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Note

Titration curves by combined isoelectric focusing—electrophoresis on a thin layer of agarose gel

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The possibility of obtaining protein titration curves by electrophoresis in a stationary pH gradient, stabilized by focused carrier ampholytes in a flat-bed polyacrylamide gel, was demonstrated by Righetti *et al.*[1] who exploited an original idea of Rosegren *et al.*[2]. These authors showed that an analysis of the shape of the pH *vs.* mobility curves of a protein and of its genetic mutants makes it possible to determine which charged amino acid has been substituted. Krishnamoorthy *et al.*[3] used this technique to study liganded states of proteins. They were able to isolate complexes of hemoglobin with different organic phosphates, measure their half-lives, the pH range of stability and the stoichiometry of the protein–ligand complexes.

Protein–protein interactions between cytochrome b_5 and hemoglobin on the one hand[4,5] and cytochrome b_5 and cytochrome b_5 reductase[6] on the other have also been studied by this technique, allowing the determination of the nature of the amino acids involved in these interactions. This technique is also useful to define a strategy for the purification of a specific protein, based on its charge properties. Furthermore, it constitutes one of the best criteria of charge homogeneity for a protein. Thus, this simple method has become a powerful tool in studying proteins.

However, polyacrylamide gel, due to molecular sieving, restricts the mobility of many large proteins and hence for them a mobility curve cannot be obtained. In this report we establish the conditions for obtaining mobility curves in large-pore-size agarose gel and discuss the difficulties encountered and the advantages of this medium over acrylamide gel for specific problems.

MATERIALS AND METHODS

Agarose IEF, Pharmalytes (pH range 3–10), gel bond plates and electrode strips (6 × 10 mm) were obtained from Pharmacia (Uppsala, Sweden). Ampholine PAG plates, pH gradient 3.5–9.5 were from LKB (Stockholm, Sweden). Sorbitol and

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Coomassie Brillant Blue R250 were from E. Merck (Darmstadt, G.F.R.). All other reagents were of the best available grade.

Agarose gels (1.3 mm thick) were prepared in the following way. Agarose and sorbitol, 1% and 12% (w/v) final, respectively, were dissolved in 15 ml of double distilled water by heating under constant stirring in a water-bath at 95°C. The solution was then allowed to cool and Pharmalytes were added (6.3%, v/v) when the temperature reached 75°C. The mixture was layered on a gel bond plastic plate disposed with its hydrophilic side upwards on an horizontal support preheated at 60°C. The dimensions of the gel were limited by a plexiglass cast (120 × 120 mm). After gelification at room temperature, the gel was left in a humid atmosphere at 4°C overnight to increase its mechanical strength. Excess of liquid was absorbed from the gel with Whatman No. 3 MM filter-paper. Electrode strips were soaked in 1 *M* sodium hydroxide solution on the cathodic site and in 0.05 *M* sulphuric acid on the anodic side. The pH gradient was preformed by applying a power of 0.5 mW/mm^3 with an LKB 2103 constant-voltage power supply for 90 min at 8°C. At this point a 10 cm long incision was made with a scalpel in the middle of the gel perpendicular to the electrode strips to be used as sample well.

Both anodic and cathodic regions were then removed by cutting the gel on the inner side of the electrode strips. Salt-free sample (7–10 μl containing 100–200 μg of protein) was applied in the well and electrophoresis was run perpendicularly to the preformed gradient using the same electrolytes. To insure a good penetration of the sample into the gel, the voltage was first maintained at 500 V for 3 min. Electropho-

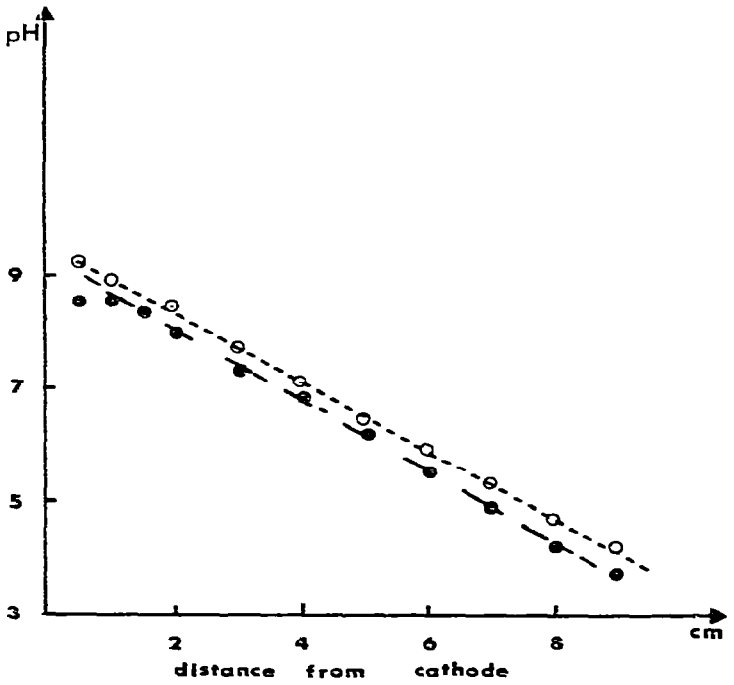

Fig. 1. Comparison of the pH gradient obtained in a PAG plate (○) and in agarose IEF gel (●).

resis was then pursued at 1000 V for 7 min. At the end of the electrophoresis, the pH gradient was measured as described previously[7] and the gel was fixed and stained according to ref. 8.

