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N^{ϵ}-(CARBOXYMETHYL)LYSINE, A CONSTITUENT OF HUMAN URINE

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Summary

An unknown urinary amino acid, present in small amounts in many children with various diseases, has been isolated and identified as N^{ϵ}-(carboxymethyl)lysine. The identity of this compound was confirmed by synthesis. Its chromatographical characteristics are described.

The compound also occurred in the urine of prematurely born infants. Even in healthy adults trace amounts could be detected.

In a few patients strikingly higher excretions were observed, up to 67 mg/g creatinine, as determined by cation-exchange column chromatography. No clear correlation with a distinct clinical picture could be established.

Speculations are given about the origin of the compound.

Introduction

In this paper we describe the isolation and identification of a ninhydrin-positive compound, which we observed when screening the urine of patients for inborn errors of amino acid metabolism. The compound appeared to be N^{ϵ}-(carboxymethyl)lysine (N^{ϵ}-CM-Lys).

When using our 5 cm × 5 cm high resolution thin-layer method the unknown compound appeared on the two-dimensional chromatogram as a distinct purple spot. Although the compound occurred in many patients with various disorders and even in normals at a trace level, it appeared to be excreted in a strikingly high amount in a few children, suggesting a possible relationship with disease. Excretory levels in these children are given. We speculate about the possible origin of the compound and its clinical significance.

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Materials and Methods

Thin-layer chromatography

Micro scale two-dimensional chromatography on 5 cm × 5 cm thin-layer chromatograms of urinary amino acids was performed as described previously [1]; some slight modifications were introduced. The solvent systems used were (1) 1-butanol/pyridine/water (1 : 1 : 1, v/v) (30 min) and (2) 88% phenol/25% ammonia/water (10 : 0.05 : 1, v/v) (50 min). The amount of the 10-fold concentrated, desalted solution applied on the chromatograms corresponds with 1 μg of α-amino-nitrogen. The chromatograms were dried at 40 °C; for the detection of the amino acids an acetone solution containing ninhydrin (0.25%, w/v), isatine (0.01%, w/v) and lutidine (1%, v/v) was used [2].

Paper chromatography

Two-dimensional paper chromatography on Whatman 3 MM (20 cm × 20 cm) was performed as described for thin-layer chromatography.

Column chromatography

Column chromatography was carried out using the standard procedures described for the Technicon NC1 and TSM1 instruments.

Isolation of the ninhydrin-positive substance from urine

800 ml of urine of patient F.K. (Table I) was filtered through a column (106 cm × 2.5 cm) of 400 ml Dowex 50, H^+ form. After washing the column with an excess of water, the amino acids were eluted with 3% ammonia; the eluate was evaporated to dryness in vacuo. The neutral amino acid fraction, including the unknown compound, was separated from the other amino acids via column chromatography over Amberlite CG-120, H^+ form (106 cm × 2.5 cm) using 1 M pyridine as eluent [3]. After evaporation of this fraction in vacuo, the dry material was dissolved in 80 ml water (solution A). Subsequently, the neutral amino acids were fractionated on a column of 516 ml Aminex-MS fraction B, H^+ form (106 cm × 2.5 cm). The column was loaded with solution A in portions of 8 ml and then eluted with 0.1 M pyridine adjusted to pH 3.5 with acetic acid. The elution was carried out at a flow rate of 2.6 ml/min at 50 °C. The eluate was monitored for the presence of the unknown compound by thin-layer chromatography. The substance was collected after 1330—1440 min, nearly free from other amino acids. The material obtained from the ten 8-ml portions was pooled and subjected once more to chromatography on Aminex-MS. The total yield of the ninhydrin-positive compound was about 10 mg.

