17 hours after which the solution was evaporated to dryness *in vacuo* under nitrogen. The crystalline residue melted at 145–150°, yield 1.215 g. (94.6%). Three crystallizations from water raised the melting point to a constant value of 150.5–151.5°. Its picrate (m.p. 164–165°) was formed in 94.4% yield in the usual manner from ethanol. Two crystallizations from water raised the melting point to 166.5–167.5°.

The preparation of 2-(β-(N-(9-phenanthrylmethyl)-carbamyl)-ethylamino)-2-thiazoline was carried out under similar conditions with the exception that the ethanolic solution of reactants was refluxed for 4 hours.

Attempts to form amides with morpholine, diisopropylamine and diethylamine under similar reaction conditions gave an 80–90% recovery of unchanged 5-keto-2,3,6,7-tetrahydro-5(H)-thiazolo[3,2-a]pyrimidine.

Methyl Ester of 2-(ε-Carboxypentylamino)-Δ²-dihydro-1,3-thiazine.—2-(ε-Carboxypentyl)-Δ²-dihydro-1,3-thiazine (6 g., 0.026 mole) in absolute ethanol (100 ml.) containing 5% hydrogen chloride was converted into its ethyl ester under the conditions described above for the preparation of 2-(β-carbethoxyethylamino)-2-thiazoline. Its solution was evaporated to dryness *in vacuo* and a yellow gummy residue was obtained. The yellow gum was dissolved in methanol (200 ml.) and passed through a column of IR-A 400 resin (150 ml. of resin in the hydroxyl form) at a rate of 8.3 ml./min. After the column was washed with methanol (250 ml.), the combined eluate and washings were evaporated to dryness *in vacuo* under nitrogen. The residue crystallized (m.p. 45–50°) on standing overnight, yield 5.21 g. (77.6%). The crude free base was extracted with hot hexane (125 ml.) and the extract was concentrated to a volume of 50 ml. Crystals (m.p. 53–55°) separated from the solution on cooling, yield 4.46 g. Another crystallization from *n*-hexane raised the melting point to 54–55° (*cf.* Table II). During the passage of the ethyl ester of 2-(ε-carboxypentylamino)-Δ²-dihydro-1,3-thiazine in methanol through the column of IR-A 400 resin transesterification occurred and the methyl ester of the acid was obtained.

A sample (220 mg.) of this product on treatment with ethanolic picric acid solution gave a crystalline picrate (m.p. 80–86°), yield 100%. One crystallization from methanol (10 ml.) and three from benzene–hexane (1:1) solution (20 ml.) gave a product with a double melting point (m.p. 82–83° and 87–87.5°).

2-(ε-(N-Methylcarbamyl)-pentylamino)-Δ²-dihydro-1,3-thiazine.—A solution of 2-(ε-carbomethoxypentylamino)-Δ²-dihydro-1,3-thiazine (3 g., 0.012 mole) in absolute methanol (250 ml.) containing methylamine (14.4 g., 0.046 mole) and sodium (0.1 g., 0.004 mole) was allowed to stand at room temperature for 120 hours. The solution, which turned cloudy after 20 hours, was taken to dryness *in vacuo* under nitrogen. A white solid residue (m.p. 115–120°) was obtained, yield 3.2 g. Crystallization of the crude product from ethanol–*n*-hexane solution gave 1.70 g. (57%) of crystals melting at 131–135°. Four additional crystallizations from the same solvent combination raised the melting point to 136.5–137.5°.

A picrate (m.p. 123–124.5°) was prepared in the usual manner from aqueous solution in 92% yield. One crystallization from ethanol–water (20:1) solution raised the melting point to 124.5–125.5°. Both the free base and the picrate are described in Table II.

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

Ion Exchange Studies of Transguanylation Reactions. II. Rearrangement of 3-Aminopropylisothiourea and N-Substituted Aminoethyl- and Aminopropylisothioureas to Mercaptoalkylguanidines and 2-Aminothiazolines or Penthiazolines

BY JOSEPH X. KHYM, DAVID G. DOHERTY AND RAYMOND SHAPIRA

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Quantitative ion-exchange chromatography was used to establish that intratransguanylation is dependent on *p*H as well as on the number of carbon atoms between the amino and isothiourea groups. The aminoethyl- and aminopropylisothioureas intratransguanylate readily at neutral *p*H but S,4-aminobutylisothiourea does not. At *p*H's 3–6, 2-aminothiazolines and -penthiazolines are formed, whereas in strong alkali, mixtures of mercaptoguanidines and mercaptoamines are formed. N-Alkyl substitution on the various N atoms alters the *p*H at which intratransguanylation takes place as well as the rate of formation and composition of the products obtained.

Introduction

S,2-Aminoethylisothiourea (AET) undergoes a series of transformations through a cyclic intermediate to yield either 2-aminothiazoline (2-AT) or 2-mercaptoethylguanidine (MEG).[2] An ion-exchange analytical procedure, developed for examination of these reactions, established that they are *p*H dependent and that only in strongly alkaline medium does AET approach the behavior of a normal isothiourea and yield mixtures of MEG, 2-mercaptoethylamine (MEA) and dicyanodiamide (DCD).[3] Color tests showed that 3-aminopropylisothiourea (APT) as well as N-alkylamine and N'-guanyl N-substituted ethyl- and propylisothioureas can also participate in these transformations.[2] Dialkylaminoalkylisothioureas and 4-aminobutylisothiourea are stable at neutral *p*H and apparently do not intratransguanylate at any *p*H.[2] The interest in this class of compounds as potential radiation-protective agents[4] rendered their chemical properties of immediate interest. We therefore investigated the transformations of the higher homologs and variously N-substituted aminoalkylisothioureas to establish the conditions necessary for the preparation of the mercaptoalkylguanidines and thiazolines or penthiazolines in a pure state and to verify the structural limitations on the intratransguanylation reaction, which were hitherto based on color tests alone. Information of this nature may also be essential for an understanding of the biological conversions of these compounds in mammalian systems and of their pharmacological properties.

Experimental

Thiol Assay of Isothiourea Reactions.—We examined the rate of intratransguanylation and thiazoline formation

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

(2) D. G. Doherty, R. Shapira and W. T. Burnett, Jr., THIS JOURNAL, **79**, 5667 (1957).

(3) J. X. Khym, R. Shapira and D. G. Doherty, *ibid.*, **79**, 5663 1957).

