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Capillary zone electrophoresis analysis of acrylamido buffers for isoelectric focusing in immobilized pH gradients

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ABSTRACT

Studies are described using fourteen weak acrylamido acids and bases (Immobilines) as buffers and titrants for isoelectric focusing in immobilized pH gradients (IPG), plus one strongly acidic (pK 1.0) and one strongly basic (pK > 12) titrants, utilized in general to reach pH extremes in extended IPG ranges. Three fundamental properties of these buffers were evaluated: (a) resistance to alkaline hydrolysis of the amido bond; (b) hydrophilicity (so as to avoid hydrophobic interactions with the matrix during electrophoretic migration); and (c) resistance to oxidation by peroxodisulphate during the polymerization step. Capillary zone electrophoresis (CZE) was used to evaluate these properties. The acidic compounds were resolved in 100 mM acetate (pH 4) whereas the alkaline species were separated in 50 mM phosphate buffer (pH 7.7) (or pH 7.2 for the weaker compounds). All the acrylamido derivatives were detected underivatized by their absorption at 214 or 254 nm. The degradation kinetics of all compounds were monitored, after hydrolysis in 0.1 M NaOH at 70°C (up to 6 h), by CZE in 0.1 M borate buffer (pH 9). The decrease in the Immobiline peak and the appearance of its hydrolytic products (acrylic acid and a diamine in the case of the acrylamido bases) could easily be monitored and quantified in CZE by using the Gold integration system. A hydrophobicity scale was constructed by measuring the partition coefficients of the basic acrylamido derivatives in 1-octanol–water. General guidelines are given for the proper use of these chemicals and for the synthesis of additional compounds.

INTRODUCTION

In contrast to conventional isoelectric focusing (IEF), which utilizes a vast number of soluble, amphoteric buffers having isoelectric points (pI) fairly evenly distributed along the pH scale [1] (carrier ampholytes, or Ampholines), immobilized pH gradients (IPG) exploit a set of a few, well defined non-amphoteric chemicals having pK values covering the pH range 3.1–10.3 [2]. Six such chemicals (acrylamido weak acids and bases) are commercially available through Pharmacia-LKB (Bromma, Sweden) as Immobilines. They are in fact "immobilized" into the polyacrylamide matrix by covalent bonding through the acrylic double bond. There are several advantages of the Immobilines over the "carrier ampholyte" buffers; most striking, however, is the fact that whereas the latter produce an interpenetrating, quasi-Gaussian steady-state profile (with a system of $n-1$ boundaries for n species), the former generate an evenly changing chemical field along the migration path. The absence of

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such boundaries in the gel bed is most beneficial to protein migration and to the focusing process. In addition, the Immobiline chemicals contain a single protolytic group, whereas Ampholines are oligoamino-oligocarboxylic acids; it turns out that oligoprotic species have a tendency to "stick" to proteinaceous surfaces, giving spurious bands [1,2].

We have recently decoded 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 pK values of 6.2 and 7.0); by introducing a thiomorpholino ring, the pK values of these compounds have been 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 pK 8.05 has also recently been synthesized, in order to close the gap in the pH 7.0–8.5 region [9]. We have also reported a pK 6.85 compound (1-acryloyl-4-methylpiperazine; AMPip) [10] and another pK 7.0 species [2-(4-imidazolyl)ethylamine-2-acrylamide or -acryloylhistamine, pK 7_{AH}] [11]. Thus, the family of acrylamido buffers is expanding: we have now described a total of sixteen monoprotic compounds and there is a report on a biprotic species, itaconic acid [12]. The formulae names, pK values and relative molecular masses (M_r) values of all the species produced so far are listed in Tables I–III.

In recent years, we have focused our attention on three most desirable properties of the Immobiline chemicals: (a) resistance to alkaline hydrolysis (particularly pronounced with the basic species, which autocatalyse the lysis of the amido bond [15]; (b) hydrophilicity (when grafted into the polyacrylamide matrix, any hydrophobic property of Immobilines will be amplified); and (c) resistance to oxidation, during the polymerization step, caused by peroxodisulphate [16]. Whereas conventional gel electrophoresis techniques are excellent for the analysis of macromolecules, they are not so readily amenable to the characterization of small molecules. Capillary zone electrophoresis (CZE) has been useful in analysing all these properties and for checking the purity of the new synthetic products.

