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TITRATION CURVES OF PROTEINS BY COMBINED ISOELECTRIC FOCUSING–ELECTROPHORESIS IN HIGHLY POROUS POLYACRYLAMIDE MATRICES

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SUMMARY

Highly cross-linked (highly porous) polyacrylamide gels as described in the literature were found to be poorly characterized support media. Diallyltartardiamide (DATD) cross-linked gels contain up to 80–90% unpolymerized DATD, which reacts with proteins and produces gluey and highly stretchable matrices. The conversion into polymer could not be affected by time or higher temperatures, since DATD is an inhibitor of polymerization, but only by adding very high amounts of persulphate. Increased levels of riboflavin actually decreased the polymerization efficiency. Highly cross-linked N,N'-methylenebisacrylamide (BIS) gels, at 40–50% C, are too hydrophobic and produce a collapsed matrix which keeps exuding water. A happy compromise are 30% C_{BIS} gels, which are stable and allow unhindered migration of globular proteins up to $5 \cdot 10^5$ daltons. BIS and DATD gels can be stabilized by covalent binding to a glass surface precoated with Silane A-174. The order of reactivity for monomers appears to be: acrylamide > BIS ≫ DATD. Polymerization conditions are described which allow better than 96% conversion of monomers into the polymer.

INTRODUCTION

By exploiting an original idea of Rosengren *et al.*[1] we have recently demonstrated the possibility of developing titration (pH–mobility) curves of proteins by isoelectric focusing (IEF) in a polyacrylamide gel followed by electrophoresis at right angles[2]. After creating a stationary pH gradient in the first dimension, the protein sample is loaded in a long trench which spans the gel from anode to cathode. When electrophoresis is performed perpendicular to the focused stack of carrier ampholytes, the protein moves in the gel according to its titration curve, since point by point along

the migration path the macromolecule "sees" the prevailing pH of the environment and picks up or releases protons according to the p*K* values of its ionizable groups.

By this technique it is possible to study interactions of proteins with ligands, and the pH dependence of the liganded state, provided the ligand has a high affinity for the protein (association constant of the order of μM)[3]. By the same method, we have also been able to detect macromolecule–macromolecule interactions (*e.g.* methemoglobin with cytochrome b_5, and hemoglobin, or α and β subunits, with haptoglobin)[4,5]. Titration curves of peptide and polypeptide chains can also be performed in 8 *M* urea, with or without 2% Nonidet P-40, in order to expose completely the charged groups to the solvent and avoid intra- and inter-chain interactions[6]. Moreover, in the case of simple, uni-valent amphoteric molecules, we have derived an equation linking the electrophoretic mobility of the titrated ions with the p*K* values of their anionic and cationic groups[7,8].

If absolute, free mobility data could be derived from our titration curves, besides representing intrinsic, macromolecular parameters for a given protein under a fixed set of experimental conditions, these data would also be of paramount importance in defining an objective fractionation route by charge-dependent techniques. Unfortunately, commonly used polyacrylamide gels (5–7% T, 4–5% C)[9], represent a sieving medium not only for macromolecules, but even for small molecules, such as buffer ions, urea and D_2O[10]. Recently, however, non-restrictive polyacrylamide gels, highly cross-linked with either N,N′-methylenebisacrylamide (BIS) or with diallyltartardiamide (DATD) have been reported for use in electrophoresis[11], isotachophoresis and IEF[12] and SDS-electrophoresis[13]. Use of these highly porous media for developing titration curves seemed therefore the next logical step. However, when we attempted to use these highly cross-linked matrices we ran into a series of unexpected difficulties, owing to the poorly characterized behavior and properties of these gels. In the present report, we shall attempt to describe the chemistry of these matrices and their successful use as a support for developing titration curves.

MATERIALS AND METHODS

BIS, DATD, acrylamide, ammonium persulphate, riboflavin and N,N,N′,N′-tetramethylethylendiamine (TEMED) were from Bio-Rad Labs., Richmond, Calif., U.S.A. Horse spleen ferritin (twice crystallized) was from Miles Res. Labs., Kankakee, Ill., U.S.A., Ampholine pH 3.5–10 from LKB, Bromma, Sweden, Silane A-174 (organosilane ester) from Union Carbide Silicones, Sisterville, W. Va., U.S.A. and acetone spectro ACS from Eastman Kodak, Rochester, N.Y., U.S.A.

Gel polymerization

Since the percentage of cross-linker (% C) was varied from 4% to 50%, while the total monomer concentration (% T) was kept constant at 6% throughout our experiments, we used a 30% stock acrylamide solution and added to it each time the required amount of solid BIS or DATD. This was particularly necessary with BIS, owing to its low solubility. The 30-ml gel solution, containing 2% carrier ampholyte, was degassed at room temperature, under stirring, for 5 min with a vacuum of 0.1 mmHg. The vacuum was interrupted with nitrogen, via a three-way stopcock, so that the solution at no time had a chance to re-equilibrate with atmospheric oxygen.

The gel slab was cast just after adding the required amount of catalysts (50 μl of TEMED and 35 mg of persulphate/100 ml of gel solution). These conditions ensure "apparent" polymerization in 5–10 min. Our polymerization conditions are considerably more drastic than the ones typically found in the literature[14,15], *i.e.* degassing with a vacuum of 10–20 mmHg at 4°, no nitrogen flushing, 10–15 mg of persulphate/100 ml of gel solution.

