[Chem. Pharm. Bull. 15(10)1446~1452(1967)]

UDC 615.849-035.1 : 547.495.9.09

184. Akira Hanaki, Tohru Hino, and Sanya Akaboshi*1 : Reactivities of Radiation-protective Aminoalkylisothiuronium Salts.*2 II. Spectrophotometric Studies on the Reductivities of 2-Mercaptoethylamine, 2-Mercaptoethyl-, and 3-Mercaptopropyl-guanidine.

(*National Institute of Radiological Sciences*)

The conjugate bases of AET and APT are transguanylated rapidly and nearly quantitatively to MEG and MPG respectively, which are determined spectrophotometrically with ferric 1,10-phenanthrolinate. The ferric complex enhanced the transguanylation of AET, but not APT. This finding suggests the possibility that AET would be easily transguanylated by metal ions or metal complexes which is contained in the body fluid, even though AET itself is injected. Aminothiols, such as MEA, MEG and MPG, displayed two maxima of the reduction abilities; one appeared at pH 3.2 and another at pH 5.0, and a minimum at pH 4.0. At those two pH regions, the reaction pathways and the products might be different. As the acidic region, the reductivities were parallell with the acidities of the aminothiols.

(Received October 15, 1966)

Aminoalkylisothiuronium salts capable of being transguanylated to mercaptoalkylguanidines have been shown to possess the protective action against a lethal dose of ionizing radiation.[1] We proposed in the previous paper that the transguanylation of 2-aminoethylisothiuronium bromide hydrobromide (AET) and 3-aminopropylisothiuronium bromide hydrobromide (APT) was accompanied with the acid-dissociation of the amino group; *i.e.*, the conjugate base of the thiuronium salt might be transformed to the sulfhydryl compound.[2] Since the dissociation constants of AET and APT were approximately 7.5 and 8.5 respectively, the rate of transguanylation was enhanced at alkaline pH region. In weakly acidic medium, on the other hand, it was shown that some of the thiuronium salts undergo a cyclization reaction. For instance, AET is cyclized to 2-amino-2-thiazoline (2-AT), which has little effect on the protection. Thus, both the transguanylation and the cyclization are main reactions in the physiological condition, but those reactions of the thiuronium salts take different meanings on the radiation-protection.

The transguanylation seems the most important and interesting reaction with regard to the protection; because the sulfhydryl compound produced in the biological test solution has been proposed to be an active agent of the thiuronium salt as a protector. Therefore, the thiuronium salts capable of being transguanylated, such as AET or APT, will be included in the radiation-protector belonging to the same category as aminothiols. The radiation-protective abilities of aminothiols would be attributed to the reactivity, especially the reductivity, of sulfhydryl group, because all of the hypotheses on the radiation-protection mechanism which have been proposed, such as

*1 Anagawa, Chiba-shi (花木 昭, 日野 亨, 赤星三弥).

*2 Part I : This Bulletin, **14**, 108 (1966).

1) *a*) L. Eldjarn, A. Pihl : "Mechanisms in Radiobiology," Vol. II, M. Errera, A. Forssber, eds., Academic Press, New York (1960). *b*) A. Hollander : "Radiation Protection and the Recovery," Pergamon Press, London (1960). *c*) S. Akaboshi : Yakugaku Zasshi, **83**, 1005 (1963). *d*) A. Hanaki, S. Akaboshi : Japan Analyst, **15**, 518 (1966).

2) A. Hanaki, *et al.* : This Bulletin, **13**, 108 (1966).

free radical scavenging,[3] reduction of oxygen tension,[4] and disulfide formation theories,[5] are related principally to the reduction of sulfhydryl group.

