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Equilibration in Aqueous Methanol Increases the Resolution of Two-Dimensional Polyacrylamide Gels

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Abstract

Equilibrating cylindrical isoelectrofocusing gels in 55% methanol rather than in aqueous buffer before starting the SDS dimension substantially increased the resolution obtained on 2-D gels. This effect may result from the *in situ* precipitation of focused proteins eliminating band diffusion prior to the second dimension.

INTRODUCTION

The technique of two-dimensional polyacrylamide gel electrophoresis (2-D gels) introduced by O'Farrel (*1*) has proved to be a very powerful tool for analyzing complex mixtures of proteins. One problem that is encountered in achieving an optimal resolution is obtaining a uniform transfer of molecules from the isoelectric focusing (IEF) to the SDS-polyacrylamide gel (SDS-PAGE) dimensions. O'Farrel used beveled glass plates to create a trough in which the cylindrical IEF gel was placed. Because the cross-sectional area surrounding the IEF gel decreases as the slab gel is approached, the resistance of the gel increases. Consequently, the voltage seen by the proteins at the trailing edge of the IEF gel is much less than that seen by those at the leading edge. This effect will thus increase the distance between the leading and trailing molecules of any given protein. Although the use of a stacking gel will partially counteract this tendancy, an increased resolution might be expected if the problem were eliminated. Several methods have been used to reduce or avoid this nonuniform electric field. Imada (*2*) reported that bent

glass plates, which would contain a much more shallow voltage gradient, increased the resolution of 2-D gels. Many authors (e.g., Refs. 3–5) have used cylindrical or slab IEF gels that are thin enough to fit between the plates of the second dimension and thus avoid the voltage gradient entirely.

We have been unable to obtain satisfactory isoelectric focusing in cylindrical gels thin enough to fit between the plates of 0.76 mm thick polyacrylamide gels. We have been reluctant to use thicker slab gels because of the resultant loss in sensitivity for the detection of ^{35}S by conventional autoradiograph and our desire to avoid the expense of fluorography. The report by Jäckel (*6*) showed that IEF gels could be fixed, stained, destained, and reequilibrated in aqueous buffers prior to SDS-PAGE. This suggested that we might be able to shrink an IEF gel by equilibrating it in aqueous methanol, and thus reduce its diameter to the point where it could be squeezed between the second dimension electrophoresis plates. This approach results in a dramatic improvement in the resolution obtained using 2-D gels.

MATERIALS AND METHODS

Two-dimension gels were prepared according to O'Farrel (*1*) with the following modifications. IEF gels were poured in 0.2 mL pipettes designed for "cotton plugging." These pipettes were chosen because the flared upper end provides a large reservoir for samples of volumes up to several hundred microliters. These pipettes were cleaned in chromic acid, rinsed, treated with 5% alcoholic KOH for 5 min, then rinsed with distilled water, and dried between uses. The IEF gel mix contained 1.6% (w/v) pH 4–6, 0.4% (w/v) pH 3–10 Ampholines (LKB), and was polymerized with 0.0025 volumes of 10% (w/v) ammonium persulfate. Gels 13–19 cm long were prepared according to the needs of the experiment. The gels were allowed to polymerize for 2 h without being overlaid with either distilled water or buffer. Only the uppermost 1–2 mm of gel solution failed to polymerize using this protocol. Gels were pre-run in the presence of a series of tracking dyes (*7*) (2.5 μg Congo Red, 2.5 μg fast green, and 5 μg Evans Blue dissolved in 20 μL of O'Farrel's lysis buffer per IEF gel) in order to identify any gels with an aberrant migration due to bubbles or other defects prior to loading samples. One million TCA precipitable cpm of [^{35}S]methionine labeled cell extracts were loaded on to each gel. The IEF dimension was electrophoresed for 460 V · h/cm of gel (30.75 V/cm for 15h). The voltage was then increased to 61.5 V/cm of gel for an additional hour.