Titration curves

The general methodology has been already described[2], except that we now routinely polymerize square-size gel slabs (12.5 × 12.5 cm, 2 mm thick) by cutting the glass slabs (12.5 × 26 cm) routinely used in the LKB 2117 Multiphor chamber. By this method we can run both dimensions by simply turning the gel slab through 90° after the IEF run and still using the same electrode cover lid. Routinely, the first dimension (IEF) is run at 10 W constant for 90 min (900 V at equilibrium) and the second dimension is run at constant voltage at 600 V/11.3 cm electrode distance, 4° and 15 min.

Covalent bonding of the polyacrylamide gel to the glass surface

Since highly cross-linked BIS gels are extremely brittle, whereas the corresponding DATD gels, while being mechanically stable, tend to swell and stretch up to twice their original size[16], we have developed a method for covalently fixing the gel slab to the glass surface by which it is supported. Just prior to casting the gel, the 1 mm thick, 12.5 × 12.5 cm glass slab routinely used in the gel moulding chamber is dipped for a few minutes in 0.1% Silane A-174 in acetone. Upon evaporating the solvent, a thin film of silane molecules (which contain a reactive double bond) is left on the glass surface. During polymerization, the layer of acrylamide molecules adjacent to the glass copolymerizes with silane so that the gel is firmly bound to the glass and cannot be peeled off or stretched during subsequent staining and washing steps. The gel is so sturdily bound to the glass, that, if the glass slab has to be recovered for further use, it has to be scraped during washing with steel-wool. The silane solution, if kept at 4° in a dark bottle in the absence of humidity, is stable for several months.

Titration of unreacted BIS and DATD

After polymerization, the gel slab was divided into four squares, each corresponding to 7.5 ml of original gel solution. One square was finely chopped, diluted to 60 ml final volume and stirred for 1 h at room temperature. Measured aliquots were acidified with H_2SO_4 (to 10 m*M* final concentration), centrifuged to remove gel debris, and titrated with 10 m*M* $KMnO_4$. The bulky, brown manganous ion precipitate was removed by repeated centrifugations and permanganate additions continued until a clear violet supernatant was detected. Under these conditions, the catalysts (TEMED, riboflavin and persulphate) did not appear to interfere with the titration as judged by titrating separately the same amount of riboflavin contained in the gel and, in the case of persulphate, by titrating gel portions electrophoretically depleted of catalyst. At least in the case of persulphate, the non-interference with the titration might be due to its conversion into sulphate during the time allotted for polymerization and gel "ripening".

RESULTS

When we started using highly cross-linked BIS and DATD gels the problems encountered were so severe that they prompted an investigation on the chemistry of these matrices. Essentially, to a lesser degree in BIS cross-linked, and to a marked extent in DATD gels, we found that the titration curves of several proteins were severely distorted, particularly in the pH range 6–8 of the pH–mobility curve. The distortion was so pronounced with ferritin, especially in DATD gels with increasing %C, that in the pH 6–8 zone the protein could not even migrate into the gel from the trench and, when it did, it moved as a highly blurred zone with strongly reduced mobility. As we were never faced with a similar problem with normal cross-linked gels, we were led to suspect an interference of BIS and DATD on the pH–mobility curves of our proteins. Therefore, a study of the parameters affecting polymerization efficiency in highly cross-linked gels was undertaken.

Polymerization efficiency vs. % C

Fig. 1 shows the degree of conversion of monomers into polymer for BIS and DATD gels at various % C up to "pure cross-link" polymers, *i.e.* gels containing only bifunctional monomer and no acrylamide. Low BIS gels (the usual 4–5%) appear to reach a better than 96% polymerization efficiency when titrated within 1h after moulding, as customary in most labs using polyacrylamide gels. However, as the

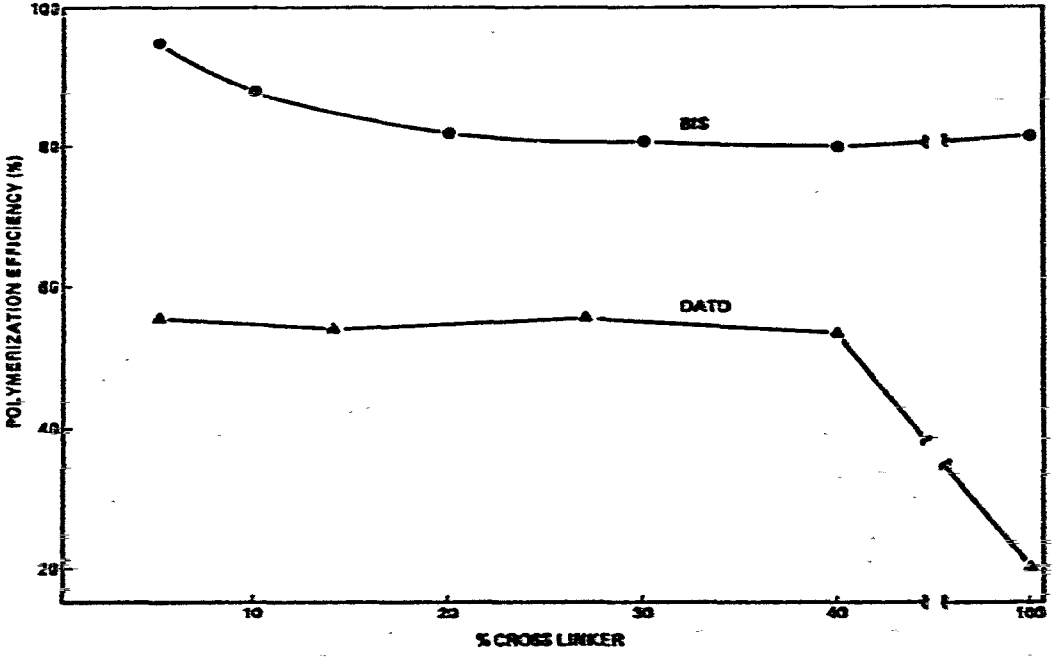


Fig. 1. Degree of polymerization of BIS and DATD gels as a function of % cross-linker. The gels were polymerized and titrated with 10 m*M* $KMnO_4$, as described under Methods. Titrations were performed 1 h after polymerizing under "standard conditions" (5 min vacuum of 0.1 mmHg, 50 μl TEMED and 35 mg persulphate/100 ml gel cocktail).

