Biochemical Pharmacology, Vol. 17, pp. 55–64. Pergamon Press Ltd. 1968. Printed in Great Britain

ACID-CATALYSED CONVERSION OF TRIETHYLENEIMINE THIOPHOSPHORAMIDE (THIO-TEPA) TO AN SH COMPOUND

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(*Received* 7 *June* 1967; *accepted* 30 *August* 1967)

Abstract—The behaviour of triethyleneimine thiophosphoramide (thio-TEPA) in acid medium has been studied. Evidence is presented of an intramolecular alkylation of the sulfur on protonation of the molecule. The five-membered ring formed in this way is hydrolyzed slowly in neutral solution at room temperature, liberating an SH group. In the SH derivative two ethyleneimine rings are retained, which were demonstrated to be more reactive towards water and sulfhydryl than were those of thio-TEPA.

OUR OBSERVATION that an SH group is formed by triethyleneimine thiophosphoramide (thio-TEPA) when it is dissolved at low pH, stimulated us to study the behaviour of the molecule in an acid medium. It has been stated previously[1] that thio-TEPA is highly unstable at pH 4·2 and 37°, 95 per cent of the alkylating groups apparently being destroyed within 30 min. However, in our hands, a decrease in alkylating activity of 1·2 per cent only was found in 30 min under these conditions. In view of the extremely rapid inactivation of the ethyleneimine rings of triethylene melamine in acid solution[2] it was interesting to find a remarkable stability against hydrolysis at low pH for two-thirds of the alkylating groups of thio-TEPA.

MATERIALS AND METHODS

Triethyleneimine thiophosphoramide (thio-TEPA) was either obtained commercially (Lederle Laboratories, American Cyanamid Company) in ampoules to which sodium bicarbonate and sodium chloride had been added or it was synthesized from phosphorus sulfochloride according to Craig *et al.*[3] The product was recrystallized from benzene-petroleum ether (b.p. 40–60°); m.p. 51·5–52°. Triethyleneimine phosphoramide (TEPA) was synthesized according to Bestian;[4] it was purified by distillation *in vacuo*. A weighed sample was found to be 98 per cent pure when titrated with sodium thiosulfate.[5] Thiolated Sephadex G 25 was prepared according to Eldjarn and Jellum;[6] it contained 0·175 μmole SH/mg (0·58%).

Alkylating groups were determined colorimetrically with 4-(4′-nitrobenzyl)-pyridine by the method of Epstein *et al.*[7] as modified by Truhaut *et al.*[8] using a saturated solution of sodium chloride to concentrate the coloured product in the organic phase. A small amount of sodium chloride solution added to the stoppered cuvette served to resolve a turbidity of the strongly alkaline organic solution by removing the carbonate produced from carbon dioxide. Thiol groups were determined colorimetrically by the method of Ellman.[9]

Chromatography: Thin layers (0·25 mm) were prepared from cellulose powder MN 300 (Macherey, Nagel and Co., Düren, Germany). Chromatograms to be developed with *n*-propanol/water (70/30, v/v) were washed with the solvent system prior to use. The alkylating products were detected by spraying the chromatogram with a 5% solution of 4-(4′-nitrobenzyl)pyridine in acetophenone, heating for 15 min at 110°, followed by alkalinization in ammonia vapour.

RESULTS

Conversion of thio-TEPA to an SH compound

From the data presented in Fig. 1, it can be seen that thio-TEPA, when dissolved in 0·1 N acid, undergoes a gradual conversion to a compound which reacts with 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB). This disulfide was introduced by Ellman as a reagent for SH groups.[9] At neutral pH no such conversion was found.

