Automated Procedure for the Determination of the Aziridine Moieties of *N,N'*-Tetramethylenebis(1-aziridinecarboxamide) and Other Compounds Containing Aziridine

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The AutoAnalyzer was used to develop an automatic colorimetric method based on the Epstein procedure for the determination of total aziridine in 25 candidate insect chemosterilants that contain aziridine. Methanolic solutions of these compounds can be analyzed at the rate of 10 to 70 samples per hour. Precision and reproducibility are excellent. Aziridine solutions differing by only 2 μg. per ml. can be easily distinguished, and the greatest variability for a particular concentration run seven times a day for 5 consecutive days was about 3%. The range of concentration that can be analyzed extends from less than 1 to more than 1000 μg. per ml.

Two problems associated with the use of chemicals instead of irradiation for sterilizing insects are the unknown hazards to wildlife exposed to the residues remaining on or in the treated insects, and the effects of pH, temperature, concentration, and other environmental factors on the stability of the parent compounds in the bait and diet formulation used to treat large numbers of insects. Thus, when programs involving the use of chemosterilants are contemplated, the insect-borne residues and handling procedures of the compounds must be assessed.

The use of 4-(*p*-nitrobenzyl)pyridine (NBP) as an analytical reagent for the quantitative determination of compounds containing aziridine and other alkylating agents was first proposed by Epstein *et al.* (1955). Since then, this method has been used by many workers (Bardos *et al.*, 1965; Beroza and Borkovec, 1964; Friedman and Boger, 1961; Klatt *et al.*, 1960) in chemotherapy and, more recently, by investigators analyzing the aziridine moiety of chemosterilants that contain aziridine (Chang and Borkovec, 1966; Maitlen and McDonough, 1967). The procedure of Epstein *et al.* (1955) is briefly as follows: The solution containing the compound is adjusted to pH 4 with a phthalate buffer, treated with a solution of NBP, heated to 95° C. for 20 minutes, and then cooled. Potassium carbonate is added to develop the color, and the resulting intensity of color is measured spectrophotometrically at 600 mμ. This absorbance can then be related to the concentration to obtain a standard curve that can be used subsequently to estimate unknown concentrations of the aziridine. The method does not distinguish between the intact molecule containing the aziridine and the partially or fully reacted species. It is only a relative measure of total aziridine, whether free, as in the reaction with ethylenimine, or incorporated into a more complex form.

However, the Epstein method has certain characteristics that make it cumbersome and prone to error when large numbers of samples must be analyzed in a short time: the reduction in the intensity of the chromophore when the mixture stands and the necessity of precise control of heating times to cause formation of the chromophoric precursor.

An automated methodology of Epstein's procedure was primarily developed to eliminate these disadvantages. Automation would also make the procedure more easily adaptable to the analysis of decidedly different aziridine-containing compounds, facilitate the simultaneous analysis of two or more aziridine-containing compounds, and increase the reliability of analysis when a large number of samples must be analyzed. Most important, the procedure could be standardized in laboratories throughout the country and be free of operator error resulting from subtle changes in the techniques. Such a standardized analytical procedure would make it possible to compare results obtained from widely separated laboratories. A preliminary report described some of the problems involved in the adaptation of Epstein's method to the AutoAnalyzer (Terranova *et al.*, 1967).

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EXPERIMENTAL

Reagents. All chemicals except those indicated were reagent grade and were used without further purification.

Two per cent (weight per volume) NBP in 0.02*M* *p*-toluenesulfonic acid (*p*-TSA) in ethylene glycol monomethyl ether–water (4 to 1). NBP recrystallized from acetone-hexane. Other percentages of NBP were prepared in the same manner; the concentration of *p*-TSA was kept constant.

Stock Base Solutions, 0.15*M* KOH; 1.20*M* K_2CO_3 in deionized glass-distilled water (prepared by dissolving 8.4 grams of KOH and 165.68 grams of K_2CO_3 in distilled water and diluting to 1000 ml.).

Working Base Solution, 40 ml. of stock base solution, 30 ml. of ethylene glycol monomethyl ether, and 30 ml. of glass-distilled water.