% C_{BIS} is increased, gels left to "age" for 1 h appear to contain more and more unreacted monomer up to 20% of the total. The situation is even more disastrous with DATD gels: at any percentage of cross-linker the polymerization efficiency is never better than 50%. Moreover, pure DATD gels contain more than 80% unreacted monomer and they are never able to form a gel, while under the same conditions pure BIS gels do form a solid matrix[17,18]. Pure acrylamide achieves a conversion rate of better than 98%, although it is unable to form a gel, for lack of cross-links, and is converted into an extremely viscous solution or "glue".

Polymerization efficiency vs. time at fixed % C and 22°

Two relatively highly cross-linked gels (20% C_{BIS} and equimolar 27% C_{DATD}) were selected for further studies at fixed % C. When the gels were left to "age" overnight at room temperature, the BIS-gels spontaneously achieved an increased polymerization efficiency, from 80% after 1 h up to better than 96% on standing overnight (see Fig. 2). Under the same conditions, DATD gels did not appear to "ripen" with time, remaining at *ca.* 50% conversion. Recently, polymerization conditions using two catalysts have been suggested ($5 \cdot 10^{-4}$% riboflavin, 0.015% potassium persulphate and 1 μl TEMED/ml)[12] so we have tried this experimental set-up, on the assumption

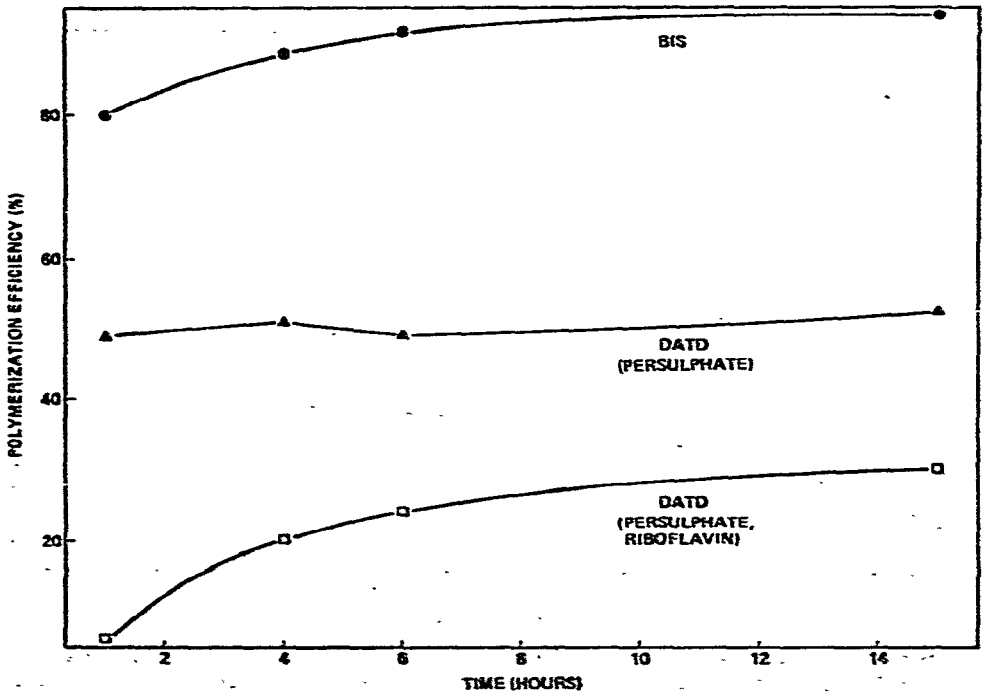


Fig. 2. Degree of polymerization of BIS and DATD gels as a function of time at room temperature (22°). ● and ▲: BIS and DATD gels, respectively, polymerized under "standard conditions" (Fig. 1). □: DATD gels polymerized as in ref. 12 ($5 \cdot 10^{-4}$ % riboflavin, 0.015% persulphate and 1 μl TEMED/ml gel mixture). In this last case, photopolymerization was continued at 22° for the time allotted for the experiment (15 h).

that our polymerization routine, even though more drastic than others, could just lack the right touch. This recipe proved to be a total failure: after 1 h ageing less than 10% DATD had reacted and the maximum conversion rate achieved was 30% after overnight photopolymerization (Fig. 2).

