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Note

Improvement of pore gradient electrophoresis by increasing the degree of cross-linking at high acrylamide concentrations

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Electrophoresis in a gel of increasing acrylamide concentration (pore gradient electrophoresis)[1,2] offers the advantage over electrophoresis in a gel of uniform concentration that optimum gel porosity is provided for all sizes of proteins in a mixture. During pore gradient electrophoresis there is a progressive decrease in mobility, as the increasing gel concentration restricts migration. If electrophoresis is continued until migration has virtually stopped, an estimate of protein size is obtained, since proteins of lower charge have an opportunity to "catch up", and the effect of charge differences is minimized. Molecular sizes can thus be determined by comparing migration distances with those of standard proteins[3–5].

However, as Rodbard *et al.*[6] point out, this empirical approach to determining molecular weight is only satisfactory in the "asymptotic case", where zone velocity approaches zero. In practice, for serum proteins with molecular weights well over 100,000, migration virtually ceases on prolonged electrophoresis[5], but migration of smaller proteins continues at a decreasing rate sufficient to interfere with size determination, unless reference proteins of comparable size and charge can be used. Attempts to further restrict pore size by further increasing total acrylamide concentration at the bottom of the gel have not been successful[5].

This article describes the use of gradient gels in which this objective has been attained by increasing the degree of cross-linking in the region of higher gel content.

MATERIALS AND METHODS

Gels were prepared in 1% Tris–0.5% borate–0.1% EDTA buffer (pH 8.3). Generally, technical-grade (Cyanamid) acrylamide and N,N′-methylenebisacrylamide (BIS) were used, but experiments with other makes and grades gave the same results. "Total gel concentration" is used to indicate acrylamide plus BIS in g/100 ml and the degree of cross-linking is given as g BIS per 100 g acrylamide plus BIS. Thus a 20% gel with 5% cross-linkage contains 19 g acrylamide and 1 g BIS per 100 ml. Solutions were polymerized with 0.1 to 0.2% dimethylaminopropionitrile and 0.5% ammonium persulfate.

Stepped gels for opacity studies were made in rectangular styrene cells. For mobility tests, the stepped gels were cast in glass cells 82 × 82 mm and 2.8 mm internal width, such as supplied with Gradipore apparatus (Gradient Pty., Lane Cove, Australia) or with Pharmacia equipment (Pharmacia Fine Chemicals, Uppsala, Sweden). The stepped slabs were cut into strips, turned sideways and embedded in 4% gel, with 5 to 10 mm of this gel above the cut edge of the test strips.

Continuous pore-gradient gels were made using a variable-geometry gradient former[7], which permits casting fourteen identical gels in one operation. A third compartment was incorporated, as shown in Fig. 1, so that gels with variable cross-linkage could be obtained. The resulting gels are of similar composition to commercially supplied Gradipore gels, which are manufactured in a scaled-up version of the laboratory apparatus. Actual gel concentrations were verified by wet and dry weights of strips cut from the gel slab, with corrections made for buffer solids[1].

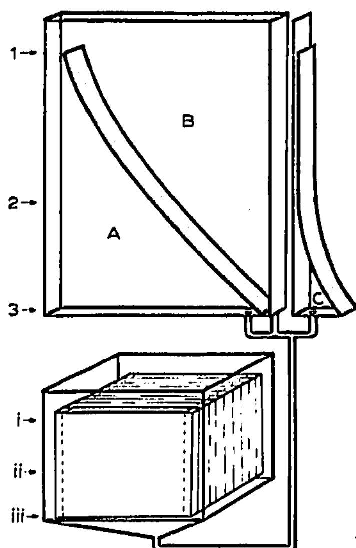

Fig. 1. Diagram illustrating the principle used to produce the pore-gradient gels with variable cross-linking referred to in Figs. 4 and 5.

RESULTS AND DISCUSSION

BIS content and gel turbidity

During the preparation of concave gradient gels (2.5 to 27% polyacrylamide including 5% cross-linkage throughout) an opalescent region at about 10% gel was consistently obtained. The opalescence could be prevented by decreasing the relative concentration of BIS (or increased by raising BIS content). Reduction of BIS content to 3% had little effect on protein migration in the upper half of the gel, but it made the gel region of over 10% polyacrylamide more permeable to smaller proteins. The degree of cross-linking in this region was therefore examined more closely.