Apparatus. The apparatus for automating the modified Epstein procedure consisted of the following components manufactured by the Technicon Corp., Ardsley, N. Y.: sampler II, proportioning pump I, variable temperature heating bath, colorimeter equipped with a 15-mm. tubular flow-through cell and 600-mμ interference filters, and a recorder loaded with absorbance chart paper. A heating bath coil of 1 minute and 45 seconds flow-through length was used. The pump tubes for the manifold assembly are described in Table I. The flow diagram (Figure 1) shows the arrangement of components, specialized glass fittings, and transmission lines needed to complete the instrumentation.

The AutoAnalyzer was operated according to standard techniques. The studies with Compound 3 (ENT-50838) were made by using cams that allowed sampling 70, 60, 50, 40, 30, or 20 samples per hour in the reverse mode. All other

Table I. Details of AutoAnalyzer Pump Tubes for Total Aziridine Manifold

Purpose	Size (I.D.), Mm.	Color Code (Shoulders)	Nominal Delivery, Ml./Min.	Type
Sample	0.056	Yellow	1.06	Solvaflex
NBP	0.045	Red	0.70	Solvaflex
Air	0.045	Red	0.80	Clear standard
Recycle	0.056	Yellow	1.06	Solvaflex
Working base	0.056	Yellow	1.06	Solvaflex
Air	0.051	Gray	1.00	Clear standard
Waste	0.073	Green	1.69	Solvaflex

compounds were sampled at a rate of 20 samples per hour (standard cam on sampler II) in the reverse mode.

Operation. The samples were placed in 5-ml. cups in the sampler tray. Operation in the reverse mode provided a cycle of 1 part of sample to 2 parts of methanol. The wash reservoir of sampler II was continuously supplied with a slow trickle of methanol from a 1000-ml. separatory funnel stationed above it. The solution of methanolic aziridine was mixed with an acidified solution of NBP as soon as it entered the AutoAnalyzer system by passing it through a double mixing coil. After the mixing, the stream was passed into a 60° ± 0.1° C. heating bath for 1 minute and 45 seconds to form the chromophoric precursor. The stream was then recycled through the proportioning pump and mixed via a double mixing coil with the working base solution to develop the blue chromophore. This solution was further mixed in a single mixing coil (the flow stream was segmented by the introduction of air to prevent diffusion of the samples), and the air bubbles were removed with a C-4 debubbler just before passage into the colorimeter.

Since the methanol caused a slight leaching of plasticizer from the new pump tubes, a 1-hour preconditioning of the manifold with all solvents was necessary to establish a smooth base line whenever the tubing was changed. One-half hour of preconditioning was done daily. Pump tubes were replaced every 5 days.

Four separate concentrations of each compound were used to establish standard curves. They were tested in the following order: high, low, intermediate I, and intermediate II.

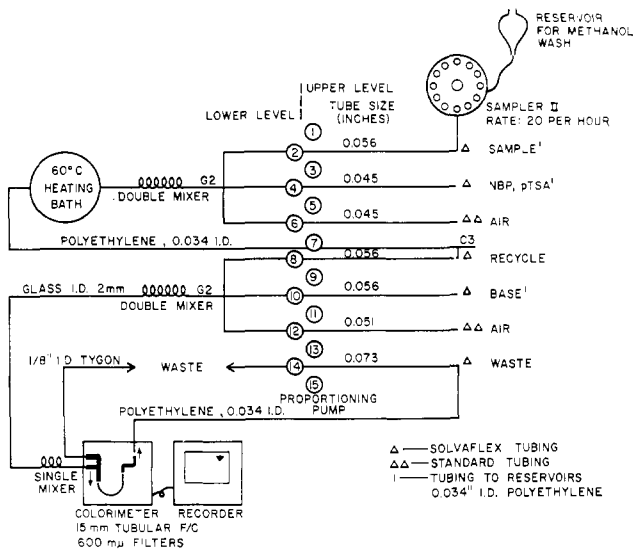

Figure 1. Flow diagram of modified Epstein procedure for automated determination of total aziridine

