[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY[1]]

Ion Exchange Studies of Transguanylation Reactions. I. Rearrangement of S,2-Aminoethylisothiourea to 2-Mercaptoethylguanidine and 2-Aminothiazoline[2]

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RECEIVED MAY 10, 1957

Quantitative ion-exchange chromatography has been used for examination of the conversion of S,2-aminoethylisothiourea (AET) to 2-mercaptoethylguanidine (MEG) and 2-aminothiazoline (2-AT). The nature and amount of the reaction products were found to be dependent on both *p*H and time. In strong acid, AET remains unchanged; at *p*H 2.5 it is converted to 2-AT, and at *p*H 7 the only product is MEG. At *p*H's above 12, the intratransguanylation reaction competes with the normal hydrolysis of the thiuronium group, and mixtures of MEG and 2-mercaptoethylamine (MEA) are formed. These reactions may be best explained through the formation of a cyclic intermediate that may then open to yield MEG or may split off ammonia to yield 2-AT.

A series of aminoalkylisothiuronium salts capable of undergoing rearrangement to mercaptoalkylguanidines have been found highly effective in the protection of mice against an otherwise lethal dose of X-radiation.[3,4] The behavior of the prototype of this series, S,2-aminoethylisothiuronium bromide hydrobromide (AET), in aqueous neutral solution, led to an hypothesis that proposed chemical change through a cycle intermediate.[4] The essential details of the mechanism of rearrangement for these compounds, now thought to involve an intratransguanylation step is the subject of another communication.[5] In strong alkali, a different type of reaction, which involves cleavage of the guanyl group of these isothiuronium compounds, can also occur.[6] Both types of reactions are shown here for AET (Fig. 1).

These reactions are in accord with known reactions of similar compounds; for example, the hydrolysis of alkylisothiuronium compounds in strong alkali produces alkylmercaptans and dicyandiamide (DCD)[6] and the oxidation of mercaptans results in the formation of disulfides. A compound similar to 2-AT (Fig. 1), 2-methylthiazoline, has been shown by Linderstrøm-Lang and Jacobsen to be in rapid equilibrium with ammonium ion to form a straight-chain mercaptan containing an amidinium ion.[7] Calvin[8] has presented conclusive evidence to show the formation of a thiazoline ring structure within the molecule of glutathione through the interaction of the mercapto grouping of the cysteinyl moiety with that of the carbonyl grouping of the γ-glutamyl residue. According to Basford and Huennekens,[9] one of the forms of coenzyme A has a thiazoline structure formed by the intramolecular reaction between the carbonyl group of the terminal amide and the thiol group of this molecule. The hydrolysis of β-hydroxyisothiuronium salts to yield an olefinic sulfide through a transguanylation mechanism similar to that presented here has been reported by Bordwell and Andersen.[10]

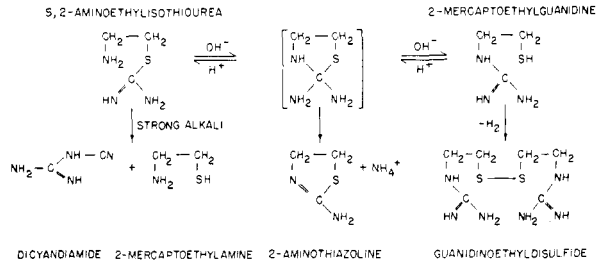

Fig. 1.

The experiments described here for AET had as a dual objective the development of a general procedure for the study of the mechanism of rearrangement of aminoalkylisothiuronium compounds that would also be suitable for the isolation of the reaction products from artificial mixtures or from solutions obtained from biological sources. It will be shown that the transformations involved are dependent on both *p*H and time. Since the products formed from these compounds in neutral or alkaline solution, as well as the compounds themselves, are stable in strong acid solution (0.2 *N* HCl), the reaction can be stopped at any given time by the addition of concentrated acid. The separation and chemical characterization of the products from AET on a strong-acid, cation-exchange resin is described in this communication. Higher homologs of AET and substituted derivatives of aminoalkylisothiuronium salts will be considered in a subsequent publication.[11]

Experimental

Materials.—S,2-Aminoethylisothiuronium·Br·HBr was prepared as previously described.[4,5] The reaction products derived from AET(NH_4^+, MEA, 2-AT, GED, MEG. DCD) were compared with authentic materials obtained from commercial sources or synthesized by known procedures.