(4) D. G. Doherty and W. T. Burnett, Jr., *Proc. Soc. Exptl. Biol. Med.*, **89**, 312 (1955); R. Shapira, D. G. Doherty and W. T. Burnett, Jr., *Radiation Research*, **7**, 24 (1957).

of several aminoalkylisothioureas in solution at various *p*H's by the previously described thiol assay[2] to select the proper times for making column analyses. Disappearance of sulfhydryl group is indicative of either disulfide or thiazoline formation. Representative curves for an unsubstituted isothiourea (APT) and an N-substituted isothiourea, S,2-methylaminoethylisothiourea (MAET), are shown in Fig. 1A and 1B, respectively.

Ion Exchange.—The ion-exchange material used in these experiments was Dowex-50 (hydrogen form), 200–400 mesh, with 8% divinylbenzene content. The column size was approximately 1 sq. cm. in cross section by 5 cm. as previously described.[3] The various isothiourea solutions were analyzed in appropriate aliquots diluted to 20–100 ml. with 0.2 *M* HCl before sorption on the exchange columns. For elutions, the concentration of HCl was increased at regular intervals; each fraction was analyzed colorimetrically for nitrogen and sulfhydryl content. Any aminoalkylisothiourea can be eluted with 500 ml. of 1.5 *M* HCl; also, any guanidinoalkylisothiourea can be eluted with more of the same reagent, or, more readily eluted by changing to 3.0 *M* HCl. At least 1500 ml. of 0.5 *M* HCl, followed by 500 ml. of 1.5 *M* HCl, does not appreciably affect the elution position of the aminoalkylisothioureas.[3] Any mercaptoalkylamine or alkylamine and NH_4^+ may be eluted with 0.2 *M* HCl and any ring compound (thiazoline or mercaptoalkylguanidine produced from AET) or any of its derivatives may be eluted with the same reagent. In the propyl series, the ring compounds (penthiazolines) and mercaptoalkylguanidines may be eluted with 0.25–0.4 *M* HCl, whereas mercaptobutylguanidine is best eluted with 0.5 *M* HCl solutions.

Characterization of Compounds.—The isothiuronium salts used were prepared as bromide hydrobromides.[2] The products obtained from these compounds through the various rearrangements to be discussed were characterized by their flavianate derivatives wherever possible. In many cases, pure derivatives could not be prepared from the complex mixtures obtained on rearrangement.

Characterization by ion exchange relies on elution position in comparison with authentic material or closely related materials when available, and on the nitrogen–sulfhydryl ratio of each isolated peak. DCD, formed in a 1:2 ratio with mercaptoalkylamine *via* the normal hydrolysis of isothioureas, is not sorbed on the exchanger.[3] Alkylamines are found as products of cyclization only in the monoalkyl N'-substituted aminoalkylisothioureas. In these, some NH_4^+ is also found. Although the ratio of NH_4^+ to alkylamine varies slightly from compound to compound, the average from a number of determinations is about 15% NH_4^+ to 85% alkylamine. NH_4^+ and alkylamine are always found together in the first peak for any of the elution sequences presented. The ratio of NH_4^+ to alkylamine is determined readily by total and inorganic nitrogen analyses. The elution position and analysis of ethyl- or methylamine and NH_4^+ arising from the N'-alkyl-substituted aminoalkylisothioureas may be determined with NH_4Cl, and ethyl- and methylamines as reference compounds. Mercaptoalkylamines are identified easily since they are the only products recovered that give a 1:1 ratio of nitrogen to sulfhydryl. The thiazolines, if present, are eluted just before the mercaptoalkylguanidines in any of the eluting systems given. The only thiazoline prepared, other than from its parent isothiourea, was 2-AT. These ring compounds are readily identified, however, since they do not give a positive sulfhydryl test and are always found in equimolar amounts with total amine or NH_4^+ or both. In each mono-N'-alkyl-substituted aminoalkylisothiourea, a mixture of two ring compounds is found in the ratio of 15 to 85%. One product is a 2-aminothiazoline, the other a 2-methyl- or 2-ethylaminothiazoline. (Since they are not separated by the ion-exchange procedure, they are given in Table II as total ring compound found in equimolar amount to total amine present.) When any of the more dilute HCl eluting agents are used, all the mercaptoalkylguanidines are the last products off the exchanger. They are characterized by nitrogen and sulfhydryl group assays, a 3:1 nitrogen–sulfhydryl ratio indicating their presence. The flavianate derivatives of the mercaptoalkylguanidines were easily prepared in most instances since, at *p*H 7.0, the mercaptoalkylguanidine form of any of the aminoalkylisothioureas is essentially the only product obtained. The stability of the isothioureas in strong acid is shown by their complete recovery, unchanged, after sorption on a column in 0.2 *M* HCl and elution by 1.5 *M* HCl. The guanidinoalkylisothioureas are more stable and show no chemical changes even if allowed to remain in 1.5 *M* HCl or at *p*H 7.0 for several days. The guanidinoalkylisothioureas do not respond to the sulfhydryl test at *p*H 7.0, but treatment with 2 *N* NaOH rapidly hydrolyzes them and the resulting mercaptoalkylguanidines are readily detectable. The test is qualitative only, since dilute sulfhydryl solutions (necessary for the test) under strongly alkaline conditions are oxidized quickly to disulfides. The elution sequences presented, and many others that were tried, do not separate diguanidinoalkyl disulfides from the guanidinoalkylisothioureas. Sulfhydryl oxidation at high concentrations and in well-stoppered vessels is small. This can be seen from Table I, where the compounds listed do not form guanidinoalkylisothioureas, only disulfides. From curve 1 of Fig. 3, a good estimate of the disulfide contamination in the guanidinoalkylisothiourea peak can be made since the actual amount of mercaptoalkylamine was high enough to permit a reliable calculation by equating the percentage of mercaptobutylamine to the percentage of mercaptobutylguanidine (see equations 1–4). Any remaining nitrogen is considered to arise from mercapto- or guanidinoalkyl disulfides. In Table II the disulfide content of the isothiourea fractions is ignored. The figures in columns 6 and 10, although representing a considerable percentage in terms of the amount of starting material, are not so reliable as the value in the other columns, since the actual nitrogen found for the mercaptoalkylamines ranged only from 100 to 800 mg. of total nitrogen.

