THE OCCURRENCE OF FREE L-PYRROLIDONE CARBOXYLIC ACID IN BODY FLUIDS AND TISSUES

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Received 9 May 1973

1. Introduction

Pyrrolidone carboxylic acid (PCA) has been known as a cyclic derivative of glutamic acid for more than 90 years [1]. An enzyme designated γ-glutamyl cyclotransferase (γ-glutamyl lactamase) was found in mammalian tissues [2] that catalyzes the conversion of L-γ-glutamyl amino acids to L-PCA and free amino acids. This reaction represents probably the main route of formation of L-PCA in mammals [3, 4]. L-PCA was found at the N-terminus of a number of peptides and proteins (for a review see [5]), and this compound was also postulated to be an intermediate of the γ-glutamyl cycle, which was proposed as a transport system for amino acids [6]. Although these considerations indicate that L-PCA is an active metabolite, there are no reports demonstrating its presence as a normal constituent of body fluids and tissues. Indeed screening methods in which gas chromatographic procedures were used for the determination of PCA as the methyl ester, failed to detect this compound in normal body fluids [7].

We report the finding of siginificant amounts of PCA in tissues and body fluids by the application of a gas liquid chromatographic procedure, utilizing electron capture detection. Analysis of the optical configuration of glutamate derived by acid hydrolysis from PCA has shown that all of the PCA present in the cerebrospinal fluid is of the L-configuration and that a substantial fraction of the PCA in urine has the D-configuration.

2. Materials and methods

All coated gas chromatographic packings were obtained from Applied Science Labs, State College, Pennsylvania. Pentafluoropropanol was purchased from PCR chemicals, Gainesville, Florida, and pentafluoropropionic anhydride from Pierce Chemical Company, Rockford, Illinois.

[^{14}C]L-PCA was prepared by cyclization of [^{14}C]L-glutamate as previously described [8]. L-Glutamic acid decarboxylase purified from *E. coli* was obtained from Sigma Chemical Company. Gas chromatography was performed on a Packard 7400 series gas chromatograph fitted with a 150 mCi tritium electron capture detector. Freshly voided urine and fresh serum samples were obtained from healthy normal human subjects. Fresh samples of spinal fluid were obtained by lumbar puncture from neurologic and psychiatric patients and patients with herniated discs.

Tissue extracts were prepared by homogenizing with 5 vol 0.4 N perchloric acid. After homogenization the protein precipitate was removed by centrifugation and the supernatant brought to pH 6.0 by addition of saturated K_2CO_3. The precipitate of potassium perchlorate was removed by centrifugation and the supernatant used for the determination of PCA. Serum samples were deproteinized by addition of perchloric acid and the samples treated similarly to the tissue extracts. Isolation of PCA was carried out from one ml aliquots of urine and cerebrospinal fluid (CSF) and from protein free solutions corresponding to either 1 g tissue or 1 ml serum. Glutamine and glutamate were removed by passing the samples through a column of AG50WX8 cation exchange resin

(100–200 mesh; 0.7 × 10.0 cm; H^+ form). The effluent was further purified sequentially by adsorption of PCA on AG1X4 (100–200 mesh; 0.7 × 15 cm) and DEAE Sephadex A25 (0.7 × 5 cm columns in the chloride forms, and carrying out the elution with 0.01 N HCl. The final effluent was evaporated to dryness by flash evaporation and PCA was converted to a derivative with a high electron capture response by reaction with a new reagent – 20% pentafluoropropanol in pentafluoropropionic anhydride [9]. In this reaction pentafluoropropionic anhydride catalyzes the esterification of the carboxyl group of PCA with pentafluoropropanol. The recovery of PCA through the procedure was 75%. Recovery was determined by adding [^{14}C]L-PCA to parallel samples (400,000 cpm; specific activity = 200 mCi/μmole), and counting the radioactivity in the final effluent. Quantitation of PCA in the samples was achieved by use of an internal standard curve constructed from the peak heights of parallel samples containing 5–20 μg added PCA. To determine the optical configuration of the PCA present in urine and CSF, the samples were purified by ion exchange chromatography and the isolated PCA determined as described above. Aliquots were removed prior to derivatization and converted to glutamate by acid hydrolysis (2 N HCl, 100°C, 4 hr). Following hydrolysis the acid was removed by evaporation under reduced pressure and the residue dissolved in acetate buffer (0.3 ml; 0.05 M, pH 5.0). L-Glutamic acid decarboxylase (0.2 ml; 1 unit) was added and the samples were incubated at 37°C for 3 hr. Samples containing L-glutamate, D-glutamate and DL glutamate were run in parallel. γ-Amino butyric acid which is formed by the action of this enzyme on L-glutamate can be derivatized with pentafluoropropanol in pentafluoropropionic anhydride and the derivative separated by gas chromatography from the derivatives of glutamate and PCA.

