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Reactivities of Radiation-protective Aminoalkylisothiuronium Salts. III.[1] Reactivities of 2-Aminoethyl- and 3-Aminopropylisothiuronium Salts

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The potentiometric titration of radiation-protective aminoalkylisothiuronium salt, such as AET or APT, was carried out in various conditions with a recording titrator. The method for the selective determination of each component in the mixtures containing the isothiuronium salt, the transguanylation and cyclization products was established first. By using this technique, it was clarified that AET was mainly transguanylated and partly cyclized during the titration and that the molar ratio of those two reaction products depends on the titration speed and temperature. APT was transguanylated nearly quantitatively without repsect to the titration speed. Both reactions may proceed through the ionized isothiuronium salt. The rate of the transguanylation was extremely fast. In the presence of 0.5 equivalent alkali, the degree of the transguanylation at 25° was as follows; AET: 0.77 in 0.5 min, 0.94 in 2 min, APT: 0.82 in 0.5 min, 0.94 in 2 min. The pH dependence of the transguanylation was postulated and discussed quantitatively.

2-Aminoethylisothiuronium bromide hydrobromide (AET) and 3-aminopropylisothiuronium bromide hydrobromide (APT), effective protective agents against a lethal dose of ionizing radiation, are transguanylated rapidly to the sulfhydryl compounds in the physiological condition.[3] The physico-chemical characteristics indicating that those compounds undergo the transguanylation are as follows; a rapid and continuous pH drop,[4] and an increase of reduction ability.[1] The potentiometric study on those compounds reveals that the titration curve, namely the ionization, behaves irreversible.[5] This property is resulted from the rapid transguanylation of the ionized (dissociated) isothiuronium salt.[5] Besides the transguanylation, AET is cyclized in weakly acidic medium, probably in neutral medium, to 2-aminothiazoline (2-AT), and ammonium ion is splitted off.[3] Therefore, the participation of the cyclization, as well as the transguanylation, should be considered during the titration. On the other hand, APT is stable against the cyclization. Thus, the reaction of the isothiuronium salt encountered during the potentiometric titration is different qualititatively between AET and APT.

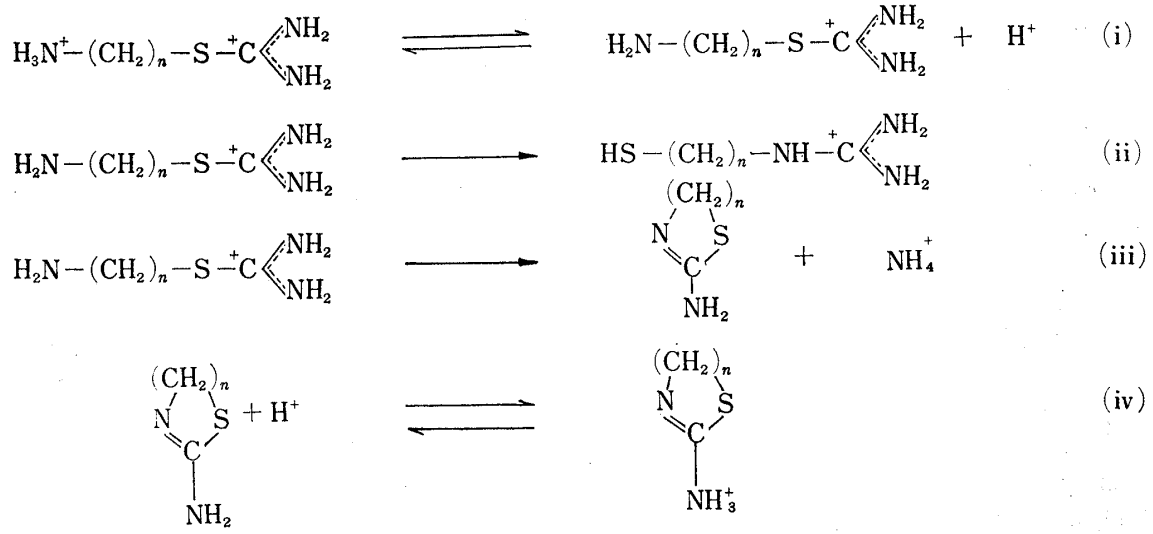
In order to investigate the reaction mechanism of the isothiuronium salt, which helps us to speculate on the protecting action of this compound, the determination of each reaction product is required first. As the method for the determination of the transguanylation product, it has hitherto been employed the following methods based on the reduction ability of sulfhydryl group; iodometric titration,[6] and spectrophotometric methods using 2,6-dichlorophenolindophenol[3] or ferric 1,10-phenanthrolinate.[1] In order to determine exactly the sulfhydryl compound, the measurement should be done strictly in such a condition that the isothiuronium salt does not undergo both the transguanylation and the cyclization during the treatment. The chemical method for the determination is not generally convenient to this purpose.

