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Note

Extension of the alkaline end of a pH gradient in thin-layer polyacrylamide electrofocusing gels by addition of N,N,N′,N′-tetramethylethylenediamine

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The use of electrofocusing for the analysis and separation of proteins in complex mixtures and the determination of protein isoelectric points is now very widespread. Polyacrylamide gels in glass tubes ("rods") are often used when a few samples are to be analysed; horizontal thin-layer polyacrylamide gels are, however, preferable when many samples are to be studied together, because of the ease of comparison of band patterns and of handling of both gel and samples.

Because the upper surface of the horizontal gel is open to the atmosphere, carbon dioxide can be absorbed, and it is difficult to produce a pH gradient extending above about pH 9.5 unless special precautions are taken[1]. Thus, electrofocusing cannot readily be used to analyse important basic proteins such as the cytochromes and histones.

In this Note, a simple and economical method for extending the alkaline end of the pH gradient in a horizontal thin-layer polyacrylamide gel is described: namely, the addition of N,N,N′,N′-tetramethylethylenediamine (TEMED), an accelerator of the polymerization of acrylamide in gels used for electrophoresis. TEMED is not required for polymerization of gels used for electrofocusing, but its addition allows the alkaline end of a nominal pH 3.5–9.5 gradient to be extended to above pH 11.

MATERIALS AND METHODS

Acrylamide and N,N′-methylenebisacrylamide (bis), both "specially prepared for electrophoresis", and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from BDH (Poole, Great Britain). Ammonium persulphate, analytical grade, was purchased from E. Merck (Darmstadt, G.F.R.). Ampholine® carrier ampholytes, pH 3.5–9.5 (LKB Cat. No. 1818-101) were employed. Cytochrome *c*, type III from horse heart, and trypsinogen, from bovine pancreas, were obtained from Sigma (St. Louis, MO, U.S.A.). For haemoglobin, a haemolysate from washed human red blood cells was used.

The gel solutions were prepared and the gels cast according to the LKB 1818-P instructions for 0.5-mm thin-layer electrofocusing polyacrylamide gels[2]. The gel solu-

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tion used to fill the mould was prepared by mixing 3.5 ml of 29.1 % (w/v) acrylamide, 3.5 ml of 0.9 % (w/v) bis, 12.0 ml doubly distilled water and 1.5 ml of LKB 1818-101 Ampholine carrier ampholytes, pH 3.5–9.5. After deaeration, 0.5 ml 1 % fresh ammonium persulphate solution (and TEMED, where used) were carefully added, mixed and the solution injected into the prepared mould.

When gels containing TEMED were run under the conditions recommended for gels prepared without TEMED, a burn line was often produced in the gel near the cathode after about 20 min. To avoid this, the LKB 2197 power supply was run at constant power with the following limiting conditions: 25 W, 1600 V and 30 mA. The running time in all cases was 80 min. Gels were run in the LKB Multiphor apparatus, cooled by water circulated at 10°C from an LKB MultiTemp thermostatic circulator. 1 *M* Phosphoric acid was used as anode solution, and 1 *M* NaOH as cathode solution.

The pH gradient across the gel was determined immediately after the power supply was switched off on completion of electrofocusing, using an LKB Multiphor surface pH electrode. The electrode was calibrated with standard buffer solution (Dr. W. Ingold AG, Urdorf ZH, Switzerland; ordering No. 9805) pH 6.841 at 10°C, which was temperature-equilibrated in a cooling jacket connected in series with the Multiphor. The pH gradient was measured at 1-cm intervals (0.5 cm near the cathode) across the 10 cm of gel between the electrode strips; the twelve points were usually measured within a total of 3–5 min. After the pH gradient had been determined, the protein bands were sharpened again by refocusing for another 10 min at the same settings.

The proteins were stained after refocusing, using Serva Blau R (equivalent to Coomassie Brilliant Blue R-250)[2].

RESULTS AND DISCUSSION

The tertiary amine, TEMED, is widely used as an accelerator in the production of polyacrylamide gels by chemical or photochemical polymerization (with ammonium persulphate or riboflavin as initiator, respectively)[3]. As early as 1973, however, it was found[4] that TEMED could be omitted from the gel solution when polyacrylamide gels containing LKB Ampholine carrier ampholytes for electrofocusing were prepared: the Ampholine itself contains enough tertiary amino groups to act as accelerator, although perhaps less efficiently than TEMED.

In our work, polymerization of a 0.5-mm thin-layer gel containing Ampholine carrier ampholytes, pH 3.5–9.5, normally takes 30–40 min in the absence of TEMED, but only 10–15 min when TEMED is present (50 μl in 20 ml of gel solution). TEMED also accelerates polymerization of the gel with Ampholines of other pH ranges, except below pH 4.5 where the addition of silver ions is necessary.