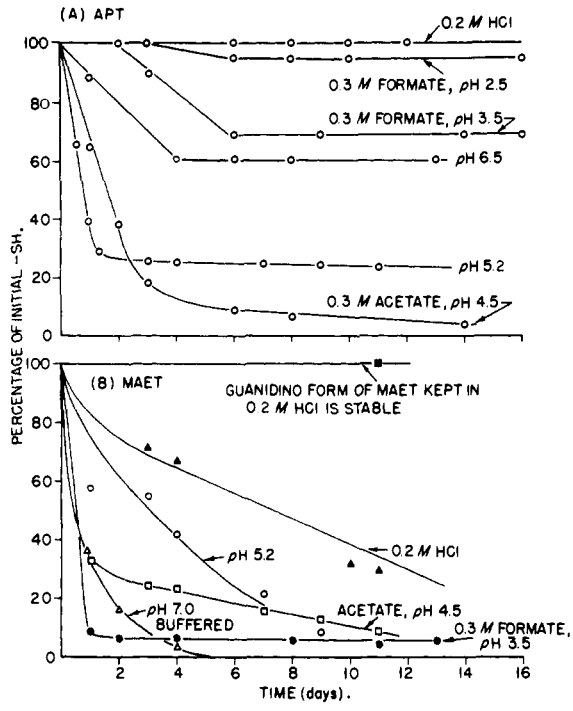


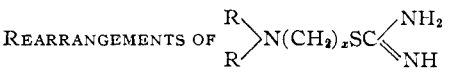
Fig. 1.—Percentage of -SH remaining *vs.* time in solution of (A) APT, 25 mg./ml., and (B) MAET, 25 mg./ml., at various *p*H's.

Preparation of Flavianic Acid Salts.—The isothiouronium salts were converted to the mercaptoalkylguanidines by the addition of one equivalent of alkali and were precipitated as the flavianates by the addition of flavianic acid. The following general procedure was used for these salts.

3-Mercaptopropylguanidine Flavianate.—Two grams of APT·Br·HBr was dissolved in 25 ml. of water, 6.8 ml. of 1 *N* NaOH was added to bring the *p*H to 7–7.5, and 7.0 ml. of a 1.0 *M* flavianic acid solution was stirred in to give an immediate precipitate. The flavianate was filtered off and recrystallized three times from water; yield 2.0 g., m.p. 187–189°.

Anal. Calcd. for $C_{14}H_{17}N_5O_8S_2$: C, 37.60; H, 3.83; N, 15.70; S, 14.32. Found: C, 37.81; H, 3.84; N, 15.79; S, 13.39.

TABLE I

REARRANGEMENTS OF 

Compound	Treatment	Length of treatment	Alkylamine or NH_4^+	Thiazoline or penthiazoline	Mercaptoalkylguanidine	Isothiourea	Disulfide	Guanidinoalkylisothiourea
APT[a]	0.2 *M* HCl	16 days				97		
2-ABT[b]		1 day				96		
MAET[c]		6 hr.				95		
MAET		8 days	58	60		36		
AET[d]		5 days	99	96				
APT	*p*H 2.5	13 days	10	10		90		
2-ABT		1 day	94	94		6		
APT	*p*H 3.5	9 days	39	37		63		
MAET		2 days	93	93				
AET	*p*H 4.5	4 days	35	34	52		3	
APT		6 days	93	95		6		
2-ABT		22 days	67	69	20		2	
MAET	*p*H 5.2	1 day	35	35	65			
MAET		9 days	53	53	7		20	
AET	*p*H 7.0, phosphate buffer	10 min.			94		5	
APT		30 min.	16	16	67	15		
4-ABT		1 day				97		
2-ABT		10 min.			90		6	
2-ABT		14 days	45	45	40		10	
MAET		10 min.			93		5	
MAET		5 days	91	87			7	
DiMAET[e]		8 days				97		
DiEAPT[f]		8 days				96		
GET[g]		1 day						94
AET	1 equiv. NaOH	10 min.			94		5	
APT		10 min.			90		10	
2-ABT		10 min.			80		20	
MAET		10 min.			90		7	
AET	*p*H 7.0, HCO_3^-	20 min.	68	68	33			
APT		20 min.	47	50	23	25		
							Mercaptoalkylamine	
4-ABT	*p*H 9.2	5 hr.			16	28	27	29
4-ABT		22 hr.			45	8	21	23
DiMAET		8 days				13	83	
DiEAPT		8 days				13	83	
GET		7 days			54			42
AET	2 *N* NaOH	10 min.			52		46	
APT		10 min.			60		44	
4-ABT		10 min.					98	
MAET		10 min.			80		12	
GET	1 *N* NaOH	10 min.			96			

[a] S,3-Aminopropylisothiourea. [b] S,2-Aminobutylisothiourea. [c] S,2-Methylaminoethylisothiourea. [d] S,2-Aminoethylisothiourea. [e] S,2-Dimethylaminoethylisothiourea. [f] S,2-Diethylaminopropylisothiourea. [g] Guanidinoethylisothiourea.

2-Mercaptoethyl-N-methylguanidine Flavianate.—Two grams of 2-methylaminoethylisothiouronium·Cl·HCl-(MAET) yielded, by the preceding method, 3.3 g. of flavianate, m.p. 201–203°. *Anal.* Calcd. for $C_{14}H_{17}N_5O_8S_2$: C, 37.60; H, 3.83; N, 15.70; S, 14.32. Found: C, 37.88; H, 3.92; N, 15.59; S, 14.07.

1-Mercaptobutyl-2-guanidine Flavianate.—S,2-Aminobutylisothiouronium·Br·HBr (2.1 g.) gave 2.5 g. of flavianate with m.p. 186–188°. *Anal.* Calcd. for $C_{15}H_{19}N_5O_8S_2$: C, 39.10; H, 4.15; N, 15.20; S, 13.91. Found: C, 37.65; H, 3.52; N, 15.35; S, 14.96.

2-Aminopenthiazoline Flavianate.—APT·Br·HBr (5.9 g.) was dissolved in 100 ml. of 0.6 *M* acetate buffer, *p*H 4.5, and allowed to equilibrate for 15 days. A 25-ml. aliquot of this solution was acidified to *p*H 1 with 1 *N* HCl, and 6.0 ml. of a 1.0 *M* flavianic acid solution was added. The precipitate was filtered off and recrystallized four times from water; yield 2.0 g., m.p. 243–245° dec.

Anal. Calcd. for $C_{14}H_{14}N_4O_8S_2$: C, 38.10; H, 3.26; N, 12.92; S, 14.78. Found: C, 38.95; H, 3.25; N, 12.98; S, 15.57.

