The Instantaneous Monitoring of Polyacrylamide Gels during Electrophoresis

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The advantages of being able to see protein zones in a gel during electrophoresis (and hence before staining) are pointed out, and a method is described which depends on local increments of refractive index in these zones. The use of local increments of refractive index in polyacrylamide gels for measuring protein concentrations in zones during electrophoresis is briefly considered; it is found that such increments are greater than would be expected from the amount of protein when sodium dodecyl sulphate is present. The enhancement depends on conditions and time of running. This makes quantitative estimates difficult, but the sensitivity of detection of protein zones by observations based on refractive-index changes is greatly increased by this property of sodium dodecyl sulphate. Methods are described for making optically uniform gels (both with uniform and with graded concentrations of polyacrylamide), necessary for observation of small changes in refractive index. A simple dark-field system of observation is described. Examples are given showing protein samples observed with the system during electrophoresis and compared with the same gel stained with Coomassie Blue after completion of the run. Under optimal conditions the optical method is comparable in sensitivity with staining. With the proteins of lower mol.wt. (approx. 15000), the optical method is not so sensitive, becoming less sensitive with longer running time. This loss of sensitivity is greatly decreased by using more concentrated polyacrylamide gels, and graded gels are therefore more suitable for optical observation than are uniform gels. The observation of protein zones during electrophoresis adds nothing to the time needed for making a stained gel and gives much information long before it can be obtained from the stained gel.

Gel electrophoresis is widely used for the examination of proteins and nucleic acids because of its sensitivity and resolution. However, unlike sedimentation runs in the analytical ultracentrifuge, electrophoresis suffers from the considerable disadvantage that in the past it has not been possible to observe the separation of the components during the run, and indeed, since many hours or even days may be taken in staining and destaining of the gels, it may be a long time before the results of an experiment are available.

I have therefore devised an optical technique which allows zones of proteins in gels to be detected during the course of the electrophoresis by the local increase in refractive index in the band. (The same use of locally enhanced refractive index is made when the schlieren technique is applied in the ultracentrifuge.) The bands are sharper than those of the subsequently stained gel because they are not broadened by diffusion, and therefore a shorter run will resolve the components. In addition to monitoring, the method allows the location of bands which are to be cut out for preparative purposes. However, the successful observation of

refractive-index changes in gels requires gels that are more uniform optically than those produced by the usual methods. In this paper I describe the production and use of optically uniform gels, and compare the sensitivity of optical methods for revealing protein zones with the conventional staining method.

Experimental

Local refractive-index increments associated with protein zones in gels

During some attempts to determine proteins quantitatively by refractive-index measurements [results reported briefly in Elliott (1974)], I made many observations on a variety of proteins, using a Michelson interferometer to observe local changes in refractive index within the bands during and after electrophoresis. An example of such an interference pattern is shown in Plate 1, and plots of patterns obtained during electrophoresis of gels containing sodium dodecyl sulphate, 50 mM-sodium phosphate buffer, pH 7.2, and protein are shown in Fig. 1. In the absence of complicating factors to be discussed

below, it would be expected that the area under the plot of fringe displacement j against the distance y along the gel would be proportional to the mass of protein within the band and that the relation for double passage of light would be

$$\text{Mass of protein} = \frac{\lambda w}{2dn/dc} \int j \, dy$$

Here λ is the wavelength of the light in the interferometer, w is the width of the specimen well normal to the light direction and to y , the direction of electrophoretic current, and dn/dc is the specific refractive-index increment for the protein (about 0.184 ml/g).

Fig. 1 shows that the value of the integrated fringe displacement for a given protein changes during the course of electrophoresis and that even the ratio of this integral for two different proteins changes during a run. Measurements in which known amounts of protein were added to gels showed that the integrated refractive index calculated from the above equation is several times larger than that corresponding to the amount of protein added. This is only partly accounted for by the sodium dodecyl sulphate, which is known to be bound to proteins (Reynolds & Tanford, 1970). At equilibrium, each

