*Journal of Chromatography*, 558 (1991) 285–295
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 427

# Synthesis of a new acrylamido buffer (acryloylhistamine) for isoelectric focusing in immobilized pH gradients and its analysis by capillary zone electrophoresis

MARCELLA CHIARI, MARIA GIACOMINI, CLAUDIA MICHELETTI and PIER GIORGIO RIGHETTI*

*Chair of Biochemistry and Department of Biomedical Sciences and Technologies, University of Milan, Via Celoria 2, Milan 20133 (Italy)*

(First received February 15th, 1991; revised manuscript received May 6th, 1991)

## ABSTRACT

By reacting acryloyl chloride with histamine a weakly basic acrylamido buffer for use in isoelectric focusing in immobilized pH gradients, acryloylhistamine [2-(4-imidazolyl)ethylamine-2-acrylamide], with a p$K$ of 7.0 (25°C) was synthesized. Even though this compound has the same p$K$ value as the commercially available p$K$ 7.0 Immobiline, it has some interesting features: (a) it is strongly resistant to hydrolysis, in contrast to the p$K$ 7.0 and 6.2 species; (b) it is hydrophilic; and (c) owing to its heteroaromatic ring, its amino groups are resistant to oxidation. The above properties (degradation kinetics, hydrophilicity, proneness to oxidation) were established in a quantitative manner by capillary zone electrophoresis analysis.

## INTRODUCTION

Isoelectric focusing (IEF) in immobilized pH gradients (IPG) 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 the 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 vast 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, under the trade name Immobiline. We have recently described the structure and given the formulae of the 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 morpholino derivatives, with p$K$ values of 6.2 and 7.0): by introducing a thiomorpholino ring, the p$K$ values of these compounds have been increased to 6.6 and 7.4, respectively, thus offering additional species buffering around neutrality, *i.e.*,

0021-9673/91/$03.50 

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 p$K$ 8.05 has also been synthesized recently, in order to close the gap in the pH 7.0–8.5 region [9]. Thus, the family of acrylamido buffers is expanding: we have described now a total of 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 acrylamido buffer, acryloylhistamine [2-(4-imidazolyl)ethylamine-2-acrylamide], having p$K$ = 7.0. Although this compound has the same p$K$ as the commercially available species (3-morpholinopropylacrylamide, p$K$ 7.0), nevertheless it exhibits some interesting properties: (a) it is strongly resistant to hydrolysis, (b) it is as hydrophilic as the p$K$ 7 Immobiline and (c) owing to the presence of an imidazole moiety, it is resistant to oxidation by peroxodisulphate.

## EXPERIMENTAL

Commercial Immobilines, Repel- and Bind-silane, Gel Bond PAG, the Multiphor II chamber, Multitemp thermostat, the Macrodrive power supply and Pharmalyte carrier ampholytes of pH 4–6 and 6–8 were purchased from Pharmacia-LKB Biotechnology (Bromma, Sweden). Non-commercial acrylamide weak acids and bases were synthesized in our laboratory as reported [5–9]. Acrylamide, N,N′-methylenebisacrylamide (Bis), TEMED, ammonium peroxodisulphate and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Labs. (Richmond, CA, USA). Acryloyl chloride and histamine were from Fluka (Buchs, Switzerland); the former was distilled just prior to use. Control and oxidized bovine serum albumin (BSA) were a gift from Professor Colonna, University of Milan. Mandelic acid, used as an internal standard in capillary zone electrophoresis (CZE) runs, was purchased from Aldrich (Steinheim, Germany).

### *Alkaline hydrolysis*

All Immmobiline buffers were dissolved (20 m$M$ each) in 0.1 $M$ sodium hydroxide solution and incubated at 70°C, under a nitrogen atmosphere, for up to 6 h. At 30-min intervals aliquots were collected and diluted in 0.1 $M$ borate buffer (pH 9.0) to 2.5 m$M$. After adding mandelic acid (2.50 m$M$) as an internal standard, the samples were analysed by CZE.

### *Capillary zone electrophoresis*

CZE was performed in a Beckman (Palo Alto, CA, USA) instrument (P/ACE System 2000) equipped with a 50 cm × 50 $\mu$m I.D. capillary. Runs were performed at 25°C in a thermostated environment in 0.1 $M$ borate (pH 9.0), except for the oxidation products (see Fig. 7), which were analysed in 50 m$M$ phosphate (pH 7.0). In all instances the migration direction was toward the negative electrode, which means that the acidic species (mandelic acid) is transported there by electroosmosis, as it migrates electrophoretically toward the positive electrode. The samples were injected into the capillary by pressure (800 kPa), usually for 10 s. A calibration graph for each

acrylamido derivative analysed was costructed with a Beckman integration system Gold, with 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 (Darmstadt, Germany), using chloroform–methanol (7:3, v/v) as eluent. The spots were revealed either with 3.5% molybdophosphoric acid in ethanol or with ninhydrin.