Nitrogen Determination.—Two-milliliter aliquots were digested in micro-Kjeldahl flasks with 0.2 ml. of 9 *M* H_2SO_4. The samples were fumed for 20 minutes, cooled, 2 drops of 7% potassium persulfate added, and the digestion was continued for 10 minutes. The samples for Nessleriza-

(1) Operated by Union Carbide Nuclear Co. for the U. S. Atomic Energy Commission.

(2) Presented in part at the Southwide Chemical Conference (Southeastern and Southwestern Sections of the American Chemical Society), Memphis, Tenn., December 6–8, 1956.

(3) D. G. Doherty and W. T. Burnett, Jr., *Proc. Soc. Exptl. Biol. Med.*, **89**, 312 (1955).

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(7) K. Linderstrøm-Lang and C. F. Jacobsen, *Compt. rend. trav. lab. Carlsberg, Sér. Chim.*, **23**, 289 (1938–1941); *J. Biol. Chem.*, **137**, 443 (1941).

(8) M. Calvin, in "Glutathione, A Symposium," eds. S. P. Colowick, *et al.*, Academic Press, Inc., New York, N. Y., 1954, pp. 3–30.

(9) R. E. Basford and F. M. Huennekens, THIS JOURNAL, **77**, 3878 (1955).

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tion were prepared by the method of Polley.[12] The digest mixture was partially neutralized with 12 *N* KOH, a few drops of gum ghatti added, diluted to volume (10 ml.), cooled in an ice-bath for 5 minutes, and the color developed by the addition of 2 ml. of cold nessler solution. A model C Beckman colorimeter with the blue filter was used for the determination of absorbancy. A linear relation between absorbancy and nitrogen content was obtained on NH_4Cl standards from 0–100 γ of nitrogen/ml.

Thiol Group Assay.—Sulfhydryl groups were determined by their oxidation with the blue dye, 2,6-dichloroindophenol. The procedure was adapted from the method reported by Basford and Huennekens.[13] The model C Beckman colorimeter was again used for these determinations, but with a red filter. The sulfhydryl test was carried out in the following manner: a dye solution having an absorbancy of approximately 1.0 was prepared from 3 mg. of the dye dissolved in 150 ml. of 0.4 *M* K_2HPO_4 buffered at *p*H 7.0. To 5 ml. of this dye solution was added 0.2 ml. of a thiol solution, and 0.2 ml. of a solution containing no thiol was added to a duplicate control tube. Both tubes were read against a water blank. Decolorization of the dye begins with the addition of the thiol solution; after about 2 minutes, a stable final reading can be obtained. This final reading subtracted from the unchanged reading of the control tube gives a difference of absorbancy (ΔA) that is proportional to the amount of sulfhydryl initially present. A linear relation between –SH content and ΔA was obtained for solutions of MEG and MEA in the concentration of 0–2 μmoles/ml. The thiols, in 0.2 *N* HCl, are stable in this concentration range whereas at *p*H 7 and higher they are readily oxidized by air to the disulfides. For example, the half-time oxidation rates of MEA and MEG at *p*H 7.0 and at a concentration of 1 μmole/ml. are, respectively, 2 and 24 hours. This use of concentrated solutions (50–100 μmoles/ml.) in stoppered vessels and preparations of the suitable test dilutions in 0.2 *N* HCl essentially eliminated oxidation. A relative extinction based on the addition of a known amount of thiol added to the dye was calculated from the equation $a = \Delta A/c$ where a is the absorptivity extinction, and ΔA is the difference in absorbancy obtained when 0.2 ml. of a solution of a thiol compound, having the concentration c, is added to 5 ml. of the dye solution. Extinctions of 0.42 unit/μmole and 0.46 unit/μmole were obtained for MEG and MEA, respectively. These values were used for empirical calculations of the concentration of any unknown MEG and MEA solutions. AET also can be estimated from these values since it is converted almost instantaneously to MEG upon addition to the buffered dye solution.

