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The Decomposition of Tryptophan in Acid Solutions: Specific Effect of Hydrochloric Acid

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The stability of tryptophan in aqueous solutions of HCl, H_2SO_4, or CH_3SO_3H under aerobic conditions was examined, and a specific effect of HCl was found. A kinetic study of the HCl-induced decomposition, tests for the free chlorine formation on heating the HCl used for the above run, *etc.* suggested that free chlorine produced by the air oxidation of HCl may have participated in the decomposition of tryptophan.

Thin-layer and ion-exchange chromatography of the decomposition products of tryptophan revealed that oxindolylalanine and dioxindolylalanine were formed by the reaction. These two compounds were also formed by treating tryptophan with ClO^- or N-chlorosuccinimide in a solution of HCl. These results support the above possibility.

Keywords——tryptophan; hydrochloric acid; free chlorine; oxindolylalanine; dioxindolylalanine

The stability of tryptophan in hot 6 N HCl has been investigated in connection with the decomposition of tryptophan during acid hydrolysis of protein. These studies[1] revealed that tryptophan was relatively stable when heated under anaerobic conditions. However, there are few papers dealing with the decomposition of tryptophan on being heated in a solution of HCl under aerobic conditions, *e.g.*, factors influencing the decomposition, the decomposition product, the mechanism involved, *etc.* In addition, it is not clear whether tryptophan is decomposed similarly when heated in other acid solutions.

In the present investigation, we examined the stability of tryptophan on being heated in solutions of HCl, H_2SO_4 or CH_3SO_3H (MSA) under aerobic conditions, and found that tryptophan was decomposed specifically in HCl solution. On the basis of this finding, further work was carried out to clarify the mode of action of HCl on tryptophan.

Experimental

Chemicals——L-Tryptophan was obtained from Kanto Kagaku Co. (Tokyo). DL-Oxindolylalanine (2,3-dihydro-2-oxo-tryptophan) was prepared by the method of Wieland *et al.*[2] and dioxindolylalanine (2,3-dihydro-3-hydroxy-2-oxo-tryptophan) by the method of Savige.[3] These amino acids were confirmed to be chromatographically homogeneous by means of an amino acid analyzer. Constant-boiling HCl (here-

after referred to as 6 N HCl) was obtained from Wako Junyaku Co. (Tokyo). All these chemicals and others used were of the best grade commercially available.

Reaction——Tryptophan was dissolved in an acid solution at a concentration of 400 μg/ml. The solution (1 ml) was put into a Pyrex glass tube (1.2×12 cm) which had been washed with hot 50% HNO_3, then thoroughly rinsed with distilled water and oven-dried. The tube was sealed with a burner and heated at 110°. When necessary, the reaction was done in an evacuated sealed tube (0.1—0.2 mmHg).

Amino Acid Analysis——Samples were prepared by evaporating each of the reaction mixtures to dryness and dissolving the residue in 5.0 ml of water when HCl was used as the acid solvent. In the cases of H_2SO_4 and MSA, 1 ml of 3.5 N NaOH was added to the reaction mixture, and the solution was made up to a final volume of 5.0 ml with 0.07 M citrate buffer (pH 2.2). These samples were chromatographed on a Hitachi KLA-5 analyzer with a 0.6×10 cm column in the usual manner.[4] Thin-layer chromatography was done on precoated plates of silica gel $60F_{254}$ (E. Merck) with a solvent system of ethyl acetate/acetic acid/water (4:1:1).

Analysis of Free Chlorine——Free chlorine in 6 N HCl was determined by a modification of the JIS method (K-8180): in a 100-ml flask which had been filled with CO_2 gas, 10 ml of 6 N HCl, 2 ml of 2.5% KI and 2 ml of CCl_4 were mixed, and the mixture was shaken well. The presence of free chlorine was judged qualitatively by viewing whether or not the CCl_4 layer was colored pink.

Results and Discussion

Table I shows the recovery of tryptophan heated in several acid solutions under aerobic and anaerobic conditions. The use of 6 N H_2SO_4 and 6 N MSA as solvents was avoided, since ion-exchange chromatographic analysis was interfered by the large quantities of sodium salts produced by the neutralization of these solvents. Under aerobic conditions, tryptophan was readily decomposed in a solution of HCl, but was relatively stable in a solution of H_2SO_4 or MSA. No change in UV spectra was observed after 48-hr heating of tryptophan in these solutions, except for HCl. As shown in Table I, aerial oxygen was clearly responsible for the decomposition of tryptophan in the solution of HCl. This result is in accord with the results reported previously.[1]

Table I. Stability of Tryptophan in Several Acid Solutions

Solvent	Recovery of tryptophan (%)	
	24 hr	48 hr
6N HCl	53.4	26.5
6N HCl (evacuated)	—	83.6
4N HCl	70.6	42.3
4N MSA	96.8	91.2
4N MSA (evacuated)	—	96.1
4N H_2SO_4	93.5	96.7

Data are averages of two experimental runs.