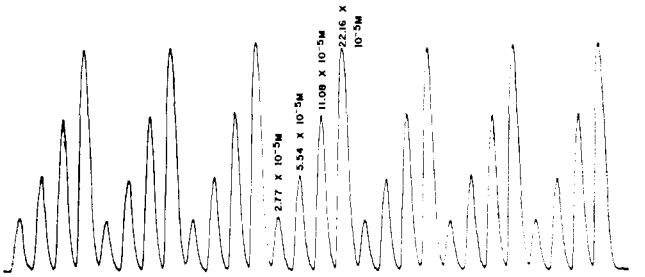

Figure 2. Portion of strip chart obtained by using AutoAnalyzer method for determination of total aziridine in Compound 3

Note reproducibility of 2.77 × 10^{-5} *M* peak following highest concentration. Samples analyzed at a rate of 20 per hour with no interposing wash cup

This series was repeated seven times to obtain replication (Figure 2) as suggested by Morgan (1967).

Sample Preparation, Extraction, and Cleanup. Individual *Phormia regina* (Meigen) were homogenized for 1 minute in 13 × 100 cm. Potter-Elvehjem tissue grinders containing 2 ml. of methanol. The contents were centrifuged and the supernatant was transferred to 10 × 0.6 cm. i.d. glass chromatographic columns containing a 2-cm. height of a 9 to 1 mixture of silica gel H–Nuchar C-190 N and eluted. This procedure was repeated two times with 2-ml. portions of methanol. As the last of the supernatant sank into the columns, an additional 1 ml. of methanol was allowed to flow through the column. The combined column eluates were taken to dryness with a stream of nitrogen, and the residue was dissolved in 4 ml. of methanol for analysis.

RESULTS AND DISCUSSION

The method was evaluated primarily for its application to the analysis of compound 3, a candidate chemosterilant that is promising for the control of the screw-worm fly, *Cochliomyia hominivorax* (Coquerel) (Crystal, 1965). The responses of the other test compounds were analogous and are not reported individually. However, Table II also lists the standard curve slope values for the compounds.

Figure 2 shows a portion of a strip chart obtained when the AutoAnalyzer method was used to determine total aziridine. A linear relationship between concentration and absorbance was obtained over the range from 1.0 to 25 × 10^{-5} *M* per liter. The results obtained when four concentrations of Compound 3 were tested for 5 consecutive days, seven times a day (after the absorbance values were converted to concentration), were as follows: 2.77 ± 0.001 × 10^{-5} *M*, 5.54 ± 0.174 × 10^{-5} *M*, 11.08 ± 0.475 × 10^{-5} *M*, and 22.16 ± 0.705 × 10^{-5} *M*. However, these particular data were obtained with one set of manifold tubes used for the entire week. The variability of the standard curve slopes (absorbance/concentration × 10^{-5} *M*) obtained with 10 weekly changes of the pump tubes was 0.0363 ● 0.0018. Thus, standard curves must be prepared for each new set of tubes, and standard solutions should be tested daily.

The effect of the rate of sampling on the attainment of steady-state absorbance is shown in Figure 3. A methanol wash cup was placed between samples, which resulted in an analysis rate one-half that of the true cam value—that is, when a sampling cam of 70 samples per hour was used, only 35 actual samples were analyzed; the other 35 consisted of methanol washes. This was necessary because at the high rates of sampling, considerable interference occurred when the highest and lowest concentrations of Compound 3 were analyzed one after the other without the interposing wash cup.

Table II. Standard Curve Slope Values of Insect Chemosterilants Containing Aziridine as Determined by the AutoAnalyzer Method for Total Aziridine

