of post tetanic potentiation of the monosynaptic reflexes in the spinal cord of cats on the water soluble proteins produced. GROSSBACH (1969, 1971) has used micro-disc electrophoresis for the analysis of chromosomal activity in the salivary glands of *Camptochironomus*. Glycoproteins of the alveolar surfactant of rat lung were analysed by REIFENRATH and ELLSSEL (1973), using micro-disc electrophoresis, and a modified PAS staining according to ZACHARIUS and ZELL (1969). 10 ng of glycoprotein can be detected and quantitatively determined in 5 µl gels. After recording the positions of the red stained glycoproteins, a second staining with amido black coloured the protein fractions blue and glycoproteins violet.

After a little practice, disc electrophoresis on the micro scale is hardly more difficult than the normal method. In addition to requiring smaller quantities of material, it has the added advantage of giving results agreeing with those obtained by the macro method in an appreciably shorter time. The lower limit for a single protein band in a 5 µl gel of 450 µm diameter is 10^{-9} g of albumin, if visualized with amido black 10B. Micro interferometric determination of amounts of proteins in separated fractions in the nanogram range is described by HYDÉN and LANGE (1968). 0.1 to 0.5 µg of a protein mixture can be fractionated by electrophoresis in 5 µl capillaries; this means that 5000–10000 estimations can performed with 1 ml solution containing 1 mg protein. The duration of electrophoresis depends on the type of protein to be fractionated, but is generally between 20 and 60 min. With gels of small diameter, staining with amido black requires only 5 min, and the decolourising process about 30 min. Electrophoretic destaining is not necessary. To give an idea of the dimensions of a microgel, Fig. 2 shows a gel with a diameter of 0.45 mm near the head of a normal household match.

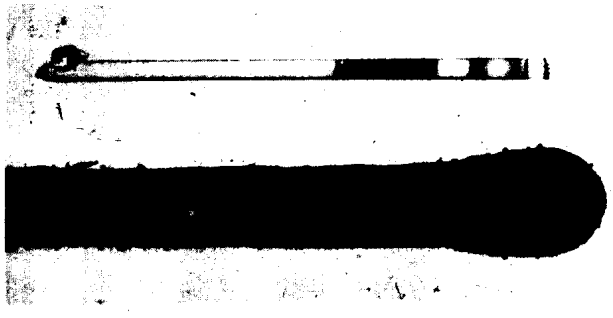

Fig. 2. Match stick and a 5 µl polyacrylamid gel. Magnification 5 ×

Micro-Disc Electrophoresis

Use of Capillaries

The Drummond Microcaps[1] introduced by GROSSBACH (1965) for micro-disc electrophoresis have proved to be extraordinarily well suited for this technique. They are obtainable in various size. 1 µl caps have an inner diameter of 0.24 mm and a length of 33 mm, 2 µl caps: inner diameter 0.28 mm, length 33 mm, 5 µl caps: inner diameter 0.45 mm, length 33 mm, 10 µl caps: inner diameter 0.56 mm, length 42 mm. The 5 µl caps are the most widely used for this method. There is sometimes a difference in length of a few millimetres between different batches of these capillaries. However, as the volume is always exact, the inner diameter of the capillaries is correspondingly larger or smaller.

For many purposes the capillaries can be used directly and filled with the gel mixture without pre-treatment. For some applications it is necessary to siliconize the capillaries. To do this, dimethyldichlorosilane is dissolved in benzene (2% v/v); capillaries which have been cleaned in chromic acid are filled up to the top by capillary attraction by dipping one end in the siliconizing solution, the solution is completely removed by placing the end of the capillary on absorbant filter paper, and the capillary is then dried for 1 hr. at 80° C. When the capillaries have been siliconized, even if the gel contains no Triton X-100 it can be expelled by applying slight pressure from a water-filled syringe.