Gels were expressed by hydraulic pressure using an adaptor made from disposable plastic micropipette tips. Approximately 1 cm was cut off the

wide end of a 200-μL disposable tip so that the opening fit snugly onto a hypodermic syringe. The narrow end of the tip then forms a good seal with the top of a variety of sizes of IEF gels. We have found that hydraulic pressure provides a much more controllable force for expressing cylindrical gels than air pressure. The gels were expressed into test tubes containing 4–5 mL of distilled water. The water was then discarded and replaced with either O'Farrel's equilibration buffer [10% (w/v) glycerol, 2% (w/v) SDS, 0.0625 *M* Tris, pH 6.8, and 5% 2-mercaptoethanol] or the same buffer containing twice the amount of SDS and diluted with methanol to a final methanol concentration of 40–70% (v/v). Gels were equilibrated for 1 h with mild continuous agitation.

The second dimension consisted of a 5-cm 5% acrylamide stacking gel poured on top of a 10-cm $12\frac{1}{2}$% acrylamide separating gel (*8*). Vinyl plastic spacers (0.03 in.) were used to make 0.76 mm thick gels. In some cases a notched beveled plate was used as described by O'Farrel (*1*), and compared to a square-cut notched plate. In these cases the IEF gel was sealed in place with a small amount of 1% agarose dissolved in equilibration buffer. In other cases the stacking gel was poured so that its upper surface was 1–2 mm below the edge of a square-cut notched plate. Before squeezing the IEF gel between the plates, this space was filled with equilibration buffer diluted with methanol. Since friction adequately held these IEF gels in place, agarose was not used. The gels were electrophoresed in parallel in Studier-type apparatuses (*9*) at 20 mA/gel with a limiting maximum voltage of 350 V. When the bromophenol blue tracking dye reached 5 mm from the bottom, (~3 h), the gels were removed, fixed for 15 min in 7% acetic acid in 40% aqueous methanol, dried under vacuum, and autoradiographed using Kodak AR medical x-ray film.

Samples were prepared from differentiated cultures of neonatal rat thigh myoblasts. Cells were labeled overnight with 100 μCi/mL of [^{35}S]methionine in methionine-free medium containing 10% fetal bovine serum, washed twice with saline, once with distilled water, then rapidly dried in a stream of air. The cells were then dissolved directly in O'Farrel's lysis buffer without further treatment. We have not found nuclease treatment (*10*) to be necessary with highly radioactive samples.

RESULTS

Figure 1 compares two gels that were either equilibrated in aqueous buffer and loaded on top of the beveled electrophoresis plates (A) or equilibrated in the same buffer containing 55% methanol and loaded between the glass plates (B). Detailed portions of these gels are shown in Fig. 2. Representative

