[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

A New Isolation of Hydroxylysine

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In 1921, Van Slyke and Hiller[2] obtained evidence of the presence of a new amino acid in gelatin by examining certain discrepancies in the nitrogen content of phosphotungstate precipitates from various proteins. Schryver, Buston and Mukherjee[3] reported the isolation of a basic amino acid from isinglass by means of the carbamate procedure.[4] The free base and various salts were obtained in a crystalline state and analyses indicated a hydroxylysine structure. Repeated attempts to obtain a crystalline picrate were unsuccessful. The authors examined various proteins for this amino acid and concluded that it was practically absent from gelatin of mammalian origin.

Hydroxylysine was first isolated from gelatin by Van Slyke, Hiller, Dillon and MacFadyen.[5] After removal of arginine and histidine from the gelatin hydrolysate, a "lysine fraction" was obtained by crystallization with phosphotungstic acid. The hydroxylysine formed a monopicrate (m. p. 225°), which was separated from lysine picrate by fractional crystallization, and a monohydrochloride was prepared from the pure picrate. No exact experimental details or yields were reported by these authors.

Martin and Synge[6] reported the isolation of a hydroxylysine monopicrate (dec. 226–227°) from hydroxylysine purified by the acetylation–benzolyation procedure of Synge.[7] The "lysine fraction" used in the work was prepared in a manner similar to that employed by Van Slyke.[5]

Heathcote[8] separated a "lysine fraction" from a protein hydrolysate by electrodialysis after arginine and histidine had been removed with flavianic acid. Pure hydroxylysine monopicrate was obtained by a fractional crystallization procedure similar to that used by Van Slyke.[5] Heathcote discusses the inherent difficulties in the method of fractional crystallization when used as a means of separating the more soluble hydroxylysine picrate from lysine picrate.

The method of isolation of hydroxylysine used in the present investigation represents a new approach to the problem; use is made of techniques which were unavailable to the early workers in this field. Ion exchange resins were used for the separation of the lysine fraction and a chromatographic adsorption on alumina separated the hydroxylysine from the lysine.

The acidic amino acids and the hydrochloric acid remaining in a concentrated gelatin hydrolysate were eliminated by the use of an acid-binding ion exchange resin. Arginine was then removed as the flavianate, and a lysine fraction was separated from the filtrate by means of a buffered carboxylic acid ion exchange resin. The separation of the lysine fraction by means of a buffered carboxylic resin was adapted from a micro procedure developed with pure amino acids by Kunin and Winters,[9] who showed that only arginine and lysine were adsorbed on the buffered carboxylic resin.

The concentrated and neutralized acid eluate from the carboxylic column was dissolved in 80% methanol and partially purified by an initial chromatographic adsorption on alumina. The flowing chromatogram[10] technique was used in a manner similar to that described by Peck[11] for the purification of streptothricin. Development was carried out with 90% methanol until the filtrate was colorless and the strongly adsorbed material was eluted with water. The development with 80% methanol removed salts, such as sodium and ammonium chloride, and some noncrystalline organic matter, but it was not carried to the point at which any appreciable quantity of lysine was removed. The water eluate from the column was further purified by crystallization to a fraction containing only lysine and hydroxylysine.

Chromatographic adsorption was again used to separate further the lysine–hydroxylysine fraction, but in this case, development was carried far enough to remove most of the lysine (phos-

(1) Swift Amino Acid Fellow, 1947–1949.

(2) Van Slyke and Hiller, *Proc. Nat. Acad. Sci., U. S.*, **7**, 185 (1921).

(3) Schryver, Buston and Mukherjee, *Proc. Roy. Soc. (London)*, **98B**, 58 (1925).

(4) Buston and Schryver, *Biochem. J.*, **15**, 636 (1921); Kingston and Schryver, *ibid.*, **18**, 1070 (1924).

(5) Van Slyke, Hiller, Dillon and MacFadyen, *Proc. Soc. Exp. Biol. Med.*, **38**, 548 (1938).

(6) Martin and Synge, *Biochem. J.*, **35**, 307 (1941).

(7) Synge, *ibid.*, **33**, 1924 (1939).

(8) Heathcote, *ibid.*, **42**, 305 (1948).

(9) Kunin and Winters, *Ind. Eng. Chem.*, **41**, 460 (1949). These workers separated artificial mixtures of arginine and lysine by adsorption of the latter on a quaternary ammonium hydroxide type ion exchange resin (Amberlite IRA-400) and inferred that eluates from the carboxylic acid resin could be treated similarly, but this was not experimentally verified.