On the chemical oxidation of mercaptoalkylguanidines, Doherty and his coworkers reported that the sulfhydryl group was determined by its oxidation with 2,6-dichlorophenolindophenol at pH 7;[6] *i.e.*, by the reduction ability of the sulfhydryl group the dye was decolorized. However, it is probable that the reductivity of the thiuronium salt estimated by Doherty's method would be approximately equal to that of the transguanylation product, because the thiuronium salt is transguanylated rapidly and almost quantitatively to the sulfhydryl compound in neutral and slightly alkaline solution. Therefore, the sulfhydryl compound determined by Doherty's method is to include both the initially presented and the transformed components during the measurement. In order to avoid the possibility that the thiuronium salt is transguanylated during the measurement, the determination should be carried out at acidic pH region. In this paper, the oxidation was examined at pH region from 2 to 6 with ferric 1,10-phenanthrolinate. By using the ferric complex, we attempted to estimate the reductivities of AET, APT, (2-mercaptoethyl)guanidine (MEG), (3-mercaptopropyl)guanidine (MPG) and 2-mercaptoethylamine (MEA) at various pH values and establish the method by which the sulfhydryl compound could be determined in the mixture containing both the transguanylation product and the thiuronium salt.

Experimental

Electronic Absorption Spectra and Acid-Dissociation——The spectrophotometric measurements were carried out with a Cary Model 14 recording spectrophotometer equipped with a thermostatted cell compartment. In the ordinary measurement, the temperature was maintained at $25 \pm 0.5°$. pH measurements were performed with a Toa-Dempa HM-5A pH meter, which was calibrated against phthalate (pH 4.01) and phosphate (pH 6.86) buffers. All pH measurements were made as the same temperature as the corresponding spectrophotometric readings.

Mercaptoalkylguanidine was prepared from aminoalkylthiuronium salt by adding 1.2 equivalents NaOH into the solution. All stock solutions of the sulfhydryl compounds were prepared immediately before the measurements. For the spectrophotometric measurement, 3.00 ml. of 5.00×10^{-3} *M* the sulfhydryl compound was added to 97.0 ml. of 0.1 *N* NaCl containing varying amounts of NaOH. The absorption spectra was measured immediately after mixing.

Reduction Ability of Aminoalkylisothiuronium Salt, Mercaptoalkylguanidine, and Mercaptoethylamine——The reductivities of the thiuronium salts and the sulfhydryl compounds against ferric 1,10-phenanthrolinate were determined spectrophotometrically with a Hitachi Model EPU-2A spectrophotometer at 25°. The reaction was initiated by adding the reductants to ferric 1,10-phenanthrolinate solution buffered with 0.2 *M* acetic acid-sodium acetate solution. Total volume of the reaction mixture was 50 ml., and the concentration of each component was as follows; 1.00×10^{-3} *M* ferric ion, 4.00×10^{-3} *M* 1,10-phenanthroline, and 0 to 5.00×10^{-5} *M* the reductants. After certain minutes reaction, an aliquot was taken from the reaction mixture, and the absorbance at 510 mμ was measured. Since the absorbance could not reach to a constant level even after several hours, because of an insufficient reduction ability of the reductant, the estimation of the sulfhydryl compound was done by measuring $\Delta E_{510m\mu}$/hr. instead of $E_{510m\mu}$.*3

Results and Discussion

Ferric 1,10-phenanthrolinate was reduced almost quantitatively with a strong and simple reducing agent, such as ascorbic acid, and a stoichiometric relation was found between the concentration of the ferric complex reduced and the reducing agent; *i.e.*, one equivalent ascorbic acid could reduce exactly one equivalent the ferric complex.

*3 The calculation of $E_{510m\mu}$/hr. was done graphically at the steady state of the reaction.

3) P. Alexander, *et al.*: Radiation Research, **2**, 392 (1955). D.G. Doherty, *et al.*: *Ibid.*, **7**, 13 (1957).
4) Z.M. Bacq, A. Pihl: "A Symposium on Oxygen in the Animal Organism," 1963, Pergamon Press, London (1964).
5) L. Eldjarn, A. Pihl: J. Biol. Chem., **223**, 41 (1956).
6) J.X. Khym, *et al.*: J. Am. Chem. Soc., **79**, 5663 (1957).

On the other hand, in order to reduce the ferric complex quantitatively with MEA, MEG and MPG, larger amounts of the reducing agent was required, probably because of the weak reducing abilities. For instance, approximately 25 equivalents MEA was required for the complete reduction of $10^{-4}M$ the ferric complex at 35° in one hour. Moreover, since a primary oxidation product of the sulfhydryl compound might have also fairly strong reduction ability to the ferric complex, the stoichiometry of the reaction appeared to be complicate.

