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POLYMERIZATION KINETICS OF POLYACRYLAMIDE GELS CONTAINING IMMOBILIZED pH GRADIENTS FOR ISOELECTRIC FOCUSING

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SUMMARY

A set of conditions is described for a reproducible incorporation of Immobiline chemicals into a polyacrylamide gel. 0.047% N,N,N′,N′-Tetramethylethylenediamine, 0.033% ammonium persulphate, pH between 7 and 8 and a polymerization time of 1 h at 50°C result in a very high conversion (84–88%) of monomers into the gel matrix with a minimum dispersion of the relative reactivity ratios among the different Immobiline chemicals. Considerably higher incorporations (93–97%) are obtained when polymerization is performed under anaerobic conditions, but this does not alter the relative reactivity ratios among the Immobiline species and considerably adds to the burden of casting an immobilized pH gradient. The different factors leading to optimal polymerization and highly reproducible Immobiline gels are critically evaluated.

INTRODUCTION

An entirely new concept for producing pH gradients for isoelectric focusing (IEF) was recently described[1], based on the principle that the buffering groups are covalently linked to the matrix used as anticonvective medium. For the generation of these types of gradients in polyacrylamide gels, non-amphoteric, buffering monomers, called Immobilines, are copolymerized with acrylamide and N,N′-methylenebisacrylamide (Bis). An Immobiline is either an acid or a base and the pK values for these buffers are such that any gradients in the interval pH 4–10 can be generated by appropriate mixtures of the available Immobilines. The pH values prevailing in the gel are defined by the concentrations and the dissociation constants of the incorporated Immobilines.

Immobilized pH gradients (IPG) have been refined into a highly sophisticated and very precise separation tool for fractionation and characterization of macromolecules. Conditions have been described for generation of ultra-narrow pH gra-

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dients (down to as little as 0.1 pH unit over the entire separation distance)[1], as well as wider gradients encompassing from 2 to 6 pH units[2–5]. A computer program has been developed which optimizes the linearity of the required pH gradient and calculates the buffering power, β, and ionic strength, I: the separations are thus conducted in a fully defined physico-chemical milieu. The preparative aspects of IPGs have also been fully explored and conditions have been described for optimizing the protein load in IPGs in terms of I, pH gradient width, gel thickness and matrix concentration, %T, *i.e.* (g acrylamide + g Bis) per 100 ml gel solution, and for detecting and recovering sample zones from IPG gels[6–8]. Applications have already been reported for peptide separation[9], hemoglobin analysis[10] and for studying the genetic polymorphism of serum proteins[11–13].

All this would be wasted if we did not have full control of the most critical step in this technique: the polymerization kinetics and incorporation efficiency of the different Immobiline chemicals during the gel casting procedure. In fact, in copolymerization chemistry it is often stated that the composition of the copolymer formed differs from the initial input[14] due to the fact that the monomers diverge in reactivity toward free radical addition. Thus, with less than 100% incorporation of monomers into the polymer, there exists a possibility that the concentration ratios between the Immobilines built into the gel will differ from the ratios in the starting gel solutions: this would have serious consequences on the pH gradient generated, *e.g.*, by changing its slope and the theoretically computed pH interval. To minimize this effect all Immobilines are acrylamide derivatives, but even with this precaution it cannot be excluded that the resulting pH values will depend to a certain extent on the polymerization efficiency.

In this work the copolymerization of Immobiline species with acrylamide monomers has been studied, and the effect of the polymerization conditions on the conversion of monomers into the polymer matrix determined. This was done as described by Gelfi and Righetti[15,16] and by Righetti *et al.*[17,18], where the reaction was allowed to proceed in a standard cuvette of a double beam spectrophotometer, by which the extent and rate of polymerization could be estimated via measurements of the absorbance decrease at 285 nm (which corresponds to the disappearance of double bonds). We have used this technique for the following purposes:

(a, b) measurement of the extent and rate of reaction of gels containing acrylamide, Bis and, separately, the different Immobilines as a function of the amounts of persulphate and N,N,N′,N′-tetramethylethylenediamine (TEMED)

(c) measurement of the same parameters, in gels containing constant amounts of persulphate and TEMED, but at various temperatures

(d) as described in (c), with constant amounts of persulphate and TEMED, at a fixed temperature, but with different pH values in the gel solution

(e) measurement of the effect of anaerobic conditions (gel solution degassing and re-equilibration under nitrogen) on the extent and rate of polymerization, all other parameters (pH, temperature, amounts of catalysts) being kept constant and at optimum level.