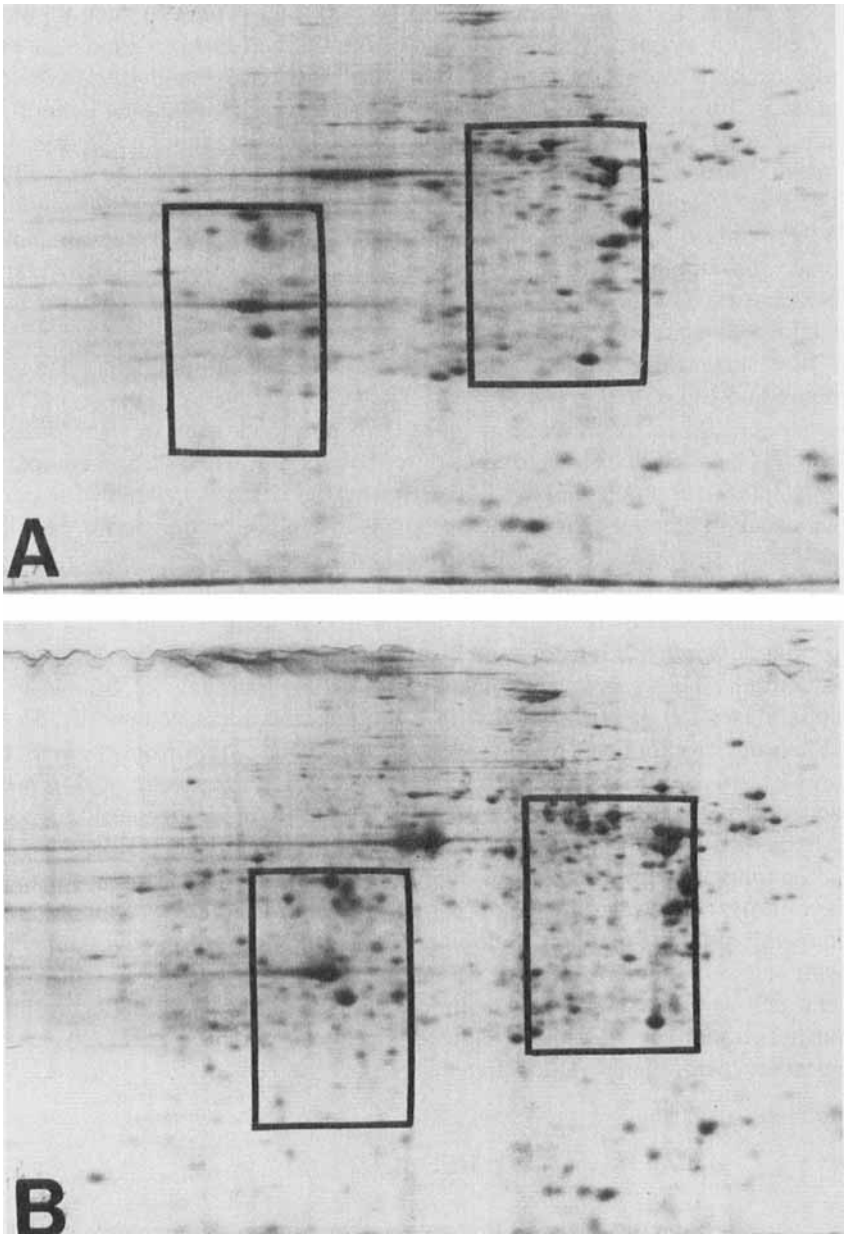

FIG. 1. Two dimensional gels of [^{35}S]methionine labeled extracts of rat thigh muscle cultures. Identical IEF gels were either incubated in O'Farrels equilibration buffer (A) or buffered 55% methanol (B) for 1 h prior to loading the gels onto the second dimension slab gel. The areas shown in boxes indicate the regions of the gel detailed in Fig. 2. The basic end of the IEF gels are to the left.