3. Results

Both CSF and urine yielded a peak with a retention time identical to authentic PCA on eight different columns coated with liquid phases of varying degrees of polarity [3% OV-1, 3% OV-17, 3% XE-60, 3% OV-225, 2% XF-1105/3% OV-17 (3/1), 3% ECNSS-M, 10% GE-SE-54, and 3% JXR (fig. 1)]. The identity of the peak was further confirmed by its conversion to glutamate following acid hydrolysis (2 N HCl, 100°C, 4 hr). Glutamate can be derivatized under the same conditions as PCA and determined quantitatively. The derivatives of these two compounds can be resolved on a number of gas chromatographic columns (fig. 2). Acid hydrolysis of urine and CSF samples prior to derivatization resulted in disappearance of the PCA peak and appearance of a glutamate peak (fig. 2). Additional proof of identity was obtained by enzymatic conversion of the L-glutamate formed from PCA to γ-aminobutyric acid (GABA) by the action of L-glutamic acid decarboxylase (see below).

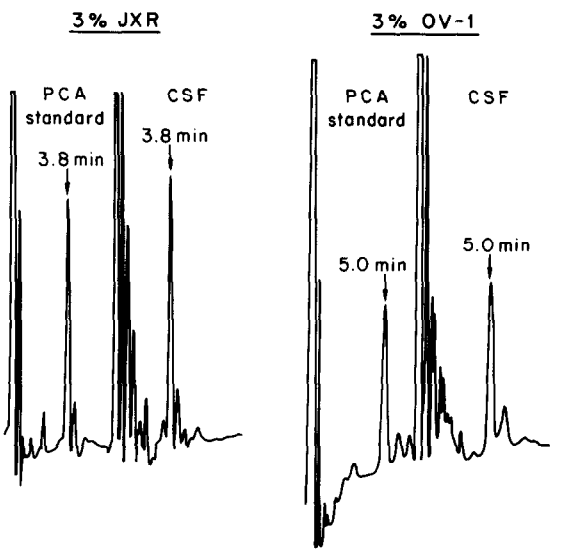

Fig. 1. Chromatograms of a CSF specimen demonstrating a peak with the exact retention time of authentic PCA. 3% JXR, 120°C, N_2 = 40 ml/min; 3% OV-1, 120°C, N_2 –30 ml/min.

Significant amounts of PCA can be demonstrated in both urine and CSF, the mean levels being, respectively, 0.19 and 0.06 μmoles/ml (table 1). Two human serum samples had PCA levels of 0.01 and 0.03 μmoles/ml, values which are lower than those found in either urine or CSF. Significant quantities of PCA were also found in a number of tissues of several species studied. In one series of experiments the following values were found in organs of the guinea pig: kidney 0.15 μmoles/g, brain 0.13 μmoles/g, lungs 0.08 μmoles/g, spleen 0.04 μmoles/g, liver 0.03 μmoles/g and heart 0.003 μmoles/g. Similar values were found in organs of the rabbit and rat.

