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On the Isolation and Structure of Hydroxylysine

SUNE BERGSTRÖM and SVEN LINDSTEDT

Department of Physiological Chemistry, University of Lund, Lund, Sweden

In 1921 van Slyke and Hiller[1] reported that an unidentified base occurred in gelatine. Later Schryver, Buston and Mukherjee[2] suggested that a basic amino acid occurring in gelatine which they isolated as a tribenzoate was an "oxylysine", *α*, *ε*-diamino-*β*-hydroxy-*n*-hexanoic acid. In 1938 van Slyke, Hiller, Dillon and MacFadyen[3] reported the isolation of a monopicrate of hydroxylysine melting at 225°. Elementary analysis, amino-N and amino acid carboxyl content corresponded to a diamino acid $C_6H_{14}N_2O_3$. van Slyke *et al.*[4, 5] later showed that alkaline periodate yielded one mole each of ammonia and of formaldehyde with the consumption of one atom of oxygen. As no indication of lactone formation was observed they suggested that the possible formulas were limited to I and II (Table 1). Martin and Synge[6] also isolated hydroxylysine from gelatine and isinglass as the picrate which decomposed at 226—27°. They used the acetylation-benzoylation method of Synge[7] on the base fraction isolated by precipitation with phosphotungstic acid.

Recently Heathcote[8] isolated the basic amino acids from gelatine hydrolysates by electrodialysis and after removal of arginine and histidine with flavianic acid studied the fractionation of the picrate of the remaining "lysine fraction". He also isolated small amounts of a picrate melting at 225°, but found this method of separation unsuitable, because the picrates like the phosphotungstates[5] of lysine and hydroxylysine apparently form solid solutions.

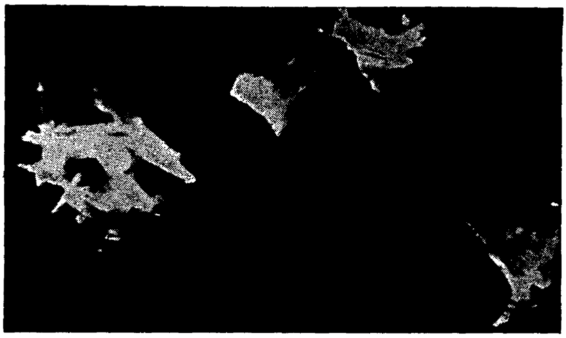
A number of workers[5, 9-15] have investigated the occurrence of hydroxylysine in various proteins with modifications of the periodate method. Collagen and gelatine have been found to contain about one per cent hydroxylysine, whereas only smaller amounts seem to be present in other proteins analyzed. Using paper chromatography, Gordon[16] recently made the interesting observation that a phosphate ester of hydroxylysine occurred in dialysate of calf embryo muscle juice.

As already mentioned in a preliminary note [17] we have utilized ion exchangers and partition chromatography to isolate pure hydroxylysine from gelatine or a preparation of dried fish skin.

After hydrolysis in 6 *N* hydrochloric acid and evaporation to dryness, the residue was dissolved in water and the remaining hydrochloric acid together with the acidic amino acids were removed with the anion exchanger Amberlite IR—4B. The basic amino acids in the effluent were then isolated by passing the solution through the carboxylic type cation exchanger IRC-50 essentially as described by Kunin and Winters [18]. However, instead of using the resin as the sodium salt we used it as the ammonium salt and eluted the basic amino acids with ammonia instead of with mineral acid. In this way the basic amino acids arginine, hydroxylysine, lysine are obtained after evaporation of the ammonia without contamination with much inorganic salts. Lysine and hydroxylysine were then absorbed on a column of the strong base anion-exchanger Amberlite IRA-400 that does not adsorb arginine [18] and eluted with hydrochloric acid. In this way a fraction was obtained consisting of a mixture of lysine and hydroxylysine in the proportion 7 to 1 contaminated with a small amount of arginine.