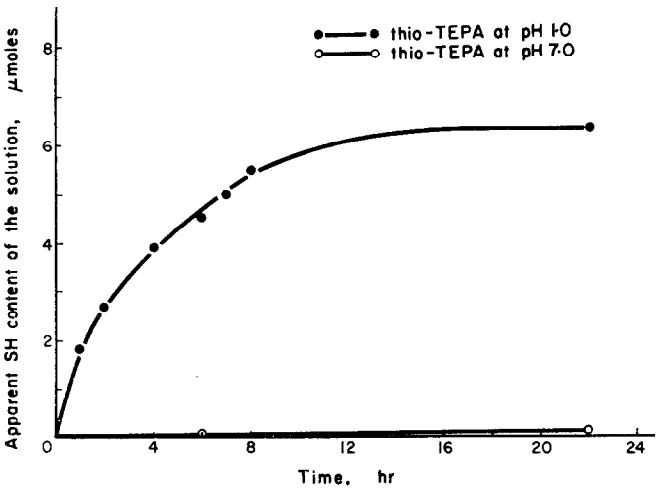


FIG. 1. Sulfhydryl groups formed from thio-TEPA in acid solution. Thio-TEPA: 6·5 mM in 0·1 N HCl. Temperature 22°. SH groups determined in neutralized aliquots by reaction with 5,5′-dithio-*bis*(2-nitrobenzoic acid).

Apparently, more than one equivalent of sulfhydryl groups was formed from thio-TEPA in the treatment with dilute acid. This can be accounted for if it is assumed that the mixed disulfide which is formed when DTNB reacts with a thiol (reaction I, where R = 3-carboxy-4-nitrophenyl) is hydrolyzed to some extent during the long time

$$\mathrm{R{-}S{-}S{-}R + XS^- \rightleftharpoons R{-}S{-}S{-}X + RS^-} \qquad \text{(I)}$$

needed for complete reaction when XSH = the "SH derivative of thio-TEPA". Such a hydrolysis (reaction II) will be followed by a dismutation in which a sulfinic acid is formed according to reaction (III). In this way "extra" SH groups are introduced

$$\mathrm{2\ R{-}S{-}S{-}X + 2\ H_2O \longrightarrow 2\ RSH + 2\ XSOH} \qquad \text{(II)}$$

$$\mathrm{2\ XSOH \longrightarrow XSH + XSO_2H} \qquad \text{(III)}$$

$$\mathrm{2\ R{-}S{-}S{-}X + 2\ H_2O \longrightarrow 2\ RSH + XSH + XSO_2H}$$

in the reaction, leading to a ratio of anion produced to SH present of more than unity.

No other reaction than a disulfide-sulfhydryl exchange seemed to occur when acid-treated thio-TEPA reacted with DTNB. This was suggested by an experiment in which an excess of the "SH derivative of thio-TEPA" was added to a limited amount of DTNB. The equilibrium then favoured the formation of two thiophenol anions per molecule of DTNB according to reactions (I) + (IV). The anions were (partially)

$$R—S—S—X + XS^- \rightleftharpoons RS^- + X—S—S—X \qquad (IV)$$

oxidized by air, after which the regenerated disulfide reacted with an excess of cysteine to give 97 per cent of the thiophenol anion production that was found when the DTNB had previously been reduced by an excess of cysteine, as can be seen from Table 1.

TABLE 1. THIOL-EXCHANGE REACTION OF 5,5′-DITHIO-*BIS*(2-NITROBENZOIC ACID) (DTNB) WITH AN EXCESS OF ACID-TREATED THIO-TEPA

Conditions	Thiophenol anions formed from 0·1 μmole DTNB (μEquiv.)	
	Acid-treated thio-TEPA (0·5 μmole)	Cysteine (0·5 μmole)
Maximum (at 2 hr)	0·144	—
Maximum (at 5 min)	—	0·184
After reoxidation (in 168 hr)	0·064	—
After reoxidation (in 24 hr)	—	0·004
After addition of cysteine (1 μmole) following reoxidation	0·172	0·177

