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# The nature of observed schlieren patterns in isoelectric focusing gels and their use for position location of banded proteins

A simple and versatile optical technique for viewing discontinuities in the thickness of horizontal isoelectric focusing gels is described. The discontinuities in the gel are reproducible and relate directly to the ampholyte distribution. Characterization of the nature of the discontinuities in the gel is presented as well as a demonstration of how they may be used for localization of focused proteins.

## 1 Introduction

The two-dimensional electrophoretic methods previously described [1, 2], when combined with other fractionation methods including zonal-centrifuge fractionation of subcellular components [3] and recycling immunosubtraction [4], allow the majority of the protein gene products of mammalian cells to be mapped and some of their proteins described. Before this can be done systematically, however, a number of problems must be solved. These include the development of parallel preparative techniques of comparable resolution which allow two-dimensional electrophoretic map spots to be identified functionally. It is not feasible to attempt the functional identification of hundreds of spots by isolating different enzymes by classical methods and then co-electrophoresing them one at a time in the presence or absence of cell extracts. Although this approach is useful for purified proteins that are already available, it is too time-consuming for practical purposes. The majority of published methods involved multistep procedures which are usually specifically tailored for each enzyme individually. We therefore ask whether a few sets of techniques can be devised, each based on different biophysical properties, which together would serve to define the function of the majority of cell proteins.

The first principle of such an effort is to use parallel techniques for sample preparation and for analysis. For example, if a preparative separation is based on solubility, an analytical technique based on the same property should be used. For complex mixtures, therefore, a series of parallel preparative and analytical methods are required, each member of each pair of methods being based on the same property of the proteins under study. Ideally, the pairs of techniques should each exploit a different, and if possible, independent property of a protein (*i. e.*, solubility, charge, molecular weight, antigenicity, or thermostability) [5, 6]. The matter of scaling preparative techniques deserves comment. When the objectives are maximum purification, identification of function (enzyme activity, *etc.*), and finally the localization of a protein in an ISO-DALT two-dimensional electrophoretic pattern, rather small amounts of material will suffice, in many cases only a fraction of a milligram. Hence, what is considered preparative for our purposes may not suffice for others. We have begun to approach the problem of the development of small scale, high resolution preparative methods for protein purification and identification by studying properties of the focused gel and by developing methods for locating bands in the slab gels.

Isoelectric focusing (IEF) gives the highest resolution of undenatured proteins currently available, with a theoretical and sometimes practical resolution of 0.01 pH unit. The problem is to achieve this resolution in a reproducible manner. The simplest method is to cut strips containing a focused protein from an isoelectric focusing slab gel and elute the protein into an appropriate buffer. Because focused protein bands are rarely straight, a portion of the gel must generally be stained for band localization. Therefore, for maximum resolution, speed, and maintenance of enzyme activity, some method for rapidly locating protein bands or pH zones is required. We have devised a simple optical system which allows the reproducible location of specific regions in an IEF slab gel by observing differences in refractive index gradients generated by the focused state of the carrier ampholytes within the gel. This method also enables us to detect instability and distortion of the pH gradient.

Available ampholyte mixtures do not posses a continuous spectrum of isoelectric points, nor is each molecular species of ampholyte present in the same concentration. As a result, schlieren patterns may be observed in IEF systems caused directly or indirectly by local differences in ampholyte concentrations. These patterns have been observed during IEF in density-gradient columns [7] and in gel slabs [8]. The schlieren patterns observed in IEF slab gels are somewhat different from those seen in density-gradient columns because, on first inspection, the slab gel patterns appear to be almost entirely due to differences in thickness (and hence in water content) of the gels. These differences are possibly related to the discontinuities of the ampholyte distribution following focusing, but the mechanisms involved are obscure. In this paper, we report our experiments designed to characterize the nature of the observed schlieren patterns and demonstrate how they may be used to localize focused proteins in slab gels.

## 2 Materials and methods

### 2.1 Apparatus and reagents

All electrofocusing was conducted on an LKB 2117 Multiphor apparatus equipped with an LKB 2103 power supply and a Lauda, K-2/R refrigerated cooler. Ampholytes were purchased from LKB, Brinkman, and Bio-Rad. Acrylamide

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Abbreviations: IEF: Isoelectric focusing; TEMED: N,N,N',N'-Tetramethylethylenediamine

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was purchased from Serva Feinbiochemica, and bis-acrylamide from Bio-Rad.

### 2.2 Thin-layer IEF

Each thin-layer gel was cast using the standard LKB Multiphor apparatus. The gel dimensions were 245 x 115 x 2 mm. The gel composition consisted of 8 ml of 31.8 % polyacrylamide containing 1.8 % bis-acrylamide, 49 ml of water, and 3.0 ml of ampholyte (40 % w/v). The solution was deaerated and polymerization initiated by the addition of 250 µl of 10 % ammonium persulfate and 30 µl of N,N,N',N', tetramethylethylenediamine (TEMED). The gel was allowed to polymerize for 2 h. In one experiment in which commercially available ampholytes were compared, a one-third volume of the above mixture was made using one ampholyte preparation, and the solution was pipetted into the caster. Butanol saturated with water was layered above the gel solution until polymerization occurred. The butanol solution was removed and the procedure repeated for the remaining two preparations of ampholytes. After polymerization, the gel was placed on the Multiphor apparatus, which had been precooled at 4 °C. Filter paper strips soaked in 1 M NaOH and 1 M $H_3PO_4$ were used for electrode wicks for the cathode and anode, respectively. Electrofocusing was performed using constant power of 25 W. For all experiments the starting potential was approximately 300 V with 90–95 mA of current. Electrofocusing was complete after 2.25 h with a final potential of 1200 V (22–25 mA).