Stewart and Nicholls kinetically investigated the decomposition of tryptophan heated in 0.1—1 N HCl, and observed that the decomposition rate was decreased by the addition of thiol compounds, but was increased by the addition of transition metal ions.[5] On the basis of these observations, they concluded that the reaction involved a free-radical autoxidation mechanism preceded by protonation of the indole ring of tryptophan. They also reported that impurities such as iron present in glass ampoules, in which the reaction had been carried out, were responsible for the initiation of the reaction. Although their experimental conditions are somewhat different from the present ones,[6] their conclusion seems to conflict with the present results. If the decomposition is initiated only by such impurities, tryptophan should be similarly decomposed in all the acid solutions. Consequently, the decomposition of tryptophan in a solution of HCl cannot be attributable to aerial oxygen and the impurities alone. To clarify the mechanism of decomposition of tryptophan in a solution of HCl, several

substances were added to the solvents and their influence on the decomposition was evaluated. Table II shows the results. Iron markedly accelerated the decomposition in a solution of HCl, but hardly affected the recovery of tryptophan when added to the other solutions. On the other hand, tryptophan was decomposed on being heated in a solution of H_2SO_4 or MSA containing NaCl (data with MSA not shown). It was, however, stable when heated in the presence of Na_2SO_4 in the same acid solution, or when heated in the presence of NaCl under anaerobic conditions. These data indicate that not only aerial oxygen but also HCl is involved in the decomposition of tryptophan. This view was supported by the following evidence. Figure 1 shows the changes of pseudo-first-order rate constant of the decomposition of tryptophan as a function of Cl^- concentration in a solution whose hydrogen ion concentration was kept constant. The plot gave a linear relation with a correlation coefficient of 0.999.

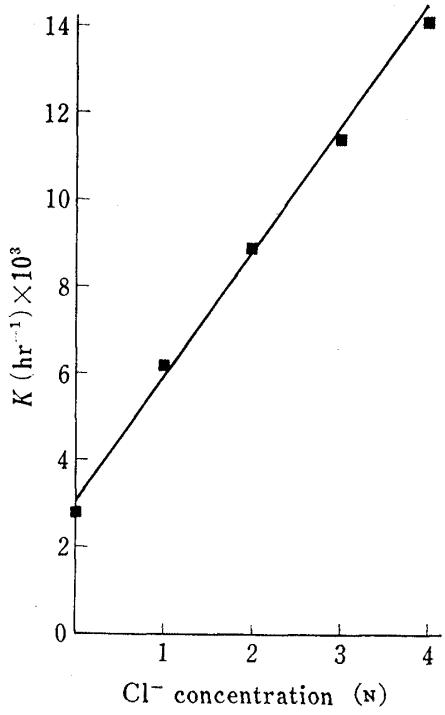

Fig. 1. Plot of the Pseudo-First-Order Rate Constant of Tryptophan Decomposition *vs.* HCl (Cl^-) Concentration in 4 N Acid Solutions

Tryptophan was heated in 4 N acid solutions containing 0—4 N HCl, and the pseudo-first-order rate constant of the decomposition was plotted against the HCl (Cl^-) concentration. The hydrogen ion concentration of the solution was kept at 4 N by mixing a solution of H_2SO_4 with that of HCl.

Free chlorine is known to be a common contaminant in commercial HCl and to be gradually produced on prolonged exposure of HCl to air.[7] The 6 N HCl used in the present study contained no detectable amount of free chlorine, but produced it in small quantities when heated under aerobic conditions. The heating of HCl in the presence of tryptophan or under anaerobic conditions gave no free chlorine. These results suggest that HCl may be oxidized by aerial oxygen in unevacuated sealed tubes to give free chlorine, which causes the decomposition of tryptophan. Addition of hydrochlorite to the solution of H_2SO_4 or MSA resulted in considerable loss of tryptophan (Table II). The inhibitory effect of thioglycolic acid, as shown in Table II, may therefore be explained by its high reactivity with free chlorine.

The decomposition products of tryptophan heated in 6 N HCl were analyzed by thin-layer

Table II. Influence of Various Substances on the Decomposition of Tryptophan

Additives	Solvent	Recovery of Trp after 24 hr (%)
$FeCl_3$ (1 ppm as Fe)	6 N HCl	17.0
	4 N H_2SO_4	98.6
	4 N MSA	94.4
NaCl (10%)	4 N H_2SO_4	83.0
	4 N H_2SO_4 (evacuated)	99.0
Na_2SO_4 (10%)	4 N H_2SO_4	95.3
$HSCH_2COOH$ (1%)	6 N HCl	87.9
NaClO (5 ppm as Cl)	4 N MSA	66.4
	4 N H_2SO_4	70.9

Data are averages of two experimental runs.