Using our mixtures, arginine and lysine could not be separated successfully by their procedure. The eluate from the carboxylic acid resin was acidic and in order not to exhaust unnecessarily the quaternary ammonium resin, a separate step was required to eliminate the excess acid. The eluate from the carboxylic resin also contained sodium chloride and apparently the sodium hydroxide formed by anion exchange with the basic resin interfered with the adsorption of the lysine. The failure of the quaternary ammonium resin to function under the conditions imposed upon it necessitated the use of flavianic acid for the separation of arginine. This procedure proved convenient and advantageous because of the elimination of extra operations and because the absence of arginine doubled the capacity of the carboxylic resin for the lysine fraction.

(10) Zechmeister and Cholnoky, "The Principles and Practice of Chromatography," John Wiley and Sons, Inc., New York, N. Y., 1941, p. 76.

(11) Peck, *et al.*, THIS JOURNAL, **68**, 772 (1946).

photungstic acid test). The water eluate was then very rich in hydroxylysine and pure hydroxylysine hydrochloride could be obtained by crystallization from water–ethyl alcohol.

The two separate chromatographic processes could not satisfactorily be combined into one step. The first adsorption on alumina removed impurities which interfered with crystallization of the fraction as a whole and the second adsorption separated the purified lysine and hydroxylysine from each other. Intermediate crystallization of the lysine fraction removed some unknown material which could not be conveniently separated from hydroxylysine by chromatography. By this process, 65–75% of the hydroxylysine in gelatin can be recovered in the pure state. This is about fifteen times greater than the highest yield previously reported. The variable values obtained for the specific rotation of hydroxylysine, and the isolation of two different picrates indicated that this product was partially racemized.

In early experiments, the basic amino acid fraction was separated by means of a sulfonic acid resin. The over-all procedure was essentially the same but the crude hydroxylysine fractions were much more difficult to purify. From 1000 g. of gelatin, only 0.6 g. of pure hydroxylysine hydrochloride was obtained.

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Experimental[12]

Removal of Free Acid and Acidic Amino Acids from a Gelatin Hydrolysate.—One kilogram of gelatin (Swampscott Brand), containing approximately 10% moisture, was dissolved in 8 l. of hot 7 *N* hydrochloric acid and heated under reflux for twenty-five hours. The dark brown solution was concentrated to a heavy dark sirup having a volume of about 1.1 l. The concentration was carried out in three passes in a single-pass, hot-tube vacuum evaporator; the volume was halved in each pass. (A similar all-glass, hot-tube, laboratory evaporator has recently been described by Bartholomew.[13])

The heavy sirup was dissolved in 2 liters of water and suspended matter was removed by filtration through Super-Cel. The filtrate was passed through a bed of conditioned[14] Amberlite IR-4B ion exchange resin (10 lb. supported in a 6-inch diameter glazed ceramic pipe) at a rate of about 1–3 liters per minute. The presence of the basic amino acids in the effluent was determined by testing with a 2% solution of phosphotungstic acid in 5% sulfuric acid. The portion of the effluent giving a white precipitate with this reagent was retained. It had a volume of approximately 14 liters and was weakly basic and chloride-free.

Removal of Arginine as the Flavianate.—The effluent was neutralized with concentrated hydrochloric acid to a *p*H of 6.5–7.0 and then concentrated to 4.5 liters. To this concentrate was added a solution of 250 g. of flavianic acid in 500 ml. of water. After standing at 0° for seventy-two hours the orange precipitate of arginine flavianate was removed by filtration and, after drying, weighed 215 g.[15] The filtrate from the arginine flavianate was passed through a small IR-4B resin exchanger (bed size 6.0 × 51 cm. in the regenerated, backwashed state) to remove hydrochloric and flavianic acids.

The Isolation of the Lysine–Hydroxylysine Fraction.—The *p*H of the effluent was adjusted to 6.5–7.0 with hydrochloric acid and the solution was passed through an Amberlite IRC-50 carboxylic acid resin in which the resin was buffered to *p*H 7.0 (bed size 6.5 × 46.5 cm., 2 lb. of resin, in the hydrogen cycle). The resin was prepared for use and buffered by the procedures of Kunin and Winters[17] and the Rohn and Haas Company.[18]

The amino acid effluent from the small IR-4B column was passed through the carboxylic column at a rate of about 2–3 liters per hour. This solution was followed on the column by 2 liters of water and then the resin was backwashed with an equal volume of water. After standing for about four hours the column was washed with water until the effluent was colorless (at this point the resin itself was almost colorless).