### 2.3 Schlieren optical system

Differences in light refraction through the gel were visualized with an apparatus designed to generate schlieren patterns corresponding to the longitudinal ridges that occur following electrofocusing. The apparatus consisted of a Mylar sheet sandwiched between two glass plates. A pattern of diagonal lines (45 °) 4 mm apart with every fifth line accentuated was drawn on the Mylar sheet. The line pattern was illuminated from below by a light box. The glass plate supporting the focused gel was positioned above the line pattern in an aluminum frame mounted on a vertical screw. This allowed the gel plate to be adjusted to varying heights above the line pattern. For our purposes a vertical distance of 10 cm was chosen as optimal. A camera mounted above the glass plate permitted the line pattern to be photographed while focusing through the gel (Fig. 1). This method is an adaptation of a technique previously described for viewing protein boundaries in centrifuge tubes [9].

### 2.4 Photography of schlieren patterns

The schlieren patterns were photographed using a Polaroid MP-4 Land camera on 4 x 5 inch Kodak Contrast Process Ortho film at f:42 with a 10 s exposure using a 215 mm f:6.3 lens. No filter was used. The negatives were developed in Kodak D-76 for 5 min, fixed, and rapidly dried. Nitrogen burst agitation was used at 20 s intervals during developing and fixing of the negatives. After drying, the negative was inserted into an enlarger that had been preset to give an image exactly the size of the original IEF gel, and a print was made on 8 x 10 inch Gavaert Rapitone P1-3 paper, and developed using a rapid processor. In less than 10 min following electrofocusing, a dried print of the schlieren pattern was available.

### 2.5 Photography of banded ampholytes

Focused ampholytes containing an attached chromophore (LKB) were contact printed on Agfa-Gevaert FO 71p Ortho transparency film using a Wratten No. 80 C filter. Positive images were obtained by contact printing on Rapitone P1-1 paper.

### 2.6 Determination of pH values

The pH values across the focused gel were determined in two ways. First, the gel was placed on the photograph of the schlieren pattern, and longitudinal strips corresponding to the major schlieren peaks were cut from the upper one half of the gel. The gel strips were placed in glass vials and covered with deaerated, glass-distilled water. The second method involved the use of a template with vertical lines drawn 3 mm apart. The glass plate supporting the remaining, lower half of the gel was placed on the template and 3 mm wide longitudinal strips were cut from the gel. These strips were then treated as described above. The vials were capped and refrigerated overnight at 4 °C. After allowing the capped vials to equilibrate to room temperature, the pH values were determined using a Beckman Model 4500 digital pH meter.

### 2.7 Sample preparation

Human erythrocyte lysate proteins were used as sample material to determine the reproducibility of protein migration. Samples of venous blood were drawn directly into evacuated tubes containing EDTA as anticoagulant. The samples were centrifuged and serum and buffy coat were removed and discarded. The erythrocytes were washed five times by resuspending in three volumes of phosphate-buffered saline, pH 7.4, and centrifuging. The washed cells were then lysed by dilution with one volume of glass-distilled water and gentle agitation for 30 min at 4 °C. Stroma and any unlysed cells were removed by centrifugation at 50 000 x *g* for 20 min. Samples (1.0 ml) to be electrofocused were applied to a 210 x 6 x 1 mm filter paper wick. The wick was placed 1 cm from the anode and electrofocusing was in the direction from anode to cathode. All other elements of the electrofocusing were as described above.

## 3 Results

### 3.1 Schlieren patterns in IEF gels

Using the apparatus shown in Fig. 1 and a pattern of 45 ° lines, schlieren peaks such as those shown in Fig. 2 were obtained. The curves represent the first derivative of the gel thickness curve and their interpretation is given diagrammatically in Fig. 3. Note that a ridge running the length of the gel (perpendicular to the electrical field) appears as two peaks, one above and one below the base line, and that the actual peak of the gel ridge is at the point where the schlieren curve crosses the base line. Scaling is accomplished by spacing the diagonal lines 1 cm apart as measured in a horizontal

