alkali required, this time was 15 sec. to 6.5 min. (11 min. for 12 dihydrochloride). The time at which pH 5.4 was reached was taken as zero time for Fig. 1. More alkali then was added automatically to maintain pH 5.4, and consumption (including that added before zero time) was plotted *vs.* time as shown in Fig. 1.

(b) **Isolation and Re-acidification of Free Bases.**—Each thiolsulfonate (*ca.* 1 mmoles) was added to water (*ca.* 10 ml.)–chloroform (20 ml.). The water contained exactly enough sodium hydroxide to convert all ammonium groups to their free bases. The chloroform extract was separated and the aqueous layer was extracted with two more 20-ml. portions of chloroform. Each of the three chloroform extracts was immediately extracted with the same portion of hydrochloric acid (an amount of *N* acid in 5 ml. of water equiv. to the acid originally neutralized). The acid extract was evaporated to dryness. "Recovery, %" (Table I) is based on the amount obtained after drying to constant weight under reduced pressure. Infrared spectra of all hydrochlorides isolated corresponded excellently with those of starting material, showing that products were pure.

Acknowledgment.—We are indebted to Doctors T. R. Sweeney and D. P. Jacobus for helpful suggestions, as well as for certain materials and for evaluation of protective activities. The research was supported by the U. S. Army Medical Research and Development Command under Contract No. DA-49-193-MD-2030.

The Stability of Tepa and Other Aziridine Chemosterilants

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Aziridine compounds, known for some time to be cancer-inhibiting agents, are useful as insect chemosterilants. Effective utilization of these chemicals required knowledge of their stability under different conditions. Investigations employing four different analytical procedures disclosed a high vulnerability of the aziridine chemosterilants to even mildly acidic aqueous solutions. Tepa in acidic or neutral solutions gave a stable degradation product, which with the aid of n.m.r. was identified as ethylenimine. The stability of ethylenimine in buffered solutions was found to decrease with increasing pH, whereas the stability of tepa increased with increasing pH of the medium. Regulation of the pH of insect diets resulted in a marked increase in activity of added chemosterilants.

The biological and physiological properties of aziridine derivatives active as cancer chemotherapeutic agents have been investigated thoroughly during the past ten years,[1] but only scattered and often conflicting reports of their chemical stability and reactivity have appeared. Interest in these compounds was intensified recently by the discovery of their activity as insect chemosterilants (antifertility agents) and their potential usefulness as new and powerful tools for insect control and eradication.[2]

Chemicals screened for sterilant activity were administered by several routes, including incorporation in the diet and addition to the drinking-water supply. Since success or failure could depend on the persistence of the compounds in the various media, experiments were set up to evaluate stability under different conditions. As a result of these investigations the use of chemosterilants has been made more effective, a better understanding of aziridine analysis has been gained, and the degradative pathway of at least one aziridinyl compound, tepa, has been demonstrated. Of particular interest is the high sensitivity of tepa and other aziridinyl compounds to even mildly acidic substrates.

Experimental

Materials. Aziridine Compounds.—These are identified in Table I and will be referred to in the text by their common names.

Tepa.—The solvent from an 85% methanolic solution of the chemical, obtained from Interchemical Corp., New York, N. Y., was removed under reduced pressure and the residue cooled to 0° for several hours. The crystals were triturated, washed with hexane, filtered, pressed dry with a rubber dam, and placed in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide. All transfers of the chemical were made in a drybox because tepa is highly hygroscopic. Our product melted at 41–43° (lit.[3] m.p. 41°).

TABLE I

Common name	Chemical name	Other designations
Tepa	Tris-(1-aziridinyl)-phosphine oxide	APO; aphoxide
Apholate	2,2,4,4,6,6-Hexahydro-2,2,-4,4,6,6-hexakis-(1-aziridinyl)-1,3,5,2,4,6-triazatriphosphorine	APN
Metepa	Tris-(2-methyl-1-aziridinyl)-phosphine oxide	MAPO; methaphoxide
Tretamine	2,4,6-Tris-(1-aziridinyl)-*s*-triazine	TEM
Ethylenimine	Aziridine or ethylenimine	

Tretamine (Chemirad Corp., East Brunswick, N. J.).—This compound was crystallized twice from ethyl acetate. It melted at about 139° dec. if the melting point was taken rapidly.[3]

Apholate[4] (Squibb Institute for Medical Research, New Brunswick, N. J.), **Metepa,** and **Ethylenimine** (Interchemical Corp.) were used as received.

Buffers for Thin Layer Chromatography.[5]—The following buffer solutions (MacIlvaine's and Sörenson's) were used: pH 3.0, 790 ml. of 0.1 *M* citric acid diluted to 1.0 l. with 0.2 *M* sodium dihydrogen phosphate; pH 5.0, 480 ml. of 0.1 *M* citric acid diluted to 1.0 l. with 0.2 *M* sodium dihydrogen phosphate; pH 7.5, 165 ml. of *M*/15 potassium monohydrogen phosphate diluted to 1.0 l. with *M*/15 sodium dihydrogen phosphate.