1-Methyl-2-aminothiazoline Flavianate.—MAET·Cl·HCl (4.2 g.) was dissolved in 100 ml. of 0.6 *M* formate buffer, *p*H 2.5, and allowed to equilibrate for two days. A 50-ml. aliquot of this solution was acidified to *p*H 1 with 1 *N* HCl and 11 ml. of a 1 *M* flavianic acid solution was added. The precipitate was filtered off and recrystallized four times from water to yield 3.1 g., m.p. 285–286° dec.

Anal. Calcd. for $C_{14}H_{14}N_4O_8S_2$: C, 38.10; H, 3.26; N, 12.92; S, 14.78. Found: C, 38.97; H, 3.10; N, 12.79; S, 15.18.

2-Amino-5-ethylthiazoline Flavianate.—2-ABT·Br·HBr (1.54 g.) was dissolved in 25 ml. of 0.6 *M* formate buffer, *p*H 2.5, and allowed to equilibrate for 11 days. The addition of 6.0 ml. of 1.0 *M* flavianic acid solution gave a precipitate, which was filtered off and recrystallized three times from water; yield 1.9 g., m.p. 194–196° dec.

TABLE II

REARRANGEMENTS OF $NH_2(CH_2)_xSC(=NH)N(H)R$

Compound	Treatment	Length of treatment	Alkylamine or NH_4	Mercaptoalkylamine	Thiazoline or penthiazoline	Mercaptoalkylguanidine	Isothiourea	Disulfide or guanidinoalkylisothiourea
AEMT[a]		6 days	24		25		73	
AEET[b]		5 days	13		14		87	
APMT[c]	*p*H 2.5	7 days					100	
APET[d]		5 days					100	
AEMI[e]		5 days					97	
AEMT		6 days	52	6	51	36	4	5
AEET		7 days	54	3	48	33	11	5
APMT	*p*H 4.5	9 days	25		21		67	
APET		6 days	10		10		90	
AEMI		6 days				27	71	
AEMT		6 days	35	3	30	65	2	4
AEET	*p*H 5.6	6 days	30	2	22	63	4	8
APMT		5 days	32	11	36	7	27	12
APET		5 days	23	6	22	6	47	12
AEMT		10 min.	7		6	93		
AEET		10 min.	4		5	90		
APMT		35 min.	15		21	33	32	9
APET	*p*H 7.0, phosphate buffer	30 min.	11		11	21	57	10
APET		5 days	34		32	56		9
AEMI		10 min.				70	28	
AEMI		5 days				99		
AEMT		10 min.		9		69		15
AEET		10 min.		16		63		14
APMT	1 equiv. NaOH	10 min.		10		70		14
APET		10 min.		20		57		17
AEMI		10 min.				84	15	
APMT	1 equiv. HCO_3^-	20 min.	30		29	2	70	
AEMI		20 min.				10	90	

[a] 2-Aminoethyl-N'-methylisothiourea. [b] 2-Aminoethyl-N'-ethylisothiourea. [c] 3-Aminopropyl-N'-methylisothiourea. [d] 3-Aminopropyl-N'-ethylisothiourea. [e] 2-(2'-Aminoethylmercapto)-imidazoline.

Anal. Calcd. for $C_{15}H_{16}N_4O_8S_2$: C, 40.72; H, 3.55; N, 12.71; S, 14.45. Found: C, 40.57; H, 3.55; N, 12.71; S, 14.62.

Results

Ion-exchange elution diagrams, representative of the systems used in this study and indicative of the separations that were obtained, are presented in Figs. 2 and 3. The analytical results obtained by column chromatography of the amino- and alkylaminoalkylisothioureas are given in Table I and the aminoalkyl-N'-alkylisothioureas in Table II. All the isothioureas are stable in 0.2 *N* HCl for at least one day; the products obtained are therefore not artifacts arising from the chromatographic procedure

APT is stable in 0.2 *N* HCl for 16 days at room temperature, and even after 12 days at *p*H 2.5 only 10% is converted to 2-aminopenthiazoline (2-PT) (Table I). At *p*H 3.5 the conversion is appreciable (Table I), but the optimum *p*H seems to be 4.5, where 31% 2-PT is formed in one day (Fig. 2, curve 1) and 95% in six days (Table I). In phosphate buffer at *p*H 7.0, APT yields a mixture of 67% 3-mercaptopropylguanidine (MPG), 18% 2-PT and some unchanged isothiourea. For complete conversion to MPG, APT must be brought to *p*H 8, either in buffer or by the addition of one equivalent of 1 *N* NaOH (Fig. 2, curve 2). Mercaptopropylamine, a product of an intertransguanylation reaction, is never found under these conditions, indicating that MPG is formed *via* an intratransguanylation process. When APT is added to an excess of 2 *N* NaOH, a mixture of 40% 3-mercaptopropylamine (MPA) and 60% mercaptopropylguanidine is obtained.

4-Aminobutylisothiourea (4-ABT) is stable in acid and at *p*H 7.0 in phosphate buffer (Table I). When 4-ABT is dissolved in borate buffer *p*H 9.2 and chromatographed after six hours, however, a mixture is obtained of all the compounds that might be expected to arise from an intertransguanylation reaction of two molecules of 4-ABT (Fig. 3, curve 1). If the mixture is allowed to equilibrate for one day, the concentration of 4-mercaptobutylguanidine (4-MBG) increases to 42% and there is a corresponding decrease in 4-mercaptobutylamine (4-MBA) and 4-guanidinobutylisothiourea (4-GBT) (Table I). The reaction scheme in Fig. 4 may be written to explain the formation of these products. We examined reaction 3 by mixing 4-ABT with MEA (in place of MBA) at *p*H 7.0 and analyzing the mixture after five hours. A mixture of starting material, 4-MBA and MEG but no 4-GBT, was found indicating that intertransguanylation takes place (Fig. 3, curve 3). Since guanidinoethylisothiourea (GET) was available from a previous experiment,[2] we studied reaction 2 by mixing GET with MEA at *p*H 7.0. Analysis after four hours