*Nuclear magnetic resonance (NMR) spectroscopy*

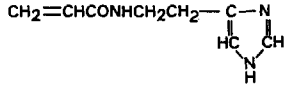
NMR analyses were carried out for solutions in deuterated methanol, using tetramethylsilane (TMS) as internal standard, with a Model EM-360 L 60-MHz spectrometer from Varian (Palo Alto, CA, USA).

*Isoelectric focusing (IEF) in immobilized pH gradients (IPG)*

IEF in IPG was performed in a 4% T, 4% C[a] polyacrylamide gel, in the pH range 4–8. The recipe for this IPG interval, utilizing both the commercially available Immobilines or the new species (acryloylhistamine), was as described previously [11]. The gel, after polymerization, washing and drying [1], was reswollen in 0.5% Pharmalyte (pH 4–8). Protein samples (20 $\mu$g each) were applied at the anodic gel side. Each run was for 2 h at 400 V, followed by 4 h at 2000 V, at 10°C. The gels were stained with Coomassie Brilliant Blue R-250 in $Cu^{2+}$.

*Synthesis of acryloylhistamine*

Histamine (3 g; 0.016 mol) was dissolved into 12 ml of 4 *M* sodium hydroxide solution and acryloyl chloride (700 $\mu$l, 0.008 mol) was added dropwise at 0°C. After stirring for 1 h, the solution was titrated to pH 8.5 and then extracted three times with chloroform. The organic phase was dried with sodium sulphate. After removing the organic solvent *in vacuo*, 250 mg of product were recovered. The material was subsequenty purified by silica gel chromatography (product-to-silica ratio 1:60, w/w) and eluted with chloroform–methanol (8:2). The recovery of the purified product was 198 mg (15% yield). $^1$H NMR ($CH_3O^2H$): $\delta$ 2.8 (2H, $=C-CH_2-$, t), 3.6 (2H, $-CH_2-NH$, m), 5.6 (1H, $-CH=$, m), 6.35 (2H, $=CH_2$, d), 6.9 (1H, NH–*CH*–CH, s), 7.6 ppm (1H, HN–*CH*=N, s). The structure of the novel acrylamido buffering compound is thus




*Synthesis of Immobiline analogues*

In order to study the proneness of the acrylamido buffers to peroxodisulphate oxidation, analogues lacking the double bond were prepared, so that exposure to peroxodisulphate would not produce concomitantly a gel phase. Two such analogues were prepared: acetylhistamine [a saturated analogue of the $pK\,7_{AH}$ (see below) species] and acetylmorpholine (morpholinopropylacetamide, a saturated analogue of the $pK\,7$ species). Both species were prepared essentially as described previously [12], except

[a] C = g N,N′-methylenebisacrylamide (Bis)/% T; T = g acrylamide + g Bis per 100 ml of solution.

that acetic anhydride (in pyridine as solvent) was used for the synthesis instead of acryloyl chloride.

*Spectrophotometric analyses*

In order to study the potential formation of N-oxides on exposure to peroxodisulphate, the two Immobiline analogues described above were subjected to UV–VIS spectrophotometry with a Cary 219 instrument (Varian). Solutions of 10 m*M* of each analogue were prepared in 100 m*M* borate buffer (pH 9) and brought to a 2% final concentration of ammonium peroxodisulphate from a stock 40% solution. Formation of N-oxides was monitored by both the spectra in the 250–450-nm range and by following the absorbance increments with time at a fixed wavelength (280 nm).

*Potentiometric titration*

The new acrylamido buffer was titrated manually under nitrogen at 25°C. A 10-ml volume of a 10 m*M* acryloylhistamine 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 buffer/titrant, which, by definition, should correspond to its p*K* value.

*Partition coefficient*

In order to establish a hydrophobicity scale, the new compound (acryloylhistamine, referred to as p$K\,7_{AH}$) and Immobilines of p*K* 6.2, 7.0, 7.4, 8.5 and 9.3 were subjected to partitioning in 1-octanol–water as described by Purcell *et al.* [13]. The partition coefficient, *P*, is defined as the ratio of the molarity of a given compound in the organic phase to that in the aqueous phase. Partitioning is performed under conditions in which the alkaline Immobilines are fully deprotonated.

