The Stability of Inosine in Acid and in Alkali

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The rate constants of the decrease in inosine at several temperatures between 50 °C and 140 °C in water were determined over the pH range from 1 to 13. They can be expressed as a sum of four simultaneous bimolecular reactions. Their thermodynamic parameters were obtained.

For the industrial isolation of inosine from the fermentation broth, information about the stability of inosine in acid and in alkali is of importance to developing the process design for the maximum yield. The kinetics of the hydrolysis of some purine nucleosides,[1-4] such as adenosine and guanosine has been reported in a limited pH range, and that for psicofuranine,[5] over the whole pH range. However, no studies concerning inosine have been reported in the literature. The present paper deals with the pH profile of the rates of the decrease in inosine at several temperatures, and with the pH dependence on the yields of the products.

Experimental

Material and Equipment. The inosine used was a commercial product of the Ajinomoto Co., Inc. The pH of the solution was measured at room temperature with a Toa Dempa HM-5A pH meter. The UV absorbance was measured with a Hitachi spectrophotometer, Model 139.

Paper Chromatography (PC). One-dimensional ascending PC was carried out on Toyo-Roshi No. 51A, the following two solvent systems being used: Solvent A, *n*-butanol–acetic acid–water (4 : 1 : 1); Solvent B, distilled *i*-butyric acid–*n*-butanol–28% ammonia aq.–water (36 : 30 : 3 : 31). The papers were developed three times if necessary for the complete separation of the components on the chromatogram. The components of the reaction mixture were detected on the paper by means of UV absorption. Usually standard solutions of inosine and hypoxanthine(H) were spotted with the reaction mixture. Several spots corresponding with the products including the remaining inosine and the standards were cut off, together with their appropriate blanks, and eluted with 0.1 M HCl, and the absorbances of the eluates were measured at 250 nm. The concentration of each component in the reaction mixture was calculated from the absorbance compared with that of each standard reference. The standard solution of 9-β-D-ribopyranosyl hypoxanthine (Y) used was that of inosine because of their similarity. The absorbance of the spot corresponding with 5-amino-1-β-D-ribofuranosyl-4-imidazolecarboxamide(X) was corrected in terms of inosine by the use of their molecular extinction coefficients.

Acid Hydrolysis. Inosine was dissolved(3 w/v%) in aq. HCl with a definite concentration, and 25 ml of the resulting solution was sealed in a glass ampoule or a stainless steel tube. It was kept for a definite period on an oil-bath at a constant temperature. The resultant solution was diluted with water to 100 ml and analyzed by means of PC in Solvent A. Table 1 lists the conditions. A steel tube was used for each run over 100 °C and a glass ampoule for each below 80 °C except that a steel tube was used for No. 6 (at 70 °C).

Degradation in Neutral Buffer Solutions. The method used was the same as that to be described with alkaline degradation except that the solvents were phosphate buffer solutions. Table 2 lists the conditions. The following buffer solutions were used: B-1, B-2, B-3, M/2 KH_2PO_4-M/2 Na_2HPO_4 (95 : 5), (30 : 60), (5 : 95); B-4, B-5, M/15 KH_2PO_4-M/15 Na_2HPO_4 (90 : 10), (40 : 60).

Alkaline Degradation. Inosine was degraded in the same manner as has been described above in the case of acid hydrolysis except that the solvent was aq. NaOH instead of HCl and except that a steel tube was used throughout all the runs. Table 3 lists the conditions. The chromatogram in Solvent A with three-times development was exhibited in the preceding paper.[6] The spot corresponding with inosine(R_f 0.52) tends to be contaminated with an unknown pale-red spot (R_f 0.55), while that corresponding with Y(R_f 0.43) is not. Therefore, the amount of Y was determined by the use of the chromatogram in Solvent A. The chromatogram in Solvent B with two-times development indicates a clear separation of a pale-red spot(R_f 0.64) from that o