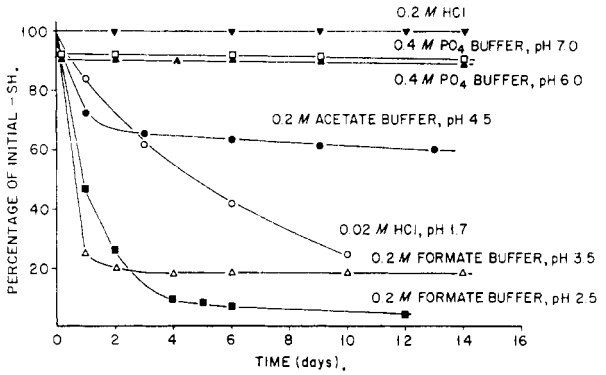

Fig. 2.—Percentage of –SH remaining *vs.* time of standing of AET solutions initially at 25 mg./ml. of AET at various *p*H's.

Ion-exchange Chromatography.—All separations were made with 200–400 mesh strong-acid cation exchanger (Dowex-50, hydrogen form). Duplicate columns 1 sq. cm. in cross section and 5 cm. high were prepared in the conventional manner.[14] Samples were absorbed on the resin columns from 0.2 *N* HCl solutions (20–100 ml.) and eluted with a succession of increasing concentrations of HCl. Fractions were collected with an automatic sample changer.

Procedure.—Solutions of AET prepared at a given *p*H were chromatographed at different time intervals for determination of chemical changes. The time chosen to make a particular column run was determined, to a large extent, from the data obtained in Fig. 2, which shows the percentage of initial sulfhydryl remaining after fresh solutions of AET (25 mg./ml.) were kept at the acidities and the time intervals shown. The reactions were stopped by the addition of acid to aliquots of these solutions, and the mixtures of products obtained were then separated on identical cation-exchange columns, the same amounts of eluting agents being used for each determination. The position of each peak was compared to a standard column run made on known amounts of authentic materials. An example of such a standard run is shown in Fig. 3, curve 1, which gives the separation of NH_4^+, 2-AT, MEG, AET and GED initially prepared as a mixture. DCD, when present, did not absorb, since it lacks a strong-basic group.[15]

The response of these compounds to a combination of several different chemical tests was a further aid to their identification when the compounds were isolated as products from AET. NH_4^+ analyzes the same as inorganic and total nitrogen, MEA gives organic nitrogen and sulfhydryl in the ratio of 1:1, and 2-AT gives only an organic nitrogen test and does not respond to the sulfhydryl test at *p*H 7.0. AET and MEG give both nitrogen and sulfhydryl in the ratio of 3:1, and the latter compound also gives a positive Sakaguchi test[16] for the guanidino group and is oxidized by I_2 or Br_2 at *p*H 7.0 to guanidinoethyl disulfide (GED), which may be assayed in column eluates through its organic nitrogen content. Further identification rested on the position of a peak with respect to the basicity of the groups, the number of ionic charges present in each compound, and the isolation of crystalline derivatives.

Preparation of Flavianic Acid Derivatives. 2-Mercaptoethylguanidine Flavianate.—One gram of AET was dissolved in 20 ml. of 0.18 *N* NaOH (final *p*H 7.0–7.5) to convert it to MEG. The addition of 4.0 ml. of 1 *M* flavianic acid gave an immediate precipitate of the MEG flavianate. The crystalline precipitate was filtered, washed successively with ice-water, ethanol and ethyl acetate, and dried *in vacuo*. Recrystallization from water or absolute alcohol gave an 80% yield of a pure product with a melting point of 170–173°.

Anal. Calcd. for $C_{13}H_{15}N_5O_8S_2$: C, 36.00; H, 3.50; N, 16.18; S, 14.79. Found: C, 36.31; H, 3.55; N, 16.37; S, 14.88.

