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The Hydrolysis of Arginine*

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The formation of ornithine from arginine is well known. The reaction is catalyzed by a number of enzymes (1), the conversion of arginine into ornithine has been recognized in the electrolytic desalting of amino acid solutions (2, 3), and ornithine may be prepared very conveniently by alkaline hydrolysis of arginine (4–7). Conversion of carboxyl-terminal arginine residues into ornithine on hydrazinolysis of protamines was observed by Felix, Goppold-Krekels, Hübner, and Yamada (8), and ornithine was formed when dried hydrolysates of wool were heated above 170° (9). It is generally believed, however, that arginine is resistant to acid hydrolysis. This view is supported by several studies of the degradation of various amino acids under conditions normally used for the hydrolysis of proteins (*e.g.* References 10–12), although a slight, but measurable loss of arginine has been reported (12).

At the beginning of a comparative study of several protamine preparations (13), it was suspected that some samples were hydrolyzed inadvertently at temperatures above 110°. Analyses made with the Beckman/Spinco automatic amino acid analyzer indicated, surprisingly, that these hydrolysates contained small quantities of lysine, but a closer examination showed that the substance was in fact ornithine. Since the proportion of ornithine in the hydrolysates (in which the arginine content was about 64 moles % of the total recovered amino acids) increased as the duration and temperature of hydrolysis were increased and was accompanied by an equivalent decrease in the proportion of arginine, it appeared that the ornithine was a hydrolysis product of arginine. The acid hydrolysis of arginine was studied in detail and it was established that arginine could be converted slowly into ornithine at elevated temperatures.

Hydrolysates of some protamine preparations contained in addition to ornithine a small quantity of citrulline. This amino acid was found only in protamine preparations that at some stage had been exposed to high concentrations of ammonia. As a prelude to a study of the affect of ammonia on protamines, the reaction between ammonia and arginine was investigated. One of the reaction products (Compound X) was eluted from an ion exchange column after arginine. This substance was identified as 3-aminopiperid-2-one which van der Horst obtained recently by treatment of arginine or ornithine with alkali (14) and which was prepared long ago by distillation of ornithine esters (15). The formation of 3-aminopiperid-2-one on alkaline hydrolysis of arginine has been observed also by Phillips.[1]

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EXPERIMENTAL PROCEDURE

Hydrolysis of Arginine with Acid—A stock solution of L-arginine monohydrochloride (200 mg) in 6 N hydrochloric acid (20 ml) was prepared. Portions of this solution (0.1 ml) were heated in evacuated, sealed tubes for periods of 24, 48, 72, 96, and 120 hours at the following temperatures: 110° (refluxing toluene), 132° (refluxing *n*-amyl alcohol), 156° (refluxing 1-hexanol), and 176° (refluxing *o*-dichlorbenzene). The contents of each tube were then evaporated to dryness in a vacuum over phosphorus pentoxide and sodium hydroxide, and the residues were used for amino acid analyses. In order to isolate the hydrolysis product, the stock solution of arginine (15 ml) was heated at 200° for 5 days in evacuated, sealed tubes. The resulting pale brown solution contained no arginine and was treated with charcoal (1 g) and evaporated to dryness. The residue was dissolved in 6 N hydrochloric acid (1 ml) and ammonium chloride was precipitated by the addition of acetone (3 ml) and was removed by filtration. The filtrate was evaporated to dryness and the residue was crystallized from 6 N hydrochloric acid (yield, 51 mg).

Hydrolysis of Arginine with Alkali—An aqueous stock solution of L-arginine monohydrochloride (2 μmoles per ml) was used for analytical experiments. Portions of this solution (1 ml) were adjusted to pH 11, 12, or 13 with aqueous ammonia or with 0.1 M aqueous barium hydroxide solution. The solutions were heated in evacuated, sealed tubes for 24 or 72 hours at the following temperatures: 37° (incubator), 60° (refluxing chloroform), 110° (refluxing toluene), 144° (refluxing *o*-xylene), and 180° (refluxing *o*-dichlorbenzene, redistilled). The samples were then evaporated to dryness in a vacuum over phosphorus pentoxide, dissolved in water and were acidified to pH 2 (with dilute hydrochloric acid) for amino acid analyses. In a preparative experiment on the isolation of Compound X, L-arginine (5 g) was refluxed for 4 hours with sodium hydroxide solution at pH 10.6 (200 ml). The cold solution was neutralized with 2 N hydrochloric acid and evaporated to low volume. The solution was made alkaline with barium hydroxide and extracted three times with hot ethanol (50 ml) and the combined extracts were evaporated to low volume. Further addition of ethanol gave a white precipitate that was removed by filtration. The pale yellow filtrate was treated with charcoal and concentrated to low volume (1 ml). Again, addition of ethanol gave a precipitate and this was removed as before. Samples of the filtrate were used for analysis (paper chromatography and ion exchange

[1] D. M. P. Phillips, personal communication.

