HYDROXYLYSINE: ISOLATION FROM GELATIN AND RESOLUTION OF ITS DIASTEREOSOMERS BY ION EXCHANGE CHROMATOGRAPHY

BY PAUL B. HAMILTON AND ROBERTA A. ANDERSON

(From the Alfred I. du Pont Institute of The Nemours Foundation, Wilmington, Delaware)

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Since the isolation of hydroxylysine as the picrate from the phosphotungstic acid-precipitable fraction of gelatin acid hydrolysates by Van Slyke, Hiller, Dillon, and MacFadyen (1), other isolation procedures have been reported (2, 3). The most recent is that of Sheehan and Bolhofer (4) who used chromatographic techniques in part to obtain analytically pure hydroxylysine from gelatin. The present paper describes a chromatographic procedure whereby, without preliminary removal of cystine or arginine, all of the hydroxylysine in a hydrochloric acid hydrolysate of 100 gm. of gelatin was separated from the other amino acids. Separation was partially effected on a column of Amberlite IR-120 and was completed on a column of Dowex 50. From the effluent of the Dowex column, hydroxylysine monohydrochloride of 98 per cent purity was isolated in yields of 80 to 90 per cent. A single crystallization from aqueous alcohol gave analytically pure but partially racemized hydroxylysine in yields of 70 to 85 per cent of the original material.

The hydroxylysine from gelatin was resolved by chromatography into hydroxylysine and allohydroxylysine, a result in accord with Piez (6) who separated a synthetic mixture of hydroxy-n-lysine from allohydroxy-n-lysine similarly. Piez also made the observation that approximately 20 per cent of the hydroxylysine in acid hydrolysates of tooth collagen was allohydroxylysine. The present study indicates that allohydroxylysine is an

1 Sheehan and Bolhofer (4) based their yields on the hydroxylysine, assayed by phosphotungstic acid precipitation, in the effluent fraction from a column of Amberlite IR-4B and obtained 65 to 75 per cent analytically pure hydroxylysine monohydrochloride. Computed on the basis of 1.2 gm. of free base per 100 gm. of protein reported by Rees (5) and which appears to be maximal, the yield of these authors is 48.4 per cent. Precipitation with phosphotungstic acid is a much less sensitive test for hydroxylysine than for lysine or arginine; hence considerable losses of hydroxylysine may occur undetected. Assay for hydroxylysine by the extremely sensitive periodate-chromotropic acid reaction (vide infra) probably accounts for the higher yields reported in the present paper. The authors are indebted to Dr. M. W. Rees, School of Biochemistry, University of Cambridge, Cambridge, England, for details and permission to use a recent unpublished method for the determination of hydroxylysine.
artifact arising through epimerization of hydroxylysine during the course of acid hydrolysis of the gelatin. In several experiments, after hydrolysis of the protein in 6 N hydrochloric acid for 16 hours, the amount of allohydroxylysine ranged from 7 to 20 per cent of the total, the amount of normal hydroxylysine being reduced correspondingly. On refluxing the hydrolysate for 96 hours, an equilibrium between the diastereoisomers appeared to be attained with 45 per cent hydroxylysine and 55 per cent allohydroxylysine.2

Isolation of Hydroxylysine from Gelatin Hydrolysates

Preparation of Acid Hydrolysates—100 gm. of gelatin (Nutritional Biochemicals Corporation) containing 8 per cent moisture were hydrolyzed under a reflux in 1500 ml. of 6 N hydrochloric acid for 16 hours. 5 gm. of carbon (Darco G-60, Darco Corporation, New York) were added and excess acid was removed in a rotating evaporator (8). The viscous residue was dissolved in water and the carbon was filtered and washed on the filter with 5 liters of water. The combined filtrate and washings, which were practically colorless, were made up to 10 liters. Prolonged evaporation of the hydrolysate gave maximal removal of free hydrochloric acid; thus the pH of the diluted solution was approximately 1.7 (glass electrode) and was suitable without further adjustment for introduction into the Amberlite column in the next step of the procedure.

Separation of Hydroxylysine Fraction on Amberlite IR-120 Column—The diluted hydrolysate was passed at a rate of 0.06 ml. per ml. of resin per minute through a 3.4 X 117 cm. (fully regenerated and back-washed) column of Amberlite IR-1203 maintained at room temperature. All of the amino acids were adsorbed on the column and the effluent was discarded. The major portions of the neutral and acidic amino acids were eluted with 1 N hydrochloric acid (at the rate of 0.01 ml. per ml. of resin per minute) and discarded. After each 100 ml. of effluent had passed through, 0.5 ml. was collected, neutralized with NaOH, and treated with periodate at pH 7.5 according to Nicolet and Shinn (9) for the determination of α-amino-β-hydroxy acids. Formaldehyde evolved from serine or hydroxylysine was determined colorimetrically with chromotropic acid according to Rees (5).