2-Aminothiazoline Flavianate.—AET (2.81 g.) was dissolved in 50 ml. of 0.6 *M* formate buffer, *p*H 2.5, and the solution allowed to stand 11 days. The flavianate salt was then precipitated from the solution by the addition of 12.0 ml. of 1 *M* flavianic acid. The precipitate was filtered off, washed with water, dried, and recrystallized from water to give an 87% yield of the product, m.p. 248–250° with decomposition. The flavianate prepared from a crystalline sample of 2-AT had the same melting point, and the mixed melting point of the two flavianates showed no depression.

Anal. Calcd. for $C_{13}H_{12}N_4O_8S_2$: C, 37.50; H, 2.91; N, 13.50; S, 15.40. Found: C, 37.43; H, 3.00; N, 13.51; S, 15.48.

Results

AET dissolved in 0.2 *N* HCl retained its available sulfhydryl content for over 14 days (Fig. 2), and when chromatographed at the end of this time, only one peak was obtained (Fig. 3, curve 2). When AET was buffered at *p*H 2.5, the sulfhydryl content decreased, as demonstrated in Fig. 2. An aliquot of solution at this *p*H taken for analysis at the end of 18 hours showed mostly unchanged AET but also considerable amounts of NH_4^+ and 2-AT (Fig. 3, curve 3). The same solution rechromatographed at

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(15) See V. Migrdichian in the American Chemical Society Monograph, "The Chemistry of Organic Cyanogen Compounds," Reinhold Publ. Corp., New York, N. Y., 1947, p. 15.

(16) See F. C. Koch and M. E. Hanke in "Practical Methods in Biochemistry," 5th Edition, Williams and Wilkins Co., Baltimore, Md., 1948, p. 51.

