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The degradation of *N*,*N*′,*N*″-triethylenephosphoramide in aqueous solutions: a qualitative and kinetic study

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Abstract

The degradation of *N*,*N*′,*N*″-triethylenephosphoramide (TEPA) in aqueous solutions has been investigated over a pH range of 3–14. Samples were analyzed using a gas chromatographic system with nitrogen/phosphorus selective detection. The degradation kinetics were studied as function of pH, sodium chloride concentration and temperature. The degradation of TEPA in buffers follows pseudo first order kinetics. The log k_{obs}-pH profile shows that TEPA is most stable in the pH range of 8–10. In the pH range 3–10 the degradation rate is higher in the presence of sodium chloride, whereas at $pH \geq 10$ chloride has no influence on the degradation rate. The degradation products were identified by gas chromatography-mass spectrometry. Mono- and dichloro adducts of TEPA are generated in the presence of sodium chloride at $pH < 9$. In the pH range of 9–11 only the monochloro adduct of TEPA could be found. No degradation products could be detected at $pH > 11$. In the absence of sodium chloride at $pH > 8$ the methoxy derivative of TEPA was formed, as a consequence of the applied procedure. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: TEPA; Chemical instability; Degradation kinetics; Sodium chloride-catalyzed degradation; GC

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1. Introduction

N,*N*′,*N*″-triethylenephosphoramide (TEPA) is the main metabolite of the alkylating anticancer agent *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA), first reported in 1960 (Mellet and Woods, 1960). TEPA also possesses alkylating activity (Hagen et al., 1990) and was found active against various solid tumors (Craig and Jackson, 1955). In the liver thioTEPA is desulfurated to TEPA by cytochrome P450 (Fig. 1; Ng and Waxman, 1990; Chang et al., 1995). After thioTEPA infusion TEPA appears rapidly in plasma (Cohen

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et al., 1986; Masters et al., 1996) and has a half life 2–7 times longer than that of the parent drug (Hagen et al., 1990; Hagen, 1991). TEPA excretion in urine accounts for 4% of the administered dose and is a factor 3–4 times higher than thioTEPA excretion (Cohen et al., 1986; Hagen et al., 1990). In a recent study two new metabolites of thioTEPA with alkylating capacity were identified as the mercapturic acid conjugate of thioTEPA and the monochloro derivative of TEPA (monochloroTEPA; Fig. 1; Maanen et al., 1999b). ThioTEPA and its metabolites accounted for 10–20% of the administered dose, indicating that the whole metabolic balance has not been elucidated yet. As the metabolic and chemical reactivity of thioTEPA were largely unknown, we previously studied the chemical reactivity of thioTEPA (Maanen et al., 1999a). This study showed that thioTEPA reacts with nucleophiles such as chloride ions and hydroxyl ions to form chloro and hydroxyl derivatives. MonochloroTEPA, identified in urine of patients treated with thioTEPA, may also be the result of chemical degradation of TEPA. It can be expected that if TEPA in vitro, under physiological conditions, reacts with different nucleophiles, these reactions, apart from enzymatic-mediated conversions, can also take place in patients, treated with thioTEPA. We started to study the degradation reactions of TEPA, which may support us in the search for at present unknown metabolites. The stability of TEPA was studied under the same conditions as earlier thioTEPA (i.e. in a broad pH range and in the absence and presence of sodium chloride) in order to compare their chemical reactivities.

2. Experimental

2.1. Chemicals

TEPA was synthesized by Dr. J.W. Zwikker (Faculty of Chemistry, Utrecht University, Utrecht, The Netherlands) as described previously (Maanen et al., 1998). The identity and purity ($>98\%$) of the reaction product were established by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). Diphenylamine, used as internal standard, was purchased from Baker Analysed® Reagent (Deventer, The Netherlands). All other chemicals used were of analytical grade.