2 Reported to the American Society of Biological Chemists, Atlantic City, New Jersey, April, 1954 (7).
3 Amberlite IR-120 (Rohm and Haas Company, Philadelphia, Pennsylvania) was conditioned by washing new resin with water until the effluent was colorless. It was converted to the hydrogen form by 50 m.eq. of hydrochloric acid per ml. of resin (10 liters of 4 N hydrochloric acid, passed at a rate of 0.133 ml. per ml. of resin per minute). The first regeneration sometimes necessitated a larger volume of acid to obtain a completely colorless effluent. The excess acid was washed out rapidly by 4 to 6 liters of water. A column of Amberlite IR-120 shrinks 15 to 18 per cent on acid regeneration but expands to its original volume with the final water wash.
This method, capable of detecting 0.01 \( \mu \)mole of formaldehyde (or amino acid equivalent) was employed to show that serine was first completely eluted by 1 N hydrochloric acid. After the passage of 9 to 10 liters, traces of hydroxylysine began to appear in the effluent. The developing fluid was then changed to 2.5 N hydrochloric acid and the effluent collected until a negative test for hydroxylysine was obtained. This was usually a little less than 3000 ml. This fraction contained all of the hydroxylysine and relatively small amounts of neutral amino acids, together with much lysine and some arginine, histidine, and ornithine. It was evaporated to dryness.

Separation of Hydroxylysine on Dowex 50 Column—The dried residue was dissolved in 1 liter of water. This solution of pH 1.7 (glass electrode) was passed at a rate of 0.002 ml. per ml. per minute through a 7.5 \( \times \) 30 cm. column of sodium Dowex 50 maintained at room temperature. The effluent was discarded. The column was then developed with 0.3 M acetate buffer, pH 5.0, and the effluent was collected in 100 ml. fractions. The position of the emerging amino acids was determined by analysis of 0.1 ml. of each fraction by the colorimetric ninhydrin method (13). As described by Moore and Stein (11), monoamino acids emerged first. Since glucosamine and tryptophan are absent from gelatin, the next major peak was hydroxylysine, followed by ornithine and lysine (cf. (10)). Ammonia and arginine were left on the column and discarded later in the alkali-regenerating fluid used preparatory to another run. The position of hydroxylysine was confirmed by the Rees procedure and that of ornithine by the method of Chinard (14). Fig. 1 shows a typical chromatogram.

As shown in Fig. 1, the separation of hydroxylysine was good and, provided that elution was kept to the recommended flow rate or the column was not overloaded, overlapping of amino acids did not occur. In other runs, ornithine was found by Hamilton and Anderson (10) in acid hydrolysates of some gelatins. It was considered an artifact which arises from alkaline degradation of arginine during the manufacturing process and does not seem to be a normal constituent of collagenous tissue.

The sodium Dowex 50 (8 per cent cross-linked, 200 to 400 mesh, The Dow Chemical Company, Midland, Michigan) was prepared according to Moore and Stein (11) and equilibrated with 0.2 M acetate buffer, pH 3.4, prior to introduction of the amino acid solution.

Stock 0.4 M acetate solution, pH 5.0, was made by dissolving 64 gm. of solid NaOH and 135 ml. of glacial acetic acid in water and making up to 4 liters. To prepare 0.2 M solution, pH 3.4, 357 ml. of 1 N hydrochloric acid were added to 1 liter of 0.4 M solution and made up to 2 liters. The 0.3 M buffer was made from the stock solution by suitable dilution with water. As recommended by Hirs, Moore, and Stein (12), detergent and sequestering agent were omitted from these buffers.

The capacity and resolving power of both the Amberlite IR-120 and Dowex 50 columns described are adequate for treatment of 100 to 300 gm. of hydrolyzed gelatin; larger quantities overload the Dowex 50 column especially and the resolution of hydroxylysine is inadequate.
some tailing of the hydroxylysine occurred, but, as these later fractions contained only a few mg. of amino acid, they were discarded. The hydroxylysine-containing fractions were combined.