TEMED was also found to increase the pH in the gel at above pH 4.5. Fig. 1 shows the effect of TEMED in the most often used pH range of 3.5–9.5. The increase in pH is greatest at the cathodic end of the gel, and depends on the amount of TEMED used: in 20 ml of gel solution, 50 μl TEMED raises the pH at 0.3 mm from the cathode by 0.7 pH units, and 100 μl TEMED by 1.3 pH units. The highest pH obtainable in the horizontal thin-layer gel system with the nominal pH range 3.5–9.5 is thus raised from pH 9.5 to above pH 11. Basic proteins such as the histones,

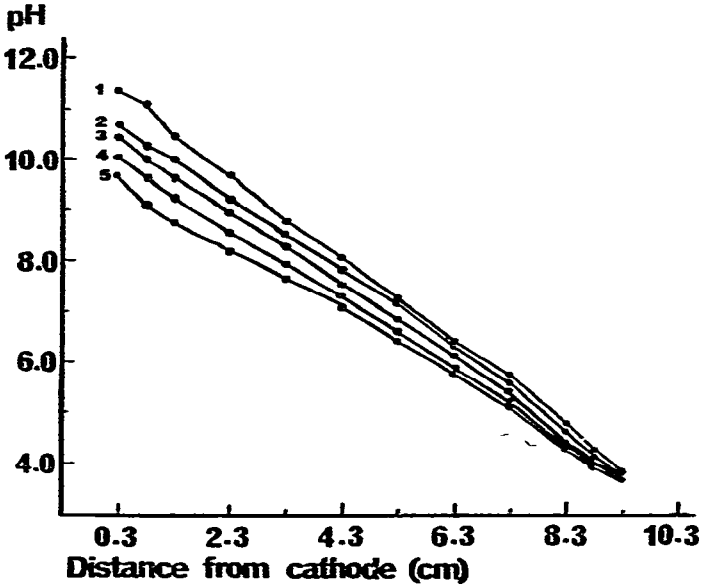

Fig. 1. Extension of a nominal pH 3.5–9.5 gradient upon inclusion of different amounts of TEMED in the gel solution. To 20 ml gel solution were added 100 μl (1), 75 μl (2), 50 μl (3), 25 μl (4) and 0 μl (5) TEMED.

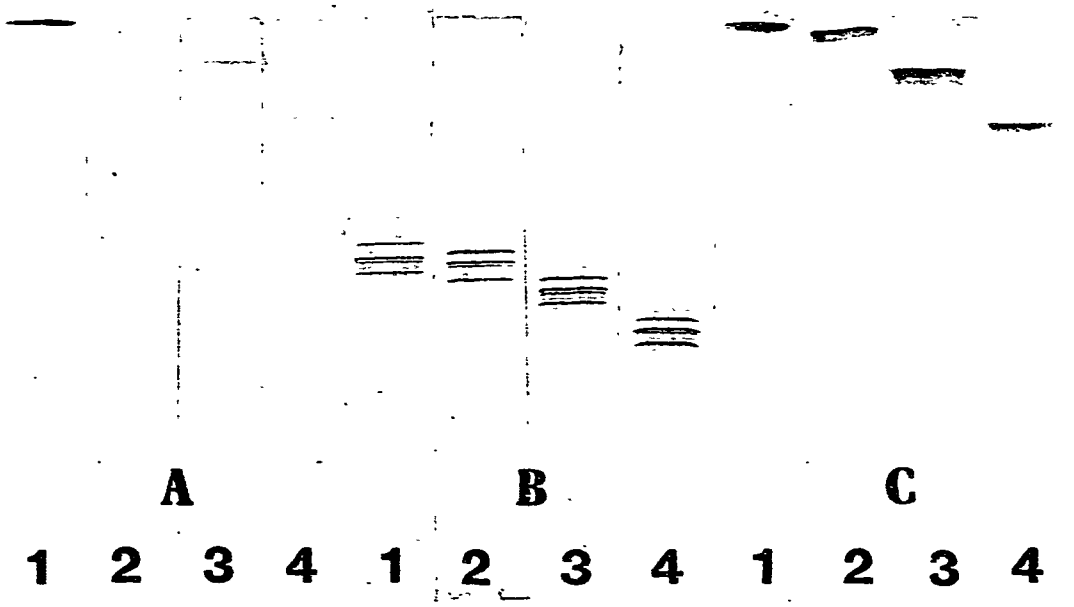

Fig. 2. Shift in the position of focusing of cytochrome *c* (A), haemoglobin (B) and trypsinogen (C) in nominal pH 3.5–9.5 gels supplemented with no TEMED (1), 20 μl (2), 50 μl (3) and 100 μl (4) TEMED. Cathode at the top.

cytochromes and lysozyme should now be easily studied by the high resolution of electrofocusing.

The results shown in Fig. 2 confirm the elongation of the pH gradient upon inclusion of TEMED in the gel solution. As the amount of TEMED is increased, the bands of the three proteins tested focus farther away from the cathode. Cytochrome *c* from horse heart focuses next to the cathode strip in the absence of TEMED, but 17 mm away from it in a gel supplemented with 100 μl TEMED. In gel A2, three bands of cytochrome *c* may be discerned, but in A3 and A4, only one: this is probably due to the fact that resolution is diminished when the pH range is extended[5]. Although elongation of the pH gradient is not necessary when haemoglobin is examined, it has been included here to show that the band pattern is not affected by the concentration of TEMED in the gel, although TEMED does slightly affect the separation of the four major bands.

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