Based on this, suitable conditions for generation of pH gradient gels are given. We have also studied how the resulting pH gradient corresponds to the polymerization efficiency, by computer simulation of the same narrow pH gradient under conditions expected to result in different conversion of monomers.

MATERIALS AND METHODS

Chemicals

Acrylamide, Bis, Immobilines p*K* 3.6, 4.4, 4.6, 6.2, 7.0, 8.5 and 9.3, Repelsilane, Bindsilane and Coomassie Brilliant Blue R-250 were from LKB Produkter (Bromma, Sweden). Ammonium persulphate (AP) and TEMED were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). All other chemicals were of analytical grade.

Spectrophotometric measurements

The extent and rate of polymerization were followed with a Cary 219 spectrophotometer (Varian, Sunnyvale, CA, U.S.A.) under a set of rigorously defined experimental conditions, and the absorbance decrease read at 285 nm. The polymerization kinetics were followed for 1 h. The spectrophotometer was connected to a Julabo thermostat. All gels were made to contain 5% T and 4% C (g Bis per 100 g T) (for a definition of T and C see ref. 19) and 10 m*M* Immobiline with various p*K* values. To investigate the influence of catalysts, various amounts of persulphate and of TEMED were used ranging from 0.015 to 0.058% for the former and from 0.024 to 0.096% for the latter. The rate of polymerization was also followed at different temperatures from 20 to 60°C, and at different pH values in the gel solution (at pH 4.5 the gels contained 80 m*M* acetate, 80 m*M* phosphate at pH 6.5 and 80 m*M* Tris–Gly buffer at pH 8.5). The gel solution was poured into a standard, 1-cm optical pathlength, quartz cuvette thermostatted at the temperature used for the experiment, before the catalysts were added. The blank cuvette contained the same chemicals as the sample cuvette, except the acrylamide monomers and Immobiline. The instrument was operated at the minimum chart speed (50 sec/cm), and at an absorbance range of 2.

Anaerobic conditions

The measurements were performed in quartz cuvettes especially designed for anaerobic kinetic studies, with 1-cm optical pathlength and facilities for adding the catalysts from two upper, separate bags fused to the cuvette stopper. This allows mixing of the catalysts with the gel solution, after degassing, without opening the closed cuvette. The gel solutions were degassed in the cuvette at room temperature for 5 min with a vacuum pump at 0.1 mmHg, the degassing being interrupted with highly purified nitrogen. The cuvette containing the gel solution was equilibrated at 50°C, whereafter the catalysts were mixed at a final concentration of 0.033% AP and 0.049% TEMED. The reaction rate was followed at a series of different pH values obtained by use of the buffers described earlier.

RESULTS

Polymerization efficiency as a function of amounts of catalysts

In this set of experiments, each Immobiline, at a level of 10 m*M*, is copolymerized in a 5% T, 4% C gel, at a constant temperature of 50°C and constant pH of 8.0. One hour after the addition of catalysts, the polymerization efficiency is expressed as [1 − (final absorbance)/(initial absorbance)] × 100 (percentile value), *i.e.*, the disappearance of double bonds is taken as a measure of the incorporation of

monomers into the gel matrix. We have first investigated the effect of different levels of persulphate over a four-fold concentration range, from 0.0145 to 0.058%. As shown in Fig. 1A, optimum conversion of Immobilines (84–88% incorporation efficiency) is obtained at a persulphate concentration of 0.033%: both below and above this level the incorporation decreases in an almost symmetrical fashion. This effect is only partially reproduced if we measure the lag time (the time lapse between the onset of polymerization after adding the catalysts) (see Fig. 1B): this is spread over 3–10 min for the different Immobilines at 0.0145% AP, but decreases to only 2–4 min at 0.033% AP; at this point, there is no further decrease in induction period with increasing level of persulphate.

