

Detection of Pyrrolidone Carboxylic Acid

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In the course of work on low-molecular constituents of plant extracts which yield amino acids on hydrolysis (cf. Synge, 1951; Synge & Wood, 1954), the question has arisen of the extent of occurrence of pyrrolidone carboxylic acid (5-oxopyrrolidine-2-carboxylic acid, pyroglutamic acid) (PCA). We have encountered this substance and partially isolated it from among the acidic components of juice from grass which had been stored at -20° for several months. We have since satisfied ourselves that PCA is at most a minor constituent of the 'bound amino acid' fraction of freshly prepared juice. In the course of this work we have observed some properties of PCA which are useful for detecting it at low concentration in biological material and which could serve as a basis for its determination. We are therefore reporting these observations independently of the progress of the work on other forms of 'bound amino acid'.

PCA, arising by decomposition of glutamine, is present in considerable amount in molasses and presumably also in cooked foods, silage, etc. Its failure to give a colour with ninhydrin and other specific chemical reactions is probably responsible for the sparseness of present knowledge of its natural distribution and metabolic behaviour.

The early isolations of PCA from biological material were by ether extraction (Štaněk, 1912, 1913, 1915; Škola, 1920; Dolinek, 1927) or by extraction of precipitates or evaporation residues with glacial acetic acid (Foreman, 1914; Bethke & Steenbock, 1923; Greenberg & Schmidt, 1936). More recently, extraction of acidified solutions with ethyl acetate has usually been employed (Wilson & Cannan, 1937; Pucher & Vickery, 1940; Leuthardt, 1940; Woodward & Reinhart, 1942; Ratner, 1944; Kögl, Barendregt & Klein, 1948; Bray, James, Raffan & Thorpe, 1949). Pucher & Vickery (1940) proposed determination of glutamine as PCA formed after heating plant extracts at pH 6.5 at 100° for 2 hr. The PCA was then extracted into ethyl acetate and determined as amino N (Van Slyke nitrous acid procedure) after acid hydrolysis (see also Sorgato & Sorenzo, 1950). However, as Pucher & Vickery reported no blank determinations and used heated extracts of dried plant tissues, their

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results have little bearing on the natural occurrence of PCA. Moreover, our own experience is that conjugated forms of glutamic acid other than PCA, as well as other conjugated amino acids, are extracted by ethyl acetate from acidified extracts of fresh plant material. Neuberger & Sanger (1942) could not obtain satisfactory results using the method of Pucher & Vickery (1940) with potato extracts.

The procedure of Schlütz (1953), which involves hydrolysis of constituents of urine soluble in aqueous ethanol, is equally open to criticism for lack of specificity.

More recently chromatography has been used for detecting or determining PCA. Kögl *et al.* (1948) mention chromatography in ethanol on alumina. Stark, Goodban & Owens (1950, 1951) and Owens, Goodban & Stark (1953) have used chromatography on ion-exchange resins. Stark *et al.* (1950; cf. Goodban, Stark & Owens, 1953), Le Quesne & Young (1952), Rowlands & Young (1952) and Simonart & Chow (1953) have used partition chromatography on paper, spraying with an indicator to locate the PCA. See also Pichler (1952).

Massa (1935) proposed a polarimetric method for determining PCA in molasses. Kanao (1948) has used a reaction of PCA with formaldehyde and formic acid for its determination. Hasse & Schumacher (1950) observed enzymic decarboxylation of PCA by preparations from radish, but gave no experimental details.

We have observed: (a) that PCA can conveniently be detected on filter paper by the chlorine-starch-iodide reaction of Rydon & Smith (1952); (b) that PCA has an anionic mobility at pH 6-7 exceeding those of glutamic acid or of any of the other components of grass juice which give a positive chlorine-starch-iodide reaction; (c) that PCA, as the free acid, is readily extractable into the phenol-rich phase of the system phenol-water and gives a zone occupying a characteristic position on water-phenol partition chromatograms (phenol phase stationary). We have further confirmed the observations of Le Quesne & Young (1952) and Rowlands & Young (1952) on filter-paper chromatography of PCA with *n*-butanol-water.

In view of our observations on the water-phenol distribution of PCA it seems likely that the observations of Austerweil & Delort-Stachenko (1952)

relate not, as they supposed, to PCA but to some other substance. PCA would be expected to move faster than glutamic acid on phenol-water filter-paper chromatograms; nor can it be revealed with ninhydrin.