After 20–24 hr at pH 1·1 thio-TEPA attained its maximal reactivity towards DTNB. By varying the time of the treatment with acid it could be shown that the transformation of thio-TEPA occurs stepwise. After acidification for a few minutes only an intermediate is formed—referred to as "first transformation product"—that is slowly converted to an SH compound after the solution has been neutralized. In one minute at pH 1·1 enough of this intermediate was formed to give rise in 144 hr at neutral pH to some 75 per cent of the SH groups maximally obtainable in 0·1N acid (Fig. 2). At any time after neutralization the conversion to the SH form could be speeded up by lowering the pH again. This resulted in an increase of the SH content of the solution to the value which was reached in 24 hr by a control sample of low pH. In the experiment of Fig. 2 loss of SH groups by oxidation in the neutral medium was suppressed by addition of thiolated Sephadex.[10]

The SH reagent *N*-ethylmaleimide[11] was also found to react with acid-treated thio-TEPA. As shown in Fig. 3, the rate of the reaction of the SH compound derived from thio-TEPA with *N*-ethylmaleimide is much slower than that of a more simple SH compound such as cysteamine. The same was found for the reaction with DTNB.

Loss of alkylating activity of thio-TEPA in acid solution

Determination with the aid of 4-(4′-nitrobenzyl)pyridine (NBP)[7, 8] showed that the alkylating activity of a dilute solution of thio-TEPA in 0·1 N hydrochloric acid decreases very rapidly to approximately 60 per cent of the original value, after which

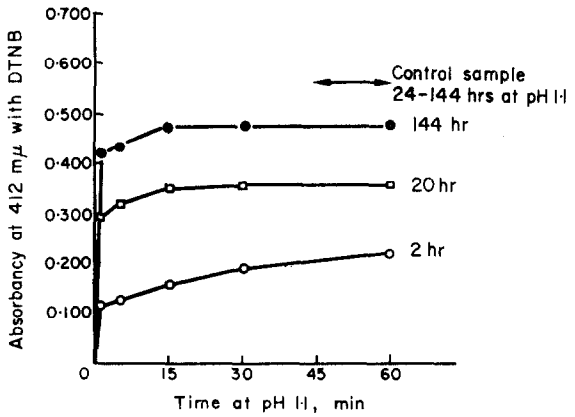


FIG. 2. SH groups produced by thio-TEPA (5·3 mM) in 2, 20 and 144 hours at neutral pH after pretreatment with acid. Pretreatment: acidification to pH 1·1 during periods from 1–60 min. Neutralization with KOH plus potassium phosphate buffer, pH 7·0. Control sample at pH 1·1 showed constant sulfhydryl level from 24 up to 144 hr. Reactions carried out at room temperature.

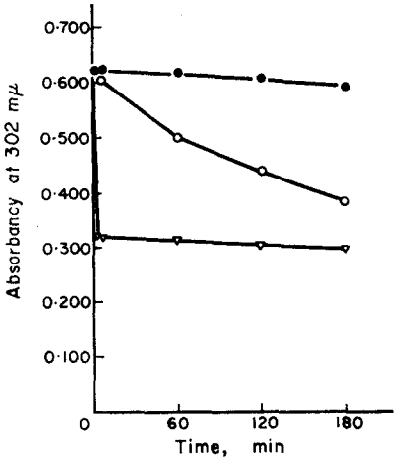


FIG. 3. Rate of reaction with *N*-ethylmaleimide (1 mM) in phosphate buffer, pH 7·0. ▽—cysteamine, 0·50 mM; ○—thio-TEPA, treated with 0·1 N HCl during 24 hr, 0·53 mM; ●—no addition.

it remains constant (Table 2). Ethyleneimine ($pK \sim 8{\cdot}0$)[12] and triethyleneimine phosphoramide (TEPA) do not show this rapid (partial) loss of alkylating activity.

By TLC in three different solvent systems it was shown that a thio-TEPA solution, kept at pH 1·1 for 5 min or for 24 hr, gives rise to a single spot with alkylating properties. The R_f values of these spots differ from those of thio-TEPA and ethyleneimine (Table 3). In the solvent systems used one cannot distinguish between the "first transformation product" and the "SH derivative" formed from thio-TEPA.