Deuterated Buffers.—A saturated solution of potassium dihydrogen phosphate in deuterium oxide was brought to a desired pH by the addition of solid sodium hydroxide. The solution was evaporated to dryness *in vacuo* and a sufficient amount of deu-

(1) L. R. Duvall, *Cancer Chemotherapy Rept.*, **8**, 156 (1960).
(2) A. B. Bořkovec, *Science*, **137**, 1034 (1962).
(3) H. Bestian, *Ann.*, **566**, 210 (1950).
(4) R. F. W. Rätz and C. J. Grundemann, U. S. Patent 2,858,306 (Oct. 28, 1958).
(5) L. R. Goldbaum and L. Kazyak, *Anal. Chem.*, **28**, 1289 (1956).

terium oxide was added to achieve solution. The evaporation was repeated 3 times. Nuclear magnetic resonance spectra of the buffer solutions showed only one peak which was consistent with the 0.2% water content of commercial deuterium oxide.

Methods. Titration of Aziridine Function.—This procedure was based on the thiosulfate method of Allen and Seaman.[6] To 5 ml. of a 20% aqueous sodium thiosulfate solution was added a 5-ml. sample containing about 0.3 mequiv. of aziridine function while a gentle stream of nitrogen blanketed the solution. The stirred mixture was titrated (pH meter) with a constant slow flow of 0.1 *N* sulfuric acid until the pH of the solution remained unchanged at 4.0 for 15 sec. Stirring and nitrogen addition, discontinued while the solution stood for 30 min., were resumed and the titration was completed by adding 0.05-ml. increments of 0.05 *N* sodium hydroxide until pH 11 was reached; pH was recorded after each addition. The end point or volume of sodium hydroxide needed to neutralize the excess acid was determined by plotting the pH change per increment of titrant *vs.* volume of sodium hydroxide.

Then

$$\% \text{ aziridine function} = 100 \times \frac{(\text{ml. } H_2SO_4)(0.1) - (\text{ml. NaOH})(0.05)}{\text{mequiv. of sample}}$$

The titration procedure does not always give theoretical results even for pure compounds. Thus, pure tepa titrated 92–96%, apholate 100%, and tretamine 96% of theory.

Colorimetric Determination of Aziridines.—The procedure of Epstein, *et al.*, was followed.[7]

Thin Layer Chromatography.—The apparatus of Stahl[8] and silica gel G (Brinkmann Instruments, Inc., Great Neck, N. Y.) were used. The gel, in layers 250 μ thick, was deposited on glass plates in accordance with the directions of Stahl.[9] After drying for 5 min., the plates were activated for 45 min. at 105–110°. About 2 to 3 μl. of a 2% w./v. chemosterilant solution (equivalent to 40 to 60 γ) in the appropriate buffer or water was applied from a micropipet at a line about 2.5 cm. from the end of the plate. After 15 min. the plate was inserted in a developing chamber at room temperature (23–25°) containing a 1-cm. depth of developing solvent and a paper lining saturated with the same solvent. After the solvent front travelled 11–13 cm. past the point of spotting, the front was marked and the plate removed. The solvent was allowed to evaporate and the plate placed in a chamber containing iodine crystals until spots appeared. A more sensitive means of making the spots visible was to spray with 0.1 *M* aqueous potassium hydrogen phthalate and then with a 5% acetone solution of 4-(*p*-nitrobenzyl)pyridine. After heating the treated plates at 110° for 30 min., they were allowed to cool and then were sprayed with *M* aqueous potassium carbonate. Intensely blue spots appeared where aziridine-containing compounds occurred. An outstanding feature of this method is that it makes visible only those compounds containing aziridine functions, whereas the iodine method responds to a great variety of organic materials.

Nuclear Magnetic Resonance Spectrometry.—The decomposition of tepa (2.5% w./v.) in deuterium oxide (D_2O) maintained within a narrow pH range with deuterated phosphate buffer was followed by observing n.m.r. spectral changes recorded with a Varian Associates Model A-60 spectrometer. Tepa's 12 equivalent protons are split into a doublet by the phosphorus atom (J_{HP} = 14 c.p.s.). Disappearance of the doublet, which appeared between +2.60 and 2.73 p.p.m., depending on the pH of the solution (between 4 and 9), served as a measure of the compound's decomposition. [All chemical shift data are in parts per million (p.p.m.) from the water peak (in D_2O), taking the high field as positive.]

In a similar manner the decomposition of ethylenimine (1.6% w./v.), which appeared as a singlet at +2.26 to 2.40 p.p.m., depending upon the pH of the solution (between 4 and 7.2), was followed in deuterated phosphate buffers by determining periodically the area under the singlet remaining.

