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# ALPHA-KETOADIPIC ACIDURIA, A NEW INBORN ERROR OF LYSINE METABOLISM; BIOCHEMICAL STUDIES*

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## Summary

Investigation of a psychomotorically retarded girl showed excretion of abnormal amounts of alpha-ketoadipic acid, alpha-hydroxyadipic acid, alpha-aminoadipic acid, 1,2-butenedicarboxylic acid and elevation of plasma alpha-aminoadipic acid levels. The identity of these metabolites was established by various methods. The excretion of alpha-aminoadipic acid correlated to the lysine intake. Degradation studies with cultured fibroblasts indicate a defect in the oxidative decarboxylation of alpha-ketoadipic acid (see Clin. Chim. Acta, 58 (1975) 271).

## Introduction

Alpha-aminoadipic acid as an intermediate in mammalian lysine degradation was first recognised by investigating guinea-pig liver [1]. It is synthesised mainly via saccharopine and delta-aminoadipic semialdehyde [2—6]. The pipecolic pathway of lysine degradation to alpha-aminoadipic acid [7] is of secondary importance in humans but may be enhanced by substrate accumulation in lysine-ketoglutarate-reductase deficiency and in premature infants [8—10]. Hyperlysinaemia caused by a defective lysine ketoglutarate reductase [11—13] and saccharopinuria [14,15] have been described as disturbances of the main lysine pathway. Two defects of the pipecolic acid pathway are known. They are the hyperlysinaemia with periodic hyperammonaemia probably caused by a defect of the lysine oxidoreductase [16,17] and a hyperpipecolataemia in which the metabolic defect is unknown [18,19].

A slightly simplified outline of lysine metabolism is given in Fig. 1.

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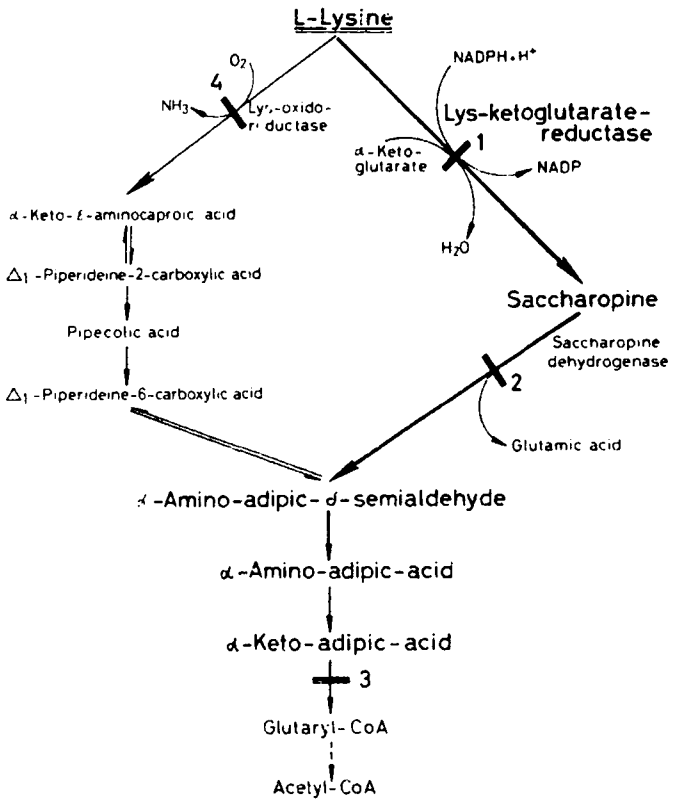


Fig. 1. Pathways of lysine degradation. 1, enzyme defect in hyperlysinaemia; 2, enzyme defect in saccharopinuria; 3, enzyme defect in the patient reported in this paper; 4, enzyme defect in lysine intolerance with periodic ammonia intoxication.

## Patients and Methods

### *I. Case report*

The patient, now 14 months old, is the first child of a debile 20-year-old mother and an unknown father. The mother has a debile brother and a normal sister. Pregnancy and birth were uneventful. The infant was a "collodium baby", later developing ichthyosiform erythrodermia. Birth weight 2950 g, length 50 cm.

A generalised convulsion was observed on the first day of life and was the reason for admittance to our hospital. No further convulsion occurred. The skin lesions responded well to symptomatic treatment and weight gain was satisfactory.