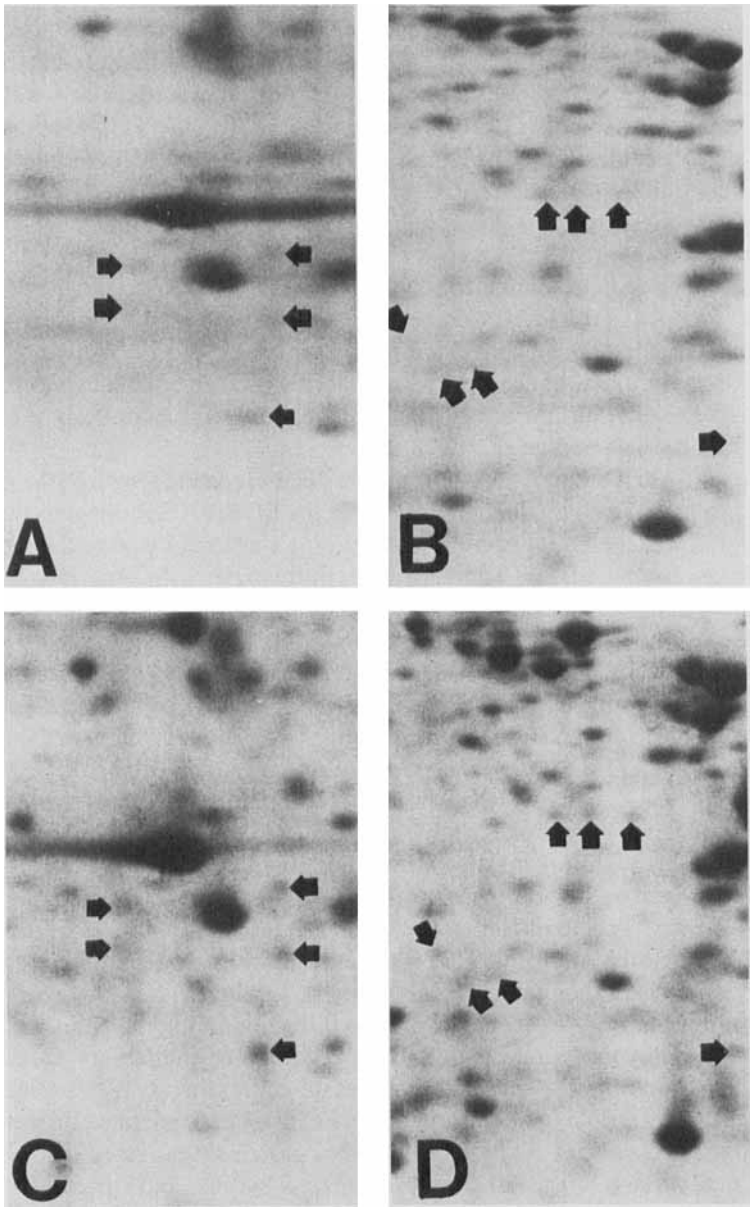

FIG. 2. Details of the gels of Fig. 1. (A) and (B): IEF gels equilibrated in O'Farrels buffer. (C) and (D): IEF gels equilibrated in buffered 55% methanol. Some representative spots are indicated by arrows in order to facilitate comparison of the gels.

spots have been indicated by arows in the two sets of gels. The increased resolution obtained under the aqueous methanol equilibration protocol is clearly evident. A careful inspection of the gels indicated that almost all of the "new" spots appearing in the methanol-equilibrated gels could be identified as faint smudges in the control gels.

Various concentrations of methanol were investigated to determine the optimal conditions. Concentrations of 70% or more caused the IEF gels to develop marked distortions (kinks and bubbles) and they became very sticky and unmanageable. Figure 3 shows the time course of gel shrinkage during equilibration in various methanol concentrations. Concentrations below 50% did not produce sufficient shrinkage to permit the gels to be squeezed between the second dimension electrophoresis plates. 50–55% methanol was chosen as the optimal concentration in that it produced adequate shrinkage and was near equilibrium after 1 h of incubation, thus permitting greater reproducibility between gels.

Aqueous 55% methanol produces a 30% decrease in the length of the gels. In order to have an equivalent gel length for the SDS-PAGE dimension, the IEF gel that was methanol equilibrated in Fig. 1 was initially poured to be 19 cm long as compared to 13 cm for the control IEF gel. This raised the possibility that the observed increase in resolution resulted from the increased resolving power of the longer IEF gel itself. This possibility was investigated by comparing equivalent 13 cm gels with or without methanol equilibration. In spite of the small size of the methanol equilibrated gel (9 cm), the increase in resolution in the final gel was equally dramatic.