2.2. Buffer solutions

For the kinetic studies the following buffer solutions were used: $3 \leq pH \leq 5$, acetate; $5 < pH \leq 8$, phosphate; $8 < pH \leq 11$, carbonate; $pH > 11$, sodium hydroxide. For the degradation studies of TEPA in the presence of sodium chloride, 1 M sodium chloride was added to the buffer

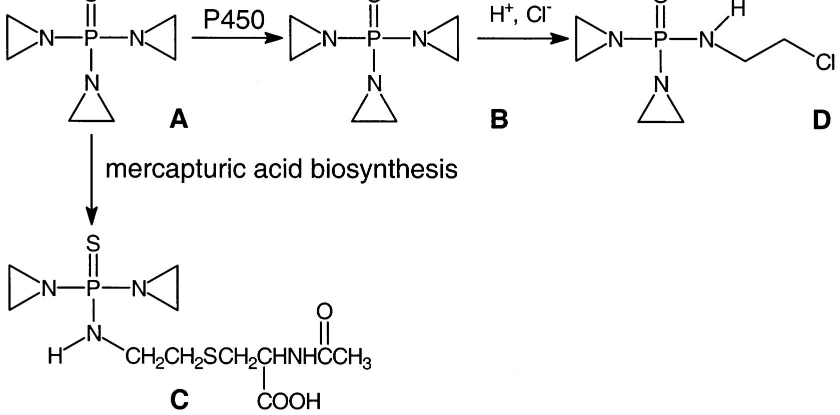

Fig. 1. Biotransformation of thioTEPA (A) to TEPA (B), thioTEPA-mercapturate (C) and the monochloro derivative of TEPA (D).

solution, if necessary the pH was adjusted with NaOH or HCl. The pH was measured with a Slim-trode pH electrode (Hamilton, Darmstadt, Germany) and a pH meter (consort P514, Turnhout, Belgium) at the temperature of study. pH values over 12 were calculated from the equation $pH = pK_w - pOH$.

2.3. Gas chromatography (GC)

A gas chromatograph (HRGC 5300, Carlo Erba Strumentazion, Milan, Italy) equipped with a split/splitless injector and thermionic N/P-selective detector was used. Separation was achieved using a 25 m × 0.32 mm CP Sil-8 CB capillary column (Chrompack, Middelburg, The Netherlands) with a film thickness of 0.25 μm. The oven temperature was programmed as follows: starting temperature 120°C, raised to 160°C with 3°C min^{-1} after which it was raised to 200°C with 20°C min^{-1} and kept for 2 min at 200°C. The injector was kept at 250°C using a split ratio of 1:20 and the detector temperature was 300°C. Helium was used as carrier gas at a flow rate of 3 ml min^{-1}. Gas flow rates were: hydrogen 4 ml/min; air 160 ml/min and make up 13 ml min^{-1}. The current was set at 2.80 A and the voltage at 3.5 V.

GC-MS measurements were performed on a Jeol JMS-AX 505W (Jeol, Tokyo, Japan). Separation was achieved on a Hewlett Packard 5890 Serie II gas chromatograph (Palo Alto, CA, USA) using a 25 m × 0.32 mm CP Sil-8 CB capillary column (Chrompack, Middelburg, The Netherlands) with a film thickness of 0.25 μm. The oven temperature was programmed as follows: starting temperature 80°C, raised to 200°C with 10°C min^{-1}. The temperature was kept at 200°C for 2 min after which it was raised to 300°C with 25°C min^{-1} where it was for 1 min. A cold-on-column injector was used. MS measurements were performed in the electron impact (EI) positive mode. The ionisation current and ion multiplier were set at 100 μA and 1.4 kV, respectively.

2.4. Kinetic measurements

The degradation reactions were initiated by adding 100 μl of a 1 mg ml^{-1} TEPA solution in methanol to 900 μl of the buffer solution to obtain a concentration of 100 μg ml^{-1}.

The reaction mixtures were kept in polypropylene microcentrifuge tubes for the degradation at 22°C. For degradation at higher temperatures, the solutions were kept in ampulla in a thermostatically controlled water bath. All experiments were performed in duplicate.

The log k_{obs}-pH profile was determined between pH 3 and 14, in the absence and presence of 1 M sodium chloride in 25 mM buffer solutions. Degradation experiments were performed at 22°C for $pH \leq 6$ and at 80°C for $pH > 6$. To determine the influence of the sodium chloride concentration on the degradation rate its molarity was varied between 0.1 and 1.2 M at pH 4. The influence of the temperature on the degradation was measured at 25, 30, 40 and 50°C in a 25 mM buffer pH 5, in the absence and presence of 1 M sodium chloride.