TABLE 1. CONDITIONS AND OBSERVED FIRST-ORDER RATE CONSTANTS FOR THE ACID HYDROLYSIS OF INOSINE

Run	°C	[HCl] (M)	hr	Obsd. pH		10^2k (hr^{-1})		
				Init.	Final			
1a, b, c	50	0.175	3, 6, 10	1.1	1.3, 1.4, 1.4	3.66,	3.33,	3.75
2a, b, c	60	0.175	3, 4, 6	1.1	1.4, 1.4, 1.4	15.4,	14.8,	15.2
3a, b, c	70	0.175	1, 3, 4	1.1	1.2, 1.4, 1.4	50.5,	49.4,	50.1
4a, b, c	70	0.015	4, 6, 8	2.1	2.3, 2.4, 2.4	5.35,	5.40,	4.61
5a, b	70	0.002	24, 48	3.0	3.2, 3.3	0.647,	0.579	
6	70	0.0002	96	4.0	4.1	0.0845		
7a, b, c	80	0.015	4, 6, 8	2.1	2.5, 2.5, 2.5	15.3,	14.1,	14.2
8a, b, c	80	0.002	6, 8, 10	3.0	3.2, 3.2, 3.2	2.53,	2.35,	2.52
9a, b	100	0.015	1, 2	2.1	2.4, 2.5	112	88.5	
10a, b, c	100	0.002	2, 4, 6	3.0	3.3, 3.4, 3.4	24.8,	15.7,	13.4
11a, b	100	0.0002	12, 24	3.5	3.8, 3.8	2.89,	2.90	
12a, b, c	120	0.0002	2, 4, 6	3.9	3.9, 3.8, 3.8	20.3,	27.1,	23.3

Table 2. Conditions and observed first-order rate constants for the degradation of inosine in the neutral buffer solutions

Run	°C	Buffer	hr	Obsd. pH Init.	Obsd. pH Final	10^2k (hr^{-1})
13a, b	120	B-1	8, 16	5.4	5.2, 4.8	1.48, 2.53
14a, b	120	B-4	10, 16	6.0	5.3, 4.7	0.770, 1.02
15a, b	120	B-5	10, 16	7.0	6.5, 6.4	0.661, 0.775
16a, b	130	B-1	4, 8	5.4	5.0, 4.7	5.68, 5.88
17	130	B-4	6	6.0	4.8	2.95
18	130	B-5	10	7.0	6.2	1.88
19	130	B-3	24	8.0	7.1	3.12
20a, b	140	B-2	3, 6	7.1	6.9, 6.7	9.77, 12.0
21a, b	140	B-3	3, 6	8.0	7.5, 7.3	14.4, 10.2

Table 3. Conditions and observed first-order rate constants for the alkaline degradation of inosine

Run	°C	[NaOH] M	hr	Obsd. pH Init.	Obsd. pH Final	10^2k (hr^{-1})
22	100	0.019	16	8.1	8.0	2.74
23	100	0.060	16	8.9	8.8	8.35
24	100	0.095	16	9.7	9.4	13.5
25	100	0.120	16	11.0	10.0	17.5
26	100	0.151	16	11.9	11.6	15.1
27	100	0.500	16	13.2	13.0	21.7
28	120	0.002	8	7.1	6.8	5.23
29a, b	120	0.019	8, 16	8.1	7.9, 7.4	16.2, 11.8
30a, b	120	0.095	8, 16	9.7	9.4, 9.1	44.7, 34.8
31a, b	120	0.151	4, 8	11.9	11.4, 10.3	70.6, 66.6
c			16		9.9	62.5
32a, b	120	0.500	8, 16	13.2	12.9, 13.0	89.1, 112
33	130	0.095	4	9.7	9.4	103

inosine(R_f 0.55), whereas the mobility of Y is the same as that of inosine. Then, the total amounts of inosine and Y were determined by the use of the chromatogram in Solvent B; the subsequent subtraction of the amount of Y determined before gave that of inosine. The amounts of H and X were determined by means of PC in Solvent A.

Results and Discussion

Inosine (I) degrades in an acid solution, giving hypoxanthine (H) and D-ribose. On the other hand, in an alkaline solution a significant amount of a ring-opened or isomerized product, such as 5-amino-1-β-D-ribofuranosyl-4-imidazolecarboxamide (X) or 9-β-D-ribopyranosyl hypoxanthine (Y), is concomitantly produced, as has been described previously[6] (Chart 1). However, apparent first-order linear plots were obtained for the acid-catalyzed degradation, and even for the alkaline treatment.