1) Part II: A. Hanaki, T. Hino, and S. Akaboshi, *Chem. Pharm. Bull.* (Tokyo), **15**, 1446 (1967).
2) Location: *Anagawa-4, Chiba-shi.*
3) J.X. Khym, R. Shapira, and D.G. Doherty, *J. Am. Chem. Soc.*, **79**, 5663 (1957); *ibid*, **80**, 3342 (1958).
4) A. Hanaki, T. Hanaki, K. Ōya, A. Andou, T. Hino, and S. Akaboshi, *Chem. Pharm. Bull.* (Tokyo), **14**, 108 (1966).
5) A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), **16**, 486 (1968).
6) R. Koch and W. Schwarze, *Arzneimittel-Forsch.*, **7**, 576 (1957).

In the previous papers, we showed, as the transguanylation proceeded, the equilibrium between the isothiuronium salt and its ionized species was disturbed and thereby hydrogen ion was liberated,[4] and that a stoichiometric relation existed between the concentration of alkali added and the sulfhydryl compound produced.[1] It was also predicted that the transguanylation product could be estimated indirectly by using the potentiometric titration technique.[5] In the present paper, we undertook to establish the potentiometric method for the selective determination of the isothiuronium salt and the transformation products in the reaction mixtures. Secondly, by using this technique, it was attempted to explain the qualitative difference of the reactivities between AET and APT.

Theoretical

The ionized species of the isothiuronium salt is the reactive form in both the transguanylation and the cyclization. Then, the reaction of the isothiuronium salt in aqueous solution can be pictured as follows (Chart 1).



n: 2 (AET), 3 (APT)

Chart 1

Owing to the basicity of 2-amino group, the cyclization product is able to associate with hydrogen ion as shown in reaction (iv), while the transguanylation product is not. The selective determination of those transformation products is based on this difference in the ability of hydrogen ion association.

The addition of alkali, less than one equivalent with respect to the isothiuronium salt, promotes both reactions. If a equivalent alkali is added, the isothiuronium salt is ionized to the conjugate base RNH_2, the concentration of which is expressed by equation (1);

$$[RNH_2]=a[R]_0+[H^+]-[OH^-] \\ =[B\text{-}OH]+[H^+]-[OH^-] \qquad (1)$$

where $[R]_0$ represents the total concentration of the isothiuronium salt, and [B–OH] indicates the concentration of alkali added. Supposing that some of RNH_2 molecule is transformed, the equilibrium of reaction (i) is disturbed and shifts to right-hand side. As a result of this reaction, hydrogen ion will be liberated. If the transformation involves only the cyclization, all of the liberated hydrogen ion associates quantitatively with the cyclization product, which is a basic compound. Since actually the transformation involves the transguanylation

as well as the cyclization, some of the liberated hydrogen ion has to be freed. This free hydrogen ion, presented as a free acid, is resulted from the transguanylation.

The ionized isothiuronium salt, though it is unstable, should be present in the reaction mixturs as far as the equilibrium (i) exists. Accordingly, if *a* equivalent acid is added newly into this reaction mixtures, the ionized isothiuronium salt will associate with the added hydrogen ion to form the conjugate acid RNH_3^+. Provided that the transguanylation product from the ionized isothiuronium salt is present in the mixture, the corresponding amount of the added acid will become surplus, and may be titrated as a strong acid. The mixture after adding *a* equivalent acid contains the following molecular species; the isothiuronium salt, the transguanylation and cyclization products, ammonium ion and free acid. Since all the species except free acid possess relatively high pK_a values, the titration of free acid can be performed easily and quantitatively. Thus, the degree of the transguanylation can be estimated by indirect titrimetry.

