Short Communication

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Enzymatic Formation of (–)-Citramalate from Acetyl Coenzyme A and Pyruvate in Respiration-deficient *Saccharomyces carlsbergensis*

Sir:

The formation or metabolism of dextro-rotatory (+)-citramalic acid has been reported in *Clostridium tetanomorphum*¹⁾ and *Pseudomonas fluorescens*,²⁾ respectively. On the other hand, the enzymatic occurrence of its optical antipode, *i.e.* laevo-rotatory (–)-citramalic acid has been described only as an intermediate in the metabolism of citraconate by a pseudomonad.^{3,4)}

In the previous papers,^{5~7)} it was reported that respiration-deficient mutants of *Saccharomyces* species accumulated a large amount of laevo-rotatory (–)-citramalic acid in their culture broths. The present communication concerns with the enzymatic formation of laevo-rotatory (–)-citramalic acid by aldol condensation between acetyl coenzyme A and pyruvate as shown in the following formula.

Respiration-deficient mutant of *Saccharomyces carlsbergensis* strain A-1 was grown on glucose in the chemically defined medium as described in the previous paper⁵⁾ except that 0.5% of urea was used instead of ammonium sul-

fate. The cells incubated for 90 to 96 hr at 30°C were harvested by centrifugation, washed thrice with 0.05 M $\text{KH}_2\text{PO}_4\text{--Na}_2\text{HPO}_4$ buffer of pH 7.3 and disrupted with French presser (Ohtake Works Ltd.) in the same buffer containing 5 mM of 2-mercaptoethanol. Cell-free enzyme preparation was obtained by removing cell debris by centrifugation at $20,000\times g$ and by dialysing it against the above buffer for 12 hr at 4°C.

The reaction mixture contained Tris-HCl buffer (pH 7.3), 200 μmoles ; MgCl_2 , 20 μmoles ; reduced glutathione, 20 μmoles ; pyruvate, 10 μmoles ; acetyl coenzyme A, 1.3 μmoles (0.4 μCi) and 0.1 ml of dialyzed cell-free extract (7.5 mg of protein) in a total volume of 2.0 ml. $1\text{-}^{14}\text{C}$ -Acetyl coenzyme A was prepared from $1\text{-}^{14}\text{C}$ acetic anhydride (The Radiochemical Centre, Amersham) and coenzyme A (Sigma Chemical Co.) by the method of Simon and Shemin.⁸⁾ Reaction was run for 60 min at 30°C and stopped by the addition of 1.0 ml of 1 N HCl. After added with authentic citramalic acid, an aliquot of reaction mixture

1) A. H. Blair and H. A. Barker, *J. Biol. Chem.*, **241**, 400 (1966).

2) H. Katsuki, N. Ariga, F. Katsuki, J. Nagai, S. Egashira and S. Tanaka, *Biochim. Biophys. Acta*, **56**, 545 (1962).

3) M. R. Raghavendra Rao, S. S. Subramanian, H. I. Rahatekar and S. V. Paranjape, *Biochem. Biophys. Res. Commun.*, **12**, 78 (1963).

4) S. S. Subramanian and M. R. Raghavendra Rao, *J. Biol. Chem.*, **243**, 2367 (1968).

5) T. Sai and M. Amaha, *J. Gen. Appl. Microbiol.*, **11**, 355 (1965).

6) T. Sai and M. Amaha, *ibid.*, **13**, 15 (1967).

7) T. Sai, *Bulletin of Brew. Sci.*, **12**, 35 (1966).

8) E. J. Simon and D. Shemin, *J. Am. Chem. Soc.*, **75**, 2520 (1953).

FIG. 1. Enzymatic Formation of Citramalic Acid from 1-¹⁴C-Acetyl Coenzyme A and Pyruvate.

(A) A paper chromatogram monitored by the use of a paper chromatogram scanner.

(B) A paper chromatogram developed with a solvent system in butanol-acetic acid-water (upper layer of 52:14:34, v/v/v) and sprayed with bromophenol blue.

(0.05 ml) was applied to paper chromatography. The radioactivity of the developed paper chromatogram was monitored with paper chromatogram scanner (Nihon Musen Co. Ltd.). As shown in Fig. 1, radioactivity appeared in the same position as that of an authentic citramalic acid.