Rates of the Decrease in Inosine. Tables 1, 2 and 3 show the conditions and observed first-order rate constants (k) corresponding to the regions for acid, for a neutral solution, and for alkali. A variation in the pH during the degradation was usually observed, and it was larger under alkaline conditions than in acid,

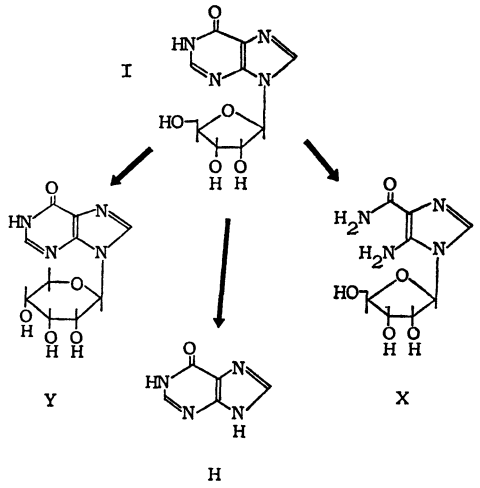

Chart 1

The longer the reaction time, the larger the variation in the pH. This variation, however, had a significant effect on the first-order rate constants. This is the reason why there are some differences in the rate constants in the corresponding experimental runs in Tables 2 and 3; for instance, between Experimental Runs No. 29a and No. 29b in Table 3 there is some difference in rate constant (16.2—11.8) resulting from the difference in the final pH (7.9—7.4). Such a variation in pH can be explained in terms of the production of considerable acid substances; for example, formic acid is released when inosine is cloven at C-2 in dilute alkali, giving X.

The observed first-order rate constant, k, at a constant pH was determined by the linear plots given by Eq. (2), which is derived from Eq. (1):

$$-d[I]/dt = k[I] \quad (1)$$

$$\ln([I_s]/[I]) = kt \quad (2)$$

where $[I_s]$ is the initial concentration of inosine and where [I] is its concentration after a certain time, t.

Figure 1 shows the profile of the overall rates of the decreases in inosine at several tempreatures. The rate is linearly dependent on the hydrogen-ion concentration in the 1—5 pH range, decreasing with an increase in the pH to a minimum of 6—7 and then

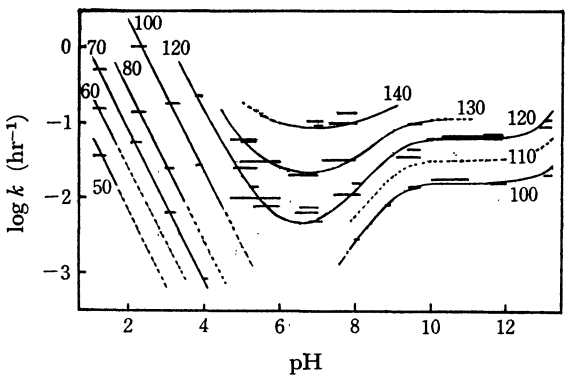

Fig. 1. Overall rate constants for the decrease of inosine in water at several temperatures (°C) as a function of pH.

Table 4. Catalytic rate constants for the decrease in inosine

Temp. °C	k_i ($l \cdot mol^{-1} \cdot hr^{-1}$)[a]			
	$i=1$	$i=2$	$i=3$	$i=4$
50	6.48×10^{-1}	—	—	—
60	2.37	—	—	—
70	8.04	—	—	—
80	2.54×10	—	—	—
100	2.12×10^{2}	—	2.73×10^{-4}	6.26×10^{-2}
120	1.42×10^{3}	2.19×10^{6}	1.07×10^{-3}	4.41×10^{-1}
130	—	1.03×10^{7}	2.01×10^{-3}	—
140	—	4.48×10^{7}	—	—

a) Rate constants for specific hydrogen ion catalysis to uncharged species (k_1), for specific hydrogen ion catalysis to mono-anionic species (k_2), for water catalysis to mono-anionic species (k_3) and for hydroxyl ion catalysis to anionic species (k_4).

increasing with an increase in the pH in the pH range of 8—9. It is insensitive to the pH in the region pH of 10—12 and then seems to increase again with an increase in the pH. This profile is similar to that obtained for aspirin by Edwards[7] with the exceptions, that the minimum of the rate constant is in the neutral region and that has a smaller value. As will be shown below, the profile of this curve can be explained in terms of four bimolecular simultaneous reactions composed of those between uncharged inosine and hydrogen ions, anionic inosine and hydrogen ions, anionic inosine and water molecules, and anionic inosine and hydroxyl ions.