Polymerization efficiency vs. time at fixed % C and 60°

In an attempt to improve the conversion of monomers into the polymer, we have studied the reaction kinetics at considerably higher temperatures. From past experience, we knew that, when we needed a gel in a hurry, incubation at 40° would bring about a very rapid polymerization. In fact, when 20% BIS gels were incubated at 60°, the conversion to 95% polymerization was achieved in only 60 min, as opposed to overnight at room temperature (Fig. 3). An effect could also be seen on 27% C_{DATD} gels, but it was rather limited since the polymerization efficiency could be increased from the usual level of *ca.* 45% to 60% but no further conversion could be obtained even on overnight incubation at 60°. We did not attempt to use higher temperatures, since under these more drastic conditions the persulphate in the gel could oxidize the carrier ampholytes. Moreover, it was reported that the water regain of the gel (and thus, presumably, the extent of polymerization) was not affected by heating the gels to 80° (ref. 13).

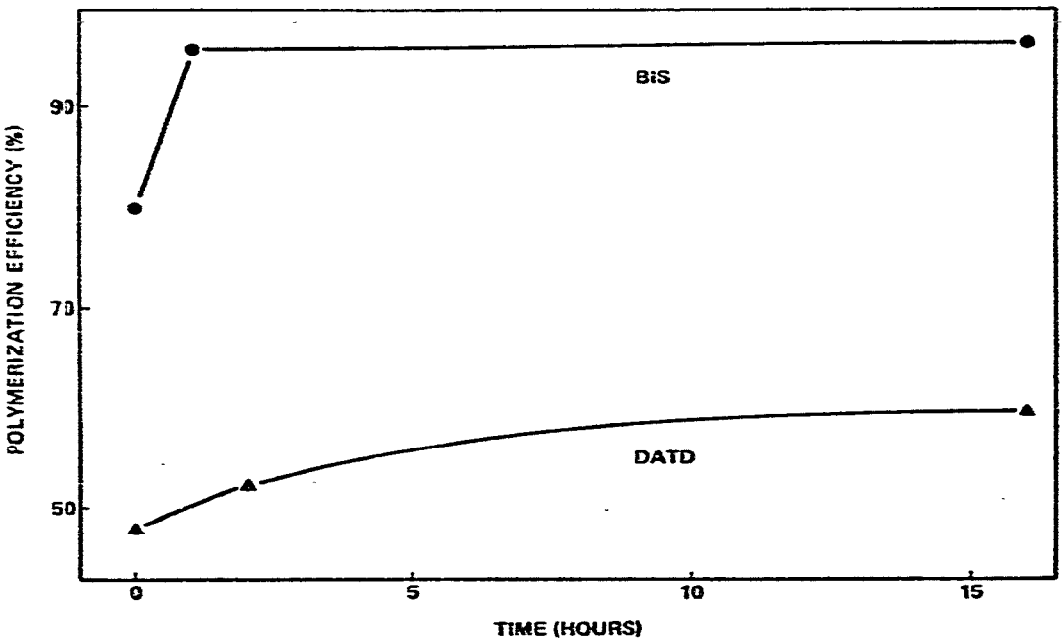


Fig. 3. Polymerization efficiency of BIS and DATD gels as a function of time at 60°. All other conditions as in Fig. 1 except that the gel slabs were incubated at 60° in a forced ventilation oven during the experimental time (16 h).

Polymerization efficiency vs. catalyst concentration

Since we have seen that BIS gels are sensitive to temperature and/or time, *i.e.* to conditions which accelerate the reaction rate, while DATD gels are practically

unresponsive to both these variables, we have continued our experiments on the assumption that it is the chemistry of the DATD molecule *per se* which interferes with the gel polymerization. Since it has been reported that alcohols inhibit acrylamide polymerization, by acting as free radical traps[13,15], it seemed logical to conclude that DATD, which is a 1,2-diol, is itself an inhibitor of gel polymerization. If this is so, only a drastic increase in the amount of catalyst in the gel, to a level capable of overcoming the inhibitory power of DATD, should bring about the desired conversion of the monomer into the polymer. In fact, as shown in Fig. 4, a 100-fold increase of persulphate above the recommended levels is required to incorporate 95% of the free DATD present into the polymer. The DATD gel thus polymerized was just as brittle and unelastic as equimolar BIS gels. At these extremely high levels of catalyst, however, no carrier ampholytes can be incorporated into the gel during the polymerization process, since they are strongly oxidized (the gel turns from colorless to deep yellow) and the polymerization efficiency is reduced. When gels containing 1 g of persulphate/100 ml of solution were polymerized in presence of 2% Ampholine and used for