Fig. 1. Plot showing fringe displacements within a gel

The displacements are caused by the presence of protein bands separated (1) after electrolysis for about 1 h, and (2) after a further 2 h. Wavelength of light used for observation of fringes was 435.8 nm. The areas under the curves for myosin and paramyosin are given in units which, though arbitrary, are the same for both curves. The horizontal scale is the distance from the position at which the proteins were applied (origin).

protein molecule is bound to 1.4 times its mass of sodium dodecyl sulphate. The remaining excess is presumably a consequence of the existence of non-equilibrium conditions and of the working of the Kohlrausch distribution function (de Wael & Wegelin, 1952), which shows that ions moving within the gel with different velocities will distribute themselves so that the total current is the same everywhere within the gel. Another phenomenon seen in gels during electrolysis is the 'buffer front', a rapidly moving region of high refractive index with a steep front and trailing edge, which Kubo *et al.* (1975) have investigated and ascribed largely to sodium dodecyl sulphate micelles.

Thus quantitative estimates of proteins or other macromolecules from electrophoretic patterns cannot be made in a straightforward way, though calibration with known amounts run in the same gel is possible, or alternatively measurements can be carried out after fixation and washing. This is not further discussed here. However, for qualitative detection the methods based on refractive-index changes become more sensitive than they would be in the absence of the factors discussed above.

Although a suitable interferometer produces easily recognized patterns corresponding to the stronger bands (Plate 2a), the weaker bands are more easily seen with other optical methods that do not need such expensive apparatus. One of the best known is the schlieren technique used in the ultracentrifuge. This is one way in which the Fourier transform of phase distribution of light transmitted by the gel is modified in such a way that phase differences (invisible to the eye) become changed to amplitude variations, which can be observed. If the zero order of the Fourier transform is cut off we have dark-field illumination; if the zero order is changed in phase we have phase contrast, and, if one-half of the Fourier transform is removed, we have schlieren patterns. I have used the first of these methods (but see remarks at end of section 'Optical viewing arrangement').

Preparation of optically uniform gels

The need for optically uniform gels will be seen if an acrylamide solution containing sodium dodecyl sulphate and buffer is polymerized in a rectangular cell (in which it can be better observed than in a cylindrical tube). Just before and during setting, vertical streaks (striae) form, showing a non-uniformity of refractive index and possibly of concentration. These persist after polymerization is complete and make the gels useless for protein-zone detection by optical methods. They are not lessened by slowing down the polymerization process (for instance, by lowering the temperature) and are evidence of an instability which develops as the temperature changes during the polymerization

EXPLANATION OF PLATE I

Interference fringes in polyacrylamide gels

Interference fringes for $\lambda = 546.1$ nm showing the principal proteins myosin (M), paramyosin (P) and actin (A) of the white adductor muscle of the oyster *Crassostrea angulata* in 4% polyacrylamide gel (6.8 mm thick) containing 100 mM-sodium phosphate buffer, pH 7.2, and 0.1% sodium dodecyl sulphate. The specimen was applied at O (origin).

EXPLANATION OF PLATE 2

Detection of protein bands in polyacrylamide gels

(a) Dark-ground illumination of 4–10% graded polyacrylamide gel (3 mm thick) containing 50 mM-sodium phosphate buffer, pH 7.2, and 0.05% sodium dodecyl sulphate after 45 min (50 V, 60 mA). Width of well 6 mm. (i) Myofibrillar proteins from rabbit psoas muscle; (ii) mixture containing approx. 8 μ g each of myosin, paramyosin, bovine serum albumin, actin and myoglobin; (iii) the same mixture with 4 μ g; (iv) the same mixture with 2 μ g. (b) As (a) after 1 h 20 min. (c) (i) Rabbit psoas myofibrils (10 μ l); (ii) the same with 5 μ l; (iii) the mixture as in (a) 8 μ g; (iv) the same mixture with 4 μ g after 2 h. (d) The gel stained in Coomassie Blue immediately after (c). Key: A, actin; BSA, bovine serum albumin; C, C-protein; M, myosin; Mb, myoglobin; P, paramyosin; TM, tropomyosin; TN-T, troponin T; α A, α -actinin; O, origin; F, buffer front.

reaction. They are related to the 'salt fingers' which appear in suitable gradients of solute mixtures possessing very different rates of diffusion (Stong, 1971).