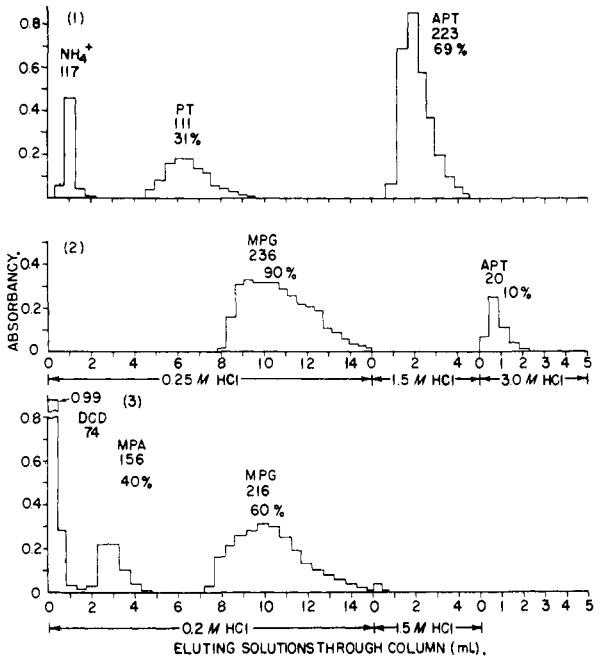


Fig. 2.—Intratransguanylation reaction of APT: exchange resin, Dowex-50-H, 1.0 × 5 sq. cm., 200–400 mesh; flow rates 0.6–0.8 ml./min.; ordinate, nitrogen concentration, determined colorimetrically by Nesslerization; numbers above peaks represent μmoles recovered; curve 1, 100 μmoles/ml. of APT in acetate buffer *p*H 4.5 for one day, 4 ml. diluted to 20 ml. with 0.2 *M* HCl and placed on column; curve 2, same concentration at either *p*H 8.1 in phosphate buffer 30 min. or in 1 equivalent of NaOH, 3 ml. diluted to 20 ml. with 0.2 *M* HCl; curve 3, same concentration in 2 *N* NaOH for 15 min., 4 ml. diluted to 50 ml. with 0.2 *M* HCl

indicated that MEG was formed almost quantitatively (Fig. 3, curve 2). GET is stable at this *p*H and gives only one peak in the expected place after 24 hours (Table I). In addition, GET rapidly transfers its guanyl group to 4-ABT at *p*H 8.0 to yield 4-GBT and MEG.

Substitution in the Alkylamino Group.—Monoalkylamino derivatives rearrange through the cyclic intermediate in a manner similar to the parent compound. Since dialkyl substitution prevents the formation of a cyclic intermediate the transguanylation reaction does not occur. This is verified by the results obtained from an ion-exchange analysis of solutions of two dialkylamino-substituted isothioureas. S,2-Dimethylaminoethylisothiourea (DiMAET) and S,3-diethylaminopropylisothiourea (DiEAPT) are stable at all *p*H's up to 7.2 for periods as long as eight days (Table I). However, at *p*H 9.2 for eight days, both compounds are hydrolyzed to yield the corresponding mercaptoalkylamine, 83% of the calculated amount of DCD and 13% starting material. MAET participates in all the usual transformations; *i.e.*, at *p*H 3.5 for 50 hours it yields 93% 2-amino-3-methylthiazoline plus an equivalent amount of NH_4^+; at *p*H 5.2 for one day it gives a mixture of 35% thiazoline and 65% mercaptoethyl-N-methylguanidine; and when neutralized to *p*H 7.0 with one equivalent of 1 *N* NaOH, it yields 90% of the mercaptoguanidine and 7% of the disulfide. In an excess of 2 *N* NaOH, MAET gives 80% of the mercaptoguanidine and 12% of the disulfide.

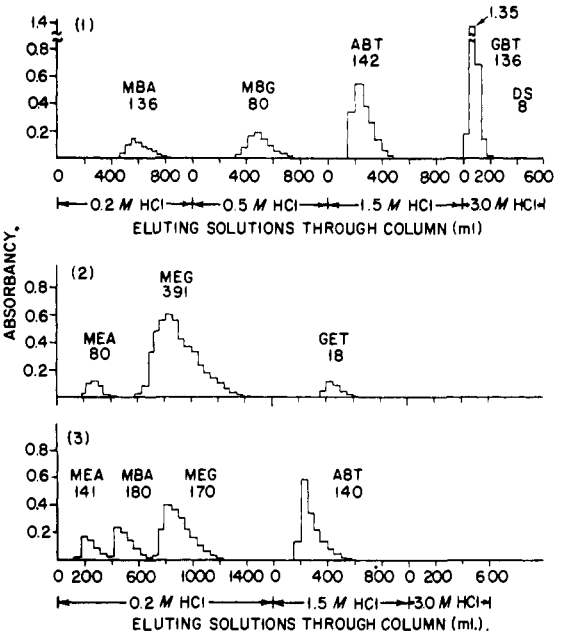


Fig. 3.—Products of intertransguanylation reactions: exchange resin, Dowex-50-H, 1.0 × 5 sq. cm., 200–400 mesh; flow rates 0.6–0.8 ml./min.; ordinate, concentration of nitrogen determined colorimetrically by Nesslerization; numbers above peaks represent μmoles recovered, disulfide (DS) concentration in GBT peak estimated; curve 1, 89 μmoles/ml. of 4-ABT in 0.4 *M* borate buffer *p*H 9.2 for 6 hours, 6 ml. diluted to 100 ml. of 0.2 *M* HCl and placed on column; curve 2, 50 μmoles/ml. of MEA and 40 μmoles/ml. of GET in 0.4 *M* phosphate buffer *p*H 7.0 for 4 hours, 6 ml. diluted to 100 ml. with 0.2 *M* HCl and placed on column; curve 3, similar to curve 2, but with 50 μmoles/ml. each of 4-ABT and MEA at *p*H 7.0 for 5 hours.

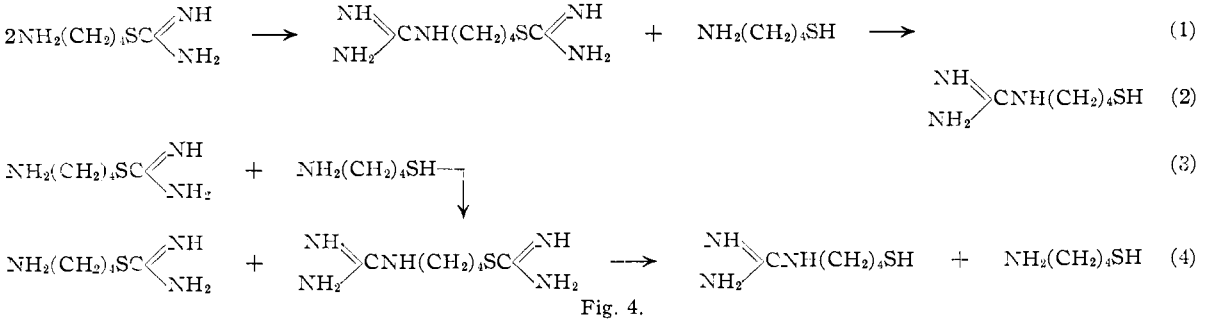


Fig. 4.