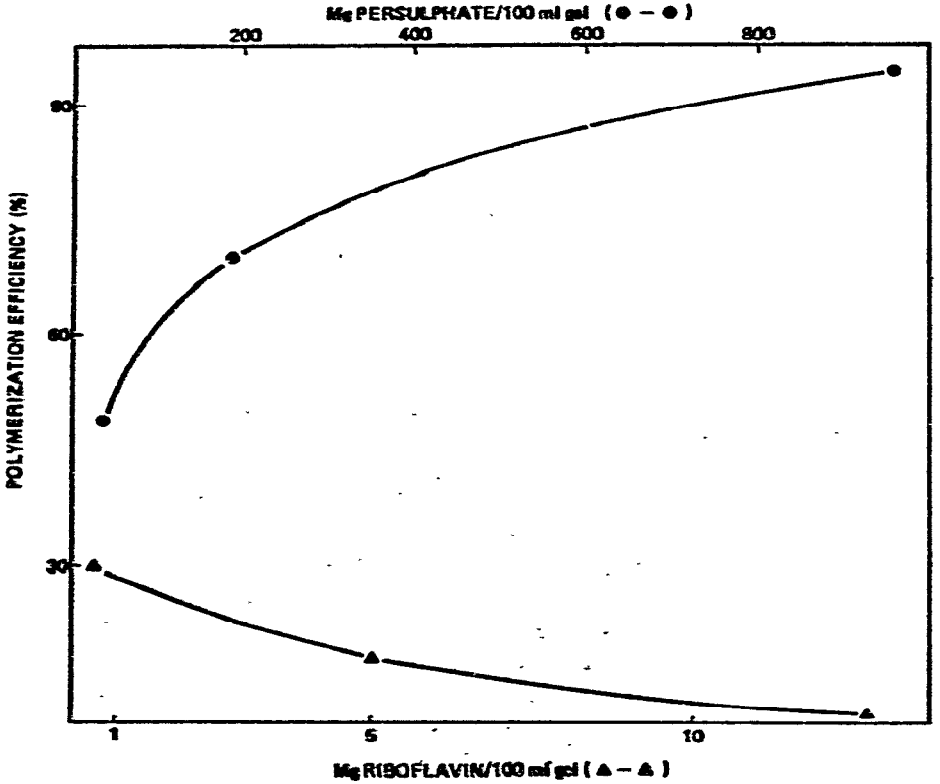


Fig. 4. Polymerization efficiency as a function of catalyst concentration. Gels were polymerized with the amounts of persulphate (●) and riboflavin (▲) indicated in the two abscissas. The "persulphate gels" did not contain any riboflavin while the "riboflavin gels" contained the standard amount of 0.015% persulphate as in ref 12. Both types of gel were titrated for unreacted material after overnight "ageing" at room temperature and under continuous illumination for the "riboflavin gels".

titration curves, the results were disastrous: two-thirds of the gel from the anodic side developed a flat plateau at pH 2; the pH then jumped suddenly to *ca.* 7, where the gel slab was greatly swollen and strongly yellow (probably oxidized Ampholine accumulated in this region); and finally, the last portion of the gel was quite alkaline. No pH gradient could be generated, and the gel matrix was quite distorted.

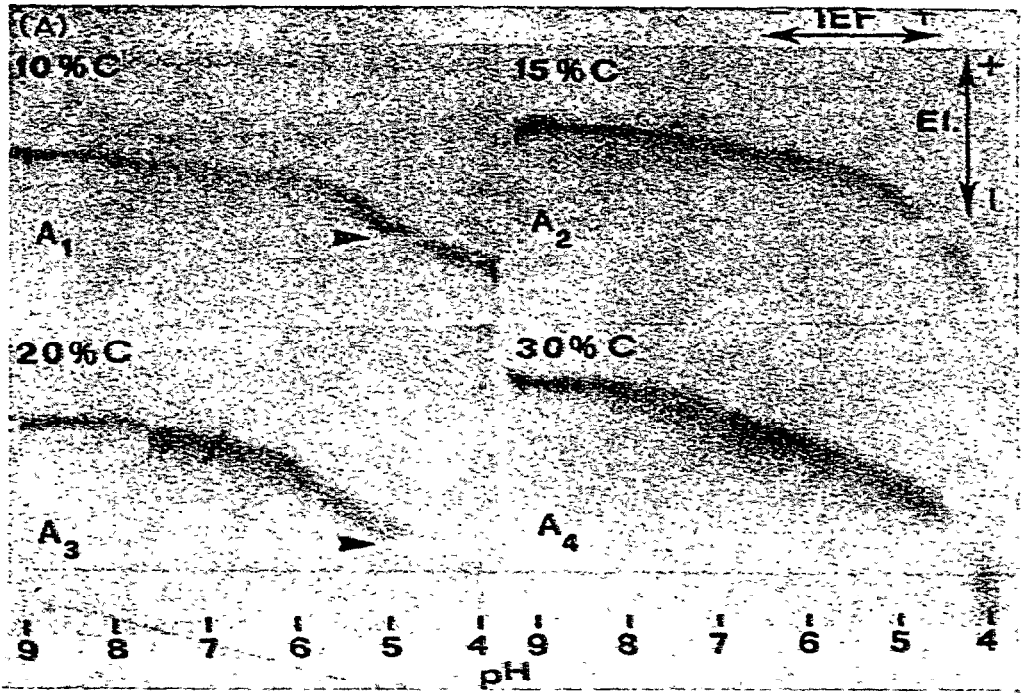
Since the conditions which achieve nearly total conversion of DATD into polymer are not quite compatible with subsequent electrophoresis or IEF, we have tried to achieve the same results by using riboflavin, which is uncharged and not an oxidizing agent as is persulphate. However, much to our surprise, as the level of riboflavin was progressively increased in the gel, up to 25 times the recommended amount, the polymerization efficiency kept decreasing from 30% conversion at low riboflavin levels down to a mere 10% conversion at high catalyst concentrations. We do not have a ready explanation for this, except to suggest that, as the riboflavin levels are increased, activated molecules can transfer their energy to molecules in the ground state, which thus act as a radical sink, much in the same fashion as increasing the level of a fluorofor in solution brings about a fluorescence decrement, by self absorption of emitted radiation.

