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Excretion of Pipecolic Acid by Infants and by Patients with Hyperlysinemia

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Extract

Pipecolic acid has been found in urine from normal infants and from children with hyperlysinemia. The reaction of pipecolic acid with ninhydrin reagent, using separation procedures in automated ion-exchange systems for amino acid analysis, produced a color constant of low value (1.21 compared with 27.6 for leucine) that could be overlooked in routine analyses of physiological fluids. Pipecolic acid was present in the urine from: four premature infants (6–23 days old) 1.2–8.1 μg/mg creatinine; two of four term infants (3–5 days old) 1.3 and 2.1 μg/mg creatinine; and one of four infants (4–11 months old) 2.1 μg/mg. No pipecolic acid was found in the urines of four infants 14–30 months old. In patients with hyperlysinemia, the amounts of pipecolic acid excreted in the urine were: 3.1 μg/mg creatinine (4-year-old girl); 5.2 μg/mg creatinine (6-year-old boy); 6.2 μg/mg creatinine (9-year-old girl); and 4.8 μg/mg creatinine (12-year-old girl).

Speculation

The low color yield of pipecolic acid, when separated by standard automated ion-exchange analysis methods, may account for past failures to detect small amounts of this substance in physiological fluids. Amounts of pipecolic acid excreted by young infants and by children with hyperlysinemia indicate that a degradation pathway for lysine, via pipecolic acid to α-aminoadipic acid, is operative in man.

Introduction

Pipecolic acid has been shown to be a product of lysine metabolism in rats [31–33], guinea pigs [5, 20, 34], and turkeys [6, 7]. It has been detected in plants [53] and is derived from lysine in some of them [15, 24]. Certain microorganisms form pipecolic acid from α-aminoadipic acid [3]; others derive it from lysine by pathways similar to those described for mammals [20, 35].

Reports of the presence of pipecolic acid in human urine are conflicting. Numerous studies of excretion of amino acids by infants, children, and adults [2, 4, 8–10, 14, 16, 19, 21–23, 25, 26, 28, 30, 36, 39, 41–45, 47, 48] make no mention of pipecolic acid as a normal urinary constituent, but Jagenburg [18] detected pipecolic acid in urine from infants less than 1 year old; however, he was unable to find it in urine of adults. Pipecolic acid also has been found in the urine of a child with hyperlysinemia [49] and in urine of patients with hyperthyroidism [38]. Ghadimi *et al.* [13], however, attempting to detect pipecolic acid in urine from normal premature infants, infants, and adults, and from patients with hyperlysinemia were unsuccessful, and

ARMSTRONG *et al.* [1] could not detect pipecolic acid in urine from normal infants and children. GATFIELD and associates [12] recently described pipecolic acid in serum, urine, spinal fluid, and tissues of a patient with mental retardation who had no demonstrable impairment of lysine metabolism, and WHITEHEAD [46] reported finding pipecolic acid in the serum of patients with kwashiorkor.

Materials and Methods

Collection and Preparation of Specimens

Twenty-four hour, nonfasting urine specimens were collected from four premature infants, four term infants, four infants less than 1 year old, and four infants over 1 year of age. The babies were fed an evaporated milk formula (20 kcal/oz): older infants were given a general diet. Overnight (12 h) urine specimens were also collected from four children with hyperlysinemia. The children did not eat 4 h before challenge or throughout the 12-h period. Thymol was added to all specimens as a preservative [37], and levels of creatinine in urine were measured promptly. The volume usually taken for analysis by the method of SPACKMAN *et al.* [40] was calculated on the basis of specific gravity (standard volume taken for analysis $= \left(\frac{0.04}{\text{sp gr} - 1.000}\right)$. A portion of urine five times this normal volume was desalted by ion exchange using a Dowex-50 column [37]. The eluate was dried in a flash evaporator, reconstituted to 1 volume, and the pH adjusted to 2.2. This fivefold desalted concentrate was frozen at —20° until analyzed.

Chromatographic Separation of Amino Acids

The automated system (54) of SPACKMAN *et al.* [40] as modified by ZACHARIAS and TALLEY [52] was used for analysis and separation of amino acids. This technique separates pipecolic acid sharply from other naturally occurring substances found in urine. A spherical resin [55] was used that slightly modified elution times for some amino acids. The 150-cm column with temperature maintained at 50° was used for separation. The buffer change (pH 3.25, 0.2 N sodium citrate followed by pH 4.25, 0.2 N sodium citrate) was at 14.75 h. With this system, the elution peak of pipecolic acid at 440 ml lies between valine (410 ml) and methionine (537 ml) and is well separated from them (fig. 1).

