ON THE ISOLATION AND IDENTIFICATION OF HOMOCITRULLINE FROM URINE*

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Received March 17, 1961

During column chromatographic analysis of free amino acids in urine by means of the Moore and Stein procedure (1951), the appearance of an unidentified component in the form of a peak between valine and the buffer change position has been observed by several workers (Fowler et al., 1957; Jagenburg, 1959a; Spackman, 1960). Jagenburg obtained evidence that the peak contained four components of which he identified one on the basis of color tests and Rf values as pipecolic acid.

In the present study on urines of infants and young children with the Beckman/Spinco Amino Acid Analyzer, following essentially the procedure as outlined by Spackman et al. (1958), an unidentified peak was frequently observed at, or just prior to the buffer change position (380 - 390 ml) on the 150 cm column chromatogram. By delaying the buffer change for 45 minutes (to 11 1/4 hrs), this peak clearly separated from cystine between 380 and 400 ml. It was still present and emerged at the same position after acid hydrolysis of the urine or after performic acid oxidation.

Isolation

Urine of a 3 month old normal infant, found to excrete the unidentified compound in unusually large amounts, was used as starting material. The urine was obtained under sterile conditions, and was kept in ice and under toluene during the 24 hr. collection period. Fifty ml was oxidized

*This work was supported in part by grants M-2730 and A-1498 from the National Institutes of Health, U.S. Public Health Service.

with performic acid (Schram et al., 1954) in order to convert cystine to cysteic acid which appears at a different place in the chromatogram. The urine was then hydrolyzed in 20% HCl for 24 hours under reflux in order to remove interfering polypeptides which in unhydrolyzed urine came off the column together with the unknown compound, but apparently developed little or no color with the ninhydrin reagent.

Batches of hydrolysate equivalent to 15 ml of urine were absorbed onto the 0.9 x 150 cm Amberlite IR 120 (Na^+) column of the Amino Acid Analyzer, and the amino acids were eluted with 0.2 M citrate buffer, pH 3.25, at the normal speed of 30 ml per hour. The temperature of the column was changed from 30° to 50° after 11 hours. The first 330 ml were discarded, and the following 120 ml of eluate were collected. The eluate, containing predominantly the unidentified compound with traces of other amino acids, was absorbed onto a 1.8 x 25 cm column of Amberlite IR 120 (H^+). After the column was washed with 200 ml water, the amino acids were eluted with 150 ml 4% ammonium hydroxide. After concentration in vacuo, combined concentrates equivalent to 150 ml of urine were again absorbed onto the 150 cm column of the Amino Acid Analyzer, and eluted as before. This time ninhydrin reagent was added to the first 330 ml of eluate and peaks of glutamic acid, glycine, alanine and valine were visible in the usual positions. A 100 ml fraction coming off after valine was collected. This fraction was desalted on an Amberlite IR 120 column and a 10 ml concentrate was obtained, containing about 30 mg of the unknown compound, calculated as leucine. Crystals were isolated from 2 ml of this concentrate by lyophilizing, dissolving the residue (9.6 mg) in 0.1 ml 1 N HCl and 2 ml of absolute ethanol, and adding absolute ether until a faint turbidity persisted. Crystallization of the hydrochloride yielded needles, m.p. 177 - 178° dec.

Identification

The compound was stable during acid hydrolysis and performic acid oxidation as stated above. On a paper chromatogram in BuAc (n-butanol:

acetic acid:water = 120:30:50) the Rf value was found to be 0.28 - 0.30; in PhAm (phenol:water:ammonia 38% = 150:50:1) the Rf value was 0.68 - 0.71. The compound reacted with ninhydrin to give a purple-red color in 20 minutes at room temperature. The color deepened considerably upon heating at 95° for 5 minutes. A pink color resulted with isatin (0.2% in acetone), and an immediate yellow color with Ehrlich's reagent. In aqueous solution the crystalline material gave a peach color reaction with diacetylmonoxime, according to the method of Archibald (1944) for the estimation of citrulline. The absorption spectrum of this reaction product had a maximum at 490 mμ, which can be considered as evidence for the presence of a ureido group. These color reactions were identical with those of citrulline and 2-amino-3-ureidopropionic acid, but both these compounds were ruled out by the Rf values and positions on the column chromatogram. Pipecolic acid was likewise ruled out, as were a number of other rare amino acids.

Alkaline hydrolysis, by refluxing in boiling 4N NaOH for 16 hours, or in saturated $Ba(OH)_2$ for 3 hours, decomposed the substance forming another ninhydrin positive compound which showed an Rf value of 0.12 in BuAc and about 0.74 in PhAm and did not give a yellow color with Ehrlich's reagent. This decomposition product was easily identified as lysine on the Amino Acid Analyzer and by paper chromatography.

On the basis of lysine formation during alkaline hydrolysis, and the above mentioned color reactions, it was concluded that the unknown substance was 2-amino-6-ureidocaproic acid (ε-carbamyllysine) or homocitrulline. This amino acid was synthesized from lysine according to the method of Kurtz (1949) as modified by Smith (1955), and was found to be identical with the unidentified compound by co-chromatography on the Amino Acid Analyzer and on paper. The unknown, and the synthetic homocitrulline gave a single and additive symmetrical peak on the column chromatogram at two different pH values (3.25 and 3.30). Furthermore, the infrared spectra (KBr pellet) of the isolated material and the synthetic homocitrulline-HCl were found to be identical, and different from the infrared spectrum of citrulline-HCl.

Discussion

In this investigation, homocitrulline was found in measurable amounts only in the urine of infants and young children. The approximate amount excreted by the infant whose urine was used in this investigation was 30 - 50 mg per 24 hours. It was either absent, or present in very low concentrations in the urine of 33 male adults examined by one of the authors during an earlier investigation (Gerritsen and Horwitt). This finding is in agreement with the presence of the unidentified peak eluted after valine in infant urine, and its absence in adult urine, as demonstrated by Dustin et al. (1955).

In view of the report by Stark et al. (1960) that homocitrulline is a hydrolysis product of ribonuclease inactivated by 8M urea at 40°, it is conceivable that the urinary homocitrulline observed in the present work might be an artifact, in the sense that it could have been formed by interaction of lysine and cyanate in the bladder. However, the comparatively low urea concentration in urine (0.3M, both in infants and adults), the low excretion of homocitrulline by adults despite a urinary lysine output comparable to that of infants (Jagenburg, 1959b.), and the fact that the majority of the urines in this investigation were collected by 24 hour catheterization, do not support this possibility. In addition, Fowler et al. (1957) observed that infants fed synthetic amino acid diets excreted urinary lysine, but failed to demonstrate the unidentified peak No. 9 detected in the 100 cm column chromatogram of urine from infants on protein containing diets.

It is unlikely that the homocitrulline in the urines can be attributed to bacterial infection. The fact that glutamine was usually present in the urines indicated that the precautions taken to prevent bacterial growth were adequate. In earlier work any growth of micro-organisms in urine was observed to result in the conversion of glutamine into glutamic acid (Gerritsen, unpublished data).

It has not yet been demonstrated that homocitrulline plays a role in animal or human metabolism (Lowenstein and Cohen 1956; Smith 1955) and we are unaware of a previous report on its natural occurrence. Further work on this subject, and on the origin of homocitrulline in urine is in progress.

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