Titration curves in highly cross-linked BIS gels

When the BIS gels at any level of cross-linker are "aged" overnight at room temperature or 1 h at 60°, unperturbed titration curves of proteins are obtained, indicating that nearly total conversion of the monomer into the polymer has been achieved (Fig. 5A). As is well known from the literature[11,17,18], we can follow the progressive increase in pore size with increasing % C. In the case of ferritin (440,000 daltons) we can see that practically free mobility is already achieved at a level of 30% BIS, since the mobility increments at any pH along the titration curve in going from 30 to 50% C are almost negligible (Fig. 5B). Thus, 30% C_{BIS} gels are non-sieving for globular molecules up to $5 \cdot 10^5$ daltons, in agreement with literature data[11] obtained by measuring retardation coefficients (K_R). A 50% C_{BIS} gel would probably be non-sieving for globular molecules up to *ca.* $3 \cdot 10^6$ daltons[11]; however, we have found that, at least in IEF, and probably in many other electrophoretic techniques, the limit value for reproducible gels and runs is centered around 30% C_{BIS}. 40% and 50% C_{BIS} gels are simply disastrous to the IEF process: the gel matrix collapses with time and this causes syneresis or exudation of liquid from the gel, with concomitant disruption of the Ampholine focused pattern. Equilibrium conditions are never reached and in fact, most of the time, the exuded liquid generates electric short circuits, and the gel starts burning on the sides. We attribute this to the fact that BIS is slightly more hydrophobic than acrylamide. Probably a 30% C_{BIS} gel represents a happy compromise between the hydrophobicity and hydrophilicity of the matrix. At higher % C the hydrophobicity prevails, the water is no longer tightly held around the polymer chains, and this results in a collapse with water loss from the matrix.

Titration curves in highly cross-linked DATD gels

As stated previously, highly distorted patterns of proteins are obtained in DATD gels (Fig. 6A). However, when the DATD is 95% converted into polymer, no deviation from the sigmoidal pattern is apparent (Fig. 6B). However, these gels seem to be of little practical utility since, in order to obtain the pattern of Fig. 6B, the



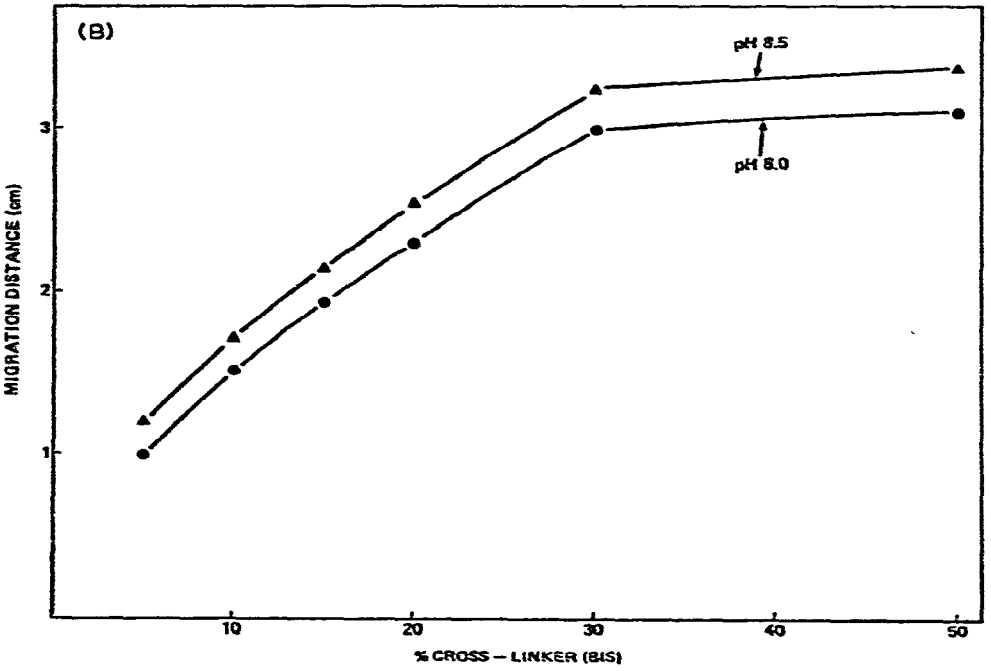
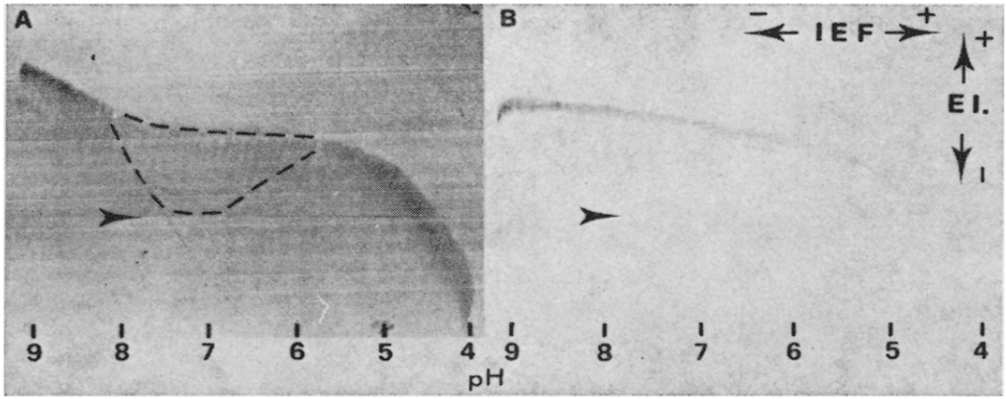


