N-Nitrosation of Triazines in Human Gastric Juice

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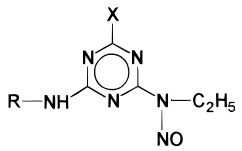
Owing to their chemical structure triazine herbicides can undergo *N*-nitrosation. In this work the formation of *N*-nitroso derivatives of atrazine, terbuthylazine, terbutryn, and terbumeton was evaluated. The kinetics of formation is discussed in relation to the structural features of the compounds and the ratio triazine/NO_2^- in amounts similar to those present in human gastric juice.

Keywords: *Gastric juice; N-nitrosoatrazine; N-nitrosoterbuthylazine; N-nitrosoterbumeton; N-nitrosoterbutryn*

INTRODUCTION

For a correct evaluation of the toxicological risk of pesticide residues in the environment and food, it is not sufficient to know their concentrations, but it is also important to obtain information about their metabolites. Since several *N*-nitroso compounds have been shown to be carcinogenic in experimental animals (Druckrey *et al.*, 1967; IARC, 1978), the possible formation of pesticide *N*-nitroso derivatives in the environment is a subject of current interest. Table 1 reports a list of the pesticides, including triazines, whose amino or amido groups could be transformed in *N*-nitroso derivatives with mutagenic and carcinogenic activity.

Much interest was concentrated in exploring the persistence, properties, and degradation of triazine herbicides, the most widely used pesticides all over the world, because some years ago a compound, supposed to be an *N*-nitroso derivative of atrazine, was identified in New Orleans drinkable water (Fine and Rounbehler, 1976; Keith, 1976). This compound was tested in a modified Ames *Salmonella typhimurium* assay with hamster liver S9 activation and in V79 assay: it produces more mutants than *N*-nitrosodimethylamine (Weisenburger *et al.*, 1988). The potential formation of *N*-nitrosotriazines in the environment, their possible relevance to human carcinogenesis, and the experimental evidence that *N*-nitroso compounds may originate from the interaction of nitrosable molecules and nitrite ingested with diet and drinking water under the acidic conditions of the gastric environment prompted us to evaluate the extent to which some triazines are converted to *N*-nitroso derivatives under conditions closely simulating those within the normal fasting human stomach. They include, beside atrazine, three triazines containing a *tert*-butyl group instead of an isopropyl group. Terbuthylazine bears on the heterocyclic ring a chlorine atom like atrazine, terbutryn a methylthio group, and terbumeton a methoxy group (Figure 1). The last two compounds are used in vineyards, while in Italy terbuthylazine has been substituted in the weed control of corn fields for atrazine, which is now forbidden.



compound	X	R
N-nitrosoatrazine	Cl	*iso*propyl
N-nitrosoterbuthylazine	Cl	*tert*butyl
N-nitrosoterbutryn	SCH_3	*tert*butyl
N-nitrosoterbumeton	OCH_3	*tert*butyl

Figure 1. Structures of the *N*-nitrosotriazines.

MATERIALS AND METHODS

The four triazines, atrazine (6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine), terbuthylazine (6-chloro-*N*-(1,1-dimethylethyl)-*N*′-ethyl-1,3,5-triazine-2,4-diamine), terbutryn (*N*-(1,1-dimethylethyl)-*N*′-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine), and terbumeton (*N*-(1,1-dimethylethyl)-*N*′-ethyl-6-methoxy-1,3,5-triazine-2,4-diamine) were purchased from Labservice. Reagents were used without purification; acetonitrile was distilled on phosphorus pentoxide.