A somewhat similar phenomenon is found when changing the concentration of TEMED over a four-fold range, from 0.024 to 0.096% (Fig. 2A). At the lowest level, the incorporation efficiency varies considerably (from 76 to 90%); there is a confluence point (84–88%) at a level of 0.047% TEMED and then a general, slight decrease of the copolymerization efficiency as the amount of TEMED is further

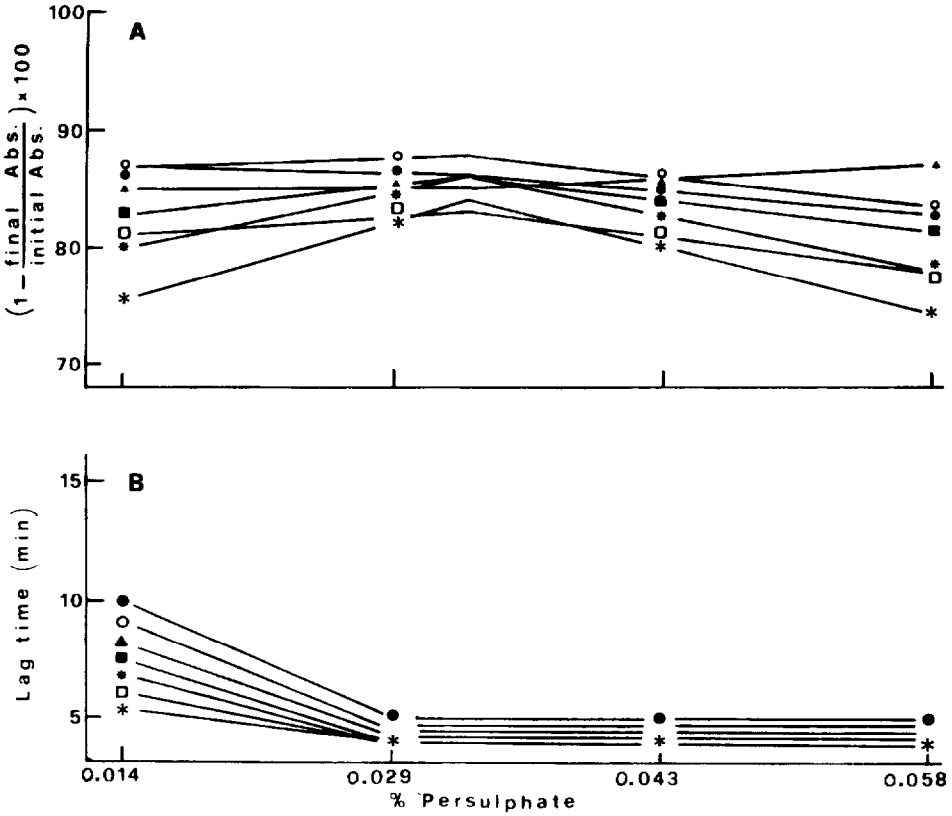


Fig. 1. (A) Efficiency of incorporation of the seven Immobiline chemicals as a function of persulphate concentration (0.0145–0.058%). (B) Lag time (induction period from addition of catalysts to the onset of polymerization) as a function of persulphate level in solution. Conditions: 10 m*M* each Immobiline in a 5% T, 4% C gel; reaction for 1 h at 50°C, pH 8.0 and 0.047% TEMED. The disappearance of double bonds was followed at 285 nm in a Cary 219 spectrophotometer with a 2A full scale. Immobiline p*K* values: 3.6 (○); 4.4 (●); 4.6 (▲); 6.2 (□); 7.0 (■); 8.5 (*); 9.3 (•).