Synthesis of N^{ϵ}-(carboxymethyl)lysine [4]

1 g poly-L-lysine hydrobromide, type VI-B, mol.wt 30 000—70 000 (Sigma Chemical Company), dried over P_2O_5 in vacuo, was added to a solution of 8 g monoidoacetic acid in 380 ml water. The reaction mixture was brought to pH 10 and kept constant with 2 *N* NaOH by using a pH stat. The solution was stirred for 2.5 h at room temperature, followed by 0.5 h at 40 °C. The mixture was then dialyzed against distilled water for 24 h. After lyophilization

the residue was hydrolyzed in 270 ml 6 *N* HCl for 20 h at 110 °C in an evacuated sealed tube. The hydrolysate was neutralized with 1 *N* NH_4OH and twice lyophilized to remove iodine. The white-yellow residue was desalted by ion-exchange chromatography on Dowex 50, H^+ form. After washing with water the amino acid fraction was collected by eluting the column with 2 *N* NH_4OH. The following products were isolated by preparative column chromatography on Aminex-MS (see above): lysine, N^{ϵ}-(carboxymethyl)lysine and N^{ϵ}-(dicarboxymethyl)lysine. The total yield of N^{ϵ}-(carboxymethyl)lysine was about 120 mg.

Synthesis of N^{α}-(carboxymethyl)lysine

N^{α}-(carboxymethyl)lysine was synthesized by coupling N^{ϵ}-(carbobenzoxy)-lysine and monobromoacetic acid, according to Biemann et al. [5] and Seitz et al. [6]. The product was purified by preparative paper chromatography on Whatman 3 MM in 1-butanol/acetic acid/pyridine/water (30 : 6 : 20 : 24, v/v) as solvent system.

Synthesis of N^{α}-(1-carboxyethyl)ornithine [7]

N^{α}-(1-carboxyethyl)ornithine was synthesized by coupling N^{δ}-(carbobenzoxy)ornithine and α-bromopropionic acid, according to Biemann et al. [5] and Seitz et al. [6]. The product was purified by preparative paper chromatography on Whatman 3 MM in 1-butanol/acetic acid/pyridine/water (30 : 6 : 20 : 24, v/v) as solvent system.

Protection of amino acids for mass spectrometry

The acylable groups of the amino acids were protected by ethoxycarbonylation with diethylpyrocarbonate as described before [8,9]. One part of the ethoxycarbonyl derivative was esterified with diazomethane in ether. The other part was esterified and permethylated with CD_3J and methyl sulphinyl carbanion in DMSO, according to Vilkas et al. [10].

Mass spectrometry

70 eV mass spectra were recorded on an A.E.I. MS-902 mass spectrometer at an ion-source temperature of 120 °C, a trap current of 500 μA and an accelerating voltage of 8 kV. High resolution mass measurements were performed with a dynamic resolving power of 10 000 and a scan speed of 16 sec per mass decade by using an A.E.I. MS-902 mass spectrometer connected on-line with a Ferranti Argus 500 computer. The exact masses measured were converted into element lists as described by Van 't Klooster et al. [11].

Results and Discussion

Characterization of the ninhydrin-positive substance

In Fig. 1 a typical two-dimensional thin-layer chromatogram (5 cm × 5 cm) of urinary amino acids including the unknown compound is given. The position of this substance is approximately symmetrical between glycine, glutamine, lysine (+ ornithine) and hydroxylysine (+ its glycosides). On Whatman 3 MM paper chromatograms (20 cm × 20 cm) the compound has a similar