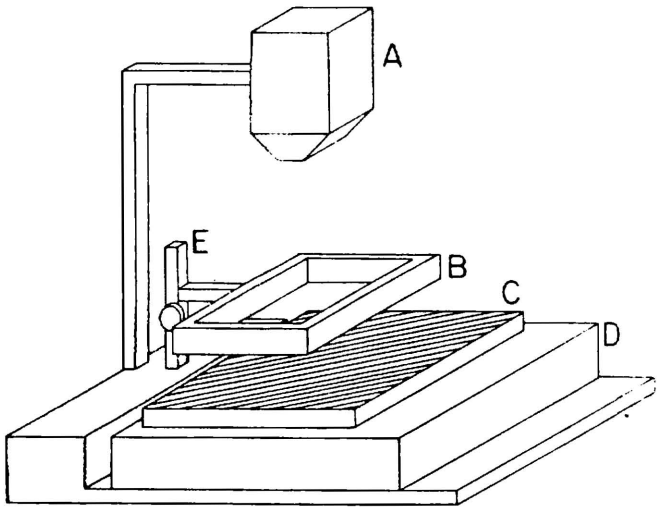


*Figure 1.* Apparatus used for obtaining photographs of schlieren patterns. (A) Camera. (B) Gel plate rack. (C) Plate with diagonal lines. (D) Light box. (E) Rack adjustment.

direction. Peak height data may then be readily converted to cm. The normalized peak height in cm, $h$, and the distance between the IEF gel and the diagonal line plate in cm, $d$, may be used to calculate the maximum angle of refraction by

$$\tan = \frac{h}{d} \quad (1)$$

For a first approximation the refractive index contribution of the ampholytes themselves may be disregarded and only the refractive index of water itself considered. The refractive index, n, is given by the equation

$$n = \frac{\sin i}{\sin p} \quad (2)$$

where i is the angle that the incident light makes with the gel surface, and p is the angle of refraction after entering. Refraction of light entering and leaving the underlying glass plate is considered self-canceling. It is thus possible to calculate the maximum slope of the gel ridges and to integrate the areas under the curve for quantitation purposes.

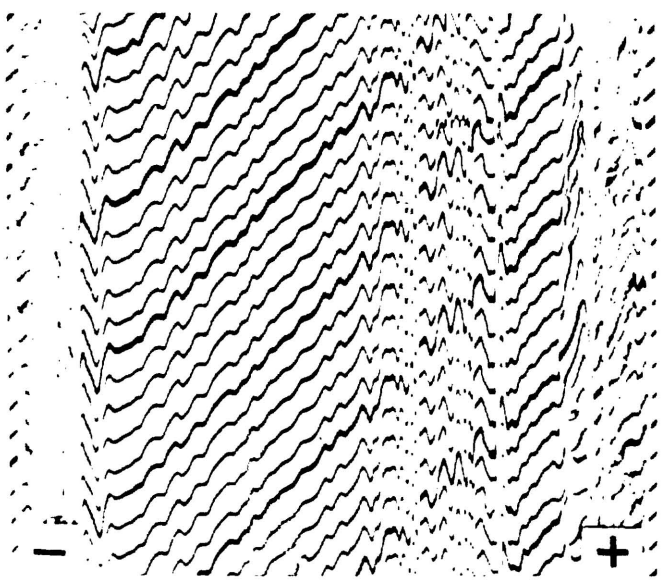


*Figure 2.* Portion of a gel showing a typical schlieren pattern obtained using Brinkman pH 2–11 wide range ampholytes.

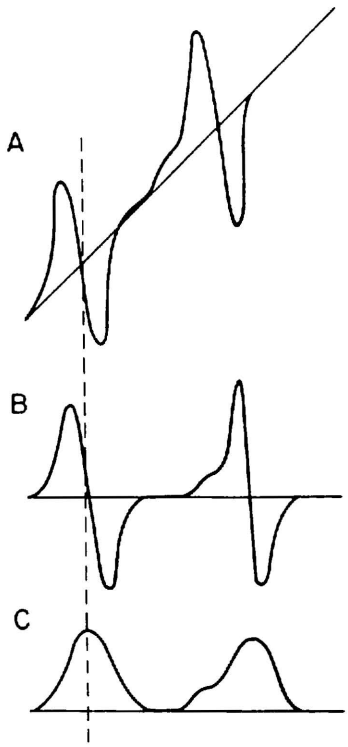


*Figure 3.* Diagrammatic interpretation of results. (A) Observed schlieren pattern. (B) Normalization of pattern to rectilinear coordinates. (C) Integration of curve B giving true gel thickness pattern.

### 3.2 Reproducibility of schlieren patterns

We have found that the schlieren patterns observed using a single batch of ampholytes is extremely reproducible between runs. However, ampholytes obtained from different sources are not alike. A comparison of ampholytes obtained from three different companies but covering approximately the same pH range is shown in Fig. 4. The gels were sequentially cast between one set of glass plates and thus the three were run in parallel. Distinct differences can be seen between A and B and between B and C. However, A and C appear to be almost identical. In all three brands of ampholytes, the acid side of the gel has a greater abundance of sharp, narrow peaks, while in the alkaline regions the peaks are much broader and less well-defined. Although no differences have been seen in our series of experiments, it is not unlikely that minor differences between ampholyte batches from the same manufacturer may also exist.