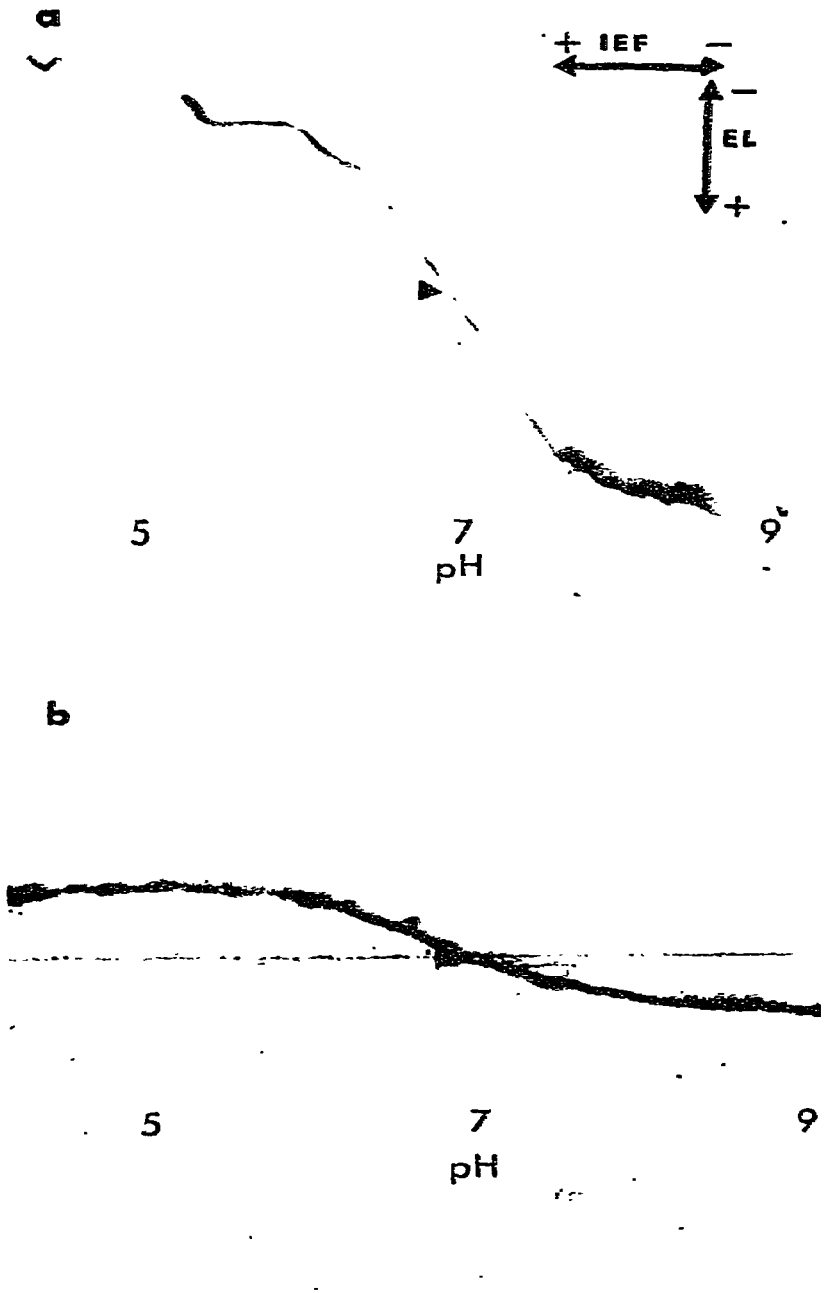

Fig. 2.