RESULTS

Fig. 1 shows the titration curve of the new, weakly basic acrylamido buffer: as the precursor was histamine, we had hoped to obtain a weakly basic buffer. The aim

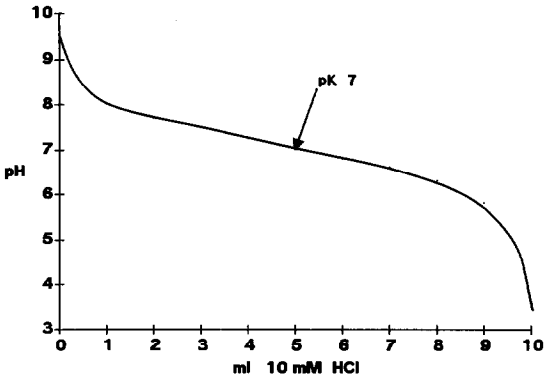



Fig. 1. Titration curve of acryloylhistamine. A 10-ml aliquot of a 10 m*M* solution of the acrylamido buffer was titrated with 10 ml of 10 m*M* HCl at 25°C under a nitrogen atmosphere. The p*K* value was determined to be 7.0.

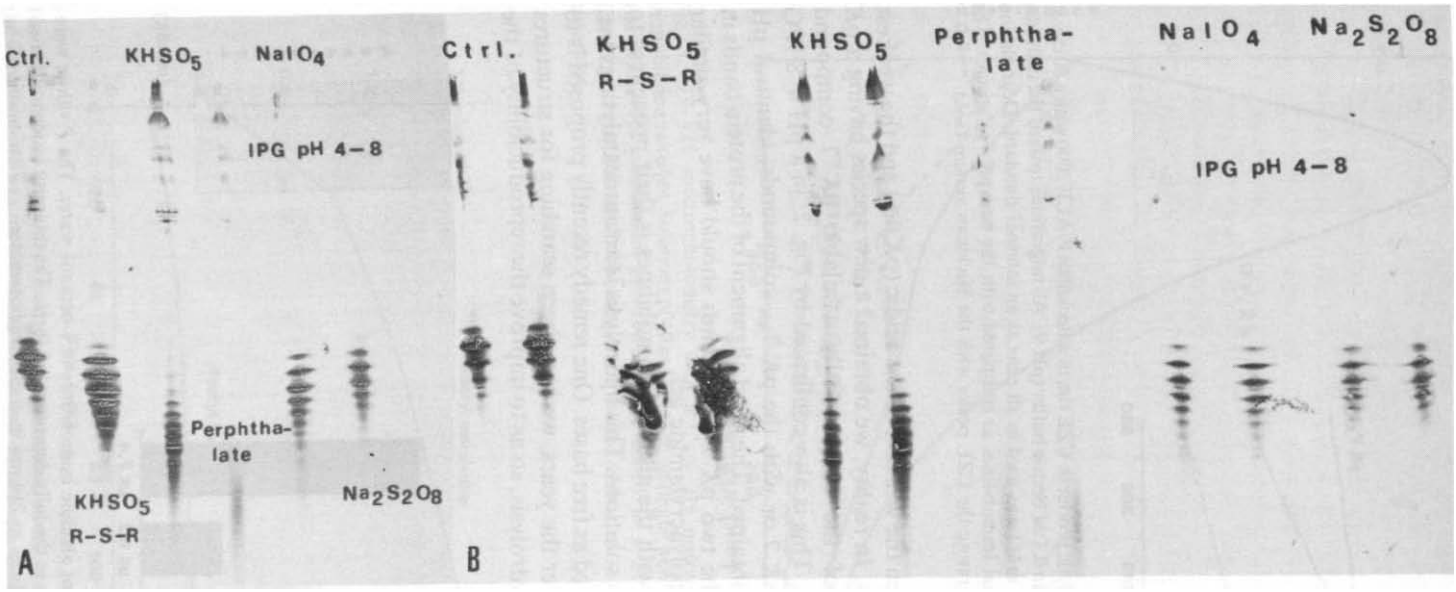



Fig. 2. Analytical IEF gel in the IPG pH 4–8 interval. The gel was a 4% T, 4% C polyacrylamide matrix, reswollen in 0.5% Pharmalyte (pH 4–8). Left side (A): formulation containing the p$K$ $7_{AH}$ compound; right side (B): recipe with the p$K$ 7.0 Immobiline buffer. Samples: Ctrl. = control bovine serum albumin (BSA); $KHSO_5$ + R-S-R = BSA oxidized by peroxymonosulphate in the presence of a sulphide compound; $KHSO_5$ = BSA oxidized by peroxymonosulphate; perphthalate = BSA oxidized by perphthalate; $NaIO_4$ = BSA oxidized by periodate; $Na_2S_2O_8$ = BSA oxidized by peroxodisulphate. All samples loaded in a 20-$\mu$g 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 $Cu^{2+}$.