The reduction abilities of the thiuronium salt and the transguanylation product were influenced remarkably by their chemical structures. The reduction rates at various pH values of the ferric complex with AET, APT, MEG, MPG and MEA were shown in Fig. 1. The reductivity of the thiuronium salt was in every case lower

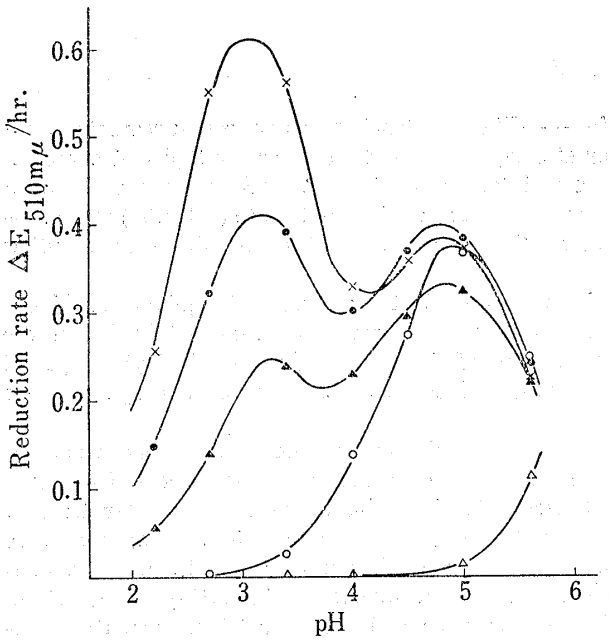


Fig.1. Reactivities against Ferric 1,10-Phenanthrolinate of AET, APT, MEG, MPG and MEA at Various pH Values
○ AET, △ APT, ● MEG, ▲ MPG, × MEA
The condition of the reaction in detail is shown in experimental part.

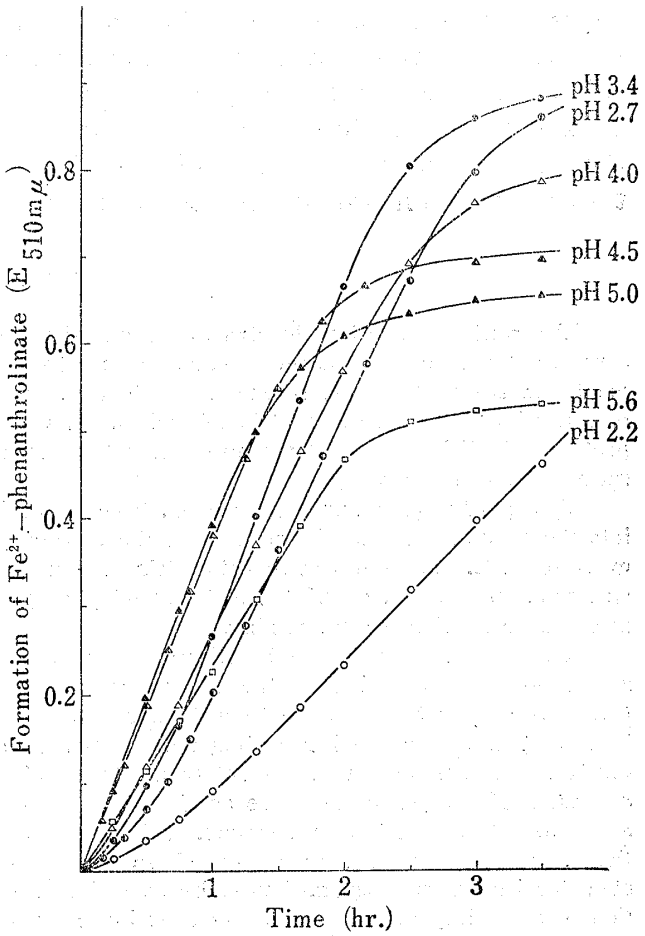


Fig. 2. Reaction Curves of the Reduction of Ferric 1,10-Phenanthrolinate with MEG at Various pH Values

than that of its transguanylation product. This fact may be attributed to the difference between the susceptibilities of sulfhydryl and thiuronium groups to the oxidizing agent. The rate by AET increased gradually from pH 3, and reached at pH 5 to a maximal level, which was nearly equal to that of MEG. The reaction curves of MEG shown in Fig. 2 appeared to be coincided completely with that of AET at the pH region beyond 5. Since pK_a of AET is approximately 7.5, larger parts of AET molecule is in the undissociated form at pH 5, and thereby the rate of transguanylation, which depends on the concentration of the conjugate base of AET, would not be rapid. Therefore,

the finding mentioned above suggests the possibility that the transguanylation of AET is enhanced by the interaction between the ferric complex and AET, which leads probably to the ionization of the amino group.*4