Compound	Chemical	No. of Manifold Changes	Range of Concn. ($\times 10^{-5}$ M)	Slope (ABS/Concn. $\times 10^{-5}$ M)	Standard Deviation ($\times 10^{-5}$ M)
1	Ethylenimine (aziridine)	10	5.98–95.77	0.0060	±0.0004
2	*N,N'*-Hexamethylenebis(1-aziridinecarboxamide)	5	1.25–20.05	0.0376	±0.0013
3	*N,N'*-Tetramethylenebis(1-aziridinecarboxamide)	10	1.31–25.00	0.0363	±0.0018
4	*N,N'*-Octamethylenebis(1-aziridinecarboxamide)	6	0.86–13.82	0.0376	±0.0015
5	*N,N'*-(1,4-Cyclohexylenedimethylene)bis(1-aziridineacetamide)	5	1.11–17.90	0.0350	±0.0014
6	1-Aziridinylphosphonic acid cyclic diester with pentaerythritol	5	3.46–65.92	0.0004	±0.0003
7	1-*p*-Toluoylaziridine	6	1.96–62.72	0.0035	±0.0001
8	Tris(1-aziridinyl)-*p*-benzoquinone	5	1.31–41.99	0.0108	±0.0009
9	2,5-Bis(1-aziridinyl)-3,6-bis(2-methoxyethoxy)-*p*-benzoquinone	6	0.95–30.65	0.0224	±0.0015
10	2,4-Bis(1-aziridinyl)-6-methyl-5-nitropyrimidine	6	1.38–44.16	0.0241	±0.0008
11	2,4,6-Tris(1-aziridinyl)-*s*-triazine	5	5.08–20.32	0.0429	±0.0020
12	2,4,6-Tris(1-aziridinyl)-2,4,6-tris(dimethylamino)-2,2,4,4,6,6-hexahydro-1,3,5,2,4,6-triazatriphosphorine	7	0.81–25.97	0.0169	±0.0008
13	*p,p*-Bis(1-aziridinyl)-*N*-ethylphosphinic amide	5	1.84–29.58	0.0112	±0.0007
14	*p,p*-Bis(1-aziridinyl)-*N*-octylphosphinic amide	6	1.21–38.94	0.0134	±0.0004
15	*p,p*-Bis(1-aziridinyl)-*N*-propylphosphinic amide	5	1.51–25.03	0.0116	±0.0005
16	*p,p*-Bis(1-aziridinyl)-*N,N*-dimethylphosphinic amide	6	1.89–60.48	0.0087	±0.0003
17	*p,p*-Bis(1-aziridinyl)-*N*-isopropylphosphinic amide	6	1.67–53.51	0.0147	±0.0008
18	Ethyl [bis(1-aziridinyl)phosphinyl]carbamate	5	1.45–28.23	0.0026	±0.0002
19	*N*-[Tris(1-aziridinyl)phosphoranylidene] benzenesulfonamide	5	4.46–35.74	0.0011	±0.0002
20	Bis(1-aziridinyl)phenylphosphine sulfide	6	1.34–42.91	0.0125	±0.0003
21	Bis(1-aziridinyl)morpholinophosphine sulfide	6	1.36–43.74	0.0025	±0.0001
22	Bis(1-aziridinyl)morpholinophosphine oxide	6	1.47–47.08	0.0043	±0.0002
23	Bis(1-aziridinyl)(hexahydro-1*H*-azepin-1-yl) phosphine oxide	5	1.78–28.48	0.0080	±0.0002
24	*p,p*-Bis(1-aziridinyl)-*N*-(*p*-methoxyphenyl) phosphinic amide	7	1.32–42.41	0.0057	±0.0003
25	1,4-Piperazinediylbis[bis(1-aziridinyl) phosphine oxide]	5	3.15–12.60	0.0079	±0.0001

The rate values thus represented in Figure 3 reflect the number of actual analyses per hour. When sampling cams of 30 and 20 samples per hour were used, interposing wash cups were not necessary. The interference observed between the highest and lowest concentrations of Compound 3 at the higher sampling rates was not present, as evidenced by no difference in the percentage of absorbance of the low concentrations when run adjacent to the higher concentrations with and without the interposing wash cup. These methanol wash cups were not used in the subsequent tests made at the rate of 20 per hour.

The results obtained when the rates of sampling were changed were in agreement with the discussion by Thiers *et al.* (1966), that all rise and fall curves in continuous-flow systems are merely parts of one basic curve that has been curtailed before steady-state absorbance has been reached. This is evident when one compares the leading edge and the trailing edge of the absorbance peaks generated by a single concentration of Compound 3 run at different sampling rates to the peak generated by that concentration for a sufficient time to reach a steady-state plateau (Figure 4). Thus, this change can sometimes be used to advantage when a response is either too low or too high to give a meaningful reading. It is easier to retest the sample at a different rate to bring the response within the desired limits than to dilute or concentrate the sample or prepare a different concentration of NBP, particularly when the sample has to be used for some other purpose or the amount is limited.