Microdetection of Aziridine Compounds.—In the following procedure as little as 1 γ of tepa and 5 γ of metepa or apholate can be detected in a drop of eluate from a silicic acid chromatographic column employing methanol as eluant. To the drop in a micro test tube was added 1 drop of sodium thiosulfate solution, 2 drops of distilled water, and 1 drop of a mixed indicator (0.07% bromocresol green and 0.05% methyl red in 95% ethanol). After stirring, the mixture became deep blue or blue green in color. Upon addition of 2 μl. of 0.1 *N* sulfuric acid, a bright red color appeared. When the test was positive, the original blue or blue-green color reappeared within 3 min.; if negative, the red color persisted.

Results and Discussion

Titrimetry, colorimetry, and thin-layer chromatography (t.l.c.) were used for the quantitative determination of the aziridinyl compounds. The first method, based on a reaction of the aziridine ring with thiosulfate ion, provides a measure of intact aziridine functions. In the case of tepa the method does not furnish a quantitative determination of the intact tepa molecule and it has to be supplemented with a method for distinguishing between unreacted, partially reacted (1 or 2 rings still intact), or fully reacted species. The colorimetric method is based on a comparison between a standard and a partially decomposed sample in which some of the aziridine functions were destroyed. Again, no distinction can be made between a mixture of intact and fully decomposed polyfunctional aziridines, or a mixture of partially reacted species. Specifically, in the degradation of tepa, neither of the two methods gives any evidence as to the stability of the P–N bond, which is of crucial importance in the decomposition.

Thin-layer chromatography provides a semiquantitative estimation of the proportion of unchanged to degraded molecules, but it does not appear to give a clear indication of the proportion of partially or wholly reacted species. Although the combination of the three methods does not give the entire quantitative picture of the degradation of tepa under different environmental circumstances, it indicates clearly the sensitivity of the aziridine ring to temperature, pH, and light.

Effect of Temperature.—Figure 1 gives the stability of 0.3% aqueous solutions of tepa and metepa as a function of temperature. Estimated time in days necessary for 50% decomposition of the imine function in tepa at various temperatures is >200 (3°), 31 (25°), 7 (50°), <0.1 (100°), and in metepa >200 (3°), 72 (25°), 16.5 (50°), and <0.1 (100°). The pH of both tepa and metepa aqueous solutions increases as their decomposition progresses and the reaction gradually slows down, probably because the decomposition rate decreases with rise in pH, as shown in Fig. 2. In the tepa solution the pH increased from 6.8 (100% imine function) to 8.45 (50% imine function remaining); similarly, the pH increase in metepa solution was from 7.05 to 8.7. In all the experiments both the thiosulfate titration and the colorimetric methods were used concurrently with no appreciable differences.

Effect of Light.—There was no difference between the rates of decomposition of aqueous tepa solutions exposed to daylight and those kept in the dark as determined by the thiosulfate titration.

The Effect of pH on the Degradation of Aqueous Chemosterilant Solutions.—Although the stability of nitrogen–mustard alkylating agents in buffered aqueous systems has been studied extensively,[10,11] similar

(6) E. Allen and W. Seaman, *Anal. Chem.*, **27**, 540 (1955).
(7) J. Epstein, R. W. Rosenthal, and R. J. Ess, *ibid.*, **27**, 1435 (1955).
(8) E. Stahl, *Pharmazie*, **11**, 633 (1956).
(9) E. Stahl, *Chemiker Ztg.*, **82**, 323 (1958).
(10) L. P. White, *Science*, **131**, 1041 (1960).
(11) O. M. Friedman and E. Boger, *Anal. Chem.*, **33**, 906 (1961).