Fig. 5. Titration curves (A) and mobility data (B) of ferritin as a function of percentage of cross-linker (BIS). (A) pH–mobility curves of ferritin in 10% (A_1), 15% (A_2), 20% (A_3) and 30% (A_4) BIS cross-linked polyacrylamide gel·150 μl containing 100 μg of ferritin were used in each titration curve. Electrophoresis conditions as under "Methods". While the first dimension varies as a function of % BIS (shorter time with increasing % C) the second run is always performed under a set of absolutely constant parameters (600 V/11.3 cm, 4° and 15 min). The two arrows and positive and negative symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (EI). The two arrowheads indicate the sample application zone (*i.e.* the zero mobility of isoelectric plane). (B) Mobility data of ferritin as a function of % C_{BIS} calculated at pH 8.0 (●) and at pH 8.5 (▲) from the titration curves of Fig. 5A. Notice the levelling of migration at 30% C_{BIS},



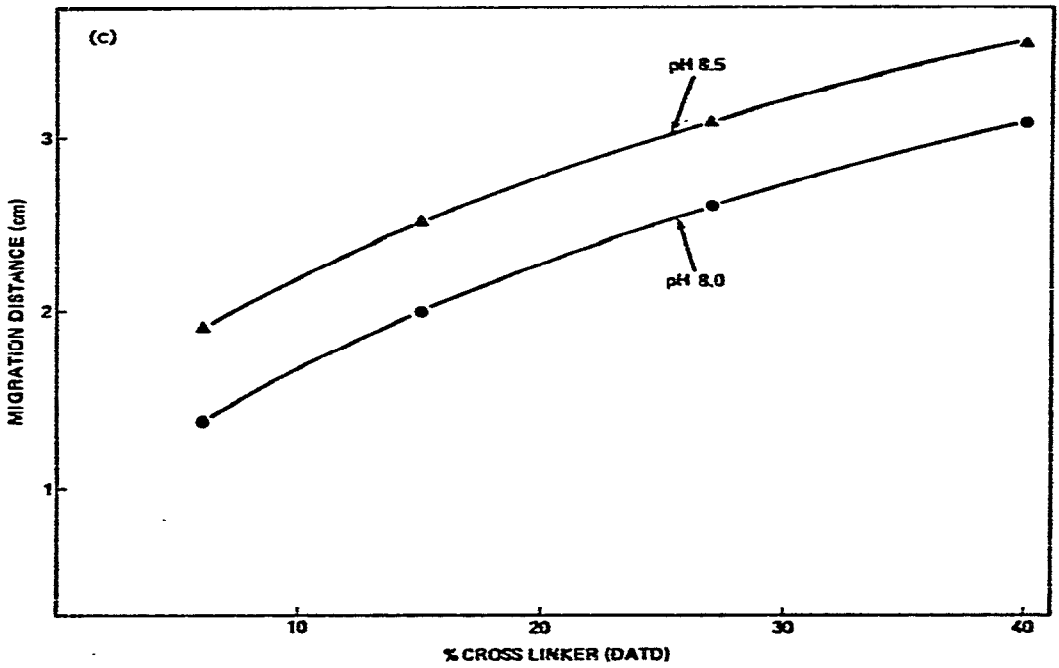


Fig. 6. Titration curves (A, B) and mobility data (C) of ferritin as a function of percentage of cross-linker (DATD). (A and B) pH–mobility curves of ferritin in 27% C_{DATD} gels polymerized under "standard conditions" (A, 50% conversion rate) and under 95% polymerization efficiency (B) after removal of persulphate and equilibration in Ampholine as described in the text. In (A), the two broken lines circumscribe the gel area where severe distortion of the ferritin titration curve occurs. Here, a blurred zone of ferritin molecules is observed, starting from the trench all the way to the outer edge of the pH–mobility curve. In (B), notice the complete absence of distortions and the much reduced mobility. (C) Mobility data of ferritin as a function of % C_{DATD} calculated at pH 8.0(●) and at pH 8.5 (▲) from a series of gels at different % C_{DATD} (not shown) polymerized under "standard conditions". Even though at these pH values the protein migrates outside the highly distorted pH zone (pH 6–8), the fact that the absolute migration distances travelled are higher than in equimolar BIS gels suggests that the protein has some amino groups reacted with DATD, resulting in a loss of positive charges.

gel had to be polymerized with very high levels of catalyst (1 g/100 ml of gel) in absence of Ampholine, the persulphate had to be washed out for 1 day and the gel equilibrated in 2% Ampholine for an additional day. This is certainly not amenable to routine application, since it is time-consuming and expensive in amounts of Ampholine used. When mobility data are plotted *vs.* % C in our routinely polymerized gels (which ensure *ca.* 50% DATD conversion) it can be seen that the ferritin mobility is still not quite leveled at 40% C_{DATD} (Fig. 6C), indicating that the average pore size is smaller than corresponding BIS gels (the mobility measurements were taken at pH 8.0 and pH 8.5, *i.e.* just outside the range of the heavy distortion due to unpolymerized DATD). Moreover, in the gel which had been polymerized to 95% efficiency (Fig. 6B), the mobility of ferritin was further decreased, indicating a further reduction in pore size. In addition, we could see that these "highly converted gels" were just as brittle and unelastic as the corresponding BIS gels of equimolar % C.