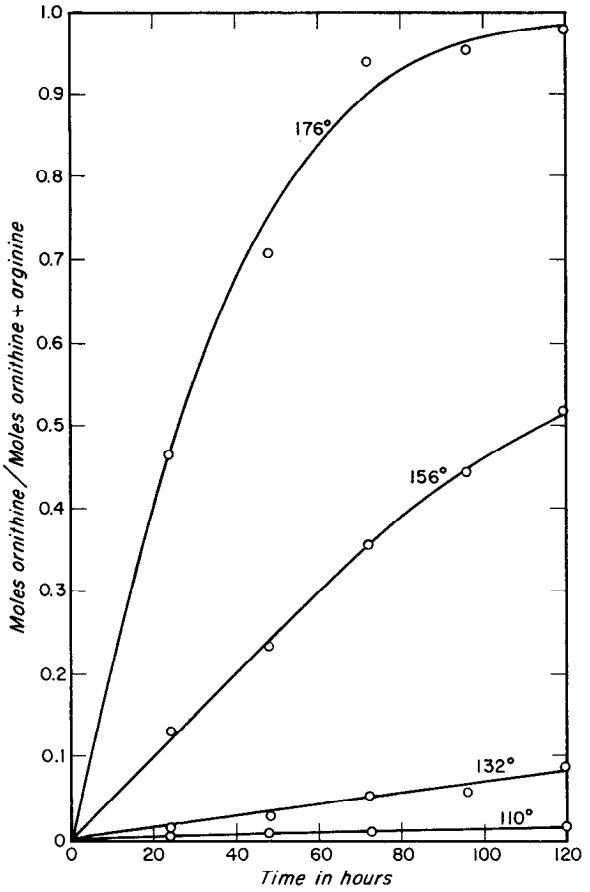

FIG. 1. Rate of hydrolysis of arginine in 6 N hydrochloric acid at various temperatures.

chromatography) and the remainder was distilled under vacuum to yield a colorless glassy oil (b.p. 98–100° at 0.03 mm of mercury). White needles separated from this oil after about 12 hours but were slowly reabsorbed after a few days at room temperature to leave a more mobile, slightly discolored oil.

Hydrolysis of Other Samples (Analytical Experiments)—Dilute solutions (about 2 mM) of arginylglutamate, citrulline, and ornithine were hydrolyzed with alkali under some of the conditions described above. An alkaline hydrolysate of arginine which contained a high proportion of Compound X was hydrolyzed with 6 N hydrochloric acid for 22 hours at 110° in an evacuated, sealed tube. All hydrolysates were evaporated to dryness, as already described, and the residues were used for amino acid analyses.

Preparation of 3-Aminopiperid-2-one from Ornithine—An aqueous solution of ornithine monohydrochloride (2.2 g in 200 ml) was adjusted to pH 10.5 with sodium hydroxide and refluxed for 6 hours. The solution was neutralized, concentrated with a flash evaporator at 40°, and was evaporated to dryness on the steam bath. The residue was mixed with saturated aqueous barium hydroxide solution (5 ml) and was extracted repeatedly with hot ethanol (30 ml). The combined ethanol extracts were concentrated, and the precipitate which formed on addition of more ethanol was filtered off; the process was repeated on the filtrate. The filtered ethanol solution was decolorized with charcoal, concentrated to low volume, and was distilled under vacuum to yield a colorless, glassy oil (b.p. 100° at 0.03 mm of mercury).

Amino Acid Analyses—The dried hydrolysates were dissolved in 0.2 M sodium citrate buffer, pH 2.2, and analyses were carried out with a Beckman/Spinco automatic amino acid analyzer. Normally, a 6.5-cm column of resin was used at 50° with 0.35 M sodium citrate buffer solution, pH 5.28, but in order to distinguish ornithine from lysine some analyses were made with 0.38 M sodium citrate buffer solution, pH 4.26, on a 50-cm column of resin and a change of temperature from 30 to 50° was made during the development of the chromatogram (16).