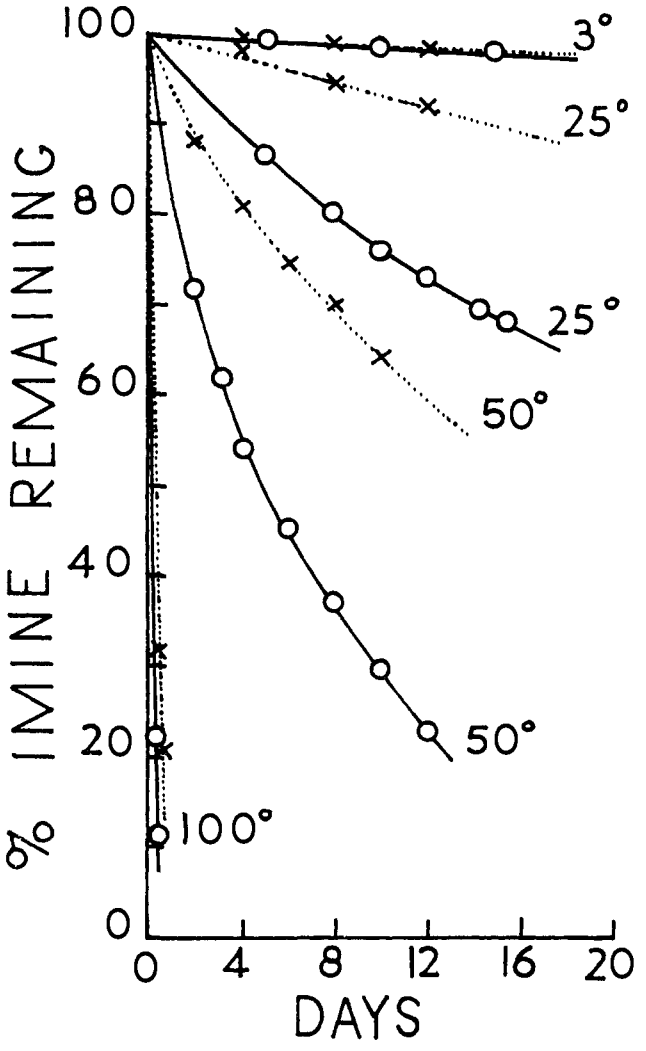


Fig. 1.—Decomposition of 0.3% aqueous solutions of tepa (———) and metepa (.....) at different temperatures as determined by titrimetric and colorimetric procedures.

studies on aziridine derivatives have not been adequate.[6] When it became apparent that the acidity of the medium in which a chemosterilant was administered influenced significantly its physiological activity, an effort was made to determine the rates of decomposition of several aziridines in variously buffered aqueous solutions. Both the thiosulfate and the colorimetric methods indicate only the disappearance of the aziridine function and it was expected that the ring opening in a polyfunctional aziridine (*i.e.*, tretamine and tepa) may not proceed simultaneously, but rather in discrete steps that may have an important bearing on the activity of the material. Thin-layer chromatography experiments confirmed this supposition only in part.

Initial t.l.c. experiments were made with tretamine. It moved as a single compact spot (R_f 0.34) with 1:1 chloroform–acetone as the developing solvent. A n.m.r. spectrum of the material recovered from the spot indicated that the compound was unaltered. An experiment was therefore set up in which 2% solutions of tretamine in water and in buffers at pH 7.5, 5.0, and 3.0 at 25° were chromatographed with the aforementioned solvent mixture 0, 0.75, 2, 4, and 24 hr. after the solutions were prepared. In the very first chromatogram (0 hr.) tretamine at pH 3.0 gave practically

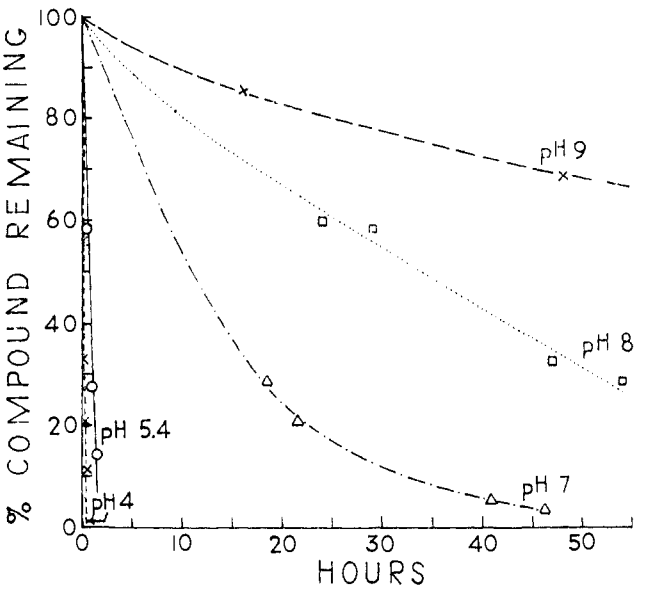


Fig. 2.—Stability of 2.5% tepa in D_2O buffered at different pH values as determined from n.m.r. spectra.

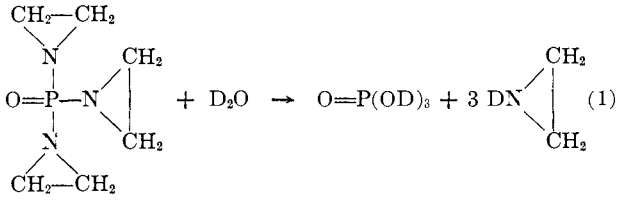
no spot at R_f 0.34 but a dark spot, undoubtedly a degradation product, at the origin. This result indicated that tretamine is degraded almost immediately at pH 3.0. It was apparent that the amount of material remaining at R_f 0.34 (and at the origin) could serve as a semiquantitative measure of breakdown. Tretamine degraded much more rapidly at pH 5.0 than at pH 7.5; the spots at pH 5.0 were about 0.5 and 0.05 the intensity of the spots at pH 7.5 after 4 and 24 hr., respectively. After 24 hr., tretamine in the unbuffered solution had degraded only slightly more than that buffered at pH 7.5, neither chromatogram indicating very much degradation.