Previously reported attempts[8,9] to adjust pore size by varying the degree of cross-linking have been frustrated by the production of turbid gels at higher BIS concentrations or of unmanageable gels at low BIS content. In general, variation in

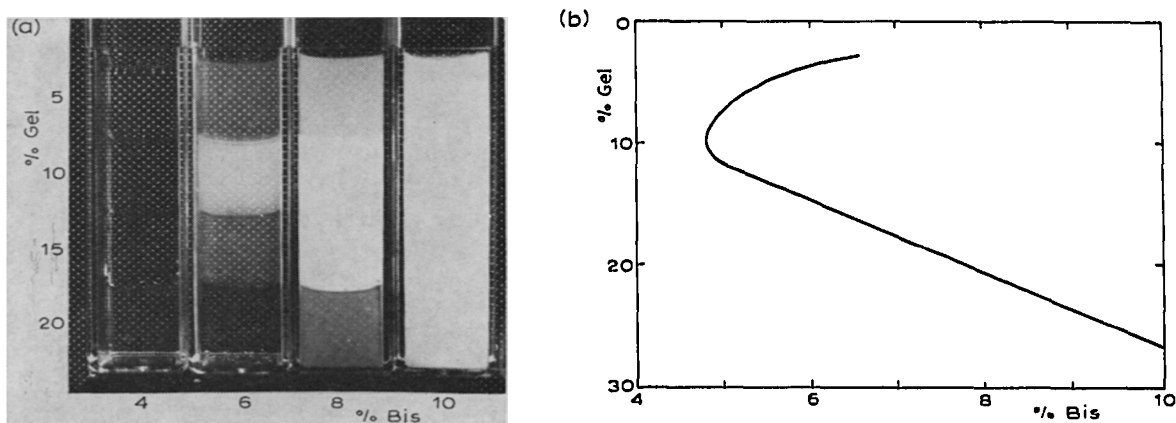

Fig. 2. Effect of BIS concentration (% of BIS + acrylamide) on gel turbidity at a range of total gel concentrations. Degrees of gel turbidities (a) are indicated by photographing with transverse illumination against a figured black background. The graph (b) shows the critical combinations of BIS content and gel concentration at which gel turbidity starts to occur. Clear gels are given by formulations to the left of the curve: turbidity is perceptible and increases to the right of the curve.

cross-linking did not affect pore size dramatically. However, these studies have not included high cross-linking at high gel concentrations.

Fig. 2 shows that at a relatively high gel content, a higher degree of cross-linking may be tolerated without production of a turbid gel. The critical BIS content, at which turbidity appears, is lowest at a gel content of about 10%.

BIS content and gel pore size

The degree of cross-linking has a more marked effect on pore size at high gel concentrations than it has for gel concentrations of less than 10%. Thus, for a uniform total gel concentration of 20%, if the proportion of BIS is doubled from 4 to 8%, the mobility of serum albumin is reduced by a factor of seven (Fig. 3). Such a reduction in mobility would hardly be possible by merely increasing total gel concentration with constant BIS content.

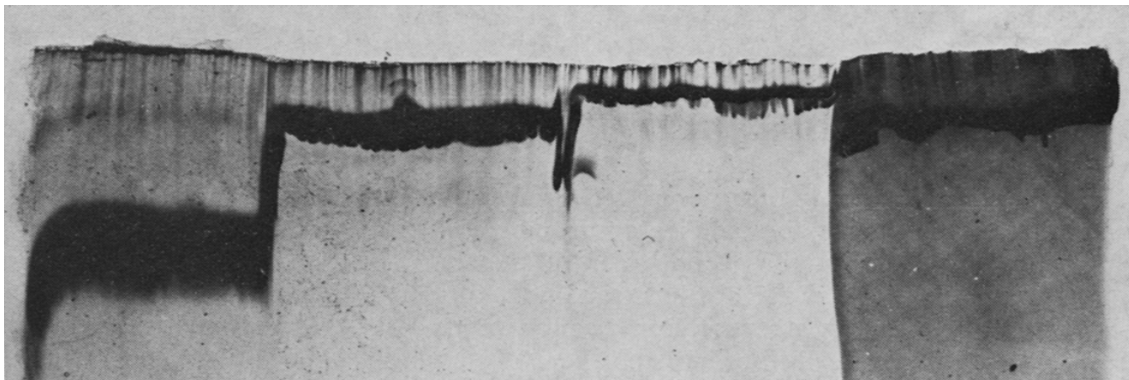

Fig. 3. Gel electrophoresis of human serum albumin in gel sections, all 20% total gel content, in which the proportion of BIS (as % of BIS + acrylamide) is 4% (left), 6%, 8%, and 12%. The electrophoresis time is 600 volt hours.