Isolation of Hydroxylysine Monohydrochloride—The combined fractions were evaporated to 100 ml. and an equal volume of concentrated hydrochloric acid was added and again evaporated. Another 100 ml. of concentrated hydrochloric acid were added to the residue and the bulky precipitate of sodium chloride was filtered and washed with three 100 ml.

portions of concentrated hydrochloric acid; the combined filtrate and washings were evaporated to dryness. The residue was stirred with 100 ml. of ethyl alcohol in which the hydroxylysine dihydrochloride dissolved slowly. Undissolved sodium chloride was removed by filtration and washed with a few ml. of alcohol and the filtrate and washings were evaporated to dryness. The residue was dissolved in 20 ml. of water and the solution neutralized with lithium carbonate to pH 6.5. This solution was evaporated to a viscous syrup. Methyl alcohol (50 ml.) was added to the

Conversion of the dihydrochloride to the monohydrochloride in aqueous solution by lithium carbonate was found preferable to conversion in alcoholic solution with pyridine or aniline. The latter procedures gave very gelatinous precipitates which stood for 24 hours in solution before granulating, and the yields were lower.
syrup and agitated with glass beads to loosen material adhering to the flask wall. The lithium chloride and some of the hydroxylysine dissolved in the methyl alcohol; the remainder of the hydroxylysine was suspended. 8 volumes of acetone added to the solution precipitated the dissolved hydroxylysine. After standing for 1 hour, the hydroxylysine was collected on a filter and washed with abundant acetone to remove residual lithium chloride. It was finally washed with 50 ml. of ethyl alcohol\(^9\) and dried \textit{in vacuo} over calcium chloride.

In typical experiments, the yields of hydroxylysine monohydrochloride ranged from 80 to 90 per cent based on the highest value reported in the literature (5) of 1.2 gm. of free base per 100 gm. of protein (1.35 gm. of hydroxylysine monohydrochloride per 100 gm. of gelatin containing 8 per cent moisture). The product melted with decomposition at approximately 210° and the specific rotation ranged from \([\alpha]_D^{25} +1.19°\) to \(+12.1°\) (\(c = 2\) per cent, 6 n hydrochloric acid) for the free base. The product was uncontaminated with other amino acids, as shown by chromatographic analysis on a 15 cm. column of Dowex 50 (11). Elementary analysis gave results within 2 to 3 per cent of theoretical. To recrystallize, the material was dissolved in 10 ml. of water, decolorized with carbon, and filtered, and ethyl alcohol was added until the cloud point was reached (approximately 60 per cent alcohol). It was seeded and stored at 4° for 12 hours. The alcohol concentration was then increased to 70 per cent. Two more additions of alcohol at 12 hour intervals brought the concentration to 90 per cent (cf. Sheehan and Bolhofer (4)). After the last addition of alcohol, crystallization was allowed to continue a further 24 hours at 4°. The crystals were collected, washed with a little 90 per cent alcohol and absolute alcohol, and then dried \textit{in vacuo} over calcium chloride. This method of recrystallization gave recoveries of 85 to 90 per cent of the crude material or 70 to 85 per cent of the hydroxylysine in the 100 gm. of gelatin. The melting point was 221-225° (literature 225° (4, 15, 16)) and the specific rotation ranged from \([\alpha]_D^{25} +6.87°\) to \(+14.1°\) (\(c = 2\) per cent, 6 n hydrochloric acid) for the free base. Hydroxylysine with a specific rotation of \([\alpha]_D^{25} +17.8°\) reported by Fones (17) for the pure L enantiomorph was never obtained for reasons detailed below.

\[
\text{C}_{4}\text{H}_{15}\text{O}_{2}\text{N}_{2}\text{Cl}.
\]

Calculated. \(C\) 36.27, \(H\) 7.61, \(N\) 14.10, \(Cl\) 17.85

Found. \(\ "\) 36.30, \(\ "\) 7.73, \(\ "\) 14.20, \(\ "\) 17.08

\textit{Chromatographic Resolution of Hydroxylysine Diastereoisomers—250 mg.}

\(^9\) The solubility of hydroxylysine monohydrochloride in absolute methyl alcohol is approximately 0.2 mg. per ml. at room temperature, and in ethyl alcohol 0.006 mg. per ml.; in acetone it is practically insoluble. Methyl alcohol was accordingly avoided for washing or drying precipitates.
of hydroxylysine monohydrochloride (from 16 hour gelatin hydrolysate), \([\alpha]_D^{25} +10.9^\circ\) (free base), were dissolved in 25 ml. of water and adjusted to pH 2 with hydrochloric acid. The solution was washed onto a 3.4 \times 115 cm. column of sodium Dowex 50, buffered at pH 3.4, and the column was developed at room temperature with 0.1 M sodium phosphate buffer, pH 7.5.\(^{10}\) The first 4.5 liters of effluent were discarded and the next 2.0 liters were collected in 10 ml. fractions.