The isothiuronium salt is transguanylated quantitatively in the presence of excess alkali.[4] Now, if excess alkali, *b* equivalent, is added into the solution which has been maintained in the presence of *a* equivalent alkali, all the isothiuronium salt including the ionized species will be transguanylated quantitatively to the sulfhydryl compound. When this solution is titrated with alkali after adding $(a+b)$ equivalent acid, free acid corresponding to the concentration of all the sulfhydryl compound can be titrated. The equivalent of free acid determined is actually less than one equivalent, because some of the isothiuronium salt has been cyclized in the presence of *a* equivalent alkali. This difference from one equivalent indicates the degree of the cyclization. The schematic explanation for the titrimetric determination of the isothiuronium salt, the transguanylation and cyclization products is pictured in Fig. 1.

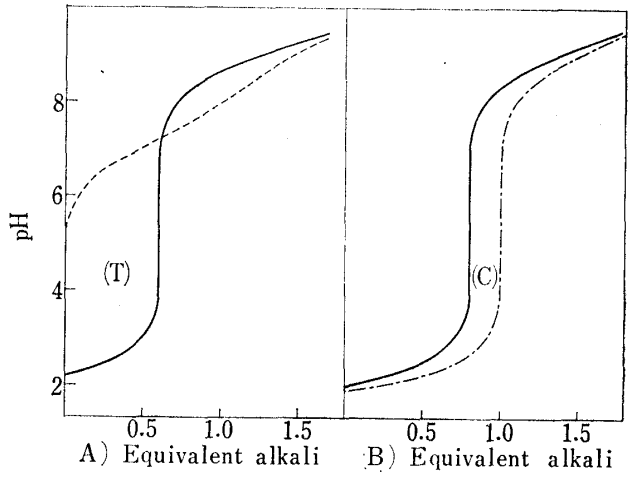


Fig. 1. Schematic Explanation for the Titrimetric Determination of Aminoalkylisothiuronium Salt, the Transguanylation and Cyclization Products

A) ------ : titration curve of aminoalkylisothiuronium salt
——— : retitration curve of the isothiuronium salt solution which has been maintained in the presence of *a* equivalent alkali
B) —·—·— : titration curve of mercaptoalkylguanidine (transguanylation product)
——— : retitration curve of the isothiuronium salt solution which is added newly *b* equivalent alkali after maintaining in the presence of *a* equivalent alkali for certain period
(*T*) : degree of the transguanylation
(*C*) : degree of the cyclization

Experimental

Materials——AET and APT prepared in this laboratory were used. For the determination of ionization constant, carbonate free KOH prepared by the method shown by Albert was used.[7] Carbonate free NaOH, which was used for the determination of the reaction products, was prepared by diluting 50% stock solution. Other chemicals were analytical grade, and used without further purification. All the solutions were prepared from twice distilled water from all glass apparatus.

Determination of Ionization Constant——The ionization constant was determined by the potentiometric titration with carbonate free KOH in a medium of 0.1 N $NaNO_3$. The titration was done in an atmosphere of nitrogen with a Radiometer TTT1c titrator and SBR2c titrigraph, and the glass electrodes 202C and 202B were used, respectively, for the measurements at 5° and 25°. The apparatus was standardized with phthalate and phosphate buffers at the corresponding temperatures to the measurements.

7) A. Albert and E.P. Serjeant, "Ionizing Constants of Acids and Bases," Methuen and Co., Ltd., London, 1962.

The titration of AET and APT was performed at a rapid speed as possible to prevent the transformation; *i.e.*, pen speed 40%/min and chart speed 30 mm/min.[8] Their ionization constants were calculated by the method described in the previous paper.[5] For the titration of other compounds, pen and chart speeds were fixed at 10%/min and 30 mm/min, respectively.

Determination of the Cyclization Product during the Titration——The solution of 5×10^{-3} M AET or APT containing 0.25 equivalent HCl was titrated with 0.1 N NaOH at 5° and 25°. The volume of the solution was exactly 20 ml at the half neutralization point. The titration speeds were selected as follows; experiment A; pen speed 40%/min and chart speed 30 mm/min, experiment B; pen speed 10%/min and chart speed 30 mm/min, experiment C; pen speed 5%/min and chart speed 4 mm/min. In the titration of AET at 25° the times for full length recording, corresponding to the titration with 2.5 equivalents NaOH (2.5 ml), were approximately 8.6, 22.0 and 66.0 min, respectively, for experiments A, B and C. After the titration with 2.5 equivalents NaOH was finished, the solution was neutralized exactly with 2.5 equivalents HCl. Then, the solution was retitrated with 0.1 N NaOH. As the end point of the titration, pH values were set at 5.5 and 5.7 at 25°, respectively, for AET and APT, and at 5.7 at 5° for AET. The degree of the cyclization was calculated from equation (2).