In order to determine if the increased resolution resulted from being able to squeeze the IEF dimension between the second dimension plates, methanol equilibrated gels were run under three different conditions: squeezed between the plates, sealed with agarose in the trough provided by beveled plates, or sealed with agarose on top of square-cut plates. All three conditions gave identical results. This implied that the increased resolution was not primarily a result of a greater voltage uniformity occurring when an IEF gel had been positioned between the plates. An alternate possibility is that the increased resolution results from a decreased protein diffusion during the equilibration process. This hypothesis was tested by examining the ability of 55% methanol in equilibration buffer to precipitate cell extracts. When aliquots were incubated at room temperature for 1 h in 55% methanol in equilibration buffer in the presence of 50 μg of cold serum proteins, 960 $\pm$ 40 dpm were precipitated versus 950 $\pm$ 30 dpm precipitated by cold 10% tricarboxylic acid. The 55% methanol equilibration buffer is thus as efficient as TCA in precipitating proteins.

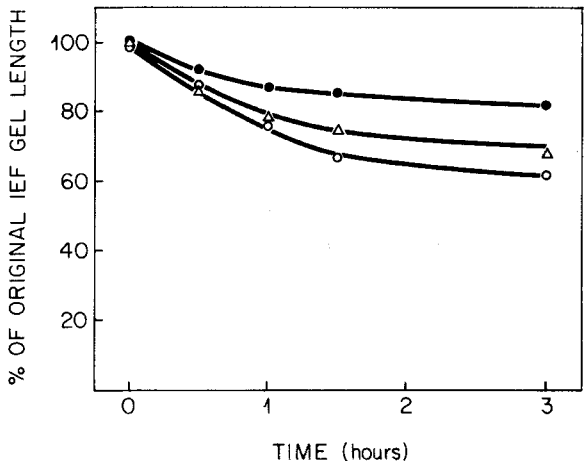

FIG. 3. Shrinkage of IEF gels in aqueous methanol. Isoelectrofocusing gels were placed in O'Farrels equilibration containing 4% SDS which had been diluted to 50% (○), 55% (△), or 60% (●) methanol. The length of each gel was then measured as a function of time.

DISCUSSION

We originally hypothesized that an increased resolution in two-dimensional gels would be obtained if one could eliminate the nonuniform voltage that occurred when a relatively large-diameter round IEF gel was placed above a thin SDS-PAGE second dimension. Equilibrating IEF gels in aqueous methanol was undertaken in order to shrink the gels to a size permitting them to be placed between the electrophoresis plates of the slab gel. This approach was very successful and resulted in a dramatic increase in gel resolving power. Further investigation suggested that the reason for this improvement resided in diffusion rather than voltage mediated effects. Loading the IEF gels onto the slab gels in configurations that might be expected to maximize nonuniform voltage effects did not significantly effect resolution. However, 55% methanol was found to precipitate essentially all of the TCA precipitable dpm. We presume that the *in situ* precipitation of focused proteins during the 55% methanol equilibration step substantially decreased diffusion without interferring with the migration of the proteins out of the IEF gel once the SDS-PAGE dimension had begun. Whatever the mechanism, a substantially increased resolution is obtained when IEF gels are equilibrated in buffer containing 55% methanol prior to SDS-PAGE.

This approach should prove valuable in studies using two-dimensional polyacrylamide gels to resolve complex mixtures of proteins.

Acknowledgments

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REFERENCES

1. P. O'Farrel, *J. Biol. Chem., 250,* 4007–4021 (1975).
2. M. Imada, *Anal. Biochem., 89,* 292–296 (1978).
3. J. I. Garrels, *J. Biol. Chem., 254,* 7961–7977 (1979).
4. N. L. Anderson and N. G. Anderson, *Anal. Biochem., 85,* 341–354 (1978).
5. K. Nikaido and G. F-L. Ames, *Biochemistry, 15,* 616–623 (1976).
6. H. Jäckle, *Anal. Biochem., 98,* 81–84 (1979).
7. A. Conway-Jacobs and L. M. Lewin, *Ibid., 43,* 394–400 (1971).
8. U. K. Laemmli, *Nature, 227,* 680–685 (1970).
9. F. W. Studier, *J. Mol. Biol., 79,* 237–248 (1973).
10. J. I. Garrels and W. Gibson, *Cell, 9,* 793–805 (1976).

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