Effects of Hydrochloric Acid on the Paper Chromatography of Amino Acids[1]

By Shin-ichi ISHII[2] and Toshio ANDO[2]

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Paper partition chromatography has been proved to be a useful and convenient tool for

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(2) Present address: Institute of Science and Technology, the University of Tokyo, Tokyo.

amino acid analysis.[3,4,5,6] R_F values of amino acids, the specific characters showing the ratios of the distances reached by them to those by the developing solvents, have been determined in their aqueous solutions developed by several solvents, and applied to the identification of each of the amino acids.. However, in practice, analysis by this method of the solution containing hydrochloric acid, especially in the hydrolyzed mixture of protein or peptide with hydrochloric acid, is very often carried out in laboratories. Repeated treatments of such solution by evaporating to dryness, with successive addition of water, can never remove hydrochloric acid completely from the solution, a quantity of the acid corresponding to the concentration of *ca.* 1 *N* being retained, although this obviously depends on the concentration of amino acids. Neutralization of the acid with alkali was often tried, but an undesirable effect of the salt produced upon the chromatogram may not then be neglected.[7] The treatment, moreover, is of very troublesome and fast impracticable in case of treating a minute volume of solution, a case often met with in the paper chromatographic method. It has never been studied whether the presence of hydrochloric acid may affect the R_F values of amino acids or not, though recently H. B. Bull *et al.*[8] and S. Aronoff[9] observed the R_F values of amino acids in the solutions of some hydrogen ion concentrations, using phenol as a solvent. The former investigators found no difference between those in neutral and alkaline or acidic solutions, while the latter found a considerable effect of hydrogen ion concentrations on the chromatogram of lysine in alkaline solution, but neither of the investigations has been extended to the range of higher acidity. As a preliminary test proved that the effect of hydrochloric acid on the chromatograms was sometimes so remarkable in cases of some solvents, the present systematic observation was carried out in order to clarify the effect in cases of several amino acids, solvents and concentrations of hydrochloric acid

Experimental Method

Amino acids used in the experiment were L-leucine, DL-valine, DL-alanine, glycine, L-arginine monohydrochloride and L-aspartic acid. Each of them was dissolved individually in 1% concentration, except L-leucine and DL-valine (both in 0.5%), in hydrochloric acid of various concentrations (0—12 *N*). A definite small volume from the solution was applied to a marked spot on each of the paper strips (the Tôyo filter paper, No. 2, 2×40 cm.). After the strip was allowed to stand in the room for about twenty hours, the chromatogram of the one-dimensional type, descending or ascending, was made as usual. As solvents, *n*-butanol, isovalerianic acid, *n*-butanol-acetic acid, phenol and collidine-lutidine were used. These were saturated with water except *n*-butanol-acetic acid and phenol. *n*-Butanol-acetic acid was composed of 4 volumes of *n*-butanol, 1 volume of acetic acid and 1 volume of water. Phenol was used as 80% (W/W) aqueous solulion.

After development and drying, the spot of amino acid was revealed by spraying with 0.2% solution of ninhydrin in water-saturated butanol followed by heating. Subsequently, the diffused area of hydrochloric acid on the strip was also revealed by spraying with 0.04% solution of bromophenol blue (B.P.B.) or thymol blue (T.B.) in 95% ethanol. The chromatograms obtained were compared with one another concerning each solvent, the results of which are as follows.

Results and Discussions

***n*-Butanol.**—In case *n*-butanol was used as a solvent, hydrochloric acid was found to

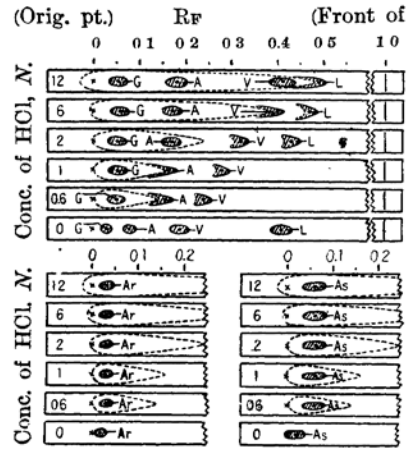

Fig. 1.—Chromatogram of amino acids in hydrochloric acid of various concentrations developed by *n*-butanol : Temperaure, 5-10°; development, descending; L, leucine; V, valine; A, alanine; G, glycine; Ar, arginine; As, aspartic acid. Areas surrounded by broken lines mean those of diffused hydrochloric acid. Chromatograms for corresponding concentrations of the acids were shown together in the same figures respectively, although experiments on each amino acid were carried out independently.