Paper Chromatography—Chromatograms were prepared on Whatman No. 1 paper by downward development with the organic phase of mixtures of butan-1-ol-ethanol-water (4:1:5), or butan-1-ol-acetic acid-water (4:1:5), or with a solution of propan-1-ol in 0.2 N aqueous ammonia (3:1). The dried chromatograms were stained with ninhydrin (0.1% in acetone).

RESULTS AND DISCUSSION

Acid Hydrolysis of Arginine—The conversion of arginine into ornithine in acid solution at various temperatures is summarized in Fig. 1. In each sample analyzed, the production of ornithine was accompanied by the release of 2 equivalents of ammonia. No trace of citrulline, or indeed of any amino compound other than arginine, ornithine, and ammonia, was found in the samples analyzed. That the product of the reaction was in fact ornithine was established by comparison of the crystalline compound obtained after hydrolysis of arginine (with hydrochloric acid at 200° for 5 days) with an authentic specimen of L-ornithine monohydrochloride. On paper chromatograms developed for 3 days with either butan-1-ol-ethanol-water or butan-1-ol-acetic acid-water the compound behaved identically with ornithine and was clearly distinguished from lysine. On ion exchange chromatography, with the automatic method of Spackman, Stein, and Moore (16), the compound again behaved identically with ornithine in two different systems; these were the use of 0.35 M sodium citrate buffer solution (pH 5.28) on a 15-cm column at 50° (here lysine and ornithine were not distinguished), and the use of 0.38 M sodium citrate buffer solution (pH 4.26) on a 50-cm column with change of temperature from 30 to 50° part way through the analysis (here lysine and ornithine were clearly distinguished). The infrared absorption spectrum of the compound was identical with that of ornithine hydrochloride. The absence of citrulline from all the samples analyzed indicates that the hydrolysis of arginine to citrulline is the rate-limiting step in the over-all conversion into ornithine.

The acid hydrolysis of arginine is of interest from two standpoints. First, because, under certain circumstances, it can cause errors in amino acid analyses on proteins, and second, as a method for the preparation of ornithine from arginine. It is clear from Fig. 1 that at 110° the rate of hydrolysis is extremely low and the loss of arginine is less than 1% in 24 hours. Therefore, under conditions usually employed for the acid hydrolysis of proteins (*i.e.* 6 N hydrochloric acid at 110° for 24 hours) the introduction of errors by conversion of arginine into ornithine will seldom be significant, as was shown by the work of Hirs, Stein, and Moore (12), although ornithine will usually be detectable. However, in the case of proteins that have a high arginine content (such as protamines or some histone fractions) the loss of arginine may be appreciable. With a different automatic system for ion exchange chromatography of the amino acids (17), in which lysine and ornithine were well resolved, Hamilton has in fact found small quantities of ornithine[2] in hydrolysates (pre-

[2] P. B. Hamilton, personal communication.

pared by reflux of the protein in 6 N hydrochloric acid under nitrogen for 24 hours) of histone fractions that had a high arginine content (calf thymus histone Fractions III and IV, Reference 18). At higher temperatures the loss of arginine obviously becomes serious; for example, at 160° about 15% of the arginine may be converted into ornithine in 24 hours. The pernicious aspect of the acid hydrolysis of arginine is that ornithine formation may easily pass unnoticed if the analysis of basic amino acids is made on an automatic ion exchange chromatography apparatus with the usual short columns of Dowex 50 resin and 0.35 M sodium citrate buffer solution, pH 5.28, for the product (*i.e.* ornithine) would not be differentiated from lysine (16). Thus, an erroneously low arginine content and a correspondingly high lysine content would be found. Modification of the conditions of chromatography to effect resolution of lysine and ornithine without resorting to the more time consuming system to which reference has already been made (17) would therefore be necessary in such cases; an appropriate modification of the analytical procedure could doubtless be made quite readily (*e.g.* Reference 6).

The only disadvantage to the preparation of ornithine by acid hydrolysis of arginine appears to be the slowness of the reaction. In order to achieve complete conversion, the reaction mixture must be maintained at about 200° for a few days. However, the reaction is very clean and conditions could be selected for the quantitative conversion of arginine hydrochloride into ornithine hydrochloride and ammonium chloride. In one experiment, arginine was heated in 6 N hydrochloric acid at 200° for 5 days. Amino acid analysis showed that arginine had been completely destroyed and the yield of ornithine was 80% of theoretical. From the optical rotation of this rather dilute solution ($[\alpha]_D$ +30°; *c*, 0.1, 6 N hydrochloric acid) it appeared that there was little, if any, racemization during hydrolysis.