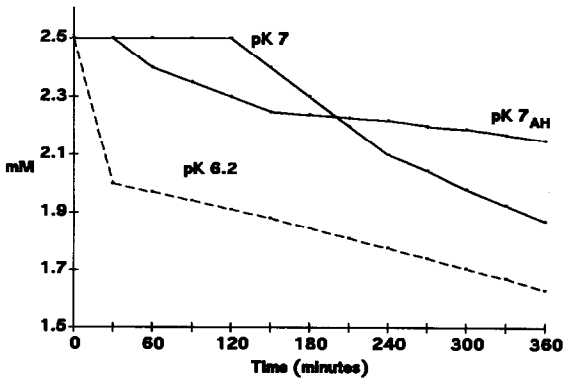



Fig. 3. CZE analysis of Immobiline hydrolytic products. CZE run in a Beckman P/ACE 2000 with a 50 cm × 50 μm I.D. capillary. Run at 15 kV, 25°C in 0.1 *M* borate buffer (pH 9). All migrations toward the cathode. Detection at 214 nm. Mandelic acid (2.5 m*M*) was used in all runs as an internal standard. Degradation kinetics of the p*K* 7.0 and 6.2 commercial Immobilines as compared with the new p*K* $7_{AH}$ species. The quantitative data were obtained by integrating the CZE peaks with the Beckman system Gold.

was in fact to close the gap between the weakest of the acidic (p*K* 4.6) and the weakest of the basic (p*K* 6.2) Immobilines. In reality, we obtained a new species having a p*K* essentially identical with that of the commercially available p*K* 7 compound (3-morpholinopropylacrylamide). This is also confirmed by Fig. 2: in a pH 4–8 IPG range, obtained either with the p*K* 7 or with the p*K* $7_{AH}$ compounds, identical pH ranges and protein patterns were obtained. The good alignment of the protein bands in the two gels suggests also that the two p*K* 7 compounds should have very similar incorporation efficiencies in the polyacrylamide gel.

One of the main problems with the alkaline Immobilines is their proneness to hydrolysis when stored in aqueous solutions. This hydrolysis is an autocatalytic event, because these chemicals are supplied as free bases. One remedy recently proposed is to store them in *n*-propanol [14]. Over the years, we have been searching for structures that would be more resistant to hydrolysis, so as to improve the reproducibility of the

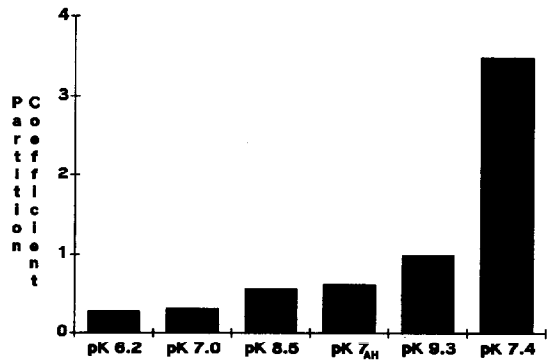



Fig. 4. Partition coefficients (*P*) of different alkaline Immobilines in 1-octanol–water. The *P* values were assessed by partitioning the different bases in the fully deprotonated form. The molarity ratios in the two phases were measured by absorbance readings at 214 nm in a spectrophotometer.