Routine laboratory investigations, including ammonia in blood, were normal, except for inconsistent metabolic acidosis with base excess values up to —9 mequiv./l, and variable disturbances of blood coagulation.

Electroencephalography, nerve conduction velocity, and ophthalmological examination were normal. Echoencephalography was normal in the first half-year of life, but now shows dilatation of the ventricular system. Bone age is slightly retarded, other roentgenological examinations were normal.

Tendon reflexes are normal, there are no pathological reflexes. Motor development is very much retarded, mental development seems to be subnormal. Short fat limbs with oedema of the dorsum of the hands and feet and definite muscular hypotonia are most striking in the appearance of the child.

Special biochemical investigations on this patient were initiated by a positive dinitrophenylhydrazine test in the urine and the appearance of an abnormal spot in TLC screening for urinary amino acids in the position of alpha-aminoadipic acid.

## *II. Methods*

Blood samples were taken, usually after an overnight fast, in heparinised syringes from a scalp or jugular vein. Plasma was deproteinised within 15 minutes after venipuncture by adding 4 volumes of 3% sulfosalicylic acid. The supernatant was kept frozen at −20° C until analysis. 24 h urine collections were done on ice and the urine kept frozen at −20° C.

### *A. Amino acids*

Two-dimensional TLC of urinary amino acids was done according to Kraffczyk et al. [20].

Quantitation of amino acids was performed on an automatic analyser (Multichrom - Beckman Instruments, Munich) using the two-column method according to Spackman et al. [21]. Separation of alpha-aminoadipic acid from alpha-amino-*n*-butyric acid was achieved by changing the initial temperature from 30 to 34° C.

For preparative purposes the separation of acidic and neutral amino acids from the basic acids was done according to the method of Kakimoto and Akazawa [22]. The pyridine eluate, containing acidic and neutral amino acids, was taken to dryness on a rotary evaporator, dissolved in a small volume of pH 2.2 sodium citrate buffer, and separated on the preparative amino acid analyser system BC 300 (LKB, Munich), using 0.2 *N* sodium acetate buffer, pH 3.25. Fractions of 18 ml each were collected and tested separately for ninhydrin-positive compounds. The maximum absorption for the unknown compound was found at 1850 ml. Appropriate fractions were combined and desalted on a Dowex 50 X8 column by the method of Carsten [23]. The isolated fraction contained small amounts of glutamic acid, glycine and alanine besides the large amounts of the unknown urinary amino acid.

For further purification preparative TLC was performed on pre-coated silica gel plates (Merck, Darmstadt) using ethanol/water (83 : 17, v/v) as the solvent. Amino acids were located by staining the outer parts of the plates with 0.2% ninhydrin in acetone/glacial acetic acid (80 : 20, v/v). The silica gel layer was scraped off at the appropriate height and taken up in water. After centrifugation the supernatant was evaporated to dryness and the residue redissolved in a small volume of water.

The purity of the isolated compound was tested by one- and two-dimensional TLC against reference substances and by rechromatography on the amino acid autoanalyser, noting the retention times at the column temperatures 30, 34, and 38° C in comparison to those of authentic test substances.

For direct inlet mass spectrometry a further purification was performed by filtering 1 ml of the solution of the amino acid obtained from the silica gel plates through a column of 2 ml of Dowex 50 W X8 (50—100 mesh). After washing the column twice with 20 ml of water the unknown amino acid was eluted by 20 ml of 2 *N* ammonia. The solution was evaporated to dryness in vacuum at 50° C. The residue was transferred to a small tube. The dry substance was subjected to direct-inlet mass spectrometry (Jeol D-100 apparatus, 75 eV, ion-chamber temperature 140—150° C).

*B. Urinary keto- and other acids:*

*Thin-layer chromatography.* Dinitrophenylhydrazones of urinary keto acids were prepared according to Cotte et al. [24] and separated by one-dimensional TLC on precoated cellulose plates (Merck) with *n*-butanol/ethanol/1 *N* $NH_4OH$ (130 : 20 : 50, v/v) as solvent and on precoated silica gel plates with benzene/methanol/glacial acetic acid (45 : 5 : 8, v/v) as solvent, together with dinitrophenylhydrazine derivates of authentic keto-acids.

*Gas chromatography.* Only trimethylsilylated compounds were studied. Extraction and trimethylsilylation were performed according to Wadman et al. [25]. Gas chromatography was slightly modified.