The effect of the concentration of NBP on absorbance is shown in Figures 5 and 6. With Compound 3, a linear relationship was obtained to about 25×10^{-5} M at all concentrations of NBP. However, absorbance readings greater than 1.0 were not reliable; although sensitivity increased with an increasing concentration of NBP, the reaction was not linear

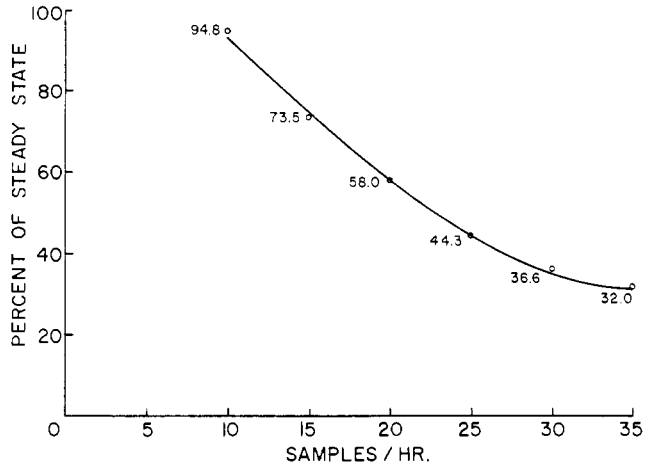

Figure 3. Percentage of attainment of steady-state absorbance (100%) when Compound 3 was analyzed at different sampling rates

Number of samples per hour represent one half actual cam rates, since a methanol wash cup was interspersed between samples

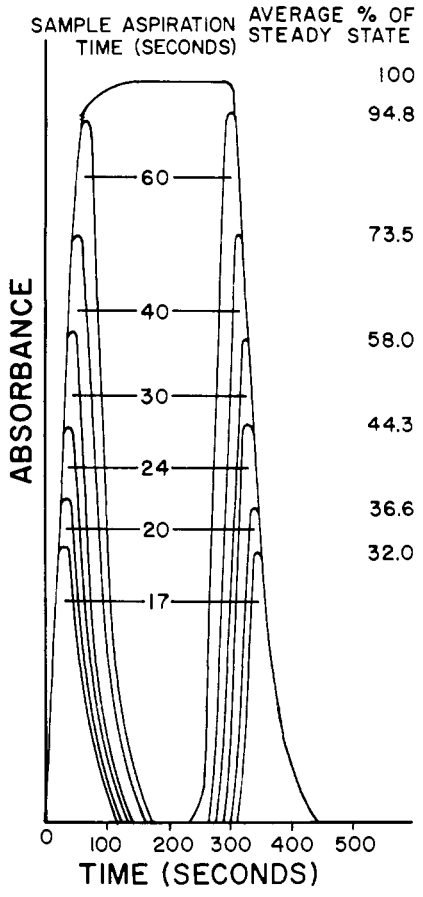

Figure 4. Effect of rate of sampling on rise and fall curves and percentage of attainment of steady-state absorbance

Note leading and trailing edges of peaks superimposed on steady-state trace

with concentrations above 2.5%. Therefore, if low concentrations of a compound containing aziridine are to be analyzed, sensitivity can be enhanced by using a higher concentration of NBP. However, when the higher concentration is used, one must determine the upper level of concentration of the alkylating agent that will keep the recorder response below 1.0.

The usefulness of this method was demonstrated for the residue analysis of aziridine-containing compounds: Individual adult *P. regina* were fortified with 20, 100, and 175 μg. of *N,N'*-tetramethylenebis(1-aziridine carboxamide) before extraction. Column blanks and insect blanks were also processed to determine if materials other than the chemosterilant eluting through the columns would interfere with the analysis. The results presented in Table III show that the extraction procedure for Compound 3 gives a recovery of about 97% of the chemical at the three levels tested, and interference from the columns or the flies is negligible.

