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Enzymic Interconversion of Δ'-Pyrroline-5-carboxylic Acid and Related Amino Acids. I. A Modified Method of Determination of Δ'-Pyrroline-5-carboxylic Acid

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As shown by Strecker and other investigators,[2-11] Δ'-pyrroline-5-carboxylic acid is an intermediate in the interconversion of glutamate, ornithine and proline. Enzymic activities such as ornithine δ-aminotransferase[11] and proline oxidase[10] are determined by the color reaction of *o*-aminobenzaldehyde with pyrroline-5-carboxylate[12] formed in the incubated mixtures. Trichloroacetic acid is combined with the *o*-aminobenzaldehyde reagent to stop enzymic activity and to remove protein from the reaction mixture.[7]

o-Aminobenzaldehyde in alcoholic trichloroacetic acid solution is very unstable in the light and it is necessary either to prepare the reagent immediately before use[7] or to keep it in darkness. A method of stabilizing the *o*-aminobenzaldehyde reagent in alcoholic trichloroacetic acid solution by adding sodium acetate is reported in the present paper.

As shown in Fig. 1, addition of sodium acetate slightly shifted the absorption maximum to a shorter wave length. The millimolar extinction coefficients obtained from the old and new reagents using a newly synthesized authentic preparation of pyrroline-5-carboxylate are shown in Table I. The reagent containing sodium acetate gave a somewhat higher value than the one without it. Strecker had reported previously that the millimolar extinction coefficient of the reaction product of *o*-aminobenzaldehyde with a relatively pure preparation of pyrroline-5-carboxylate was 2.14 at 430 mμ.[8] In a following paper, it was calculated

Table I. Millimolar Extinction Coefficients of Newly Synthesized Authentic Preparation of Pyrroline-5-carboxylate

	Reagent without sodium acetate	Reagent with sodium acetate
Millimolar extinction coefficient	2.817	2.918
λ_{max}	445 mμ	438 mμ
Standard deviation	0.017 ($n=5$)	0.046 ($n=10$)
Coefficient of variation	±0.6%	±1.6%

The procedure is in the text.

1) Location: *1-8, Kandasurugadai, Chiyoda, Tokyo, 101, Japan.*
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12) The abbreviation used is: pyrroline-5-carboxylate, Δ'-pyrroline-5-carboxylic acid.

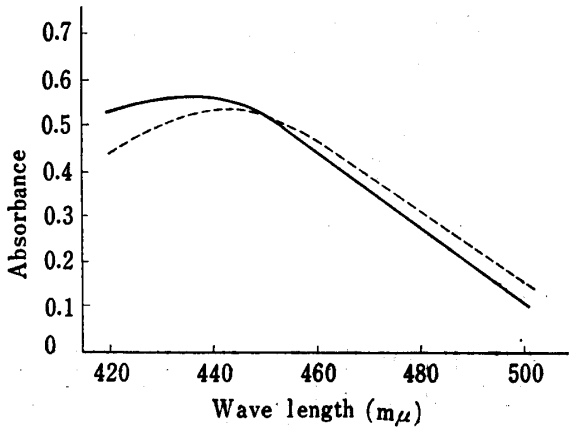

Fig. 1. The Absorption Spectra of the Reaction Mixtures from Pyrroline-5-carboxylate and *o*-aminobenzaldehyde Reagents

The procedure is in the text.
-------: Reagent without sodium acetate
———: Reagent with sodium acetate

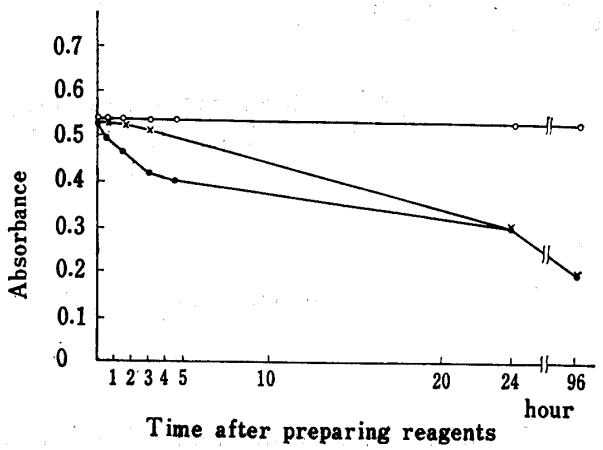

Fig. 2. Absorption of Pyrroline-5-carboxylate with *o*-Aminobenzaldehyde Reagents Prepared at Several Time Intervals

The procedure is in the text.
—●—: Reagent without sodium acetate was kept without shading from light. Wave length is 445 mμ.
—×—: Reagent without sodium acetate was kept in the dark. Wave length is 445 mμ.
—○—: Reagent with sodium acetate was kept without shading from light. Wave length is 438 mμ.

from the enzyme reaction with ornithine δ-aminotransferase that the value was 2.71 at 443 mμ.[11] The values in the present paper are greater than the values which reported by Strecker. We have no explanation for the differences.