Apparatus: F and M 810, dual column, double FID, with Hewlett-Packard integrator 3370 A.

Columns: Stainless steel, 8 ft × 1/8 inch, 5% GESE-52 on Chromosorb W AW DMCS, 100—120 mesh.

Temperatures: Oven: 75° C isothermal for 10 minutes, then 75—220° C (2° C/min), finally 220° C isothermal for 10 minutes. Injection port: 190° C. Detector: 220° C.

Gasflow: $N_2$ : 27 ml/min; $H_2$ : 28 ml/min; air: 450 ml/min.

Range: $10^2$ ; attenuation: 8; chartspeed: 1/2 inch/min.

Sample size: 1 µl of final solution. Internal standard: phenylbutyric acid.

*Gas chromatography-mass spectrometry.* For this technique the Jeol D-100 GC-MS combination was used, equipped with the same column and temperature programming as described above. Helium was used as a carrier gas instead of $N_2$ .

*C. Dietary studies*

To establish the influences of lysine intake on the presumed metabolic defect the following dietary regimes were used:

1. Normal diet with a protein intake of 2.5—4.2 g/kg body wt/day, corresponding to 210—360 mg L-lysine/kg body wt/day.

2. Synthetic diet on the basis of a lysine-free amino acid mixture (Maizena, Hamburg), equivalent to 2.3 g protein/kg body wt/day, with 170 mg L-lysine/kg body wt/day added.

3. The same synthetic diet without additional L-lysine.

An oral lysine load test was done in the patient and a normal child of the same age, with 100 mg L-lysine/kg body wt. Urine was collected in 6-hour portions, starting 6 hours before the load and continuing for 48 hours in both children. Blood samples were taken at 0, 30, 60, 120, 180, 240, 720, and 1680 minutes after the load in the patient.

Fig. 2. Two dimensional urinary amino acid screening on cellulose plates. Solvents: I, pyridine/dioxane/ammonia/water (35 : 35 : 15 : 15, v/v); II, *n*-butanol/acetone/glacial acetic acid/water (35 : 35 : 10 : 20, v/v). The alpha-aminoadipic acid spot indicated by the arrows.

## Results

### *A. Amino acids*

Two-dimensional TLC of urine revealed a large spot in the position of alpha-aminoadipic acid (Fig. 2). Column chromatography of plasma and urine showed a peak between alanine and alpha-amino-*n*-butyric acid (Fig. 3). TLC of the alpha-aminoadipic acid containing fractions after isolation with preparative column chromatography showed identical Rf values with test substances (Table I).

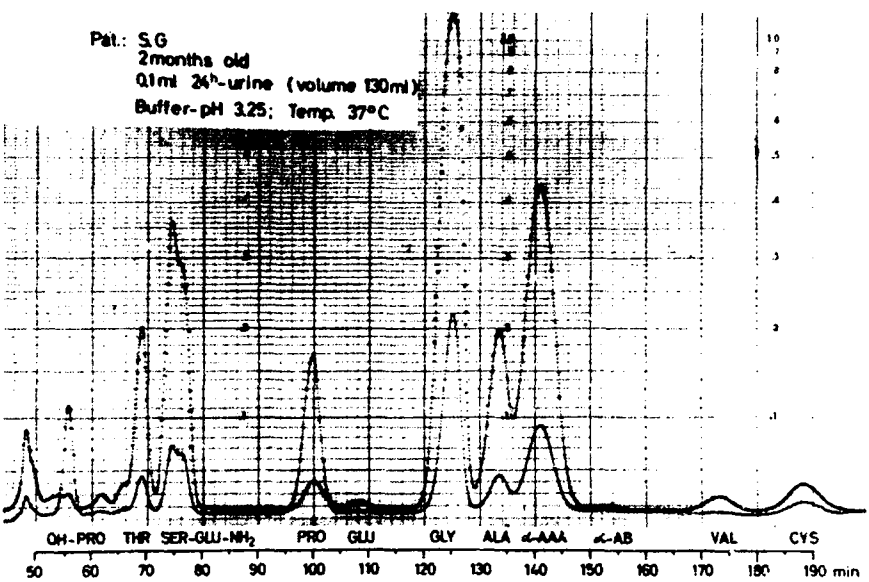


Fig. 3. Column chromatographic separation of the urinary amino-acids of the patient. Gly, glycine; Ala, alanine; α-AAA, alpha-aminoadipic acid; α-AB, alpha-amino-*n*-butyric acid.