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. Noncommercial acrylamido weak acids and bases were synthesized in our laboratory as reported [3–11]. Acrylamide, N,N′-methylenebisacrylamide (Bis), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium peroxodisulphate and Coomassie Brilliant Blue were obtained from Bio-Rad Labs. (Richmond, CA, USA). Acrylic acid was purchased from Fluka (Buchs, Switzerland) and was distilled just prior to use. Horse heart myoglobin was purchased from Sigma (St. Louis, MO, USA) and haemoglobin mutants were a gift from Dr. A. Mosca (University of Milan). Mandelic acid, used as an internal standard in capillary zone electrophoresis (CZE) runs, was purchased from Aldrich (Steinheim, Germany).

TABLE I

ACIDIC ACRYLAMIDO BUFFERS

pK^a	Formula	Name	M_r	Source[b]
1.0	$CH_2=CHCONH-C(CH_3)(CH_2SO_3H)$	2-Acrylamido-2-methylpropanesulphonic acid	207	A
3.1	$CH_2=CHCONHCH(OH)COOH$	2-Acrylamidoglycolic acid	145	B
3.6	$CH_2=CHCONHCH_2COOH$	N-Acryloylglycine	129	C
4.4	$CH_2=CHCONH(CH_2)_2COOH$	3-Acrylamidopropanoic acid	143	C
4.6	$CH_2=CHCONH(CH_2)_3COOH$	4-Acrylamidobutyric acid	157	C

[a] The pK values for the three Immobilines and for 2-acrylamidoglycolic acid are given at 25°C; for AMPS (pK 1.0) the temperature of pK measurement was not reported.
[b] A, Polysciences, Warrington, PA, USA; B, ref. 6; C, Pharmacia–LKB.

Alkaline hydrolysis

All acrylamido derivatives and Immobiline buffers were dissolved (20 mM each) in 0.1 M NaOH and incubated at 70°C, under a nitrogen atmosphere, 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 mM. Mandelic acid (2.50 mM) was then added and the mixtures were analysed by CZE.

Capillary zone electrophoresis

CZE was performed in a Beckman (Palo Alto, CA, USA) P/ACE System 2000 instrument equipped with a 50 cm × 75 μm I.D. capillary. All runs were performed at 25°C in a thermostated environment. Analysis of acidic acrylamido compounds was

TABLE II

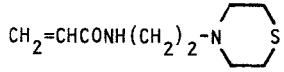
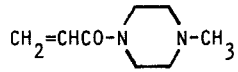
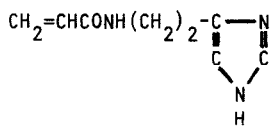
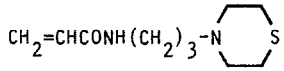
BASIC ACRYLAMIDO BUFFERS

pK^a	Formula	Name	M_r	Source[b]
6.2	$CH_2=CHCONH(CH_2)_2-N$ 	2-Morpholinoethylacrylamide	184	A
7.0	$CH_2=CHCONH(CH_2)_3-N$ 	3-Morpholinopropylacrylamide	198	A
8.5	$CH_2=CHCONH(CH_2)_2N(CH_3)_2$	N,N-Dimethylaminoethylacrylamide	142	A
9.3	$CH_2=CHCONH(CH_2)_3N(CH_3)_2$	N,N-Dimethylaminopropylacrylamide	156	A
10.3	$CH_2=CHCONH(CH_2)_3N(C_2H_5)_2$	N,N-Diethylaminopropylacrylamide	184	B
>12	$CH_2=CHCONH(CH_2)_2-\overset{+}{N}(C_2H_5)_3$	N,N,N-Triethylaminoethylacrylamide	198	C

[a] All pK values (except for pK 10.3) measured at 25°C. The value of pK 10.3 refers to 10°C.
[b] A, Pharmacia–LKB. B, Ref. 13. C, IBF, Villeneuve La Garenne, France.

TABLE III

NEW BASIC ACRYLAMIDO BUFFERS

pK^a	Formula	Name	M_r	Ref.
6.6		2-Thiomorpholinoethylacrylamide	200	8
6.85		1-Acryloyl-4-methylpiperazine	154	10
7.0		2-(4-Imidazolyl)ethylamine-2-acrylamide	165	11
7.4		3-Thiomorpholinopropylacrylamide	214	8
8.05	$CH_2=CHCONH(CH_2)_3N(CH_2CH_2OH)_2$	N,N-Bis(2-hydroxyethyl)-N′-acryloyl-1,3-diaminopropane	200	9

[a] All pK values measured at 25°C.

performed in 100 m*M* acetate (pH 4.0) at 20 kV and 23 μA, of the commercial basic Immobilines in 50 m*M* phosphate buffer (pH 7.7) at 13 kV and 80 μA, of the weakly basic acrylamido buffers in 50 m*M* phosphate buffer (pH 7.0) at 20 kV and 100 μA and for checking the hydrolytic products in 0.1 *M* borate (pH 9.0) at 15 kV and 55 μA. 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 into the capillary by pressure from a nitrogen tank (*ca.* 0.8 MPa), usually for 10 s. The calibration graph for each acrylamido derivative analysed was constructed with the Beckman Gold integration system, with concentration points of 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 [17].

