cysteine found was 96.9%. Both samples I and II were analyzed by direct iodimetric titration in a medium of phosphoric acid–sodium dihydrogen phosphate of pH 2; the results were 96.2 and 97.5%, respectively (*16*, *17*). While these comparisons are instructive, especially in view of the fact that iodimetric methods have been so extensively used, the results cannot be considered as giving a reliable indication of accuracy. Doubts concerning the stoichiometric exactness of the reaction with iodine have been expressed many times, and the experience of these authors confirms them.

As a check on the absolute accuracy of the method could not be obtained in this way, samples of cystine were reduced and analyzed. Cystine is available commercially in nearly pure form, and, in contrast to cysteine, is stable. The optical rotation of the sample used (−208°) indicates a purity of 97.7% (*5*). The cysteine solution in 1*M* hydrochloric acid was reduced with sodium amalgam, a procedure which has been used before (*10*, *18*) for the same purpose. The technique used in this work was somewhat different from that previously described, and may be found advantageous. The results are shown in Table I, sample III; the −SH titer is in excellent agreement with that indicated by the optical rotation.

The work of Anson (*1*) had indicated that other amino acids would not interfere, but this was checked directly. The −SH titer found in the presence of a mixture of amino acids (aspartic acid, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), each in the same concentration as the cysteine, gave 96.6% cysteine, compared with 97.0% in the absence of added amino acids. Serine and tyrosine were tested individually because they are more likely to interfere. Cysteine found, in the presence and absence of added serine, was 97.0 and 96.7%, respectively; in the presence and absence of added tyrosine, 97.7 and 97.9%, respectively.

Table I. Summary of Analyses

| Sample | Taken, Mmole | No. of Determinations | % Cysteine | Av. Dev., % |
|---|---|---|---|---|
| I | 0.4 to 0.9 | 8 | 96.8 | 0.18 |
| | 0.015 to 0.055 | 6 | 95.8 | 0.52 |
| II | 0.3 to 0.6 | 15 | 96.4 | 0.47 |
| | 0.03 to 0.05 | 6 | 96.9 | 0.21 |
| | 0.0015 to 0.0025 | 4 | 96.9 | 0.15 |
| III | 0.0016 to 0.6 | 13 | 97.5 | 0.35 |

Adequate protection of the samples from oxidation is essential for satisfactory analytical work in this field. In the authors' experience, this requires considerable practice. It is well to standardize and continually to check the technique employed with cysteine samples as small as the lowest amounts it is desired to determine.


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# Polarographic Study of Cytotoxic Nitrogen Mustards


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**Pharmacologically active ethylenimonium ions formed by the cyclization of β-chloroethyl groups of aliphatic nitrogen mustards are reducible at the dropping mercury electrode. The diffusion current obtained upon reduction is linearly related to the concentration of the electroactive species over certain ranges of concentration. Polarographic procedures are described for the quantitative estimation of ethylenimonium intermediates, and for determining the rate at which the initial cyclization process occurs. The polarographic waves appear to be irreversible and a two-electron reduction is postulated.**


THE cytotoxic nitrogen mustards have enjoyed some degree of success in the chemotherapy of cancer (*11*). The pharmacologically active intermediate of this class of compounds is believed to be the ethylenimonium (aziridinium) cation, which is capable of alkylating the functional group of many compounds of biological importance (*12*).

Ethylenimonium ions are formed from parent compounds by the internal cyclization of β-chloroethyl groups in near neutral aqueous solutions (*6*). The initial cyclization process undergone by methyl-bis(β-chloroethyl)amine, a typical nitrogen mustard, is illustrated by the following equation:

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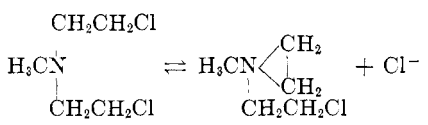
Table I. Rate of Initial Cyclization of MBA in Borate Buffer, pH 7.3, at 25° C.