2.5. Analytical procedures

2.5.1. GC

At appropriate time intervals 100 μl samples were withdrawn from the reaction solutions and 100 μl of a 0.5 M phosphate buffer pH 8 was added to bring the solutions to a neutral pH. For degradation at 80°C, ampulla where taken from the waterbath. After cooling down, 100 μl of a 0.5 M phosphate buffer pH 8 was added to 100 μl of the solution. To 100 μl of this mixture, 100 μl water, 20 μl of a 500 μg ml^{-1} diphenylamine solution in methanol, 10 μl 1 M NaOH and 500 μl of a 10% v/v mixture of 1-propanol in chloroform were added. The mixture was vortexed for 10 s and centrifuged 3 min at $1100 \times g$. The organic layer was transferred into a 0.7 ml amber glass vial (Chromacol, Trumbull, USA). The organic layer was evaporated to near dryness and reconstituted in 50 μl MeOH; 1 μl was injected into the gas chromatograph.

2.5.2. GC-MS

GC-MS measurements were performed on degradation samples of TEPA to identify the degradation products. To 1 ml of the degradation sample 100 μl 1 M NaOH and 3 ml of a 10% v/v 1-propanol in chloroform mixture were added. The mixture was vortexed for 10 s and centrifuged for 3 min at 1100 × *g*. The organic layer was evaporated to near dryness and the residue was dissolved in 50 μl MeOH; 1 μl was injected into the gas chromatograph.

3. Results and discussion

3.1. Analysis and identification of the degradation products

Thusfar no reports have been presented covering the chemical degradation of TEPA, the main metabolite of thioTEPA, whereas the chemical degradation of thioTEPA in the absence and presence of sodium chloride is described in several studies (Mellet and Woods, 1960; Benckhuijsen, 1968; Maxwell et al., 1974; Zon et al., 1976; Cohen et al., 1984; Pyatigorskaya et al., 1987; Xu et al., 1996; Murray et al., 1997; Maanen et al., 1999a).

GC was used to perform the analysis of the degradation kinetics of TEPA, whereas the low specific absorption of TEPA made it impossible to use HPLC with UV detection.

To develop an appropriate isolation procedure for the degradation products, reaction solutions were applied to solid phase extraction (SPE) and liquid–liquid extraction (LLE). Normal phase, reversed phase and ion exchange SPE was applied and for LLE organic solvents with a wide range of polarities were tested. The highest recovery yield of degradation products was obtained with SPE C18 cartridges, but extractions were not reproducible. Reproducible results were obtained, however, upon extraction with 10% 1-propanol in chloroform. This method gave also a good yield of the formed degradation products and was thus used during the kinetic study.

