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Structure–stability relationship of Immobiline chemicals for isoelectric focusing as monitored by capillary zone electrophoresis

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ABSTRACT

An acrylamido buffer for isoelectric focusing in immobilized pH gradients, 1-acryloyl-4-methylpiperazine (pK = 6.85 at 25°C), was synthesized. As it is a disubstituted amide, it was thought that it would be much more resistant to alkaline hydrolysis. In reality, it degraded rapidly in 0.1 M sodium hydroxide solution at 70°C (86% in 6 h). Therefore, the stability of Immobiline buffers of pK 9.3, 8.5, 7.0 and 6.2 was investigated. Under the same hydrolysis conditions, the degradation was 11%, 22%, 26% and 34%, in order of decreasing pK values. The kinetics of degradation were monitored by capillary zone electrophoresis in 0.1 M borate buffer (pH 9). The decrease of the main Immobiline peak and the appearance of its hydrolytic products, acrylic acid and a diamine, could be easily measured. The following general rules were derived: when the nitrogen engaged in the amido bond is inserted into a cyclic structure (*e.g.*, 1-acryloyl-4-methylpiperazine) there is very little protection against hydrolysis; and conversely, when this nitrogen carries flexible, fairly long substituents (4–5 atoms long) much stronger shielding and protection of the amido bond can be obtained. These findings helped in designing new acrylamido derivatives strongly resistant to chemical degradation.

INTRODUCTION

Isolectric focusing (IEF) in immobilized pH gradients (IPGs) represents perhaps the most powerful development in electrokinetic separations, with an unrivalled resolving power and a very high load ability in preparative runs [1]. The power and precision of IPG rely on the quality of the buffers used to generate and maintain the pH gradient in the electric field. Unlike conventional IEF, where the pH gradient is obtained by electrophoretic sorting of a large number of soluble amphoteric buffers, called carrier ampholytes [2], the IPG technique uses a set of a few, well defined chemicals available commercially as crystalline powders or liquids. We have recently

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decoded the structures and given the formulae of acidic [3] and basic [4] Immobiline chemicals. In addition, we have proposed over the years a number of additional compounds for expanding the fractionation ability of IPGs: both more acidic [5,6] and more alkaline [7] compounds have been produced in our laboratory. We have also synthesized analogues of the weakest Immobiline bases (the morpholine derivatives, with pK values of 6.2 and 7.0) : by introducing a thiomorpholino ring, the pK values of these compounds were increased to 6.6 and 7.4, respectively, thus offering additional species buffering around neutrality, *i.e.*, in a region which normally lacks suitable buffering groups and where the bulk water conductivity reaches a minimum [8]. A new hydrophilic Immobiline with a pK of 8.05 has also been synthesized recently, in order to close the gap in the pH 7.0–8.5 region [9]. Hence, the family of acrylamide buffers is expanding: we have now described fourteen monoprotic compounds and there is a report on a biprotic species, itaconic acid [10]. Nevertheless, we have continued the search for new chemicals, especially for compounds buffering around neutrality, so as to increase the versatility and flexibility of the IPG technique.

We report here the synthesis of a new weakly basic acrylamide buffer, 1-acryloyl-4-methylpiperazine (AMPip), with pK = 6.85 (at 25°C). This compounds was chosen because it has a disubstituted amido group, in the hope that it would show a high stability against alkaline hydrolysis. These expectations, although in line with current chemical understanding, proved to be fallacious: AMPip turned out to be the most unstable of all alkaline Immobiline chemicals synthesized so far. In an attempt to understand this phenomenon, we reinvestigated the hydrolysis kinetics of the most common Immobiline species available, *i.e.*, those with pK = 9.3, 8.5, 7.0 and 6.2. The results contributed to the understanding of the mechanism of their degradation and to the formulation of future, highly stable chemicals.

EXPERIMENTAL

Commercial Immobilines, Repel- and Bind-silane, Gel Bond PAG, the Multiphor II chamber, Multitemp thermostat, the Macrodrive power supply and Pharmalyte carrier ampholytes (pH 7–9) were purchased from Pharmacia–LKB Biotechnology (Bromma, Sweden). Non-commercial acrylamido weak acids and bases were synthesized as reported [5–9]. Acrylamide, N,N'-methylenebisacrylamide (Bis), TEMED, ammonium persulphate and Coomassie Brilliant Blue were obtained from Bio-Rad Labs. (Richmond, CA, USA). 1-Acryloyl-4-methylpiperazine (AMPip) and acryloylmorpholine (AMorph) were a gift from Dr. M. C. Tanzi (Institute of Organic Chemistry, School of Engineering, Milan, Italy). Acrylic acid was from Fluka and was distilled just prior to use. AMPip was synthesized and purified as described [11]; the synthesis of AMorph has been reported by Artoni *et al.* [12]. Horse heart myoglobin was purchased from Sigma (St. Louis, MO, USA) and haemoglobin mutants were a gift from Dr. A. Mosca (University of Milan, Milan, Italy). Mandelic acid, used as an internal standard in capillary zone electrophoresis (CZE) runs, was purchased from Aldrich (Steinheim, Germany).

