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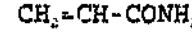
Developments in the use of pH and electric field strength gradients for the electrophoretic analysis of biomolecules

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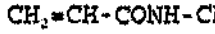
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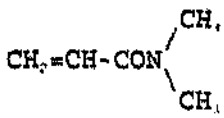
The above general rules can be made clearer by means of examples. First of all, let us look at acrylamide:



The amide group confers an hydrophilic nature to this compound and its polymers. However, the amide group is unshielded from attack from hydroxyl anions and is hence quite susceptible to alkaline hydrolysis. Adding a methyl group (*N*-methylacrylamide) would increase its stability due to steric protection:



The addition of a second methyl group (*N,N*-dimethylacrylamide) would afford a greater shielding effect:



Increasing the length of the *N*-alkyl groups (*N,N*-diethylacrylamide) would shield the amide group even more, due to oscillation of the alkyl chains in the surrounding space:

Although they recently brought out a nonlinear pH 3.5-10 gradient for 2-D maps, it is supplied bathed in a slightly acidic buffer, which has to be washed out prior to use.

Hence, it is apparent that polyacrylamide derivatives of similar ease of polymerization, but much more stable to hydrolysis would be a significant advance in electrophoretic technology. In general, because of steric protection of the amide group, *N,N*-disubstituted amides are more stable than *N*-monosubstituted amides, which are in turn more stable than unsubstituted amides. Research by Righetti's group on the relationship of the structure of acrylamide derivatives with their hydrolytic stability (Chiari *et al.*, 1991a,c; Gelfi *et al.*, 1992) has shown that there are other, more subtle mechanisms governing stability, from which they have derived the following rules:

(i) to afford protection of the amido bond, the most important parameter is not the degree of substitution of the amide nitrogen (mono- or disubstituted) but the type of substituent;

(ii) in particular, rigid ring structures are inefficient in protecting the adjacent amido bond, as their rigidity prevents them from oscillating in the surrounding space and thus shielding the amido plane;

(iii) flexible chains bound to the nitrogen of the amido bond are capable of protecting the amido plane, as they can fluctuate in the nearby space and shield the amido group;

(iv) 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.

electrophoretic analysis of proteins and small- to medium-sized nucleic acids, being of especial importance in DNA sequencing. Its popularity as an electrophoretic support stems from some fundamental properties, such as:

(i) optical transparency, including the ultraviolet

(ii) electrical neutrality, due to the absence of charged groups

(iii) possibility of synthesizing gels in a wide interval of porosities[1].

However, polyacrylamide's main drawback is its instability at alkaline pH values (most electrophoretic separations occur at alkaline pH values for both proteins and nucleic acids). Thus partial hydrolysis of the amide groups of the polyacrylamide gel to carboxylic acids will occur during the course of an electrophoretic run, especially in DNA sequencing gels which are normally run at 70°C in order to prevent reannealing of the DNA stands. The presence of negative charges on the polyacrylamide matrix results in unpleasant phenomena such as electroosmosis and gel swelling, rendering the gel unusable for further electrophoretic separations. This limits the use of polyacrylamide gels to single electrophoretic runs - adding to the time, labour and expense of projects such as the sequencing of the human genome, where the availability of reusable matrices would greatly shorten the analysis time. To date Pharmacia-LKB Biotechnology AB have commercialized only ready-made polyacrylamide gels containing acidic IPG intervals.

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[1] Gels may be prepared containing from 3% to 30% acrylamide, corresponding to pore sizes of 0.5 nm and 0.2 nm diameter respectively. In general terms a 30% gel is suitable for the separation of compounds having molecular masses around 10^4 daltons whereas a 3% gel will separate compounds having molecular masses around 10^7 daltons.

fully protonated (at least 1 pH unit below the p*K* of the basic Immobiline). Alternatively, all oxidations can be prevented by washing of the IPG gels with a dilute solution of a reducing agent such as ascorbic acid, 2-mercaptoethanol or dithiothreitol after the polymerization step.