The reduction abilities of MEG, MPG and MEA displayed two maxima; one appeared at pH 3.2, and another at pH 5.0. Important factors which govern the rate of oxidation would be the electron density at the oxidizable atom,[7] the concentration of the reactive species, *i.e.*, the oxidizing agent and the oxidizable molecule, and the steric factor of the reaction. In the oxidation of cysteine catalyzed by ferric ion, Taylor, *et al.* found that the most reactive form of cysteine is the first ionization-product.[8] The ionization of cysteine is rather complicate as compared with simple aminothiols.[9] In the simple aminothiol like MEA, the step-wise ionization was measured between amino and sulfhydryl groups, and the first ionization occurred solely in the sulfhydryl group.[10] However, because of the electronic effect due to carboxylate group in cysteine, the basicity of amino group is weakened and then the first ionization-product is a mixture of $^{+}H_3N\text{-}CH(CH_2S^{-})\text{-}COO^{-}$ and $H_2N\text{-}CH(CH_2SH)\text{-}COO^{-}$. The ionization constant of sulfhydryl group itself can be determined by spectrophotometric method. The finding by Noda that alkylmercaptans absorb ultraviolet light at 230~240 mμ in strongly alkaline,[11] but not in acid, solution suggests a means of measuring spectrophotometrically the concentration of the dissociated sulfhydryl group, RS^{-}. The spectrophotometric

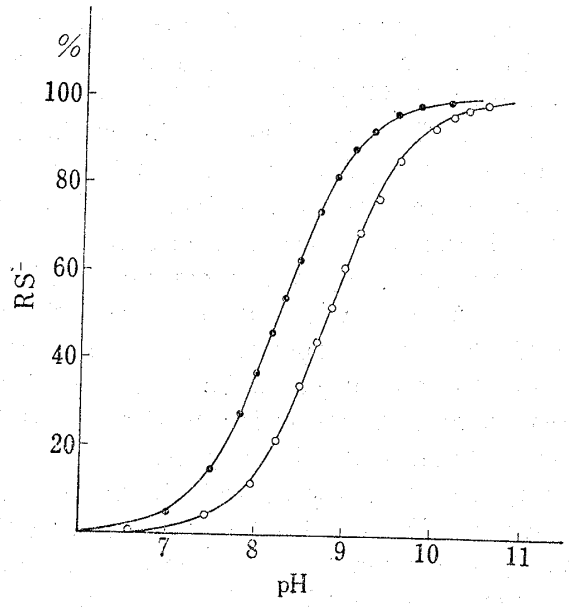


Fig. 3. Spectrophotometric Titration Curves of MEG and MEA
○ MEG, ● MEA
Concentration; $1.50\times10^{-4}M$, Temperature; 25°, Ionic Strength; 0.10 (NaCl)
The line is calculated from equation 1 using pKa of MEG (8.8) and MEA (8.25), respectively.