The resulting radioactive citramalic acid isolated by paper chromatography was diluted with carrier authentic DL-citramalic acid and recrystallized from ethyl acetate-ligroin to constant radioactivity. No change of radio-

activity was observed by the subsequent recrystallization. The labelled citramalate was not formed, when boiled enzyme was used or pyruvate was omitted from the reaction mixture.

Furthermore, the recrystallized radioactive citramalic acid was degraded according to the following reaction sequences in the same manner as described by Strassman and Ceci.⁹⁾ Carbon dioxide formed in the second decarboxylation reaction represents the β -carboxyl group of citramalic acid molecule. Whole

9) M. Strassman and L. N. Ceci, *J. Biol. Chem.*, **238**, 7 (1963).

TABLE I. OPTICAL NATURE OF ENZYMATICALLY FORMED CITRAMALIC ACID

Substance	Total Radioactivity**	Specific Activity
DL-Citramalic acid*	13,900 dpm	139 dpm/mg
(-)-Citramalic acid fraction (Solution)	15,020	—
(+)-Citramalic acid fraction (Crystal)	—	1

* The recrystallized radioactive citramalic acid (100 mg, 0.68 mmoles, 13,900 dpm) and brucine hydrate (125 mg, 0.32 mmoles) are dissolved in 1 ml of water.

** Radioactivity was measured with a Packard Tri-Carb Liquid Scintillation Counter (Packard Instruments Co., LaGrange, Ill.). Samples (1.0 ml in Water) were placed into vials containing 0.2 ml of hyamine 10-X (hydroxide) and 10 ml of liquid scintillator: 10% naphthalene, 1% PPO (2,5-diphenyloxazole), and 0.025% POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene) in dioxane.

radioactivity of labelled citramalic acid was recovered only by decarboxylation of β -carboxyl group and at the same time acetone formed by reaction (3) gave its 2,4-dinitrophenylhydrazone of no radioactivity.

Whether the enzymatic product of this condensation reaction is (-)- or (+)-citramalic acid, was then determined as follows. Optical resolution of the enzymatically formed radioactive citramalic acid mixed with optically inactive DL-acid was carried out according to Barker's brucine method.¹⁰⁾ The recrystallized radioactive citramalic acid (0.68 mmoles) together with brucine hydrate (0.32 mmoles) was dissolved in 1 ml of boiling water. After this clear solution was allowed to stand overnight at 5°C, crystals of the (+)-acid brucine salt which showed no radioactivity was obtained because of its lower solubility and the remaining filtrate solution contained all of the (-)-acid in which the radioactivity entirely resided as shown in Table I. Since all the radioactivity was found only in the filtrate solution, i.e. in (-)-citramalic acid fraction, the enzymatically formed citramalic acid is well assessed as its laevo-rotatory enantiomorph.

The enzymatic formation of citramalic acid from pyruvate and acetyl coenzyme A is known in yeast¹¹⁾ or in *Pseudomonas ovalis* Chester.¹²⁾ However, in those cases, the

optical nature of the formed citramalic acid was not determined. From the above-described experimental results, it was concluded that an enzyme from respiration-deficient mutant of *Saccharomyces carlsbergensis* could produce laevo-rotatory (-)-citramalic acid by aldol condensation of acetyl coenzyme A and pyruvate. It is conceivable that the formation of (-)-citramalic acid by respiration-deficient mutant of *Saccharomyces* species due to this enzyme and the chemical pathway of its formation may be different from those of (+)-acid in mesaconase system participating in both glutamate and itaconate dissimilation.

It should be further clarified whether primary reaction product of enzymatic condensation of acetyl coenzyme A to pyruvate is free (-)-citramalic acid or (-)-citramalyl coenzyme A, i.e. the formation of citramalic acid is catalyzed by a single enzyme or by two separate enzymes via citramalyl coenzyme A as an intermediate as shown in formula (1).

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10) H. A. Barker and A. H. Blair, *Biochem. Preparations*, **9**, 25 (1962).

11) M. Losada, J. L. Canovas and M. Ruiz-Amil, *Biochem. Z.*, **340**, 60 (1964).

12) C. T. Gray and H. L. Kornberg, *Biochim. Biophys. Acta*, **42**, 371 (1960).

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