It has recently been shown that photopolymerization of acrylamide, eg. with riboflavin, is essentially non-oxidizing (Chiari *et al.*, 1992), so that no reducing step is needed. Unfortunately, riboflavin-catalyzed photopolymerization of acrylamide requires at least 8 hours of illumination to ensure 95% conversion of the acrylamide monomers (Righetti *et al.*, 1981*b*). Moreover, with riboflavin, proper polymerization is only obtained in the pH 4.0-7.0 range. A novel method of photopolymerization has recently been introduced, using methylene blue as the photoinitiator and a redox couple (sodium toluenesulfinate as the reductant and diphenyliodonium chloride as the oxidant) (Lyubimova *et al.*, 1993). Methylene blue-catalyzed photopolymerization is as fast as persulfate-catalyzed polymerization, but with the advantage of being non-oxidizing. Moreover, it performs well over the pH 4.0-10.0 range (Lyubimova and Righetti, 1993; Caglio and Righetti, 1993), whereas persulfate-catalyzed polymerization performs well only in the pH 7.0-10.0 range. Hence it appears that photopolymerization will become the preferred method for the preparation of IPG gels.

D.4 DEVELOPMENT OF ACRYLAMIDE DERIVATIVES OF IMPROVED PERFORMANCE

Polyacrylamide, since its introduction as a gel matrix for zone electrophoresis in 1959 by Raymond and Weintraub, has remained the most popular support for the

over only one pH unit. It has been calculated that IEF using 0.1 pH unit-wide IPG gels can resolve isoforms with a difference in pI of only 0.001 (Bjellqvist *et al.*, 1982; Righetti, 1990). Although it is possible to perform isoelectric focusing in very narrow carrier ampholyte-generated pH gradients, as high resolution is generally not attained, since there are insufficient ampholyte species per pH unit[1] to provide an even pH gradient (Charlionet *et al.*, 1979).

D.3 OXIDATION OF THE BASIC IMMOBILINE' CHEMICALS

Biochemists performing isoelectric focusing in IPG gels noticed that oxidation of cysteine residues readily occurred (Altland *et al.*, 1988), altering the pI values of the affected proteins and hence leading to the generation of spurious banding patterns. This phenomenon was found to be caused by oxidation of the tertiary amine groups of the basic Immobilines' by persulfate during the polymerization, with the formation of *N*-oxides (Righetti *et al.*, 1989*b*). These R,N→O species in turn oxidize the sulfhydryl groups (-SH) of cysteine, transforming adjacent -SH groups into disulphide (-S-S-) bridges. Since only the free base form of tertiary amines is susceptible to oxidation, oxidation can be prevented by performing the polymerization at a pH at which the tertiary nitrogen atoms of the basic Immobilines' are

[1] In order for two proteins of very close pI values to be resolved in a carrier ampholyte-generated pH gradient, there must be at least one carrier ampholyte species present of an intermediate pI value in order for physical separation of the two proteins to occur (Rilbe, 1976). Hence in order for two proteins differing in pI of only 0.02 to be separated, carrier ampholytes differing in pI of 0.01 would have to be present, ie. at least 100 discrete carrier ampholytes species per pH unit would be required.

The Immobiline® chemicals were originally supplied by LKB in their pure form, to which distilled water was added by the purchaser to make a 0.2 M solution. However, it soon became apparent that the basic Immobilines® were not stable, even when the solutions were stored at -20°C (Righetti, 1990; Astrua-Testori *et al.*, 1986). One problem was that the amide bond of the basic acrylamido derivatives hydrolysed rather rapidly at the high pH of the 0.2 M solutions. Moreover, the basic Immobilines® on storage showed a marked tendency to autopolymerize. The resulting oligomers formed complexes with proteins during electrofocusing, leading to precipitation (Rabilloud *et al.*, 1987; Fawcett and Chrambach, 1988; Fawcett *et al.*, 1988). These problems were solved only in 1988 (Gåveby *et al.*, 1988), when it was found that the use of *n*-propanol as a solvent prevented both the hydrolysis and autopolymerization. LKB now market the Immobiline II® range, in which the acidic p*K* 3.6 and 4.6 Immobilines® are provided as 0.2 M solutions in distilled water, with a trace of 4-methoxyphenol as a polymerisation inhibitor, and the basic p*K* 6.2, 7.0, 8.5 and 9.3 Immobilines® as 0.2 M solutions in *n*-propanol.