TABLE I

$R_f$ VALUES OF THE ISOLATED UNKNOWN AMINO ACID AND TEST SUBSTANCES

| | Cellulose plates | |
|---|---|---|
| | Pyridine/dioxane/ammonia/water (35 : 35 : 15 : 15, v/v) | *n*-Butanol/acetone/glacial acetic acid/water (35 : 35 : 10 : 20, v/v) |
| Glycine | 0.43 | 0.30 |
| Alanine | 0.59 | 0.44 |
| Alpha-aminoadipic acid | 0.19 | 0.44 |
| Isolated compound | 0.19 | 0.44 |

Rechromatography of the isolated compound on the amino acid analyser gave retention times of 178, 161, and 150 min at 30, 34, and 38°C, respectively, compared to 178, 161, and 150 min for authentic alpha-aminoadipic acid and 179, 170, and 167 min for alpha-amino-*n*-butyric acid.

The mass spectrum of the isolated product was identical with that of synthetic alpha-aminoadipic acid. It is shown in Fig. 4.

Parent peak $M$ = 161 not visible; $M-H_2O$ = 143; $M-COOH$ = 116, $M-H_2O-COOH$ = 98; $[CH_2\text{-}CH=C=O]^+$ = 55.

*B. Organic acids*

TLC of urinary dinitrophenylhydrazones showed excretion of abnormal amounts of alpha-ketoadipic acid compared to a normal child (Fig. 5). $R_f$ values of urinary keto acids and of the compound isolated by preparative TLC were identical to those of test substances (Table II).

In Fig. 6 a gas chromatogram of TMS derivatives of urinary acids is shown. GC-MS proved the presence of alpha-ketoadipic acid ($RR_t$ 1.244, peak area 39.369 IU/0.01 ml urine). The concentration is approximately 232 mg/l or 214 mg/g of creatinine. The mass spectrum, identical with that of the tri-TMS derivative of the synthetic product, is given in Fig. 7.

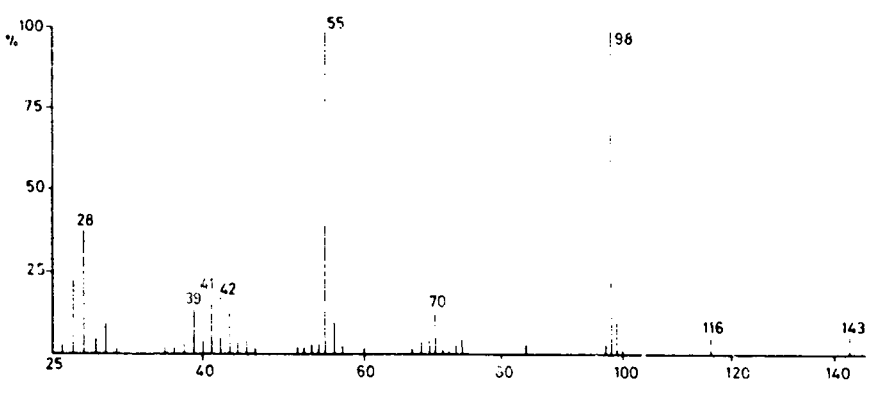


Fig. 4. Mass spectrum of alpha-aminoadipic acid, isolated from the urine of patient S.G.

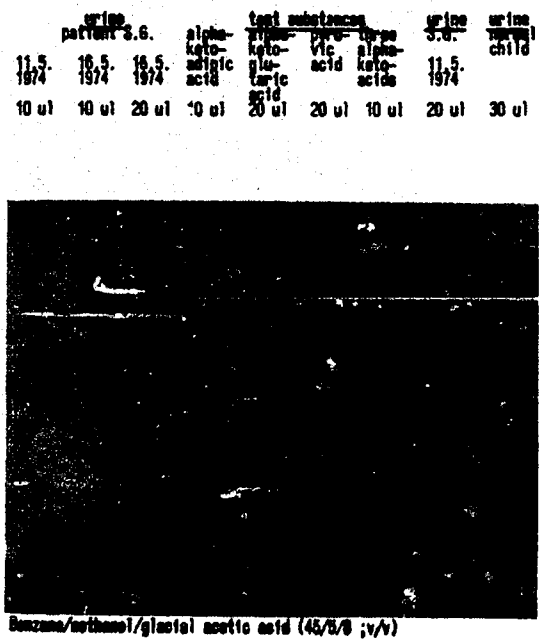


Fig. 5. TLC of the urinary keto-acids of the patient as dinitrophenylhydrazones on silica gel plates.