It is known that the acid-catalyzed hydrolysis of nucleosides giving nucleic bases and sugars can be explained in terms of a bimolecular reaction between nucleosides and hydrogen ions.[2] The mechanism of the reaction has also been discussed.[2] A decrease in the inosine in the acid solution in the present study can be simply explained by this reaction.

In the pH range of 1—5 plots of log k *vs.* pH show a linear relation with a slope of unity (Fig. 1), and the rates of the hydrogen ion-catalyzed reactions are given by Eq. (3):

$$-d[I]/dt = k[I] = k_1[H^+][I^\circ] \quad (3)$$

where k_1 is the catalytic rate constant for a specific hydrogen-ion catalysis and where [I°] is the concentration of the uncharged species of inosine, being equal to the total inosine [I] in this pH range. The intercepts, log k_1, at the several temperatures derived from the acid branch in Fig. 1 are given as k_1 in Table 4.

The Arrhenius plots according to the classical relation of the specific rate constant, k or k_1, are given by:

$$k_1 = A \cdot \exp(-E_a/RT) \quad (4)$$

The plots of log k_1 *vs.* $1/T$ were linear, and the activation energy, E_a and the frequency factor, A, were determined as is shown in Table 5 using Eq. (4), where R is 2 $cal \cdot deg^{-1} \cdot mol^{-1}$ and where T is $(273+t)$K.

In the pH region over 6 the dissociation of inosine to the anion (pK_a 8.75[8]) must be taken into consideration; that is,

$$[I^\circ] \rightleftharpoons [I^-] + [H^+] \quad (5)$$

$$[I^-]/[I^\circ] = K_a/[H^+] \quad (6)$$

$$[I] = [I^\circ] + [I^-] \quad (7)$$

where $[I^-]$ is the concentration of anionic inosine and where K_a is the ionization constant of the equilibrium given in Eq. (5). The plots of log k *vs.* pH in this region are not linear. However, a ready explanation can be found for this curve by employing other terms, including a water-catalytic reaction independent of both hydrogen and hydroxyl ions, in addition to a usual hydrogen-ion- or hydroxyl-ion-catalyzed reaction for anionic inosine. Such a solvent attack on a degradable species is known to be frequently acceptable. The observed rate expression in this region is given by Eq. (8):

$$-d[I]/dt = k[I] = k_2[H^+][I^-] + k_3[H_2O][I^-] + k_4[OH^-][I^-] \quad (8)$$

where k_2 and k_4 are the catalytic rate constants for hydrogen-ion and hydroxyl-ion catalysis respectively and where k_3 is that for water catalysis.

The isomerization or ring-cleavage is observed in this region besides usual hydrolysis, and three main UV-absorbing reaction products, illustrated in Chart 1, were found, along with several minor products, as has been described in a previous paper.[6] However, these three main reaction products seem not to correspond simply with the above three bimolecular reactions. This problem will be briefly discussed later. The complete mechanisms of these three reactions will be clarified in the future. In spite of the above uncertainty, Eq. (8) is useful in estimating the stability of inosine over a wide pH range with a view to obtaining information about the decrease in a marked compound.

The rate constants over the whole pH range can be expressed by a sum by Eq. (3) and Eq. (8). This resultant equation may be rearranged by the use of Eq. (6) and Eq. (7) as follows:

$$k = \frac{k_1[H^+]}{1+K_a/[H^+]} + \frac{k_2[H^+]+K_3[H_2O]+k_4[OH^-]}{1+[H^+]/K_a} \quad (9)$$

The term including k_1 predominates over the others below pH 5, the term including k_2 does so at pH 6—7, k_3 does so at pH 8—12, and k_4, over pH 13.