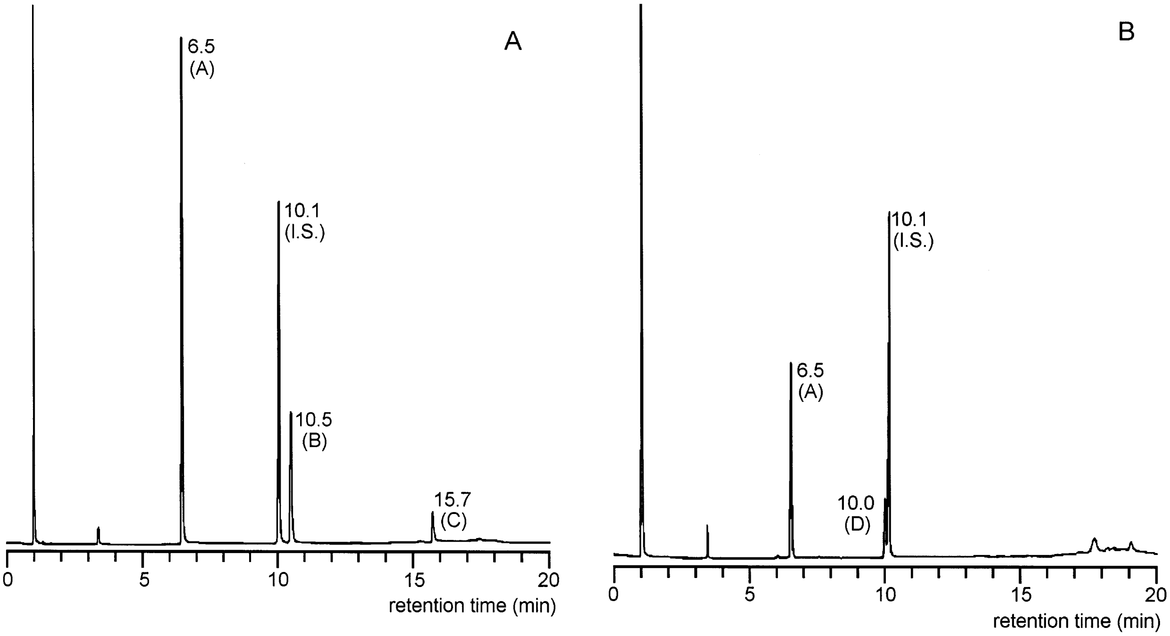

Fig. 2. A: GC chromatogram of a degradation sample of TEPA. TEPA was degraded for 20 min at pH 4.0 in the presence of 1 M sodium chloride at ambient temperature. The peak eluting at 6.5 min and 10.1 min originate from TEPA and diphenylamine (I.S. = internal standard); the other peaks (B and C) eluting at 10.5 and 15.7 min are decomposition products. B: GC chromatogram of a degradation sample of TEPA. TEPA was degraded for 16 h at pH 11.0 at 80°C. The peaks eluting at 6.5 and 10.1 min originate from TEPA and diphenylamine, respectively; the peak (D) eluting at 10 min originates from a decomposition product.

In the pH range of 3–8 no degradation products were detected in the absence of sodium chloride. Two products with retention times of 10.5 min (product B) and 15.7 min (product C) were detected in the presence of sodium chloride (Fig. 2A). The retention time of TEPA was 6.5 min (product A). At pH > 8 in the absence of sodium chloride only one product with a retention time of 10.0 min (product D) was detected (Fig. 2B). In the presence of sodium chloride only product B was detected. In Fig. 3 the decrease in TEPA concentration and formation of the degradation products as function of time is plotted. Mass spectra of the degradation products are depicted in Fig. 4. The mass spectrum of TEPA shows a molecular ion at *m*/*z* 173 (Fig. 4A) and the loss of a single aziridine function (*m*/*z* 131). The mass spectrum of product B showed a molecular ion at *m*/*z* 209 with an isotope at 211 (Fig. 4B). The ratio 3:1 for *m*/*z* 209 and 211 indicates the presence of a chlorine atom. The mass spectrum shows loss of chlorine (*m*/*z* 174; same mass as protonated TEPA), aziridine (*m*/*z* 167), chloromethyl (*m*/*z* 160), and chloroethylamine (*m*/*z* 131); *m*/*z* 42 originates from an aziridine group. Product C revealed a molecular ion at *m*/*z* 245 with isotopes at 247 and 249 in the ratio of 9:6:1, which indicates the presence of two chlorine atoms (Fig. 4C). The mass spectrum showed the loss of chlorine (*m*/*z* 210), aziridine (*m*/*z* 203), chloromethyl (*m*/*z* 196) and chloroethylamine (*m*/*z* 167). These results indicate the formation of the mono- and dichloro adduct of TEPA during the degradation in the presence of sodium chloride. This is in agreement with the chemical properties of TEPA. TEPA is classified as an activated aziridine and will undergo ring opening in the presence of a nucleophilic reagent. In acidic environment in the presence of a halogenide, the halogenide is coupled to the aziridine with ring-opening to form a 2-haloethylamine (Bestian, 1950; Dermer and Ham, 1969).