For electrophoresis on polyacrylamide gradients, even new capillaries must be carefully cleaned before being charged with polyacrylamide. For this purpose 200–300 microcaps are transferred to a suction flask which is half-filled with chromic acid. The capillaries are completely filled with the chromic acid by creating a vacuum by means of a waterpump. In order to fill the capillaries completely with the acid, it is necessary to release the vacuum repeatedly by opening the tap quickly. It is recommended that the capillaries should stay in the acid overnight. The acid is then poured off and the capillaries are transferred to a suitable sintered-glass filter-funnel over which a separating funnel fitted with a stopper is placed (see Fig. 3). The separating funnel is filled with distilled water, and the whole system is connected to a vacuum line via a filter funnel. On closing the funnel tap the pressure in the sintered funnel is reduced; when the tap connecting the separating funnel to the sintered glass filter is opened suddenly, water enters the glass funnel rapidly. By frequent repetition, the chromic acid is completely removed from the capillaries. The capillaries are then rinsed several times with absolute ethanol and finally with acetone. To ensure complete drying, the capillaries are left over night in drying cabinet at 37° C.

Capillaries which have already been used may be cleaned for re-use by the following procedure: when the gels have been pressed out of the capillaries after an electrophoresis run, the empty capillaries are collected in a beaker full of water; when a reasonable quantity of used capillaries has been collected, cleaning is performed as above. It is advantageous to precleanse the used capillaries in undiluted potassium hypochlorite (JAVELLE'S solution) to dissolve any remaining

[1] Drummond Scientific Co., U.S.A.

polyacrylamide before transferring them to the chromic acid. Since the capillaries are relatively expensive cleaning is probably worth while.

JAVELLE'S solution is also suitable for cleaning capillaries which are completely filled with polyacrylamide gel. It is advantageous to transfer the capillaries to a suction flask half filled with JAVELLE'S solution and connected to a vacuum pump.

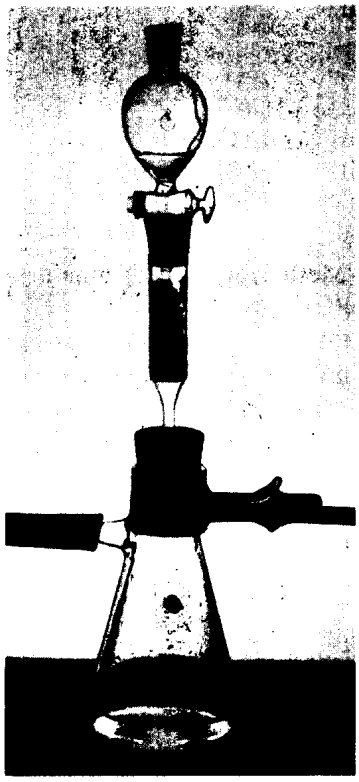

Fig. 3. Equipment for cleaning capillaries

The suction flask should have a perforated stopper with metal tube and a magnetic valve that is operated by a synchron motor, so that the vacuum is interrupted at short intervals and the gas formed by the action of the potassium hypochlorite is immediately removed from the capillaries. If this is not done, the gas bubbles will remain between the solution and the acrylamide in the capillaries, and stop the gel disolving.

The dissolution of a complete gel in a 5 µl capillary takes about 10 hrs. The dissolution time can be reduced to about 2 hrs. if the capillaries containing the gels are first dryed for several days at approximately 100° C. In this case the JAVELLE'S solution can enter the space between the dryed gel and the capillary wall so increasing the surface of action.

Preparation of Gels

The polyacrylamide gels are made up from the following stock solutions:

Stock A: 860 mg Tris
+8 ml H_2O
+0.063 ml N,N,N′,N′-Tetramethyl-ethylendiamine (TEMED)
+3.6 N H_2SO_4 to pH 8.8 (ca. 0.45 ml)
+H_2O ad 10 ml

Stock B: 2.85 g Tris
+25 ml H_2O
+8.7 M H_3PO_4 to pH 7
+1 M H_3PO_4 to pH 6.7
+H_2O ad 50 ml

Stock C for 20% Gel:
20 g Acrylamide
+200 mg N,N′-Methylene-bis-acrylamide
+3.75 mg $K_3Fe(CN)_6$
+H_2O ad 37.5 ml

Stock D: 70 mg Ammonium peroxydisulphate
+25 ml 4% Triton X-100 in H_2O
+25 ml H_2O