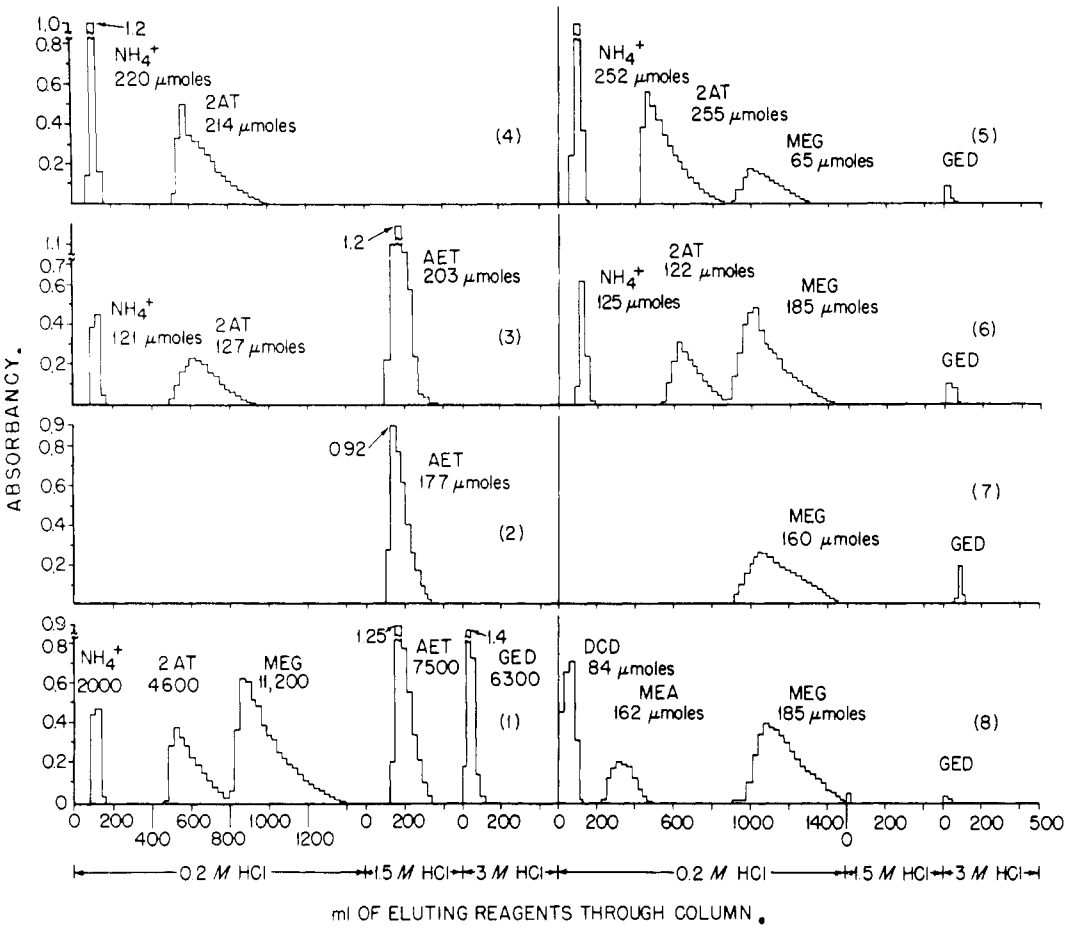

Fig. 3.—Ion-exchange analysis of products obtained from AET; columns 1.0 cm.^{2} × 5 cm. Dowex-50-H$^+$, 200–400 mesh, flow rates 0.6–0.8 ml./min. Ordinate, concentration of nitrogen determined colorimetrically by Nesslerization; abscissa, milliliter of eluting solutions through column as shown. Curve 1, NH_4^+, 2-AT, MEG, AET and GED prepared as a mixture in 0.2 *M* HCl, in the amounts shown on the graph expressed as total micrograms of nitrogen. MEG was prepared by adding 1 equiv. of NaOH to a freshly prepared water solution of AET. This solution was added to the 0.2 *M* HCl solution containing the other products. Curve 2, 2 ml. of AET in 0.2 *M* HCl taken at the end of 14 days; curve 3, 4 ml. of the *p*H 2.5 solution at the end of 18 hours; curve 4, 2½ ml. of the *p*H 2.5 solution at 5 days; curve 5, 4 ml. of the *p*H 3.5 solution at 4 days; curve 6, 4 ml. of the *p*H 4.5 solution at 4 days; curve 7, 2 ml. of the *p*H 7.0 solution at the end of 10 minutes; curve 8, 4 ml. of AET dissolved in 2 *N* NaOH for 15 minutes. Aliquots of the equilibrating solutions (all initially as 25 mg./ml. of AET) were adjusted to 20 ml. with 0.2 *M* HCl prior to sorption on the columns. In curve 8, the influent volume was adjusted to 100 ml. All influent volumes are included as part of the 0.2 *M* HCl eluting reagent. Recoveries ranged from 90 to 97%.

the end of 5 days demonstrated essentially the complete conversion of AET to equimolar amounts of NH_4^+ and 2-AT (Fig. 3, curve 4). A solution of AET equilibrated at *p*H 3.5 for 4 days gave rise to a small amount of MEG (Fig. 3, curve 5) but the major chemical change was still the production of equimolar amounts of NH_4^+ and 2-AT, in agreement with the decay curve of Fig. 2 for this *p*H. The equality between NH_4^+ and 2-AT was maintained at *p*H 4.5, but there also appeared, after the solution had equilibrated for 4 days, a quantity of MEG larger than that produced at *p*H 3.5 in the same length of time (Fig. 2, and Fig. 3, curve 6). The same solution of *p*H 4.5 analyzed after only 20 minutes contained 53 mole % of unreacted AET; the other materials present were NH_4^+, 2-AT and MEG (run not shown in Fig. 3). At *p*H 7.0, the only product obtained other than a small amount of GED produced by oxidation was MEG. The experiment shown in curve 7 of Fig. 3 was begun after AET had been dissolved in phosphate buffer of *p*H 7.0 for 10 minutes, at which time the amount of GED produced amounted to only 5% of the initial MEG nitrogen. At the end of 12 days, the amount of GED was increased only slightly (about 19% of the MEG nitrogen). The same results were obtained when 1 equivalent of NaOH was added rapidly to freshly dissolved AET dihydrobromide in water. The final *p*H in this case was 7.0 and the column run was carried out immediately after the addition of the base. MEG was also prepared in a different manner. Guanidinoethylisothiourea was synthesized and then hydrolyzed in alkaline solution to MEG and DCD. The ion-exchange properties of this MEG were identical to MEG obtained from AET.