Alkaline Hydrolysis of Arginine—When one of the alkaline hydrolysates of arginine was analyzed with the automatic amino acid analyzer and the duration of chromatographic development was prolonged, the presence of a compound which was eluted considerably after arginine was revealed. Such a chromatogram is illustrated in Fig. 2 and the material eluted after arginine was called Compound X. Analyses of the products of alkaline hydrolysis, under various conditions, of arginine and some related compounds are listed in Table I; the result of acid hydrolysis of Compound X is included in the same table. These results show that the formation of Compound X is dependent on temperature and pH. It is difficult to assess a quantitative dependence on these factors, but the compound is formed more readily at higher temperatures. It is apparent that Compound X was found only in hydrolysates that contained ornithine. Since Compound X was formed also from citrulline and ornithine, and since only ornithine was produced on acid hydrolysis of Compound X, it appeared very probable that this compound was 3-aminopiperid-2-one, *i.e.* the lactam of ornithine. The facile cyclization of appropriate amino carboxylic acids to yield lactams is well known. Van der Horst has shown the presence of 3-aminopiperid-2-one (β-aminopiperidone) on paper chromatograms of cattle rumen specimens and has shown that the compound is readily formed from ornithine (and hence from arginine) on warming dilute alkaline solutions (14).

Compound X was isolated from an alkaline hydrolysate of arginine and was compared with a sample of 3-aminopiperid-2-one prepared by treatment of ornithine with alkali. The two substances behaved identically on ion exchange chromatography

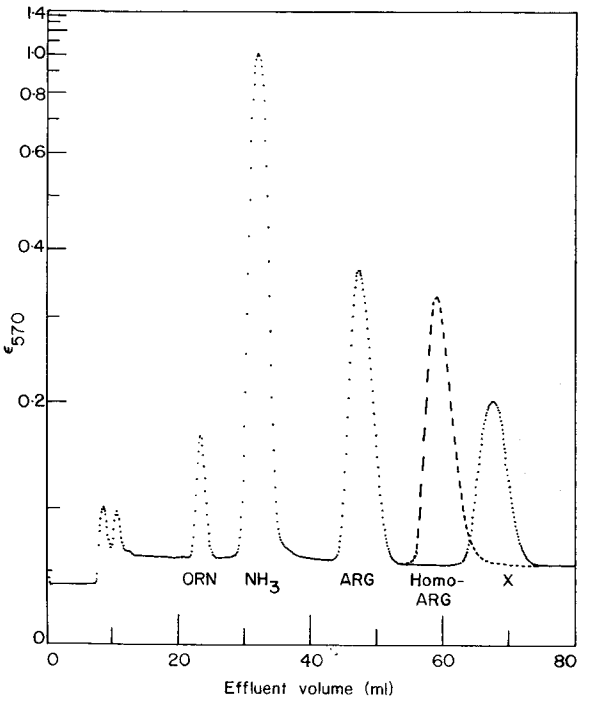

FIG. 2. Amino acid analysis of an alkaline hydrolysate of arginine. A 6.5-cm column of Dowex 50 ion exchange resin was used on an automatic amino acid analyzer: 0.35 M sodium citrate buffer solution, pH 5.28, flow rate 30 ml per hour; flow rate of ninhydrin solution, 15 ml per hour. The elution position of homoarginine (taken from a separate analysis), indicated by the *dashed line*, is included for reference. X is 3-aminopiperid-2-one.

(both were eluted in the position indicated in Fig. 2) and on paper chromatography in the solvent systems butan-1-ol-acetic acid-water, and propan-1-ol-ammonia. Each compound gave R_F values of 0.41 and 0.60 in the respective systems at 23°; values of 0.38 and 0.58, respectively, were quoted for β-aminopiperidone by van der Horst (14). The boiling points of Compound X and 3-aminopiperid-2-one were the same, and the compounds furnished identical infrared absorption spectra in which strong absorption bands at 1650 cm^{-1} were consistent with displacement of the absorption band of the 2-carbonyl group due to hydrogen bond formation with the 3-amino function. The infrared absorption spectrum of the acetate of Compound X was virtually the same as that obtained by van der Horst for his "Compound X" which proved to be the acetate of β-aminopiperidone.[3] The elemental analysis of Compound X and the analysis calculated for $C_5H_{10}ON_2$ are given below.

$C_5H_{10}ON_2$

Calculated: C 52.6, H 8.8, N 24.8
Found: C 51.8, H 8.6, N 25.9

Thus it was established that Compound X was 3-aminopiperid-2-one.