Fig. 4 demonstrates the pH dependence of the partial loss of alkylating activity which thio-TEPA undergoes in acid solution. It also shows the coupling of this process with the appearance of SH groups.

The ethyleneimine rings of both the "first transformation product" and the "SH derivative" of thio-TEPA differ from those of the original molecule in the rate of their reaction with NBP. Fig. 5 shows the development of colour with time at 100° for the different compounds. They were tested in a molar ratio of two to three for thio-TEPA and its derivatives, respectively, the data of Table 2 being taken to indicate

TABLE 2. LOSS OF ALKYLATING ACTIVITY OF THIO-TEPA, TEPA AND ETHYLENEIMINE IN SOLUTIONS OF LOW pH

Time at pH 1·1 (min)	Alkylating activity (percentage)		
	Thio-TEPA	TEPA	Ethyleneimine
0	100	100	100
5	57	—	99
15	57	99	99
24 hr	56	100	88

Conditions: thio-TEPA, 6·0 mM; TEPA, 7·5 mM; ethyleneimine, 14·0 mM. Solute: 0·1 N hydrochloric acid. Neutralization with KOH at times indicated. Temperature 22°.

TABLE 3. THIN-LAYER CHROMATOGRAPHY OF ALKYLATING PRODUCTS DERIVED FROM THIO-TEPA ON ITS DISSOLUTION IN 0·1 N ACID

Solvent system	Thio-TEPA after 5 min at pH 1·1*	Thio-TEPA after 24 hr at pH 1·1*	Thio-TEPA	Ethyleneimine
	R_f	R_f	R_f	R_f
Propanol–water (70/30, v/v)	0·96	0·96	0·90	0·57
Butanol, water saturated	0·97	0·95	0·89	0·09
Acetone–water (70/30, v/v)	0·97	0·97	0·95	0·63

Spots detected in ammonia vapour after spraying with 4-(4′-nitrobenzyl)pyridine solution (see Methods).

* Concentration of thio-TEPA: 6 mM. Temperature 22°.

that one third of the alkylating groups of thio-TEPA is lost at pH 1·1. The non-linearity found for the "first transformation product" and the "SH derivative" suggests that these compounds are more heat-labile than is thio-TEPA itself. This lability precludes the estimation by this method of the exact amount of alkylating groups which is left after dissolving thio-TEPA at pH 1·1. Titration of the alkylating groups with sodium thiosulfate[5] could not be used either because the sulfhydryl groups interfered in this method.

As is shown in Table 4, the alkylating groups of the "first transformation product" and of the "SH derivative" are hydrolyzed more rapidly at neutral pH than those of thio-TEPA. At pH 1·1, however, hardly any hydrolysis of the two remaining ethyleneimine rings of the thio-TEPA derivatives occurs.

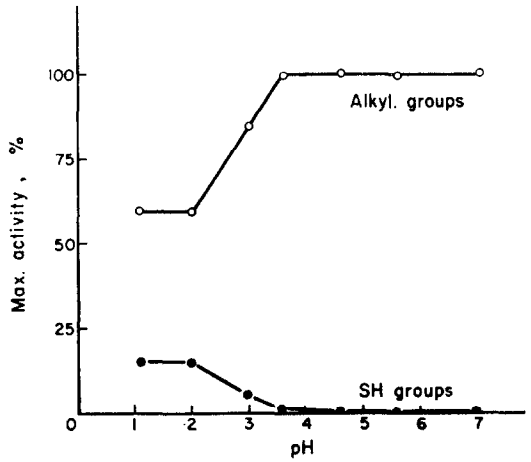


FIG. 4. pH dependence of SH production and of loss of alkylating groups from thio-TEPA (5·3 mM). Glycine buffer, 0·05 M, pH 1·1, 2·0 or 3·0; sodium acetate buffer, 0·05 M, pH 3·6, 4·6 or 5·6; potassium phosphate buffer, 0·05 M, pH 7·0. Temperature 22°. Alkylating groups and sulfhydryl measured after 15 min.