TABLE II

$R_f$ VALUES OF THE ISOLATED URINARY KETO-ACID, TEST SUBSTANCES, AND TOTAL URINARY KETO-ACIDS AS DINITROPHENYLHYDRAZONES

| | Silicia gel plates Benzene/methanol/ glacial acetic acid (45 : 5 : 8, v/v) | Cellulose plates *n*-Butanol/ethanol/ ln $NH_4OH$ (130 : 20 : 50, v/v) |
|---|---|---|
| Alpha-ketoadipic acid | 0.47 | 0.19 |
| | | 0.27 |
| Alpha-ketoglutaric acid | 0.40 | 0.21 |
| Total urinary keto-acids | 0.40 | 0.21 |
| | 0.47 | 0.27 |
| Isolated keto-acid | 0.47 | 0.19 |
| | | 0.27 |

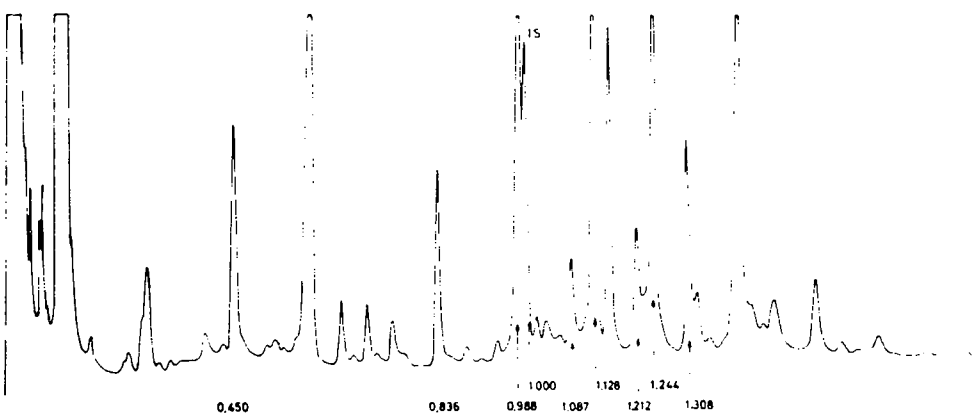


Fig. 6. Gas chromatogram of TMS-derivatives of urinary acids in patient S.G. with alpha-keto adipic aciduria.

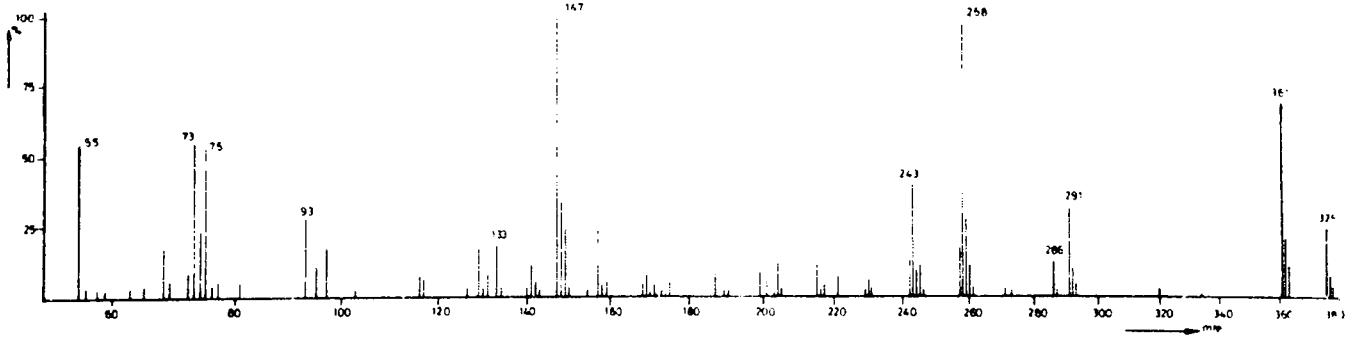


Fig. 7. Mass spectrum of the TMS-derivative of urinary alpha-keto adipic acid in patient S.G.

