RESOLVING POWER OF ISOTACHOPHORESIS AND ISOELECTRIC FOCUSING FOR IMMUNOGLOBULINS

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1. Introduction

Among the various techniques which are able to discriminate between individual members of a protein family like the immunoglobulins, isoelectric focusing (IEF) (for a recent review, see [1]) has been very frequently used since the introduction of polyacrylamide slab-gels. The advantages of IEF include high resolving power and flexibility with regard to sample detection. One of the drawbacks of IEF in polyacrylamide gels is the restriction exerted on very large molecules, but this problem has recently been solved by the use of polymerizable thin layers containing Sephadex as anti-convective medium [2]. Another disadvantage is of a more serious nature, namely the low ionic strenght in the medium in which focusing is carried out. The sensitivity of many proteins to low ionic strenght and their tendency to precipitate at a pH near their isoelectric point therefore preclude the analysis of an appreciable number of proteins by IEF.

We have tried to overcome these problems by employing isotachophoresis (ITP), an electrophoretic method, which has so far found only very limited application in biochemistry, especially in the analysis of protein molecules. The two most attractive features of ITP derive from its theoretical basis [3–7]: the ionic strenght in the system can be regulated over a wide range and the sample ions carry a net charge. Thus, precipitation of proteins at their isoelectric point is avoided.

Usually, ITP is done either in columns or in capillary tubes, both of which make a comparison of similar samples difficult and time-consuming. We therefore introduce here a simple and inexpensive slab-gel system for ITP, which makes a comparison of different samples easy.

We have compared the immunoglobulins secreted by two myeloma lines and four cloned sublines derived from myeloma X spleen cell fusions by ITP and IEF. Because of their sensitivity to low ionic strenght, two of the immunoglobulins studied could not be focused but they exhibited clearly different mobilities in ITP in the presence of spacer ampholytes. Although the resolving power of IEF for immunoglobulins seems to be generally superior to that of ITP, our results show, for the first time, that there exist some cases where the reverse is true.

2. Materials and methods

Sperm whale myoglobin was obtained from Calbiochem, USA. [^{14}C]Lysine-labeled culture supernatants from immunoglobulin-secreting myeloma lines or myeloma X spleen cell hybrids were prepared as described before [8]. All cell lines employed in this study with their respective secreted immunoglobulins are listed in table 1.

Carrier ampholytes 'Ampholine' of pH range 3.5–10 were purchased from LKB produkter AB, Stockholm, Sweden; acrylamide (twice crystallized) and *N,N'*-diallyl tartardiamide (DADT) from Serva Entwicklungslabor, Heidelberg, Germany; Tris-(hydroxymethyl)–aminomethane (Tris) and 6-aminocaproic acid (6-ACA) from Fluka AG, Buchs, Switzerland; all other chemicals employed were of analytical grade.

IEF was carried out in polyacrylamide slab-gels

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Table 1
Cells lines and their respective IgGs employed in this study

Cell lines	Subclasses of IgGs secreted
P3-X63Ag8	γ1; κ
P1Bul	γ2a; κ
[a]Sp2/HL	γ2b; κ
Sp2/HK	γ2b; κ
Sp3/HK	γ1; κ
Sp3/HLK	γ1; κ ; κ

[a]Sp-lines are cloned sublines from myeloma × spleen cell fusions. Immunoglobulins consist of heavy and light chains, named H and L when derived from the spleen cell and G and K (for gamma (γ) and kappa (κ)) when derived from the myeloma parent (in this study always P3-X63Ag8).

containing 6 M urea with a pH 5–9 gradient as described [8].

ITP was done in slabs (length, 14 cm; width, 15.5 cm; thickness, 0.1 cm). A slab-gel apparatus identical to those usually employed for SDS-electrophoresis [9] was used. The buffer system had 0.3 M 6-ACA adjusted to pH 9.1 with Tris as terminating electrolyte; the leading electrolyte was 0.05 M phosphate adjusted to pH 6.5 with Tris [10]. Gels contained 4% acrylamide, 0.7% DATD and 0.05 M phosphate/Tris pH 6.5; 0.075% *N,N,N',N'*-tetramethylethylene diamine and 0.075% ammonium persulphate initiated the polymerization. ITP was carried out at room temperature for different periods of time depending on the amount of 'Ampholine' spacers present, but the current was always kept constant at 5 mA for the entire electrophoresis. The slab-gels were stained and destained as described before [2], dried on Whatman 3 MM paper and then auto-radiographed (Kodak RP Royal X-Omat).