In order to ascertain if PCA occurs in fresh grass, we took freshly expressed juice, acidified to pH 2, and extracted it with phenol in a simple counter-current train. Such a procedure yields in the phenol extract most of the 'bound amino acid' constituents (cf. Synge & Wood, 1954). Filter-paper electrophoresis showed that no more PCA was present in the phenol extract than might be expected from decomposition of the glutamine present under the acid conditions of the extraction (cf. Thierfelder & von Cramm, 1919; Melville, 1934; Ratner, 1944). PCA added initially to the juice was recovered in apparently good yield. PCA, derived from glutamine, was likewise found on applying the same procedure to juice which had been heated at neutral pH.

In view of the amount of PCA in freshly prepared grass juice being at most small compared with other forms of 'bound amino acid', we have not worked out any procedure for its exact determination. However, some zone-electrophoretic technique (see Tiselius & Flodin, 1953) could be adapted to take advantage of PCA being the nitrogenous compound which migrates most rapidly towards the anode at neutral pH. This differentiates PCA from other forms of 'bound glutamic acid' extractable with organic solvents, such as those at present being studied as well as, presumably, such known compounds as pteroylglutamic acid, phenacetylglutamine, etc. Extraction from acidic aqueous solution into organic solvent is clearly desirable for preliminary concentration of PCA and elimination of free glutamic acid, plant acids, carbohydrates, etc. However, this must not be done in the presence of glutamine, in view of its decomposition to PCA under such conditions. Glutamine would probably be best eliminated by initially isolating acidic constituents from the crude extract on anion-exchangers or by electrical transport in a diaphragm cell at neutral pH.

EXPERIMENTAL

L-Pyrrolidone carboxylic acid (PCA)

Partially racemized PCA was prepared by heating 15 g. *L*-glutamic acid ($[\alpha]_D^{25} + 30.3^\circ$ in 9% (w/v) HCl, $c=2$, $l=0.5$) for 2 hr. at 150–158° (cf. Abderhalden & Kautzsch, 1910). The melt was crystallized from 15 ml. water. Yield, 3.9 g. For analysis, the product was dried *in vacuo* at room temp. over H_2SO_4 and soda lime. $[\alpha]_D^{25} - 5.4^\circ$ in water ($c=1.96$, $l=0.5$). N (Kjeldahl), 10.5. Calc. for $C_5H_9O_3N$: N, 10.9%. Carboxyl N (conditions of Synge, 1951), < 1% of total N. No colour was observed after the heating with ninhydrin. When a hydrolysate (24 hr. in 6N-HCl at 105°) was evaporated to dryness and analysed under the same

conditions, carboxyl N was 110% of total N. Presumably the low optical rotation was due to not collecting a high yield of crystals, to avoid contamination with glutamic acid. Abderhalden & Kautzsch (1910) noted that the DL compound is less soluble than the L enantiomorph.

Detection and partial isolation of PCA in extract of stored grass

Countercurrent distribution of diffusate with phenol-water. Italian rye grass which had been stored for 4 months at -20° (11 kg.) yielded on treatment with ether as before 1560 ml. of juice; this was dialysed in cellophan against chloroform water (10 l.) for 5 days at $0-2^\circ$ (cf. Synge, 1951). The diffusate (9.6 l. containing 1.45 g. N) was evaporated *in vacuo* to 200 ml. This was placed in the first of a train of five separating funnels; the other four contained each 200 ml. of the water-rich phase of the system water-phenol (freshly distilled from A.R. grade). Countercurrent distribution was effected by passing eight 200 ml. portions of the phenol-rich phase of the same system through the train. Separation of the phases was hastened by centrifuging when necessary. The eight portions of phenol-rich phase emerging from the train were pooled and evaporated *in vacuo* with repeated addition of water (cf. Synge & Wood, 1954).

Fractionation in diaphragm cell. The residue was taken up with water, adjusted to pH 6–7 with NH_3 and fractionated (two batches) in the four-compartment diaphragm cell according to Synge (1951). Material from the acetic acid compartment contained 0.83% of the N of the original diffusate. This was evaporated to dryness *in vacuo* repeatedly with addition of water to remove acetic acid (fraction P 4 A).

Water-phenol partition chromatography. A portion of P 4 A (10.55 mg. N, 605 mg. dry matter) was taken up in 8.5 ml. of water-rich phase of the above phenol-water system and chromatographed (cf. Synge & Wood, 1954) on a column (5.4 cm. diam., 10.8 cm. long) made from 87 g. kieselguhr (Hyflo Supercel, Johns Manville Co., London), which had been treated with dichlorodimethylsilane according to Howard & Martin (1950), mixed with 58 ml. phenol-rich phase and packed as a slurry in water-rich phase using the perforated-disk tool of Howard & Martin (1950). The chromatogram was developed with water-rich phase in a cool room (14°) without special temp. control. Successive fractions of effluent were collected, weighed and evaporated in tared flasks to dryness *in vacuo* below 40° , with repeated addition of water until phenol was removed. The residues were weighed and the distribution of dry matter in the effluent was plotted. Since the resulting curve showed no well-marked peaks, the fractions were pooled into six successive groups accounting for roughly equal portions of the dry matter (and N) recovered. Of these, group 3 (P 4 A-3), comprising the effluent from the column (counted from moment of applying sample for analysis) between 253 g. and 373 g. of effluent, contained 106.4 mg. dry matter and 3.08 mg. N.