Alkaline hydrolysis

All acrylamide derivatives and Immobiline buffers were dissolved (10 mM each) in 0.1 M sodium hydroxide solution and incubated at 70°C, under a nitrogen atmo-

sphere, for up to 6 h. At hourly intervals aliquots were collected and diluted in 0.1 *M* borate buffer (pH 9.0) to 2.5 m*M*. Mandelic acid (2.50 m*M*) was then added, followed by CZE analysis.

Capillary zone electrophoresis

CZE was performed in a Beckman (Palo Alto, CA, USA) instrument (P/ACE System 2000) equipped with a 50 cm × 75 μm I.D. capillary. All runs were performed at 25°C in a thermostated environment in 0.1 *M* borate (pH 9.0). In all instances the migration direction was toward the negative electrode, which means that the acidic species (acrylic and mandelic acid) are transported there by electroosmosis, as they migrate electrophoretically toward the positive electrode. The sample was injected in the capillary by pressure, usually for 10 s. The calibration graph for each acrylamido derivative analysed was constructed with the Beckman Gold integration system, with the concentration points 0.25, 0.50, 1.00, 1.25, 2.00, 2.50 and 3.50 m*M*. In each run mandelic acid (2.50 m*M*) was used as an internal standard.

Thin-layer chromatography (TLC)

TLC was performed on silica gel $60F_{254}$ plates from Merck using chloroform–methanol (7:3, v/v) as eluent. The spots were revealed either with 3.5% molybdophosphoric acid in ethanol or with nihydrin. The reaction products formed during alkaline hydrolysis were analysed every 2 h and after prior extraction in chloroform.

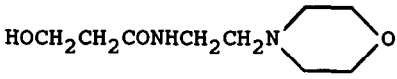
Column chromatography

As all monosubstituted acrylamide buffers revealed, after hydrolysis, in addition to two major components (acrylic acid and diamine) a small, unidentified peak, preparative purification of this unknown compound was attempted. A 1-g amount of p*K* 6.2 Immobiline (2-morpholinoethylacrylamide, as a representative compound) was hydrolysed at 70°C for 6 h and then extracted with chloroform. The 400 mg recovered were purified on a silica gel column (Merck 60, 230–400 mesh) with a ratio 1:80 (w/w) of product to silica gel [elution system chloroform–methanol–water (15:10:2, v/v/v)]. A 40-mg amount of the unknown product (see spot 3 in Fig. 3) was recovered and analysed by NMR and IR spectrometry.

NMR spectrometry

The unknown degradation product of the p*K* 6.2 Immobiline (see spot 3 in Fig. 3) was purified and subjected to NMR spectrometry. NMR analyses were carried out for solutions in [^{2}H]chloroform, C^2HCl_3 using tetramethylsilane (TMS) as internal standard, with a Model AM-500 (at 500 MHz, for ^{1}H) and an AC-200 (at 50.3 MHz, for ^{13}C) NMR spectrometer (Bruker, Rheinstetter, Germany). The ^{1}H and ^{13}C NMR spectra lacked signals for olefinic function. The presence of the CONH function was shown in an IR spectrum (a band at 1670 cm^{-1}), in an ^{13}C NMR spectrum (δ 170 ppm for CO) and in a ^{1}H spectrum, due to the presence of a quartet at δ = 3.345 ppm; the value of δ was as expected for a methylene group linked to an amide function (in the diamine precursor the corresponding signal was at δ = 2.7 ppm). After exchange of mobile NH hydrogen with deuterium oxide, the signal was transformed to a triplet (J = 6.4 Hz, due to the vicinal coupling with a CH_2). The ^{1}H NMR spectrum (with exclusion of 2 mobile hydrogens) showed only three groups of

signals: the first at δ 3.75–3.65 ppm (6H), the second at 3.345 (2H) and a third at 2.5–2.42 ppm (8H). A two-dimensional ^1H homonuclear shift correlation experiment (COSY) showed that the CH_2NHCO signal at δ = 3.345 ppm is coupled with a signal in the third group (centred at 2.48 ppm) and that the two parts of the first group of signals (centred at 3.73 and 3.68 ppm) are coupled with signals in the third group (centred at 2.48 ppm). These findings suggest the following structure, due to a nucleophilic addition of water to the conjugated double bond of the acrylamide moiety:



The first group (δ = 3.75–3.65 ppm) is due to the primary alcoholic methylene and the morpholino CH_2–O–CH_2 groups, whereas the third group is due to the three methylenes of the tertiary amine function and to the CH_2CO group.