The separation of these amino acids was effected by partition chromatography with 0.1 *N* hydrochloric acid saturated with phenol supported on "Hyflo Super-Cel" as the stationary phase and phenol saturated with 0.1 *N* hydrochloric acid as the moving phase. With the latter medium on paper chromatograms lysine moves about twice as fast as hydroxylysine while arginine is still faster. In accordance with this all arginine and practically all lysine had left the aforementioned column before hydroxylysine appeared in the effluent. The fractions containing only hydroxylysine were combined, dilute hydrochloric acid was added and the phenol extracted with ether. Hydroxylysine can be crystallized in low yield directly from the residue but a good yield was obtained by benzoylation of the whole fraction in alkaline solution when a N,N′-dibenzoate of hydroxylysine was obtained (m. p. 166—67°) with the composition $C_{20}H_{22}O_5N_2$. The methylester (m. p. 138—39°) was prepared with diazomethane. Benzoylation of this ester with benzoylchloride in pyridine yielded a tribenzoate (m. p. 171—73°). The composition of these derivatives all corresponded to the one expected for a monohydroxydiaminohexanoic acid. $C_6H_{14}O_3N_2$. Fifty per cent of the nitrogen was liberated with periodate [4, 5]. Hydrolysis yielded the crystalline monohydrochloride of hydroxylysine, (Fig. 1).

In order to determine the configuration of the grouping split by periodate we oxidized the dibenzoate (III or IV) with an excess of chromic trioxide in 97.5 per cent acetic acid at 50° and determined the excess of oxidant left after

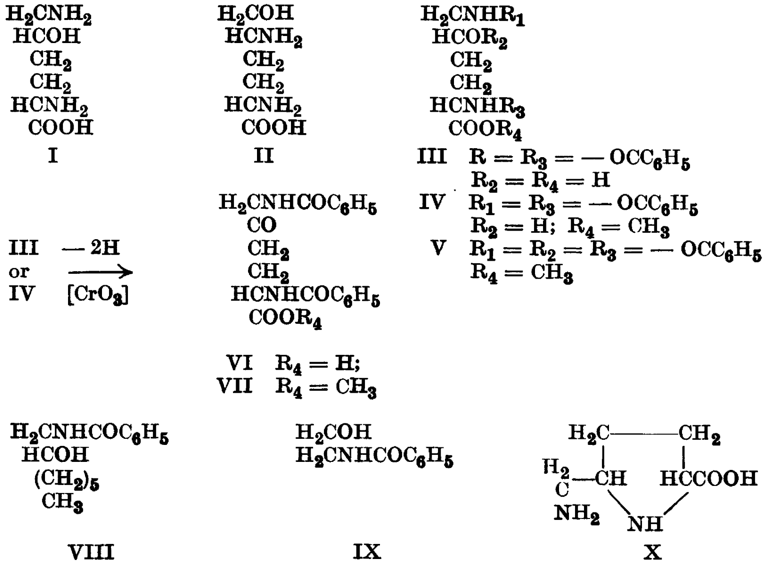


Hydroxylysine hydrochloride from aqueous ethanol (30 ×).

different times. The dibenzoate rapidly consumed two equivalents corresponding to the dehydrogenation of a secondary alcohol to a ketone (VI or VII) as shown in figure 2. 1-Benzamido-2-octanol (VIII) also consumed 2 equivalents whereas 2-benzamido-1-ethanol (IX) consumed 4 equivalents with the formation of a carboxyl group. The group reacting with periodate thus clearly has the configuration corresponding to VIII and hydroxylysine should have the configuration I of the two formulas proposed by van Slyke *et al.*[4, 5]. The reaction product from the oxidation of IV was isolated (m. p. 134—35°) and characterized. However, the configuration of the carbon chain of hydroxylysine had not been proved by van Slyke *et al.* We subjected a small sample of hydroxylysine to reduction with hydroiodic acid and red phosphorous at 160° for 4 hours. With paper chromatography it was found that no hydroxylysine could be found in the reaction mixture but instead a compound behaving like lysine had been formed together with another compound giving a strong yellow spot with ninhydrin. The yellow spot that represents the main reaction product is possibly caused by the cyclic derivative 5-aminomethyl proline (X) that is formed in analogy with the proline synthesis from α-amino-γ-hydroxy or γ-halogene pentanoic acid[19-21]. That the reaction mixture contained L-lysine was further strengthened by microbiological assay with *Leuconostoc mesenteroides* P-60. According to this analysis 5—10 per cent of the expected amount of L-lysine had been formed. Hydroxylysine does not support the growth of this organism.

The optical activity of the N,N′-dibenzoate and corresponding methyl esters of L-lysine and hydroxylysine are collected in Table 2 together with the δ-keto lysine derivative (VII) prepared from natural hydroxylysine in which the asymmetry of the δ-carbon atom has been destroyed. From these data it appears highly probable that hydroxylysine has the same configuration

Table 1.



as L-lysine. This is also supported by the recent work of Weisiger[29] with the enzymatic synthesis of the anilide from natural hydroxylysine.