(3) R. Consden, A.H.Gordon and A. J.P. Martin, *Biochem. J.*, **38**, 224 (1944).
(4) R. Consden, *Nature*, **162**, 359 (1948).
(5) A. J. P. Martin, *Ann. N. Y. Acad. Sci.*, **49**, 249 (1948); *Ann. Repts. Progress Chem.*, **45**, 267 (1949).
(6) D. L. Clegg, *Anal. Chem.*, **22**, 48 (1950).
(7) A. H. Gordon, *Nature*, **162**, 180 (1948).
(8) H. B. Bull, J. W. Hahn and V. H. Baptist, *J. Am. Chem. Soc.*, **71**, 550 (1949).
(9) S. Aronoff, *Science*, **110**, 590 (1949).

increase the R_F value of each amino acid with an increase in the concentration of hydrochloric acid. The results were shown in Fig. 1. In extreme cases, where the concentration of hydrochloric acid was 12 *N* or 6 *N*, the position of valine and alanine was moved to the usual one of leucine and valine respectively. Accordingly, the identification of these amino acids may possibly be often mistaken without the knowlegde of this fact.

The diffused area of hydrochloric acid revealed by B.P.B. spread more extensively as the concentration of the acid increased, though its size was independent of the kind of amino acids. The area was shown surrounded by broken lines in the figure. Hydrochloric acid seemed to be accompanied thus far with *n*-butanol as a result of its considerable solubility in the latter. The reason why each amino acid travels abnormally for a longer distance may be understood from the fact that amino acid is more soluble in *n*-butanol containing hydrochloric acid than in it alone. The relation between the acid areas and the location of amino acids was fairly interesting in this case. The spots of amino acids of higher R_F values went just ahead of the field of hydrochloric acid, forming a distinct saddle shape colored by ninhydrin as strongly as usual. The further the areas of hydrochloric acid extended, the further the spots of amino acids were pushed forward with them. When the formers went too far for the amino acids of lower R_F values to ride on the top of them, the latters were left behind the formers and were included inside of them. The shapes of them were then changed into ovals, while their color became a little paler.

When a chromatogram of a sample containing an unknown concentration of hydrochloric acid is obtained, the paper ought to be sprayed with an alcoholic solution of B. P. B. to show the area of the acid. On comparing this with the figures already obtained, the concentration of the acid and its probable effect upon the chromatogram therefore may also approximately be supposed.

Isovalerianic acid.—When isovalerianic acid was used as a solvent, contrary to the case of *n*-butanol, hydrochloric acid was found to decrease remarkably the R_F value and also the strength of coloring of each amino acid, as shown in Fig. 2, with an increase in the concentration of hydrochloric acid. The diffused area of hydrochloric acid revealed by T. B. was now limited to the neighborhood of the original point and did extend no more. The fact seems to be resulted from the difficult solubility of hydrochloric acid in isovalerianic acid, contrary to that in *n*-butanol. Hydrochloic acid thus retained in the stationary phase near the original point may likely prevent the amino acids from moving.

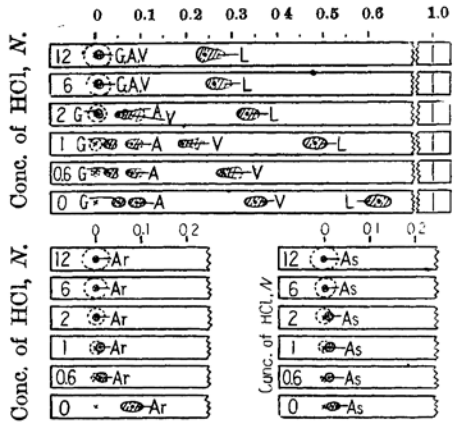

Fig. 2.—Chromatograms of amino acids in hydrochloric acid of various concentrations developed by isovalerianic acid: Temperature, 5-10°; developement, ascending.
Other notes are as Fig. 1.

***n*-Butanol-acetic acid.**—In this case, on spraying of an alcoholic solution of B. P. B. two acidic areas were revealed on a paper of the chromatogram, one was shown extended from one end of the strip to R_F value of about 0.2, the other ahead of the former, as shown in Fig. 3 a (the former was not represented in the figure to avoid confusion). As the size of the former is independent of the concentration of hydrochloric acid, it is inferred that this area does not indicate the presence of hydrochloric acid but that of remaining acetic acid. On the contrary, as the size of the latter area grows larger with an increase in the concentration of hydrochloric acid, obviously this area shows the diffused one of hydrochloric acid. The latter acid was found to increase slightly the R_F values of amino acids which were located near its field, namely alanine, glycine and aspartic acid (see Fig. 3 a). On the other hand the R_F value of arginine was rather decreased, the results of which observed in various concentrations of hydrochloric acid were separately given below, to avoid confusion in the figure. Leucine, located far from the acidic area, was scarcely affected.