Isoelectric focusing in immobilized pH gradients

IEF in IPGs was performed in a T4%, C4% polyacrylamide gel[a] in the pH range 6.5–8.5. The recipe for this IPG interval, utilizing the commercially available Immobilines, was as given by Gianazza *et al.* [13], whereas the corresponding recipe for the same pH interval utilizing the new acrylamide buffer (p*K* 6.85) instead of the p*K* 7.0 Immobiline was as follows (in μl of 0.2 *M* Immobiline solutions per 15 ml of gel):

pH 6.5	p*K*	pH 8.5
380 μl	3.6	94 μl
138 μl	6.2	76 μl
92 μl	6.85	139 μl
269 μl	8.5	181 μl

The gel, after polymerization, washing and drying [1], was reswollen in 0.5% Pharmalyte (pH 6–8). Protein samples (20 μg each) were applied at the anodic gel side. The run was for 2 h at 400 V, followed by 4 h at 2000 V, 10°C. The gels were stained with Coomassie Brilliant Blue R-250 in Cu^{2+}

Synthesis of 1-acryloyl-4-methylpiperazine (AMPip)

AMPip was synthesized according to Barbucci *et al.* [11], modified as follows: 5 g (0.05 mol, 5.5 ml) of N-methylpiperazine were dissolved in anhydrous toluene and added dropwise to an acryloyl chloride solution (5.6 mol, 0.07 *M*) in 40 ml of the same solvent, at 0°C. A white precipitate was recovered by filtration. After suspending the precipitate in chloroform, 6.8 g (0.05 mol) of K_2CO_3 were added with stirring for 20 min. After eliminating precipitated salts by filtration and solvent evaporation, 2.0 g of product were recovered. This material was purified on a silica gel column (1:30 ratio of product to silica) and eluted with chloroform–methanol (9:1, v/v). The yield of purified product was 30% (1.5 g).

[a] C = g Bis/%T; T = g acrylamide + g Bis per 100 ml of solution.

Potentiometric titration

The new acrylamide buffer (AMPip) was titrated manually under nitrogen at 25°C. A 10-ml volume of a 10 m*M* AMPip solution was titrated with 10 ml of 10 m*M* hydrochloric acid. The p*K* value was independently assessed also by measuring the pH of a 2:1 molar solution of AMPip–titrant, which, by definition, should correspond to its p*K* value. The p*K* value was found to vary as a function of temperature as follows: 25, 20, 15 and 10°C, p*K* = 6.85, 6.88, 6.90 and 6.93, respectively.

RESULTS

Fig. 1 shows the titration curve for the synthesized weakly basic acrylamide buffer: it has a p*K* of 6.85 at 25°C, which is very close to that of the commercial Immobiline of p*K* 7.0 (3-morpholinopropylacrylamide). When this new compound was substituted, in an IPG pH 6.5–8.5 interval, for the p*K* 7.0 species, the two gels exhibited essentially identical protein patterns (Fig. 2), indicating that the two weakly basic acrylamide buffers are interchangeable. The reason why we synthesized this new compound, however, was in the hope of obtaining a highly stable derivative, resistant to alkaline hydrolysis, as AMPip is a disubstituted amide. It is well know that hydrolytic stability increases in the order acrylamide < methacrylamide < N-substituted acrylamide < N-substituted methacrylamide < N,N-disubstituted acrylamide [14]. In addition, we had previously reported the hydrolysis kinetics of alkaline Immobiline buffers and found them to be unstable [15] (they are all monosubstituted amides). Much to our surprise, however, when we performed some preliminary hydrolysis experiments, we found that AMPip degraded rapidly.

We therefore decided to study the degradation kinetics of all alkaline Immobiline buffers (p*K* 9.3, N,N-dimethylaminopropylacrylamide; p*K* 8.5, N,N-dimethylaminoethylacrylamide; p*K* 7.0, 3-morpholinopropylacrylamide; and p*K* 6.2, 2-morpholinoethylacrylamide) and of a neutral monomer (4-acryloylmorpholine), in the hope of elucidating the mechanism of their proneness or resistance to hydrolysis.