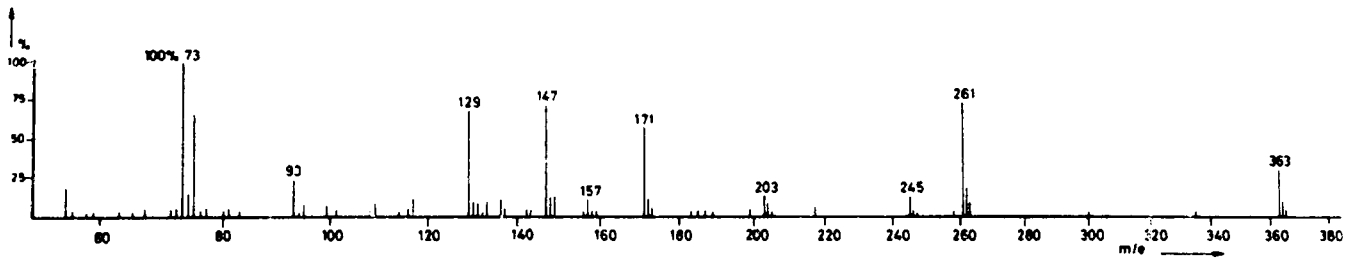


Fig. 8. Mass spectrum of the TMS-derivative of urinary alpha-hydroxy adipic acid in patient S.G.

Parent peak $M$ = 376; $M$—$CH_3$ = 361; 291 unexplained; $M$—TMSOH = 286; $M$—HCOOTMS = 258; 258—$CH_3$ = 243.

Another unknown compound ($RR_t$ 1.212; peak area 11415 IU/0.01 ml urine) occurred, its concentration being nearly one third of that of the alpha-ketoadipic acid. Its mass spectrum is identical with the tri-TMS derivative of alpha-hydroxyadipic acid. The latter compound was synthesized from alpha-aminoadipic acid by treatement with nitrous acid. The mass spectrum is given in Fig. 2.

Parent compound $M$ = 378 (just visible but not drawn); $M$—$CH_3$ = 363; 335 unexplained; $M$—COOTMS = 261; 335—TMSOH = 245; 261—TMSOH = 171.

A third unknown peak ($RR_t$ 0.988, peak area 39 069 IU/0.01 ml urine) was present. In the gas chromatogram this is the position of the TMS-derivative of adipic acid; but the mass spectrum pointed to the di-TMS derivative of 1,2-butene dicarboxylic acid $M$ = 288. In this peak a second compound is probably present the structure of which has still to be proved.

Other acids which were increased in this sample are listed below; their identity was also established by mass spectrometry:

Beta-hydroxybutyric acid ($RR_t$ ± 0.450, peak area 16 860 IU/0.01 ml urine, corresponding to 106 mg/l or 97.7 mg/g of creatinine).

Glutaric acid ($RR_t$ 0.836, peak area 15116 IU/0.01 ml urine, corresponding to 65 mg/l or 59.9 mg/g of creatinine.

Alpha-hydroxyglutaric acid ($RR_t$ 1.087, peak area 7971 IU/ml urine).

Alpha-ketoglutaric acid ($RR_t$ 1.128, peak area 39112 IU/0.01 ml urine, corresponding to 345 mg/l or 318.0 mg/g of creatinine).

Aconitic acid ($RR_t$ 1.308, peak area 19 727 IU/0.01 ml urine, corresponding to 240 mg/l or 221 mg/g of creatinine).

*C. Dietary studies*

Table III shows the alpha-aminoadipic acid and lysine levels in plasma in

TABLE III

ALPHA-AMINOADIPIC ACID AND LYSINE PLASMA LEVELS IN RELATION TO PROTEIN INTAKE

| | Alpha-amino-adipic acid (μmol/ml plasma) | Lysine (μmol/ml plasma) |
|---|---|---|
| Patient S.G. | | |
| Age 1.5 months | | |
| protein intake 4.1 g/kg body wt/day | | |
| lysine intake 390 mg/kg body wt/day | 0.063 | 0.177 |
| Age 10 months | | |
| protein intake 1.9 g/kg body wt/day | | |
| lysine intake ca. 140 mg/kg body wt/day | 0.079 | 0.194 |
| Age 13 months | | |
| protein intake 1.3 g/kg body wt/day | | |
| lysine intake ca. 80 mg/kg body wt/day | 0.050 | 0.062 |
| Normal children* | | |
| (age 4—30 months old) | — | 0.227 ± 0.091 |

* Ghadimi and Pecora, 1964.