The foregoing experiments were repeated with tepa, one of the most effective insect chemosterilants, except that absolute methanol was used as the t.l.c. developing solvent. The results paralleled those with tretamine very closely and if anything, tepa appeared to be more susceptible to decomposition by acid than tretamine. The R_f of tepa was 0.53. Both tretamine and tepa yielded only two reproducible spots on the t.l.c. plates. The spot at the origin contained all of the degradation products and the advancing spot corresponded to the pure starting material. In order to determine whether the high concentration of buffer at the origin would interfere with the appearance of the intermediate spots, a 3.1% tepa solution was brought to pH 4 by the addition of N H_2SO_4 and the pH was maintained approximately constant (3.8–4.3) for 3 hr. Periodically, samples were withdrawn and their aziridine-function content determined by thiosulfate titration. At the same time the samples were chromatographed, but instead of making the spots visible by exposure to iodine vapor, the more sensitive and specific colorimetric method described under the t.l.c. procedure was used. The results of the titration showed a rapid decrease of aziridine function within the first 30 min., but then the decomposition slowed down considerably. The samples analyzed by t.l.c. indicated a complete disappearance of the tepa spot after 1.5 hr., but the presence of aziridine function in the spot at the origin persisted for 24 hr. It was thus established

that at about pH 4.0 tepa decomposed rather rapidly to an unknown aziridine intermediate(s) relatively stable toward hydrolysis.

In order to elucidate the nature of the intermediate (s), n.m.r. spectrometry was employed. Tepa was dissolved in deuterium oxide (D_2O) in the presence of deuterated phosphate buffer so that only the protons of the aziridine ring were visible in the n.m.r. spectrum. It was anticipated that the tepa doublet would diminish with time in direct proportion to the disappearance of the aziridine function as determined by the chemical methods. The results obtained with tepa in pH 4.0 buffer are illustrated in Fig. 3. The tepa doublet disappeared almost completely in 1 hr. with the concomitant rise of a new strong singlet.

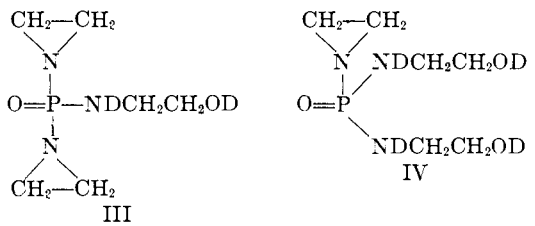
Tepa (I) appears as a doublet because of the spin coupling between the 12 equivalent protons and the phosphorus atom. As long as the P–N bond is intact a singlet cannot be obtained. Only through cleavage of the P–N bond is it possible to obtain a singlet, which presumably would be due to N-deuterioethylenimine (II) generated as shown in the following equation.



I II

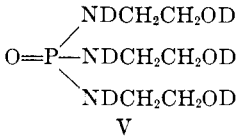
By injecting N-deuterioethylenimine into the 1-hr. old solution of tepa in pH 4.0 buffer, the singlet peak immediately increased in intensity, thus establishing unequivocally the identity of the singlet-producing degradation fragment.

However, as may be seen from Fig. 3, ethylenimine is not the only decomposition product observable in the n.m.r. spectrum at the 63-min. interval. The broad multiplets of indefinite character show that a substantial amount of degradation products other than ethylenimine is found. These degradation products cannot arise from ethylenimine because, as will be shown later (Fig. 7), ethyleimine at pH 4.0 is rather stable, producing no more than a small percentage of decomposition product in 63 min. Instead, these products must arise mainly from hydrolytic cleavages other than those of the P–N bond. Such cleavages would be expected to give rise to the following compounds



III

IV



V

Eventually all of the degradation products will be hydrolyzed to deuteriophosphoric acid and D_2NCH_2-CH_2OD.

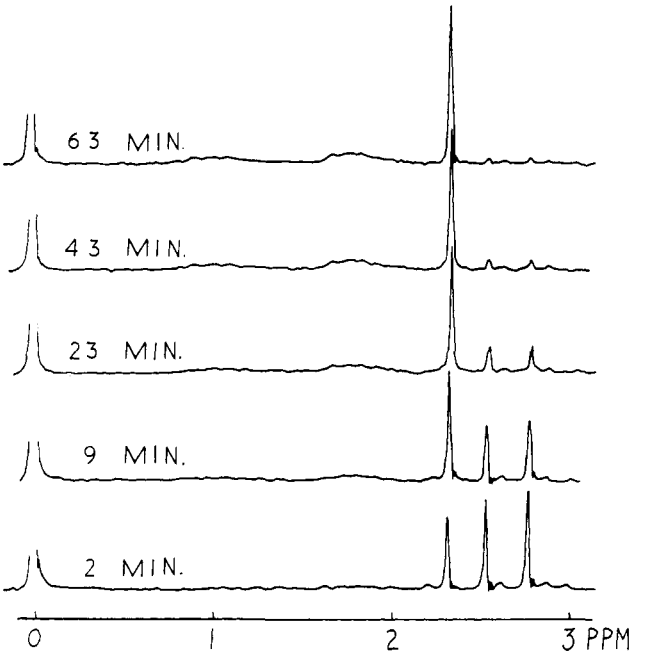


Fig. 3.—N.m.r. spectra of 2.5% tepa in D_2O buffered at pH 4.0; final pH was 5.32.