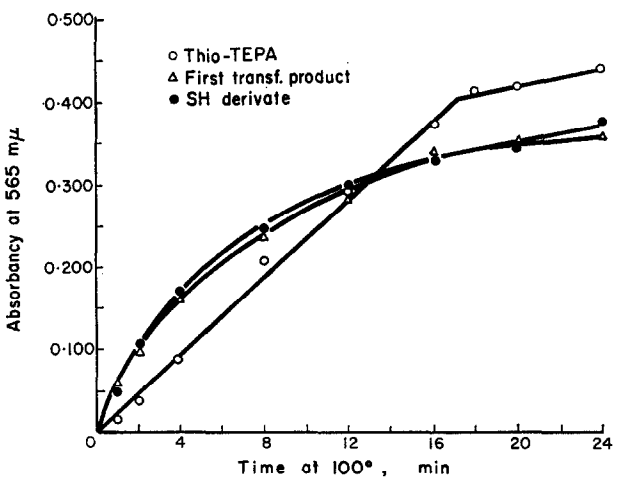


FIG. 5. Rate of reaction with 4-(4′-nitrobenzyl)pyridine at 100°. The compounds were tested in a molar ratio of two to three for thio-TEPA to thio-TEPA derivatives, it being assumed that two ethyleneimine rings are retained after the treatment with acid (*cf.* Table 2).

In Fig. 6 it is shown that the "first transformation product" formed from thio-TEPA in acid solution, reacts more easily with cysteine-SH than does thio-TEPA itself. In In this experiment anaerobic conditions were employed to prevent oxidation of SH groups. In Table 5 experiments are reported which showed a reaction of both the "first transformation product" and the "SH derivative" with cysteine-SH. In these experiments disulfide formation was prevented by addition of thiolated Sephadex.[10] In a control experiment it was shown that the SH groups of the Sephadex reacted to only a small extent with the alkylating groups.

TABLE 4. HYDROLYSIS OF THE ALKYLATING GROUPS OF THIO-TEPA AND ITS TRANSFORMATION PRODUCTS IN NEUTRAL AND ACID MEDIUM

Alkylating agent (5·3 mM)	Conditions (pH)	Alkylation in % of activity at zero time*	
		After 24 hr	After 48 hr
Thio-TEPA	7·0	97	98
1st transf. product†	7·0	75	62
Thiol-derivative‡	7·0	72	63
1st transf. product†	1·1	100	93
Thiol-derivative‡	1·1	95	95

* Zero time = time of neutralization.
† Prepared from thio-TEPA by dissolving at pH 1·1 for 5 min (22°).
‡ Prepared from thio-TEPA by dissolving at pH 1·1 for 24 hr (22°).

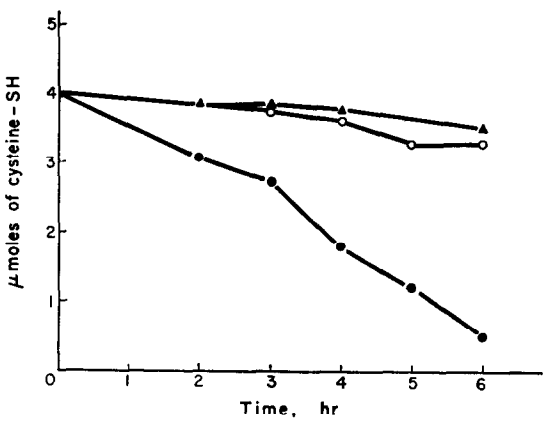


FIG. 6. Loss of cysteine-SH under anaerobic conditions from a 4 mM solution. ▲—cysteine alone; ○—with thio-TEPA, 5·3 mM; ●—with thio-TEPA (5·3 mM) which had been kept at pH 1·1 for 5 min Medium: potassium phosphate buffer, 0·1 M, pH 7·4; EDTA, 0·1 M. Temperature 22°.