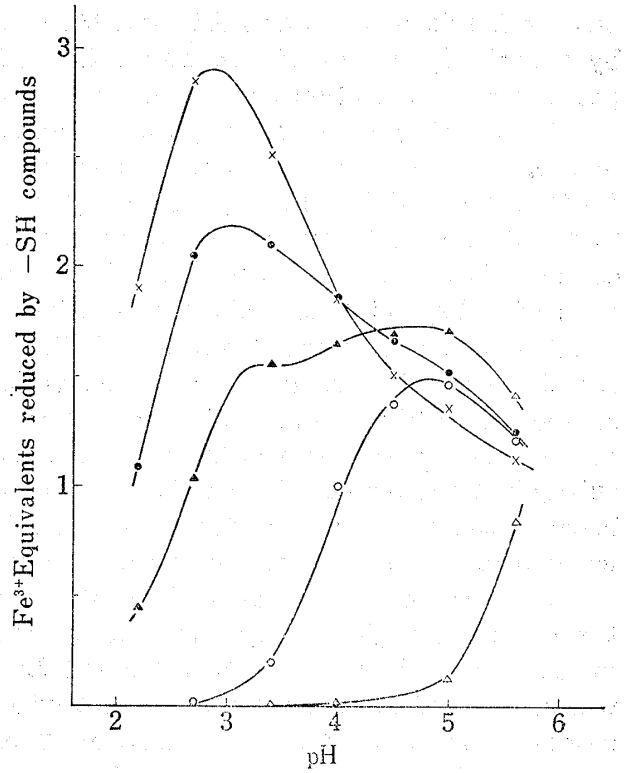


Fig. 4. Extent of the Reduction of Ferric 1,10-Phenanthrolinate with AET, APT, MEG, MPG and MEA at Various pH Values
○ AET, △ APT, ● MEG, ▲ MPG, × MEA

*4 The transguanylation as well as the oxidation was stimulated also by cupric ion.

7) M. Ishidate, A. Hanaki: Nature, **192**, 1198 (1961).
8) J.E. Taylor, *et al.*: J. Am. Chem. Soc., **88**, 1663 (1966).
9) J.T. Edsall: Biochemistry, **4**, 28 (1965).
10) R.E. Benesch, R. Benesch: J. Am. Chem. Soc., **77**, 5877 (1955).
11) L.H. Noda, *et al.*: *Ibid.*, **75**, 913 (1953).

titration curves plotted $E_{232m\mu}$ due to RS^- against pH were shown in Fig. 3. The curve of MEG displayed a continuous sigmoid-shape, which was resemble to that of MEA. In the ionization of MEA, because of the large difference between the basicities of sulfhydryl and amino groups, the sulfhydryl group is ionized completely prior to the acid-dissociation of the amino group. In general, the basicity of guanyl group is stronger than that of amino group; *i.e.*, pK_a of the former is higher than that of the latter. Therefore, a larger separation is expected between the ionization of sulfhydryl and guanyl group of MEG. Then, it is possible to treat that MEG is practically a mono-basic acid, though it is a di-basic acid thermodynamically. The ratio of the ionized to the total sulfhydryl group, which can be determined by spectrophotometric method, is written as follows;

$$(RS^-)/(R)_0 = 1/(\alpha+1) \qquad (1)$$

where α represent $[H^+]/K_a$, and (RS^-) and $(R)_0$ the concentration of the ionized and the total sulfhydryl groups, respectively. A solid line in Fig. 3 is the theoretical curve computed by using equation 1. A good coincidence between the theoretical and the observed curves indicates clearly that the first ionization of MEG depends completely on the sulfhydryl group. Therefore, it is also obvious that all of the ionized MEG and MEA in weakly alkaline solution are zwitter ion but not neutral species. The zwitter ion formations of MEG and MEA at pH 7.5 are respectively 5 and 15% computed by using pK_a values of 8.8 and 8.25. The ionization constant calculated from the potentiometric titration curve was approximately 9.3.

At the pH region below 4, the reduction abilities of the compounds examined, shown in Fig. 1, were widely spreaded. The reductivities were arranged as follows; MEA, MEG, MPG, AET and APT. The higher reducibility of AET than APT is attributed to the difference between the transguanylation rates. The abilities of the sulfhydryl compounds are correlated with the ionizability of the sulfhydryl group. At the higher pH region beyond 4, the similar reaction abilities were observed between MEA and MEG, but not between MEG and MPG. As pH increases, the concentration of the reactive ionized sulfhydryl compound, which is determined by the ionizability of the compound, might become unimportant; the important factor which control the activities of the reaction system would be the reactivity of the active species, *i.e.*, the ionized sulfhydryl compound. The reactivities of the ferric complex would be also important, because the complex is hydrolyzed as pH increases, and its catalytic activity would be weakened. A detailed discussion on the reaction mechanism is in progress.