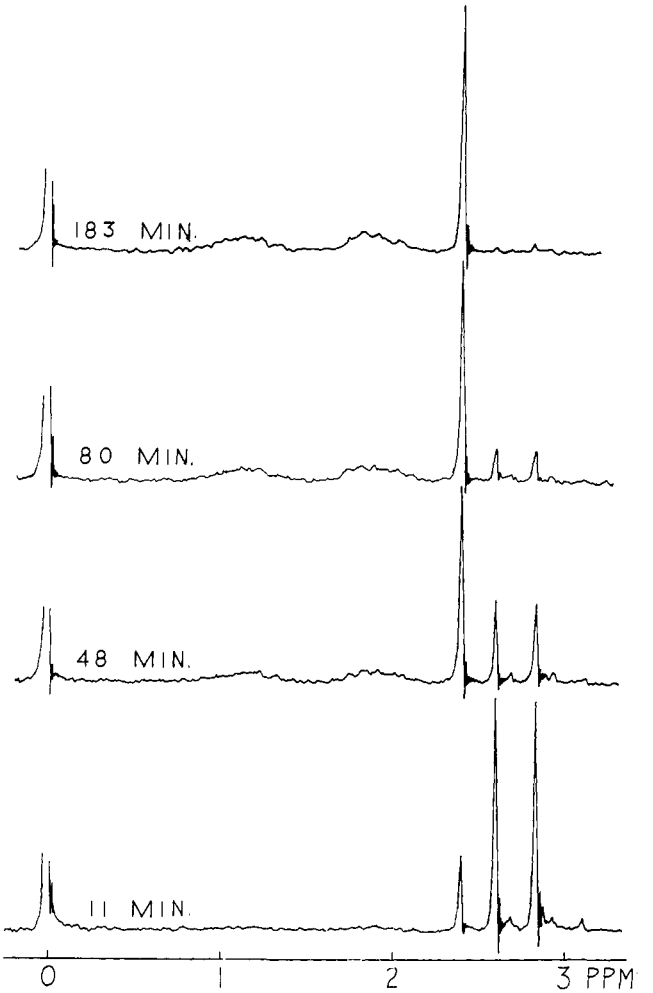


Fig. 4.—N.m.r. spectra of 2.5% tepa in D_2O buffered at pH 5.4; final pH was 5.62.

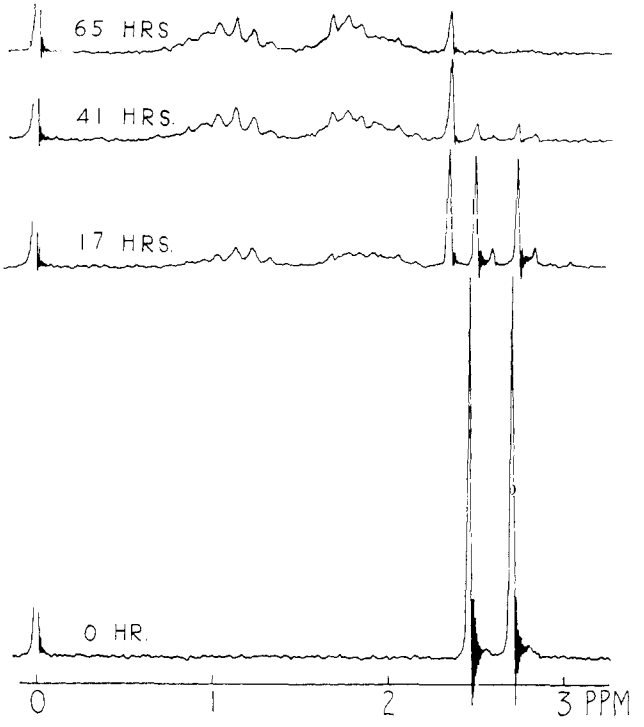


Fig. 5.—N.m.r. spectra of 2.5% tepa in D_2O buffered at pH 7.0; final pH was 7.10.