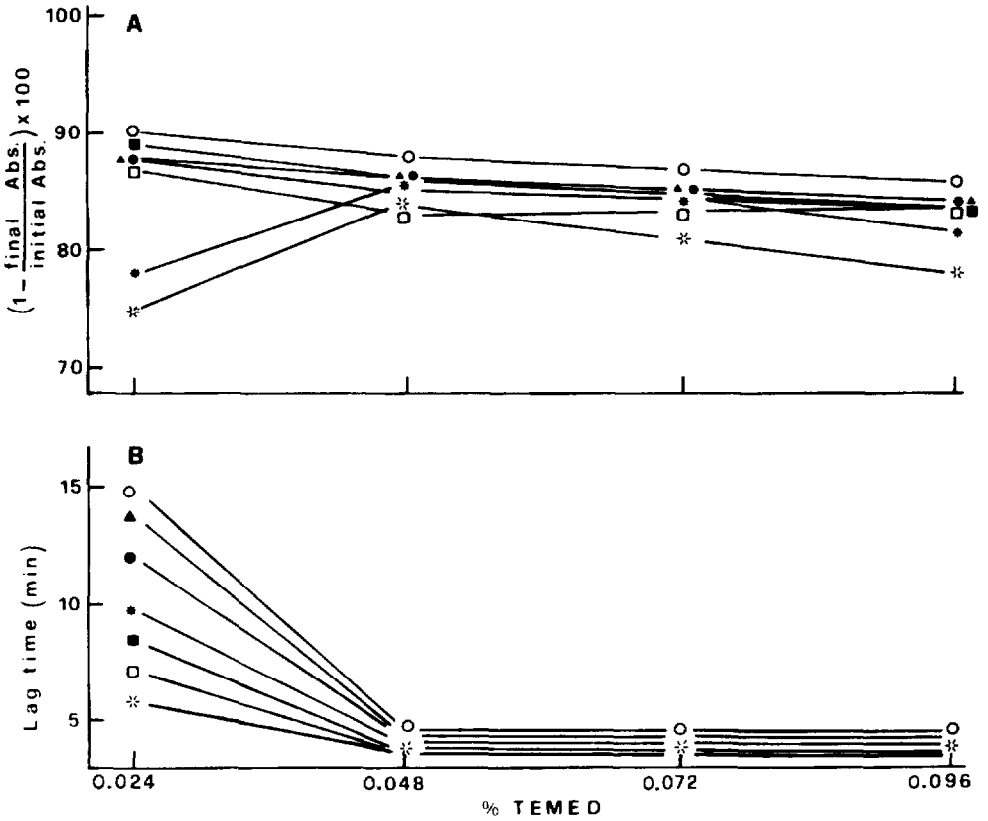


Fig. 2. Incorporation efficiency (A) and lag time (B) for the seven Immobiline chemicals as a function of TEMED concentration (0.024 0.096%). All conditions as in Fig. 1, except that the amount of persulphate was kept constant at 0.033%.

increased. The lag time pattern is practically identical to the one exhibited in the case of persulphate: an induction period over 5–15 min for the different Immobilines at the lowest TEMED level, which is reduced to only 2–4 min at a 0.047% concentration (Fig. 2B). The system tends to a plateau and no further effects are seen upon increasing amounts of TEMED. Interestingly, with both catalysts, the largest effects of varying their amounts are produced on the two most basic Immobilines, those with p*K* 8.5 and 9.3 (see Figs. 1A and 2A).

Effect of pH

Another parameter which could affect the efficiency of incorporation of the Immobiline monomers into the gel matrix is the pH prevailing in solution during polymerization. It is known that radical reactions with the couple AP–TEMED do not progress well at acidic pH values[20]. To investigate this aspect, the polymerization kinetics were followed at pH 4.5 in 80 m*M* acetate, at pH 6.5 in 80 m*M* phosphate and at pH 8.5 in 80 m*M* Tris–Gly buffers, while keeping all other parameters constant: 0.033% AP, 0.047% TEMED and 50°C for 1 h. As shown in Fig. 3A, the incorporation efficiency is substantially decreased, and differs considerably for the