$$\text{Degree of the Cyclization} = 1 - (\text{Equivalent of Free Acid}) \qquad (2)$$

Determination of the Rate of the Transguanylation——Half equivalent NaOH, corresponding to 0.8 ml, was added rapidly as possible ,*i.e.*, within 10 sec, into 20 ml of 8.00×10^{-3} M AET or APT, which had been thermostatted at 25°. After keeping 0.5 or 2.0 min, 0.8 ml of 0.1 N HCl was added rapidly into this solution. Then, the reaction mixture was retitrated with 0.1 N NaOH. The end point pH values for the titration were set at 4.8 and 5.8, respectively, for AET and APT. The equivalent of alkali titer, namely of free acid, indicates the degree of the transguanylation.

Results

The potentiometric titration of AET and APT was done in different conditions; *i.e.*, at the different titration speeds and temperatures. The behaviors of the titration curves against the varying speeds of the titration had been described in the preceeding paper.[5] The retitration curves of both compounds displayed the characteristic pattern indicating the existence of free acid as shown in Fig. 2. It was confirmed from the retitration curve that the transguanylation was occured undoubtedly, during the course of the titration. The degree of the transguanylation estimated from the generation of free acid ought to increase with the reaction time; in the present experiment, with the time required for the titration. However, it was found unexpectedly that the concentration of free acid appeared to reduce with the decreasing of the titration speeds as shown in Table I. From those findings, we had to imagine the participation of another reaction, probably the cyclization. It may be expected that the cyclization competes with the transguanylation during the titration, because both reactions might proceed through the same intermediate. If the condition, in which the titration is done, is favorable to the cyclization, the ratio of the cyclization to the transguanylation might increase.

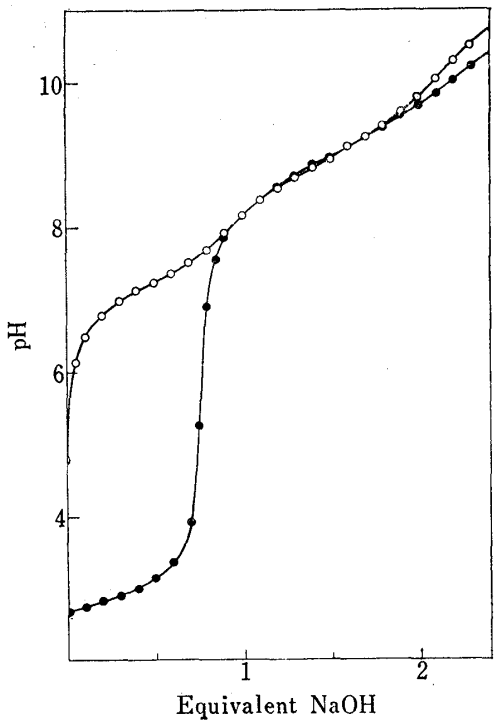


Fig. 2. Titration and Retitration Curves of AET

—○—○— : titration curve (same titration speed as experiment A)

—●—●— : retitration curve

$[AET]_0$: 5.00×10^{-3}M

In order to titrate precisely the free acid generated, the end point of the titration curve

8) The derivery of the standard alkali solution is controlled by both pen and chart speeds.

Table I. Relationship between Free Acid Generation and Titration Speed

Experiment	Free acid (10^{-3} M)	Titration speed[a] (min)
A–1	3.68	8.7
A–2	3.81	8.5
B–1	3.36	21.8
B–2	3.35	22.0
B–3	3.24	22.1
C–1	2.95	66.0
C–2	2.90	66.0

a) time required for the titration with 2.5 equivalents NaOH
$[AET]_0$: 5.00×10^{-3} M temperature: 25°

should be set up rigidly. For simplicity, let us consider two components mixtures consisting of free acid and a very weak acid. The end point for the titration of free acid corresponds to the inflection point of the curve displaying a sigmoid shape. This inflection point can be calculated from equation (3);

$$a=\frac{[OH^-]-[H^+]}{[A]_0}+\frac{K_a}{K_a+[H^+]} \qquad (3)$$

where a, $[A]_0$, and K_a represent, respectively, alkali equivalent, the concentration of a very weak acid, and its ionization constant. In the acidic region, where the weak acid can not ionized, a is negative. As the ionization begins, a becomes positive. Accordingly, the inflection point is found at $a=0$. In the multi-components system consisting of free acid and several weak acids, K_a in equation (3) can be represented by the smallest value of all. The pK_a values of the isothiuronium salt and its transformation products were tabulated in Table II. In column 3 of Table II, the inflection point of the titration curve was presented.