| Time, Minutes | Determination 1 $i_t$, μa. | Determination 1 $(i_\infty - i_t)$, μa. | Determination 1 $k$, Minute$^{-1}$ | Determination 2 $i_t$, μa. | Determination 2 $(i_\infty - i_t)$, μa. | Determination 2 $k$, Minute$^{-1}$ |
|---|---|---|---|---|---|---|
| 0 | 2.16 | 3.52 | ... | 2.14 | 2.48 | ... |
| 5 | 3.76 | 1.92 | 0.123 | 3.21 | 1.41 | 0.119 |
| 10 | 4.64 | 1.04 | 0.132 | 3.84 | 0.78 | 0.140 |
| 15 | 5.14 | 0.54 | 0.118 | 4.23 | 0.39 | 0.124 |
| 20 | 5.38 | 0.30 | 0.126[a] | 4.41 | 0.21 | 0.112[b] |
| 25 | 5.52 | 0.16 | ... | 4.50 | 0.12 | ... |
| ∞ | 5.68 | | | | | |

[a] Av., 0.125 ± 0.004. $k$ from slope, 0.123.
[b] Av., 0.124 ± 0.008. $k$ from slope, 0.125.



In dilute aqueous solutions this reaction proceeds practically to completion, and cyclization of the secondary β-chloroethyl group may occur only after the antecedent cyclic structure has undergone hydrolysis and the initial cyclization process is well advanced (*4*).

Ethylenimonium ions may be determined quantitatively by reaction with thiosulfate. The usual procedure is to add an excess of standard thiosulfate solution to an aliquot solution of ethylenimonium ions and then to determine the unreacted thiosulfate iodometrically—the amount of thiosulfate consumed is equivalent to the concentration of ethylenimonium ions (*4*, *6*).

This present work is concerned with the polarographic behavior of ethylenimonium ions in solutions of aliphatic nitrogen mustards. It has been found that the quaternary nitrogen of certain ethylenimonium intermediates is polarographically reducible and well-formed polarographic waves are obtained upon reduction. Based on these findings, a method has been developed for the quantitative assay of ethylenimonium ions. This method has been applied to a study of the kinetics of the initial cyclization process for certain aliphatic nitrogen mustards.

### APPARATUS AND MATERIALS

A Sargent Model XXI visible recording polarograph, with a dropping mercury electrode and a Lingane-Laitinen H-type polarographic cell (*8*) containing a saturated calomel reference electrode, was used. In certain experiments, in which it was desirable to prevent chloride ion contamination from the salt bridge, a Carritt-type H-cell was employed (*2*). Dissolved oxygen was removed from test solutions with tank nitrogen, which was purified by passing the gas through a train of vanadous sulfate solution (*10*) and then washed with distilled water. All sample solutions were degassed for 15 minutes. The resistances of the polarographic cells used in this study, as determined by a conductivity bridge, were all less than 1000 ohms and no correction for $iR$ drop was made in measuring $E_{0.5}$ values.

The capillary used in the study of methyl-bis(β-chloroethyl)amine (2,2′-dichloro-*N*-methyl-diethylamine, MBA), and tris(β-chloroethyl)amine (2,2′,2″-trichloro-triethylamine, TBA), had a constant $m^{2/3}t^{1/6}$ value of 1.904 mg.$^{2/3}$ sec.$^{-1/2}$ in the base solution at −1.00 volt *vs.* S.C.E. The capillary used in the study of ethyl-bis-(β-chloroethyl)amine (2,2′-dichlorotriethylamine, EBA) had a constant $m^{2/3}t^{1/6}$ value of 1.884 mg.$^{2/3}$ sec.$^{-1/2}$ in the base solution at −1.20 volts *vs.* S.C.E. The base solution consisted of a borate buffer system having a pH of 7.3 (*1*) and containing 0.002% of Triton X-100 as maximum suppressor.

Analytical reagent grade chemicals were used throughout. The nitrogen mustards were obtained as the hydrochloride salts. MBA, observed melting point of 109–10° C. (corrected), was supplied by Merck & Co., Inc., Rahway, N. J.; TBA, observed melting point of 130–31° C. (corrected), was obtained from the Sloan-Kettering Institute for Cancer Research; EBA, observed melting point of 140–41° C. (corrected), was supplied by the Army Chemical Center, Md. The observed melting points correspond closely to the values reported in the literature. All of the nitrogen mustards were recrystallized from acetone solutions with ether and dried over phosphorus pentoxide in a vacuum desiccator.