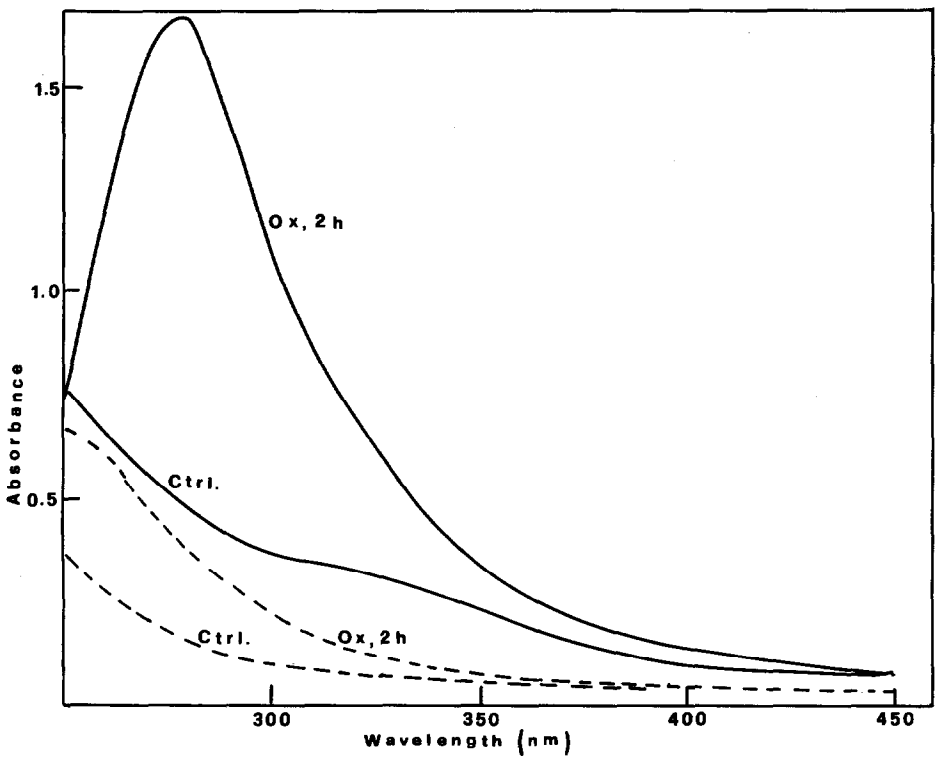



Fig. 5. UV–VIS spectrophotometry with a Cary 219 instrument of peroxodisulphate oxidation of Immobiline analogues. Solutions of 10 m*M* of each analogue were prepared in 100 m*M* borate buffer (pH 9) and with 40% ammonium peroxodisulphate added to a 2% final concentration. Note the strong chromophore at 280 nm for the acetylmorpholine derivative. — = p*K* 7.0, morpholine; - - - = $pK\ 7_{AH}$, histamine.

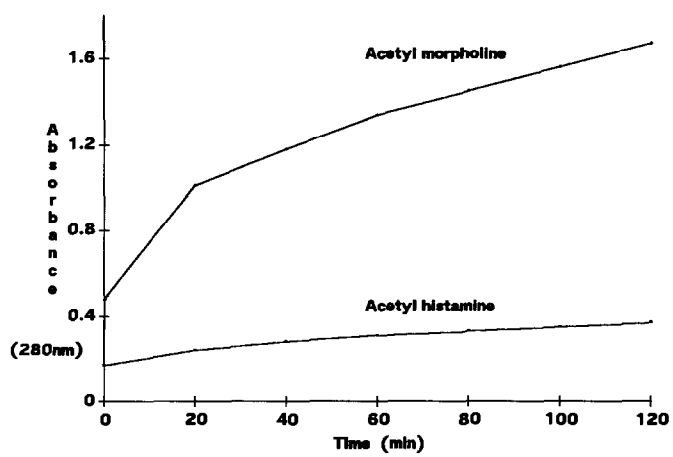



Fig. 6. Kinetics of Immobiline oxidation. Experimental conditions as in Fig. 5, except that the appearance of the chromophore was monitored at a constant wavelength (280 nm).

IPG technique. We therefore subjected the p$K$ $7_{AH}$ compound and commercial Immobilines with neighbouring p$K$ values (the p$K$ 7.0 and 6.2 species) to forced ageing and measured the extent of hydrolysis by CZE. As shown in Fig. 3, the new p$K$ $7_{AH}$ compound exhibits much better resistance to hydrolysis than the other two species.

Another desirable property of the Immobiline buffers is their hydrophilicity: hydrophobic interactions are usually deleterious during electrophoresis, as the analyte proteins could bind to the grafted buffers and give pronounced smearing. We have thus created a hydrophobicity scale of several of the basic acrylamido buffers that we had available by partitioning them in 1-octanol–water. The results are shown in Fig. 4: it is seen that most Immobilines are hydrophilic, the hydrophobicity increasing with increasing chain length (thus reaching a higher value with the p$K$ 9.3 buffer). However, a huge hydrophobicity increment is obtained when the oxygen of the morpholino ring in the p$K$ 7 buffer (3-morpholinopropylacrylamide) is replaced with a sulphur atom (p$K$ 7.4, 3-thiomorpholinopropylacrylamide).