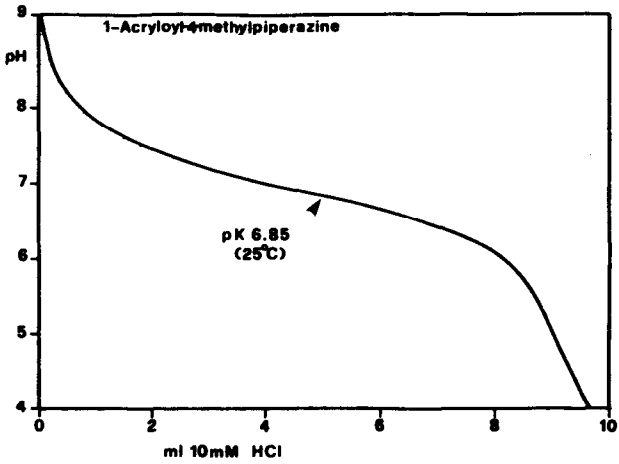


Fig. 1. Titration curve of 1-acryloyl-4-methylpiperazine. A 10-ml aliquot of a 10 m*M* solution of AMPip was titrated with 10 ml of 10 m*M* HCl at 25°C under a nitrogen atmosphere. The p*K* value was determined to be 6.85.

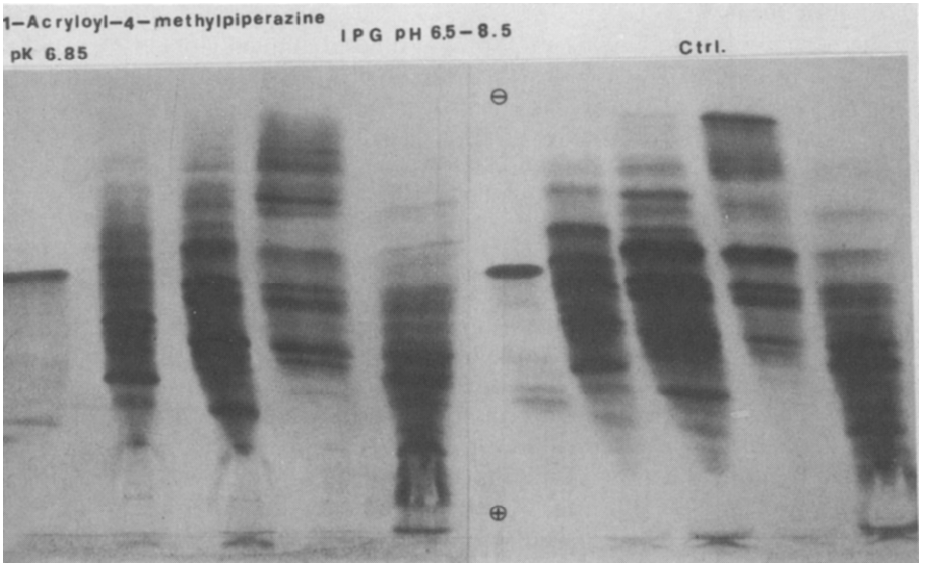


Fig. 2. Analytical IEF gel in the IPG pH 6.5–8.5 interval. The gel was a T4%, C4% polyacrylamide matrix, reswollen in 0.5% Pharmalyte (pH 6–8). Left, formulation containing the p*K* 6.85 chemical; right, recipe with the p*K* 7.0 Immobiline buffer (control, Ctrl.). Samples: 1 = horse myoglobin; 2 = haemoglobin A/S; 3 = hemoglobin A/lepore; 4 = hemoglobin A/C; 5 = hemoglobin A/S Paris. All samples loaded in a 20-μg amount at the anodic gel side. Run: 2 h at 400 V followed by 4 h at 2000 V. Stain: Coomassie Brilliant Blue R-250 in Cu^{2+}.

As an example of what happens to these chemicals on alkaline attack, Fig. 3 shows the TLC pattern of the p*K* 6.2 Immobiline before and after extended hydrolysis. The two main hydrolytic products are spots 1 (the diamine) and 2 (acrylic acid), *i.e.*, the two precursors used to synthesize the acrylamide derivative. There was a third, unidentified spot (No. 3), which we purified on a preparative scale and subjected to NMR analysis. The results of structural investigation suggested this to be the p*K* 6.2 species non-hydrolysed, but with a molecule of water added to the double bond (see Discussion). This latter compound represents a small percentage of the total hydrolytic products, so this degradation pathway is decidedly a minor one. All of the acrylamido buffers analysed showed the formation of the same type of degradation products.

CZE was instrumental in assessing the identity of the hydrolytic products and quantifying them. Fig. 4 shows a representative CZE run of (A) a control and (B) an extensively hydrolysed sample of 4-acryloylmorpholine, a neutral acrylamide monomer possessing a disubstituted amido group. It is seen that no acrylic acid is present in the control; at the end of the 6-h hydrolysis period, the monomer peak has greatly diminished and a large peak of acrylic acid has appeared. In all runs, mandelic acid (2.5 m*M*) was added to each sample as an internal standard for quantification purposes. The CZE pattern is representative of all the runs performed with all the chemicals investigated. After analysing all samples in CZE, we could construct curves representing the degradation kinetics of each species.