The reaction curves at various pH values appeared to show complicate profiles. At the lower pH region, a lag phase of the reaction was observed for 30 or 40 min. of the initial stage. While, as pH increased beyond 4, the formation of ferrous complex, which was indicated by the increment of $E_{510m\mu}$, was increased linearly with time. The reaction curves of MEG at various pH values were shown in Fig. 2. The concentration of the ferric complex reduced by the sulfhydryl compounds can be calculated by using molar absorptivity at 510 mμ of ferrous 1,10-phenanthrolinate, 1.06×10^4. In Fig. 4, it was shown the relation how many molecules of the ferric complex were reduced with one molecule of the sulfhydryl compounds for 3 hr. Those results suggest that the oxidation of the sulfhydryl compound is not terminated at the stage of the primary product, probably disulfide, and further oxidation might be proceeded. The most interesting finding is that the oxidation involves probably two different pathways. One is undergone predominantly in acidic medium, maximum at pH 3.2, and invloves probably at least three equivalents oxidation. Another is in weakly acidic medium,

maximum at pH 5.0, and may be 1.5 or 2 equivalents reaction.

Since mercaptoalkylguanidines possess the weak reduction ability against ferric 1,10-phenanthrolinate and then the reaction mechanism is complicate mentioned above, it is impossible to derive a stoichiometric relationship between the concentration of the sulfhydryl compound and the ferric complex reduced. However, it is possible to expect a definite and reproducible relation between the concentration of the sulfhydryl compound and the ferric complex reduced for a certain period. In general, Lambert-Beer's law can be rewritten as follows;

$$\Delta E/\Delta t = (\Delta\varepsilon/\Delta t)Cl = \varepsilon' Cl \qquad (2)$$

where E, ε, C and l represent optical absorbance, molar absorptivity, concentration in mole/liter and cell length, respectively. Following the above modified equation, the concentration can be estimated by measuring $\Delta E/\Delta t$ instead of E. In order to determine the amount of MPG in the mixture of APT and MPG, the measurement could be done at any pH lower than 5. On the other hand, the determination of MEG in the mixture of AET and MEG should be done at the pH region lower than 3.5, because AET which is susceptible to the catalytic transguanylation is to show a significant reduction ability at the higher pH region. A calibration curve for the determination of MEG in the mixture of AET and MEG was shown in Fig. 5.

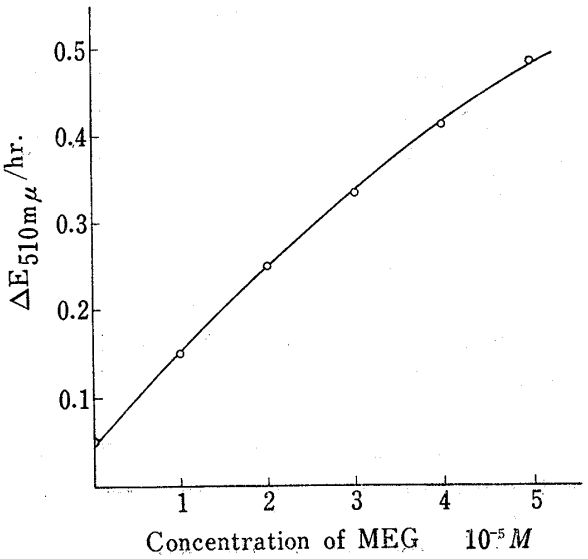
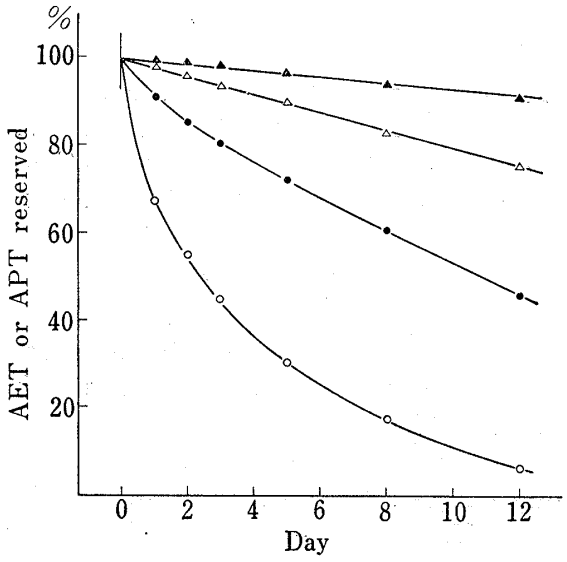