TABLE IV

URINARY ALPHA-AMINOADIPIC ACID AND LYSINE EXCRETION IN RELATION TO LYSINE INTAKE WITH THE FOOD

| | Alpha-aminoadipic acid | | Lysine | |
|---|---|---|---|---|
| | μmol/min/ 1.73 $m^2$ | μmol/24 h | μmol/min/ 1.73 $m^2$ | μmol/24 h |
| Patient S.G. | | | | |
| Age 1.5 months | | | | |
| protein 4.5 g/kg body wt | | | | |
| lysine 390 mg/kg body wt | 3.153 | 519.7 | 0.736 | 121.3 |
| Age 5.5 months | | | | |
| protein 2.3 g/kg body wt | | | | |
| lysine 170 mg/kg body wt | 0.601 | 155.1 | 0.205 | 52.9 |
| Age 6 months | | | | |
| protein 2.3 g/kg body wt | | | | |
| lysine 0 | 0.189 | 48.8 | 0.056 | 14.5 |
| Controls | | | | |
| Male, age 5.5 months | 0.082 | 20.9 | 0.133 | 33.9 |
| Female, age 9 months | 0.088 | 28.5 | | |
| Male, age 16 months | 0.058 | 20.9 | 0.144 | 51.6 |
| Values from the literature | | | | |
| Stein (1953), adults [27] | | 62 | | |
| McMurray (1963), no age given [30] | | 12 | | |
| Freeman (1970), 3—7 months [31] | | trace to 8.1 | | 3 to 146 |
| Scriver (1965), 3—10 years [28] | | | 0.113 | 51.6 |
| Brodehl (1968), 0.5—4 months [29] | | | 0.171 | |
| Brodehl (1968), 2—13 years [29] | | | 0.151 | |

the patient in relation to lysine administration with the food. There are no reference values of measurable amounts of alpha-aminoadipic acid in plasma in the literature. In the plasma of four healthy adults and of four healthy infants we could not detect alpha-aminoadipic acid using an amino acid analyser and the method mentioned before. In the patient, apparently, plasma-aminoadipic acid levels do not correlate with lysine intake as does lysine itself.

Table IV shows the urinary excretion of alpha-aminoadipic acid and lysine in relation to lysine intake, compared to three healthy children (5, 16 and 9 months of age) on a normal diet, and also to values taken from the literature [26—31]. Dependency of alpha-aminoadipic acid excretion on lysine content of the food is obvious in the patient. There was no alpha-aminoadipic acid demonstrable by column chromatography in the cerebrospinal fluid of the patient.

The oral lysine load in the patient resulted in acute diarrhoea not influencing the clinical status of the child, in very high and prolonged lysine increases in plasma and urine, in a marked elevation of alpha-aminoadipic acid in plasma and urine (Figs 9 and 10) and a compensated metabolic acidosis for more than 24 hours. The same load in the normal control produced a very small rise in urinary lysine and alpha-aminoadipic acid excretion.

The mother of the patient excretes 77 μmoles/24 hours of alpha-aminoadipic acid.

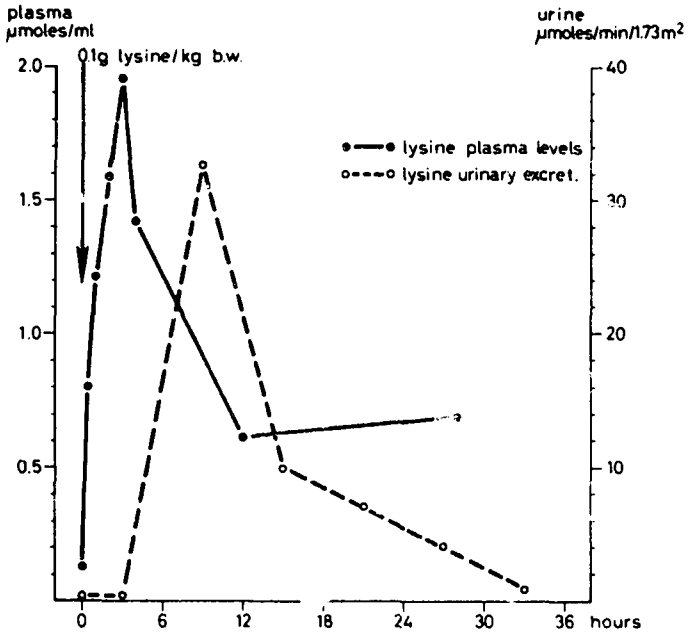


**Fig. 9.** Results of oral lysine load: lysine concentrations in plasma and lysine urinary excretion. The urinary lysine excretion of the control child was too low for the scale used in this figure.