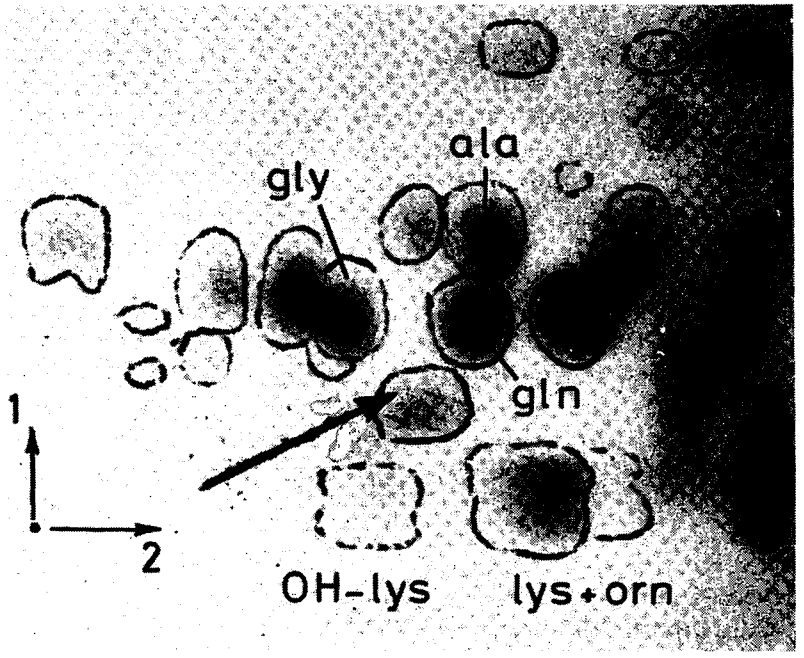

Fig. 1. The position of the unknown compound among other urinary amino acids on the 5 cm × 5 cm thin-layer chromatogram. First solvent: butanol/pyridine/water (1 : 1 : 1, v/v). Second solvent: 88% phenol/25% ammonia/water (10 : 0.05 : 1, v/v). The unknown compound is indicated by the arrow.

position. On column chromatography using the Technicon NC1 standard procedure the compound is present between isoleucine and leucine; in the Technicon TSM1 standard procedure the substance coincides with isoleucine.

The unknown product is stable under normal hydrolysis conditions used for the cleavage of peptide bands (6 *N* HCl; 24 h; 105 °C). Therefore, it is not a peptide. From the isolation procedure it follows that, in all probability, we have to deal with a neutral amino acid or amino acid derivative.

By spot tests, it was established that the compound does not contain a free phenolic or unsubstituted imidazolic group (diazotized sulphanilic acid), an indole group (*p*-(dimethyl)aminobenzaldehyde/HCl) or SCH_3, SH and CSC groups (H_2O_2 oxidation).

After isolation on a preparative scale, the substance was investigated by low- and high resolution mass spectrometry. For this purpose the compound was ethoxycarbonylated to protect primary and/or secondary amine groups. Subsequently, one part of the ethoxycarbonylated material was treated with diazomethane for the esterification of carboxyl groups (Ec-Me derivative) and the other part was trideuteriomethylated with CD_3J and methylsulphinyl carbanion resulting in the esterification of carboxyl groups and the methylation of amide groups (Ec-PdMe derivative). Figs 2 and 3 show the low resolution mass spectra of the Ec-Me and the Ec-PdMe derivative of the unknown product, respectively. The mass spectrum of the Ec-Me derivative gives rise to a parent peak at *m/e* 376 ($C_{16}H_{28}O_8N_2$). For the Ec-PdMe derivative a parent peak at *m/e* 399 ($C_{17}H_{21}D_9O_8N_2$) was found. Based on the difference in mass be-

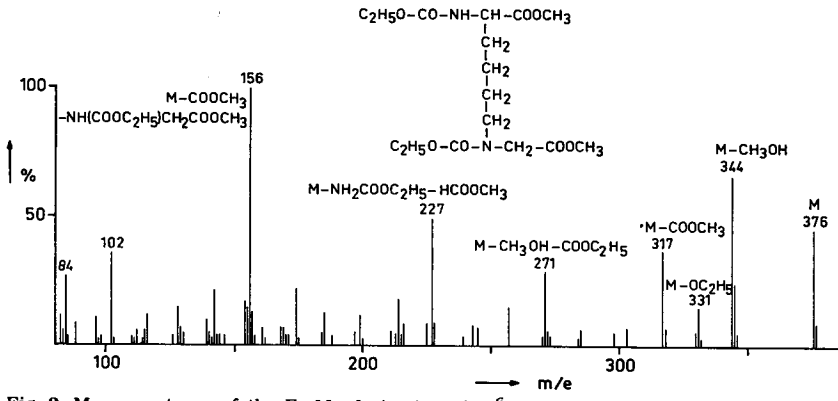

Fig. 2. Mass spectrum of the Ec-Me derivative of N^{ϵ}-(carboxymethyl)lysine. Only mass values > *m/e* 80 ıd intensities ⩾3% are given.