The position and shape of the resulting peaks were established by ninhydrin colorimetric analysis of 0.1 ml. samples taken from alternate fractions. The resolution obtained was similar to that shown at A (Fig. 2), with 91 per cent of the recovered hydroxylysine in the fore peak and 9 per cent in the after peak.\(^{11}\) (Recovery of hydroxylysine from the column in this experiment was 96 per cent.) Fractions comprising the fore and after peaks, respectively, were combined; fractions that overlapped were discarded. The combined fore and after peak fraction solutions, each approximately 300 ml., were passed through 2.2 \times 30 cm. columns of Amberlite IR-120 in the hydrogen form, conditioned as previously described. Hydroxylysine and sodium were retained on the column, while some detergent (BRIJ 35) and phosphoric acid appeared in the effluent. The remainder of both these latter compounds was washed out with 2000 ml. of water. All but a trace of sodium was preferentially eluted as sodium chloride with 450 ml. of 1 N hydrochloric acid: the hydroxylysine was retained on the column as described by Stein (18). The hydroxylysine was then eluted with 200 ml. of 4 N hydrochloric acid and the effluent evaporated to dryness. Hydroxylysine monohydrochloride was isolated and crystallized as previously described. The specific rotation of the fore peak material was \([\alpha]_D^{25} +16.5^\circ\) (c = 2.0 per cent, 6 N hydrochloric acid) for the free base and was unchanged after recrystallization. The specific rotation of the after peak material was \([\alpha]_D^{25} -27.1^\circ\) (c = 2.31 per cent, 6 N hydrochloric acid) for the free base. Fones (17) reported \([\alpha]_D^{25} +17.8^\circ\) for the hydroxy-L-lysine free base and \([\alpha]_D^{25} -32.1^\circ\) for the allohydroxy-D-lysine free base. The values obtained in the present work indicate that the larger fore peak was hydroxy-L-lysine and the smaller after peak was allohydroxy-D-lysine. The relative positions assigned to the normal and allo forms are in agreement with those of hydroxylysine and allohydroxylysine observed by Piez (6).

A commercial synthetic racemic hydroxylysine was resolved similarly into 40 per cent hydroxy-\(DL\)-lysine and 60 per cent allohydroxy-\(DL\)-lysine;\(^{10}\) As a detergent, 10 ml. of BRIJ 35 (Atlas Powder Company, Wilmington, Delaware) solution (11) were added to each 1000 ml. of buffer.\(^{11}\) In other similar experiments the allohydroxylysine in 16 hour hydrolysates ranged from 7 to 20 per cent of the total hydroxylysine. In 25 hour hydrolysates the values were more consistent and agreed closely with the results found after hydrolysis of hydroxy-\(DL\)-lysine for the same length of time.
approximately 20 per cent only of this preparation was therefore hydroxy-L-lysine.

_Inversion of Hydroxylysine and Allohydroxylysine—_Commercial racemic hydroxylysine was resolved chromatographically into hydroxy-DL-lysine.

![Fig. 2. Chromatograms showing inversion of hydroxylysine (A and C) and allohydroxylysine (B and D) refluxed for 24 and 72 hours in 6 N hydrochloric acid. For experimental details see the text. A, hydroxylysine 68 per cent-allohydroxylysine 32 per cent; B, hydroxylysine 32 per cent-allohydroxylysine 68 per cent; C, hydroxylysine 53 per cent-allohydroxylysine 47 per cent; D, hydroxylysine 45 per cent allohydroxylysine 55 per cent.](https://www.jbc.org/)

![Fig. 3. Inversion of hydroxy-DL-lysine (A) and allohydroxy-DL-lysine (B) on refluxing in 6 N hydrochloric acid. The ordinates give the percentage of each diastereoisomer present at the times indicated (abscissas).](https://www.jbc.org/)
and allohydroxy-DL-lysine and each diastereoisomer isolated as described above. Both were chromatographically homogeneous. Respective portions were refluxed for 24, 48, 72, and 96 hours in 6 N hydrochloric acid and then rechromatographed. Chromatograms for the portions refluxed for 24 and 72 hours are shown in Fig. 2. The results are summarized in Fig. 3.