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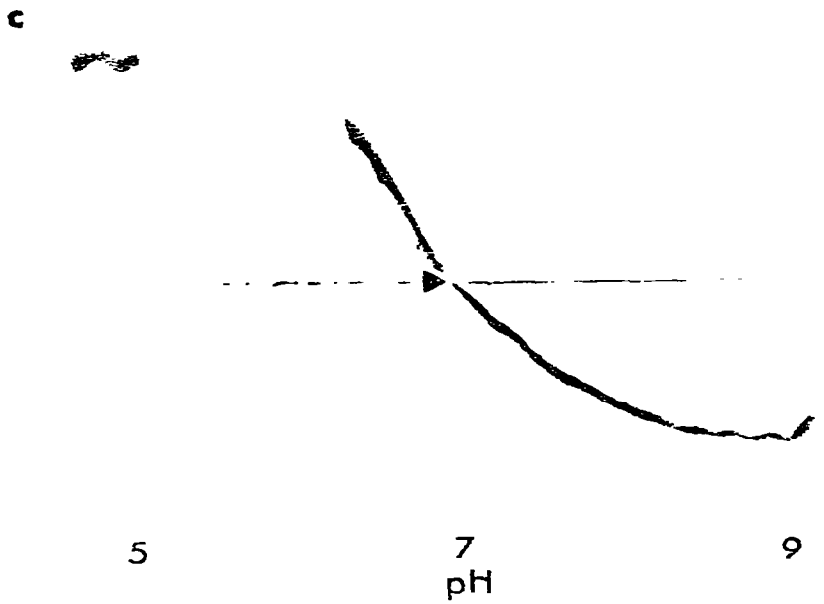

Fig. 2. Titration curves of total red blood cell lysates. The bidirectional arrows and the − and + symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (EL). The position at which the titration curve crosses the application well (indicated by an arrow) represents the zero-mobility point of the macromolecule, *i.e.*, its isoelectric point (p*I*). Gel fixing and staining are as described[11]. a, Agarose IEF gel. Conditions: IEF in the first dimension as described in the text; EL in the second dimension for 3 min at 500 V then for 7 min at 1000 V (constant voltage). b, PAG plate gel, pH gradient 3.5–9.5. Anode electrode solution: 1 *M* H_3PO_4. Cathode electrode solution: 1 *M* NaOH. Conditions: IEF in the first dimension at 7 W (constant power) for 90 min, 700 V at equilibrium; EL in the second dimension for 3 min at 250 V then 7 min at 400 V (constant voltage). Temperature = 4°C. c, PAG plate gel, pH gradient 3.5–9.5. Conditions as in Fig. 2b, except that EL was performed for 3 min at 250 V and then for 25 min at 400 V (constant voltage) to obtain a curve with a comparable mobility as in Fig. 2a.

RESULTS

As a first step the formation of the pH gradient in the agarose gel matrix was compared with that obtained in polyacrylamide gel. As shown in Fig. 1 a pH gradient can be generated along the gel. The shape of the gradient is similar and linear in both cases.

A total red blood cell lysate was used to show the feasibility of obtaining titration curves in agarose IEF gel. Fig. 2a shows the results obtained using the conditions described above. A sharp titration curve is generated which crosses the sample well at pH 6.95 corresponding to the p*I* of hemoglobin A. This result has been compared to those from a PAG plate LKB gel, pH gradient 3.5–9.5.

Fig. 2b shows the titration curves obtained after an electrophoresis of the same duration (10 min) as that used in agarose IEF gel, whereas in Fig. 2c a longer electrophoresis duration (28 min) had to be used to achieve a similar mobility as in agarose IEF gel.