Table III. Recovery of *N,N'*-Tetramethylenebis(1-aziridinecarboxamide) (Compound 3) from Individual *Phormia regina* (Meigen) Adults Fortified at Several Levels

Sample	Compound 3, μg. Added	Found	Recovery, %
Column blank	0	0	—
Standard	50	50 ± 1.7	100 ± 3.4
Extracted standard	50	48.8 ± 2.3	97.6 ± 4.6
Phormia blank	0	0.7 ± 0.2	—
Fortified *Phormia*	20	19.2 ± 0.6	96.0 ± 3.0
Fortified *Phormia*	100	96.3 ± 2.7	96.3 ± 2.7
Fortified *Phormia*	175	171.4 ± 3.9	97.9 ± 2.2

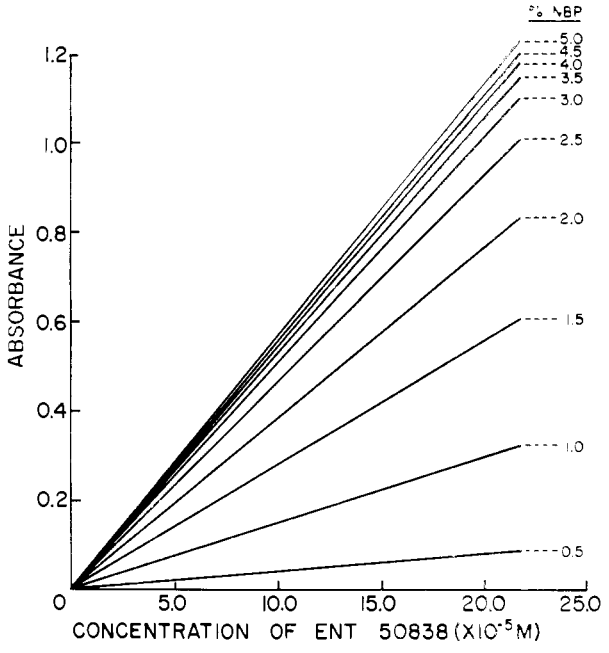

Figure 5. Linearity of response of Compound 3 at different concentrations of NBP

The method should prove useful in the analysis of residues of compounds containing aziridines that are used in and on insects and other biological materials. The manipulatory errors associated with manual techniques are practically eliminated with the AutoAnalyzer, because the variability is negligible between samples for each separate step of the analysis. Sensitivity can be adjusted from less than 1 to more than 1000 μg. per ml. by simply changing the rates of sampling or adjusting the concentration of NBP, a matter of only a few

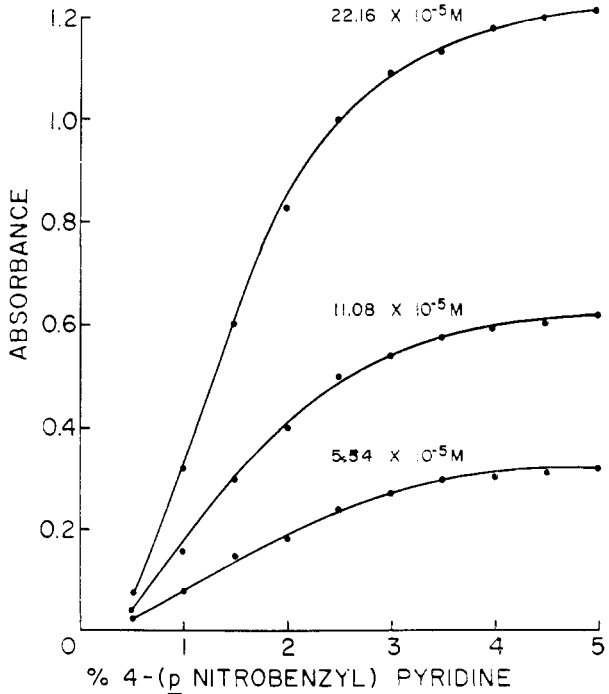

Figure 6. Effect of increasing concentrations of NBP on response of Compound 3

moments. The ability to analyze from a few to as many as 250 samples per 8-hour working day is another useful feature. Moreover, the method could be invaluable in the study of rate of alkylation and other kinetic phenomena associated with the chemistry of alkylating agents. Such studies are under way; they will be described in future reports.

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