When the *o*-aminobenzaldehyde reagent which lacked sodium acetate was kept without shading from light for 30 minutes or more, the color value of the reaction product decreased. When it was kept in the dark, stability was maintained for several hours but nevertheless gave only two-thirds of the initial value with pyrroline-5-carboxylate after keeping for 24 hours. On the other hand, the reagent containing sodium acetate kept in the room light did not change after 24 hours and even after several days. These results are shown in Fig. 2.

It is concluded from these observations that sodium acetate in the reagent protects the *o*-aminobenzaldehyde reagent from the deteriorating effect of light and perhaps also from other unknown chemical effect such as oxidation or polymerization during the storage of the reagent for fairly long period. The intensity of the color reaches the maximum within 20 minutes after adding the new reagent to the pyrroline-5-carboxylate solution, and does not change for 2 hours or more.

Table II. Results of Assay of Ornithine δ-Aminotransferase

	Reagent without sodium acetate	Reagent with sodium acetate
Absorbance	0.443	0.446
	0.440	0.460
	0.438	0.445
	0.430	0.448
	0.438	0.451
Average	0.436	0.456
Standard deviation	0.00415	0.0072

Two kinds of reagents were added to similar mixtures of enzyme reaction.
The procedure is in the text.

The usefulness of the reagent for assay in an enzymic reaction was tested by adding to incubation mixtures in order to determine ornithine δ-aminotransferase in tissue homogenates.[10] The results are shown in Table II. Sodium acetate did not appear to have any adverse effect on the capacity of trichloroacetic acid to stop the enzymic reaction and clarify the reaction mixture.

Thus alcoholic *o*-aminobenzaldehyde solution with trichloroacetic acid and sodium acetate appears to be a useful reagent for determining pyrroline-5-carboxylate and the activities of enzymes forming this product. A millimolar extinction coefficient for the reaction product of 2.91 at 438 mμ should be used in calculating these activities.

Experimental

Materials——Pyrroline-5-carboxylate was prepared as previously reported by Strecker.[8,10] In the work described in the present report, freshly synthesized pyrroline-5-carboxylate was used as a aqueous solution. *o*-Aminobenzaldehyde reagent was freshly prepared as described by Smith, *et al.*[13] from *o*-nitrobenzaldehyde, which in turn was prepared according to Tsang, *et al.*[14] The components of the reagents follow:

Reagent I (wihthout sodium acetate):
0.5% *o*-aminobenzaldehyde, 5% trichloroacetic acid in ethyl alcohol
Reagent II (with sodium acetate):
0.5% *o*-aminobenzaldehyde, 5% trichloroacetic acid and 1.5% sodium acetate in ethyl alcohol

Color Reaction of *o*-Aminobenzaldehyde Reagent with Authentic Pyrroline-5-carboxylate——Two ml of an aqueous solution of pyrroline-5-carboxylate were mixed with 2 ml of *o*-aminobenzaldehyde reagent. After standing for 30 minutes at room temperature, the absorption spectra of the reaction mixture was determined with the Hitachi Photoelectric Spectrophotometer Type EPS, Type EPU was used for other determinations using cuvettes of 10 mm light path. Reference solutions were prepared by mixing 2 ml of water and 2 ml of *o*-aminobenzaldehyde reagent.

Enzyme Assay——Rat liver was homogenized with 0.25 M sucrose, pH 7, and soniciated. Each incubation mixture contained 70 μ moles of L-ornithine, 7.5 μ moles of α-ketoglutaric acid, 100 μ moles of potassium phosphate (pH 7.1) and the enzyme solution in a total volume of 2 ml. The mixtures were incubated with shaking for 30 minutes at 37°, and 2 ml of *o*-aminobenzaldehyde reagents were added and mixed. After 30 minutes the mixtures were centrifuged and the absorptions of the supernatant were measured at each absorption maximum.

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