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Note

Isoelectric focusing of insulins in polyacrylamide gel

Isoelectric focusing in sucrose gradients, when applied to the separation of insulin and similar proteins, has the disadvantage that precipitation occurs before the isoelectric points are reached. Separations by isoelectric focusing in polyacrylamide gels are not disturbed by precipitation, however, even though microgram amounts of samples are used. There have been several reports of the successful use of isoelectric focusing in rods of polyacrylamide[1–4], although some difficulties have been experienced in achieving reproducible pH gradients.

This paper describes both an improved disc electrophoresis apparatus for gel isoelectric focusing and buffer systems that produce reproducible pH gradients over the nominal range. Under the conditions described, the high resolving power of isoelectric focusing enables the minor components in insulin preparations of different species to be compared.

Material and methods

Ampholyte solutions, pH range 3–10 and 4–6, were obtained from LKB Producter AB, Bromma, Sweden. N,N,N',N'-Tetramethyldiaminoethane, acrylamide and N,N'-methylenebisacrylamide were obtained from Serva, Heidelberg, G.F.R., and the latter two were recrystallized from acetone and chloroform, respectively. Bovine insulin (normal Prod. Charge and standard grade) and porcine insulin (Ch. 010172) were generously supplied by VEB Berlin-Chemie. Pork insulin (Lot No. S 21 166 and Act B 66) were supplied by Novo, Copenhagen, Denmark. Human insulin was prepared from human pancreas[5]. Proinsulin and intermediate were isolated from commercial bovine insulin[6,7]. Bovine and porcine insulin were freed from proinsulin by gel chromatography on Sephadex G-50$_{fine}$ (Pharmacia, Uppsala, Sweden) in 5 *M* acetic acid–0.15 *M* sodium chloride solution.

Apparatus. We improved the apparatus that is commonly used for disc electrophoresis (Fig. 1). This apparatus provides efficient cooling of the gels and minimises pH and concentration gradients between the gel and electrodes by using small volumes of electrolytes. In order to prevent the accumulation of gas bubbles at the cathodic end of the gels, the platinum wire was mounted on a frame of polyacrylate (Fig. 1, lower part).

Electrofocusing conditions. Acrylamide gels were prepared as described by CONWAY-JACOBS AND LEWIN[8], except that the volumes of the solutions used in our glass tubes (6.0 mm I.D. and 120 mm long) were doubled and 0.2 ml of glycerol was added to each tube.

The gel tubes were mounted as shown in Fig. 1 and the anodic and cathodic chambers were filled with 1% phosphoric acid and 1% ethylenediamine solution, respectively (see also legend to Fig. 3). For separations in the pH gradient 4–6, solutions of 0.01 *M* H_3PO_4 and 0.02 *M* NaOH were used.

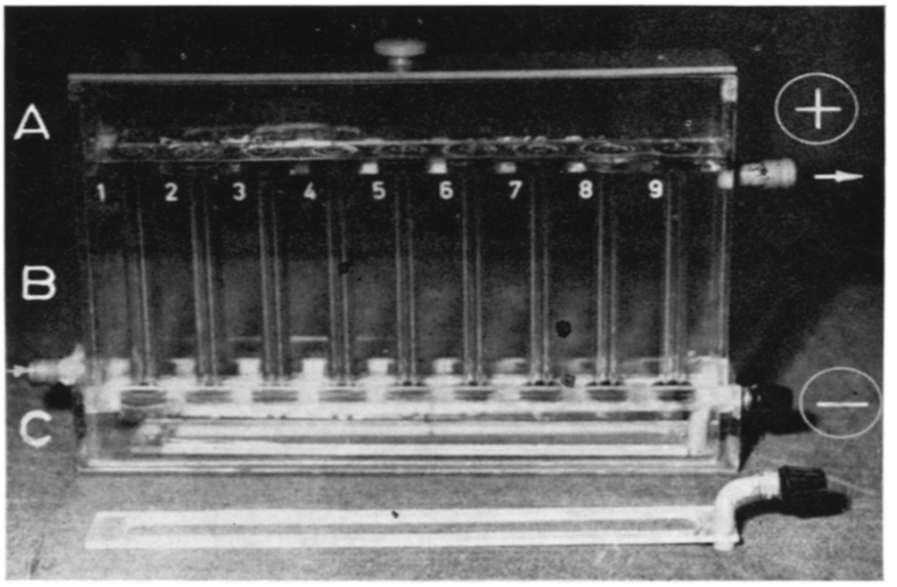

Fig. 1. Gel electrofocusing apparatus. Nine gel tubes are held in a water chamber (B) with rubber grommets. The lower electrode chamber (C) is removable. The platinum electrodes are mounted on a frame of polyacrylate and can be placed in the electrode reservoirs (A and C).

The current was regulated at 0.67 mA per tube until a potential of 320 V was reached, which was maintained for the remainder of the experiment. A total period of 9 h was used during which the gels were kept at 14° by circulating cooling water.

Measurement of the pH gradient. The gel columns were sectioned as described by CATSIMPOOLAS[2] and the pH was measured at 22° with a micro-electrode (Radiometer, Type G 2222 C-K 4112) in conjunction with a Radiometer pH meter, PHM 28.