Partition coefficient

In order to establish a hydrophobicity scale, the available alkaline Immobilines (p*K* 6.2, 7.0, 7_{AH}, 7.4, 8.05, 8.5, 9.3 and 10.3) were subjected to partitioning in 1-octanol–water as described by Purcell *et al.* [18]. The partition coefficient *P* is defined as the ratio of the molarities of a given compound in the organic and the aqueous phase. Partitioning is performed under conditions in which the alkaline Immobilines are fully deprotonated. For the p*K* 10.3 compound, as partitioning was executed at pH 11.6 (borate buffer, under nitrogen), a correction factor was applied to account for the small degree of protonation at this pH [18]. After partitioning, both the water and 1-octanol phases were monitored at 254 nm to measure the concentration in each phase. As an independent assessment, the water phase was also analyzed by CZE and the peak area measured with the Beckman Gold integration system. The two sets of data agreed within a relative standard deviation of 5% ($n = 8$).

RESULTS

CZE analysis of acrylamide weak acids and bases

Fig. 1a shows the separation of five acidic Immobilines (p*K* 4.6, 4,4, 3.6, 3.1 and 1.0) and of a contaminant, acrylic acid, added to the mixture. It is seen that the conditions used are able to separate these species completely, and are clearly optimum for the weaker acids (p*K* 4.6, 4.4 and 3.6 and acrylic acid). Owing to its high mobility, the p*K* 1.0 (2-acrylamido-2-methylpropanesulphonic acid) peak is highly skewed, as its anodic transport competes strongly with the cathodic electroosmotic flow. To a lesser extent, this is also true for the p*K* 3.1 peak. In fact, these two compounds show fronting, as opposed to very slight tailing of the other peaks.

Fig. 1b shows an interesting application of the CZE technique: by plotting the p*K* values of the four Immobiline weak acids (p*K* 4.6, 4.4, 3.6 and 3.1) *versus* their respective migration times, a straight line is obtained. This can be used as a calibration graph for measuring unknown p*K* values of compounds with intermediate mobilities. Thus, by entering the calibration graph with the mobility of acrylic acid (vertical arrow), a p*K* value of 4.2 (horizontal arrow) is obtained, which compares well with literature data (p*K* 4.25) [14].

Fig. 2a shows the separation of the four commercially available basic Immobilines (p*K* 6.2, 7.0, 8.5 and 9.3) in phosphate buffer (pH 7.7). In this instance, both the