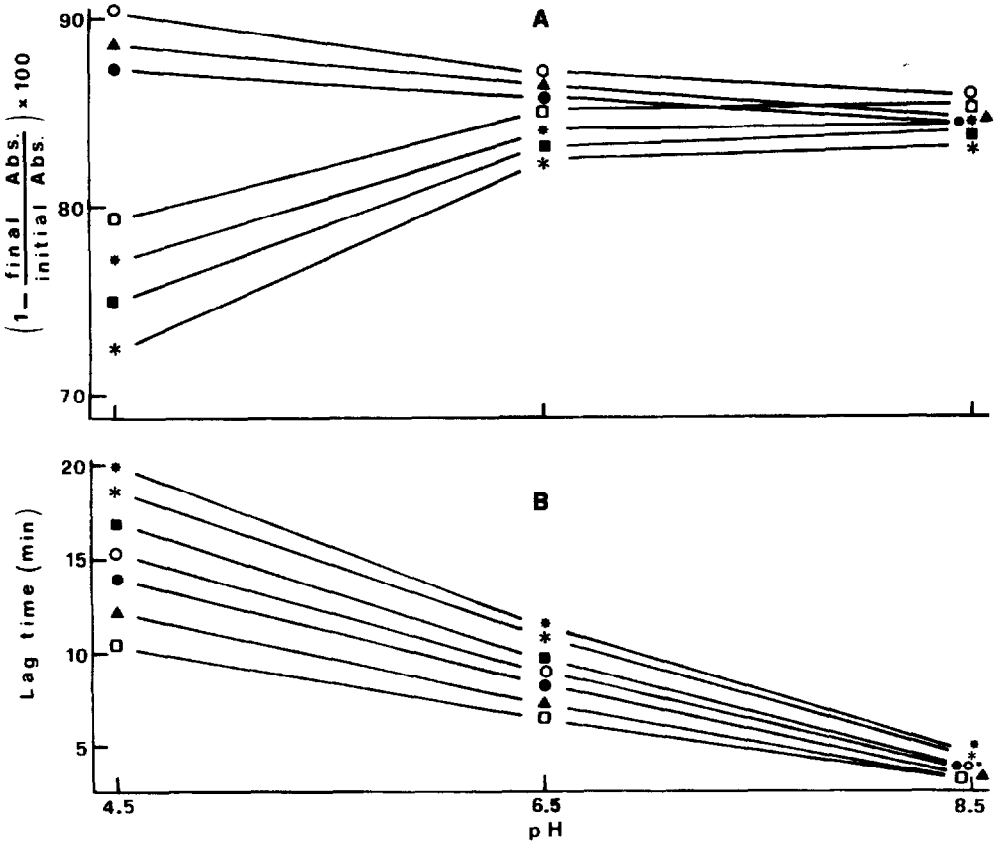


Fig. 3. Efficiency of incorporation (A) and lag time (B) for the seven Immobiline chemicals as a function of pH in solution: pH 4.5, in 80 m*M* acetate buffer; pH 6.5, in 80 m*M* phosphate buffer; pH 8.5, in 80 m*M* Tris–Gly buffer. Conditions: 10 m*M* each Immobiline in a 5% T, 4% C gelling solution; reaction for 1 h at 50°C, 0.047% TEMED and 0.033% AP.

various Immobiline chemicals, at pH 4.5; there is a marked improvement at pH 6.5, after which little is gained by increasing the pH to 8.5. However, the lag time pattern (Fig. 3B) shows a markedly different behaviour: the induction period is very long (12–20 min) at pH 4.5, is reduced at pH 6.5 (5–10 min) but reaches a minimum (2–4 min) only at pH 8.5. In view of these findings, we recommend that Immobiline gels be polymerized at any pH around neutrality or slightly alkaline: routinely, we have adopted pH 8.0 for casting our gels even though any pH between 7.0 and 8.5 will be satisfactory.

Effect of temperature

Temperature is another variable which significantly affects radical reactions; even in the absence of Immobilines, Gelfi and Righetti[16] have demonstrated that 50°C is in general needed to produce homogeneous gels, by breaking up clusters of cross-links, especially in the case of Bis. We have therefore explored the effects of temperature by polymerizing Immobiline gels at between 20 and 60°C, using 10°C

increments, all other parameters being kept constant at the optimal level: 0.033% AP, 0.047% TEMED and pH 8.0. As shown in Fig. 4, Immobiline gels are no exception to the above rule: at 20°C the polymerization efficiency is rather poor and highly variable for the seven Immobiline species. Curiously, at 60°C, the incorporation efficiency is slightly lower for some compounds (notably the alkaline ones, pK 8.5 and 9.3). Polymerization for 1 h at 50°C, as previously suggested for conventional gels[16], appears to be the optimum: all Immobilines seem to come to a confluence point at this temperature, exhibiting very similar reactivity ratios and incorporation efficiencies (84–88%). Therefore, in all our Immobiline work, we have adopted an incubation time of 1 h at 50°C in a forced-ventilation oven as a standard gel casting procedure.

Aerobic vs. anaerobic conditions

Even when all experimental variables were optimal, we have never been able to achieve greater than 84–88% incorporation. Previously, Bianchi Bosisio *et al.*[21] described conditions leading to greater than 96% conversion of monomers into the gel matrix. The main difference between the two experimental set-ups is that in the present case the gels are polymerized under aerobic conditions since they are cast with the aid of a gradient mixer equilibrated in air. We have therefore repeated our experiments by degassing all gel solutions for 5 min at 0.1 mmHg and then equilibrating them with O_2-free nitrogen, all other experimental conditions being kept