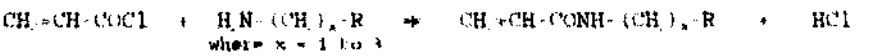
D.2 CONDUCTIVITY ASPECTS OF IPG GELS

Since there are no free buffer ions in an IPG gel (after polymerization the gels are extensively washed in distilled water to remove all dissolved ions), the conductivity is very low, as the current can only be carried by H_3O^+ and OH^- ions. The positive aspect of the very low conductivity of IPG gels is that IEF can be performed at voltages as high as 5 kV without overheating, since the power consumption is minimal (power = voltage x current). Hence very sharp focusing of proteins is achieved, even with pH gradients extending

Appendix D: Aspects of the chemistry of the Immobiline' compounds and of the physical chemistry of immobilized pH gradients in general[1]

D.1 CHEMISTRY OF THE IMMOBILINE' CHEMICALS

The synthesis of the Immobiline' chemicals is very simple, involving the reaction of acryloyl chloride with an amino acid or a diamine to produce the acidic and basic Immobiline' buffers respectively (Righetti, 1990; Chiari *et al.*, 1989a,*b*):



acryloyl chloride | amino acid/ diamine | acrylamido buffer | hydrochloric acid

Notwithstanding their ease of synthesis, the Immobiline' chemicals are, as is the case with most speciality chemicals, overpriced. An illuminating example can be seen in the 1995/1996 Fluka catalogue: 2-acrylamido-2-methylpropane-1-sulfonic acid, a strongly acidic acrylamide derivative, costs $14.85 for 250 g. Yet the identical chemical, listed elsewhere in the catalogue as Acrylamido buffer p*K* 1 costs $339.65 for 5 g, ie. $16982.50 for 250 g - over one thousand times the price!

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[1] The authoritative reference work on the field of immobilized p*H* gradients (IPG's) is Righetti (1990). This book probably contains all the information of papers, published up to 1990, that are related to the field of IPG's. Practical protocols for IEF-IPG are given in Righetti *et al.* (1990) and Westermeier (1993).

according to their molecular masses, as in discontinuous SDS-PAGE, first introduced by Laemmli in 1970. The chloride-glycine boundary can be seen as a refractive line moving ahead of the proteins; or if a marker dye such as bromophenol blue is used, a chloride-bromophenol blue-glycine moving boundary will be observed.

3. **Ionic strength of buffer** - the resolving gel buffer (0.060 M HCl - 0.361 M Tris, pH 8.8) is of a higher ionic strength, and hence conductivity, than the stacking gel buffer (0.060 M HCl - 0.063 M Tris, pH 6.8). Hence the voltage gradient is lower in the resolving gel, causing the velocity of the proteins to drop (see Equation (B-7) in Appendix B) as they enter the resolving gel. This leads to a further sharpening of the protein bands, and hence increasing the resolution obtained.

4. **Nature of the buffer ions** - the stacking and resolving gel buffers should not contain glycine, nor should the upper electrode buffer contain chloride, as this decreases the electric field strength gradient across the chloride-glycine steady state moving boundary. (See Section 1.2.3 of the Introduction for a discussion on the effect of mixed ion zones in steady state moving boundary systems.)

C.4 FURTHER ASPECTS OF THE DISCONTINUOUS ELECTROPHORESIS SYSTEM OF ORNSTEIN AND DAVIS

The discontinuity between the stacking and separating gels in based on four parameters (Williams and Reisfeld, 1964; Westermeier, 1993):

1. **Gel structure** - the stacking gel has large pores in order not to impede the migration of the proteins, thereby enabling them to stack behind the moving boundary. The separating gel, on the other hand, has small pores in order to separate the pre-stacked protein bands by molecular sieving.