Filter-paper ionophoresis. Material from the various chromatographic fraction-groups was subjected to filter-paper ionophoresis in 0.04M sodium phosphate buffer (pH 7.1) for 4 hr. at 10 v/cm. according to Kunkel & Tiselius (1951), using glass plates smeared lightly with Silicone Fluid M.S. 550 (Hopkin and Williams Ltd., Chadwell Heath, Essex) and sealed at the edges with Dow Corning Stopcock Grease (Dow Corning Corp., Midland, Mich.). Three sheets of paper were used in each run, the same samples (ca. 1 mg.

dry matter in 5 μ l. water) being applied at corresponding positions on each sheet. It was found that the silicone interfered with the Cl_2 -starch-KI reaction of Rydon & Smith (1952). Accordingly, the middle sheet was always used for this test.

Material from each of the fraction-groups 1-6 on ionophoresis showed anionic material of varying mobilities giving positive Cl_2 -starch-KI reaction. However, only P4A-3 showed the presence of a strong spot migrating faster towards the anode than any of the other Cl_2 -starch-KI reacting components of any of the six fraction-groups. This component gave no colour with ninhydrin. Its isolation was attempted as follows.

Silica-jelly ionophoresis. Procedure B of Consden, Gordon & Martin (1946) was adopted, using a trough 55 cm. long, 4 cm. broad and 0.7 cm. deep. For the main jelly, 9.4 ml. of a 1:2 (by vol.) dilution of waterglass (140° Tw., Jos. Crosfield Ltd., Warrington) was diluted with water and neutralized (bromothymol blue) with acetic acid to final vol. 150 ml. The inlay (near cathode end of jelly) was made by neutralizing a sample from P4A-3 (80 mg. dry matter, 2.32 mg. N) with diluted waterglass to give the same pH and silica concentration. The cathode was perfused with a solution of 0.22M sodium acetate in 0.011M acetic acid and the anode with 0.22M sodium acetate. 100 ma was allowed to pass through the apparatus (without special cooling precautions) for 10.75 hr. A print was then taken and coloured with Cl_2 -starch-KI. This revealed the centre of the main zone 15.5 cm. to the anode side of the inlay. Two other, obviously complex, zones occupied most of the space between the inlay and the principal zone. The jelly from 12.5-19.0 cm. on the anode side of the inlay was cut out. Obvious impurities present in this section (not coinciding exactly with the principal zone) were a trace of brown pigment, a zone fluorescing pale blue in u.v. light and a zone giving a purple colour on treating the print with Cl_2 (before spraying with starch-KI).

The cut-out slab of jelly was stirred with water and the resulting slurry transferred to the specimen compartment of the four-compartment diaphragm cell. The fractionation was as usual (pH 6-7) except that NaOH was used instead of NH_3 in the cathode compartment and for maintaining pH in the specimen compartment. After 6 hr. the contents of the acetic acid compartment was removed and evaporated to dryness *in vacuo* repeatedly with water. The residue was taken up in water, filtered from a little insoluble material (silica) and again evaporated (11.0 mg. dry matter; 0.54 mg. N, representing 6.8% of the N of P4A, i.e. 0.057% of the N of the original diffusate). Subsequent work showed that the very low yield of PCA was due to not having acidified the diffusate before the phenol extraction.

Characterization of partially isolated PCA

The product, analysed after drying *in vacuo* over H_2SO_4 -soda lime at room temp., consisted of crystals embedded in syrup and was freely soluble in water. $[\alpha]_D^{19} - 11.3^\circ$ in water ($c = 1.7$, $l = 0.5$); N (Kjeldahl), 4.95%; acid equiv. wt. 237. Carboxyl N was < 1%, but after hydrolysis 87% of total N (conditions as above; no colour on heating unhydrolysed material with ninhydrin). The acid hydrolysate on 2-dimensional filter-paper chromatography (conditions of Synge, 1951) showed a strong spot corresponding to glutamic acid and very faint spots corresponding to glycine and other neutral amino acids.