tween both parent peaks and their total formulae the unknown amino acid contains two carboxyl groups, one primary amine group and one secondary amine group. Furthermore, the low- and high resolution spectra suggest the presence of several methylene groups. Supposing that the unknown amino acid has one of the normally occurring amino acids as basic structure, the following possibilities were taken into account: N^{α}-(carboxymethyl)lysine, N^{ϵ}-(carboxymethyl)lysine, N^{α}-(1-carboxyethyl)ornithine, N^{α}-(2-carboxyethyl)ornithine, N^{δ}-(1-carboxyethyl)ornithine and N^{δ}-(2-carboxyethyl)ornithine. N^{α},N^{δ}-Bis-ethoxycarbonylornithine methyl ester and N^{α},N^{ϵ}-bisethoxycarbonyllysine methyl ester can be discriminated between, because the mass spectrum of the first derivative shows as base peak *m/e* 142 ($C_7H_{12}O_2N$) and the latter derivative *m/e* 156 ($C_8H_{14}O_2N$). In the mass spectrum of the Ec-Me derivative of synthetic N^{α}-(1-carboxyethyl)ornithine (N^{α}-octopinic acid) *m/e* 142 was found as the base peak. It was concluded that the presence of *m/e* 142 as base peak is characteristic of compounds with ornithine as basic structure. The base peak in the spectrum of the Ec-Me derivative of the unknown amino acid (Fig. 2) was detected 14 m.u. higher at *m/e* 156, pointing to a compound with lysine as asic structure.

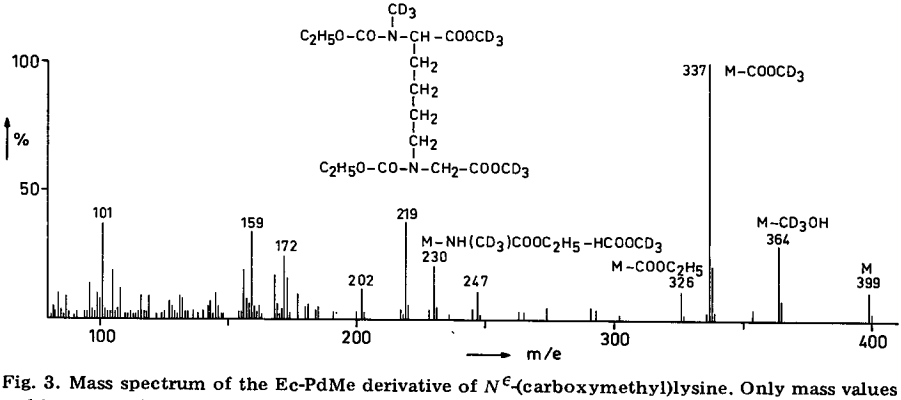

Fig. 3. Mass spectrum of the Ec-PdMe derivative of N^{ϵ}-(carboxymethyl)lysine. Only mass values > *m/e* 80 and intensities ⩾2% are given.

Comparison of the data of synthetic N^{α}- and N^{ϵ}(carboxymethyl)lysine with those of the unknown amino acid demonstrates that the amino acid is identical to N^{ϵ}-(carboxymethyl)lysine. Thin-layer chromatography of both synthetic compounds gave rise to spots at the same position as the unknown amino acid. By column chromatography it was found that only N^{ϵ}-(carboxymethyl)-lysine coincides with the unknown substance. The mass spectra of the Ec-Me and Ec-PdMe derivatives of N^{ϵ}-(carboxymethyl)lysine were identical to those of the amino acid, whereas the spectra of the N^{α}-(carboxymethyl)lysine derivatives showed a number of significant deviations.

Colorimetric determinations

The colour yield of N^{ϵ}-(carboxymethyl)lysine in the column chromatographic NC 1 procedure was 86% of that of norleucine on a basis of equimolar concentrations. The absorbances were measured at 570 nm. The ninhydrin/hydrazine sulphate reagent was used. The ratios $A_{570\,nm}/A_{440\,nm}$ were 3.08 for N^{ϵ}-(carboxymethyl)lysine, 3.17 for lysine and 7.01 for norleucine.