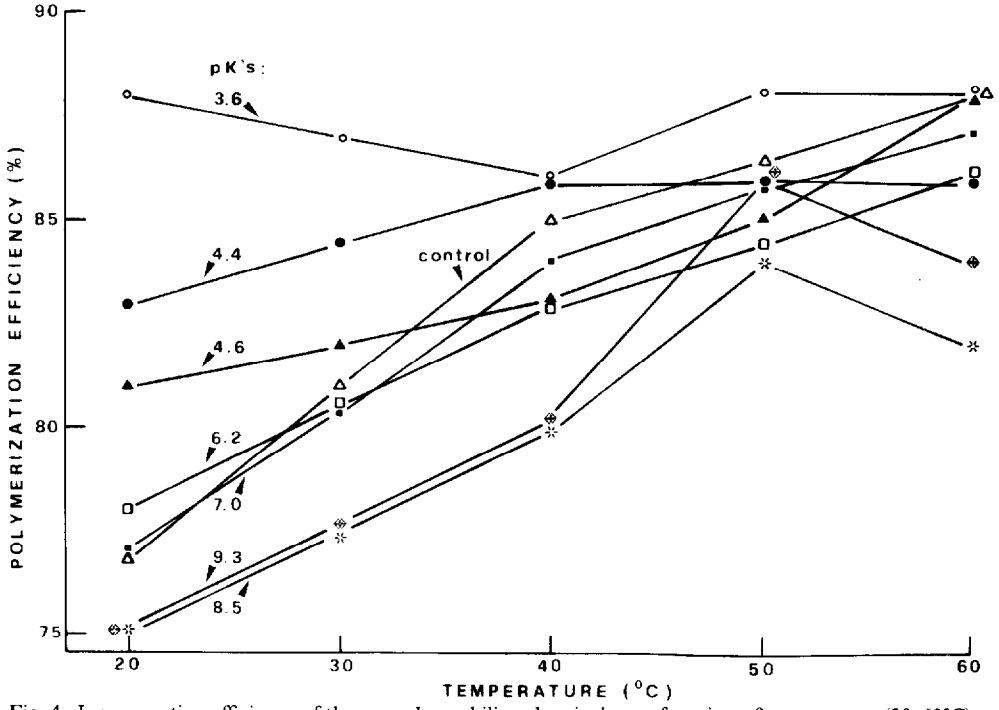


Fig. 4. Incorporation efficiency of the seven Immobiline chemicals as a function of temperature (20–60°C). Conditions: 10 mM each Immobiline in a 5% T, 4% C, pH 8.0 gelling solution; reaction for 1 h, 0.047% TEMED and 0.033% AP.

constant at the optimized levels, as described in previous sections. The results are summarized in Table I: in the absence of oxygen the incorporation efficiency is indeed increased for all Immobiline species by about 9–10%, thus reaching levels of the order of 93–97% as compared with 84–88% under standard conditions. This is the best that can be achieved with this reaction. We have also built a gradient mixer with which the solutions can be degassed *in situ* and re-equilibrated under oxygen; however, due to the more cumbersome experimental manipulations involved, we suggest, for the great majority of cases, that the standard polymerization procedure be adopted for Immobiline gels under aerobic conditions (see Discussion).

Simulation of an Immobiline gradient under "proper" and "poor" polymerization conditions

In order to study the effect of different polymerization conditions on a theoretically predicted and, in principle, highly reproducible, Immobiline gradient, we have selected two cases in which the couple buffer–titrant either converge or maximally diverge in incorporation efficiency. We have therefore neglected the case of "aerobic" *vs.* "anaerobic" conditions, since here it is only the overall conversion level which is increased, leaving practically unaltered the ratio between the two Immobilines defining a given, narrow pH gradient. Among the different polymerization conditions described, the one having the most significant effect is the temperature (see Fig. 4). We have thus compared the expected pH gradient, and the accompanying buffering power and ionic strength, for an ideal gel (100% incorporation) or in the case of polymerizations at 50°C and 20°C. The couple selected is Immobiline pK 8.5 (buffering species), titrated in the pH interval 8.2–9.2 with Immobiline pK 3.6, since they have the most different incorporation efficiencies at both 20°C and at 50°C. (For calculations, see ref. 22, bearing in mind that at 10°C in the gel the pK of the buffer is still 8.5.) The data are presented in Table II: when polymerizing at 50°C, the two values which are most greatly affected are β and I, which are about 14% less then

TABLE I

POLYMERIZATION EFFICIENCY OF IMMOBILINES UNDER AEROBIC AND ANAEROBIC CONDITIONS

Each Immobiline was 10 mM in a 5% T, 4% C gel solution, under optimal polymerization parameters: 0.033% AP, 0.047% TEMED, 1 h at 50°C, pH 8.0. The disappearance of double bonds is taken as a measure of polymerization efficiency (percentile).