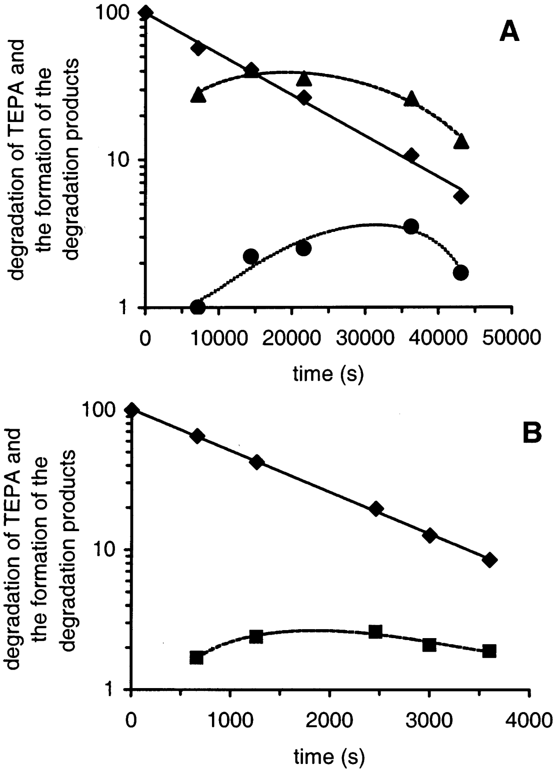

Fig. 3. A: Degradation of TEPA and the formation of the degradation products of TEPA as function in time at pH 6 in the presence of sodium chloride (◆ TEPA, t_r 6.5 min; ▲ product B, t_r 10.5 min; ● product C, t_r 15.7 min). The amount of degradation products was calculated as TEPA equivalents, relative to the initial amount of TEPA. B: Degradation of TEPA and the formation of the degradation product of TEPA as function in time at pH 13 in the absence of sodium chloride (◆ TEPA, t_r 6.5 min; ■ product D, t_r 10.0 min). The amount of degradation product was calculated as TEPA equivalents, relative to the initial amount of TEPA.

The mass spectrum of product D gave a molecular ion at *m*/*z* 205 (Fig. 4D) and showed the loss of methyl (*m*/*z* 190), methylmethoxy (*m*/*z* 160), methoxyethylamine (*m*/*z* 131), and is consistent with the methoxy derivative of TEPA. Product D is not a degradation product, but is the result of the applied method. To initiate the degradation, an aliquot of a TEPA solution in methanol is added to the buffer solution. Methanol, as a nucleophile gives a substitution reaction with TEPA (Fig. 5; Cohen et al., 1984). If a solution of TEPA in ethanol (10% v/v) is used, a product with a retention time of 11.3 min was formed. The mass spectrum of this product showed a molecular mass of *m*/*z* 219 and loss of ethyl (*m*/*z* 190), methylethoxy (*m*/*z* 160), ethoxyethylamine (*m*/*z* 131), and is consistent with the ethoxy derivative of TEPA (data not shown).

As can be seen in Fig. 3 the formed products during the degradation of TEPA in the presence of sodium chloride are not end-products. Chloro adducts were also formed during the chemical degradation of thioTEPA in the presence of sodium chloride (Maanen et al., 1999a). One of the end products of thioTEPA formed in the presence of sodium chloride is *N*,*N*′,*N*″-trichloroethylthiophosphoramide. Considering the structural resemblance with thioTEPA, the trichloro derivative of TEPA will also be formed, but could not be detected. The other products formed during the degradation of thioTEPA in presence of sodium chloride were *N*-ethylene,*N*′-2-chloroethyl,*N*″-2-hydroxyethylthiophosphoramide and *N*,*N*′-2-chloroethyl,*N*″-2-hydroxyethylthiophosphoramide. Similar products were not detected for TEPA. Ring opening of an activated aziridine in the presence of a nucleophilic reagent can also occur in basic and neutral media (Dermer and Ham, 1969). A side reaction that can occur during ring opening reactions is polymerization as depicted in Fig. 6. The ring opened product contains a weakly basic nitrogen atom, which can undergo a nucleophilic substitution reaction with TEPA, catalyzed by nucleophiles (Dermer and Ham, 1969). No such products were detected during the degradation of TEPA, but the assay was not designed to pick up these type of compounds. In Fig. 7 the degradation scheme of TEPA in acidic environment in the presence or absence of sodium chloride is presented, as verified in our study. The formed products are not the end-products of the degradation reaction, the remaining aziridine moieties are probably subjected to further degradation.

3.2. Kinetic studies

The degradation rate of TEPA at various pH values with or without sodium chloride can be described by pseudo first order kinetics. This can be concluded by the linear character of the plots of the natural logarithm of the residual TEPA concentrations against time (Fig. 3). The observed rate constants (k_{obs}) for the overall degradation have been extracted from the slopes of these plots.