Occurrence of N^{ϵ}-(carboxymethyl)lysine and its excretory values

Of the occurrence of N^{ϵ}-CM-Lys in plasma very little can be said. We never observed this compound among the serum amino acids on two-dimensional chromatograms, which probably means that the concentration is below the limit of detectability. The substance may be readily cleared from the blood by the kidneys, like saccharopine in patients with saccharopinuria [12,13].

The occurrence of N^{ϵ}-(carboxymethyl)lysine seems to be not associated with disease per se. Traces were also found in the urine of normal adults. The compound is already excreted at a young age. It was found to be present in the urine of 26 out of 55 'healthy' prematures. In 9 of the excretors the concentration was evaluated by two-dimensional chromatography as 'positive' and in 17 as 'on a trace level'.

Out of 105 patients of ages varying from 4 months to 16 years, whose urines were presented to our laboratory for screening for amino acid abnormalities, 12 excreted at a trace level, 38 were positive, and out of the latter 4 had strikingly high concentrations. On the whole in some 50% of all the children the compound occurred at some level.

The fact that in some patients high excretory levels occur may indicate a relationship with disease. In Table I excretory levels are given, which were strikingly high at first inspection of the two-dimensional chromatograms. From the list of clinical main characteristics we cannot conclude that a high excretion of N^{ϵ}-CM-Lys correlates with a pronounced clinical condition. The compound attracted our attention when investigating patient H.D. (Table I), who was mentally retarded. However, a clear correlation with a deficient mental development could not be established. Neither did an obvious relationship with liver-, renal- or bone disease appear until now.

It seems that we are not dealing with an inborn error of metabolism. The natural distribution in a variety of patients and in normals does not point in that direction. Also high excretory levels appeared to be a transient phenomenon. In patient H.D. the excretory level was much lower five years after the first analysis. F.K. (from whose urine the compound was isolated for identifica-

TABLE I

N^{ϵ}-(CARBOXYMETHYL)-LYSINE IN HIGH EXCRETORS

	Patients	Gene	Age (years, months)	N^{ϵ}-(CM)-Lys (mg/g creatinine)	Creatinine (mg/l)
1	H.D.	m	1,6	67	916
2	F.K.	m	1,7	25	700
3	E.v.E.	m	3,10	22	560
4	S.S.	m	2,5	13	1000
			2,9	12	640
5	W.B.	f	3,3	15	1100
6	A.W.	f	0,7	26	265
7	D.K.	m	0,2	62	187
8	Je.B.	m	0,4	33	145
9	J.V.	m	2,2	24	380
10	Jo.B.	m	0,8	15.9	270
11	D.R.	m	0,7	24.5	200
12	O.D.	f	2,3	15.5	750

(1) Pancreas annulare. Dystrophy. Mental retardation. EEG: focal epileptical fits.
(2) Multiple epiphysical dysplasia. Psychomotoric retardation.
(3) Syndrome of Prader-Willi.
(4) Mental retardation, e.c.i.
(5) Severe mental retardation. Complicated congenital heart disease.
(6) Glycogenosis due to phosphorylase deficiency.
(7) Premature with transient liver disease.
(8) Chondrodystrophia calcificans congenita.
(9) Progressive muscular dystrophy.
(10) Scoliosis of the thoracal spine.
(11) Hypotonia gravis, e.c.i.
(12) Rickets due to vitamin-D deficiency.

tion) excreted much less one year after the first analysis. Probably, we are dealing with a transient metabolic event, which may be a developmental stage or may be connected with temporal disease. A dietary origin of N^{ϵ}-CM-Lys seems unlikely as many but not all premature infants and young children on a milk formula appeared to excrete this compound. On the other hand, a theoretical possibility may be that the product originates from the breakdown of dietary Maillard compounds.

We questioned whether N^{ϵ}-CM-Lys is a direct metabolite of lysine. Theoretically this compound may be formed from saccharopine by β-oxidation and decarboxylation. Whether patients with saccharopinuria are N^{ϵ}-CM-Lys excretors remains to be investigated more closely. Saccharopine and N^{ϵ}-CM-Lys travel almost in the same position in the chromatographic systems used, so the presence of N^{ϵ}-CM-Lys in patients with saccharopinuria may be overlooked.