Table II. Ionization Constants of AET, APT and Their Transformation Products

Compound	pK_a		Inflection point pH	
	5°	25°	5°	25°
AET	8.3	7.5	5.3	4.9
MEG[a]	9.20	8.73	5.7	5.5
2-AT	9.47	8.78	5.8	5.5
APT	9.5	8.6	5.8	5.4
MPG[b]	9.81	9.31	5.9	5.7
2-PT	10.79	9.99	6.3	6.0
NH_4^+	10.01	9.26	6.0	5.7

a) mercaptoethylguanidine b) mercaptopropylguanidine

The rate of the transguanylation, which had been postulated to be extremely fast, was measured at 25° in the presence of half equivalent alkali. Immediately after the addition of alkali, approximately half of the isothiuronium salt should be ionized, as shown in equation (1), to the monoionic conjugate base, which is the reactive species against the transguanylation. The results were presented in Table III, where the degree of the transguanylation was expressed as the ratio to the reactive species being present initially. As indicated in Table III, the rate is extremely rapid; more than half of the conjugate base is transguanylated within 0.5 min, and more than 90 percent within 2 min. APT appeared to be more susceptible to the transguanylation. A detailed discussion on the rate will be published in the following paper.

Table III. Rate of the Transguanylation at 25°

Reaction time (min)	$[R'SH]^{a)}/[RNH_2]_0$	
	AET	APT
0.5	0.770	0.825
2.0	0.940	0.940

a) R'SH: transguanylation product $[RNH_2]_0$: 4.00×0^{-3} M

In APT, which resists against the cyclization, the amounts of free acid generated during the titration is equal to approximately one equivalent without respect to the titration speed. However, in the solution of APT, which had been stored at room temperature for a few week, the cyclization product, 2-aminopenthiazoline (2-PT), was detected undoubtedly by using the potentiometric titration technique.[1] Moreover, the formation of 2-PT is reported to increase with the increasing of pH between 2 and 5.[3] If APT were titrated at a extremely slow rate, the production of 2-PT would be confirmed. The reason why APT is hardly cyclized during the titration will be discussed in the following part. On the other hand, AET is cyclized at a fairly rapid rate during the titration. The degree of the cyclization was shown in Table IV in connection with the titration speed. In column 4 of Table IV, the time required for the titration with one equivalent alkali was presented. The ratio of the transguanylation to the cyclization was affected also by temperature. Considering the pH dependence of pK_a, the initial pH, which is measured immediately after the addition of a definite amount of alkali, should increase according to the decrease of temperature. Therefore, a participation of hydroxide ion is expected in the transguanylation.

Table IV. Relation between the Cyclization of AET and Titration Speed

Experiment	Temperature (°C)	Cyclization (equiv.)	Titration speed[a] (min)
A	5	0.135	3.5
A	25	0.250	3.7
B	5	0.180	9.7
B	25	0.340	10.3
C	5	0.265	29.3
C	25	0.415	30.7

a) time required for the titration with 1 equivalent NaOH
$[AET]_0$: 5.00×10^{-2} M

Discussion

Though the isothiuronium salt is mainly transguanylated in neutral and weakly alkaline medium, why does the degree of the cyclization increase with the decreasing of the titration speed? One reason might be ascribed to the pH dependence of the reaction rate. As described above, the isothiuronium salt is transguanylated immediately after the addition of alkail, and the pH drop of the solution follows it. From a microscopic viewpoint, the acid-base titration means that a very small amount of alkali is added into the acid solution at a constant interval. Each time alkali is added, pH of the isothiuronium solution has to be lowered continuously. The pH drop depending on the interval of alkali addition may be observed during the titration with one equivalent alkali. Accordingly, the titration curve moves to acidic side with the decreasing of the titration speed.[5] If the rate of the transguanylation depends on the concentrations of both the ionized isothiuronium salt and hydroxide ion as

indicated in equation (4), the reaction is retarded in accordance with the lowering of pH. In equation (4), k_1 represents the rate constant of the transguanylation.