3. Results and discussion

The IEF patterns of six different (as judged by SDS-electrophoresis and IEF of reduced heavy and light chains) IgG-containing culture supernatants are depicted in fig.1. The myeloma lines, P3-X63 Ag8 (fig.1a) and P1Bul (fig.1f) both give 3–5 clearly distinguishable bands upon IEF. P1Bul secretes also free κ-chains [11]. The different Sp3-derived immunoglobulins (fig.1d,e) possess very similar isoelectric points. As observed previously [8], the Sp2/HL and Sp2/HK proteins (fig.1b,c) precipitate and do not give detectable patterns in IEF, because they are sensitive to low ionic strength. We were, therefore, interested whether it would be possible to discriminate these two proteins by ITP.

The choice of a slab-gel system for the analysis

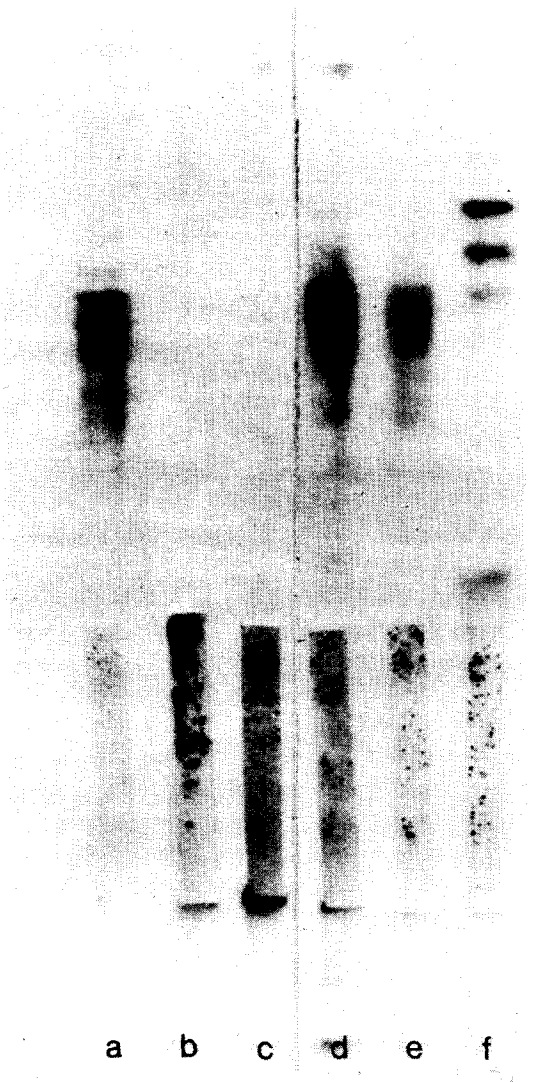

Fig.1. Isoelectric focusing of [^{14}C]lysine-labeled immunoglobulin-containing culture supernatants. 50 μl of each supernatant was applied to Whatman 3 MM paper strips. The cathode is on top. (a) P3-X63Ag8 (b) Sp2/HL (c) Sp2/HK (d) Sp3/HK (e) Sp3/HLK (f) P1Bul.

of immunoglobulins was dictated by the necessity of comparing very similar molecules side by side. To ensure that the gel exerted no steric hindrace on immunoglobulins of the IgG-class, we chose a polyacrylamide gel heavily crosslinked with DATD [12].

When run without 'Ampholine' spacers (fig.2), all labeled protein-species migrate as two sharp zones; the one with the lower mobility (probably IgG, see below) stacks about 0.5–1 mm behind the serum albumin (culture supernatants contain 10% horse serum). Whale myoglobin (as coloured marker protein) stacks at about the same height as the immunoglobulins (not shown). Since two labeled protein bands could already be distinguished, in spite of the absence of 'Ampholine' it is possible that horse serum contains anions which can act as spacers in this system.