The k_3 value is easily obtained from the apparent first-order rate constant, k, by the use of Eq. (9) at pH 11, where k is completely independent of the pH (Fig. 1) and where the other three terms are negligible. The Arrhenius plots for k_3 are also easily obtained by the use of Eq. (4). The determination of k_2 and k_4 were not so simple because of the considerable influence from the other terms. They were obtained so as to fit with the observed values of the total rate constants, k, by the use of Eq. (9). The activational energy and the frequency factor for them were also obtained so as to get the best fit with the experimental data. These are summarized in Tables 4 and 5, where K_a is 1.8×10^{-9}.

The curves in Fig. 1 were drawn by the use of Eq. (9) and the constants in Tables 4 and 5; they show a good agreement with the observed values. The ioniza-

Table 5. Thermodynamic parameters for the decrease in inosine in four contributing reaction types

Reaction types[a)]	The activational energy E_a (kcal·mol^{-1})	The frequency factor A (hr^{-1})
1	27.9	3.7×10^{18}
2	49.0	2.6×10^{33}
3	20.0	1.2×10^{8}
4	28.6	2.8×10^{15}

a) Correspond to k_i (i=1—4).

tion of inosine for the cation and the dianion has little effect on the present study because their pK_a values are estimated[9] to be 1.2 and 12.3 respectively. However, some disturbances are probable at both ends of our pH range; especially at the end of the alkaline region it is necessary to assume that [I$^-$] includes the dianion. It may be supposed that Eq. (9) gives a somewhat larger value if it is applied in the pH region below 1.

When the rate constants for acid hydrolysis are compared with those of guanosine as measured by Zoltewicz *et al.*,[3] inosine is found to be hydrolyzed about 2 times faster than guanosine at 100 °C in the 1—4 pH range. This is consistent with our previous estimation[10] that inosine would be hydrolyzed faster than guanosine in a weak acid, while it would be hydrolyzed slower in a strong acid.

Yields of Products. In the acid region the decrease in the mole amount of inosine was equal to amount of H produced, and no side-reaction was observed. On the other hand, in the alkaline region three major UV-absorbing products, H, X and Y, were obtained. The sum of these three products and the inosine remaining was often below 100% when a long reaction time was employed. This indicates the presence of other parallel or sequential degradations.

The representative pH dependences of the three major UV-absorbing products are exhibited in Fig. 2 in the case of 120 °C, corresponding to Experimental Runs No. 29—32. There is an optimum near pH 10 for the production of X and Y, while H increases with an increase in the pH. These convex curves for X and Y correspond with that calculated from the percentage for the mono-anionic species of inosine, as is shown in Fig. 2. The rate-determining term near pH 10 in Eq. (9) included k_5; this term represents a bimolecular reaction between the mono-anionic inosine and the water molecule. Therefore, the rate-determining step of such a reaction that produces X or Y must involve the attack of water on the mono-anionic inosine.

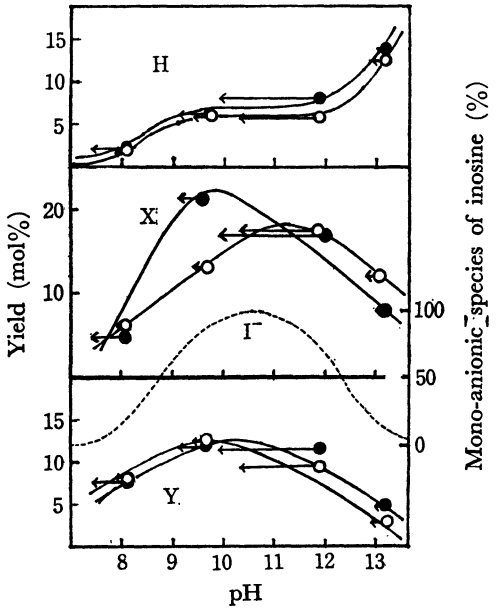

Fig. 2. Yields of UV-absorbing products at 120 °C, and a calculated curve of the development of mono-anionic inosine.
○: Reacted for 8 hr. ●: For 16 hr.
←: Variation in pH during the reaction.
H: Hypoxanthine.
X: 5-Amino-1-β-D-ribofuranosyl-4-imidazolecarboxamide.
Y: 9-β-D-Ribopyranosyl hypoxanthine.
I$^-$: Mono-anionic species of inosine.

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