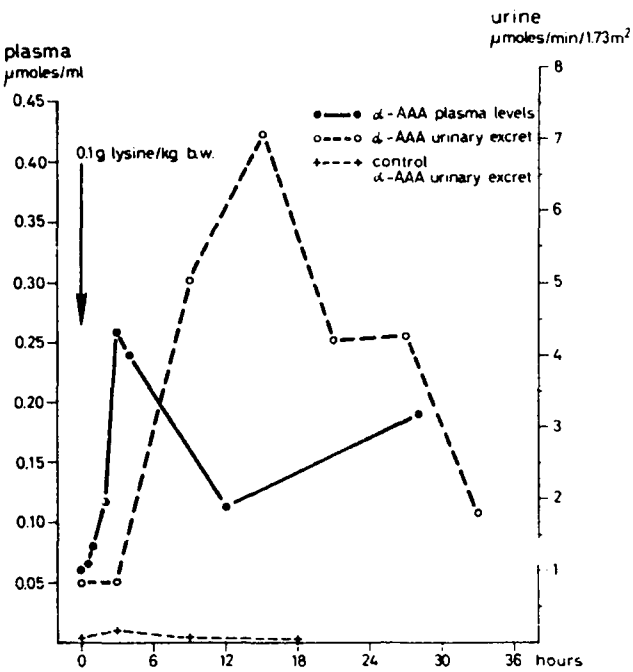


**Fig. 10.** Results of oral lysine load: alpha-aminoadipic acid in plasma and urine of the patient and in the urine of a normal control.

## Discussion

On the data presented in this paper and the degradation studies in fibroblasts, which are published separately [34], we believe this child to suffer from a hitherto unknown genetic defect of lysine degradation. This defect leads to a markedly increased urinary excretion of alpha-aminoadipic acid, alpha-ketoadipic acid, alpha-hydroxyadipic acid and (quantitatively less) 1,2-butenedicarboxylic acid, and to elevated alpha-aminoadipic acid concentrations in plasma. We could not find elevated concentrations of pipecolic acid, homocitrulline, alpha-*N*-acetyl-lysine or epsilon-*N*-acetyl-lysine in plasma or urine, neither of saccharopine.

Small amounts of alpha-aminoadipic acid regularly occur in human urine [27,32]. The normal excretion is, however, much lower than that found in this child. Alpha-ketoadipic acid has not previously been reported as a normal urinary constituent. In the sample analysed here its concentration (320 mg/l) was of the same order of magnitude as that of the alpha-aminoadipic acid (380 mg/l). Having demonstrated the presence of the keto acid, the alpha-hydroxyadipic acid could also be expected to occur. Its concentration appeared to be lower. The 1,2-butene-dicarboxylic acid may result from oxidative de-amination of alpha-aminoadipic acid or from alpha-hydroxyadipic acid by loss of one molecule of water. The increase of glutaric acid, alpha-ketoglutaric acid, alpha-hydroxyglutaric acid and aconitic acid is less easy to explain; they are probably secondary phenomena.

Compared to a healthy child the urinary excretion of alpha-aminoadipic acid increased markedly after an oral load of L-lysine. There was also a much higher increase and a slower elimination of the lysine in plasma after the oral load. After eliminating lysine from the diet the urinary excretion of alpha-aminoadipic acid dropped to near normal values. As has to be expected, these results demonstrate a direct relation of urinary alpha-aminoadipic excretion to the lysine intake.

The patient exhibits, with definitely retarded motor development, marked muscular hypotonia and a moderate metabolic acidosis. The mental development seems to be less impaired. At the age of 14 months the child shows some ventricular dilatation, which was not present shortly after birth. However, the observation of a single patient with a specific metabolic disorder does not provide definite proof for a relationship between metabolic abnormality and clinical symptoms.

The mother of the child is mentally retarded and shows a definite urinary excretion of alpha-aminoadipic acid. Because the father of the patient is with great probability a near relative it remains open whether the mother is also homozygous with respect to this metabolic defect. A further metabolic investigation of the mother has been refused by the family.

## Acknowledgement

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