2. **pH of buffer** - During the stacking phase the pH of the terminating zone is regulated at pH 9.0, giving glycine a net mobility of -4.9×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$. This is slower than the net mobilities of most proteins at the same pH, ensuring that stacking of the proteins occurs between the chloride leading ions and glycine terminating ions. During the separating phase the pH of the terminating zone is regulated at pH 9.5, increasing the net mobility of glycine to -12.2×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$. The glycine ions now overtake the protein bands. The chloride-glycine moving boundary migrates ahead of the proteins, and the proteins are left behind in a homogeneous buffer medium (0.046 M glycine - 0.347 M Tris, pH 9.5), each effectively in an extremely thin starting zone. Migration of the sample proteins will now occur as in ordinary zone electrophoresis, ie. according to their charge, shape and size. Since the higher pH gel is normally of smaller gel pore size, molecular sieving of the sample proteins will occur. If a denaturing buffer is used, then the proteins will be resolved solely

1.	$C_{chloride^-}$	= 0.060 M	8.	pH_T	= 9.9
2.	$C_{AMPD^+(L)}$	= 0.060 M	9.	$\bar{m}_{glycine^-}$	= -21.6×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$
3.	$C^{total}_{AMPD(L)}$	= 0.361 M			
4.	$C^{total}_{glycine}$	= 0.045 M	10.	κ_L	= 6.43 $mS \cdot cm^{-1}$
				κ_T	= 2.79 $mS \cdot cm^{-1}$
5.	$C^{total}_{AMPD(T)}$	= 0.364 M			
6.	$C_{glycine^-}$	= 0.026 M	11.	I_L	= 0.060 M
				I_T	= 0.026 M
7.	$C_{AMPD^+(T)}$	= 0.026 M			

Composition of the leading zone:
0.060 M HCl - 0.361 M AMPD, pH 9.5

Composition of the terminating zone:
0.045 M glycine - 0.346 M AMPD, pH 9.9

When the chloride-glycine steady state moving boundary crosses the stationary pH boundary between the stacking and separating gels, the composition of the leading electrolyte is changed from 0.060 M HCl - 0.066 M AMPD, pH 7.8 to 0.060 M HCl - 0.361 M AMPD, pH 9.5. This causes the ionic composition of the terminating electrolyte to be altered from 0.046 M glycine - 0.052 M AMPD, pH 9.3 to 0.046 M glycine - 0.346 M AMPD, pH 9.9. Hence, in effect, the proteins in the separating gel migrate in a 0.046 M glycine - 0.347 M AMPD buffer, pH 9.5. The net mobility of the glycine terminating ions becomes -21.6×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$.

Composition of the leading zone:
0.060 M HCl - 0.066 M AMPD, pH 7.8

Composition of the terminating zone:
0.045 M glycine - 0.052 M AMPD, pH 9.3

Electrode buffer:
0.077 M glycine - 0.013 M AMPD, pH 8.8

Although the initial composition of the terminating zone (electrode buffer) is 0.077 M glycine - 0.013 M AMPD, pH 8.8, once electrophoresis is commenced the concentrations of glycine and AMPD in the terminating zone become regulated against the concentrations of chloride and AMPD in the leading zone, so that the composition of the terminating zone becomes 0.045 M glycine - 0.052 M AMPD, pH 9.3. The net mobility of the glycine terminating ions is -9.1×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$.

Ionic parameters of leading and terminating zones during separating phase

The following ionic parameters were calculated for the separating phase of discontinuous electrophoresis, using the equations in section C.2. The total molar concentration of the chloride leading ion is 0.060 M, and the pH of the leading zone (pH_L) is 9.5.

This causes the ionic composition of the terminating electrolyte to be altered from 0.046 M glycine - 0.049 M Tris, pH 8.9 to 0.046 M glycine - 0.361 M Tris, pH 9.5. Hence, in effect, the proteins in the separating gel migrate in a 0.046 M glycine - 0.347 M Tris buffer, pH 9.5. The net mobility of the glycine terminating ions becomes -11.9×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$.

C.3.2 Tamura-Ui system

Ionic parameters of leading and terminating zones during stacking phase

The following ionic parameters were calculated for the stacking phase of discontinuous electrophoresis, using the equations in section C.2. The total molar concentration of the chloride leading ion is 0.060 M, and the pH of the leading zone (pH_L) is 7.8.