A third problem with the Immobiline buffers is their proneness to oxidation during the peroxodisulphate-induced polymerization process [14–16]. In order to quantify this phenomenon, two analogues (acetylmorpholine and acetylhistamine of

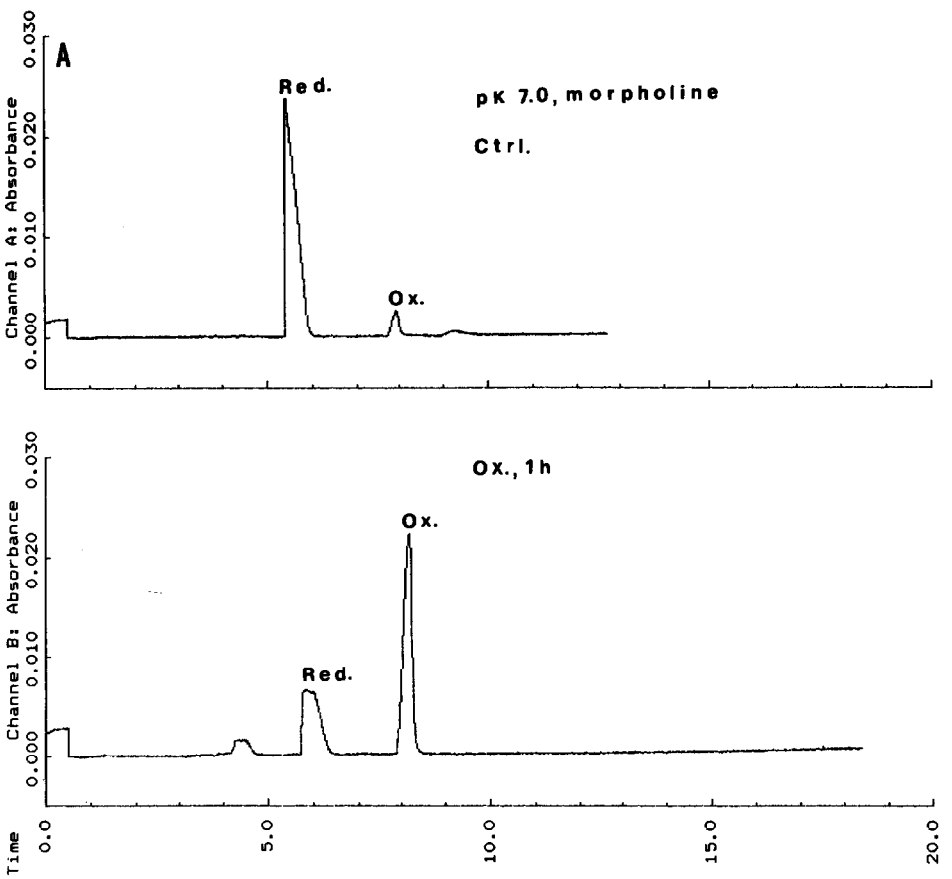



Fig. 7.