Degradation experiments at $\mathrm{pH} < 6$ were performed at 22°C. At higher pH degradations were performed at 80°C. To construct the overall $\log k_{\mathrm{obs}}$-pH profile, reaction rate constants determined at 22°C were recalculated at 80°C, using the Arrhenius equation:

$$\ln k_{\mathrm{obs(T)}} = \ln A - E_a/RT$$

in which A is the frequency factor (s^{-1}), E_a is the energy of activation (J mol^{-1}), R is the gas constant (8.31 J K^{-1} mol^{-1}) and T is the absolute temperature (K). A and E_a (Table 1) are calculated from Arrhenius plots in which the $\mathrm{k_{obs}}$ of the degradation of TEPA at pH 5 with or without sodium chloride were plotted against the inverse of the absolute temperature. The $\log k_{\mathrm{obs}}$-pH profiles of the TEPA degradation at 80°C in presence and absence of sodium chloride are depicted in Fig. 8. The linear character of the plot at $\mathrm{pH} < 6$ indicates no change in reaction mechanism within this pH range.

The pH profiles can be divided in three parts: a proton catalyzed part ($2 < \mathrm{pH} < 7$), a solvent catalyzed part ($6 < \mathrm{pH} < 12$) and a hydroxyl catalyzed part ($\mathrm{pH} > 11$) according to the following formula:

$$k_{\mathrm{obs}} = k_0 + k_{\mathrm{H}^+}[\mathrm{H}^+] + k_{\mathrm{OH}^-}[\mathrm{OH}^-] + k_{\mathrm{NaCl}}[\mathrm{NaCl}] + k_{\mathrm{buffer}}[\mathrm{buffer}]$$

in which k_0 is the rate constant of the solvent catalyzed reaction; k_{H^+} is the rate constant of the proton catalyzed reaction; k_{OH^-} is the rate constant of the hydroxyl catalyzed reaction and k_{NaCl} is the rate constant of the sodium chloride catalyzed reaction. For $\mathrm{pH} < 7$ and $\mathrm{pH} > 11$ the slopes were calculated from the $\log k_{\mathrm{obs}}$-pH profile. The slope at $\mathrm{pH} < 7$ was −1.01 in the absence of sodium chloride, indicating a specific proton catalyzed reaction. In the presence of sodium chloride the slope at $\mathrm{pH} < 7$ was −0.80, due to the influence of sodium chloride. The slopes at $\mathrm{pH} > 11$ were + 1.16 and + 1.13 in the absence and presence of sodium chloride, respectively, indicating no influence of sodium chloride on the degradation reaction. In the presence of sodium chloride the k_{obs}-values of TEPA are in