However, if BIS content in a 20% gel (Fig. 3) is increased beyond 8% and past the level which produces a turbid gel, then the relative mobility of this protein increases again. Thus, even if gel clarity were to be sacrificed, there is no advantage in respect to pore size reduction in venturing into formulations that lead to turbid gels. Apparently, the factors that lead to gel turbidity also produce a slightly more open gel network. Possibly excess BIS is incorporated in localized centres where there is a high degree of cross-linking. Such regions would account for gel turbidity without further reducing the overall pore size.

Gradient gels with variable cross-linking

On the basis of these results, gradient gels were prepared with 3.8% cross-linking from 3 to 15% total gel concentration, and with the degree of cross-linking rising progressively to 6.5% in the region from 15 to 30% total gel content as shown in Fig. 4.

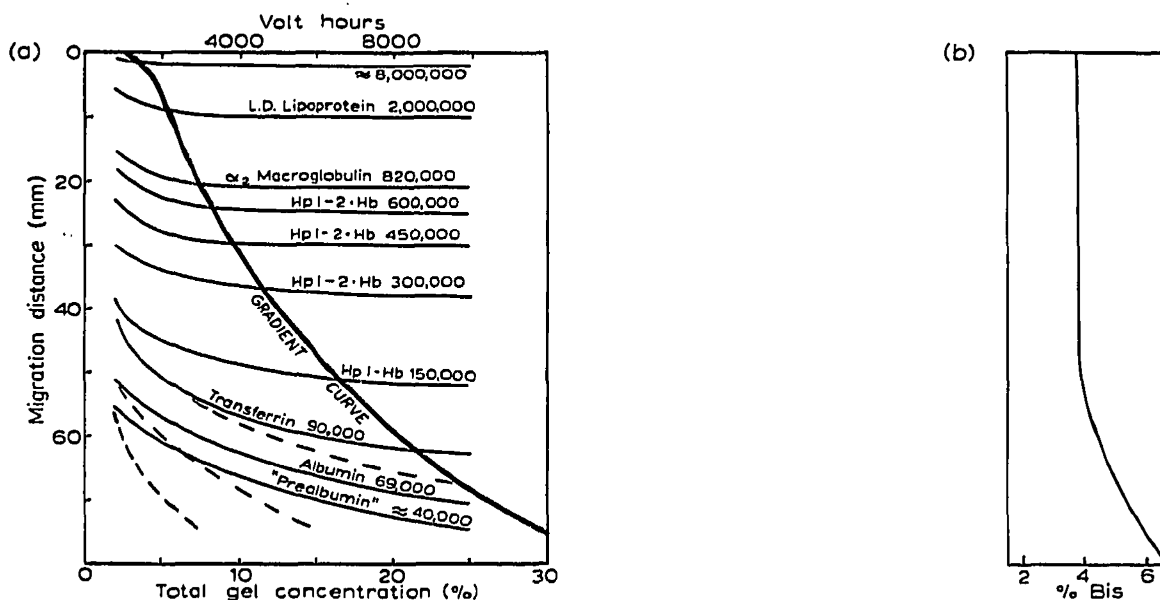

Fig. 4. Plots of the mobilities of serum proteins against time of electrophoresis using pore gradient gels with uniform 5% cross-linking (dashed lines) or variable cross-linking, as indicated by side diagram (b). For both types of gel, the bold transverse plot indicates the variation in total gel concentration (acrylamide plus BIS). The curve indicating slowest mobility represents some very-low-density lipoproteins and γ M polymers with molecular weights in the region of 8,000,000.

The upper portion of Fig. 1 shows the gradient-forming device[7], which drains into the gel-forming tower[1] (below) for polymerization. The flat rectangular vessel of the gradient former is divided into two chambers, A and B, by a curved partition (shaded). Compartment A is filled with a 30% acrylamide–BIS mixture (3.8% BIS, 96.2% acrylamide). B contains diluent buffer. Chamber C contains a 30% acrylamide–BIS mixture (20% BIS, 80% acrylamide) and is designed to superimpose variation in degree of crosslinking on the main gradient. As the three chambers are slowly drained