It is well established that nonenzymic cyclization of glutamine is substantial even at 37°C. Glutamate, gluta-

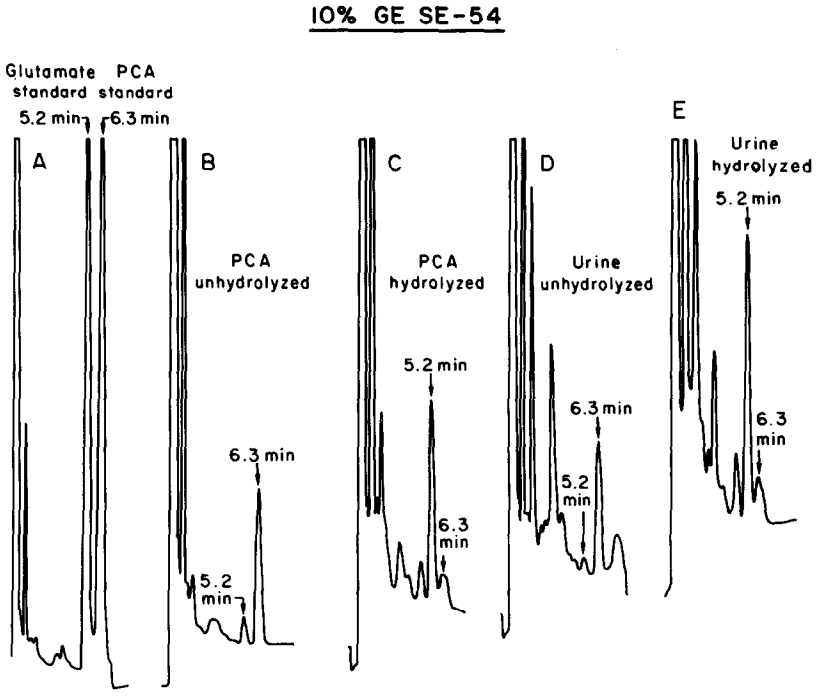

Fig. 2. Identity of urinary PCA. Chromatographic conditions: 10% GE SE-54, 158°C, N_2 –60 ml/min. A) Separation of pentafluoropropionate derivatives of PCA and glutamate. B) Authentic PCA carried through procedure and derivatized. C) Authentic PCA hydrolyzed with 2 N HCl for 4 hr at 100°C before derivative formation. D) Urine sample carried through procedure. E) Aliquot of sample D hydrolyzed with 2 N HCl for 4 hr at 100°C prior to derivative formation.

Table 1
Levels of pyrrolidone carboxylic acid in urine and cerebrospinal fluid.

Sample	No. of specimens	Mean ± S.D. (μmoles per ml)	Range (μmoles per ml)
Cerebrospinal fluid	25	0.06 ± 0.02	0.03–0.11
Urine	31	0.19 ± 0.11	0.04–0.58

thione and γ-glutamyl amino acids are much more resistant to cyclization than glutamine. The possibility that the levels of PCA found in body fluids were derived from the nonenzymic cyclization of glutamine at some stage of the determination was ruled out by processing solutions of glutamine in concentrations equivalent to those in serum and CSF. Only a trace of material with the retention time of PCA was observed (< 0.001 μmoles/ml). We therefore conclude that PCA is a normal constituent of body fluids and that it is probably derived from normal intermediary metabolic processes.

The reported presence of D-PCA in urine and the finding that D-glutamate is converted to D-PCA *in vivo* and excreted in the urine [10] led us to determine the optical configuration of the PCA present in urine and CSF as described above. The results of one such experiment are presented in table 2. As can be seen all the PCA present in CSF can be converted after acid hydrolysis and incubation with the enzyme to GABA, and must therefore be of the L-configuration. The same result was obtained with four other cerebrospinal fluids. In contrast only about 44% of the PCA in urine was on the L-configuration (table 1). In five other urines the amount of L-PCA was found to vary between 30 and 40%. Thus although D-PCA seems to prevail in the urine, significant amounts of the L-isomer can also be detected.

Table 2
Determination of optical configuration of pyrrolidone carboxylic acid in urine and cerebrospinal fluid.