The fivefold concentrate of amino acids in the urine specimens was freed of ammonia and applied to the 150-cm by 0.9-cm column. With buffer flow at 30 ml/h, the stream-dividing pump was set to divert 6 ml/h to the spectrophotometer and the remainder of the column eluate was collected in 2-ml fractions.

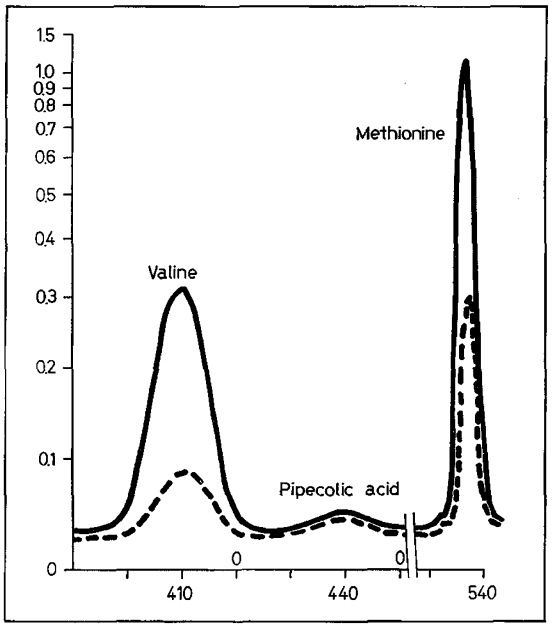

Fig. 1. Absorption curves of 1 μmole of valine, pipecolic acid, and methionine separated by the ZACHARIAS and TALLEY modification of the method of SPACKMAN *et al.* [40]. Upper trace at 570 mμ, lower at 440 mμ.

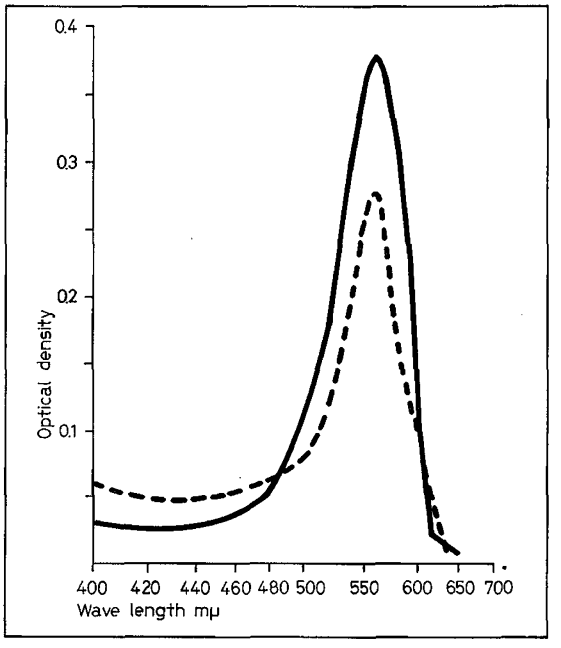

Fig. 2. Absorption spectra, using acid ninhydrin reagent of PIEZ *et al.* [29] of 0.1 μmole of DL-pipecolic acid (solid line) and of pooled and concentrated fractions of peak eluted at 440 ml by the ZACHARIAS and TALLEY method [52] from the urine of a hyperlysinemic child (dashed line). Beckman DU with 1-cm cell.

Detection and Measurement of Pipecolic Acid

Preliminary studies using standard amino acid calibration mixtures suggested reasons why pipecolic acid can easily escape detection when present in small amounts in physiologic fluids. Figure 1 shows the absorption peak produced by 1 μmole of pipecolic acid that was separated from a calibration mixture by the method of ZACHARIAS and TALLEY [52]. The area below this peak reflects the low color constant produced by the reaction of pipecolic acid with the ninhydrin reagent designed for the recorder (1.21 compared with 27.6 for leucine). Because this procedure achieved a poor color yield, pipecolic acid was isolated from the urine and confirmed and quantitated by other means. Accordingly, the fractions of column eluate containing pipecolic acid (430 through 448 ml) were pooled, desalted by ion exchange on Dowex-50, and concentrated by vacuum evaporation. An aliquot of this concentrate was applied to Whatman no. 1 paper and chromatographed with butanol–acetic acid–water (12:3:5) and with phenol–water–ammonia (160:40:1). Standard DL-pipecolic acid [56] was run simultaneously. Material from fractions corresponding to the ion-exchange elution peak at 440 ml formed discrete spots on the chromatograms. These had R_F values, in each solvent, identical with those of known pipecolic acid. Following the reaction with ninhydrin (0.2% in acetone), the spots from the column peak exhibited the red fluorescence under ultraviolet light that is characteristic of pipecolic acid [26]. Comparison of the size and density of the pipecolic acid-ninhydrin spots with those produced by standard pipecolic acid permitted semiquantitative estimates of the amount of pipecolic acid in the urine.