1. $c_{chloride}$ = 0.060 M
2. $c_{AMPD+(L)}$ = 0.060 M
3. $c^{total}_{AMPD(L)}$ = 0.066 M
4. $c^{total}_{glycine}$ = 0.045 M
5. $c^{total}_{AMPD(T)}$ = 0.052 M
6. $c_{glycine^-}$ = 0.011 M
7. $c_{AMPD+(T)}$ = 0.011 M
8. pH_T = 9.3
9. $\overline{m}_{glycine^-}$ = -9.1×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$
10. κ_L = 6.43 $mS \cdot cm^{-1}$
    κ_T = 1.18 $mS \cdot cm^{-1}$
11. I_L = 0.060 M
    I_T = 0.011 M

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CH_2=CH-CONH-$CH_2CH_2CH_2$-OH

Research on the chemical properties of AAP by Prof. Righetti's group (Simò-Alfonso *et al.*, 1996*a*,*b*) showed it to combine high hydrophilicity (1-octanol:water partition coefficient of 0.10 *versus* 0.13 and 0.2 for AAEE and acrylamide respectively) with extreme hydrolytic stability - slightly better than that of AAEE. Moreover aqueous solutions of AAP, stored at room temperature in plain daylight, showed no autopolymerization even after two months. Resolution of DNA by capillary electrophoresis in entangled solutions of linear poly(APP) showed excellent reproducibility (Gelfi *et al.*, 1996). It is expected that poly(AAP) will also be of great potential in the field of biochromatography for the preparation of hydrophilic, hydrolytically-stable gel media.

AAEE even more hydrophilic than acrylamide (1-octanol:water partition coefficient of 0.13 *versus* 0.2), but it is very resistant to hydrolysis - poly(AAEE) being 500 times more stable towards alkaline hydrolysis than polyacrylamide. Entangled solutions of linear poly(AAEE) were shown to show superior performance compared to linear polyacrylamide for capillary electrophoresis (Chiari *et al.*, 1994*b*). Isoelectric membranes composed of poly(AAEE) (Bossi *et al.*, 1994*a*; Chiari *et al.*, 1994*c*) were superior to acrylamide membranes for preparative isoelectric membrane electrofocusing (PrIME).

Unfortunately AAEE, stored as a 1:1 (v/v) solution in water at 4°C, was shown to undergo an autopolymerization reaction, possibly caused by the reaction of dissolved oxygen with the ether groups to form peroxides. Moreover, molecular modelling studies of AAEE found that transfer of a hydrogen atom from the double bond to the ether oxygen was favoured, with the consequent formation of free radicals (Simó-Alfonso *et al.*, 1996*a*).

Since ether groups can no longer be introduced into the *N*-alkyl chains, this means the *N*-alkyl chains may not be more than four carbon atoms long or else the acrylamide derivatives will be too hydrophobic. The rules for derived by Chiari *et al.* (1994*a*) require any hydroxyl groups present to be separated by at least three carbon atoms from the amido group so as to prevent formation (*via* hydrogen bond with the free -OH group) of 5- and 6-membered rings closing onto the amido bond, which favour hydrolysis *via* a mechanism of *N,O*-acyl transfer. It is apparent that the only acrylamide derivative fulfilling these requirements is *N*-acryloylaminopropanol (AAP):

hydrolytic stability, results in compounds like dideoxytrisacryl and *N,N*-dimethylacrylamide that are too hydrophobic to use for preparing hydrophilic matrices for gel electrophoresis. These results provoke a dilemma: hydroxyl groups are necessary for hydrophilicity, yet catalyze the rate of hydrolysis of the amido bond. How does one solve this paradoxical situation?

The answer is obtained (Chiari *et al.*, 1994*a*) by fulfilling the following requirements:

(i) *N*-mono- and disubstituted acrylamides are generally more resistant to hydrolysis due to steric protection of the amido group. The longer the chains, the better.

(ii) Hydroxyl groups are necessary for hydrophilicity. In order to combine both hydrolytic stability and hydrophilicity, the necessary -OH groups should be at a distance from the amido group so as to prevent formation (*via* hydrogen bond with the free -OH group) of 5- and 6-membered rings closing onto the amido bond, which favour hydrolysis *via* a mechanism of *N,O*-acyl transfer.