Since the development of striae is a consequence of differences of specific gravity, it can be prevented if the cell containing the uniform mixture is rotated about a horizontal axis during polymerization so reversing the effect of gravity. A speed of rotation of about 1 rev./s is suitable. This requires that the cell containing the reactants should be totally enclosed and free from air bubbles. Gels free from striae can be made in this way with a ratio of NN' -methylenebisacrylamide to acrylamide of 1:20 (w/w), but they may have other small irregularities of refractive index near the boundaries and they scatter light; these defects are greatly decreased by changing this ratio to 1:40.

The cell for uniform gels is shown in Fig. 2. The faces are of sheet glass from cleaned photographic plates; the other parts are of polymethyl methacrylate (Perspex). The glass plates are selected to give a uniform field in the dark-field apparatus described below; as mentioned by Fries (1976), one orientation of the plates (related to the direction of drawing the sheet glass) will be better than others. No grease or cement is used, but the glass plates are carefully ground to size and have parallel edges. Two strong rubber bands encircle the cell, keeping the glass plates in contact with the side separators. The

Fig. 2. Cell for uniform gels (thickness exaggerated)

Usual dimensions of the gel are 70mm×70mm×3mm. The number of specimen wells may be eight for this size of cell.

Fig. 3. Box for cell shown in Fig. 2

The whole box is filled with the solution to be polymerized after the empty cell has been inserted.

lower member, which holds the side separators in position, is pierced by holes, and electrophoresis is carried out without removing this member. The comb for casting the sample wells shown above the cell in Fig. 2 may have 1 mm holes as shown, with a steel pin fitting each hole during filling and polymerization. The pins are withdrawn before removal of the comb, to facilitate the entry of air. The pins are not absolutely necessary, but their use lessens the possibility of damage to the gel during comb extraction. When in position, their ends should be flush with the under-side of the comb.

Of the various methods of sealing the cell which have been used, the most satisfactory is to put the cell within a rectangular Perspex box with well-fitting top and bottom (Fig. 3) and to fill the box and cell completely. The cell is a loose fit, but is prevented from moving in the box by a piece of soft rubber compressed between the bottom of the cell and the bottom of the box. If the ends of the box are carefully ground flat, the top and bottom can be secured in an air-tight manner by means of eight nuts on each. The bottom end has a boss for mounting the whole on the horizontal spindle of a low-speed motor. This end is pierced by two large holes covered with dental-rubber dam to accommodate volume changes when the gel sets and to lessen strain from such changes. The top has a plain tube for entry of the reactants and also an exit tube over a toroid-shaped dome by means of which the whole of the air in the box can be expelled. The de-aerated solution is fed in with the box standing on the narrow side marked

'A' in Fig. 3, and when nearly full the box is rotated to the position shown in Fig. 3 before filling is completed. This procedure minimizes the formation or lodging of air bubbles, which may otherwise be troublesome. Rubber tubes on the inlet and exit ports are closed (after filling) with burette clips.

After polymerization (during which the box is rotated), the cell is gently pushed out of the Perspex box, excess of gel is removed and the comb carefully extracted.

Production of uniformly graded gels

Suitably graded gels in which the smaller proteins are observed in a more concentrated gel (smaller pore size) than those of greater molecular weight give protein zones that are of more uniform width than those run in gels of constant concentration. Their preparation has been described by Margolis & Kendrick (1968), who used two cylindrical pots connected at their lowest points, one containing a high, the other a low, concentration of reactant mixture. Liquid from the less-concentrated mixture is run into the lower part of the cell first, followed by progressively more concentrated mixture.

I have used a similar linear mixer to feed the reactants into the cell. The cell base and comb

Fig. 4. Comb and base for graded gel

The same glass plates and side members which in Fig. 2 forms parts of a cell for uniform gels may be used interchangeably for graded gels.

are shown in Fig. 4 and are used with the glass plates and side-spacers of Fig. 3. The mixed liquid enters the cell base via a plug (shown separately), which has two small holes to direct the liquid entering the cell horizontally. Uniformly graded gels without striae can be made provided that the initiator (tetramethylethylenediamine) is graded in the same way as the acrylamide. In this respect my procedure (which ensures that polymerization proceeds from bottom to top sequentially) differs from that recommended by Margolis & Kendrick (1968). The buffer and ammonium persulphate are not graded. I have generally used the buffer system described by Weber & Osborn (1969), but it should be used at half-strength, i.e. 0.05M-sodium phosphate and 0.05% sodium dodecyl sulphate in gel and tank, otherwise striae may be formed.