More accurate measurement of pipecolic acid in the fractions could be obtained when the ninhydrin method of PIEZ *et al.* [29] was applied to the concentrate of column eluate. Pipecolic acid absorbs maximally at 565 mμ with this reagent. Using known pipecolic acid mixtures as standards, the acid ninhydrin reaction was used to determine the amount of pipecolic acid in the concentrate of the pooled fractions comprising the 440-ml peak. As with the standards, the pooled fractions from this peak showed maximal absorption with acid ninhydrin at 565 mμ (fig. 2).

Results

Identification

The conclusion that the substance in the peak eluted at 440 ml that reacted with ninhydrin is pipecolic acid appears justified because its behavior is identical with that of known pipecolic acid with regard to: R_F values by paper chromatography, elution behavior on ion-

Table I. Pipecolic acid levels in urine from control subjects

Control subjects	Age	Urine		Paper chromatography		Acid ninhydrin method, μg/mg creatinine
		Sp gr	Creatinine, mg/ml	Pipecolic acid spot[1]	Ultraviolet fluorescence	
Premature	6 days	1.004	0.18	+	+	1.2
Premature	6 days	1.006	0.12	+	+	4.6
Premature	9 days	1.004	0.10	+	+	1.2
Premature	23 days	1.005	0.10	+	+	8.1
Neonate	3 days	1.022	2.18	0	0	0
Neonate	4 days	1.009	0.13	+	+	2.1
Neonate	5 days	1.011	0.79	+	+	1.3
Neonate	5 days	1.004	0.22	0	0	0
Infant	4 months	1.008	0.23	0	0	0
Infant	5 months	1.010	0.28	0	0	0
Infant	8 months	1.009	0.17	+	+	2.1
Infant	11 months	1.027	0.73	0	0	0
Infant	14 months	1.020	0.38	0	0	0
Infant	24 months	1.020	0.54	0	0	0
Infant	24 months	1.013	0.53	0	0	0
Infant	30 months	1.012	0.32	0	0	0

[1] Ninhydrin reaction and R_F value identical with those of known pipecolic acid.

Table II. Pipecolic acid levels in urine from hyperlysinemic patients

Hyperlysinemic patients	Age, yr	Urine		Paper chromatography		Acid ninhydrin method, μg/mg creatinine
		Sp gr	Creatinine, mg/ml	Pipecolic acid spot[1]	Ultraviolet fluorescence	
Sister	9	1.015	0.70	+	+	6.2
Brother	6	1.013	0.07	+	+	5.2
Sister	4	1.018	0.05	+	+	3.1
Cousin	12	1.010	0.35	+	+	4.8

[1] Ninhydrin reaction and R_F value identical with those of known pipecolic acid.

exchange column analysis, cherry-red fluorescence of the ninhydrin product upon exposure to ultraviolet light, and maximal absorption at 565 mμ after reaction with acid ninhydrin reagent.

Pipecolic Acid Excretion by Normal Infants

Table I shows the amounts of pipecolic acid excreted in urine from four of four premature infants, three of four term neonates, and one of four infants under 1 year of age. No pipecolic acid was excreted by four older infants, 14–30 months of age. The amounts excreted varied from 8.1 μg/mg creatinine (in a premature infant) to 1.3 μg/mg creatinine (in a term infant).

Pipecolic Acid Excretion by Patients with Hyperlysinemia

Table II shows the levels of pipecolic acid in urine from four patients with hyperlysinemia; values range from 4.8 to 6.2 μg/mg creatinine.

Discussion

Pipecolic acid was eluted by 100-cm ion-exchange column chromatography [41] from the urine of a 1-year-old child [18]. The eluate emerged as a peak (designated as G) between valine and methionine. Also, pipecolic acid was found by paper chromatography [18] in urine of premature and term infants less than 76 days old, but the frequency of detection was not reported. FOWLER *et al.* [11], using column chromatography [41], found a peak (which they called no. 9) between valine and methionine; however, they did not identify it. The peak occurred in urine from two of three premature infants, two of two neonates, and three of three infants, but was not found in any subject over 5 months old. GATFIELD *et al.* [12] used methods almost identical with those described here and reported finding pipecolic acid in serum of normal children 2–15 years of age, but did not detect pipecolic acid in the urine. In their 18-month-old patient with elevated serum pipecolate, pipecolic acid in the urine was found to average 5.2 μg/mg creatinine (0.04 μmole/mg creatinine). When their patient and four control subjects (3–7 years old) were given 200 mg/kg of L-lysine by mouth, there was no alteration in the level of pipecolic acid in the serum or urine after 6 h.