Substitution of the Guanyl Nitrogen of the Isothiourea Group.—In a similar manner, the transformations of the aminoalkyl-N′-alkylisothioureas were examined using as prototypes 2-aminoethyl-N′-methyl- (AEMT) and -N′-ethylisothiourea (AEET) and 3-aminopropyl-N′-methyl- (APMT) and -N′-ethylisothiourea (APET). As can be seen from Table II, intratransguanylation through a cyclic intermediate occurs although at higher *p*H's than the parent compounds AET and APT. In all cases, mixtures of varying complexity were obtained, but neither the thiazoline nor the mercaptoalkylguanidine could be gotten as a single component in solution. In addition, at *p*H 5.6 and above, the disulfide peak contained sulfhydryl-positive material, presumably guanidinoalkylisothiourea, as well as a peak of mercaptoalkylamine, which indicated an intertransguanylation reaction between 2 moles of compound.

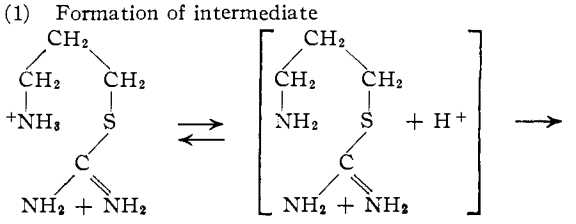
Reversible Reaction of Mercaptoalkylguanidines to Form Thiazolines.—MAET and 2-ABT are the only two isothioureas among the many examined that, after conversion to the mercaptoalkylguanidine, revert to the corresponding thiazoline and ammonia after long periods in solution. Both MAET and 2-ABT show a 90% conversion to the mercaptoalkylguanidine after five minutes in phosphate buffer at *p*H 7.0. The same solution of MAET in five days yields 90% thiazoline and ammonia, 10% disulfide and no mercaptoalkylguanidine. The 2-ABT solution in 14 days yields 45% thiazoline and ammonia, 40% mercaptoalkylguanidine and 10% disulfide. Both mercaptoalkylguanidines are stable indefinitely if, immediately after formation at *p*H 7, the solution is readjusted to *p*H 2.0.

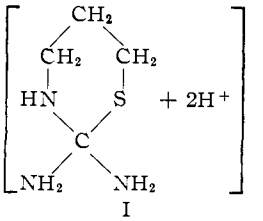
Transimidazolination.—2 - (2′ - Aminoethylmercapto)-imidazoline (AEMI) prepared from bromoethylamine and ethylenethiourea transfers its imidazoline ring intact to the amino group when neutralized to *p*H 7.0. The compound is stable in both 0.2 *N* HCl and 0.4 *M* formate buffer, *p*H 2.5; after six days in 0.4 *M* acetate buffer at *p*H 4.5, only 27% is converted to 2-(2′-mercaptoethylamino)-imidazoline (MEAI), the remainder being recovered as unchanged starting material. The conversion is rapid at *p*H 7.0, 70% of the mercaptoimidazoline being formed in ten minutes. After five days, no starting material remains and the conversion to the mercapto form is complete.

Discussion

The data indicate that APT can undergo intratransguanylation in a manner analogous to its lower homolog, AET.[2] In addition, CO_2 seems to catalyze the formation of 2-aminopenthiazoline in aqueous solution. These reactions may be visualized as proceeding through a six-membered cyclic intermediate according to the equations

(1) Formation of intermediate





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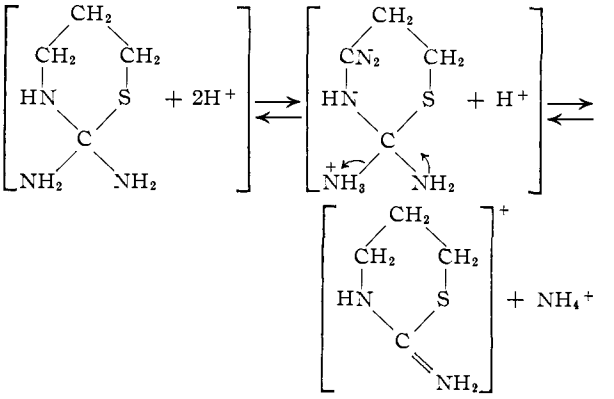
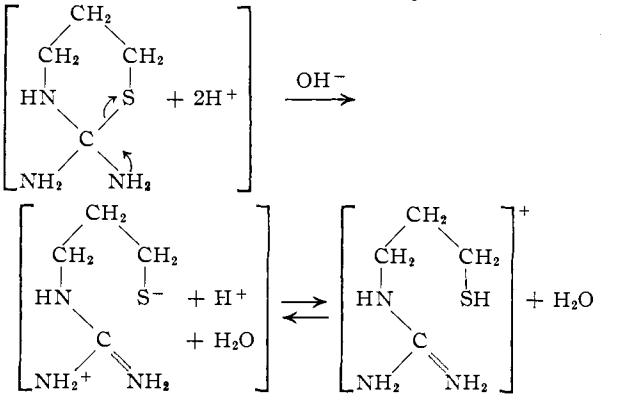
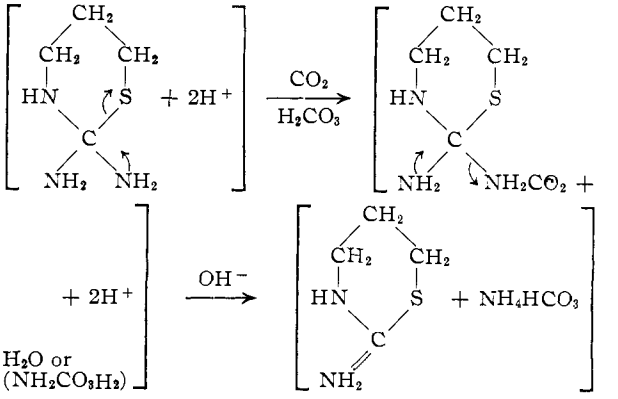
(2) Acid-catalyzed formation of 2-PT$^+$ by elimination of NH_4^+