It is seen that commencing with either hydroxylysine or allohydroxylysine after 96 hours an equilibrium appeared to be attained with 46 per cent hydroxylysine-54 per cent allohydroxylysine. After refluxing in 1 N barium hydroxide for 24 or 48 hours, the relative proportions were 44 per cent hydroxylysine-56 per cent allohydroxylysine.

**DISCUSSION**

The evidence presented indicates that optical inversion of hydroxylysine occurs readily in boiling 6 N hydrochloric acid. It was also found that there was no marked difference between hydroxy-L-lysine (from gelatin) and hydroxy-DL-lysine (synthetic) with respect to rate of inversion and equilibrium attained. For example, a sample of hydroxy-L-lysine kindly supplied by Dr. John C. Sheehan, Massachusetts Institute of Technology, isolated from gelatin that had been hydrolyzed for 25 hours, was resolved into 35 per cent allo- per cent normal hydroxylysine. After being refluxed for a further 71 hours (96 hours total) in 6 N hydrochloric acid, it was resolved into 56 per cent allo-44 per cent normal hydroxylysine. Similarly, a calf skin gelatin hydrolyzed for 96 hours was chromatographed and resolved into 55 per cent allo-45 per cent normal hydroxylysine (see synthetic hydroxy-DL-lysine in Fig. 3, A). It seems justifiable to conclude that, since allohydroxylysine in gelatin hydrolysates approximates the amount found after refluxing hydroxylysine the same length of time in 6 N hydrochloric acid, the former is an artifact which arises during the course of acid hydrolysis of the protein. To obtain maximal values, resolution without isolation would be preferable because of the greater solubility of allohydroxylysine in water and alcohol and because of the partial separation of the diastereoisomers which occurs on crystallization from water-ethanol mixtures. For example, crude hydroxylysine, [α]_D^{25} +1.16° (free base), was crystallized in successive crops from the same solution as the alcohol concentration was raised from 60 to 70, 80, and 90 per cent; specific rotations were +7.06°, +3.50°, -8.04°, and -9.56°, respectively. The presence of 20 per cent of allohydroxylysine in tooth collagen hydrolysates as reported by Piez (6) seems to be adequately explained by the present findings.

Concerning the site of optical inversion, the data are consistent with the view that epimerization at the α-carbon atom predominates. The change from hydroxy-L-lysine to allohydroxy-D-lysine is compatible with the
specific optical rotations observed, $+16.5^\circ$ and $-27.1^\circ$, respectively, for material isolated (after separation of the diastereoisomers) from the same source. Insufficient material was available to undertake resolution of the diastereoisomers into their optical antipodes to prove conclusively that racemization (i.e. epimerization at the $\delta$-carbon atom) had or had not occurred. That both of the rotations given above are lower than those reported by Fones (17) for the pure substances indicates that some epimerization at the $\delta$-carbon atom might have taken place. Without doubt, however, the major site of inversion would appear to be the $\alpha$-carbon atom. This is in agreement with the evidence in the literature concerning racemization of amino acids containing two asymmetric centers (19).

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**SUMMARY**

1. A method is described whereby hydroxylysine was first partially separated from the bulk of the amino acids in a hydrochloric acid hydrolysate of gelatin on a column of Amberlite IR-120 cationic exchange resin. Resolution was completed on a column of sodium Dowex 50 with 0.3 M acetate, pH 5.0, as eluent. Hydroxylysine monohydrochloride (98 per cent analytically pure) was isolated in yields which ranged from 80 to 90 per cent of that originally present in gelatin. A single crystallization from water-ethanol gave an analytically pure product in yields of 70 to 85 per cent of the original material.

2. The diastereoisomers of hydroxylysine were resolved on 100 cm. columns of sodium Dowex 50 with 0.1 M phosphate, pH 7.5, as eluent.

3. Inversion of hydroxylysine and allohydroxylysine was shown to take place rapidly in boiling 6 N hydrochloric acid. After 96 hours, an apparent equilibrium of the diastereoisomers was attained with 46 per cent hydroxylysine-54 per cent allohydroxylysine. Hydroxy-L-lysine (from gelatin) and hydroxy-DL-lysine (synthetic) are inverted at approximately the same rate and attain similar equilibrium values.

4. Hydroxylysine from gelatin was resolved into hydroxy-L-lysine and allohydroxy-D-lysine. Inversion took place by epimerization predominantly at the $\alpha$-carbon atom. It was not proved conclusively that epimerization at the $\delta$-carbon atom did not occur.

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