General Procedure for the Synthesis of *N*-Nitroso Derivatives: *N*-Nitrosoterbumeton. In a flask equipped with a $CaCl_2$ valve and a rubber septum, $NOBF_4$ (0.85 g, 7.28 mmol) was dissolved in acetonitrile (4 mL) and cooled at 0 °C. Terbumeton (0.50 g, 4.80 mmol) was added and then pyridine (0.38 mL, 4.8 mmol) in acetonitrile (0.1 mL) through a syringe during 5 min. The mixture became yellow. The reaction was monitored by TLC on silica gel 60 using 7:3 hexane:ethyl acetate as eluent. After 1 h the solvent was evaporated, and the residue was diluted in ethyl acetate, washed with cold brine, dried, and evaporated. *N*-Nitrosoterbumeton was purified by flash chromatography on silica gel; 45% yield, mp 89−91 °C. NMR: δ 1.08 (3 H, t, $J = 7$, CH_3), 1.5 (9 H, s, t-Bu), 4.1 (2 H, q, $J = 7$, NCH_2), 4.05 (3 H, s, OCH_3), 5.8 (1 H, broad, NH). MS *m*/*z* (%): 254 (49), 239 (26), 224 (100), 210 (47), 209 (51), 194 (76), 169 (84), 168 (64), 154 (49).

The other compounds were prepared in the same way.

N-Nitrosoatrazine was obtained in 97% yield, mp 88−90 °C (Mirvish *et al.*, 1991). NMR: δ 1.05 (3 H, t, $J = 7$ Hz, CH_3), 1.2 (6 H, d, $J = 7$ Hz, CH_3), 4.1 (2 H, q, CH_2), 4.3 (1 H, m, CH), 5.8 (1 H, broad, NH). MS *m*/*z* (%): 246 (15), 244 (55), 229 (5), 214 (100), 199 (15), 184 (30), 172 (36).

N-Nitrosoterbuthylazine was obtained in 70% yield, mp 115 °C (dec.). NMR: δ 1.05 (3 H, t, $J = 7$, CH_3), 1.43 (9 H, s), 4.0

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Table 1. Pesticides That Are Known To Undergo *N*-Nitrosation

compd	ref	compd	ref
benzthiazuron	Eisenbrand et al. (1975)	dodine	Lasciafari (1981)
carbaryl	Eisenbrand et al. (1975)	cymoxanil	Cova et al. (1986)
propoxur	Eisenbrand et al. (1975)	atrazine	Weisenburger et al. (1987)
simazine	Eisenbrand et al. (1975)	cyanazine	Zwickenpflug and Richter (1994)
thiram	Andrews et al. (1980)		

Table 2. Comparison of the Chemical Shifts (ppm) of Some Relevant Peaks of the NMR Spectra of the Parent Triazines and the *N*-Nitroso Compounds

	atrazine		terbuthylazine		terbumeton		terbutryn	
assignment	parent	*N*-nitroso	parent	*N*-nitroso	parent	*N*-nitroso	parent	*N*-nitroso
CH_3CH_2N	1.13	1.05	1.13	1.05	1.13	1.08	1.13	1.05
CH_3CH_2N	3.45	4.10	3.38	4.03	3.40	4.10	3.40	4.05
$(CH_3)_3CN$			1.38	1.43	1.40	1.50	1.38	1.48
$(CH_3)_2CHN$	1.25	1.20						
$(CH_3)_2CHN$	4.18	4.30						

(2 H, q, $J = 7$, CH_2), 5.8 (1 H, broad, NH). MS m/z (%): 260 (10), 258 (28), 243 (27), 228 (100), 213 (73), 198 (61), 172 (45).

N-Nitrosoterbutryn was obtained in 43% yield, mp 91−92 °C. NMR: δ 1.05 (3 H, t, $J = 7$, CH_3), 1.48 (9 H, s, t-Bu), 2.55 (3 H, s, SCH_3), 4.05 (2 H, q, $J = 7$, CH_3), 5.5 (1 H, broad, NH). MS *m*/*z* (%): 270 (25), 255 (3), 240 (100), 225 (27), 210 (24), 184 (22).

Nitrosation in Human Gastric Juice. Gastric juice samples were extracted under standardized conditions from untreated fasting individuals. Their pH values were determined by the mean of a digital pH meter (1.5−2.0). Incubations were carried out at 37 °C over 1.5, 3, 4.5, 6, and 12 h with continuous shaking. Gastric juice was added with $NaNO_2$ (0.5, 1.5, 3 mM) and various concentrations of triazines, ranging from 0.05 to 1 mM. Residual nitrite was destroyed at the end of each incubation by adding excess sulfamic acid. Blank determinations of *N*-nitroso compounds were made by omitting the triazines and/or the NO_2^-, to account for the small contribution of gastric juice itself. At the end of the incubation, aliquots of the samples were analyzed. Each experiment was replicated three times.