When pipecolic acid was used with the ninhydrin reagent employed for automated ion-exchange amino acid separations, the color-yield of pipecolic acid was so low that 1 μmole recorded an unimpressive peak. Because color values are low, routine analytic ion-exchange methods permit pipecolic acid to be easily overlooked. Using paper chromatographic methods and 0.2% ninhydrin in acetone [37] for color development, amounts of pipecolic acid below 0.3 μg seldom can be detected on one-dimensional chromatograms. Such small amounts yield bluish spots, but red fluorescence under ultraviolet light, which is characteristic of pipecolic acid, does not usually occur with amounts less than 0.4 μg. Thus, small quantities may be missed by conventional screening methods using paper chromatography whenever dependence is placed on ultraviolet fluorescence for confirmation.

Studies bearing on lysine metabolism by the human [1, 12, 17, 50, 51] indicate that alternate pathways for lysine degradation exist (fig. 3), and that the pathway (II) via conversion of lysine to saccharopine by lysine-ketoglutarate reductase is probably the major one. Detection of pipecolic acid in urine from four children with hyperlysinemia demonstrated that the pathway via pipecolic acid [2] was functioning in these hyperlysinemic patients. The small amounts of pipecolic acid that were excreted in the urine, when compared with the large amounts of lysine simultaneously excreted by these patients, suggest that this path was normally a secondary one, which in these patients, had been pressed into maximal utilization by substrate loading. This would be comparable to the situation in

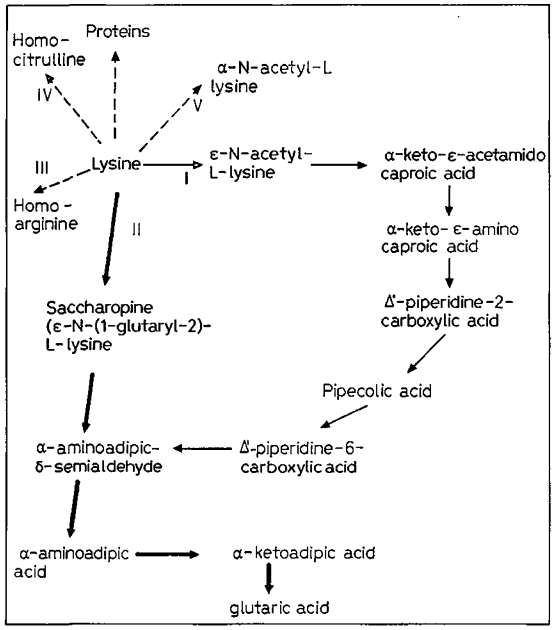

Fig. 3. Lysine metabolic pathways for man.

phenylketonuria and histidinemia where alternate pathways are utilized.

The presence of pipecolic acid in the urine of premature infants and neonates, and absence from urine of older infants, coupled with the observation that premature infants and neonates excrete more lysine than older subjects [8, 9, 27, 39], implies that lysine-ketoglutarate reductase activity may be reduced in the newly born and be induced by substrate loading during the initial weeks of life. Since GATFIELD and colleagues [12] found renal tubular reabsorption of pipecolic acid to be very efficient in normal children and in the patient with hyperpipecolatemia, the pipecolic aciduria observed in neonates and in patients with hyperlysinemia might result from a renal tubular reabsorption defect, age-related in the neonates and secondary to the inhibition of enzyme activity by an accumulated metabolite in the hyperlysinemic patients.

Summary

Pipecolic acid has been measured in the urine of premature and term neonates and of infants. One of four infants less than 1 year old, two of four term neonates, and four of four prematures excreted pipecolic acid in the urine. It was not found in urines of four infants over 1 year of age. Four children with hyperlysinemia excreted amounts of pipecolic acid that demonstrated that a pathway for lysine degradation, via pipecolic acid to α-aminoadipic acid, was functioning in these patients, that this pathway had been pressed into maximal utilization by the high tissue lysine levels of these patients, and that in hyperlysinemic patients this pathway was a secondary one for lysine degradation. These findings support the thesis [12, 17, 50, 51] that the major path of lysine degradation in man is through conversion (by lysine-ketoglutarate reductase) of lysine to saccharopine en route to α-aminoadipic acid.

Neonates excrete pipecolic acid at levels exceeding those found for older infants, suggesting that lysine-ketoglutarate reductase activity may be reduced in the newly born and become enhanced by substrate loading during the first weeks of life.

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