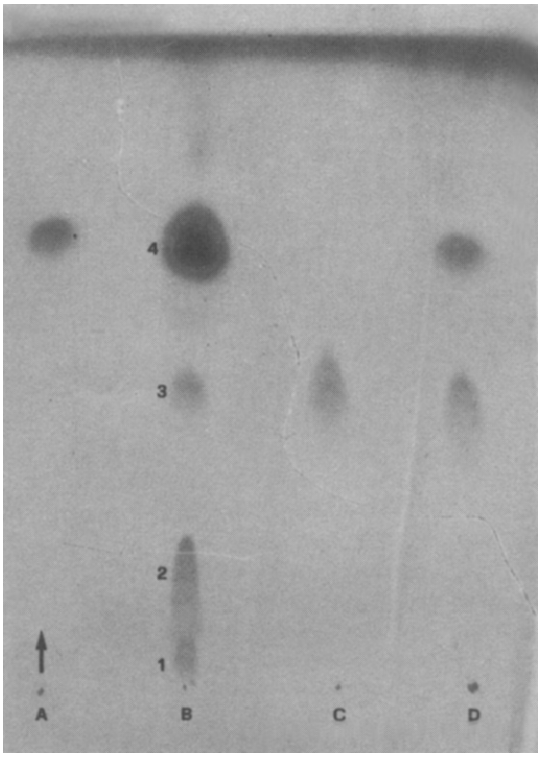


Fig. 3. TLC of the pK 6.2 Immobiline before and after degradation. TLC was performed on silica gel $60F_{254}$ plates using chloroform–methanol (7:3, v/v) as eluent. Samples (from left to right): A = control, undegraded pK 6.2; B = pK 6.2 after 6-h hydrolysis (at 70°C in 0.1 M NaOH); C and D = purified spot No. 3 (in two different degrees of purification). Spots: 1 = diamine; 2 = acrylic acid; 3 = unidentified product; 4 = undegraded pK 6.2 Immobiline. The vertical arrow indicates the migration direction of the eluent.

Fig. 5 shows the destruction rate of two such species, the neutral monomer 4-acryloylmorpholine and the charged species 1-acryloyl-4-methylpiperazine. Even though they both contain a disubstituted amide, they were found to degrade extensively (86%). Conversely, when the pK 9.3 and 8.5 Immobilines were subjected to the same hydrolysis conditions (70°C, 0.1 M NaOH, up to 6 h), much reduced degradation rates were observed (Fig. 6): only 11% for the pK 9.3 Immobiline and 22% for the pK 8.5 Immobiline. The last two compounds investigated, the pK 7.0 and 6.2 Immobilines (both containing a morpholino ring at different distances from the amide bond), were found to have intermediate degradation kinetics with degradations of 26% and 34%, respectively (Fig. 7).

The data on the degradation kinetics of all the species investigated (five charged and one neutral monomer) are shown in the bar graph in Fig. 8, expressed as a percentage of undegraded compound remaining at the end of a 6-h hydrolysis time. It is interesting that the four commercial Immobiline have degradation kinetics inversely proportional to the pK value.

DISCUSSION

We have previously performed an extensive investigation on the stability of the Immobiline buffers used for IEF in IPGs [15], and reported that indeed the alkaline species would degrade rapidly as a function of pH and temperature. On hydrolysis, free acrylic acid is produced, which is incorporated into the polyacrylamide gel instead of the original basic compound, resulting in totally offset pH gradients. The situation became so problematic that an Immobiline II generation was proposed [16], by which the alkaline Immobilines were stabilized by dissolving them in *n*-propanol, while the acidic species were prepared as 0.2 *M* solutions in water containing traces of inhibitor. Simultaneously, we found other degradation pathways, namely autopolymerization on storage to oligomers and *n*-mers [17] and oxidation during the polymerization process by persulphate to produce N-oxides [18]. All of these problems, connected with the alkaline Immobiline buffers, spurred us to search for suitable acrylamido weak bases, resistant to hydrolysis. Disubstituted amides were deemed to be suitable compounds, as it is amply documented [14] that such compounds are stable, probably because the two substituents on the nitrogen engaged in the amido bond sterically protect the latter against reactants approaching the plane of the