Immobiline pK	$(1 - \frac{Final\ absorbance}{Initial\ absorbance}) \times 100$	
	Aerobic	*Anaerobic*
3.6	88	97
4.4	85	94
4.6	84.5	94
6.2	85	94
7.0	85	94
8.5	84	93
9.3	85	95

TABLE II

ALTERATION OF pH GRADIENT, β AND I AS A FUNCTION OF POLYMERIZATION CONDITIONS

A pH 8.20–9.20 gradient was simulated with pK 8.5 as buffering and pK 3.6 as titrating Immobilines (calculations made at 10°C in the gel phase). β = buffering power in mequiv. l^{-1} pH^{-1}; I = ionic strength in mequiv. l^{-1}.

Immobiline pK	*Theoretical (100% polymerization efficiency)*				*50°C polymerization*				*20°C polymerization*			
	mM	*pH*	β	I	*mM*	*pH*	β	I	*mM*	*pH*	β	I
8.52 3.58	9.14 6.15	8.20 (acidic chamber)	4.63	6.15	7.68 5.40	8.15	3.70	5.40	6.85 5.40	7.95	2.63	5.40
8.52 3.58	11.99 2.07	9.20 (basic chamber)	3.95	2.08	10.07 1.82	9.18	3.44	1.83	8.99 1.82	9.11	3.35	1.83

theoretically predicted, in view of the average incorporation of the two Immobilines of *ca.* 86%. However, the pH gradient span is only slightly different from the computed one since the ratio of the two Immobilines incorporated into the gel does not diverge greatly from the theoretical case; the actual pH span is shifted from 8.2–9.2 to 8.15–9.18. When polymerizing at 20°C, on the other hand, the situation is considerably worsened: not only the average β and I values are further decreased by *ca.* 20% on the average, but also the experimental pH interval becomes unacceptably different from the computed one (from 8.2–9.2 to 7.95–9.11). This is because at 20°C Immobiline pK 8.5 is only 75% incorporated, whereas the titrant (pK 3.6) exhibits an incorporation efficiency of 88%: the resulting pH gradient is therefore quite acidic. It should be noted that the slight alteration in pH gradient exhibited at 50°C, although small, is still the widest possible one: the other Immobiline species encompass a rather narrow conversion interval (84–86%), thus ensuring that their ratio in the gel will be quite similar to their ratio in solution.

DISCUSSION

Many variables can affect copolymerization of Immobilines into a polyacrylamide gel. We will try to evaluate critically some of them.

Hydrogen bonds

Hydrogen bonds are about 0.3 nm in length and 1.5–6 kcal/mol in strength. They have been demonstrated to exist at low temperature and in non-ideal solvents for most cross-linking agents, notably Bis: they lead to inhomogeneities by clustering (opaque gels)[15,16]. This phenomenon is fully abolished by polymerizing at 50°C; in any event, there is no evidence at present that Immobiline chemicals are hydrogen-bonded in solution.

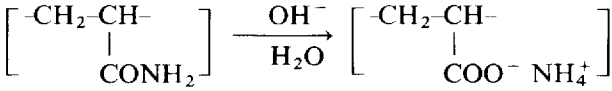
Ionic bonds

Ionic bonds are 0.2–0.3 nm in length and 10–20 kcal/mol in strength. As Im-

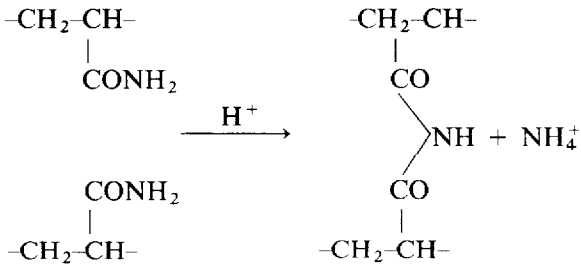
mobilines are monovalent ions, the probability that basic and acidic species will be ionically bonded in aqueous solution (especially at 50°C) is practically nil. However, whether they can interact ionically within the polyacrylamide matrix is unknown: this will depend on the charge density of the polymer coil (ratio of acrylamide to Immobiline), the spatial relationship among the charged species and on the flexibility of segments within the matrix network (degree of cross-linking, which can restrict the motion of longer or shorter polymer coils).