Stock E: 5.98 g Tris
+50 ml H_2O
+0.46 ml TEMED
+8.7 M H_3PO_4 to pH 7
+1 M H_3PO_4 to pH 6.7
+H_2O ad 100 ml

Stock F: 200 mg Ammonium peroxydisulphate
+5 ml 4% Triton X-100
+5 ml H_2O

Electrodebuffer:
3.0 g Tris
+14.4 g Glycine
+H_2O ad 500 ml
pH 8.4

Bromophenol blue:
100 mg/5 ml H_2O

Fluorescein:
saturated solution in H_2O

Amido black 10B:
1.0% in 7.5% CH_3COOH

pH adjustment must be carried out *after* the addition of TEMED, since this reagent is alkaline. Potassium ferricyanide is used in solution C to retard the polymerisation and give a better quality gel. Triton X-100 is necessary to

facilitate the removal of the gels from the capillaries by water pressure, but does not effect the separation quality of the gel. However, Triton X-100 interferes so strongly with SDS (sodium dodecyl sulphate) that Triton-containing gels are unsuitable for SDS electrophoresis.

Gels which are to be dissolved for radioactivity measurements are made up with ethylene diacrylate in place of bisacrylamide (CHOULES and ZIMM, 1965). Ethylene diacrylate does not markedly affect the resolution of the gel. Gel slices are cut according to the stained protein bands and are transferred to counting vials which contain ca. 1 ml conc. ammonia. When the slices are completely dissolved, the ammonia is allowed to evaporate. In order to disperse remaining traces of water, 0.5–1 ml of absolute ethanol (according to the quantity of water) is added, and finally the vial is filled with scintillation solution.

Apart from solution D, all solutions can be stored at 0–4° C for 2–3 months. Gels prepared from the stock solutions listed above were introduced for the fractionation of brain proteins (NEUHOFF, 1968), but they can also be used for many other fractionations. Proteins with molecular weights between 700000 and 20000 can be fractionated on these gels; however, the optimal pH and gel concentration should be determined for each protein mixture. All substances used for preparing the gels should be of the purest quality and should be stored in brown bottles at 4° C.

It is not always necessary to work with freshly recrystallized acrylamide or bisacrylamide. However, if preparations have been stored for a long time, or for the electrophoresis of particularly "sensitive" proteins, it is advisible to recrystallize the acrylamide or bisacrylamide. 70 g of acrylamide are dissolved in a litre of chloroform (analytical grade) at 50° C, filtered warm, and recrystallized at −20° C. The crystals are filtered off, washed with ice-cold chloroform, and dried in air. 10 g of bisacrylamide are dissolved in a litre of acetone (analytical grade) at 50° C, filtered hot, and allowed to crystallize slowly. The solution is first cooled to room temperature, then to 4° C, and left at −20° C. The crystals are filtered off, washed with ice-cold acetone, and dried in air.

To prepare 20% gels, 0.5 ml of solution A is mixed carefully with 1.5 ml of solution C. When mixing, care must be taken that no air bubbles enter the liquid. To 1 ml of this mixture, 1 ml of solution D is added. 25% gels are prepared by adding 100 mg acrylamide to 2 ml of a 20% gel mixture. Gel concentrations lower than 20% are obtainable by replacing a corresponding aliquot of solution C by water. The smaller the capillary diameter, the higher the polyacrylamide concentration needed in order to achieve a similarly good fractionation with the same protein mixture. For instance, if a 20% gel in 5 µl capillaries gives a good separation of a particular mixture, the polyacrylamide concentration must be raised to 25–30% for fractionation in 2 µl or 1 µl capillaries.

The sharpness of the protein bands can be improved by adding 10 mg hydantoin to 2 ml of the gel mixture (NEUHOFF, 1968; NEUHOFF and LEZIUS, 1967, 1968). This is due to the ionic nature of the compound (see Fig. 4); by adding hydantoin the total ion concentration of the gel mixture is increased. The glycine of the electrode buffer can be completely replaced by hydantoin; the hydantoin/H_2SO_4 buffer system gives the same separation of a protein mixture as the glycine/H_2SO_4 buffer system.