(iii) In addition, in order to lessen the hydrophobicity of the *N*-substituted alkyl chains, an oxygen atom should be present along these chains, preferably every two carbon atoms.

An acrylamide derivative meeting these requirements is *N*-acryoylaminoethoxyethanol (AAEE):

$$CH_2{=}CH\text{-}CONH\text{-}CH_2CH_2\text{-}O\text{-}CH_2CH_2\text{-}OH$$

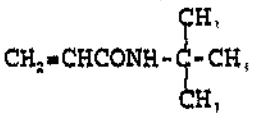
introduced in 1994 by Prof. Righetti's group at the University of Milan (Chiari *et al.*, 1994*a*). Not only is

Table 18: Effect of structure on hydrophobicity and stability of acrylamide derivatives (Data from Gelfi et al., 1992)

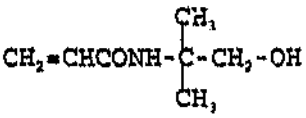
Acrylamides	Formula	Stability[1]	Hydrophobicity[2]
N,N-dimethylacrylamide	$CH_2=CHCON(CH_3)_2$	185	0.50
Dideoxytrisacryl[3]	$CH_2=CHCONH-C(CH_3)_2-CH_2OH$	130	0.86
Acrylamide	$CH_2=CHCONH_2$	111	0.20
N-Acryloylmorpholine	$CH_2=CHCON(CH_2CH_2)_2O$	80	0.79
Trisacryl[3]	$CH_2=CHCONH-C(CH_2OH)_2-CH_2OH$	15	0.01

[1] Half-life in minutes for degradation of acrylamide compound in 0.7 M NaOH at 70°C.
[2] Partition coefficient of acrylamide compound between 1 octanol and water at room temperature.
[3] Registered trademark of IBF Corporation, France (Boschetti, 1989)

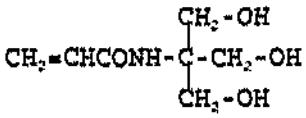
Surprisingly, the hydroxymethyl groups of Trisacryl[3], instead of protecting the amido group sterically from hydrolytic attach by hydroxyl anions, actually increase its rate of hydrolysis. This is due to the fact that the hydroxymethyl groups can form a hydrogen-bonded ring with the carbonyl of the amido group, and, for electronic reasons, autocatalyze the rate of hydrolysis (by a mechanism of *N,O*-acyl transfer). Yet removal of the hydroxyl groups, while increasing



However, *N*-(tert-butyl)acrylamide is not suitable for use in electrophoresis due to its extreme hydrophobicity. The addition of hydroxyl groups is the obvious solution:

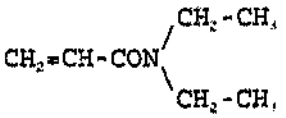


Dideoxytrisacryl'

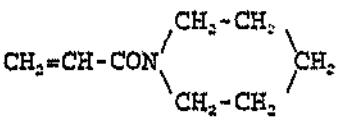


Trisacryl'

The monohydroxylated and trihydroxylated derivatives of *N*-(tert-butyl)acrylamide - Dideoxytrisacryl' and Trisacryl' respectively, are commercially available chemicals (Boschetti, 1989). However, as can be seen in the following table, based on data from Gelfi *et al.* (1992), the hydrolytic stability of Dideoxytrisacryl' and Trisacryl' is very poor indeed:



However, if the *N*-alkyl chains are in the form of a rigid ring structure (*N*-acryloylpiperazine), then little steric protection is afforded to the amide group, as oscillation of the alkyl chains in the surrounding space can no longer occur:



The following acrylamide derivative, *N*-(*tert*-butyl)acrylamide, prepared by the reaction of acrylonitrile and *tert*-butyl alcohol under strongly acidic conditions (Plaut and Ritter, 1951), is extremely resistant to alkaline hydrolysis, requiring prolonged heating under drastic conditions to hydrolyse the amide group[1]:

[1] Compounds of similar structure require refluxing in concentrated potassium hydroxide in ethylene glycol for 48 hours for quantitative hydrolysis of the amide group to be achieved (Minieri and Ritter, 1948).

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