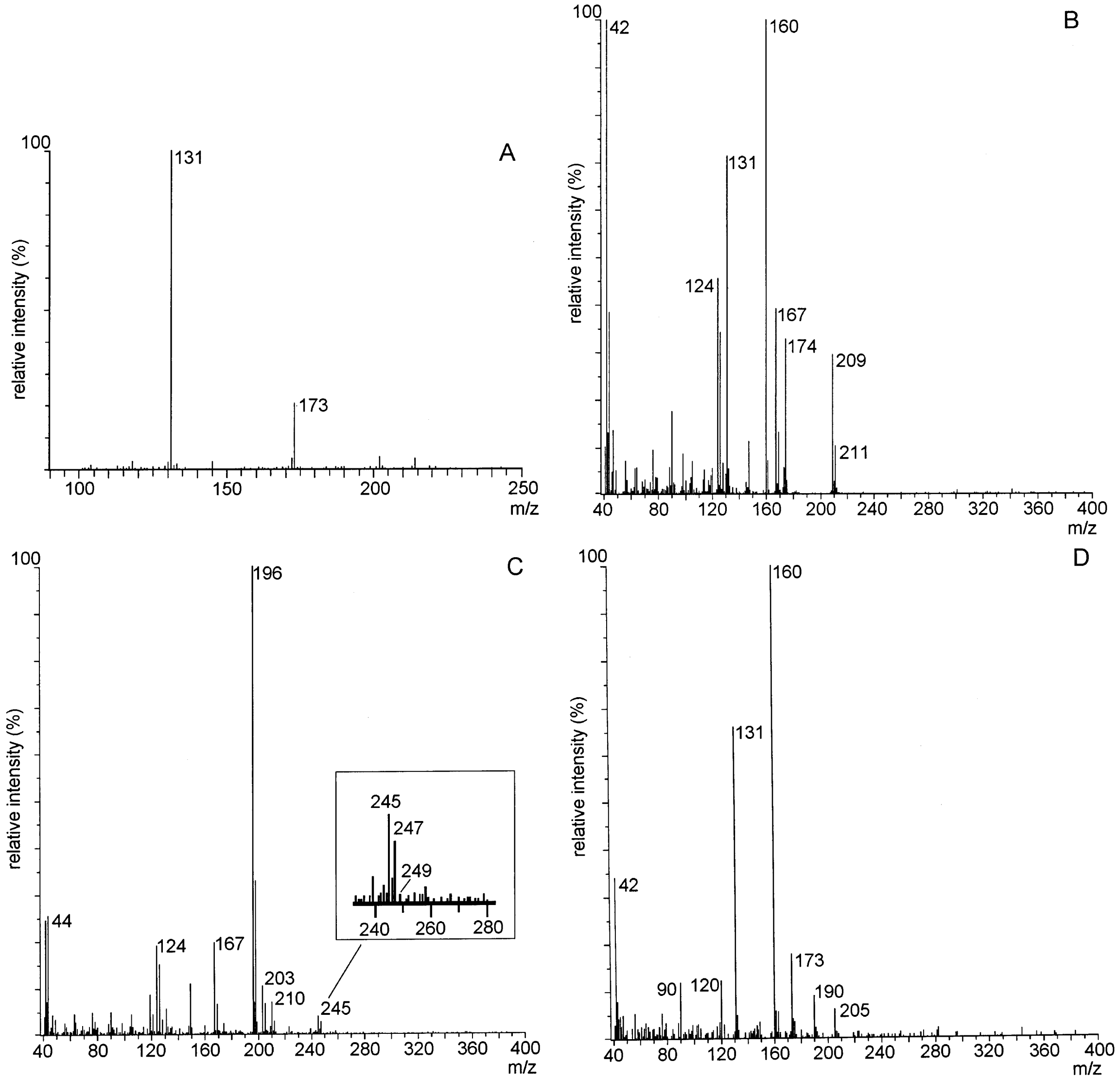

Fig. 4. Mass spectra of TEPA (A) and its degradation products with t_r 10.5 min (B), t_r 15.7 min (C) and t_r 10.0 min (D).

the same order of magnitude as the k_{obs} of thioTEPA (Maanen et al., 1999a). At pH < 7 in the absence of sodium chloride TEPA is more reactive than thioTEPA. The influence of sodium chloride was, similar to the degradation of thioTEPA, only observed at pH < 7. In Fig. 9 the

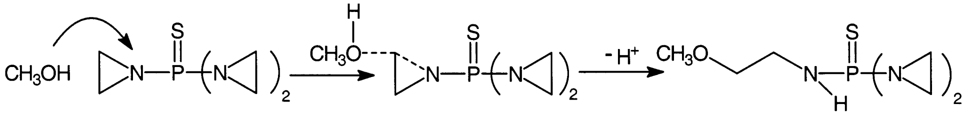

Fig. 5. Reaction of TEPA with methanol (Dermer and Ham, 1969).

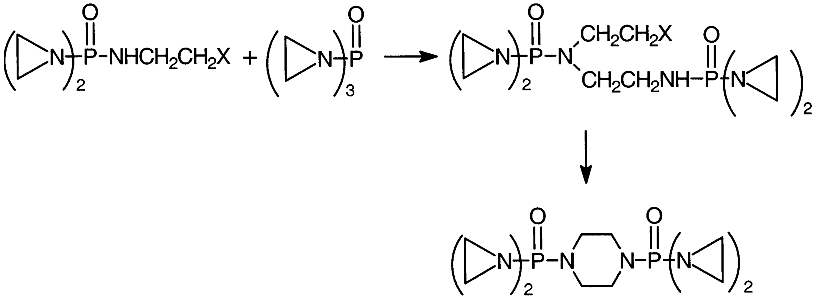

Fig. 6. Polymerization of TEPA, catalyzed by nucleophiles (Dermer and Ham, 1969).