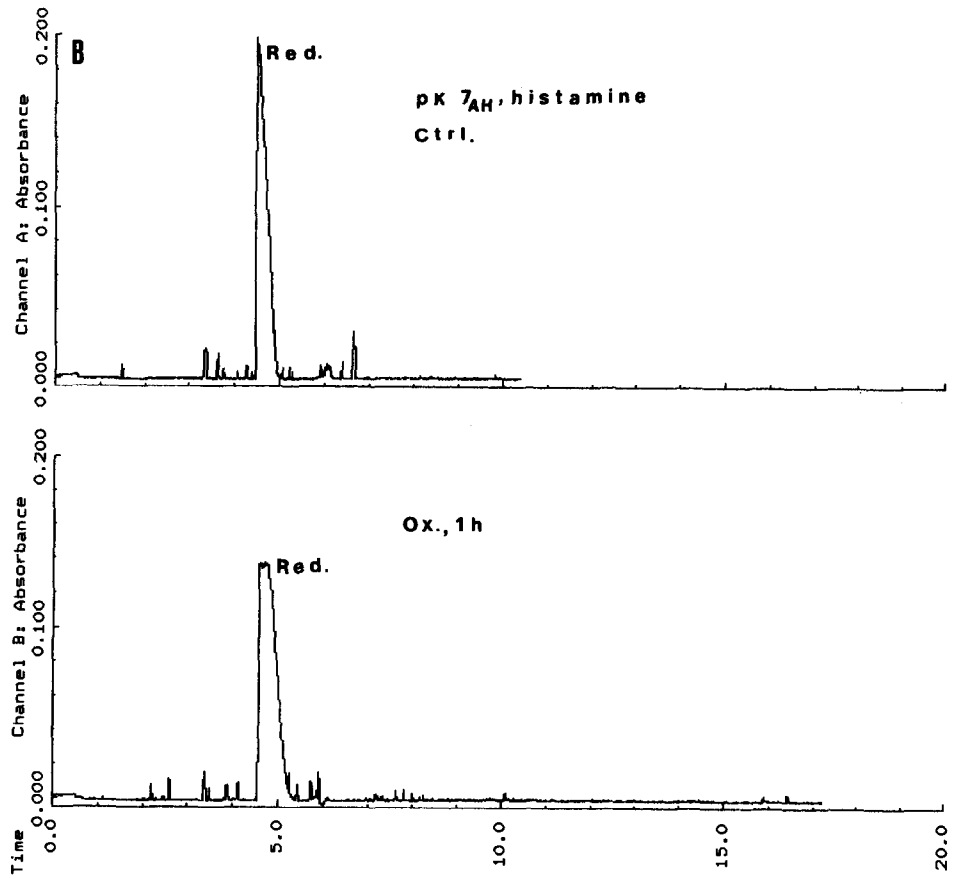



Fig. 7. CZE analysis of Immobiline oxidation by peroxodisulphate. The two Immobiline analogues [acetylhistamine (B) and acetylmorpholine (A)] were incubated with 2% peroxodisulphate at room temperature for 1 h and then analysed by CZE in 50 m*M* phosphate buffer (pH 7). CZE run in a Beckman P/ACE 2000 with a 50 cm × 50 μm I.D. capillary at 15 kV, 25°C. All migrations toward the cathode. Detection at 280 nm. Mandelic acid (2.5 m*M*) was used in all runs as an internal standard. The quantitative data were obtained by integrating the CZE peaks with the Beckman system Gold. The slow-migrating peak with acetylmorpholine is assumed to be the N-oxide, as such a species can no longer be protonated. Time in min.

the two p*K* 7 buffers) were incubated in a spectrophotometric cell with 2% peroxodisulphate and spectra recorded at different time intervals up to 2 h. As shown in Fig. 5, while the commercial p*K* 7 Immobiline is strongly oxidized, with the appearance of a marked chromophore at 280 nm, little modification is seen with the acetylhistamine derivative. The kinetics of such an oxidative process (followed on the 280-nm chromophore) is shown in Fig. 6: at the end of a 2-h period, it is seen that the p$K$ $7_{AH}$ species has undergone only a very modest oxidation in comparison with the commercial derivative. These data, however, do not allow us to have a quantitative picture of the phenomenon, as the molar absorptivity of the putative oxidation product is not known. CZE has been instrumental in such an assessment: as shown in

Fig. 7, when the analogue of the commercial p$K$ 7 species is analysed at pH 7 (where it would be 50% protonated), it is easily separated from the oxidized form, as the latter cannot be protonated. After incubation for 1 h in peroxodisulphate, as much as 50% of the acetylmorpholine appears to be oxidized. Note that, owing to its proneness to oxidation, even the control contains a small amount (5%) of N-oxide. Conversely, with the new compound proposed here, hardly any modification by peroxodisulphate can be detected (Fig. 7B).

## DISCUSSION

Starting from the original set of six acrylamido buffers, commercially available as Immobilines (two acids, with p$K$ 3.6 and 4.6, and four bases, with p$K$ 6.2, 7.0, 8.5 and 9.3), we have developed, over the years, a considerable number of new acrylamido buffers and titrants, covering the pH interval 1–12. With the experience we have accumulated in this period, we are now able to define the desirable properties of ideal buffers for use in the IPG technique. They are essentially three: (a) resistance to chemical degradation (especially spontaneous hydrolysis in solution); (b) hydrophilicity (so as to minimize hydrophobic interaction with proteins under analysis); and (c) resistance to oxidation by peroxodisulphate.

The p$K$ $7_{AH}$ species described here fulfils most of these requirements. It is clearly more resistant to hydrolysis than the two Immobiline species with similar p$K$ values. At the end of the 6-h degradation period, the p$K$ 6.2 has degraded as much as 35%, the p$K$ 7.0 30% and the p$K$ $7_{AH}$ only 15%. As all three of them are monosubstituted amides, this resistance to hydrolysis might not be due so much to steric protection of the amido bond, but rather to electronic factors.

The p$K$ $7_{AH}$ species is also hydrophilic: although, in the hydrophobicity scale of Fig. 4, it is located between the p$K$ 8.5 and 9.3 derivatives, its partition coefficient (0.6) is so close to that of the commercial p$K$ 7.0 compound (0.4) that they can be considered equivalent. In fact, it was surprising to us that the $P$ value should be slightly higher, considering that the substituent chain on the amido group is shorter by two carbon atoms compared with the p$K$ 7.0 compound. Conversely, a huge hydrophobicity increment is noted when the oxygen in the morpholino ring of the p$K$ 7.0 Immobiline is replaced with a sulphur atom (p$K$ 7.4 compound, 3-thiomorpholinopropylacrylamide).