Source	PCA (μmoles per ml)	D-glutamate* (μmoles per ml)	γ-Aminobutyrate* (μmoles per ml)
Urine	0.57	0.30	0.24
Cerebrospinal fluid	0.07	<0.001	0.065

* Values were obtained after acid hydrolysis and incubation with L-glutamic acid decarboxylase. For details see the text.

4. Discussion

These studies provide evidence that PCA is a natural constituent of body fluids and tissues and that the levels found cannot be derived from the non-enzymatic cyclization of glutamine. It may be thus assumed that PCA is derived from normal intermediary metabolic processes, most probably from the operation of the γ-glutamyl cycle, the only known pathway in which large amounts of L-PCA can be formed [6]. The cycle postulates that the reabsorption of amino acids in the kidney is linked to the synthesis and degradation of glutathione and thus to the renal synthesis of ATP. This idea became attractive [11] when it was discovered that γ-glutamyl transpeptidase, the enzyme that transfers the γ-glutamyl moiety of glutathione to amino acids [12] is heavily concentrated in the brush border of the proximal convoluted tubules and other sites where transport could be anticipated [13–15]. Supporting evidence for the functioning of the γ-glutamyl cycle in amino acid transport has recently been obtained in studies of a metabolic disorder termed pyroglutamic aciduria discovered in a 20-year-old mentally retarded patient who excretes approx. 30 g PCA per 24 hr in the urine [7]. Biochemical studies of this disorder [16] seem to indicate that the patient has a block in the enzymatic step of the γ-glutamyl cycle in which L-PCA is converted in an ATP requiring reaction to L-glutamate [17].

The relatively low levels of PCA found by us may reflect rapid turnover of PCA and efficiency of the enzymic system that catalyzes its conversion to glutamate. It is of interest in this respect that the levels of PCA in CSF are considerably higher than in plasma. It remains to be elucidated whether these levels result from the operation of the γ-glutamyl cycle in the brain where the activity of the enzyme that converts L-PCA to L-glutamate is relatively low [17].

Acknowledgements

This work was supported in part by a Research Career Development Award #1K04GM40793-04 to S.W. and grants by the National Institute of Health and the National Institute of Mental Health.

References

[1] L. Haitinger, Monatch. Chem. 3 (1882) 229.
[2] G.E. Connell and C.S. Hanes, Nature 177 (1956) 377.
[3] M. Orlowski, P.G. Richman and A. Meister, Biochemistry 8 (1969) 1048.
[4] M. Orlowski and A. Meister, J. Biol. Chem. 248 (1973) 2836.
[5] M. Orlowski and A. Meister, The Enzymes 4 (1971) 123.
[6] M. Orlowski and A. Meister, Proc. Natl. Acad. Sci. U.S. 67 (1970) 248.
[7] E. Jellum, T. Kluge, H.C. Barresen, O. Stokke and L. Eldjarn, Scand. J. Clin. Lab. Invest. 26 (1970) 327.
[8] M. Orlowski and A. Meister, J. Biol. Chem. 246 (1971) 7095.
[9] S. Wilk and E. Watson, Federation Proc. 32 (1973) 798.
[10] S. Ratner, J. Biol. Chem. 152 (1944) 559.
[11] M. Orlowski, Arch. Immun. et Therap. Exptl. 11 (1963) 1.
[12] C.S. Hanes, F.J.R. Hird and F.A. Isherwood, Nature 166 (1956) 288.
[13] Z. Albert, M. Orlowski and A. Szewczuk, Nature 191 (1961) 767.
[14] G.G. Glenner, J.E. Folk and P.L. McMillan, J. Histochem. Cytochem. 10 (1962) 481.
[15] Z. Albert, M. Orlowski, Z. Rzucidio and J. Orlowska, Acata Histochem. 25 (1966) 312
[16] L. Eldjarn, E. Jellum and O. Stokke, Clin. Chim. Acta 40 (1972) 461.
[17] P. van der Werf, M. Orlowski and A. Meister, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2982.