Filter-paper ionophoresis (as above) showed with Cl_2 -starch-KI a main spot identical in mobility (mixed spot) with authentic PCA. The product from grass also showed a faint spot migrating 0.83 times as fast as the main spot. PCA had mobility towards the anode, measured from a spot of *N*-2:4-dinitrophenylethanolamine which served as a control for electroendosmosis, etc. (Mould & Synge, 1954), 1.13 times that of L-glutamic acid. The authentic PCA preparation gave a faint spot, having 1.24 times the anionic mobility of the main spot, which may be due to a dimeric anhydride of glutamic acid.

On descending chromatography with *n*-butanol-water on Munktel OB paper (Grycksbo Pappersbruk AB, Grycksbo, Sweden), the product from grass and PCA behaved identically (confirmed by mixed chromatogram). The spots (Cl_2 -starch-KI) had leading edge at R_f ca. 0.5 and diffuse tails (cf. Le Quesne & Young, 1952; Rowlands & Young, 1952).

Authentic PCA was chromatographed on a water-phenol column as above (1 cm. diam.; 3 g. silane-treated kieselguhr). The Cl_2 -starch-KI reaction was applied to spots on filter paper from successive effluent fractions. The peak of the zone was found at $R = 0.73$, the leading edge of the zone being much more diffuse than the tailing edge. The corresponding chromatographic fraction in the isolation (P4A-3) represented the cut $R = 0.98$ to $R = 0.66$. $R = 0.73$ was calculated (Martin & Synge, 1941) to correspond to partition coefficient $\alpha = 3.0$ in favour of the stationary phenol-rich phase of the system phenol-water.

We conclude that the isolated product was PCA contaminated with much N-free but only little nitrogenous impurity.

Detection of PCA in fresh grass juice

Control experiments in which PCA was added to grass juice showed that recovery in the phenol extracts was inadequate unless the juice was acidified before extraction.

Juice was squeezed from freshly picked Italian rye grass after treatment with ether, ether was removed by a short evaporation *in vacuo* in the cold and the juice was acidified to pH 2 (glass electrode) with 6N- H_2SO_4 . Two 100 ml. portions of acidified juice (each 124 mg. total N) were taken. To one was added PCA (1.07 mg. N). The portions were then separately subjected to the countercurrent distribution as described above, using 100 ml. portions of phenol-water phases. The extraction lasted approx. 4 hr. It was done at room temp. since the crystallization of a phenol hydrate prevented working at 0-2°.

A suitable portion of the combined phenol extract from each distribution was evaporated as above and the residues were taken up with water to give a slurry which was applied for filter-paper ionophoresis of spots. Each spot represented 153 μ g. N of original grass juice, and, for the specimen with added PCA, 1.32 μ g. N of added PCA. A control spot (1.32 μ g. N of PCA) was run in parallel. After ionophoresis as described above, Cl_2 -starch-KI treatment revealed a streak of staining material which had migrated towards the anode, as well as much neutral staining material in the grass extracts. None of this material migrated faster than PCA; the spot in the corresponding region, though present, was much less intense than the PCA control spot with the extract of untreated juice, whereas the extract of juice to which PCA had been added showed a spot in this region of comparable intensity to the control spot. The PCA present in

the fresh juice was therefore much less than 0.86% of the N of the juice. Assuming 1-2% decomposition of the glutamine present (cf. Thierfelder & von Cramm, 1919; Melville, 1934; Ratner, 1944) and using the figure for glutamine given below, 0.1-0.2% of the total N of the juice might be expected to have been transformed into PCA.

The juice was also adjusted to pH 6.8 with NaOH and heated at 100° for 3 hr. It was then subjected to acidification, phenol extraction and filter-paper ionophoresis as above. A spot much more intense than the PCA control spot was found in the same position. Glutamine was determined by estimating carboxyl N on the heated and unheated juice (procedure of Syngé, 1951. Cf. Neuberger & Sanger, 1942; Hamilton, 1945). Carboxyl N was 41.8% of total N originally, 30.5% after heating. This corresponds to glutamine N 22.6% of total N (cf. Syngé, 1951), and to the formation of PCA amounting to 11.3% of the total N of the grass juice.

SUMMARY

1. Observations on ionophoresis and partition chromatography of pyrrolidone carboxylic acid (PCA) are reported. PCA is conveniently detected by the procedure of Rydon & Smith (1952). PCA was detected in, and partially isolated from, Italian rye grass that had been stored at -20°.

2. In extracts of fresh grass the amounts of PCA are negligible compared with those of other forms of 'bound amino acids'.

3. Methods hitherto used for detecting and determining PCA are reviewed and possible improvements are suggested.

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