The adsorbed basic amino acids were eluted with 6 *N* hydrochloric acid, about 4 liters being required before the effluent gave a negative test with the phosphotungstic acid test solution. The acid eluate was concentrated under reduced pressure by means of a hot-tube evaporator to a volume of 500 ml., and 220 g. of sodium chloride was removed by filtration. The salt was washed with a small amount of concentrated hydrochloric acid followed by methanol and the filtrate and washings were concentrated to a heavy sirup to remove most of the excess hydrochloric acid. The residue was dissolved in 100 ml. of water, the solution cooled to 0°, and neutralized to *p*H 6 with 2 *N* sodium hydroxide (*ca.* 100 ml. required). This solution was concentrated to a heavy sirup which crystallized even while hot (in some experiments crystallization was not observed but subsequent manipulations were not affected). The weight of crystalline residue was 157 g. including, of course, an unknown amount of water and salt.

The residue was dissolved in 100 ml. of water and methanol was added to bring the volume to 1000 ml. The solution was allowed to stand at room temperature for a few hours and then filtered to remove a small amount of flocculent material and sodium chloride. The filtrate was divided into six portions of approximately 170 ml. each.

One portion of the solution, 1000 ml., of 80% methanol, 550 ml. of water and 550 ml. of 80% methanol were consecutively passed through an alumina column (bed size 60 × 3.8 cm.). The solution which was collected until the water was added to the column was considered methanol eluate and thereafter the effluent was considered water eluate.[19]

The six methanol eluates were combined and concentrated; the residual viscous sirup was dissolved in 85% methanol (200 ml.) and 39 g. of sodium chloride was removed by filtration. The filtrate was passed through the alumina column in the same manner as described for the portions above. The methanol eluate from this adsorption contained 0.4 g. of hydroxylysine hydrochloride and 40 g. of salt and other amino acids. It was discarded.

The seven water eluates were combined and concentrated to about 500 ml. Analysis of an aliquot of the

(12) We are indebted to Mr. S. M. Nagy and his associates for the microanalyses. All melting points are corrected.

(13) Bartholomew, *Anal. Chem.*, **21**, 527 (1949).

(14) Laboratory Manual, Amberlite Ion Exchange Resins, Rohm and Haas Company.

(15) The theoretical yield of arginine flavianate from 1000 g. of 90% gelatin is 220 g. using the value of 8.7%[16] for the arginine content of gelatin.

(16) Sahyun, "Outline of the Amino Acids and Proteins," Reinhold Publ. Corp., New York, N. Y., 1944, p. 67.

(17) Kunin and Winters, *Ind. Eng. Chem.*, **41**, 460 (1949).

(18) Mimeographed notes entitled "Amberlite IRC-50," Rohm and Haas Company.

(19) The alumina used in the column was not activated. The ordinary 48 to 100 mesh activated alumina marketed by the Aluminum Ore Company was washed with 5% acetic acid to remove residual basicity. It was then thoroughly washed and a slurry was poured into the column which was partially filled with water. When the material was in place and had settled, the water was displaced with 80% methanol.

solution with periodate[20] indicated the presence of 9.50 g. of hydroxylysine hydrochloride. The solution was treated with decolorizing carbon and the filtrate was concentrated to a heavy sirup weighing 65 g., which crystallized while still hot.[21]

After standing at room temperature for twelve hours and at 0° for twenty-four hours, the crystalline residue was broken up and stirred vigorously with 300 ml. of methanol, until the product was a homogeneous suspension of fine crystals. After standing at 0° for another twenty-four hours, the product was collected by filtration, washed with methanol and dried; weight 44.0 g. (20.4% hydroxylysine hydrochloride).

The mother liquor (which was discarded) contained 12.4 g. of solid material, of which 0.70 g. was hydroxylysine hydrochloride.

The crystalline product was dissolved in 40 ml. of water and after the addition of 100 ml. of methanol, the solution was allowed to stand at room temperature for six hours with occasional shaking. An additional 250 ml. of methanol was added and the mixture was allowed to stand for twenty-four hours at 0°. The product weighed 34.5 g. and contained 20.9% hydroxylysine hydrochloride.

The filtrate and washings were concentrated to a glass weighing 13.0 g., which was dissolved in 5 ml. of water and then diluted with 75 ml. of methanol. Crystals separated very slowly from the solution and after twenty-four hours storage at 0°, 6.0 g. of a product containing 21.8% hydroxylysine hydrochloride was obtained. The discarded filtrate contained 0.23 g. of hydroxylysine hydrochloride and 6.25 g. of other material.