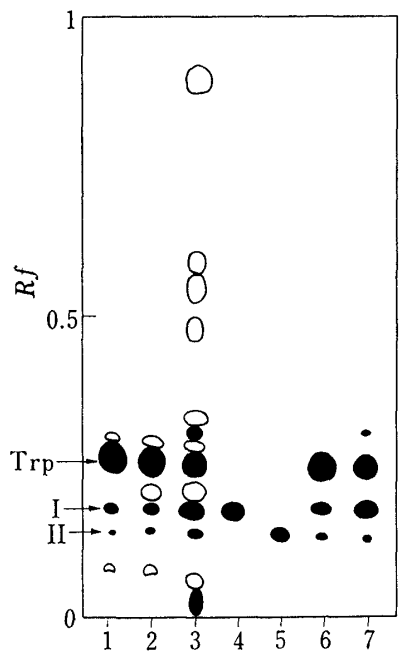

Fig. 2. Thin-Layer Chromatogram of the Decomposition Products of Tryptophan

Tryptophan was heated in 6 N HCl for 1 hr (1), 6 hr (2) and 24 hr (3), or was allowed to react with equimolar amounts (25 μmol) of NaClO (6) and N-chlorosuccinimide (NCS) (7) in 6 N HCl for 1 hr at room temperature. (4); oxindolylalanine, (5); dioxindolylalanine. The chromatogram was visualized under UV light (254 and 365 nm). Dark-shaded spots were also visualized with ninhydrin reagent.

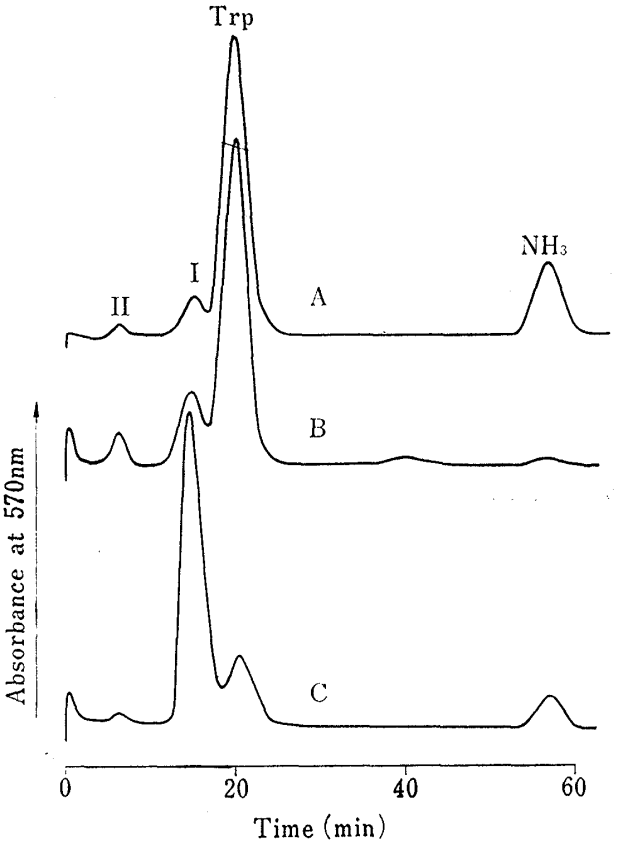

Fig. 3. Ion-Exchange Chromatogram of the Decomposition Products of Tryptophan

Samples were prepared as in Fig. 2. (A); tryptophan heated for 24 hr, (B); tryptophan+NaClO, (C); tryptophan+NCS.

chromatography (Fig. 2) and ion-exchange chromatography (Fig. 3). At least 12 compounds were found to be produced after 24-hr heating. Of these compounds, two were identified as oxindolylalanine (I) and dioxindolylalanine (II) by comparing their elution times and *Rf* values with those of the authentic compounds. These two compounds were also formed by treating tryptophan with active chlorine compounds such as hypochlorite or N-chlorosuccinimide in a solution of HCl (Fig. 2 and Fig. 3). Halogenation of 3-substituted indoles in hydroxylic solvents often leads to oxindole derivatives.[8] The reaction of tryptophan with periodate in 1 N HCl is known to give dioxindolylalanine.[9] Consequently, free chlorine is likely to participate in the decomposition of tryptophan in HCl solution. The possibility that active oxygen species such as singlet oxygen and the superoxide ion, which is known to participate in biological and photochemical oxidation of tryptophan to formylkynurenine,[10] may be produced during the heating of HCl in contact with air must also be considered. Kynurenine, the hydrolysis product of formylkynurenine, was not detected in the present experiment, but further investigations seem necessary to clarify whether or not active oxygen participates in the decomposition of tryptophan.

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