Since our preliminary report of this work appeared[17], the isolation of pure hydroxylysine has also been announced from three different laboratories[27-29]. Sheehan and Bolhofer[27] have furthermore described the conversion of hydroxylysine to an optically inactive derivative, methyl-α,ε-diphthalimido-δ-keto-D,L-hexanoate which they also prepared from glutamic acid by an unambigous synthesis. Weisiger[29] has synthesized hydroxylysine and separated it into the L and D isomeres with aid of the enzymatic anilid formation.

Table 2.

R	R $\vert$ $HCNHCOC_6H_5$ $\vert$ $COOCH_3$	R $\vert$ $HCNHCOC_6H_5$ $\vert$ $COOH$
$-CH_2CH_2CH_2CH_2NHCOC_6H_5$	− 120	− 101
$-CH_2CH_2CO\cdot CH_2NHCOC_6H_5$	− 84	
$-CH_2CH_2CHOH\cdot CH_2NHCOC_6H_6$	− 156	− 119

M_D in pyridine

The configuration of the second asymmetric carbon atom in hydroxylysine is not yet known. The question remains if partial or total racemisation might have taken place during the preparation of gelatine or during the isolation procedure that would explain the differences in the reported specific rotations of hydroxylysine [27, 29].

EXPERIMENTAL

Isolation of the "lysine" fraction from gelatine hydrolysates

As a source of hydroxylysine Difco Bacteriological gelatine was first used. It was found, however, that this material contained some ornithine, probably originating by partial destruction of arginine in the alkali treatment during the manufacturing of the gelatine. As ornithine was difficult to separate from hydroxylysine in the chromatographic procedure, a commercial preparation of dried fish skin was used for some isolation experiments, because it was found to be free from ornithine. The material was hydrolyzed with about fifty times its weight of boiling 6 *N* hydrochloric acid for 20 hours. Excess hydrochloric acid was removed by repeated evaporation *in vacuo* with water. The "lysine" fraction was isolated with the aid of a series of ion exchange resins, essentially according to the method of Winters and Kunin [18]. The amino acids were dissolved in water and passed through a column of the weak base anion exchange resin Amberlite IR—4B which had been pretreated with one normal sodium hydroxide and then washed with distilled water until the effluent was neutral. This removed the hydrochloric acid left and most of the dicarboxylic amino acids. The effluent was then directly passed through a column with the carboxylic type cation exchange resin Amberlite IRC-50. This column had been converted to salt form by treatment with *N* ammonia instead of sodium hydroxide as recommended by Winters and Kunin. The column was then treated with ammonia-ammonium chloride buffer of pH 7.5 and finally water until the washings were free from chloride. With this treatment only amino acids with an isoelectric point higher than that of histidine were adsorbed, *i. e.* arginine, lysine, hydroxylysine, ornithine.

The column was then eluted with *N* ammonia in fractions. By using ammonia as the regenerating and eluting reagent, the formation of salts in the eluate was avoided. Attempts to obtain a complete fractionation of the basic amino acids by using ammonia of increasing strength were not successful, though hydroxylysine was enriched in the first fractions. The fractions containing hydroxylysine were combined and evaporated to dryness *in vacuo*, redissolved in water and passed through a column of the strong base anion-exchange resin Amberlite IRA-400 which had been converted to hydroxyl form by treatment with sodium hydroxide and washed to neutral reaction. As Winters and Kunin have shown, arginine passes through while amino acids that are weaker bases are adsorbed. After washing with water until the washings showed a negative Sakaguchi reaction, the column was eluted with *N* HCl and the eluate taken to dryness repeatedly with water to remove excess hydrochloric acid.

In some preparations the hydrolysis was performed with 6N sulfuric acid which was removed with barium hydroxide or barium carbonate. In these cases the first column with Amberlite IRA-4B was not used.

The Amberlite columns used in the following examples were pretreated as follows:

1. Amberlite IR-4B (28 × 500 mm). The column was washed with one liter of *N* hydrochloric acid and then with water until the effluent was chloride free.