(3) Rearrangement to MPG at neutral *p*H



(4) CO_2-catalyzed formation of 2-PT by elimination of NH_4HCO_3



The formation of the unstable intermediate I (reaction 1) common to all further reactions, by the interaction of the carbonium ion and the amino group, is probably the rate-limiting step. In accord with the slightly increased distance between the carbonium ion and the amino group as well as the increased degrees of freedom in APT, this and all subsequent transformations occur at a lower pro-

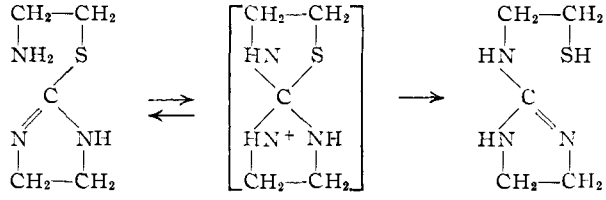
ton concentration in APT than in AET. Thus AET is converted to 2-aminothiazoline at *p*H 2.5, whereas APT requires a *p*H of 4.5 for the comparable conversion to 2-aminopenthiazoline. Similarly, the conversion of APT to MPG (reaction 3) also occurs at lower proton concentrations, *p*H 7.5 and above being required for the formation of MPG only. The effect of CO_2 and HCO_3^- is similar to that observed for AET and can be explained by the formation of an addition complex as indicated in reaction 4. Thus neutralization of APT with bicarbonate yields mixtures of penthiazoline, MPG and unchanged isothiourea, but CO_2 under pressure at *p*H 4–6 yields only penthiazoline.

Increasing the separation of the amino and guanyl groups by the introduction of a methylene group in the chain yields a compound, 4-ABT, that is stable at *p*H values up to 8.0. It does not participate in either intratransguanylation or heterocyclic ring formation since interaction of the amino and guanyl groups would require formation of an unlikely seven-membered cyclic intermediate. This observation is analogous to that of Fishbein and Gallaghan,[5] who found that oxazolines were formed from 1-(hydroxyalkyl)-guanidines and -nitroguanidines only when the alkyl chain was ethyl or propyl, the butyl compound being stable. At higher *p*H values, 4-ABT exhibits normal isothiourea reactions, guanylating itself by a bimolecular, concentration-dependent intertransguanylation process at *p*H 9.2 and hydrolyzing completely to 4-MBA in dilute NaOH (Table I). Indeed, 4-ABT acts as a guanylating agent when mixed with mercaptoethylamine at *p*H 7.0 to form mercaptoethylguanidine and mercaptobutylamine (Fig. 2, curve 3). This observation, and the absence of ring compounds at low *p*H's, and the sequence of products obtained with increasing time at *p*H 9.2 (Table I) establish the 4-ABT reaction as one of intertransguanylation.

In agreement with the previous report,[2,4] we found that at least one displaceable hydrogen atom on the amino group was necessary for the rearrangement of the aminoalkylisothioureas through a cyclic intermediate. The methylamino derivative of AET MAET, intratransguanylates to yield the expected products as readily as AET or APT, whereas the disubstituted aminoalkylisothioureas, DiMAET and DiEAPT, are stable up to *p*H 7.0 and at 9.2 slowly hydrolyze in a normal manner to the mercaptoalkylamine and dicyandiamide. It is interesting to note that in MAET, ammonium ion rather than methylamine is split out at acid *p*H's, yielding 1-methyl-2-aminothiazoline in accord with the hypothesis of a cyclic intermediate.

Substitution of alkyl groups on the guanyl nitrogen atoms of the aminoalkylisothioureas reduces to some extent the ability of these compounds to rapidly form a cyclic intermediate, as evidenced by their slower conversion to cyclic products at acid *p*H's and by the appearance of mercaptoalkylamines at neutral *p*H's, which was indicative of a competing intertransguanylation reaction. In the formation of the heterocyclic rings, elimination of alkylamine is favored over ammonia so that the products are predominantly 2-aminothiazoline or 2-aminopenthiazoline rather than the corresponding 2-alkylamino derivatives. The optimum *p*H ranges for the various conversions of these N-substituted compounds are, however, closely related to the parent compounds AET and APT.

The final case of group transfer examined was the formation of 2-(2-mercaptoethylamino)-imidazoline from 2-(2-aminoethylmercapto)-imidazoline illustrated in the reaction



In this instance, however, although the formation of a cyclic intermediate is as likely as in the previous cases, the formation of a thiazoline is improbable since it would involve at least the rupture of the imidazoline ring. Indeed, only the starting material and the mercaptoimidazoline could be isolated in any of the column analyses. Similarly to AET, the conversion to the mercaptoimidazoline begins in the *p*H range 3.5 to 4.5 and is rapid at *p*H 7.0. As might be expected, there is no effect of the HCO_3^- on the reaction and the mercaptoimidazoline is stable for at least five days at *p*H's up to 9.0.

Additional support for the postulation of a common cyclic intermediate for thiazoline and mercaptoguanidine formation is furnished by the finding that, in some instances, part of the reaction is reversible. Thus the mercaptoalkylguanidines prepared from MAET and 2-ABT will, in solution for long periods at *p*H's 5 to 7, be converted to the corresponding thiazolines with the loss of ammonia. Indeed, we found that the same equimolar mixture of ammonia and thiazoline is obtained from these compounds at a given *p*H at equilibrium regardless of whether the initial compound was the aminoisothiourea or the mercaptoguanidine. At present it is not clear why all the mercaptoalkylguanidines examined in this study do not undergo the reverse reaction to form thiazolines. However, as pointed out by Benesch, Benesch and Rogers,[6] the reactivity of the sulfhydryl group may be appreciably altered by formation of hydrogen bond or by the steric hindrance of neighboring groups, factors that would affect the *p*H's of both the guanido and sulfhydryl groups.

It is interesting to note that even in strong alkali the intratransguanylation process competes favorably with the normal hydrolysis reaction in those aminoalkylisothioureas capable of rearrangement. Thus AET, APT and MAET (Table II) all yield mixtures richer in mercaptoguanidine than mercaptoamine, but aminoalkylisothioureas incapable of intratransguanylation (*e.g.*, 4-ABT and GET) yield only the corresponding mercaptoamine and dicyandiamide. Rough colorimetric tests showed

(5) L. Fishbein and J. A. Gallaghan, *J. Org. Chem.*, **21**, 434 (1956).