Fixing and photography. The gels were fixed in 12.5 % aqueous trichloroacetic acid and photographed without staining. Transmitted light was used.

Results

Effect of electrolytes and ampholyte concentration on pH gradients. With the commonly used ampholyte concentration of 1 %, we were not able to obtain stable and linear gradients. When carrier ampholytes of nominal pH range 4–6 and an electrolyte system of 0.02 *M* NaOH–0.01 *M* H_3PO_4[4] were used, the pH range extended from 4.0 to 9.7 (Fig. 2, lower curve). Incorporation of ampholytes to a final concentration of 2 %, however, gave linear gradients over the desired range.

An ampholyte concentration of 2 % also improved the linearity of the pH gradients, but it proved to be impossible to obtain the nominal range from 3.0 to 10.0 when using 0.25 m*M* NaOH and 0.36 m*M* H_2SO_4 as electrolytes (Fig. 2, upper curve). Fig. 3 shows that 1 % phosphoric acid at the anode and 1 % ethylenediamine solution at the cathode gave smooth gradients over the expected range.

Effect of electrolytes on the resistance in the electrofocusing cell. Because of Ohm's law, the potential depends upon resistance at constant current and was therefore determined during focusing. It can be seen from Fig. 4 that the resistance in the gel did not increase linearly with time and was dependent on the electrolyte system

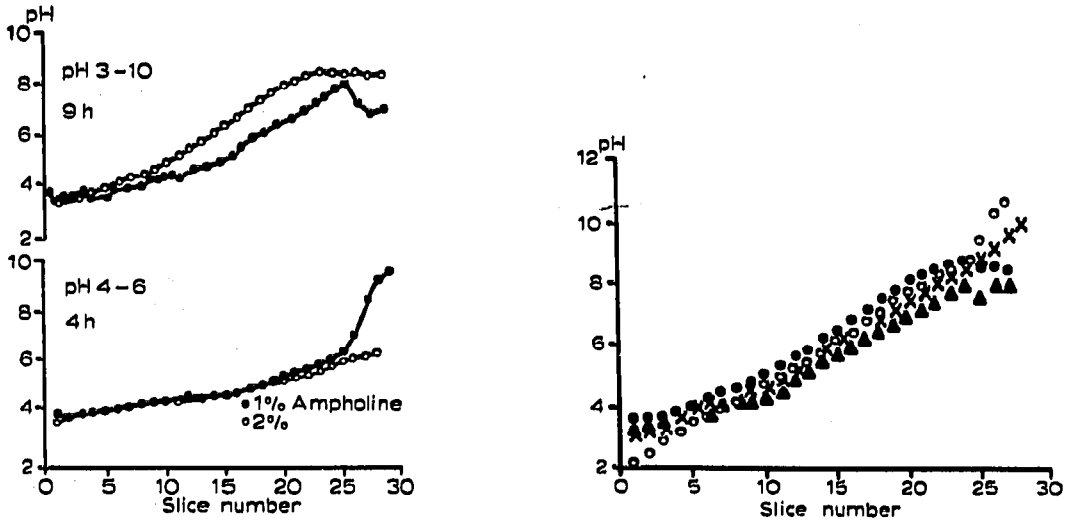

Fig. 2. Effect of ampholyte concentration on the range and shape of pH gradients. Acrylamide gels containing 1% (●) or 2% (○) Ampholyte LKB 8141 (pH 3–10) or Ampholyte LKB 8152 (pH 4–6) were subjected to focusing for either 9 or 4 h. The gels were then sliced and eluted as described.

Fig. 3. Effect of some electrolyte systems on the range of pH gradients. Acrylamide gels containing 2% Ampholyte LKB 8141 (pH 3–10) were focused for 12 h: ○, 5% H_3PO_4–5% ethylenediamine; ×, 1% H_3PO_4–1% ethylenediamine; ▲, 0.4% H_2SO_4–0.4% triethanolamine; ●, 0.36 m*M* H_2SO_4–0.25 m*M* NaOH.

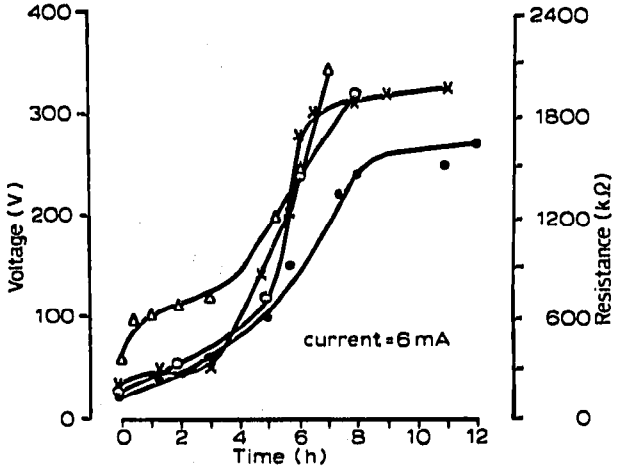

Fig. 4. Effects of various electrolyte systems on resistance in the gels containing 2% Ampholyte LKB 8141 (pH 3–10) at a constant current of 0.67 mA per tube. ●, 2.5% H_3PO_4–5% ethylenediamine; ×, 1% H_3PO_4–1% ethylenediamine; ○, 0.4% H_2SO_4–0.4% triethanolamine; △, 0.36 m*M* H_2SO_4–0.25 m*M* NaOH.