The Separation of Lysine and Hydroxylysine by Alumina Chromatography.—The two crops were combined and by adsorption on an alumina column, 89.5% of the hydroxylysine was recovered in a 10.20 g. fraction containing 71.2% hydroxylysine hydrochloride. The chromatographic adsorption on alumina was carried out in the following manner:

A 10.0-g. sample (24.9% hydroxylysine hydrochloride) was dissolved in 60 ml. of water and 240 ml. of methanol added. This solution was passed through an alumina column (48–100 mesh, 60 × 3.8 cm.) which was developed with 6 liters of 80% (by vol.) methanol. The column was eluted with 600 ml. of water and 600 ml. of 80% methanol and the eluate was concentrated to 2 ml. To this solution was added 15 ml. of methanol and, after standing at 0° for eighteen hours, 0.90 g. of crystals (84.4% hydroxylysine hydrochloride) was collected. The development solutions were reworked until a 90% recovery of hydroxylysine was obtained.

Hydroxylysine Monohydrochloride.—The 10.20 g. fraction of 71.2% material was dissolved in 17 ml. of water and absolute ethanol was added (approx. 15 ml.) until the cloud point was just reached at 0° (at higher temperatures, less ethanol is required to reach the cloud point). The solution was seeded and, after storage at 0° for three days, 2.3 g. of 99% hydroxylysine hydrochloride was obtained.[22] The mother liquor was concentrated and the adsorption–crystallization cycle was repeated.

By this continued reworking of development and mother liquors, 6.4 g. of 98% hydroxylysine hydrochloride was obtained. The remainder of the hydroxylysine in the starting fraction can be accounted for in manipulatory losses, control titrations, and discarded fractions. After two recrystallizations from water and ethanol, the product decomposed at 215–220°.

Anal. Calcd. for $C_6H_{15}O_3N_2Cl$: C, 36.27; H, 7.61; N, 14.10. Found: C, 36.60; H, 7.79; N, 14.01.

Pure hydroxylysine prepared by this process is partially racemized. The specific rotation of various samples of the free base in 1 *N* hydrochloric acid (2% solution) ranged from −4.5 to +14.9°.

Hydroxylysine Picrate.—The picrate was very easily prepared by dissolving the hydroxylysine hydrochloride in 20 volumes of water and adding twice its weight of picric acid. From samples having a high specific rotation, two different crystalline substances were obtained. A light yellow picrate, which crystallized in clusters of needles, separated faster than a darker yellow picrate which crystallized as coarse, individual needles. By filtration before crystallization was complete, and recrystallization of each fraction thus obtained, both forms of the picrate were readily isolated in a pure state.

The darker yellow form was found to be a monopicrate having a decomposition point of 227°. It appeared to be the same compound reported by Van Slyke,[5] Martin and Synge[6] and Heathcote.[8] A weighed portion of this picrate was added to a known quantity of 1 *N* hydrochloric acid and, after one extraction with nitrobenzene, the rotation of the aqueous solution was determined and the specific rotation calculated for the free base: $[\alpha]^{20}D$ −19.4° (2% solution).

The light yellow picrate was optically inactive and, by periodate analysis, it was found to be a dipicrate which had not been reported previously. It apparently crystallized in two different modifications which could not be visually distinguished. One form melted gradually in the range 115–130° without decomposition, while the other modification decomposed sharply at 195°. The factors affecting the formation of either form are not known; preferential seeding was without reproducible effect. The low-melting modification was easily converted to the form possessing the high decomposition point merely by rubbing the crystalline product on a glass plate with a spatula. This dipicrate was obtained even from solutions containing half the theoretical amount of picric acid required for its formation.

Anal. Calcd. for $C_6H_4O_3N_2 \cdot 2C_6H_3O_7N_3$: C, 34.85; H, 3.25; N, 18.07. Found: C, 34.66; H, 3.27; N, 18.18.

Summary

A new method for the isolation of hydroxylysine from a gelatin hydrolysate is reported. After removal of free acid and acidic amino acids, by an acid-binding resin, arginine was precipitated as the flavianate. The lysine–hydroxylysine fraction was separated by means of a carboxylic acid ion exchange resin, and pure hydroxylysine hydrochloride was obtained by alumina chromatography and fractional crystallization. By this method, 60–75% of the hydroxylysine originally present is conveniently isolated in pure form.

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(20) Van Slyke, Hiller and MacFayden, *J. Biol. Chem.*, **141**, 681 (1941).

(21) It is necessary that crystalline material be obtained at this point. No difficulty has been experienced in achieving this, but the presence of too much water lowers the yield, and too rapid a concentration, leaving too little water, results in a glass which crystallizes poorly.

(22) This crystallization is very slow and it has been found that, if methanol is added in amounts not exceeding the quantity of ethyl alcohol, after the cloud point is reached crystallization is complete in twenty-four hours in higher yields. This device must be used with care, and preferably only with samples containing 85% or more of hydroxylysine hydrochloride, as methanol causes crystallization of lysine hydrochloride.