### KINETIC STUDIES

Kinetic studies were performed on the initial rate of cyclization of MBA and EBA. Polarographic data showed the rate of formation of the reducible species to be first order. This constituted strong evidence that ethylenimonium ions were being reduced, as the initial reaction of aliphatic nitrogen mustards in aqueous solutions has been shown to be a first-order cyclization process (*3*).

Kinetic studies were carried out in borate buffered solutions, pH 7.3, at 25° C. Carritt-type polarographic cells were used throughout these studies. All solvents were first equilibrated to a temperature of 25° C. prior to actual use. These kinetic experiments were conducted on the assumption that the limiting current was proportional to the concentration; this presumption was later verified. The general procedure was to prepare a solution of the nitrogen mustard in degassed base solution and then to follow the increase in concentration of the reducible species polarographically as a function of time. This was done by applying a constant potential (−1.5 volts *vs.* S.C.E.) corresponding to a point on the plateau of the reduction wave and measuring the increase in current with time. The reaction was considered to be complete when the current reached a steady state and no longer increased with time. Measurements of time were made with an electric timer. The results obtained using different initial concentrations of MBA are given in Table I.

The values for the velocity constants given in Table I were calculated from the following equation:

$$K = \frac{1}{(t - t')} \ln \frac{(i_\infty - i_t')}{(i_\infty - i_t)}$$

where $i_\infty$ represents the current value when the reaction was considered complete for all practical purposes, and $i_t$ and $i_t'$ current measurements at times $t$ and $t'$, $t$ being greater than $t'$. The accepted velocity constant was determined graphically from a plot of $\ln(i_\infty - i_t)$ against time. A straight line was drawn through the experimental points by inspection and the velocity constant determined from the slope of the line (*5*).

An average velocity constant of 0.313 min.$^{-1}$ for the initial cyclization process of TBA was obtained when determined graphically by the above method. Although a similar type of current-time curve was obtained for EBA as that for MBA and TBA, the cyclization process occurred at a rate which was too rapid to permit an accurate determination of the velocity constant by the above procedure. The difference between the initial current reading and the reading when the current reached a steady state never exceeded 1.2 μa. Consequently, a calculation of the velocity of the initial cyclization process of EBA did not appear to be possible with any degree of accuracy or precision.

From the data obtained by polarographic kinetic studies, it may be concluded that the initial cyclization process is first order and that the electroactive species is quaternary nitrogen in the form of ethylenimonium intermediates.

### POLAROGRAPHIC DETERMINATION OF ETHYLENIMONIUM IONS

The following procedure was used to construct wave height–concentration plots for ethylenimonium ions in solutions of aliphatic nitrogen mustards. Henceforth, quaternary nitrogen in the

form of ethylenimonium derivatives will be referred to as EI.

Aliquots of a nitrogen mustard hydrochloride stock solution ($5.00 \times 10^{-3}M$ in the case of MBA and TBA, and $4.00 \times 10^{-3}M$ in the case of EBA) were transferred to a 100-ml. volumetric flask containing 50.0 ml. of a borate buffer concentrate, such that when the solution was diluted to 100 ml. the resulting solution had a pH of 7.3. This standard solution was degassed for 15 minutes and electrolyzed over a potential span of approximately −0.40 to −1.60 volts *vs.* S.C.E. When 50% of the potential span was traversed, an equal aliquot of the stock solution was transferred to a second 100-ml. volumetric flask which also contained 50.0 ml. of buffer concentrate. This solution was titrated to a pH of 4 with $0.4N$ sulfuric acid (in order to arrest further cyclization) using methyl orange indicator. The end point color was determined by comparison to a known buffer solution, pH 4.0, containing methyl orange. To this solution a known excess volume of standard $1.25 \times 10^{-2}M$ sodium thiosulfate was added while stirring, and the solution diluted to 100 ml. The solution was then degassed for 15 minutes and the residual thiosulfate determined polarographically, over a potential span of −0.40 to +0.14 volt *vs.* S.C.E. (*9*). A blank solution containing an equal volume of thiosulfate was also run. The amount of thiosulfate consumed was determined by subtracting the diffusion current of the residual thiosulfate in the standard solution from the diffusion current obtained by electrolyzing the blank solution, and then determining the equivalent concentration of thiosulfate from a previously constructed calibration curve of thiosulfate. Experimental data used in the construction of the thiosulfate calibration curve were obtained under identical conditions at which the thiosulfate was made to react with EI.