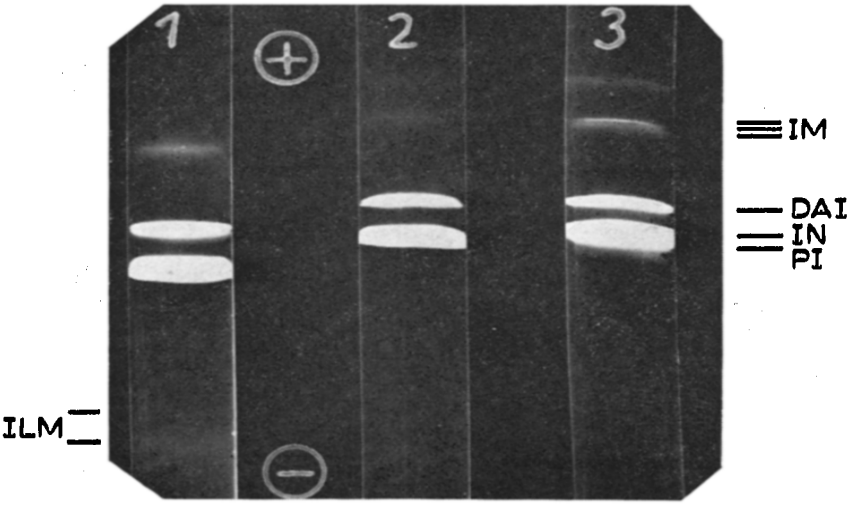

Fig. 5. Isoelectric focusing pattern of (1) human insulin (crystallized once), (2) porcine insulin (crystallized twice), (3) bovine insulin (crystallized twice) in 7.5% acrylamide gels containing 2% Ampholyte LKB 8417 (pH 3–10). Total electrolysis period, 9 h. PI = proinsulin; IM = intermediate forms; DAI = desamidoinsulin; IN = insulin; ILM = insulin-like material.

used. The asymptotic nature of the resistance with time was demonstrated for 11 h.

Heterogeneity of insulin. Fig. 5 shows that for bovine insulin, two main bands containing proinsulin and insulin, and desamidoinsulin, and three narrow bands due to intermediate forms, occurred whereas with porcine insulin one of the intermediate bands was missing. The subsidiary band in bovine and porcine insulin at the anodic side could not be identified clearly. Human insulin gave two diffuse bands at the cathodic side, which represent insulin-like material[9]. The most intense band from all three preparations consists of two merged bands that could be resolved by means of an ampholyte of narrow pH range (4–6) (results not shown).

Discussion

Because of difficulties experienced in achieving stable pH gradients, isoelectric focusing in polyacrylamide gels has not yet been sufficiently standardized for routine use, in contrast with disc electrophoresis.

The results described here show that the electrolyte composition, the concentration of the carrier ampholytes and the purity of the reagents used are important factors in gel electrofocusing.

Recrystallization of acrylamide and N,N'-methylenebisacrylamide improved the separation profile considerably. By using ampholytes with a concentration of 2%, sufficiently stable pH gradients and sharp protein boundaries were obtained. The use of higher ampholyte concentrations decreased the migration rate of the proteins without giving improved separation. As Fig. 4 shows, the use of very dilute solutions of H_2SO_4, NaOH and triethanolamine as electrolytes caused a rapid increase in the resistance with subsequent overheating of the gel. The use of phosphoric acid and ethylenediamine as electrolytes at the concentrations used in this study prevented this increase in resistance by providing sufficient electrical conductivity.

When the time of focusing exceeds the point at which the change of potential (ΔV) is small with change of time (Δt), *i.e.*, $\Delta V/\Delta t$ tends to zero, condensed bands and a cathodic shift of the pH gradient were observed.

Up to seven components (proinsulin, various intermediate forms, arginine, insulin and desamidoinsulin) could be clearly differentiated by using our procedure, whereas in disc electrophoresis only four bands were observed at the same load of 100 μg of protein per gel column. By using other experimental conditions, the heterogeneity of insulin has already been shown by isoelectric focusing[10–12].

We extended these studies to human insulin, and were able to determine the correct positions of the minor components by comparison with the purified proinsulin and intermediate forms[12] isoelectrically focused under the same conditions. Even ten-times crystallized and proinsulin-free bovine and pork insulins gave at least three clearly separated fractions. The identification of the minor components in commercial insulins is important with regard to the antigenicity of the preparations.

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Zentralinstitut für Diabetes "Gerhardt Katsch", Bereich experimentelle Diabetesforschung, 2201, Karlsburg (G.D.R.)

K.-D. KOHNERT
E. SCHMID
H. ZÜHLKE
H. FIEDLER

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