Effect of pH

We have demonstrated that the highest degree of polymerization is obtained in the interval pH 7.0–8.0. At pH > 9 and high temperatures (50°C), there is a hydrolysis of the amide bonds in the matrix, according to the reaction[23]



while at very low pH values (below pH 2.5) the reaction proceeds via loss of ammonia and formation of imido groups:



While the latter conditions are very rarely used in acrylamide gel polymerization, the former (pH > 9) should also be avoided when casting Immobiline gels, since conditions which would lead to hydrolysis of the matrix could also lead to degradation of Immobilines into their constituents, free acrylic acid and free buffering ion.

The fact that there is no further improvement of conversion efficiency above pH 8.0 is in agreement with the data of Pegon and Quincy[24]; moreover, the fact that the lag time is reduced to a minimum at pH 8.5 suggests that TEMED is already sufficiently deprotonated to play its rôle as an accelerator. In addition, the finding that at low pH the reaction is impaired is of great practical significance: when operating with wide Immobiline ranges (> 2 pH units), in order to ensure adequate and reliable polymerization efficiency, the acidic end of the pH gradient should be titrated to the same pH as the basic one.

Effect of catalysts

The effects of TEMED and persulphate are similar: at low levels, the lag time is considerably increased (up to 15 min) and the relative reactivities of the Immobilines are considerably different (from 75 to 88%). At the optimum levels (0.047%

TEMED and 0.033% AP) high reactivities are found, with a minimum of dispersion (84–88% conversion). (It should be noted that the most critical parameter for immobilized pH gradients is not so much the absolute incorporation level of each species, as the maintenance of a constant ratio of incorporation among the different species, which should be kept as close as possible to unity.) At higher catalyst levels, the reactivities again diminish somewhat. This is in agreement with the findings of Richards and Lecanidou[25] that: (a) excess of catalyst leads to short polymer chains; (b) excess of catalyst can inhibit gel formation. These authors suggest that it is best to use equimolar concentrations of ammonium persulphate and TEMED, generally in the 1–10 m*M* range.

Temperature

High temperature too is known to reduce the average length of polyacrylamide chains[18], but this should be of no consequence to charge separations, such as conventional IEF and IPG. The increase in conversion at 50°C could be due to several factors: (a) increased rate of decomposition of AP with consequent increase in the concentration of free radicals at the steady-state (they are in general present at very low levels, usually in the nanomolar range); (b) decreased viscosity of the liquid phase, which increases the rate of diffusion of the bulky Immobilines (N-substituted acrylamides are known to have lower reactivity rates, proportional to the number and size of the substituents); (c) decrease of the activation energy barrier (Arrhenius equation).

Aerobic vs. anaerobic conditions

Anaerobic polymerization is definitely superior to casting a gel in air, since the incorporation efficiency is the highest possibly achievable (93–97%) and therefore, in principle, it should be the preferred procedure. In practice, it should be evaluated also in terms of how much more difficult the technique becomes. In fact, not only a special gradient mixer, enabling degassing *in situ* and re-equilibration under nitrogen, should be built but also the gel-casting cassette should be kept either under a glovebox saturated with nitrogen, or it should be purged with argon (which will stay in the cassette, since it is denser than air). The practical difficulties of such a casting procedure do not make it very attractive for routine purposes. Moreover, since the most critical parameter in IPG gels is the ratio buffering/titrating Immobiline, and not so much their absolute incorporation efficiency, for most practical purposes it will suffice to polymerize at 50°C for 1 h under aerobic conditions (pH and catalyst levels being kept constant at the optimum) and then recalculate the β and I values (and, if necessary, a minute correction in pH gradient) on the basis of the incorporation efficiencies of each Immobiline as found in Fig. 4.

CONCLUSIONS

The use of the appropriate pH, temperature and amounts of TEMED and persulphate ensures that the Immobiline matrix will polymerize as a random copolymer, with a distribution of monomeric units approaching the statistical one. Inappropriate polymerization conditions, especially the use of low temperatures (< 20°C), could drive the equilibrium towards the formation of a "pure block" or a "graded

block" copolymer[14], *i.e.*, one in which the Immobilines, by virtue of their strongly reduced reactivities, would attach themselves as a string at the end of a pure polyacrylamide coil. The practical consequences of this would be disastrous: the pK values would be significantly altered, and thus the resulting pH range in the gel would be quite different. Moreover, highly charged gel zones could strongly bind to the proteins to be separated and would also strongly absorb the dye during the process of staining and destaining, giving a spotted gel.

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