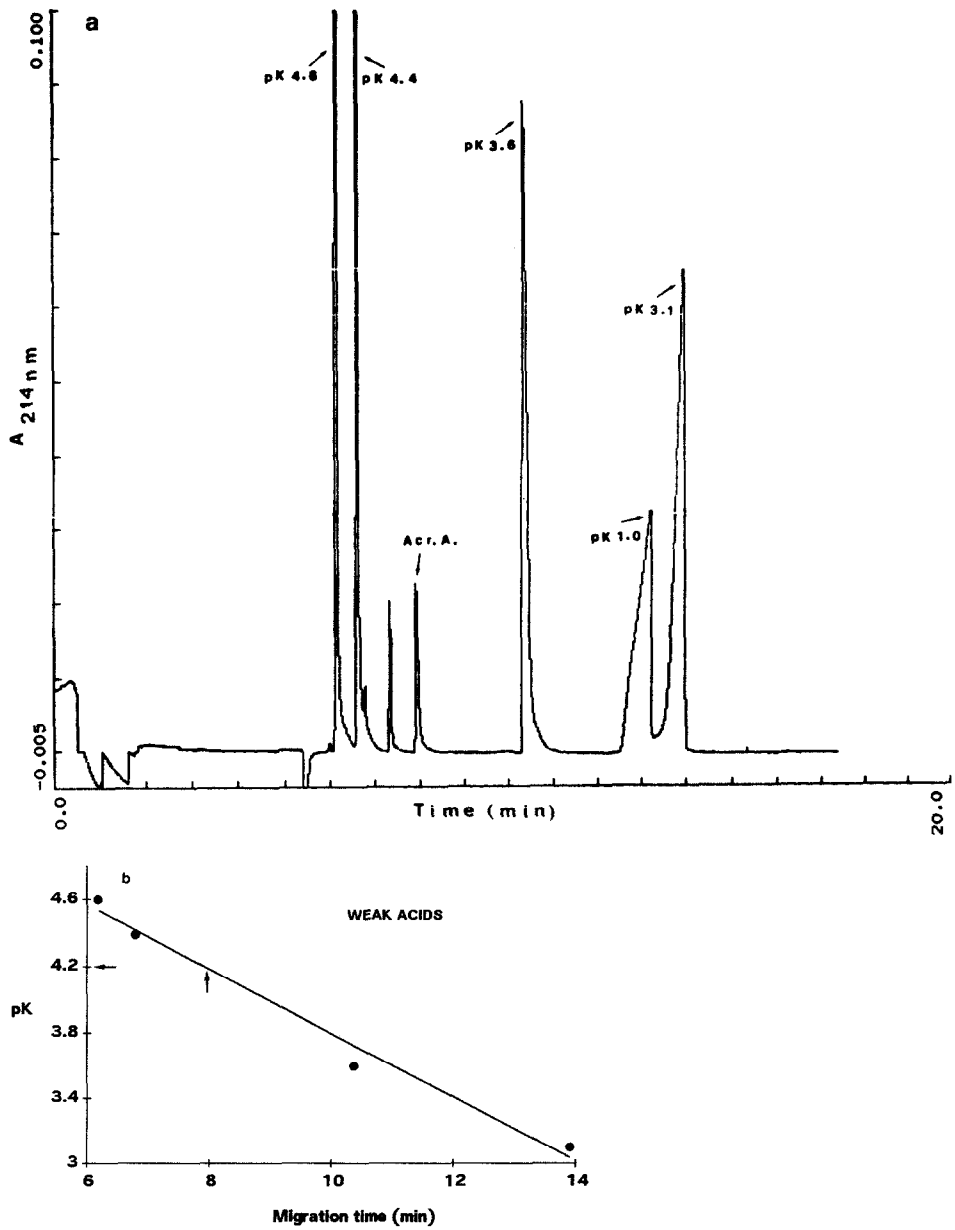


Fig. 1. Analysis of acidic Immobilines by CZE. A mixture of 2.5 m*M* each of Immobilines of p*K* 4.6, 4.4, 3.6, 3.1 and 1.0 was introduced by pressure (10 s) into a 50 cm × 50 μm I.D. capillary in the Beckman P/ACE System 2000. The run was at 25°C in 100 m*M* acetate buffer (pH 4.0) at 20 kV and 23 μA. Detection was by UV absorption at 214 nm. The sample was contaminated with 2.5 m*M* acrylic acid (Acr. A.). Migration toward the cathode by electroosmosis. (a) CZE profile; (b) correlation between p*K* values and migration times. The vertical arrow indicates the migration time of acrylic acid and the horizonal arrow its corresponding p*K* value as derived from the plot.