Analyses were conducted in HPLC by a reverse phase method on the column Lichrospher 100 RP-18 (5 μm, 4 × 125 mm) on an instrument equipped with a Jasco PU-980 pump and a spectrophotometric detector Perkin-Elmer LC75. The analytical conditions were the following: $\lambda = 254$ nm; mobile phase, 70:30 acetonitrile/water; flow, 1 mL/min. All of the solvents were analytical grade. Recovery of all of the triazines in gastric juice were in the range of 86 and 90%.

RESULTS

Purified samples of *N*-nitrosotriazines were prepared for an accurate quantification. As these derivatives are in general very unstable and sensitive to acids, very mild conditions were preferred for their synthesis. Nitrosonium tetrafluoborate ($NOBF_4$) has been used only seldom for the preparation of *N*-nitrosoamines (Nagasawa *et al.*, 1973), but it can be very useful because it works in mild conditions in anhydrous acetonitrile. The recovery of the *N*-nitroso derivative is easy: concentration of the solvent gives the crude *N*-nitroso derivatives which can be purified by flash chromatography on silica gel without detectable decomposition. The purity of the compounds, assessed by HPLC, was >95%.

All of the compounds studied have two different secondary amino groups which can be nitrosated, but in our conditions (equimolar amount of nitrosating agent) only one isomer was formed. The structure of the compounds was assigned by comparison of ^{1}H-NMR spectra with those of the mother compounds (Table 2). In atrazine only the quartet of the methylene of the group CH_2CH_3 at 3.45 ppm is shifted to 4.1 ppm in the *N*-nitroso derivative, while the multiplet of the CH is unchanged. This indicates that the ethyl bearing nitrogen was nitrosated, because nitrosation strongly deshields α-protons (Karabatsos and Taller, 1964). Mirvish *et al.* (1991) observed the same selectivity using $NaNO_2$ in acidic water. Traces of a second more lipophilic yellow compound were observed (possibly the *N*-isopropyl-*N*-nitroso derivative Zwickenpflug and Richter, 1994), but during the workup of the reaction the compound disappeared.

Table 3. Mass Fragmentation of *N*-Nitrosotriazines

	% abundancy			
structure	terbuthylazine (a)	terbumenton	terbutryn	atrazine[a]
M	28	49	25	55
M − CH_3	27	26	3	5
M − NO	100	100	100	100
M − NO − CH_3	73	51	27	15
M − NO − C_2H_5 − H	61	76	24	30
M − NO − CH_2=$C(CH_3)_2$	45	64	22	36[b]

[a] Only % abundancy of ions containing ^{35}Cl are reported. [b] M − NO − CH_2=$CHCH_3$.

In the nitrosation of the triazines containing a *tert*-butyl group an analogous downshift of the methylene was observed which indicates that the nitrosation occurs in the same way. The explanation of the complete regiospecificity of the reaction can be found in the higher positive inductive effect which increases the basicity of the nitrogen, precedent literature suggests that less basic amines are better nitrosated than more basic ones (Mirvish, 1975). It is possible that in this case the steric hindrance of the *tert*-butyl substituent may have a contribution, too.

The mass spectra of the *N*-nitroso derivatives are characterized by a great similarity (Table 3). The base peak comes from the loss of NO, which, in less intense peaks, is accompanied by loss of CH_3, CH_2CH_3 + H, or CH_2=$C(CH_3)_2$.

Table 4 collects the data of the four triazine *N*-nitrosation in human gastric juice at pH 1.5-2.0. Among them, terbuthylazine rapidly undergoes nitrosation: after 1.5 h the conversion maximum is reached, while the peak for the conversion of the other triazines is reached after 3 h of incubation (Figure 2a).