2. Amberlite IRC-50 (28 × 300 mm) was converted to free acid with one liter *N* hydrochloric acid and washed chloride free with water. The resin was then converted to the ammonium salt with one liter of N ammonia and then buffered with 750 ml ammonia-ammonium chloride buffer of pH 7.5 and subsequently washed with 750 ml water.

3. Amberlite IRA-400 (28 × 400 mm) was treated with 2 liters of *N* sodium hydroxide followed by one liter of water.

Example 1. 50 g Difco Gelatine was hydrolyzed with 3.5 l boiling 6 *N* hydrochloric acid for 24 hours and then repeatedly evaporated to dryness *in vacuo* after addition of water. The residue was dissolved in 750 ml water and passed through the IR—4B column followed by 750 ml water. The effluents were combined and directly passed through the IRC-50 column. The column was then washed with about one liter of water. The adsorbed basic amino acids were displaced by 0.1 *N* ammonia. The effluent was collected in fractions of 250 ml and the fractions giving a positive ninhydrin (fraction 2—5) were combined and evaporated to dryness *in vacuo*. The residue was dissolved in 750 ml and passed through the IRA-400 column that was then washed with one liter of water. The adsorbed lysine and hydroxylysine were then eluted with 500 ml *N* hydrochloric acid. The residue from the hydrochloric acid eluate weighed 2.72 g and contained[4,5] 0.34 g hydroxylysine calculated as the free base corresponding to a content of 0.68 per cent hydroxylysine of the starting material. Paper chromatography showed that the fraction consisted of lysine and hydroxylysine contaminated with some arginine and a little ornithine.

Example 2. 70 g of a commercial fish skin preparation was hydrolyzed with 3 l boiling 6 *N* hydrochloric acid for 24 hours and the hydroxylysine isolated using the same columns.

The isolated fraction weighed 2.0 g and analyses showed the presence of 0.145 g hydroxylysine. Hydroxylysine nitrogen constituted 12.6 per cent of the total nitrogen. Paper chromatography indicated that a little arginine was the only ninhydrin positive compound present except lysine and hydroxylysine.

Partition chromatography of the "lysine" fraction

Freshly distilled phenol was mixed with enough 0.1 N hydrochloric acid to yield a small aqueous phase over the phenolic phase. These solutions are stable and can be kept at room temperature for weeks. 30 ml of the aqueous phase was added to 60 g Hyflo Super-Cel. The mixture was worked with a spatula until homogenous. Enough of the phenolic phase was then added to give a suspension that could be poured into the chromatographic tube made by annealing a glass tubing (i. d. 35 mm, length 350 m) to a fritted glass funnel with the same inner diameter (Corning medium). The column was filled under a slight vacuum. When the upper surface was almost dry, the suction was discontinued, enough dry sand poured on top of the column to form a horizontal layer of about 10 mm depth. The solution containing the amino acids, generally 5—15 ml, was immediately carefully added with a pipette without disturbing the surface of the sand. The solution was washed into the column with several 5 ml portions of the phenolic phase.

When the "lysine" fractions were dissolved in the phenolic phase, an aqueous phase separated and an emulsion was formed by shaking the sample immediately before adding it to the column. The layer of dry sand helps in keeping the aqueous phase dispersed in small droplets. The column was then fastened over an automatic fraction collector[22] that changed the receiving vessel every half hour. A layer of fresh phenolic phase about 10 cm high was maintained by hanging a 1 liter separatory funnel with the tip of the stem at this height over the surface of the sand. The phenolic phase was then poured into the funnel, the stopper was inserted and the stop cock carefully opened. Each half hour fraction was 16—20 ml. The amino acids present in the fractions were ascertained by running one dimensional paper chromatograms with the same acid phenolic phase as moving phase.

Any arginine present first appeared in the effluent followed by lysine. When almost all lysine had left the column, hydroxylysine appeared in the effluent. When the fractions only contained hydroxylysine the addition of phenol was stopped and the column washed with water to collect the hydroxylysine left on the column. If ornithine was present, it appeared in the later lysine and earlier hydroxylysine fractions.