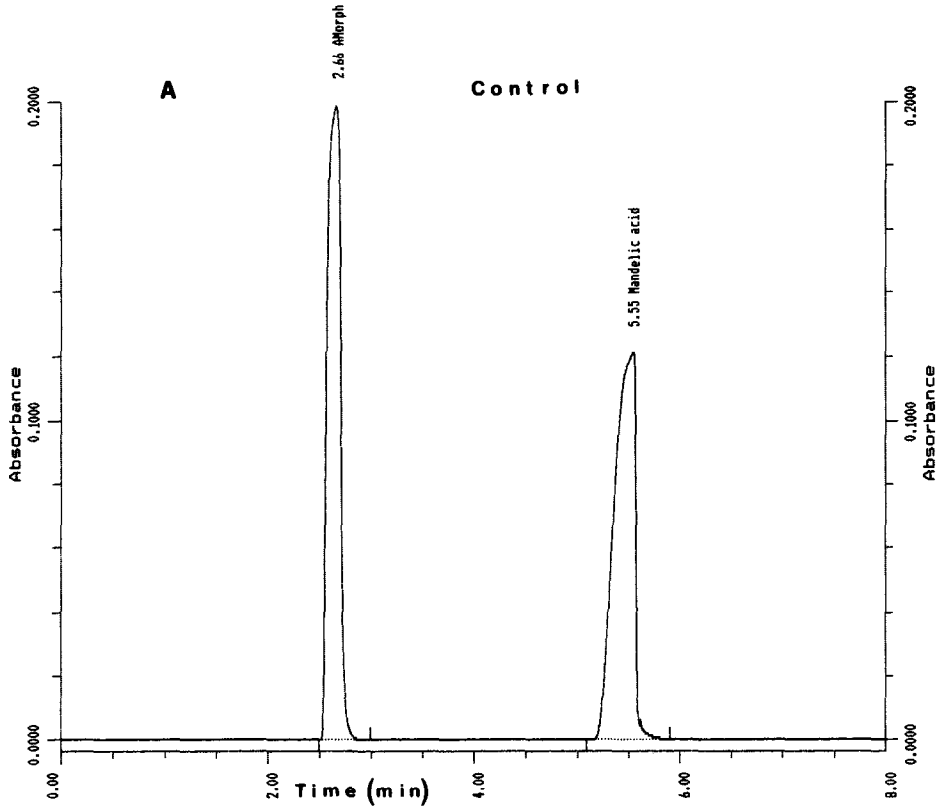


Fig. 4.

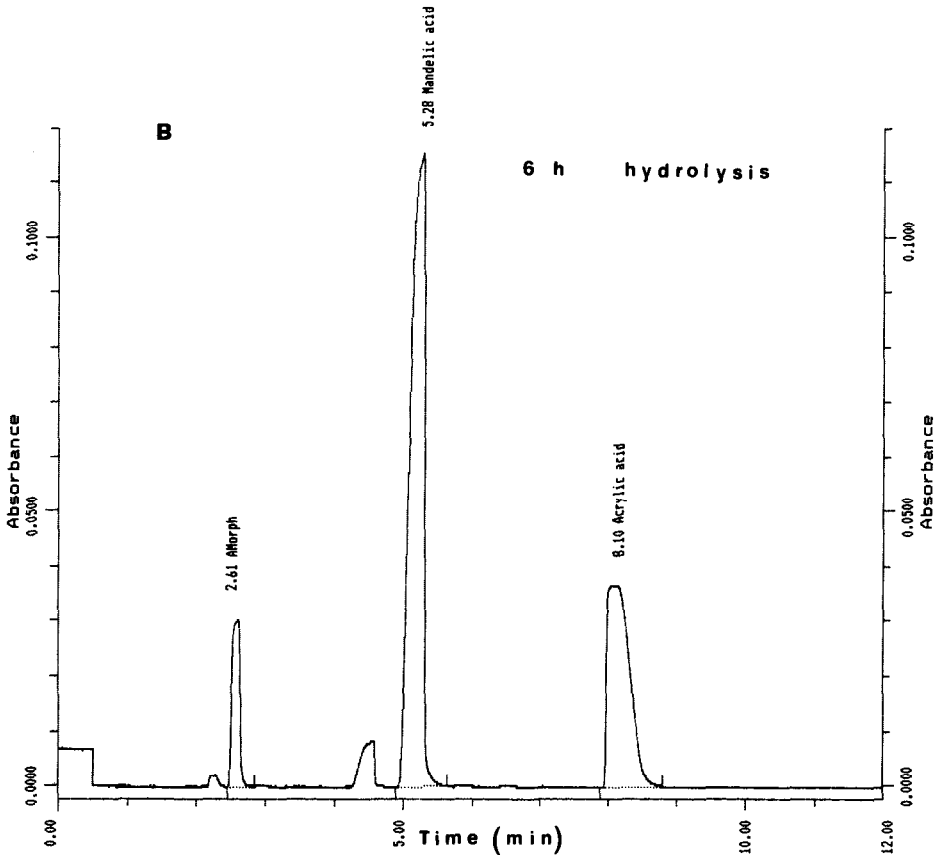


Fig. 4. Representative CZE run for analysis of Immobiline hydrolytic products. CZE run in a Beckmann P/ACE 2000 with a 50 cm × 75 μm I.D. capillary. Run at 15 kV, 25°C in 0.1 *M* borate buffer (pH 9). All migrations toward the cathode. Detection at 214 nm. Mandelic acid (2.5 m*M*) was used in all runs as an internal standard. (A) Control; (B) after 6 h of hydrolysis. Sample injected: 4-acryloylmorpholine.