### 3.3 Reality of ridges

Two experiments were done to explore the possibility that the ridges and the corresponding schlieren patterns observed in the gel might be entirely due to differences in the concentration of carrier ampholytes through the gel and that the thickness of the gel was actually constant. In the first experiment, a focused gel was placed on a black background and photographed using oblique light. A pattern of ridges was observed as shown in Fig. 5 A. The ridges correspond to the illuminated areas while the shadowed areas correspond to valleys. The second experiment was to determine whether the

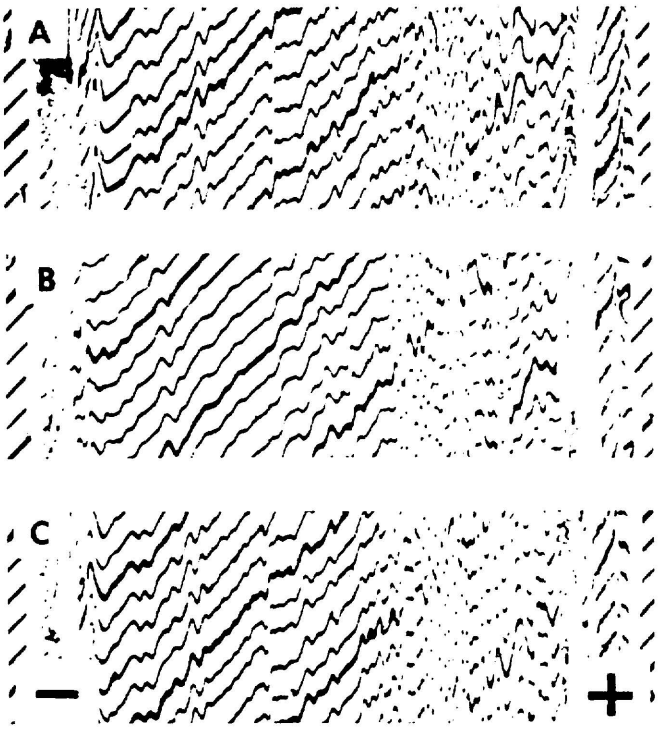


*Figure 4.* Section from a gel in which three commercially availabe wide-range ampholytes were run in parallel. (A) Brinkman pH 2–11, (B) LKB pH 3.5–10. (C) Bio-Rad ph 3–10.

schlieren pattern was due directly to the concentration of ampholytes. A shallow glass tray was arranged so that the gel could be photographed while immersed in water. If the refractive index changes are due directly to differences in ampholyte concentration in the gel, then the schlieren peaks should remain the same. The results of this experiment, shown in Fig. 5B, suggest that the schlieren pattern is due primarily to localized hydration of the gel and only secondarily to the distribution of focused carrier ampholytes. As will be shown later, the concentration of ampholytes, as expected, directly affects the degree of localized hydration and consequently the observed peak height.

### 3.4 Time course of ridge formation

To monitor the formation of the ridges and hence the pH gradient, photographs of the developing schlieren pattern were taken as shown in Fig. 6. Ridge formation proceeded rapidly after the first hour of electrofocusing and continued for an additional 75 min. Extending the focusing time resulted in extreme condensation at the top and bottom edges of the gel (results not shown) and frequently led to electrical problems. Although no cathodal displacement of the pattern was observed under our conditions using relatively short focusing time, it has been clearly demonstrated with prolonged focusing as described by Jonsson *et al.* [7]. Since a given batch of ampholytes always gave the same pattern with time, the quality of a given experiment may easily be monitored by comparing schlieren patterns with previous runs. Disturbances in the gel and hence the pH gradient can be readily observed.

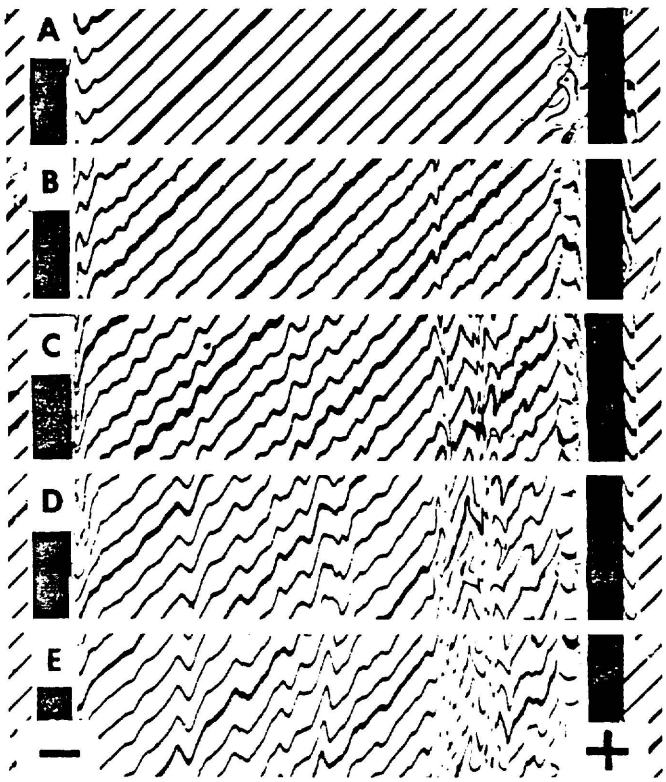


*Figure 6.* The time course of ridge formation, demonstrated by photographs of the gel during electrofocusing. (A) 30 min, (B) 60 min, (C) 90 min, (D) 120 min, and (E) 135 min. The dark bands at the edges are electrode strips which were not removed for photography.