Another desirable property of alkaline Immobilines is their resistance to potential oxidation by peroxodisulphate during the polymerization process. We have recently found that, by contact with peroxodisulphate, the alkaline Immobilines, when deprotonated, are converted into N-oxide derivatives, which are then able to oxidize SH groups in proteins during the focusing process [14–16]. With the p$K$ $7_{AH}$ species, the fact that the protolytic nitrogen atom is part of a heteroaromatic ring should protect it against potential oxidation processes, as reported in the literature. This appears in fact to be so: as shown in Figs. 5 and 6, little oxidation is seen in the p$K$ $7_{AH}$ compound on exposure to 2% peroxodisulphate, whereas the corresponding p$K$ 7 commercial species (3-morpholinopropylacrylamide) seems to be substantially modified. Such a phenomenon had been already reported for all Immobiline chemicals [15] and was shown to apply also to the soluble carrier ampholytes used in conventional isoelectric focusing. The presence of such amine oxides ($R_3N^+O^-$) is deleterious to protein

analysis: when focusing proteins in alkaline IPG ranges, free SH groups would be oxidized to S–S bonds by the immobilized N-oxides, generating artefactual, higher p*I* bands. In a model system, in which free cysteine was incubated anaerobically, at pH 9.0, with a crushed IPG gel, 100% oxidation to cystine was found in a 12-h period [15]. Such oxidation of free SH groups in proteins (*e.g.*, globin α-chains) could be demonstrated even in conventional IEF in the presence of soluble, amphoteric buffers, as the latter also can be oxidized by excess of peroxodisulphate added during the polymerization step. Hence, even when the peroxodisulphate is discharged at the anode in a prefocusing step, a harmful oxidation power remains in the gel in the form of N-oxides of carrier ampholytes [16]. Clearly, the availability of new Immobiline chemicals resistant to peroxodisulphate oxidation would be greatly beneficial to the IPG technique.

ACKNOWLEDGEMENTS

This work was supported in part by grants from Agenzia Spaziale Italiana (ASI), Progetto Finalizzato Chimica Fine II, CNR (Rome) and Ministero della Pubblica Istruzione. We are greatly indebted to Drs. R. Montini and S. Di Biase of Beckman Italia for the kind loan of the CZE instrument.

REFERENCES

1 P. G. Righetti, *Immobilized pH Gradients: Theory and Methodology*, Elsevier, Amsterdam, 1990.
2 P. G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983.
3 M. Chiari, E. Casale, E. Santaniello and P. G. Righetti, *Appl. Theor. Electrophoresis*, 1 (1989) 99–102.
4 M. Chiari, E. Casale, E. Santaniello and P. G. Righetti, *Appl. Theor. Electrophoresis*, 1 (1989) 103–107.
5 E. Gianazza, F. Celentano, G. Dossi, B. Bjellqvist and P. G. Righetti, *Electrophoresis*, 5 (1984) 88–97.
6 P. G. Righetti, M. Chiari, P. K. Sinha and E. Santaniello, *J. Biochem. Biophys. Methods*, 16 (1988) 185–192.
7 C. Gelfi, M. L. Bossi, B. Bjellqvist and P. G. Righetti, *J. Biochem. Biophys. Methods*, 15 (1987) 41–48.
8 M. Chiari, P. G. Righetti, P. Ferraboschi, T. Jain and R. Shorr, *Electrophoresis*, 11 (1990) 617–620.
9 M. Chiari, L. Pagani, P. G. Righetti, T. Jain, R. Shorr and T. Rabilloud, *J. Biochem. Biophys. Methods*, 21 (1990) 165–172.
10 R. Charlionet, R. Sesboüé and C. Davrinche, *Electrophoresis*, 5 (1984) 176–178.
11 E. Gianazza, F. Celentano, G. Dossi, B. Bjellqvist and P. G. Righetti, *Electrophoresis*, 5 (1984) 88–97.
12 P. G. Righetti, M. Chiari, E. Casale and C. Chiesa, *Appl. Theor. Electrophoresis*, 1 (1989) 115–121.
13 W. P. Purcell, G. E. Bass and J. M. Clayton (Editors), *Strategy of Drug Design: a Guide to Biological Activity*, Wiley-Interscience, New York, 1973, pp. 126–143.
14 B. M. Gåveby, P. Petterson, J. Andrasko, L. Ineva-Flygare, U. Johannesson, A. Görg, W. Postel, A. Domscheit, P. L. Mauri, P. Pietta, E. Gianazza and P. G. Righetti, *J. Biochem. Biophys. Methods*, 16 (1988) 141–164.
15 M. Chiari, C. Chiesa, P. G. Righetti, M. Corti, T. Jain and R. Shorr, *J. Chromatogr.*, 499 (1990) 699–711.
16 G. Cossu, M. G. Pirastru, M. Satta, M. Chiari, C. Chiesa and P. G. Righetti, *J. Chromatogr.*, 475 (1989) 283–292.