In the text experiments, the influence of increasing amounts of spacer ampholytes of the pH range 3.5–10 on the separation of IgGs was tested. Initially, 20 μl of 'Ampholine' (8 mg) was added to each sample, and the results (fig.3) show that some of the IgG-molecules can now easily be discriminated. As in IEF, the two myeloma lines (fig.3a,f) give two clearly different patterns; the IgG1 of P3-X63 Ag8 has a

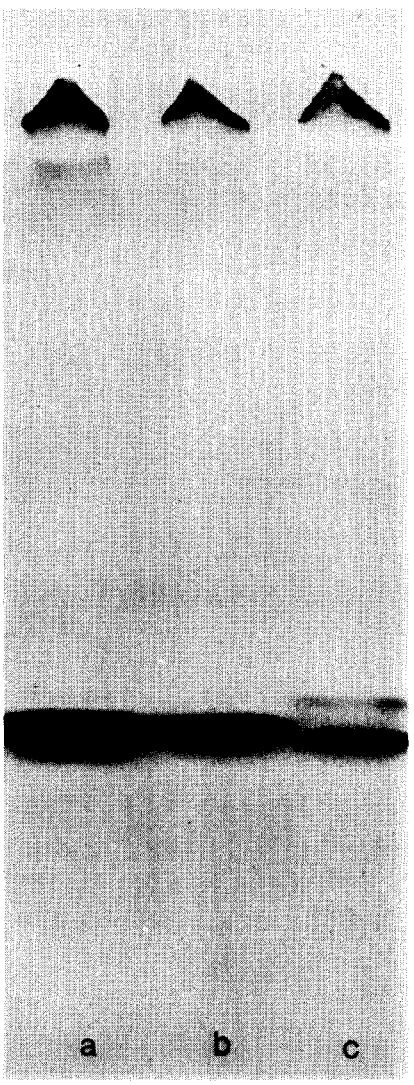

Fig.2. Isotachophoresis of [^{14}C]lysine-labeled immunoglobulin-containing culture supernatants in a slab-gel without 'Ampholine' spacers. 50 μl of each culture supernatant was mixed with 50 μl of a solution containing 50% (w/v) sucrose and 0.01% bromophenol blue in water. The solutions were applied to individual slots of the slab-gel and overlaid with terminating buffer. The gel was run for 14 h. The cathode is on top. (a) Sp3/HK, (b) Sp3/HLK, (c) P1Bul.

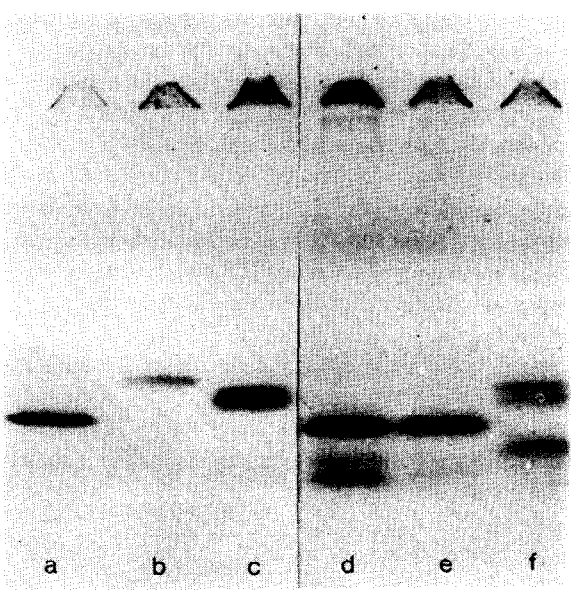

Fig.3. Isotachophoresis of [^{14}C]lysine-labeled immunoglobulin-containing culture supernatants in the presence of 'Ampholine' spacers in a slab-gel. 50 μl of each culture supernatant was mixed with 100 μl of a solution containing 25% (w/v) sucrose and 0.005% bromophenol blue in water, then 20 μl of pH 3.5–10 'Ampholine' (40% w/v) was added. The mixtures were applied to different slots and overlaid with terminating buffer. The electrophoresis was carried out for 18 h. The cathode is on top. (a) P3-X63Ag8 (b) Sp2/HL (c) Sp2/HK (d) Sp3/HK (e) Sp3/HLK (f) P1Bul.