$$\text{Rate (transguanylation)} = k_1[RNH_2][OH^-] \quad (4)$$

On the other hand, the rate of the cyclization which would be indifferent to hydroxide ion may be expressed in equation (5);

$$\text{Rate (cyclization)} = k_2[RNH_2] \quad (5)$$

where k_2 represents the rate constant of the cyclization. Equations (4) and (5) can be rewritten as follows;

$$\text{Rate (transguanylation)} = k_1\frac{K_a}{[H^+]+K_a}[R]_0[OH^-] \quad (6)$$

$$\text{Rate (cyclization)} = k_2\frac{K_a}{[H^+]+K_a}[R]_0 \quad (7)$$

where $[R]_0$, and K_a represent the total concentration of the isothiuronium salt and its ionization constant, respectively. By using those equations, the pH dependece of the reactions may be evaluated. A schema indicating the relationship between the reaction rates and pH was shown in Fig. 3. It was adopted tentatively in Fig. 3 that $[R]_0$ is 1×10^{-2} M, pK_a 7.5 at 25°, k_2 unity and k_1 10^7 k_2. It may be understood that the condition at higher pH is convenient to the transguanylation.

By combining equations (6) and (7), the ratio of the transguanylation to the cyclization is expressed by equation (8).

$$\text{Ratio (transguanylation/cyclization)} = \frac{k_1}{k_2}[OH^-] \quad (8)$$

The equation indicates that the ratio is controlled by the following two factors; the ratio of the rate constants, and the concentration of hydroxide ion. The rate constant might depend on the physical and chemical characteristics of the reactant RNH_2 and the activated complex. In the transformation of the isothiuronium salt, those factors are assumed to be the energetic difference between the reactant and the complex, and the stability of the complex. Since both the transguanylation and the cyclization might proceed through the same activated complex, the stability of the complex would be an only

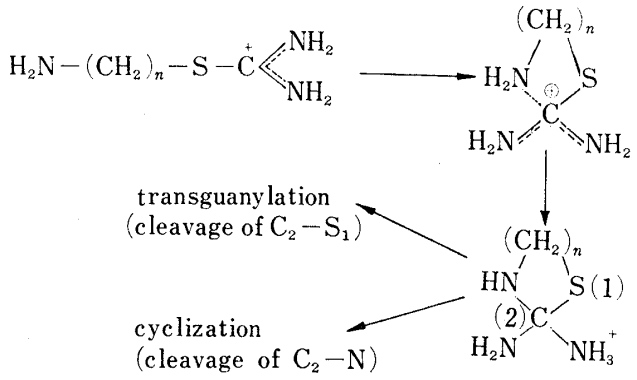


Chart 2

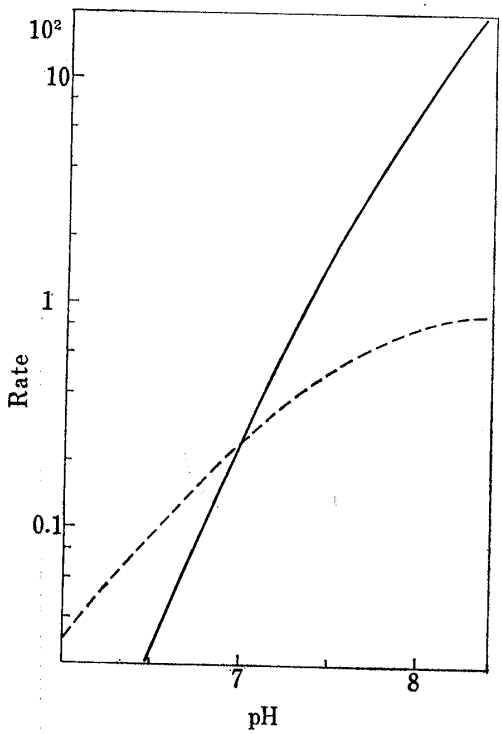


Fig. 3. pH Dependence of the Transguanylation and the Cyclization

——— : transguanylation calculated from equation (6)

– – – – – : cyclization calculated from equation (7)

important factor which decides the reaction pathway. Especially, the relative strength of C_2-S_1 and C_2-N (amino) bonds would take a great role in order to decide which pathway is convenient to a compound. For instance, if the ring of the intermediate in the activated state is extremely labile, the transguanylation may be predominant. On the other hand, if C_2-S_1 bond is relatively stable, the cyclization may be predominant. The term $[OH^-]$ is participated only in the transguanylation, and its contribution becomes increasingly important in alkaline region.