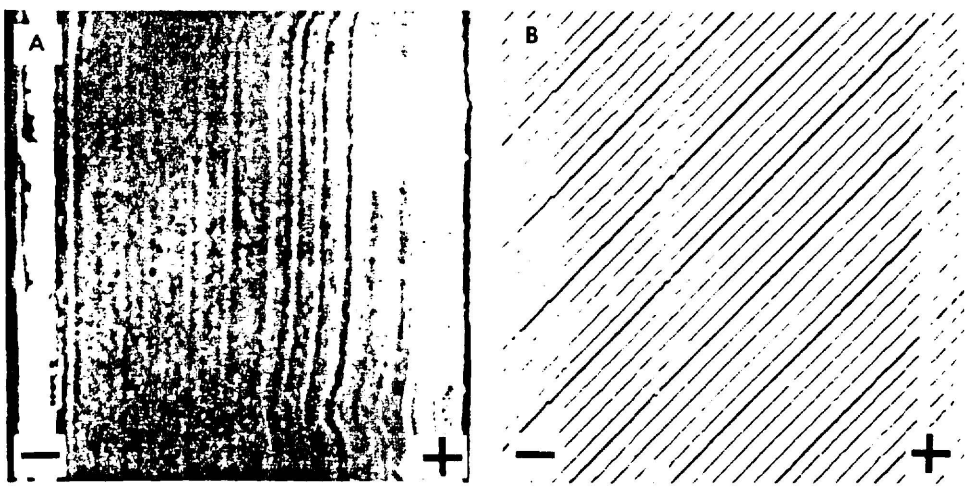


Figure 5. (A) Photograph of a section of a gel using oblique lighting. Ridges and valleys can be seen as alternating light and dark bands. (B) Photograph of a gel submerged in water. The slight schlieren pattern remaining visible is presumably due to focused ampholytes.

### 3.5 Patterns without evaporation

If the conductivity of the gel varies inversely with ampholyte concentration, then greater resistance heating should occur between species of focused ampholytes producing excess evaporation. To determine whether evaporation produced valleys of dehydrated areas, experiments were run in which evaporation was prevented. The top half of the IEF slab gel was carefully covered with Saran film over the entire area, excepting that required for electrode wicks. The gel was run in the normal manner and the schlieren pattern was shown to be almost identical in the two portions (Fig. 7). However, a significant amount of evaporation does occur during electrofocusing. By carefully weighing the gel and plate before and after a typical run, it was found that 4.5 % of the initial weight of the gel was lost due to evaporation. Nevertheless, this loss does not appear to contribute significantly to the observed formation of the ridges.

### 3.6 Ampholyte concentration versus peak height

If the banding of carrier ampholytes electrophoresed into zones causes the observed schlieren pattern, then the peak height observed in the pattern should directly reflect the concentration of ampholytes. To explore this possibility, a gel was cast in which one half of the gel contained the normal concentration of ampholyte (2.0 % w/v) while the other portion contained 2.4 times the normal amount (4.8 % w/v). After electrofocusing in the normal way the schlieren patterns were observed. The schlieren peaks obtained with the greater concentration of ampholyte were higher, sharper, and more well-defined throughout the gel, and especially in the neutral and alkaline regions (Fig. 8). Increases in peak height were therefore directly a result of increased ampholyte concentration.

### 3.7 Visualization of banded ampholytes

To demonstrate that the ampholytes have focused into discrete bands under the conditions employed, ampholytes containing an attached chromophore were electrophoresed in the normal manner. Distinct banding of isoelectrically focused ampholyte species was observed, as shown in Fig. 9. The separation between some of the bands appeared to be complete, suggesting that the ampholyte mixture did not possess a continuous spectrum of isoelectric points.

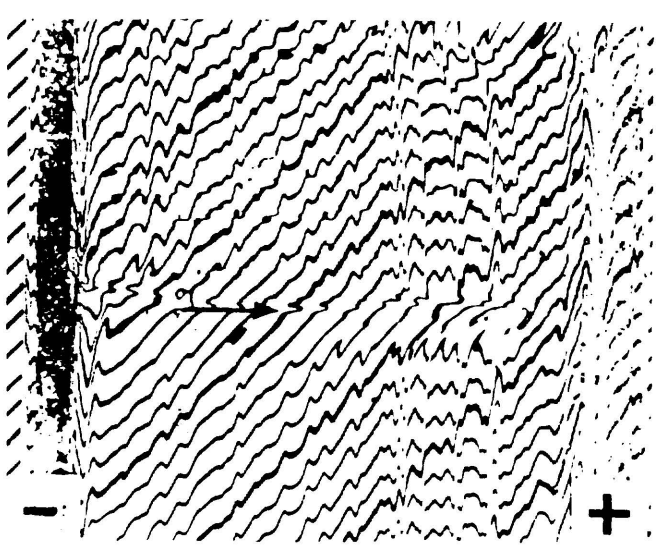


*Figure 7.* Section of a gel in which evaporation was prevented. Top portion of the gel was covered with film while bottom portion was run under normal conditions. The arrow shows the boundary between the two portions of gel. Ampholytes were Brinkman pH 2–11.