mobility intermediate between those of the intact IgG2a and the excess light chains which are both secreted by the P1Bul line. This resembles the IEF pattern, where the IgG1 has an isoelectric point lower than the IgG2a, but higher than the κ-chain. The Sp3-derived sublines secrete immunoglobulin-species which are indistinguishable by ITP. The most striking feature of fig.3 is the patterns obtained with immunoglobulins of the two Sp2-derived sublines, which do not band at all in IEF, but in ITP give patterns different from all the other IgGs analyzed. Apparently these two intact proteins do not precipitate under the electrophoretic conditions employed, and they are distinguishable here, although they differ from each other only in the variable part of their light chains. In all samples, the aforementioned labeled band with high mobility, now being spaced into 2–3 weak bands, can be seen. These protein-species do not show any heterogeneity in mobility from sample to sample, presumable they do not represent immunoglobulins.

In a further experiment, 50 μl of 'Ampholine' (20 mg) was added to each sample. After 18 h the whale myoglobin showed 4 bands, closely reminiscent of the IEF pattern [2]. The bands continued to migrate towards the anode but the pattern did not change during the next 6 h, after which the experiment was terminated. The autoradiography (fig.4) indicates that spacing of protein, as expected, is increased in comparison to fig.3; but also the protein bands have become thicker, probably because of the presence of spacer ampholytes with identical mobility. the spacing efficiency of 'Ampholine' is not uniform over the whole mobility range, since the distance of the two bands of the IgG2a molecule remains approximately constant (figs.3f,4f), while the distance between (for example) the IgG1- and the Sp2/HL-proteins is clearly increased upon addition of more 'Ampholine' (figs.3a,b;4a,b).

Some IgG-species can be discriminated by IEF, like the IgG1- and Sp3/HLK-proteins, but give practically indistinguishable patterns after ITP.

Summing up, our experiments provide evidence supporting the following conclusions:

(1) Antibody molecules which cannot be focused because of their solubility properties can be discriminated in ITP with 'Ampholine' spacers.

(2) The addition of small amounts of 'Ampholine' spacers, thought to be not advantageous at present [13], is essential in analytical ITP in order to obtain a good resolution.

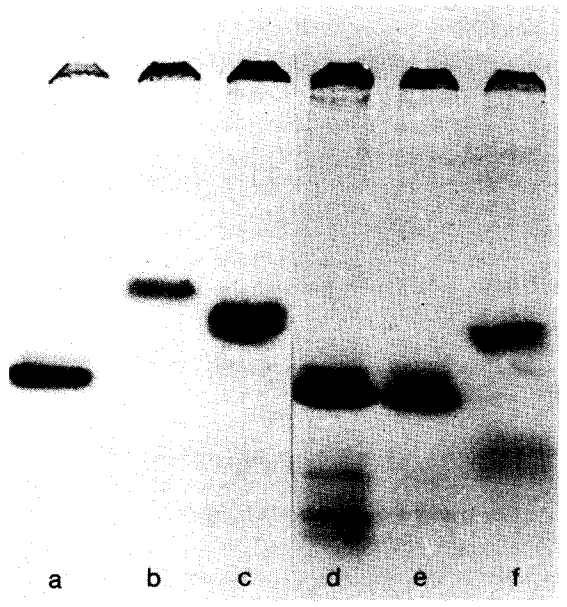

Fig.4. Isotachophoresis as in fig.3, but 50 μl pH 3.5–10 'Ampholine' (40% w/v) was added to each sample. The gel was run for 24 h, and the cathode is on top. (a) P3-X63Ag8 (b) Sp2/HL (c) Sp2/HK (d) Sp3/HK (e) Sp3/HLK (f) P1Bul.

(3) The number of bands per clone in this form of ITP is reduced in comparison with IEF, thus making it (possibly) easier to count clones in a heterogeneous mixture of antibody molecules, and finally,

(4) the simple slab-gel system described here makes this electrophoretic method much less tedious than before and allows one to measure the pH-gradient in the gel and to run the proteins at room temperature, but in addition leads to a significant reduction in cost of an experiment, since about 4 times less 'Ampholine' is needed for ITP as for IEF.

We are currently investigating the possibility of separating different, intact IgM-molecules in an undenaturated form by ITP.

Acknowledgements

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