(Conc. of HCl):	(0 *N*)	(0.6 *N*)	(1 *N*)	(2 *N*)	(6 *N*)	(12 *N*)
Arginine:	0.19	0.18	0.17	0.16	0.16	0.16

By supplementary experiments, it was also proved that the effect on proline and glutamic acid was similar to that on alanine. Above

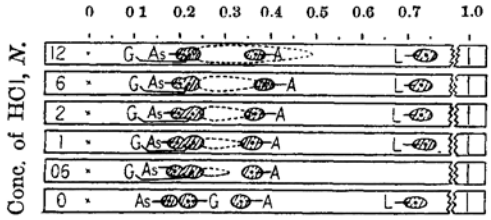

Fig. 3 a

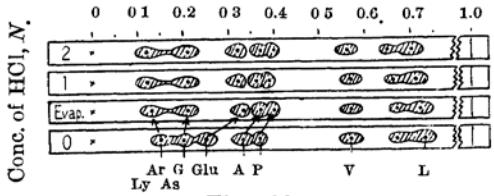

Fig. 3 b

Figs. 3 a and 3 b.—Chromatograms of amino acids in hydrochloric acid of various concentrations developed by *n*-butanol-acetic acid. Temperature, *ca.* 10°; development, descending; P, proline, Glu; glutamic acid; Ly, lysine. Other notes as Fig. 1.

all, the effect on glutamic acid was the most remarkable. These were shown in Fig. 3 b, where 10% solutions of the dried digestion product of casein with trypsin in 2 *N* and 1 *N* hydrochloric acid or merely in distilled water respectively, were used for chromatography as usual. On the one hand, the solution of the above product in 6 *N* hydrochloric acid was also prepared and evaporated to dryness on steam-bath repeatedly four times to remove hydrochloric acid and chromatographed, the result of which was shown in the third strip of the figure. One may notice that a similar effect also appears on it as that of 2 *N* or 1 *N* acid.

Phenol.—When phenol was used as a solvent the effect of hydrochloric acid hardly appeared on the chromatograms of amino acids of higher Rf values, namely leucine, valine, alanine and arginine, as shown in Fig. 4.

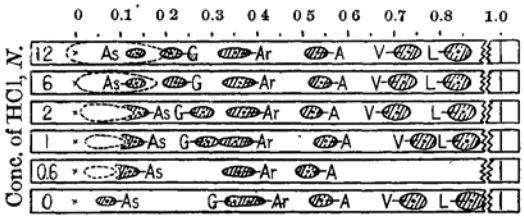

Fig. 4.—Chromatograms of amino acids in hydrochloric acid of various concentrations developed by phenol. Temperature, *ca.* 20°; development, ascending.
Other notes are as Fig. 1.

However, as the diffused area of hydrochloric acid was here revealed near the original point on the strip by B. P. B. (see Fig. 4), the amino acids of comparatively lower Rf values and hence located about this area, namely glycine and aspartic acid, were affected by hydrochloric acid obviously as expected; the former decreased, while the latter increased.

Collidine-lutidine.—On developing with collidine-lutidine, a yellow separate band, perhaps connecting with the hydrochloric acid,[10] usually appeared just ahead (at *ca.* 0.65 in Rf) of the location of leucine as shown in Fig. 5, by revealing with B. P. B. The shape of

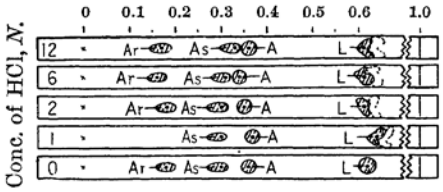

Fig. 5.—Chromatograms of amino acids in hydrochloric acid of various concentrations developed by collidine-lutidine. Temperature, 20°; development, ascending.
Other notes as Fig. 1.

leucine was distorted by this band, but its Rf value and coloring hardly varied in any concentration of hydrochloric acid. As to the other amino acids, almost none of the effect of hydrochloric acid was found on the Rf value, shape and coloring of the chromatogram (see Fig. 5). Among them, glycine was omitted from the figure to avoid confusion, and its Rf values observed in various concentrations of hydrochloric acid were given below:

(Conc. of HCl):	(0 *N*)	(1 *N*)	(2 *N*)	(6 *N*)	(12 *N*)
Glycine:	0.32	0.34	0.33	0.33	0.35

Therefore, it is the most desirable way to use collidine-lutidine as the solvent in case some hydrochloric acid may be contained in the sample to be analyzed by paper chromtography.

Summary

To explain what effect hydrochloric acid, contained in the sample to be analyzed, gives upon the paper chromatograms of amino acids, Rf values of some amino acids dissolved in hydrochloric acid of various concentrations were observed with different kinds of solvent.

(10) Probably the hydrochloric acid composes a salt with collidine-lutidine.

In case of *n*-butanol, hydrochloric acid was found to increase the R_F value of each amino acid with an increase in the concentration of hydrochloric acid, while in isovalerianic acid, on the contrary, to decrease that. On the one hand, only the amino acids of lower R_F values were affected by hydrochloric acid in the case of phenol. In butanol-acetic acid, on the other hand, some effect was also found, but in collidine-lutidine it was negligible. In all cases, the relation between these effects and the diffused areas of hydrochloric acid on the chromatograms was observed with great interest.

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Radiation Chemistry Research Institute,
the University of Tokyo, Tokyo