Total yields of *N*-nitroso compounds increase with the highest nitrite concentrations, while with use of 0.5 mM $NaNO_2$ only a very low detectable amount is formed (about 1−5% conversion). With 3 mM $NaNO_2$, atrazine and terbuthylazine nitrosation reaches a maximum

Table 4. Percentage Formation of *N*-Nitroso Derivatives from Triazines (Concentration = 0.05 mM) in Human Gastric Juice[a]

compd	$NaNO_2$ concn (mM)	treatment time 1.5 h	3 h	4.5 h	6 h	12 h
atrazine	0.5	0	2 ± 0.11	1 ± 0.05	0	0
	1.5	14 ± 1.1	18 ± 0.95	12 ± 1.1	4 ± 0.3	3 ± 0.06
	3.0	18 ± 1.1	23 ± 1.7	16 ± 0.8	14 ± 1.3	5 ± 0.7
terbuthylazine	0.5	3 ± 0.15	1 ± 0.05	0	0	0
	1.5	20 ± 1.3	16 ± 1.05	15 ± 1.3	8 ± 0.89	4 ± 0.31
	3.0	35 ± 1.9	26 ± 1.7	20 ± 1.1	14 ± 1.1	6 ± 0.9
terbutryn	0.5	1 ± 0.06	6 ± 0.8	4 ± 0.8	2 ± 0.07	2 ± 0.05
	1.5	39 ± 2.1	42 ± 2.3	30 ± 2.1	15 ± 1.05	9 ± 1.1
	3.0	41 ± 2.9	46 ± 3.1	37 ± 2.9	19 ± 1.1	11 ± 0.9
terbumeton	0.5	0	4 ± 0.31	2 ± 0.13	0	0
	1.5	32 ± 1.9	38 ± 2.7	27 ± 2.1	7 ± 0.8	6 ± 0.9
	3.0	36 ± 2.4	45 ± 2.9	33 ± 1.9	30 ± 2.1	11 ± 1.3

[a] Values are the average of three determinations.

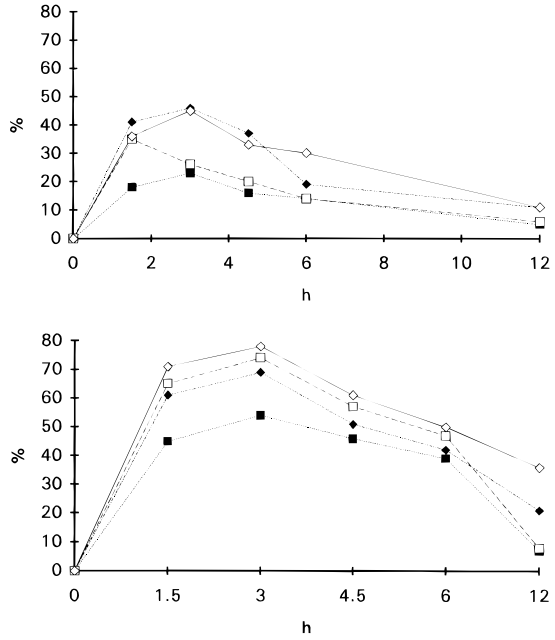

Figure 2. Kinetics of the formation of the *N*-nitrosotriazines: (■) *N*-nitrosoatrazine, (□) *N*-nitrosoterbuthylazine; (♦) *N*-nitrosoterbutryn; (◇) *N*-nitrosoterbumeton. (a, top) Experiments conducted with 0.05 mM triazines and 3 mM $NaNO_2$. (b, bottom) Experiments conducted with 1 mM triazines and 3 mM $NaNO_2$.

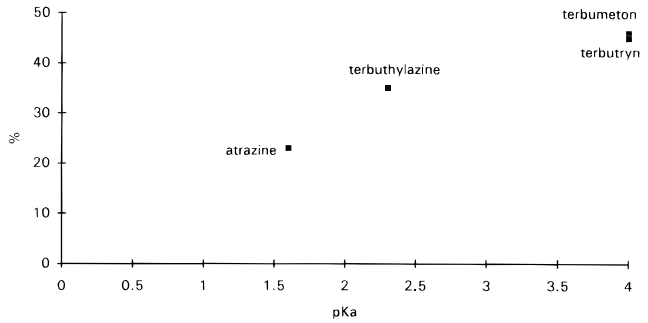

Figure 3. Dependence of the maximum percentage conversion of each triazine into *N*-nitroso derivative with respect to pK_a.

conversion of 23 and 35%, respectively, while for terbutryn and terbumeton the percentage nitrosation is higher with a value of about 45%. In the experiments conducted in the presence of 1 mM substrate and 3 mM nitrosating agent, from the first hours of incubation until the sixth, the percentage conversions to *N*-nitroso derivatives are constantly higher, as reported in Figure 2b.