Fig. 5. Calibration Curve for the Determination of MEG in the mixture of AET and MEG
Fe^{3+}; $1.00\times10^{-3}M$, Phen.; $4.00\times10^{-3}M$, pH; 3.5 (Acetate Buffer) Temperature; 25°, [AET]+[MEG]; $5.00\times10^{-5}M$



Fi. 6. Stabilities of AET and APT against the Cyclization Concentration of the Thiuronium Salt; $2.00\times10\,M$
AET : in the presence of 1 equivalent HCl ● and absence of HCl ○ at 20°
APT : in the presence of 1 equivalent HCl ▲ and absence of HCl △ at 40°

The transguanylation of aminoalkylisothiuronium salt has shown to proceed solely in the presence of alkali. The above mentioned method for the determination of sulfhydryl compound was applied to confirm the stoichiometry on the transguanylation by alkali. The relation between the amounts of alkali added and the formation of MPG from APT was described in Table I. The formation of MPG appeared to correspond approximately to the amounts of alkali added. In the presence of an equivalent caustic alkali, the concentration of the ionized thiuronium salt, RS^-, is

TABLE I. Relationship between the Amounts of Alkali added and the Transguanylation of APT

Experimental 1[a]		Experimental 2[b]	
Alkali equivalent	$\Delta E_{510\,m\mu}$/hr.	MPG $10^{-5}M$	$\Delta E_{510\,m\mu}$/hr.
0	0.040		
0.2	0.390	1.00	0.385
0.4	0.474	2.00	0.476
0.6	0.538	3.00	0.543
0.8	0.571	4.00	0.559
1.0	0.590	5.00	0.590

a) For the determination of MPG produced in experimental 1, the stock solutions containing $5.00\times10^{-4}M$ APT neutralized respectively with 0, 0.2, 0.4, 0.6, 0.8 and 1.0 equivalent NaOH were prepared. From the stock solution, 5 ml. was pipetted, and mixed with 5 ml. of $1\times10^{-2}M$ Fe^{3+}, 10 ml. of $2\times10^{-2}M$ 1,10-phenanthroline, and 20 ml. of $0.5M$ acetate buffer, pH 5, solutions. Finally the solution was diluted to 50 ml. with distilled water. The reaction mixture was thermostatted at 30°, and after 10, 20, 30, 40 and 50 min., $E_{510\,m\mu}$ was measured.

b) Experimental 2 was done on the assumption that the ionized APT was transguanylated quantitatively to MPG. The reaction mixture contained MPG and ferric 1,10-phenanthrolinate, but not APT.

calculated as follows;

$$[RS^-]=a[R]_0+[H^+]-[OH^-] \qquad (3)$$

where $[R]_0$ represents the total concentration of the thiuronium salt, $a[R]_0$ is equal to the amounts of alkali added, and the term, a is always less than one equivalent. In the case of APT, since the concentration of a R_0 is large excess as compared with the term, $[OH^-]-[H^+]$, equation 3 can be rewritten as follows;*5

$$[RS^-]=a[R]_0=[NaOH]$$

Therefore, the information obtained from Table I indicates that the transguanylation proceeds through the ionized APT, which is an intermediate of the reaction, but not through APT itself.

The cyclization is a main reaction of aminoalkylisothiuronium salt itself. By adding excess alkali, the thiuronium salt in the test solution is transformed quantitatively to the sulfhydryl compound, while the cyclization product formed during the storage is undergone the ionization, but not the transformation. Therefore, the concentration of the cyclization product can be determined by subtracting the concentration of the sulfhydryl compound from the total concentration of the thiuronium salt. By this principle, the rate of cyclization was measured. The extent of the cyclization of AET and APT in various condition was shown in Fig. 6. AET was less stable than APT against the cyclization, and both were stabilized by adding mineral acid. The factor which decides the stability against the cyclization might be the probability or ability of the cyclic intermediate formation.

*5 For instance, the total concentration of APT shown in Table I is $5\times10^{-4}M$. If 0.6 equivalent alkali is added, a R_0 corresponds to $3\times10^{-4}M$ and the term, $[OH^-]-[H^+]$, is less than $5\times10^{-6}M$.