Once the relationship between the limiting current produced by the reduction of EI and concentration of EI is established, the determination of residual thiosulfate is no longer necessary, because the concentration of EI can be obtained directly from the height of polarograms of EI by using previously constructed wave height-concentration curves. Table II illustrates the proportionality between the limiting current and the concentration of EI in solutions of nitrogen mustards.

**Table II. Calibration Data for Determination of Ethylenimonium Ions (EI) in Solutions of Nitrogen Mustards, pH 7.3**

| Thiosulfate Consumed, Mmole Liter$^{-1}$ | $i_d$, μa. | $E_{0.5}$ *vs.* S.C.E. of EI, Volts | $\frac{i_d}{C}$ |
|---|---|---|---|
| MBA Solutions | | | |
| 1.02 | 8.84 | −1.14 | 8.67 |
| 0.71 | 6.24 | −1.12 | 8.79 |
| 0.57 | 4.98 | −1.12 | 8.74 |
| 0.38 | 3.30 | −1.08 | 8.68 |
| 0.28 | 2.48 | −1.08 | 8.86 |
| EBA Solutions | | | |
| 1.04 | 7.44 | −1.08 | 7.15 |
| 0.90 | 6.56 | −1.07 | 7.29 |
| 0.76 | 5.46 | −1.06 | 7.18 |
| 0.63 | 4.47 | −1.04 | 7.10 |
| 0.42 | 3.00 | −1.02 | 7.14 |
| 0.25 | 1.80 | −1.01 | 7.20 |
| TBA Solutions | | | |
| 2.10 | 11.6 | −0.97 | 5.52 |
| 1.68 | 9.30 | −0.96 | 5.54 |
| 1.56 | 8.56 | −0.96 | 5.49 |
| 1.25 | 6.96 | −0.96 | 5.57 |
| 1.18 | 6.54 | −0.95 | 5.54 |

**Table III. Variation of Half-Wave Potentials of EI with pH in Solutions of Nitrogen Mustards**

| pH[a] | $E_{0.5}$ *vs.* S.C.E., Volts | $i_d$, μa. |
|---|---|---|
| MBA Solutions | | |
| 7.31 | −1.14 | 8.84 |
| 8.10 | −1.17 | 8.80 |
| 9.25 | −1.24 | 8.48 |
| EBA Solutions | | |
| 7.35 | −1.03 | 3.96 |
| 8.10 | −1.08 | 3.72 |
| 9.25 | −1.10 | 3.68 |
| TBA Solutions | | |
| 7.25 | −0.96 | 7.62 |
| 8.30 | −1.02 | 7.10 |
| 9.15 | −1.10 | 6.70 |

[a] Measurements of pH were made with a Beckman Model GS pH meter operated as a standard Model G meter.

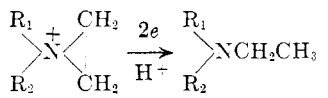
### ANALYSIS OF POLAROGRAPHIC WAVES

The dependence of the $E_{0.5}$ values of various ethylenimonium forms on pH is illustrated in Table III. In all cases, the $E_{0.5}$ values shift to more negative potentials with increasing pH. This indicates that hydrogen ions are involved in the reduction process. The test solutions were prepared by the same procedures outlined for the preparation of EI standard solutions. Polarograms were recorded in the usual manner one hour after the solutions were prepared.

The fact that the $E_{0.5}$ values of the polarographic waves obtained by electrolyzing solutions of nitrogen mustards vary with the concentration of the reducible EI species at a constant pH indicates that the electrode reaction is irreversible (*7*).

### POSTULATED MECHANISM OF REDUCTION

Because of the complex nature of the reducible species in a solution of a nitrogen mustard, it is difficult to formulate the net electrode reaction in the form of a general reduction equation which would apply to all the cases studied. However, from theoretical considerations, a two-electron transfer is postulated in the reduction of each ethylenimino group. The reduction of a quaternary nitrogen intermediate may be represented as follows:

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