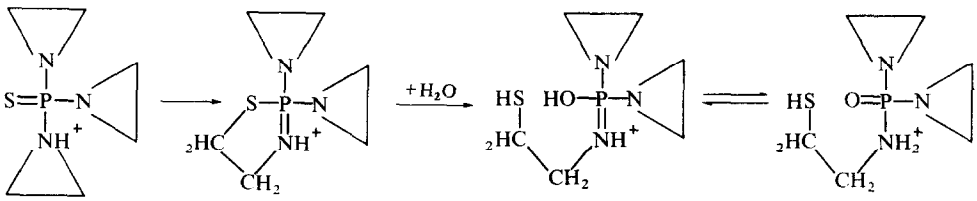
TABLE 5. LOSS OF CYSTEINE-SH IN THE PRESENCE OF THIO-TEPA OR ITS TRANSFORMATION PRODUCTS FORMED AT LOW pH

Additions	Cysteine-SH groups present* (μmole)	
	After 6 hr	After 24 hr
None	8·30	8·33
Thio-TEPA	8·19	7·65
Thio-TEPA, kept at pH 1·1 for 5 min	6·05	5·91
Thio-TEPA, kept at pH 1·1 for 24 hr	5·57	5·10

Conditions: cysteine, 8·3 μmole; thio-TEPA, 5·3 μmole; phosphate buffer, pH 7·0, 0·08 M. Thiolated Sephadex, 30 mg Total volume of fluid, 1·5 ml. Temperature 22°.
* Mean data from 5 expts.

DISCUSSION

From the data presented above it can be inferred that addition of a proton to the thio-TEPA molecule (A) does not simply lead to a hydrolytic cleavage of the protonated ethyleneimine ring. The formation of an SH group by thio-TEPA in a dilute solution of low pH suggests that the protonated ring has reacted with the sulfur atom. We propose the formation of the isomer B, followed by hydrolytic cleavage of the P—S bond to give compound C or D.



It has been shown by several investigators (for review see Heine[13]) that 1-aziridinyl compounds which contain either a double-bonded oxygen or sulfur atom adjacent to the ethyleneimine ring can undergo isomerization to oxazolines and thiazolines, respectively, on treatment with acid. Bardos *et al.*[14, 15] postulated the occurrence of an analogous intramolecular rearrangement during hydrolysis of one of his "dual antagonists": ethyl[*bis*(2,2-dimethyl-1-aziridinyl)phosphinyl]carbamate.

The structures B and C or D account for the properties of the two products (*cf.* Fig. 2) derived from thio-TEPA in 0·1 N acid, the "first transformation product" and the "thiol derivative", respectively. The alkylating activity which is left after dissolving thio-TEPA in 0·1 N acid (suggestedly two-thirds of that originally present) cannot be attributed to free ethyleneimine, as demonstrated by chromatography (Table 3). Moreover, the SH group which is ultimately formed, is carried by a molecule which reacts very much slower with *N*-ethylmaleimide than does cysteamine (Fig. 3), indicating that the SH group must be attached to the parent molecule.

The conversion of B to C or D was found to be much faster at low pH, as can be seen from Fig. 2. Protonation of the *N* of the reacted ring, increasing the polarity of the P—S bond, will facilitate opening of the five-membered ring by hydrolytic cleavage of this bond.