The fractions containing only hydroxylysine and the final water fraction were combined, a little hydrochloric acid was added and the phenol was extracted with ether. The aqueous phase was then taken to dryness *in vacuo.*

Benzoylation of the hydroxylysine-fraction in alkaline solution

The residue from a chromatogram was dissolved in a minimum of water (hydroxylysine content calculated from periodate-ammonia: 180 mg) and after addition of 5 ml 2 *N* sodium hydroxide it was benzoylated at room temperature by the method of Carter and Stevens[23] with 1.8 ml benzoyl chloride and 18 ml 2 *N* sodium hydroxide added in 9 equal portions during 3 hours. The reaction mixture was then acidified and filtered. After the dried precipitate had been exhaustively extracted with hot petroleum ether the residue weighed 470 mg. Crystallization from hot absolute ethanol yielded 252 mg of colorless needles, m. p. 165—67°. After recrystallization from ethanol or acetone the *N,N'-dibenzoate of hydroxylysine* melted at 166—67°.

$C_{20}H_{22}O_5N_2$ (370.4)

Calc.	C 64.85	H 5.99	N 7.56
Found	» 64.61, 65.00	» 5.98, 5.88	» 7.37, 7.35

Equiv. wt. found 376.9 (microtitration).

Rotation: $[\alpha]_D^{20°} = -31° \pm 2°$ (C = 0.05; pyridine; microtube).

The *methyl ester of the N,N'-dibenzoate of hydroxylysine* was prepared by treating the acid in methanol with an ethereal solution of diazomethane. The methyl ester was recrystallized from methanol-ether; m. p. 138—39°.

$C_{21}H_{24}O_5N_2$ (384.4)	Calc.	C 65.61	H 6.29	N 7.29
	Found	» 65.59	» 6.36	» 7.24

Rotation: $[\alpha]_D^{24°} = -40.7° \pm 1.9°$ ($C = 0.355$; pyridine; microtube).

Methyl ester of tribenzoate of hydroxylysine (V)

32 mg of the dibenzoylmethyl ester (IV) was dissolved in 2 ml pyridine. 0.1 ml benzoylchloride was added and the solution left for 20 hours at 30°. Two drops of water were added and after 2 hours the reaction mixture was evaporated to dryness. Dilute hydrochloric acid and ether were added and the ether phase was washed with dilute hydrochloric acid, sodium carbonate, water, dried over sodium sulphate and evaporated to dryness. The residue was recrystallized from methanol-water yielding clusters of needles m. p. 171—73°.

$C_{28}H_{28}O_6N_2$ (488.5)	Calc.	C 68.84	H 5.78	N 5.74
	Found	» 69.14	» 6.25	» 5.77

Hydroxylysine monohydrochloride

350 mg of the hydroxylysinedibenzoate was hydrolyzed by refluxing 20 hours in 40 ml 5 normal hydrochloric acid. The solution was evaporated to dryness and redissolved in hot water, the benzoic acid was extracted with ether and the aqueous phase taken to dryness. The residual sirup that consisted of the dihydrochloride crystallized partly. It was dissolved in a few drops of water and one tenth ml of pyridine was added. Ethanol precipitated an oil that rapidly crystallized. Recrystallization was effected by dissolving in water and carefully adding ethanol when the monohydrochloride of hydroxylysine rapidly crystallized in clusters of needles. 159 mg was obtained decomposing at 220°.

$C_6H_{15}N_2O_3Cl$ (198.66)	Calc.	C 36.27	H 7.61	N 14.10	Cl 17.85
	Found	» 36.07	» 7.47	» 14.23	» 18.25

Rotation: $[\alpha]_D^{22} = + 7.5° \pm 1.0$ ($C = 0.4$; N HCl microtube).

Oxidation of N,N′-dibenzoyl hydroxylysine, 1-benzamido-octanol and 2-benzamido-1-ethanol with chromic trioxide

One to 2 mg samples of the substance were weighed into test tubes (18 × 200 mm) with a weighing tube. The samples were then dissolved in 1 ml glacial acetic acid purified by distillation from chromic trioxide. Exactly 1 ml of a stock solution containing approximately 50 mg chromic trioxide per 100 ml of 95 per cent acetic acid was then added. These tubes together with an equal number of blanks without any added substance were then placed in a water thermostat at 50°. A sample and a blank were taken out after times indicated, 1 ml 10 per cent potassium iodide solution was added and the liberated iodine titrated with 0.01 *N* sodium thiosulphate. The results are plotted in Fig. 2. *N*-benzoyl-ethanolamine rapidly consumed two atoms of oxygen whereas N benzoyl-1-amino-2-octanol only consumed one atom. The N,N′-dibenzoate of hydroxylysine also consumed only one atom oxygen per mole.