amide. As it turned out from our data, the only two disubstituted amides we had available (AMorph and AMPip) were, on the contrary, extensively degraded, in fact almost completely destroyed in a 6-h period at 70°C in 0.1 *M* NaOH. Conversely, all other acrylamide buffers (with p*K* values of 9.3, 8.5, 7.0 and 6.2) showed only moderate to medium degradation, even though they are all monosubstituted amides.

While we agree with the general knowledge on the stability of disubstituted amides, it is clear from our results that there are other, more subtle mechanisms governing such stability. On the basis of our data, and of the known structures of the acrylamide derivatives, we derived the following rules:

(a) to afford protection of the amide bond, the most important parameter is not the degree of substitution in the nitrogen engaged in the amido plane (primary secondary or tertiary) but the type of substituent;

(b) in particular, rigid ring structures (as in AMorph and AMPip) are complete-

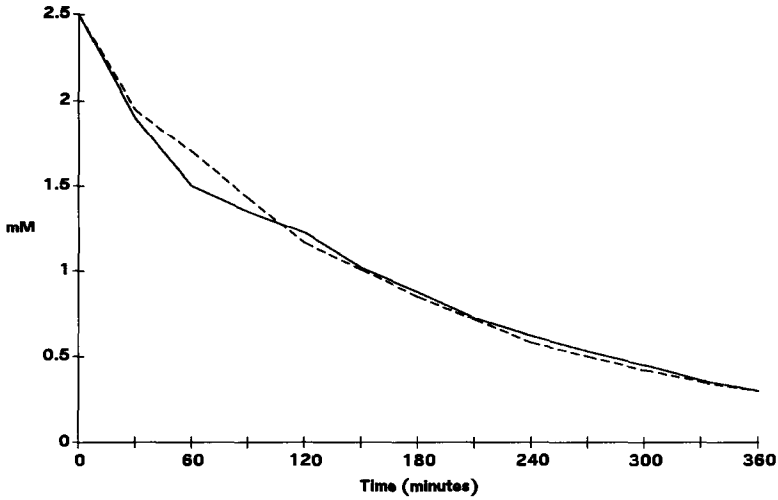


Fig. 5. Degradation kinetics of 4-acryloylmorpholine (----) and of 1-acryloyl-4-methylpiperazine (———). The quantitative data were obtained from analytical CZE runs as exemplified in Fig. 4. All integrations done with the Beckman Gold system. Note that here the molarity scale goes from 0 to 2.5 m*M*; note also the almost complete destruction of the two chemicals under the hydrolysis conditions (70°C, 0.1 *M* NaOH).

ly inefficient in protecting the adjacent amide bond, as their rigidity prevents them from oscillating in the surrounding space and thus shielding the amido plane;

(c) flexible chains bound to the nitrogen of the amido bond are efficient in protecting the amido plane, as they can oscillate in the surrounding space and shield the amido group;

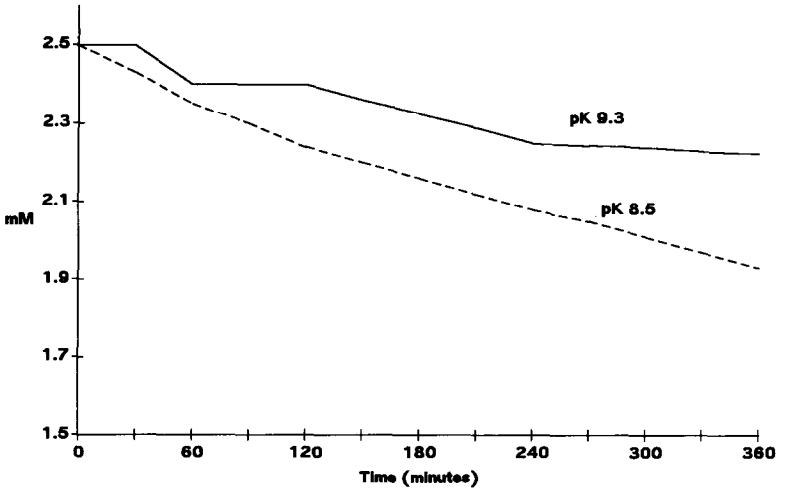


Fig. 6. Degradation kinetics of the p*K* 9.3 and 8.5 Immobilines. The quantitative data were obtained from analytical CZE runs as exemplified in Fig. 4. All integrations were done with the Beckman Gold system. Note that here the molarity scale goes from 1.5 to 2.5 m*M* (as opposed to 0 to 2.5 m*M* in Fig. 5); note also the very modest destruction of these two chemicals under the hydrolysis conditions used (70°C, 0.1 *M* NaOH).