Only at very high *p*H's does the transguanylation

of AET, through a cyclic intermediate to form MEG, yield to a side reaction. In 0.4 *N* NaOH, the major product recovered was MEG (80% of the total initial nitrogen) along with a small amount of MEA and formed DCD *via* the normal alkaline hydrolysis of isothiuronium compounds (see Fig. 1). When S,2-aminoethylisothiourea was dissolved in 2 *N* NaOH and chromatographed 15 minutes later, only 52 mole % of the initial AET was recovered as MEG. The other products were stoichiometric amounts of DCD and MEA (Fig. 3, curve 8).

Discussion

In accordance with the chemical properties of AET, both the alkylamino and guanylamino groups of this compound would be positively charged in strongly acidic solutions. Consequently, any interaction of these two terminal groups, initiating chemical change, would be unlikely. This concept is thoroughly demonstrated by the fact that AET retained completely its available sulfhydryl content in 0.2 *N* HCl and only unchanged material was recovered when aliquots of such solutions were analyzed by the ion-exchange procedure either immediately or at the end of 14 days (Fig. 3, curve 2). As the *p*H is increased, the proton of the alkylamino group of AET competes for this amino group with the proximate guanylamino carbonium ion, and formation of the unstable cyclic intermediate can then occur. In the *p*H range 2.5–3.5 (Fig. 3, curves 3, 4, 5), the favored reaction is the splitting of ammonia off the cyclic intermediate to yield 2-AT as the major product. Apparently, the driving force toward the formation of these two products in this *p*H range is the release of ammonia from the cyclic intermediate and its subsequent neutralization by excess acid present, resulting in the formation of ammonium ion and leaving cationic 2-AT as the other major product. At increasingly higher *p*H's, this driving force lessens and the alternative is the formation of the strongly basic, highly resonating structure of the guanidino group present in MEG. At *p*H 7.0 (Fig. 3, curve 7), the transguanylation reaction is predominant, the cyclic intermediate opening to yield exclusively MEG. MEG remains the only product up to a *p*H of about 12. Above that level, MEA begins to appear, about 10% being found at an apparent *p*H of 13.5. Even in an excess of 2 *N* NaOH, the transguanylation reaction competes with hydrolysis and an approximately equal mixture of MEA and MEG is obtained.

The transformation of AET in solutions ranging from strongly acidic to neutral indicates that the mechanism of change is through the cyclic intermediate according to the reactions seen in Fig. 1. Although the reverse reaction to re-form components through the cyclic intermediate has not yet been found for AET, it has been found in higher homologs and substituted alkylamino derivatives of AET. This finding of reversibility lends support to the proposed cyclic intermediates. A mercaptoalkylguanidine would have similar equilibrium forms of the type reported by Benesch and Benesch[17] for aminothiols (see Fig. 4). In strong acid

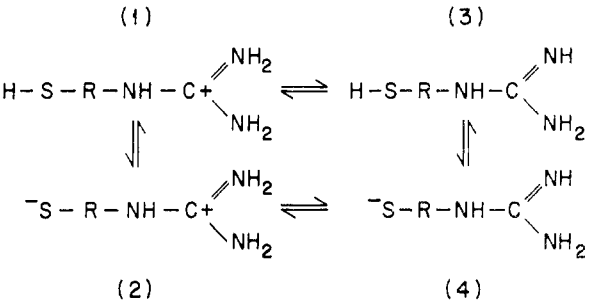

Fig. 4.

solution, form (1) would predominate and would remain a stable structure owing to the association of the thiol and the resonating structures of the guanido group. As the *p*H is increased, the other forms would begin to appear and at some preferred *p*H it is most likely that form (2) is the structure best adapted to ring closure, since there is an attraction of a positive and negative charge that would result in bringing the carbon of the guanido grouping in close proximity to the sulfide ion. Once the *p*H is raised too high, this attractive force would again be lost. Initial studies on derivatives of AET have supported this reasoning.[11]

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