The glass plates (held by spring clips) are made watertight by a thin layer of aq. 1% agar and 4% polyvinyl alcohol (mol.wt. about 125000). This, unlike agar alone, spreads easily and is easily removed by hot water. The seal between glass cell and Perspex base is made with the same mixture retained by a wide strong rubber band. This band in turn is held lightly by polyurethane foam lining a clamp which presses along the straight portion of the rubber band. A very small Perspex support projecting from the base into the cell prevents bending of the glass plates (not shown in Fig. 4).

The plain comb shown in Fig. 4 should be a fairly tight fit when the spring clips holding the glass plates to the side-spacers are in position. The liquid is allowed to rise in the cell (which is tilted very slightly round an axis perpendicular to the cell face) until near where the lower end of the comb will be. Any bubbles are removed from the surface with a drawn-out Pasteur pipette, the comb is introduced and the filling completed in this way without bubbles under the comb. The cell base is then gently levelled, propanol saturated with water is layered on the top free surface and the gel is left to set.

Running conditions

In order to use a sensitive optical viewing arrangement, the windows of the electrolytic tank may either be of plate glass or they may be cut away (from a Perspex tank) to give a view of the cell. I have used both, but prefer the second alternative. This requires that only the top few millimetres of the gel cell can project into the upper buffer tank. A hole is cut in the base of this tank with rounded ends to match the shape of the gel cell, with a minimum of clearance. The cell must be clamped so that it cannot move within the hole; for this purpose four Perspex lugs are cemented on to the under-side of the tank, two on either side of the cell. Nylon screws passing through tapped holes in the lugs

hold the cell, as in a vice. For sealing, molten 1% agar solution is used coloured with dye to facilitate inspection. The cell does not pass right through the hole in the tank, but stops short of the upper face of the tank bottom. This provides a ledge in which the molten gel may be run. By sealing in this way, the hydrostatic pressure of the buffer will force the congealed agar gel into the gap to be filled. Only in such a way is it possible to use so weakly adhering a substance as agar gel. When the main body of the cell is in air, a blower or fan may be used to maintain a uniform temperature and so uniform velocity of bands in the several compartments of the gel.

Optical viewing arrangement

Quite crude optical systems will serve to reveal strong protein bands in a gel (which can often be seen simply by positioning the eye suitably), but to see the weaker bands some refinement is needed. The system shown in Fig. 5 is a simple but sensitive one for dark-field illumination which has been used to provide the examples shown in Plate 2. A tungsten-filament lamp with a straight horizontal filament (such as a 12V 36W automobile lamp) is used in a housing which carries an adjustable tube at the end of which is a $\times 6.5$ microscope objective of 0.25 numerical aperture. This produces a reduced image of the filament. A wide slit screens off the image of the hot filament supports. The filament image is at the principal focus of a collimating lens which directs a parallel beam through the gel. A good quality achromat large enough to cover the whole of a gel is expensive (two are required), and I have used objectives made for field glasses. The largest that I have found is of 20cm focal length and 6.4cm diameter. This enables three of four specimens within the gel to be seen at once, over a length of 5cm, and other parts are viewed by sliding the cell containing the gel. The advantage of using objectives from manufacturer's stock are obvious. The light transmitted by the gel is focused by an identical second lens to a horizontal line, where a horizontal stop (a wire of diameter about 0.2mm) is placed to

intercept the main beam if dark-field illumination is used. The light diffracted by the bands within the gel is not intercepted, and these bands may be seen either by placing the eye just behind the stop, or more conveniently by looking at the ground-glass screen of a camera which is focused on the gel. As shown in Plate 2, these bands appear bright on a dark ground.