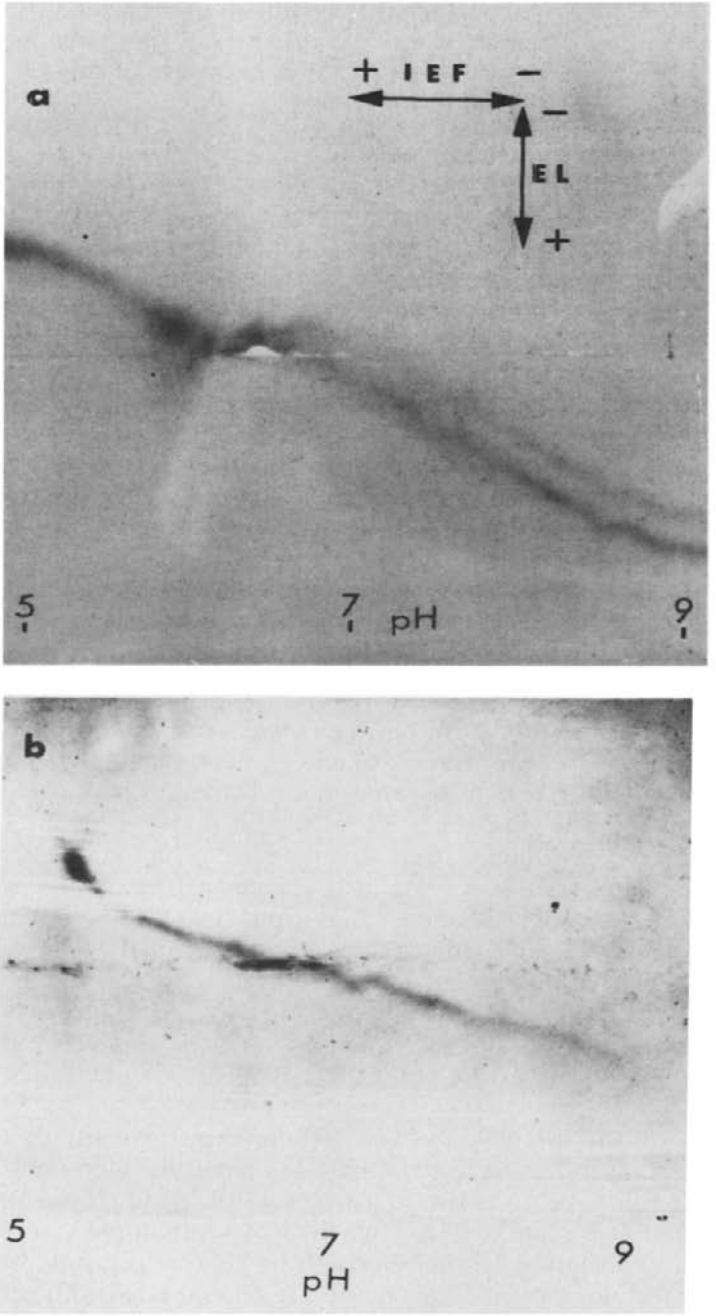

Fig. 3. Titration curves of a mutant of NADH cytochrome b_5 reductase (Diaphorase). The sample was the non-heme protein fraction purified from total red blood cell lysates according to ref. 12. Specific staining for NADH cytochrome b_5 reductase was performed according to the method of Kaplan and Beutler[13]. a. Agarose IEF gel, pH gradient 3–10. Electrical conditions as described in the text. b. PAG plate, pH gradient 3.5–9.5. Conditions as in Fig. 2b. A p*I* of 6.8 has been found in both experiments.

The method has been used to analyze genetically substituted proteins and the superiority of agarose IEF gel as compared to polyacrylamide gel has been demonstrated in specific problems. An example is provided by a mutant of cytochrome b_5 reductase (diaphorase)[9,10].

The same sample from a heterozygote patient was run on agarose IEF gel (pH gradient 3–10) and on a PAG plate gel (pH gradient 3.5–9.5). After specific staining the following patterns were obtained: on agarose IEF gel (Fig. 3a) a single band on the acidic side of the p*I*, and two bands on the basic side with different migrations; on PAG plates (Fig. 3b) a single band along the whole pH gradient.

DISCUSSION

A reliable interpretation of electrophoretic mobility curves first requires a stable linear gradient and an efficient migration which itself must take place in a relatively short time so as not to disrupt the gradient.

In polyacrylamide gels migrations of high-molecular-weight molecules is slowed down by a molecular sieving effect, due to the pore size of the gel, and to compensate for this a prolonged migration time is required which partially destabilizes the gradient. We therefore developed, as an alternative electrophoretic support, the use of agarose gel which has larger pores.

One problem is related to electro-osmosis[14] of ordinary agarose which carries negative charges. This can be solved by using highly purified agarose IEF, where selective positive charges neutralize the existing negative charges[15]. The electro-osmotic effect was also minimized by the use of sorbitol which increases the viscosity of the gel, decreasing the osmotic flow as a secondary effect and by overnight storage of the gel at 4°C in a humid chamber which increases its mechanical strength. As seen in Fig. 1, these conditions provide a linear gradient.

The results shown in Fig. 2 clearly demonstrate that the resolution obtained in generating titration curves on agarose gel is as good as that achieved by the use of polyacrylamide as a support and in a shorter time of migration. It should be noted that the sample well is replaced by a slit made with a scalpel, thus reducing the volume of the sample; this results in a sharper and better resolved curve.

We found it essential that the electrophoresis be run initially at low voltage in order to allow the sample to penetrate into the gel. If a high voltage is applied from the beginning, the sample migrates at the surface of the gel, and is washed out during the staining procedure.

Fig. 3 gives an example of the usefulness of this technique in the study of a mutant of cytochrome b_5 reductase. When analyzed on a PAG plate, the mixture of the substituted enzyme and its wild type gives rise to a single visible band (Fig. 3b). On the agarose gel, the same sample is separated into two distinct bands at pH values above the p*I* (6.8), Fig. 3a. One explanation for the behaviour on agarose gel could be a more efficient penetration of the chromogenic agents into the gel. Other advantages of agarose IEF gel have also to be considered, *e.g.*, the ease, rapidity and reliability of gel preparation, the aerobic polymerization, the rapidity of staining and destaining, the use of non-toxic chemicals, the absence of chemically reactive components in the gel and the unrestricted mobility for large proteins. All these advantages together make agarose IEF a suitable support for isoelectric focusing experiments.

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