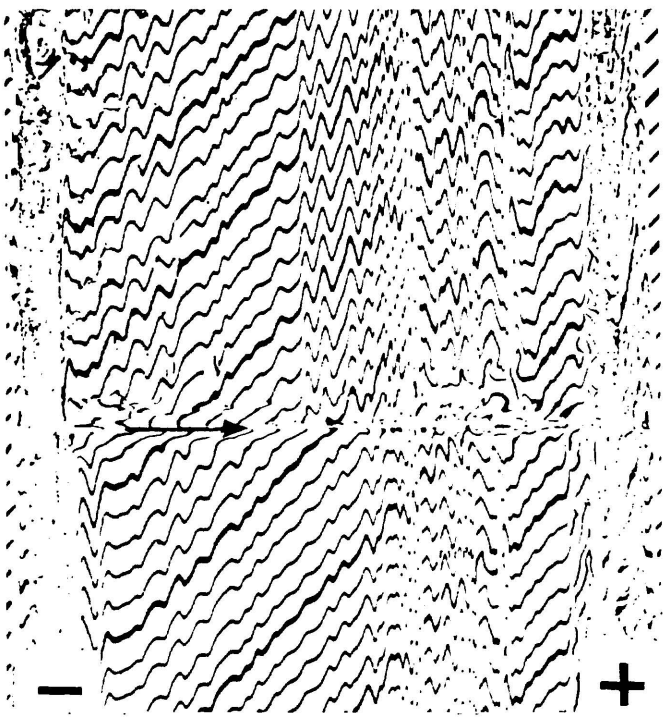


*Figure 8.* Section of a gel in which two concentrations of ampholytes were electrofocused. The top portion of the gel contained 4.8 % w/v of Brinkman pH 2–11 ampholytes while the bottom portion contained 2.0 % w/v. The arrow marks the junction between the two portions of the gel.

### 3.8 Schlieren patterns as guides to protein positions

In order to utilize the schlieren pattern as a guide to protein banding, a number of criteria must be met. First, the pH gradient across the gel should be consistent between runs. Second, a given schlieren peak should always represent the same pH region, and third, the same protein should always band in the same position relative to a given peak. The pH gradient of a typical run is given in Fig. 10, which shows the curve determined from 3 mm gel slices as described in section 2.6. Values of pH in the respective fractions were reproducible between runs. The reproducibility of protein banding was determined by using human red blood cell lysates as a source of protein and the position of the hemoglobin observed is shown in Fig. 11. Ridges containing focused hemoglobin bands were cut out as described in section 2.6. The pH values of the three bands were consistently (A) 7.64, (B) 7.32, and (C) 7.13. Fig. 12 illustrates the differences in the banding characteristics of hemoglobin when ampholytes from two sources were compared. In all experiments, regardless of the source of ampholytes, the well-focused, minor bands of hemoglobin always migrated to the same relative positions. Identical results were obtained in numerous experiments, suggesting that the schlieren pattern can serve as a relibable guide to the position of focused proteins.

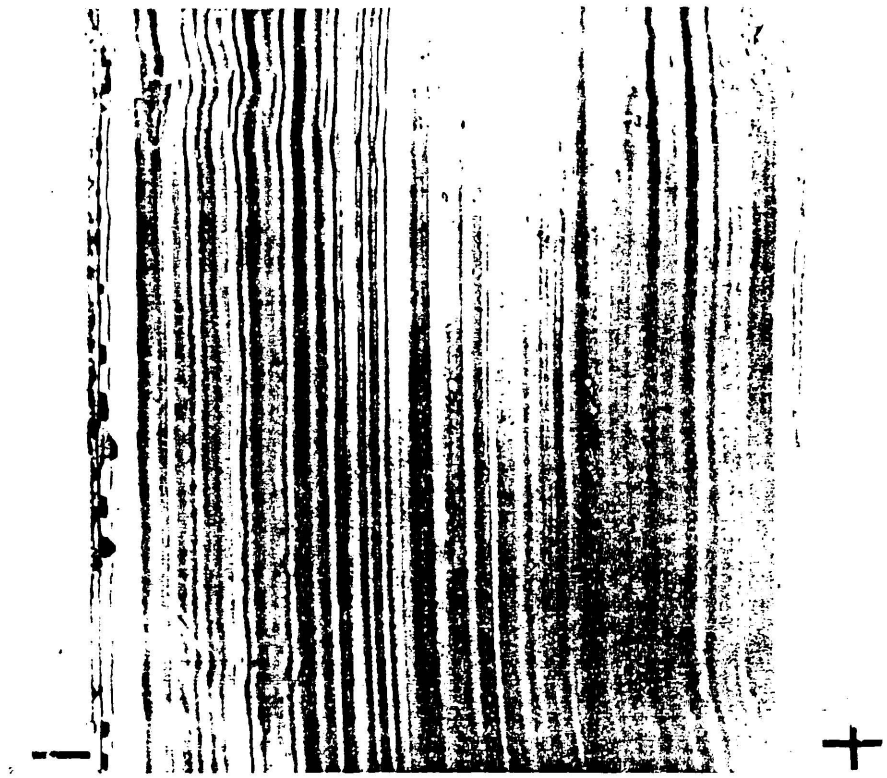

*Figure 9.* Section of a gel focused with LKB pH 3.5–10 ampholytes containing an attached chromophore.