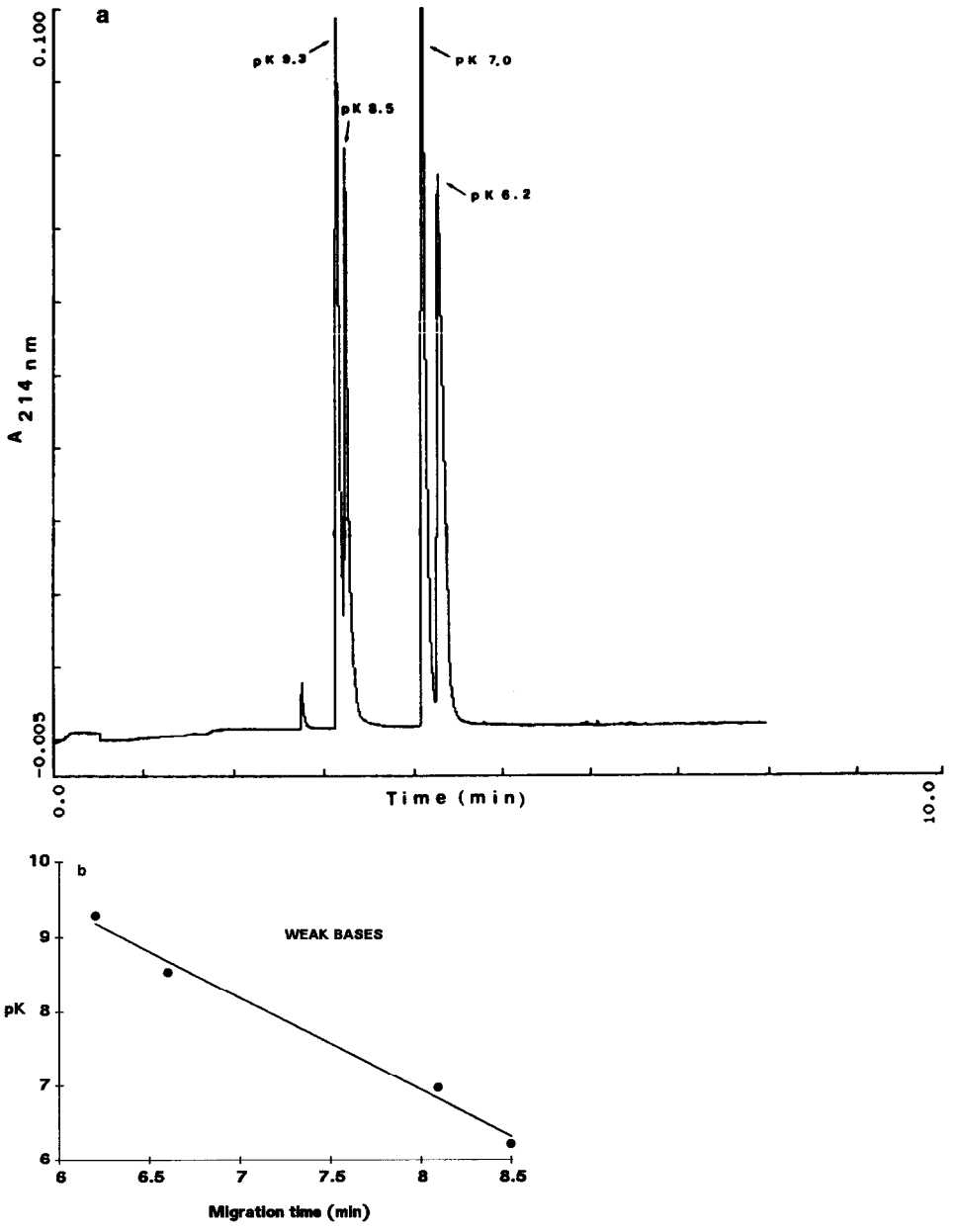


Fig. 2. Separation of alkaline Immobilines by CZE. A mixture of 2.5 m*M* each of Immobilines of p*K* 9.3, 8.5, 7.0 and 6.2 was run in the P/ACE System 2000 in 50 m*M* phosphate buffer (pH 7.7) at 13 kV and 80 μA. All other conditions as in Fig. 1. Cathodic migration. (a) CZE profile; (b) correlation between p*K* values and migration times.

electrophoretic migration and electroosmotic flow are in the same direction, and the peaks are sharp, with a much reduced separation time (less than 5 min, as opposed to 15 min in Fig. 1). Here too, a plot of pK values *vs.* migration time gives a straight regression line, suggesting that this also can be used as a calibration graph for measuring the pK values of unknown compounds (Fig. 2b).

As we have recently synthesized two analogues of the weak bases of pK 6.2 and 7.0 (with a thiomorpholino substituting the morpholino ring), it was of interest to examine the separation of these compounds. By decreasing the operating pH, this analysis is easily accomplished (Fig. 3a). Note that as the pK values are regularly spaced at 0.4 pH unit intervals, so is the peak distance in the electropherogram. In fact, a plot of pK *vs.* migration time (Fig. 3b) gives a straight regression line with all the points on the line.

Hydrolytic stability of the basic acrylamido buffers

It is known that the alkaline acrylamido buffers are the species most prone to hydrolytic degradation, as the high pH of these solutions (supplied as free bases) auto-catalyse the lysis of the amido bond. Thus, structures which stabilize these chemicals against degradation are highly desirable. We subjected all the alkaline Immobilines to hydrolysis in 0.1 M NaOH at 70°C for up to 6 h and analysed the degradation products by CZE. A summary of these data is presented as a bar graph in Fig. 4, which gives the percentage of undegraded material left at the end of the 6-h period. Curiously, it is seen that the stability, contrary to expectations, is in order of pK values: the higher the pK, the more stable is the chemical. This order is, however, merely accidental, as the stability is not so much a function of the pK value but rather of the type of substituents on the nitrogen of the amido group (see Discussion).