The stop should have a fine vertical adjustment as well as movement along the axis of the lens. With a perfect optical system and the stop located at the focal distance from the lens, the field seen on the camera screen should darken uniformly as the stop is brought into the beam from above or below. The width of the stop should be the smallest that will darken the field completely with the gel removed. Although the objectives used are colour-corrected, it is found necessary to use a colour filter to remove the residual colour aberrations. A green filter such as Wratten 61 is suitable. In practice it may be found that the weakest bands are most easily seen when the stop is displaced slightly from the central dark-field position. This was done for the photographs shown in Plates 2(a), 2(b) and 2(c), the adjustment being chosen to give best visibility of the weakest bands.

It might be thought that the presence of a concentration gradient (and hence of refractive index) within the gel would interfere with the observation of the local changes in refractive index caused by protein bands. This is not so; a linear gradient of refractive index behaves like a prism with a small refracting angle and merely deviates the whole beam slightly. This is not noticed when the stop is adjusted for maximum visibility of the bands.

Any camera which can be used to focus on the gel may be used, if necessary with an auxiliary lens added to enable such a near object to be focused. It is convenient to use plates or cut films in cassettes so that exposures can be processed immediately. I have used a quarter-plate camera with a repeating back, allowing several exposures on one film.

A system using two concave mirrors has been described by Fries (1976) for viewing gels (see below). This arrangement is of course quite free

Fig. 5. Optical viewing arrangement

from colour aberrations, but takes up a great deal of bench length. An auto-collimating concave-mirror system using a much shorter length (and only one curved mirror) would probably be practicable, however.

Discussion

Typical results obtained with the procedures described above are given in Plates 2(a)–2(d) which shows a 4–10% graded gel with phosphate buffer [Weber & Osborn (1969), used at half-strength] photographed during a 2h run, after which it was stained with Coomassie Brilliant Blue G-250 (Serva, Heidelberg, West Germany), as described by Weber & Osborn (1969). All protein solutions were boiled with 1% β -mercaptoethanol to disrupt S–S bonds. One specimen was a mixture of approximately equal amounts of the heavy chains of myosin, paramyosin, bovine serum albumin, actin and myoglobin, which chain weights respectively 200 000, 110 000, 68 000, 42 000 and 17 200. The specimen wells measured 5.5 mm $\times$ 3 mm, and loads of approx. 2, 4 and 8 μ g of each protein species were applied. All can be seen clearly after 45 min (Plate 2a) and after 1 h 20 min (Plate 2b). After 2 h (Plate 2c), the myoglobin band has broadened and weakened and can be seen only on the 8 μ g loading. It is typical of the method described for observing bands in sodium dodecyl sulphate gels that the sensitivity diminishes after the bands have run some distance. This is much more marked in gels of uniform concentration than in graded gels, which are on this account (as well as others) to be preferred.

The other specimen shown is a solution of myofibrils from the rabbit psoas muscle. The pattern of bands is not properly resolved after 45 min (Plate 2a), but after 1 h 20 min (Plate 2b) the troponin T band is resolved from the neighbouring (much stronger) actin and tropomyosin. The minor bands α -actinin and C-protein can be easily seen.

The sensitivity of the method here described is greater than when sodium dodecyl sulphate is not present and when there are striae. While this paper was being written, a description of optical methods for viewing such gels appeared (Fries, 1976). Quantitative comparison is difficult; Fries (1976)

used 100 mm-wide wells, and the minimum mass of protein that he could detect (100 μ g) would be decreased with narrower wells, but in the presence of striae there is a limit to the decrease possible when the background is 'noisy'.

With gels containing sodium dodecyl sulphate which have been prepared free of optical irregularities by the methods here described, the sensitivity of optical methods under the best conditions is comparable with that obtained by staining. The further a band runs in the gel, the less easily it is detected optically. In Plates 2(c) and 2(d) the unidentified bands between the origin and myosin are more easily seen optically than by staining, between myosin and paramyosin the two methods are about equally sensitive and for the lighter proteins staining is more sensitive. Early observation and the use of a high concentration of gel are favourable to detection by an optical method.

In conclusion, much information can be obtained during electrophoresis from observation of the kind here described; no additional time is needed and the gel can be stained after electrophoresis in the usual manner. Optically uniform gels (whether examined before or after staining) would be expected to give densitometer records relatively free from noise.

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