together through their common outlet, they remain in hydrostatic equilibrium. At any level, the proportions of the three liquids in the effluent mixture correspond to their respective surface areas at that level. There is thus an increasing contribution of acrylamide solution from A corresponding to the shape of the partition between A and B. On the other hand, compartment C only contributes significantly to the gradient during the latter part of draining when the acrylamide content is higher (15–30%). The increasingly dense effluent is fed by gravity from below, up into a moulding tower which contains a row of fourteen empty glass cells. Each of these consists of two 82-mm squares of glass taped together and separated at the vertical edges by two 3-mm-thick strips of glass. After the contents of the gradient former have emptied, concentrated sucrose solution is poured down the outlet tube to displace the gradient up into the glass cells. When this has been done, the proportions of A, B, and C at levels 1, 2 and 3 of the gradient former correspond exactly to actual compositions at levels (i), (ii) and (iii) of the electrophoresis plates, respectively. The apparatus is then left for polymerization to take place. Refs. 1 and 7 provide full details for practical construction and use of the basic apparatus, together with instructions for varying the gradient shape.

Fig. 5 shows a series of electrophoretic separations of human serum proteins using a gel made with the apparatus shown in Fig. 1. During the time allowed for

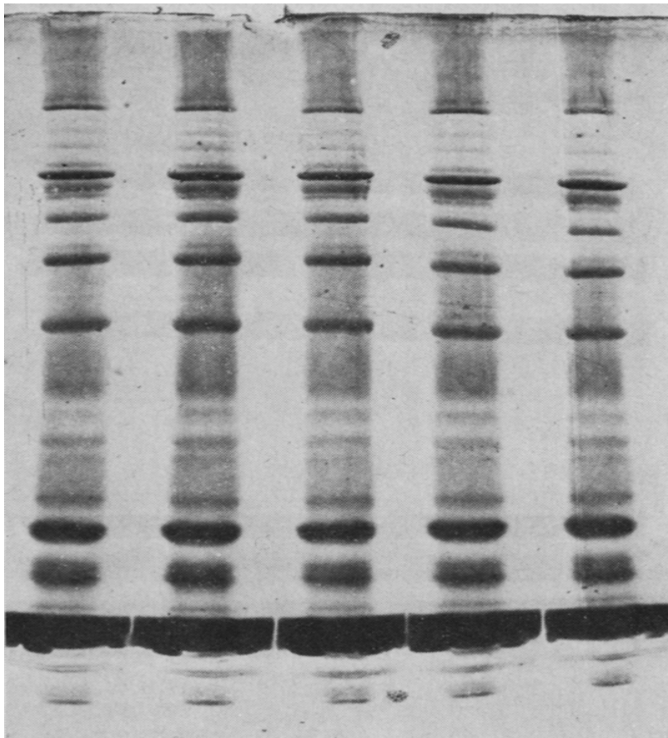

Fig. 5. Electrophoretic separations of replicate samples of human serum with added hemoglobin in a pore gradient gel slab with variable cross-linking, for 2,000 volt hours. The sideways spread of the heavy albumin band is characteristic of protein in high concentration approaching pore-limit conditions.

this run, the albumin zone for example, would have run off the gel if the BIS content had been constant throughout at, say, 3.8%. Increasing the degree of cross-linking at the bottom of the gel has retained not only the albumin, but also the prealbumin bands.

Molecular weight estimations

Progress curves of serum proteins (Fig. 4) show that increased cross-linking has effectively reduced pore size in the denser region of the gel, so that plots (solid lines) for smaller proteins come much closer to being asymptotic than equivalent plots (broken lines) with uniform 5% cross-linking.

This new type of gel thus offers a much better basis for estimation of molecular weights by comparison with proteins of known sizes. An electrophoretic time equivalent to about 2000 volt hours is suggested as a satisfactory stage to compare migration distances in gauging protein size. It represents a compromise between convenience in performing the experiment and approach to "pore limit" conditions. For example, it allows adequate time for the less highly charged molecule hemoglobin to catch up to albumin, which is very similar in size.

CONCLUSION

These results illustrate the contrast between dynamic gel electrophoresis and pore limit electrophoresis. In the former case, the gel network offers frictional resistance to migration, thus implicating protein size in mobility, but protein charge also contributes significantly. On the other hand, pore limit electrophoresis represents the limiting case where molecules have penetrated a pore gradient so far that they become "jammed" in the gel network: migration distance is a reflection of size and partly shape, but not charge. This article describes the means of approaching conditions of pore limit electrophoresis to facilitate molecular weight comparisons between proteins in the native state.

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