Hydrophobicity of the basic acrylamido buffers

Owing to the types of substituents on the nitrogen of the amido bond, the alkaline Immobilines have a much greater chance of presenting a hydrophobic surface than the acidic species, as the latter are in general less substituted and contain a carboxyl group, which is decidedly more hydrophilic than an amino group. Therefore, our efforts to construct a hydrophobicity scale were concentrated solely on the alkaline compounds. Partitioning of the deprotonated species was performed in 1-octanol–water mixtures and the concentration in each phase read at 254 nm. The data in the water phase were double checked by CZE and peak integration. A summary of these data is presented as a bar graph in Fig. 5, which gives the partition coefficients (P) of the alkaline Immobilines studied (the maximum P value being 6). It is seen that most species are fairly hydrophilic, having P values below 1. However, in the morpholino ring, when the oxygen is substituted with a sulphur atom, for synthesizing the thiomorpholino analogues (pK 6.6 and 7.4) the hydrophobicity greatly increases. The same applies to the pK 10.3 compound, which contains two N-ethyl groups as opposed to the two N-methyl groups in the pK 9.3 buffer.

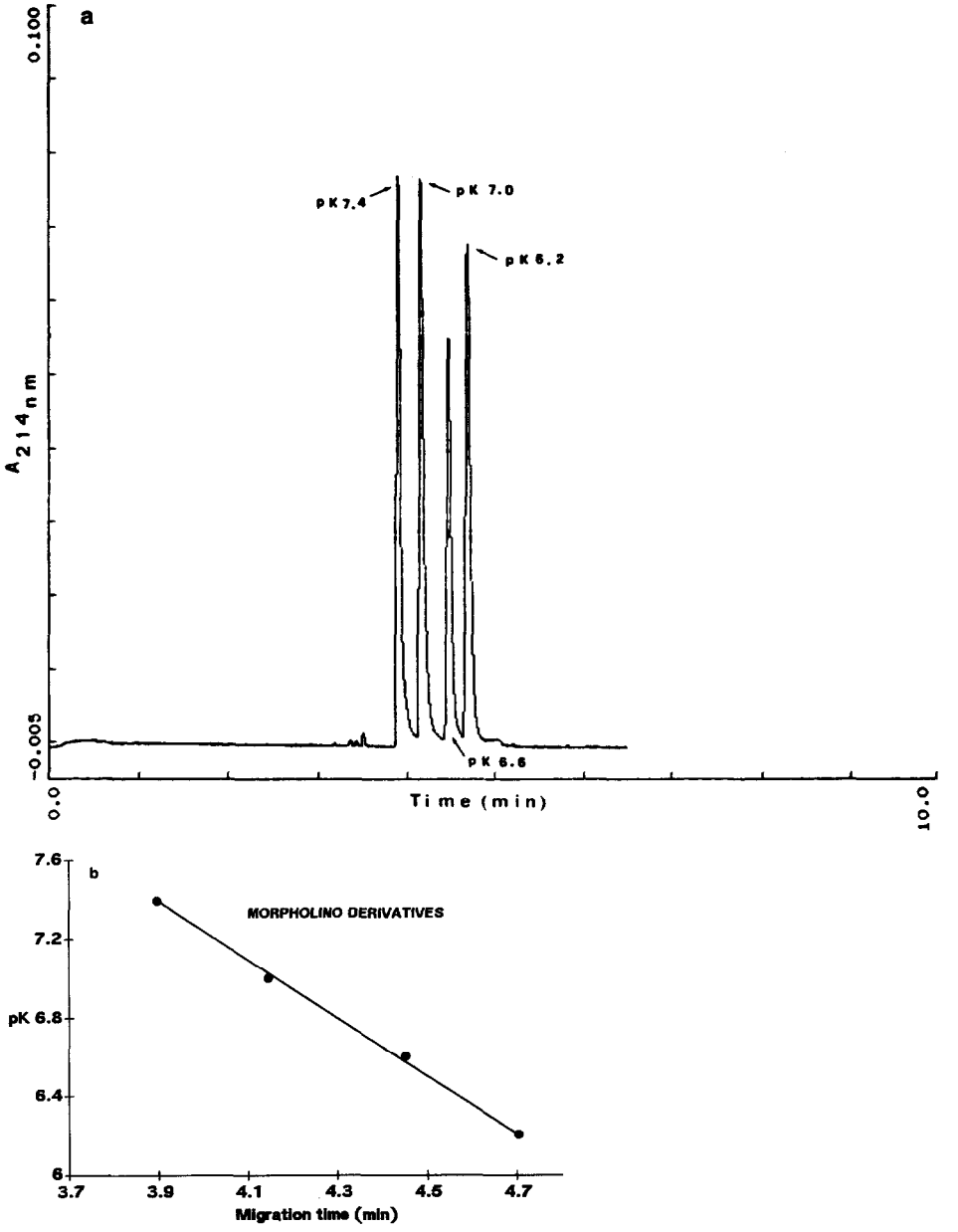


Fig. 3. Separation of weakly basic Immobilines by CZE. A mixture of 2.5 m*M* each of Immobilines of p*K* 7.4, 7.0, 6.6 and 6.2 was run in the P/ACE System 2000 in 50 m*M* phosphate buffer (pH 7.0) at 20 kV and 100 μA. The p*K* 7.4 and 6.6 buffers are the thiomorpholino derivatives of the commercial morpholino species (p*K* 7.0 and 6.2, respectively). All other conditions as in Fig. 1. (a) CZE profile; (b) correlation between p*K* values and migration times.