## 4 Discussion

Isoelectrically focused slab gels can provide a high resolution preparative procedure for protein purification. One of the major drawbacks to the use of this method has been the necessity for staining at least a portion of the gel to visualize focused material. We have devised a method, based upon simplified schlieren optics, whereby a given region of the gel can be reproducibly located without prior staining. Schlieren patterns observed in IEF slabs can provide a rapid, simple, and inexpensive method for localizing focused iso-pH lines or regions (and hence focused proteins), and for assessing the quality of the pH gradient during and following electrofocusing. Although differences in schlieren patterns occur between commercially available mixtures, the patterns for a given batch are consistently reproducible. The observed schlieren patterns result from differences in the thickness of the gel due to localized hydration caused by focusing of the carrier ampholyte. We have shown that ridge formation is not the result of resistance heating only, which would produce differential evaporation, nor are they directly due to differences in ampholyte concentrations across the gel. Therefore, more complex mechanisms must exist to explain the observed ridge formation.

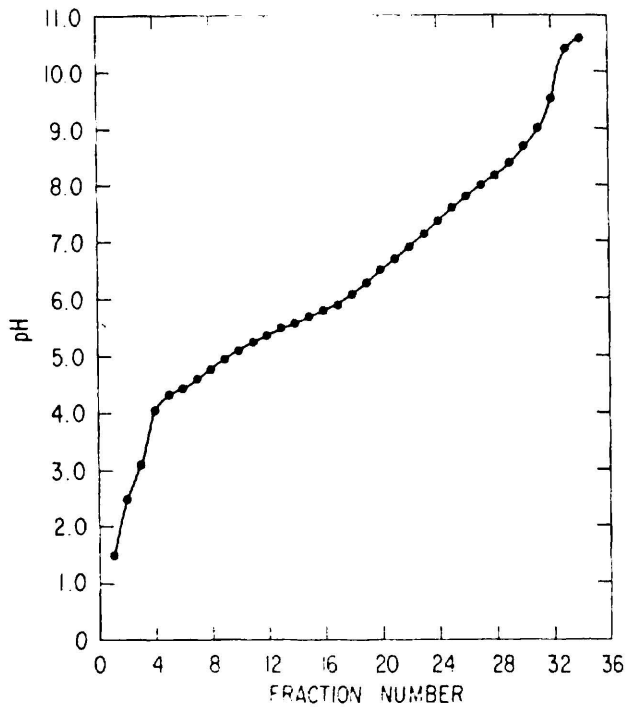


*Figure 10.* pH Gradient obtained using Brinkman pH 2–11 wide range ampholytes. Values were determined on 34 gel slices of 3 mm width. Gel from the center of each electrode to the edge was discarded.

One explanation of ridge formation may arise from consideration of endosmotic flow in ampholyte gradients. The molecular weights of ampholytes are such that they do not band as sharply with the voltage gradients used as do proteins. Hence, the distribution of a given ampholyte results from an equilibrium between diffusion, tending to spread an ampholyte band, and IEF in a pH gradient in the presence of an electrical field. In this equilibrium condition, ampholytes on the acid (andode) side of the pI assume a slight positive charge, while the opposite effect occurs on the cathode side. The net effect is similar to that of fixed charges attached to a gel, which is to induce ionization in the water, and endosmotic flow away from the center of concentration of the ampholytes in both directions. Electroendosmosis in the alkaline region of IEF gels in well known, and may be explained by residual negative charges in the acrylamide gels [10]. Differential water movement as observed here cannot

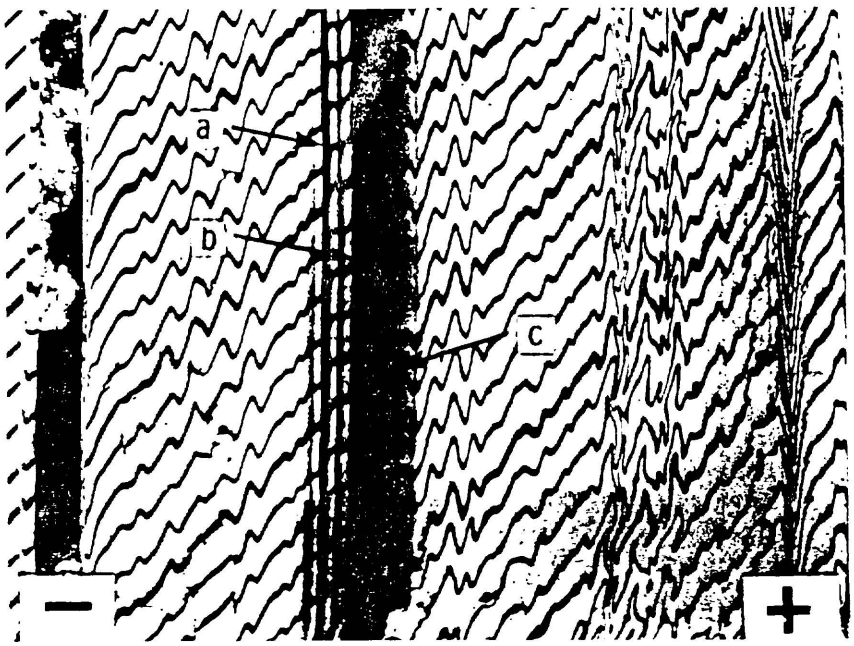


*Figure 11.* Section of a gel showing banding of hemoglobin after focusing using LKB pH 3.5–10 wide-range ampholytes. Peaks containing the indicated hemoglobin bands were removed and pH values determined. (A) 7.64, (B) 7.32, and (C) 7.13.