(6) R. Benesch, R. E. Benesch and W. I. Rogers, in "Glutathione, A Symposium," eds. S. P. Colowick, *et al.*, Academic Press, Inc., New York, N. Y., pp. 31–41.

that this generalization holds for all the other isothioureas examined in this study.

It is apparent from the experimental results reported here that amino and variously N-substituted aminoalkylisothioureas with an amino nitrogen to sulfur distance of not more than three carbon atoms are rather labile compounds in aqueous solution and that the products obtained may be controlled, to some extent, by a suitable choice of *p*H, time of reaction and nature of the base used for neutralization. These facile transformations make possible the preparation of a wide variety of mercaptoalkylguanidines, 2-aminothiazolines and 2-aminopenthiazolines in good yields from relatively simple and readily available starting materials. In addition, aminoisothioureas such as 4-ABT and GET, which are incapable of the intramolecular reaction, should be useful protein guanylating agents in the neutral *p*H range.

OAK RIDGE, TENN.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

The Structure of Etamycin

BY JOHN C. SHEEHAN, HANS GEORG ZACHAU[1a] AND WILLIAM B. LAWSON[1b]

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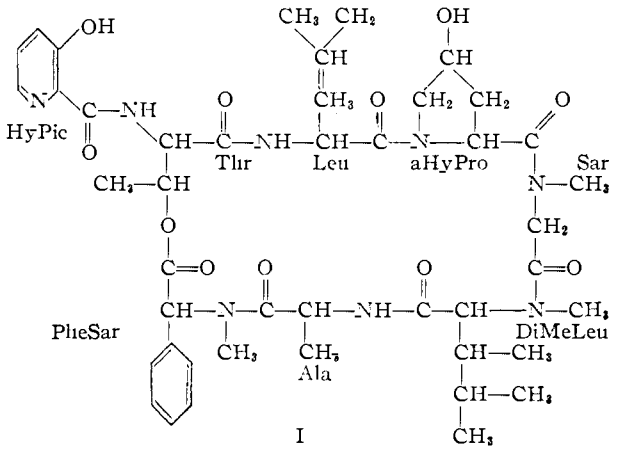
The structure of the antibiotic Etamycin is shown to be a macrocyclic peptide lactone, represented by formula I. Etamycin is an unusual peptide containing eight amino acids, only two of which are known as components of animal protein. Four of the amino acids, 3-hydroxypicolinic acid, *allo*-hydroxy-D-proline, L-α-phenylsarcosine (IIb) and L-β,N-dimethylleucine (III) have not been encountered previously in nature.

The isolation of the peptide antibiotic Etamycin from culture broths of a Streptomyces species was described in 1954 by Heinemann, *et al.*[2] Simultaneously, Bartz, *et al.*,[3] reported an antibiotic termed Viridogrisein, obtained from *Streptomyces griseus*. Etamycin and Viridogrisein were shown subsequently to be identical.[4] The antibiotic exhibits considerable activity against gram-positive



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organisms and *Mycobacterium tuberculosis;* in addition it causes a reversible leucopenia in dogs.[2,5,6] Etamycin possesses unusual solubility properties for a peptide, being freely soluble in benzene and carbon tetrachloride. The values reported[2,3] for the molecular weight varied from 530 to 982, though most were in the range 800–900. After acid hydrolysis 3-hydroxypicolinic acid (HyPic), D-leucine, *allo*-hydroxy-D-proline and L-alanine were isolated,[3] and threonine was identified (paper chromatography[2]).

This paper reports the isolation from Etamycin hydrolysates of three additional components. These are sarcosine and two previously unknown amino acids, L-α-phenylsarcosine (PheSar, IIb) and L-β,N-dimethylleucine (DiMeLeu, III). On the basis of degradation experiments the structure of Etamycin is formulated as I.[7]

Amino Acid Composition.—Two dimensional paper chromatograms of Etamycin total hydrolysates, developed with ninhydrin at 100°, showed three spots in addition to those corresponding to alanine, threonine, hydroxyproline and leucine. When the color was developed at 60° or below these spots either were very faint or absent. These three spots were due to amino acids rather than to peptides since after elution and vigorous hydrolysis they reappeared at the same places on a two dimensional chromatogram. Two of the amino acids gave the red color test with *p*-nitrobenzoyl chloride–pyridine characteristic of N-alkylamino acids.[8] The third amino acid behaved chromatographically like sarcosine, which is known not to give this color test.[8c] That the three unknown amino acids are N-methylamino acids was confirmed by an N-methyl determination (three N-methyl groups per molecule of Etamycin).

The three N-methylamino acids were isolated by preparative paper chromatography. Deamination of an Etamycin total hydrolysate with nitrous acid[9] destroyed alanine, threonine and leucine. After reconstitution from the nitroso derivatives by heating with hydrochloric acid,[9] the secondary amino acids were separated on Whatman No. 3

(1) (a) Aided by a grant from the National Institutes of Health; (b) National Institutes of Health Postdoctoral Fellow, 1956–1957.

(2) B. Heinemann, *et al.*, *Antibiotics Annual*, **2**, 728 (1954–1955).

(3) Q. R. Bartz, *et al.*, *ibid.*, **2**, 777–784 (1954–1955).

(4) The identity of Viridogrisein with Etamycin was established in the laboratories of the authors of refs. 2 and 3 and at M.I.T. We wish to thank Dr. Murray Goodman for his part in this work.

(5) H. L. Dickison, K. M. Cull and D. E. Tisch, *Antibiotics Annual*, **2**, 733 (1954–1955).

(6) J. Ehrlich, *et al.*, *ibid.*, **2**, 790 (1954–1955).

(7) A preliminary report of the work appeared in THIS JOURNAL, **79**, 3933 (1957).

(8) (a) E. Waser, *Mitt. Lebensm. Hyg.*, **20**, 260 (1929); *C. A.*, **24**, 1601 (1930); (b) S. Edlbacher and F. Litvan, *Z. physiol. Chem.*, **265**, 241 (1940); (c) P. A. Plattner and U. Nager, *Helv. Chim. Acta*, **31**, 2203 (1948).

(9) B. Witkop and C. M. Foltz, THIS JOURNAL, **79**, 192 (1957).