The *N*-nitroso derivatives are unstable and after 6−12 h of incubation revert to the parent triazines, at this time the detectable amounts of *N*-nitroso compounds are very low.

DISCUSSION

The actual *N*-nitrosotriazine concentration in the medium is the result of two competitive reactions: their formation and decomposition, which produces almost quantitatively the parent triazine. The data reported in Table 4 and Figure 2 demonstrate that *N*-nitrosotriazines have a maximum formation peak around the third hour with a percentage conversion that in the case of the triazines bearing a *tert*-butyl group is higher than in the case of atrazine. This behavior is particularly clear in the case of terbutryn and terbumeton which reach a double concentration with respect to atrazine either at 3 h or at 4.5 h, while the increase is less pronounced in the case of terbuthylazine. Both atrazine and terbuthylazine bear a chlorine atom on the heterocyclic ring and differ only in the substituent on the amino group not directly involved in nitrosation. In terbutryn and terbumeton, on the contrary, the chlorine atom is substituted by SCH_3 and OCH_3, respectively. The difference of the formation kinetics of *N*-nitroso derivatives, therefore, appears to be connected to the electronic properties of the substituent on the heterocyclic ring. The σ_{meta} of the three substituents are 0.37 for Cl, 0.12 for SCH_3, and 0.15 for OCH_3, respectively. Thus, the presence of a less electron-withdrawing group on the heterocyclic ring could have a positive effect on the stability of the intermediate cation deriving from the addition of NO^+ onto the amino group or disfavor the decomposition of the *N*-nitroso derivative.

Another feature of the compounds connected to σ_{meta} is basicity. The pK_a of the hydrolysis of the ammonium salt are in the following order: atrazine = 1.76, terbuthylazine = 2.08, terbutryn = 4.36, and terbumeton = 4.47. Thus, the strongest bases give the highest maximum conversion (Figure 3).

It must be noted that, in *N*-nitrosation of amines in general, the reaction takes place preferentially on the less basic amino group (Mirvish, 1975). This is clearly in contrast with our results, but the cited experiments have been performed in simple aqueous solutions in the presence of a strong acid (i.e., HCl, AcOH) and high concentrations of both substrate and sodium nitrite. On

the contrary, our experiments were conducted in a complex medium as gastric juice, whose acidity is buffered by the presence of mucopolysaccharides, etc. Moreover our experiments were performed at a very low concentration, 0.05 mM in substrate, which can promote the decomposition of the *N*-nitroso derivatives. In fact after 4.5 h (Figure 2a) the percentage of the *N*-nitroso derivatives in the reaction medium diminishes slowly. At 1 mM substrate concentration and 3 mM $NaNO_2$, the conversion is higher, perhaps owing to mass-law effect.

There is clearly a need of more work to extend the present results to the risk assessment projections for triazines. The actual *in vivo* hazard seems to depend on the concentration of nitrite and substrate in the gastric environment. Nitrite concentration is rather low in the gastric juice of fasting healthy individuals, but it is known that after a meal containing 38 mg of NO_3^- kg^{-1}, gastric nitrite rises to 14 mg L^{-1} (about 0.3 mM), a concentration of the same magnitude as the ones used in this work (Walters *et al.*, 1979). Water naturally contains these ions, and if their amount is higher than 100 mg L^{-1}, 70% of the daily nitrate intake comes from this source (Fraser and Chilvers, 1981). Thus, drinking water may significantly raise the risk of formation of *N*-nitrosoamines and other *N*-nitroso compounds, when high nitrate concentrations are present. Nitrates are nitrosating agents themselves, but they usually are reduced by microrganisms in the soil or in the gastrointestinal tract to nitrite ions, the most specific nitrosating species.

Our results, even if performed *in vitro*, may provide evidence concerning the importance of nitrosation reactions and suggest the usefulness of the assessment of the *in vivo* formation of these *N*-nitroso intermediates in the evaluation of human carcinogenesis.

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