be due to these residual charges, as this would mean that a new version of electroendosmosis would be envoked as follows. Each ampholyte species is driven to form a sharp band by the pH differences existing on either side of its isoelectric point. However, zones are formed between different ampholyte species by diffusion, and these zones define the pH gradient. If we consider one species of ampholyte present in excess, it is evident that only the center portion of its band will be at its isolelectric point, while the remainder of that species will be on either side of this point. The net effect of ampholyte on the acid side of its isoelectric point would be to produce excess positive charges, which in turn would produce an electroendosmotic flow toward the anode. The reverse occurs on the cathode side, with the result that water flows away in both directions from the isoelectric point.

Another possible explanation for localized hydration of the gel is that differences in ampholyte concentration produce marked differences in osmotic pressure across the gel, which lead to a redistribution of water; *i. e.*, water is forced to flow toward areas of high ampholyte concentration. With substances of relatively low molecular weight, such as ampholytes, the focused band may be quite broad resulting in only a shallow gradient in osmotic pressure.

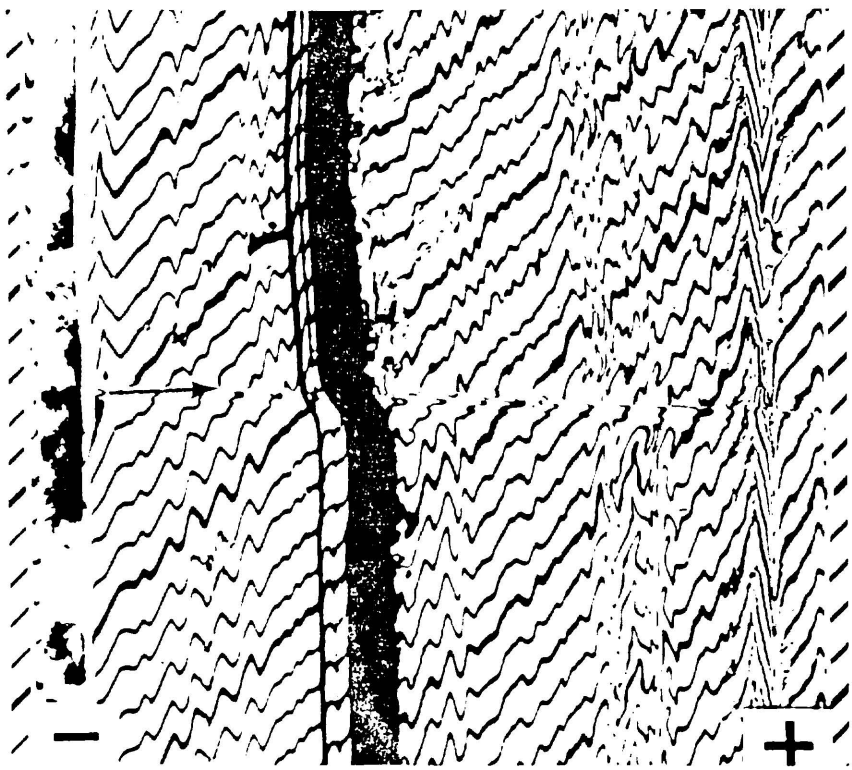


*Figure 12.* Banding of human hemoglobin proteins in two commercially available ampholytes. Top portion of the gel contained Brinkman pH 2–11 ampholytes while the lower portion contained LKB pH 3.5–10 ampholytes. The samples were focused in parallel. The arrow marks the junction between the two gel sections.

The question, therefore, is the relative importance of the electroendosmotic and osmotic effects. The electroendosmotic effect, being opposite on the two sides of the focused ampholyte, would result in a depression where the ampholyte is found since the water would be drawn away from the center. The osmotic effect is quite the opposite, and would result in a raised water-rich ridge where the ampholyte is focused.

We have not conclusively determined which of these effects plays the primary role in ridge formation. However, based upon preliminary interpretation of the schlieren patterns, and on the localization of focused protein bands relative to the schlieren curve, it appears that the osmotic pressure exerted by the focused ampholyte species draws water from adjacent regions where the ampholyte concentration is lower. Additional experimentation is planned to further elucidate the mechanisms involved in ridge formation. Although the exact nature of the observed ridges and their formation is not known, the use of this optical technique will [1] provide a method for the evaluation of a given ampholyte batch, [2] emphasize irregularities present in a focused gel, [3] offer a means for evaluating the reproducibility of focusing between runs, and [4] provide a rapid and reproducible method for localizing a particular region of the gel without prior staining or pH determination.

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