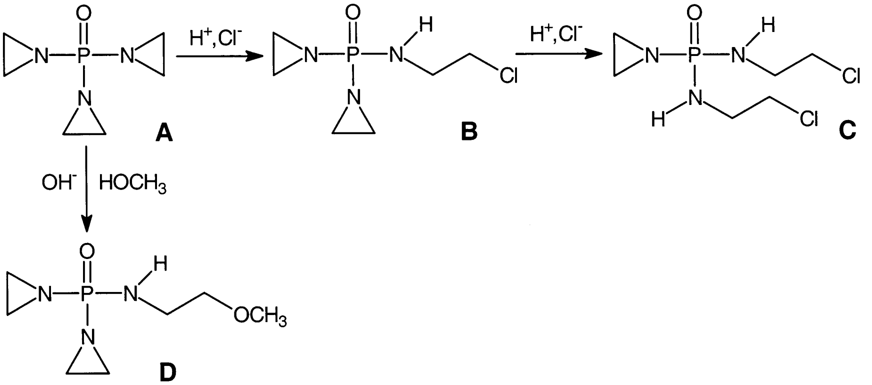

Fig. 7. Degradation scheme of TEPA in the presence of sodium chloride, with TEPA (A), mono- (B) and dichloro derivatives of TEPA (C). The methoxy derivative of TEPA (D) is formed as consequence of the applied sample pretreatment.

influence of the sodium chloride concentration on the k_{obs} at pH 4 is depicted. The reaction rate constant of the sodium chloride catalyzed reaction (k_{NaCl}) was 8.61×10^{-4} M^{-1} s^{-1} in the range of 0.1–0.7 M. At higher sodium chloride concentrations no linear relationship was observed.

4. Conclusion

During the degradation of TEPA several products are formed. In the pH range of 3–8 in the presence of sodium chloride the mono- and dichloro derivatives of TEPA are formed. The formation of chloro adducts was also seen during the degradation of thioTEPA (Maanen et al., 1999a). In the absence of sodium chloride no degradation products were detected. At pH > 8 in the presence of sodium chloride only the monochloro derivative of TEPA was detected. In the absence of sodium chloride a methoxy derivative of TEPA is formed, due to the presence of methanol in the reaction medium. TEPA is most stable in the pH range 8–12 and 9–12 in the absence or absence of sodium chloride, respectively. The degradation of TEPA follows first order kinetics. In the presence of sodium chloride the k_{obs} of TEPA are in the same order of magnitude as the k_{obs} of thioTEPA. The influence of sodium chloride was, similar to the degradation of thioTEPA, observed at pH < 7. In the absence of

Table 1
Parameters calculated from the Arrhenius plot

pH 4	r^2	E_a (J mol^{-1})	A (s^{-1})
With NaCl	0.9994	4.47×10^4	2.45×10^4
Without NaCl	0.9970	3.74×10^4	2.82×10^2

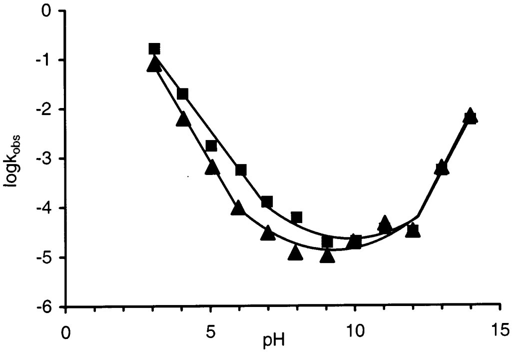

Fig. 8. Log k_{obs}-pH profiles of the degradation of TEPA in the presence and absence of sodium chloride in 25 mM buffers at 80°C (-■- with sodium chloride; -▲- without sodium chloride).

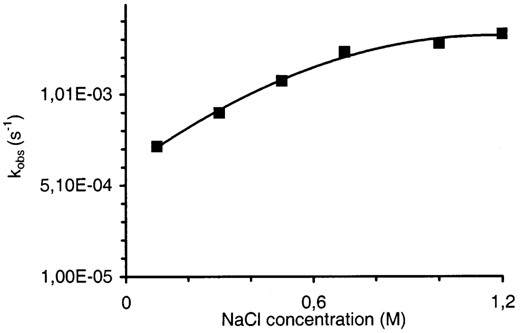

Fig. 9. Influence of the sodium chloride concentration on the observed reaction rate constant in 25 mM sodium acetate buffer pH 4 at 22°C.

sodium chloride at pH < 7, TEPA is more reactive than thioTEPA.

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