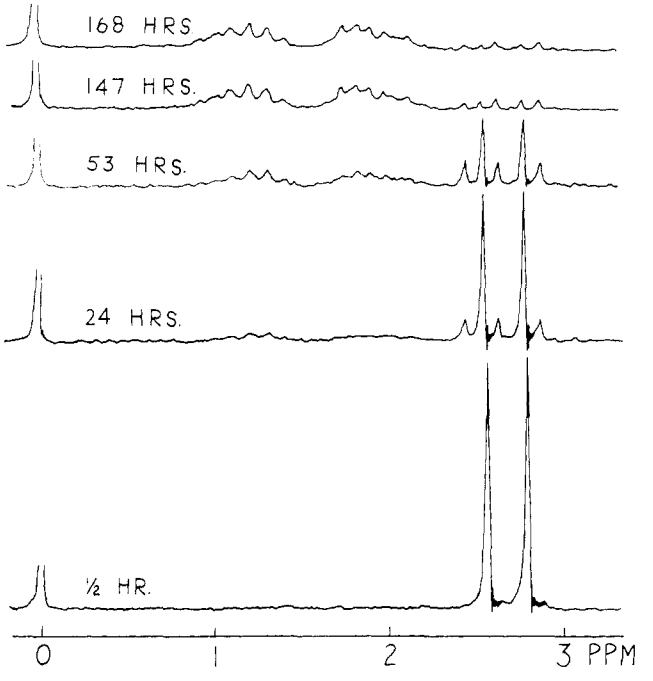


Fig. 6.—N.m.r. spectra of 2.5% tepa in D_2O buffered at pH 8.0; final pH was 8.15.

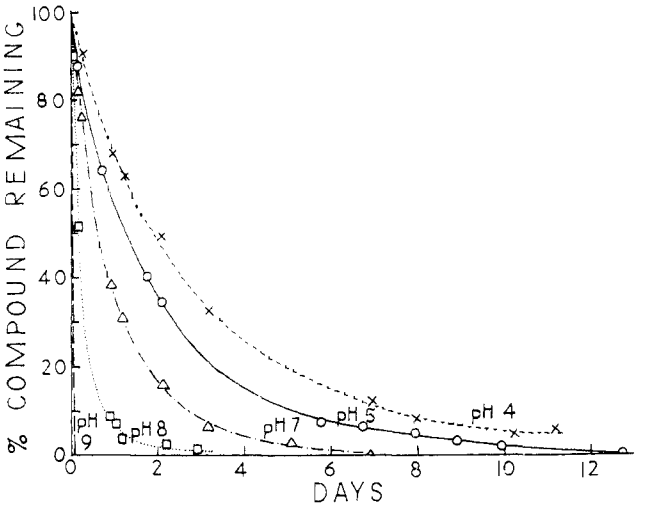


Fig. 7.—Stability of 1.6% ethylenimine in D_2O buffered at different pH values as determined from n.m.r. spectra.

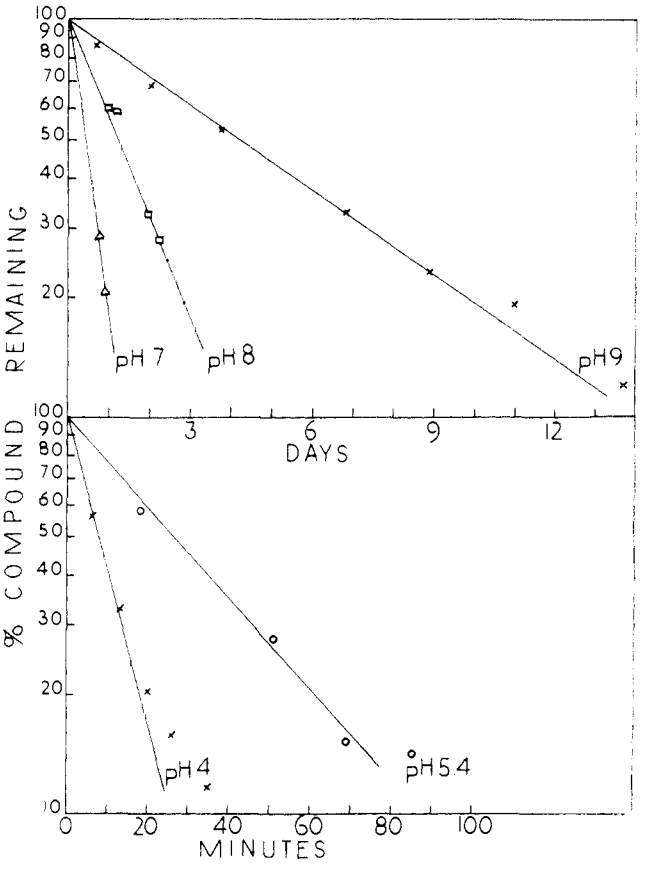


Fig. 8.—Stability of 2.5% tepa in D_2O buffered at different pH values as determined from n.m.r. spectra; log concentration *vs.* time.

In order to maintain the pH of the tepa solutions even approximately constant, saturated solutions of buffers had to be employed which surely led to some reaction between the buffer anion (phosphate) and the basic degradation products. Consequently, structures III to V are suggestive of the general type of products rather than of actual entities isolated.