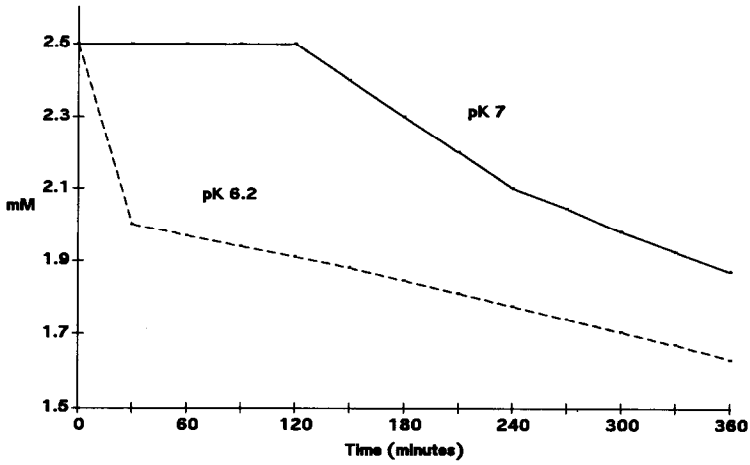


Fig. 7. Degradation kinetics of the pK 7.0 and 6.2 Immobilines. The quantitative data were obtained from analytical CZE runs as exemplified in Fig. 4. All integrations were done with the Beckman Gold system. Note that here the molarity scale goes from 1.5 to 2.5 mM (as opposed to 0 to 2.5 mM in Fig. 5); note also the more extensive destruction of these two chemicals under the hydrolysis conditions used (70°C, 0.1 M NaOH) compared with those of Fig. 6.

(d) if rigid structures are present in the nitrogen substituents, they should be removed from the plane of the amido bond: this is why the pK 7.0 Immobiline (3-morpholinopropylacrylamide) degrades substantially less than the pK 6.2 (2-morpholinoethylacrylamide);

(e) if a simple, flexible chain is present as a substituent on the nitrogen of the amido bond, greater protection of the latter is afforded by a longer chain; this is why the pK 9.3 Immobiline (N,N-dimethylaminoethylacrylamide) is more resistant than the pK 8.5 (N,N-dimethylaminoethylacrylamide) derivative.

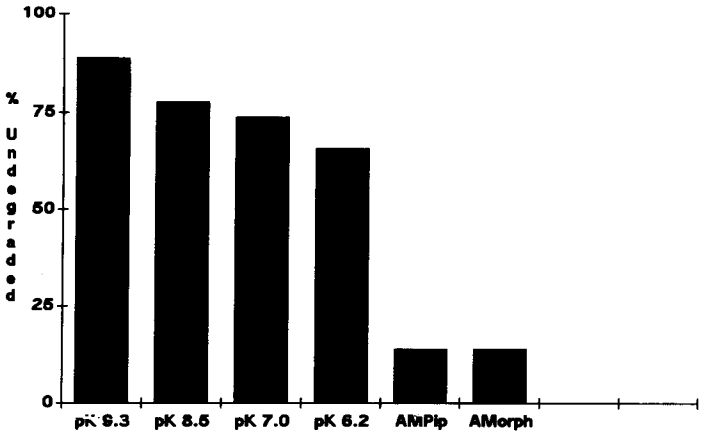


Fig. 8. Summary of the degradation kinetics of the five acrylamido bases and the neutral monomer studied. The vertical bars represent the amount of undegraded product remaining after 6 h of hydrolysis at 70°C in 0.1 M NaOH. AMorph = 4-acryloylmorpholine; AMPip = 1-acryloyl-4-methylpiperazine.

These general findings have helped us in designing a general strategy for the synthesis of new monomers that are extremely resistant to chemical attack (their synthesis will be reported elsewhere). Another finding of great interest is the unknown reaction product found in all acrylamido derivatives tested (and exemplified in Fig. 3, spot 3, in the case of the pK 6.2 derivative). After preparative purification and NMR analysis, it was found to be the product derived from the addition of water to the acrylic double bond. Given the relatively mild hydrolysis conditions (6 h at 70°C in 0.1 M NaOH) this was unexpected. However, on a literature search, we were able to locate a few examples of such reaction (nucleophilic attack of water on the carbon–carbon double bond); *e.g.*, one way of preparing β-hydroxypropionic acid is by the action of alkali on acrylic acid [19]. Although the extent of conversion is minute, it opens up new perspectives in the chemistry of acrylamide, certainly the most popular monomer in the field of biochemical separations.

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