It was observed by van der Horst (14) that the lactam was degraded quite readily by hot, aqueous alkali or acid, but that in alkaline solution an equilibrium between ornithine and its lactam was established. The results presented here confirm this conclusion, and suggest that there was less tendency for the compound to be formed (or to survive) as the pH of the reaction

[3] We are greatly indebted to Dr. C. J. G., van der Horst for a copy of the infrared absorption spectrum of this compound.

TABLE I

Products obtained under various conditions of alkaline hydrolysis of arginine and some related compounds

Compound hydrolyzed	Reagent	pH of reaction	Temperature	Time	Total recovered amino acids			
					Citrulline	Ornithine	Arginine	Compound X
				hrs	*moles %*			
Arginine	Ammonia	11	37°	24	0	0	100	0
Arginine	Ammonia	11	37	72	1	1	98	0
Arginine	Ammonia	11	110	24	4	6	68	22
Arginine	Ammonia	11	110	72	1	6	29	63
Arginine	Ammonia	11	145	24	2	8	0	89
Arginine	Ammonia	11	145	72	0	27	1	73
Arginine	Ammonia	12	37	24	0	0	100	0
Arginine	Ammonia	12	37	72	1	1	98	0
Arginine	Ammonia	12	110	24	1	90	0	8
Arginine	Ammonia	12	110	72	3	45	0	52
Arginine	Barium hydroxide	12	37	24	0	0	100	0
Arginine	Barium hydroxide	12	37	72	4	4	92	0
Arginine	Barium hydroxide	12	110	24	4	41	1	55
Arginine	Barium hydroxide	12	145	24	3	25	0	73
Arginine	Barium hydroxide	12	145	72	0	19	0	81
Arginine	Barium hydroxide	13	60	72	13	72	15	0
Arginine	Barium hydroxide	13	180	72	0	33	0	67
Arginine	Sodium hydroxide	10.6	100	4	18	25	29	28
Arginylglutamate	Barium hydroxide	13	60	72	52*	39	9	0
Citrulline	Ammonia	12	145	72	6	55		39
Ornithine	Ammonia	12	145	72		74		26
Ornithine	Barium hydroxide	13	60	72		100		0
Ornithine	Barium hydroxide	13	180	72		16		84
Ornithine	Sodium hydroxide	10.5	100	6		59		40
Compound X	Hydrochloric acid, 6 N		110	22		100		0

* Citrulline plus glutamic acid.

mixture was raised. Apparently the lactam is readily formed under mild alkaline conditions, but its formation leads to an increase in pH which eventually favors the reverse of the reaction, and an equilibrium state is reached. An observed increase in pH (from 10.5 to 11.3) on refluxing a solution of ornithine in aqueous sodium hydroxide is consistent with this suggestion.

It is clear from the work of van der Horst (14), and from the studies described here, that when arginine is converted into ornithine by alkaline hydrolysis there will usually be a concomitant formation of 3-aminopiperid-2-one. This should be considered when an analysis of basic amino acids is required on such a solution, or on an alkaline hydrolysate of a protein, for example. If such an analysis is to be made by means of an automatic ion exchange chromatography apparatus it is desirable to extend the period of chromatographic development in order to ensure the elution and detection of 3-aminopiperid-2-one. The quantity of this compound may be calculated, for its leucine color equivalent in the ninhydrin reaction (19), or "C value" (16) was 10.0 under conditions where the corresponding values for lysine, ammonia, and arginine were 20.2, 16.5, and 18.1, respectively. However, when alkaline hydrolysis is effected for prolonged periods under strongly alkaline conditions (*e.g.* 24 hours reflux in 0.5 N sodium hydroxide) formation of the lactam of ornithine may not occur to any great extent.

SUMMARY

The hydrolysis of arginine to ornithine in acid solution at high temperatures has been established. The reaction is very slow, amounting to less than 1% under the conditions usually used for the hydrolysis of proteins. Under certain circumstances, however, the conversion can become significant and may be rendered quantitative if the temperature is raised and the time of heating extended. From studies of the alkaline hydrolysis of arginine the lactamization of ornithine to yield 3-aminopiperid-2-one has been confirmed. The behavior of 3-aminopiperid-2-one on ion exchange chromatography is described. These aspects of the hydrolysis of arginine may sometimes be of importance in the analysis of amino acids (particularly protein hydrolysates) by automatic methods of ion exchange chromatography.

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