The effects of pH on the stability of tepa and ethylenimine solutions determined by the n.m.r. method are summarized in Fig. 2–9. In saturated phosphate buffer solutions the rate of tepa decomposition increases with decreasing pH, whereas the rate of ethylenimine decomposition decreases with decreasing pH. In the acidic and neutral pH range ethylenimine was established as an intermediate in the tepa degradation.[12] It is not clear, however, whether the same is true in the alkaline pH range. At pH 8 the prominent ethylenimine peak is missing in Fig. 6 and even a cursory inspection of Fig. 2, 7, 8, and 9 reveals that ethylenimine

(12) Ethylenimine intermediate in tepa degradation was suggested previously by A. W. Craig and H. Jackson, *Brit. J. Pharmacol.*, **10**, 321 (1955), but no experimental evidence for this hypothesis was given.

decomposes faster than tepa and that no appreciable accumulation of it could be expected even if it were an intermediate in the tepa degradation. Unfortunately, the n.m.r. method is not sufficiently sensitive to enable us to decide whether in alkaline solution the P–N bond cleavage in the tepa molecule precedes the C–N cleavage in the attached aziridine ring. Other approaches, which could resolve this important problem, are currently being investigated. Figures 8 and 9 indicate that the degradation of tepa and of ethylenimine in buffered solutions follows first-order kinetics. The obvious deviation from the straight line (Fig. 8, pH 4) was undoubtedly caused by the inability of the buffer to maintain a constant pH throughout the reaction.

Both the t.l.c. and the n.m.r. methods gave information concerning the disappearance of tepa, but only the n.m.r. revealed the structure of the intermediate ethylenimine. The other two analytical methods, thiosulfate titration and colorimetry, provided only quantitative data on the disappearance of the total aziridine function.

Recognition of the pH effects led us to examine the acidity of the diets being used to screen insect sterilants. The diet for the Mexican fruit fly, *Anastrepha ludens* (Loew), contained orange juice crystals,[13] which are known to be highly acidic owing to the high citric acid content. Setting up an experiment to check quantitatively the stability of tepa with orange juice crystals required certain modifications of the usual procedure, since tepa could not be titrated in the presence of the acidic crystals. It was determined that almost 90% of the tepa could be recovered from aqueous citric acid solutions by saturating the latter with sodium chloride, extracting three times with equal volumes of chloroform, and evaporating the chloroform. One per cent tepa added to orange juice crystals was quickly ground and exposed to a relative humidity of 50% at 25°. After 1 hr. at least 90% of the tepa had decomposed. On the basis of these investigations, the diet of the Mexican fruit fly was replaced with one that was neutral. The results were dramatic in that many aziridinyl-containing compounds that were ineffective became potent chemosterilants.

Honey was used in a diet for sterilizing mosquitoes. A 50% aqueous solution of honey was found to have a pH of 3.5, which is about normal. However, even though it is acid, a potentiometric titration showed that it contained but a small quantity of acid, 0.06 mequiv. of base being required to bring a 5-g. sample to pH 7.0. This small amount of acid did not cause much decomposition of the sterilant, but it undoubtedly caused some. For example a honey solution, initially at pH 3.6, was found to be at pH 5.8 1 hr. after mixing with tepa. The large pH shift could only be brought about by degradation of the sterilant in part to an amine. In subsequent experiments honey was replaced with sugar water.

(13) McKee's® instant orange juice crystals. Mention of this proprietary compound does not necessarily imply endorsement of this product by the U. S. Department of Agriculture.

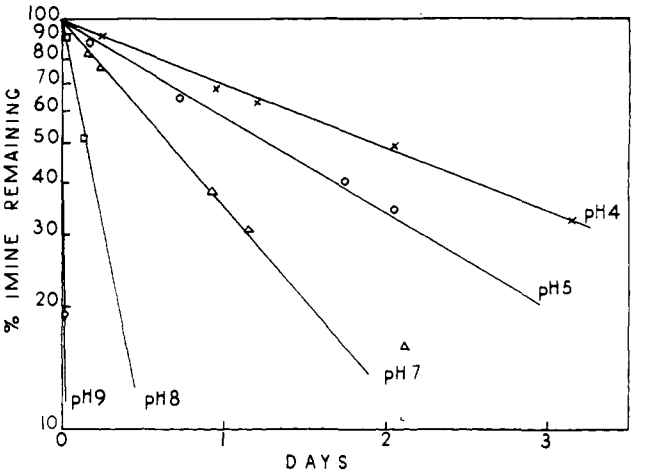


Fig. 9.—Stability of 1.6% ethylenimine in D_2O buffered at different pH values as determined from n.m.r. spectra; log concentration *vs.* time.

It is apparent that the initial pH of the solution may be less important than the amount of acid available insofar as the stability of chemosterilants is concerned. The orange-juice crystals required 6.83 mequiv. of base to bring a 5-g. sample to pH 7.0, about 100 times the amount required for honey.

T.l.c. investigations yielded other useful findings. The fact that only the pure material migrated from the origin suggested that it was only necessary to filter the chemosterilants through a layer of the silicic acid to recover the pure material from partially decomposed samples. This procedure works, but recovery is not complete. As part of this study the qualitative method described under "Methods" was developed for detecting as little as 1 to 5 γ of aziridine-containing chemosterilants in one drop of liquid.

Acknowledgment.—We wish to thank Mr. E. L. Gooden for the spectra and Miss A. M. Limburg and Mr. R. T. Brown, all of this Division, for performing the analyses.