DISCUSSION

We finally seem to begin to understand the behavior of highly cross-linked polyacrylamide matrices. Especially in the case of DATD gels as prepared according to the original protocol of Anker[19] and others[12,13], all the strange properties reported, namely: gel swelling and stretching up to twice the original size[16], softness or gluey appearance of the gel, levelling of increase of pore size at 15% C (ref. 15) or invariance in pore size in the range 7.26 to 27% C_{DATD}[13] and concave and diffuse protein bands[13], can now be understood if we assume that a large amount of the cross-linker in the gel was actually never converted into polymer. At least in one case, where clear polymerization conditions were detailed[12], we have demonstrated that in fact 80–90% of the original DATD present in the gel is still left monomeric and unchanged after gelation. No wonder then that no progressive increase in pore size could be measured with progressive increase in % C_{DATD}: the gel was already a loose framework of fibers sparingly cross-linked to start with, even at low % C. These vast amounts of free cross-linker in the gel could have serious deleterious effects on the proteins to be separated. It is in fact known that acrylamide can react with free amino groups (especially the amino terminus of a protein chain, which usually has a low p*K*), thiol residues as well as hydroxyl groups of tyrosine. Since, in the case of ferritin, the strongest modifications are seen in the pH 6–8 range, this suggests reaction of DATD with terminal amino groups (there are 24 subunits in ferritin) and also with free, reactive thiol groups of cysteine residues. If we assume that there is no extensive charge alteration of the protein molecule, then the marked mobility decrease in the pH region 6–8 could be due to DATD acting as a cross-linker over different ferritin molecules, grafting them together into a high-molecular-weight aggregate. At the moment, however, we have not attempted to measure variations in ferritin molecular weight or the stoichiometry of ferritin/DATD. Highly cross-linked DATD gels are also quite dangerous for laboratory personnel handling them, owing to the high levels of unreacted monomer, which is known to have neurotoxic effects. "Highly cross-linked DATD gels" so far reported in the literature should in fact be renamed "highly un-cross-linked gels".

At the moment, the only acceptable highly porous gels for titration curves are 30% C_{BIS} gels. They allow unhindered migration of globular proteins up to $5 \cdot 10^5$

daltons, thus allowing measurements of practically free mobilities at any pH value. The fact that they are brittle (however, just as brittle as properly polymerized DATD gels) should no longer be a problem, since by the Silane A-174 casting procedure the gel stays firmly bound to the glass slab used for gel moulding and can be conveniently stained and destained without breaking into a thousand pieces. The problem in fact arises if the gel has to be detached from the glass surface for drying or storage. In this case, after a final equilibration step in 5% glycerol, the gel is firmly pressed against a sheet of Whatmann 3MM. As the filter paper is gently lifted, most of the gel can be sliced away from the glass surface with the aid of a long, sharp knife. Now the paper-pasted gel can be dried in the air.

In terms of general properties of highly cross-linked gels, DATD gels appear to be fully compatible with water, as seen by the ready solubility of DATD in water and as predicted from its 1,2-diol structure. On the other hand, BIS, which contains an additional methylene group for each two molecules of acrylamide, seems to be markedly more hydrophobic than DATD and acrylamide. It is sparingly soluble in water, and as seen in our experimental conditions, it causes the gel matrix to collapse and exude water above 30% concentration, a behavior typical of an hydrophobic polymer. Moreover, the gel surface in 40% and 50% C_{BIS} gels has a "dry" appearance and feels "dry" to the touch. The hydrophobicity of BIS could also be responsible for the peculiar way in which these gels polymerize. Above 10% BIS, the polymerization does not start and propagate uniformly throughout the gel, as customary in regular gels, but it begins in scattered points along the gel chamber. These "nucleation events" are clearly seen since droplets of opaque, polymerizing material appear suddenly through the chamber and begin to sediment, being denser than the surrounding medium. Hydrodynamic streaming is produced in their wake as these particles sediment. This probably results in some inhomogeneity of the gel matrix, but luckily this effect must be rather limited as we cannot see any disturbance in our titration curves.

Perhaps hydrophobic 50% C_{BIS} or pure BIS gels could be used as a medium for hydrophobic interaction chromatography or maybe for affino electrophoresis of membrane proteins. From a point of view of general behavior of highly cross-linked gels, at levels of 20% C_{BIS} or higher, the focusing time in the first dimension is practically halved (45–50 min as opposed to 90 min in regular gels) suggesting that routinely used gels (5–7% T, 4–5% C) retard the migration of even carrier ampholytes species (average molecular weight 800 daltons), as already reported for buffer ions[10]. In terms of reactivity, the scale appears to be: acrylamide > BIS ≫ DATD. The last molecule, in fact, seems to be effectively an inhibitor of polymerization.

An ideal matrix for titration curves would be a 0.5–1% agarose gel, which has an exclusion limit of *ca.* $1.5 \cdot 10^8$ daltons and thus would allow practically unhindered migration of macromolecules in the multimillion molecular weight range. However, even the best purified brand of agarose recommended for IEF (agarose EF from LKB), while satisfactory for equilibrium IEF, still contains enough charges to produce severe distortions on our titration curves (unpublished experiments with Dr. K. Ek, Stockholm, Sweden). Work is in progress to develop a purified agarose suitable for titration curves.

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NOTE ADDED IN PROOF

While this work was in press, it was brought to our attention by Dr. D. Halpern (Polysciences, Warrington, Pa., U.S.A.) that indeed allyl derivatives are generally poorly reacting species. We have been unable to find direct data on DATD; however, it has been reported that the C_M value (transfer constant to monomer) for most allyl derivatives is usually 1000 times higher than for acrylamide[20]. Moreover, the r_1 value (monomer reactivity ratio), which gives the extent to which a monomer either propagates a chain by reacting with itself or by reacting with another, different monomer present in the mixture, is, for allyl derivatives, usually 100 times smaller than for acrylamide and, in quite a few cases, it is altogether zero[21]. This is in complete agreement with our experimental data and further confirms the widening gap between the biochemical and chemical literature.

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