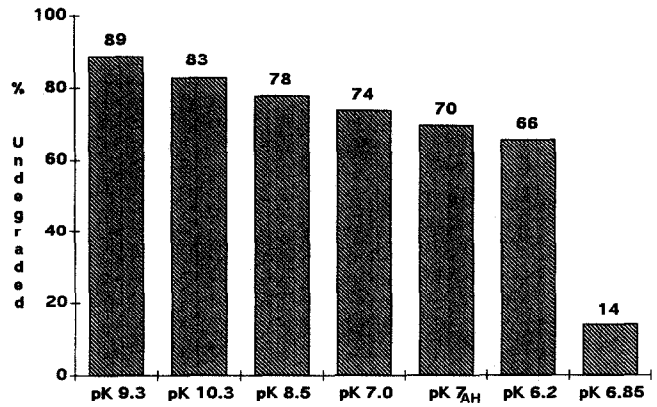


Fig. 4. Summary of the degradation kinetics of the seven acrylamido bases studied. The vertical bars represent the amounts of undegraded product remaining after 6 h of hydrolysis at 70°C in 0.1 *M* NaOH. p*K* 6.85 = 1-acryloyl-4-methylpiperazine; p$K\,7_{AH}$ = acryloylhistamine (p*K* 7.0). All data obtained by CZE in a Beckmann P/ACE 2000 with a 50 cm × 75 μm I.D. capillary. Run at 15 kV and 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 as an internal standard in all runs. Peak integrations were done with the Beckman system Gold. The numbers on each bar give the reading on the ordinate (% intact compound).

DISCUSSION

Determination of pK values

Although our data would appear to be limited to the analysis of acrylamido buffers, some results could have wider implications. Noteworthy is the fact that there appears to be a linear relationship between p*K* values (of both weak acids and bases) and the migration (or transit) times. This could lead to a simple and rapid method for

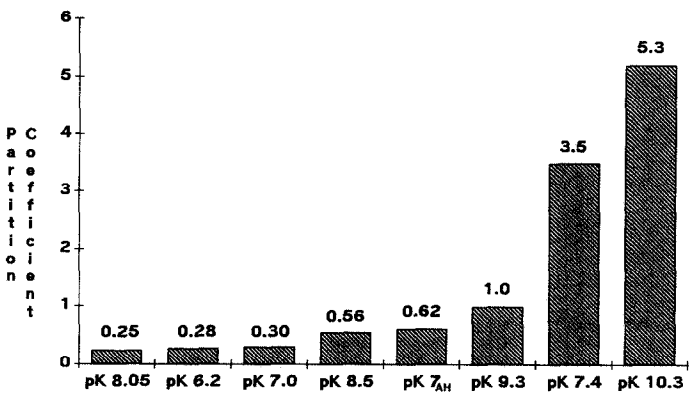


Fig. 5. Partition coefficients (*P*) of different alkaline Immobilines in 1-octanol–water. The *P* values were assessed by partitioning the different bases in the fully deprotonated form. The molarity ratios in the two phases were measured by absorbance readings at 214 nm in a spectrophotometer. For the water-phase values, all data were double checked by CZE under the same conditions as in Fig. 4. The data presented are the averages of the spectrophotometric readings and the CZE peak integrations. The number on top of each bar is the *P* value.

the determination of the pK values of unknown compounds. The Immobiline weak acids and bases (well characterized compounds) could be used to construct calibration graphs in certain pH regions for the rapid assessment of unknown pK values. The problem of determining ionic mobilities and dissociation constants of weak acids and bases has been extensively studied in capillary isotachophoresis [19–21]. Most workers have elaborated methods that combine computer simulations with experimental measurements of conductivities of the isotachophoretic zones in the steady state: if agreement between simulated and experimental data for pK and mobility was achieved, such data were considered to be valid [19–21]. More recently, Pospichal *et al.* [22] built a micropreparative isotachophoresis unit in which, in addition to an on-line measurement (relative effective mobility), an off-line determination (pH) could be performed in the collected zone. In a linear plot of 1/mobility *vs.* $[H^+]$, the K (and thus pK) value could then be accurately assessed. In a different approach, Beckers *et al.* [23] proposed a double detector system in zone electrophoresis for measuring absolute mobilities of cation and anions. In the latter instance, the experimental and literature data agreed to within 1%.