In Table II a distinct, but not exaggerated excretion of N^{ϵ}-CM-Lys is shown in a patient, B.H., with hereditary hyperlysinemia probably due to a lysine-α-ketoglutarate reductase deficiency (still to be proven) and in a patient with a hereditary hyperlysinuria (who excreted small amounts of α-amino, α-keto-, and α-hydroxyadipic acids). A patient, S.G., who had a defect of lysine breakdown at the level of α-keto-adipic acid decarboxylase resulting in an overflow of α-amino-, α-keto-, and α-hydroxyadipic acids [14] did not excrete N^{ϵ}-CM-Lys at a detectable level.

TABLE II

N^{ϵ}-(CARBOXYMETHYL)-LYSINE IN A PATIENT WITH HYPERLYSINEMIA AND IN A PATIENT WITH HYPERLYSINURIA

Patient B.H.: psychomotoric retardation, epilepsia and abnormal behaviour; hereditary hyperlysinemia.
Patient M.K.: hepatomegaly, growth retardation; hereditary hyperlysinuria.

	Serum lysine (mg/100 ml)	Urinary lysine (mg/g creatinine)	Urinary N^{ϵ}-(CM)-Lys. (mg/g creatinine)	Urinary creatinine (mg/l)
B.H. m, 7 years 5 months				
Samples 30-4-'73	21.0	679	7.2	960
Samples 4-5-'73	21.8	351	6.3	1330
M.K. f, 3 years 9 months				
Sample 28-7-'71	decreased or normal	499	5.0	865

Another possibility is that N^{ϵ}-CM-Lys originates from the breakdown of collagen or elastin cross-link fragments, such as dehydrohydroxylysinonorleucin [15,16] and related compounds [17]. Overexcretion may then reflect excessive breakdown of connective tissue protein rich in such cross-links or a diminished catabolism of the cross-link residues or both.

Whatever the origin of N^{ϵ}-CM-Lys may be and whatever a high urinary excretion of this compound may mean, we hope that other investigators will pay attention to this urinary constituent. Possibly its clinical significance may then be elucidated.

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References

1 S.K. Wadman, H. Fabery de Jonge and P.K. de Bree, Clin. Chim. Acta, 25 (1969) 87
2 Ch.R. Scriver, E. Davies and A.M. Cullen, Lancet, ii (1964) 230
3 Y. Kakimoto and S. Akazawa, J. Biol. Chem., 245 (1970) 5751
4 H.G. Gundlach, W.H. Stein and S. Moore, J. Biol. Chem., 234 (1959) 1754
5 K. Biemann, C. Lioret, J. Asselineau, E. Lederer and J. Polonsky, Bull. Soc. Chim. Biol., 42 (1960) 979
6 E.W. Seitz and R.M. Hochster, Can. J. Bot., 42 (1964) 999
7 A. Ménagé and G. Morel, C.R. Acad. Sci. Paris, 261 (1965) 2001
8 J.P. Kamerling, W. Heerma and J.F.G. Vliegenthart, Org. Mass Spectrom., 1 (1968) 351
9 J.F.G. Vliegenthart and L. Dorland, Biochem. J., 117 (1970) 31 P
10 E. Vilkas and E. Lederer, Tetrahedron Lett., (1968) 3089
11 H.A. van 't Klooster, J.S. Vaarkamp-Lijnse and G. Dijkstra, Org. Mass Spectrom., 8 (1974) 303
12 N.A.J. Carson, B.G. Scally, D.W. Neill and I.J. Carre, Nature, 218 (1968) 679
13 O. Simell, J.J. Visakorpi and M. Donner, Arch. Disease Child., 47 (1972) 52
14 H.J. Bremer, S.K. Wadman, U. Wendel, H. Przyrembel, I. Lombeck, 12th Ann. Meeting of the S.S.I.E.M., Heidelberg, 11—14 September, 1974, to be published
15 A.J. Bailey, C.M. Peach and L.J. Fowler, Biochem, J., 117 (1970) 819
16 A.J. Bailey and C.M. Peach, Biochem. J., 121 (1971) 257
17 P.M. Gallop, O.O. Blumenfeld and S. Seifter, Ann. Rev. Biochem., 41 (1972) 617