APT is transguanylated nearly quantitatively without respect to the titration speed, while AET is mainly transguanylated and partly cyclized and the ratio of the reactions depends on the titration speed. The difference in this ratio between AET and APT can be explained satisfactorily by equation (8). The ionization constant of APT is approximately 10 times smaller than that of AET. This fact means that the concentration of hydroxide ion is approximately 10 times larger in APT than in AET at any corresponding point of the titration curve between zero and one equivalent alkali. Accordingly, the ratio in equation (8) is approximately 10 times larger in APT, even though the ratio k_1/k_2 is not greatly different between both compounds.

The intermediates in the activated state have been postulated to from five- and six-membered rings, respectively, for AET and APT. The intermediate having six-membered ring might be convenient to the transguanylation, because C_2-S_1 bond weakens by virtue of a strain of the ring. On the other hand, the intermediate of five-membered ring, which might be less labile than the six-membered ring, undergoes both the transguanylation and the cyclization. Therefore, the ratio k_1/k_2 is larger in APT than AET, and thereby the transguanylation is predominant in APT. Because of the larger contribution of both k_1/k_2 and $[OH^-]$, the transformation of APT is inclined to the quantitative transguanylation.

Appendix

In the two components system consisting of free acid and a very weak acid, the reaction (9) is in equilibrium;

$$AH^+ \rightleftarrows H^+ + A \qquad (9)$$

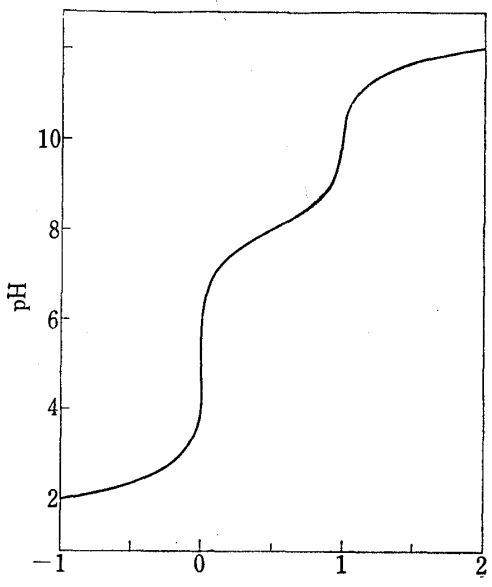


Fig. 4. Calculated Titration Curve of Equimolar Mixture Constisting of Free Acid and Very Weak Acid (pK_a 8) at 25°

$[A]_0$: 1.00×10^{-2} M

where AH^+ and A represent the weak acid and its conjugate base, respectively. The equilibrium constant of this reaction is

$$K_a=[H^+][A]/[AH^+] \qquad (10)$$

where K_a represents the ionization constant, and the square brackets indicate equilibrium concentrations. For electroneutrality requirement, equation (11) should be satisfied;

$$[AH^+]+[B\text{–}OH]+[H^+] = [A]_0+[H-X]+[OH^-] \qquad (11)$$

where $[A]_0$, [H–X] and [B–OH] represent the total concentrations of the weak acid, free acid and alkali added, respectively. For simplicity, let us employ a $[A]_0$ in place of [B–OH]−[H–X], where a represents equivalent alkali with respect to the weak acid. Then, equation (11) can be rewritten as follows:

$$[AH^+]=(1-a)[A]_0+[OH^-]-[H^+] \qquad (12)$$

Since $[A]_0$ is equal to the sum of $[AH^+]$ and [A], [A] is expressed as follows:

$$[A]=a[A]_0-[OH^-]+[H^+] \tag{13}$$

By combining equations (10), (12) and (13), the relation in equation (3) is obtained.

$$a=\frac{[OH^-]-[H^+]}{[A]_0}+\frac{K_a}{K_a+[H^+]} \tag{3}$$

The theoretical titration curve of an acid A can be calculated from equation (3). As a typical example, a titration cruve at 25° of equimolar mixture of free acid and a weak acid, pK_a 8, is shown in Fig. 4.

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