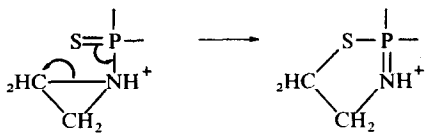
The stability in dilute acid solution of the two remaining ethyleneimine rings is comparable to that of the alkylating groups of triethyleneimine phosphoramide (TEPA) and ethyleneimine under similar experimental conditions (*cf.* Table 2). However, it is in contrast to the rapid loss of alkylating activity which triethylene melamine (TEM) undergoes in 0·05 N acetic acid.[2]

As judged from the rates of reaction with water (Table 4), NBP (Fig. 5) and the SH group of cysteine (Table 5), the chemical reactivity of the ethyleneimine rings in B and C or D at neutral pH is somewhat higher than that of thio-TEPA. A low rate of reaction with SH groups has been reported previously for TEM[16] and was also found for TEPA (unpublished experiment).

It is difficult to see how the sequence of reactions initiated by the addition of a proton to the thio-TEPA molecule is related to its higher therapeutic index, which

was found by Ross and Mitchley[17] in the treatment of the Walker rat carcinoma after pretreatment of the animal with glucose to induce a slightly acid pH in the tumor. It is clear from Fig. 4 that addition of a proton occurs only at low pH. The ethyleneimine rings, retained after the intramolecular alkylation, react only very slowly with water at low pH. A direct acid-catalysis of the alkylating activity of thio-TEPA therefore seems doubtful. Only when a transitional binding of a proton has occurred and has been followed by alkylation of the sulfur atom, a somewhat enhanced reactivity of the remaining ethyleneimine rings is to be expected as an indirect effect. As suggested by Connors *et al.*,[18] the higher therapeutic index of ethyleneimine derivatives after glucose pretreatment may also be only an apparent one, being caused by a decrease in urinary excretion of the drug due to anuria following the glucose pretreatment.

The isomerization of thio-TEPA *via* A to B might occur by the following mechanism:



Schaefer, who demonstrated an isomerization of triethylene melamine to 2,3,6,7,10,11-hexahydro-trisimidazo-(1,2-1′,2′-1″,2″-)-*s*-triazine by heating it in acetonitrile in the presence of triethylamine hydrochloride, postulated a similar reaction mechanism for this closely related rearrangement.[19]

In this scheme a carbanion would react with a relatively positive sulfur atom. It supposes that electron displacement from the double bond towards the protonated nitrogen dominates over an increase in polarity of an N—C bond of the ethyleneimine ring. Polarization of an N—C bond leading to reaction *via* a carbonium ion was assumed by Heine *et al.*[20] to be the mechanism of the acid-catalyzed isomerization of several of his 1-aziridinyl compounds. However, in the presence of a double-bonded sulfur atom adjacent to an ethyleneimine ring, preference might exist for reaction *via* a carbanion. The striking difference between the rate of hydrolysis of ethyleneimine in its protonated form and the rate of the intramolecular alkylation of the sulfur by a protonated ethyleneimine ring in thio-TEPA, points to such an influence of the sulfur atom. In triethyleneimine phosphoramide (TEPA), where an oxygen atom replaces the sulfur, intramolecular alkylation apparently does not occur (Table 2).

It was found by Heine *et al.*[20, 21] that nucleophiles such as iodide ions can also catalyze the ring widening of 1-aziridinyl compounds to give thiazolines or oxalines. Preliminary experiments with thio-TEPA dissolved at low concentration in acetone, showed that sodium iodide catalyzes the formation of a derivative which on addition of distilled water is slowly converted to an SH compound. Thus, there might exist a mechanism by which compound B could also be formed under neutral conditions.

Acknowledgements—The author wishes to thank Dr. E. Kriek and Dr. J. Links for helpful discussion and Dr. P. Emmelot for his interest and his criticism during the preparation of the script. Thanks are also due to Mr. A. Fung Kim for the synthesis of thio-TEPA and TEPA and to Miss M. J. Molenaar for skilful technical assistance.

A gift of pure thio-TEPA from Lederle Laboratories, American Cyanamid Company, Wayne, New Jersey, U.S.A. is gratefully acknowledged.

The author was a Senior Fellow of the "Koningin Wilhelmina Fonds" during performance of the work.

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