What we propose here is a simple approach by which a pK value can be determined by interpolation on a calibration graph, which is obtained in a run simultaneously with the unknown sample. Constructing a plot of pK *vs.* migration time is thus similar to a plot of log M_r *vs.* K_{av} used for M_r determination in gel filtration [24]. The rule in gel permeation is that a solute must have a K_{av} between V_e (totally excluded) and V_t (totally included), preferably in fact K_{av} should be on the linear portion of the curve (between 0.2 and 0.8). Thus, for a correct assessement of pK values from our proposed calibration graph, the unknown sample should be dissolved at a pH encompassing the interval p$K \pm 0.5$. In a similar approach, Nishi and Terabe [25] proposed the concept of t_o and t_{mc} for measuring the partition coefficient of a solute in micellar electrokinetic chromatography.

This is a simple approach to the problem of assessing a true thermodynamic dissociation constant, which represents a complex system of equilibria, involving first a K_i, *i.e.*, a thermodynamic constant of the true ionization process of an ionogenic molecule, followed by a K_d, *i.e.*, the constant of the dissociation process on the basis of which the solvated ion pairs are finally separated into solvated ionic species by action of the solvent molecules [26]. Nevertheless, CZE could prove useful not only as a simple tool for practical pK determinations, but also for discriminating substances with very small ΔpK values. In fact, Terabe *et al.* [27] have demonstrated, in the separation of oxygen-isotopic benzoic acids (BA), that three isotopes ($^{16}O_2$[BA]; $^{16}O^{18}O$[BA] and $^{18}O_2$[BA]) differing in pK values by only 1% could still be effectively separated.

Hydrolytic stability of alkaline Immobilines

All Immobilines analysed here are monosubstituted amides, except for the pK 6.85 (AMPip) species, which is disubstituted.Although we agree with the general knowledge on the greater stability of such compounds in comparison with unsubstituted 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 these acrylamido derivatives (see Tables II and III), we can thus derive the following rules.

(a) To afford protection of the amido bond, the most important parameter is not the degree of substitution in the nitrogen engaged in the amido plane (mono- or disubstituted) but the type of substituent.

(b) In particular, rigid ring structures (as in AMPip) are completely inefficient in protecting the adjacent amido 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.

(d) If rigid structures are present in the nitrogen substituents, there should be some distance from the plane of the amido bond. This is why the pK 7.0 (3-morpholinopropylacrylamide) degrades substantially less than the pK 6.2 (2-morpholinoethylacrylamide) derivative.

(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 (N,N-dimethylaminopropylacrylamide) is more resistant than the pK 8.5 (N,N-dimethylaminoethylacrylamide) derivative.

Hydrophobicity scale

Generally, most of the Immobilines that we have analysed are fairly hydrophilic, as they have a P value below 1(on a scale of up to 6). There is a trend of increasing hydrophobicity with increasing pK value, no doubt due to the increasing complexity of the molecules and the longer aliphatic chains as substituents on the nitrogen of the amido bond (in a homologous series). Some small exceptions can be found, *e.g.*, the pK 7_{AH} species, which in the hydrophobicity scale of Fig. 5 is located between the pK 8.5 and 9.3 derivatives, with a partition coefficient of $P = 0.62$. It was surprising that the P value should be higher than that of the commercial analogue pK 7.0 ($P = 0.3$), considering that the substituent chain on the amido group is two carbon atoms shorter than that in the pK 7.0 compound. Conversely, a huge hydrophobicity increment is noted when the oxygen in the morpholino ring of the pK 7.0 Immobiline is replaced with a sulphur atom (pK 7.4 compound, 3-thiomorpholinopropylacrylamide). The highest hydrophobicity jump in the scale is, however, exhibited by the pK 10.3 buffer (an analogue of the pK 9.3 species with two ethyl, instead of methyl, substituents on the tertiary nitrogen); in the deprotonated form, *ca.* 95% of it is extracted into the 1-octanol phase ($P = 5.3$). Hence, the addition of only two carbon atoms (compared with the pK 9.3 compound) has the effect of increasing the partition coefficient by a factor of > 5 (see Fig. 5). In the light of these data, it appears worthwhile to search for new, basic Immobilines with more hydrophilic substituents able to lower the P value. In fact, IPG in very alkaline ranges has often been plagued by the adsorption of protein on the gel matrix [28]. A balance will have to be obtained between he hydrophobicity and hydrophilicity of such alkaline compounds, because the addition of two terminal OH groups (see the formula of the pK 8.05 species) lowers the pK value of the pK 10.3 species by more than 2 pH units.

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