N-Benzoyl-1-amino-2-octanol

960 mg 1-nitro-2-octanol, prepared according to Spray and Degering[24], was hydrogenated with W 6-Raney nickel[25] in ethanol at room temperature. After 1.5 hours 410 ml hydrogen had been adsorbed, the catalyst was filtered off and the solution was taken

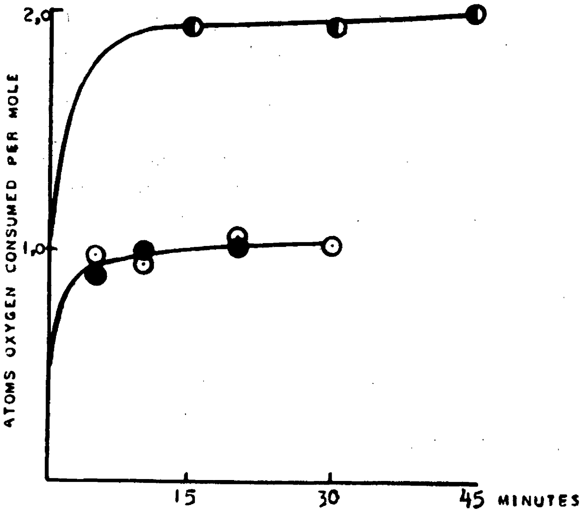


Fig. 2. Oxidation with CrO_3 in 97.5 per cent acetic acid at 50°

○-○ *NN'-Dibenzoylhydroxylysine*
●-● *1-Benzamido-2-octanol*
◐-◐ *2-Benzamido-ethanol*

to dryness. The residue was dissolved in 10 ml dry pyridine and 2 ml benzoyl chloride was added. After 16 hours at room temperature a few drops of water were added and the solution was taken to dryness *in vacuo*. Ether and water were added and the ether phase was washed with dilute hydrochloric acid, water, dilute alkali, water, and dried over sodium sulfate. The residual oil obtained after evaporating the ether weighed 1.96 g.

The oil was dissolved in 25 ml ethanol and 15 ml 0.4 N barium hydroxide was added and the solution left over night at room temperature. The solution was then neutralized with 0.2 ml *N* HCl and concentrated somewhat *in vacuo* when the *N*-benzoate crystallized.

The crystals weighed 550 mg and melted at 97—98°. Recrystallization from aqueous ethanol raised the m. p. to 99—101°.

$C_{15}H_{23}O_2N$ (249.3)	Calc.	C	72.25	H	9.30	N	5.62
	Found	»	72.12	»	9.31	»	5.55

N-benzoyl-amino-2-ethanol IX[26]

This compound was prepared from amino ethanol (Schering, A. G.) by benzoylation and partial saponification as described above. The product melted at 60°.

$C_9H_{11}O_2N$ (165)	Calc.	N 8.5	Found	N	8.63

Preparative oxidation of the methylester of N,N′-dibenzoylhydroxylysine (IV) to ketone (VII)

115 mg of the dibenzoylmethyl ester (IV) was dissolved in 30 ml glacial acetic acid (distilled from chromic trioxide). 30 ml of a solution of 70 mg CrO_3 in 50 ml 95 per cent acetic acid was added and the mixture was placed in a water bath at 38° C. After 50 minutes the calculated amount of oxidant had been consumed. A little methanol was added and the solution evaporated to a syrup *in vacuo*. The residue was dissolved in 25 ml methanol, water, and ether was added and the aqueous phase was extracted with about 500 ml ether in six portions. The residue after evaporation of the ether was diluted with a few drops of methanol and left at — 15°. 85 mg of colorless needles were obtained. Recrystallization from methanol-ether yielded 68 mg of needles m. p. 134—135°.

$C_{21}H_{22}O_5N_2$ (382.4)	Calc.	C	65.95	H	5.80	N	7.33
	Found	»	65.70	»	5.80	»	7.44

$[\alpha]_D^{24} = -21.9 \pm 1.8$ (pyridine; C = 0.0223; microtube).

SUMMARY

Hydroxylysine has been isolated from gelatine and fish skin and a number of derivatives have been prepared.

The structure has been shown to be *α,ε*-diamino-*δ*-hydroxy-*n*-hexanoic acid.

Hydroxylysine probably belongs to the L-series.

The microanalyses have been made at the microanalytical laboratory of the Department of Medical Chemistry at Upsala.

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