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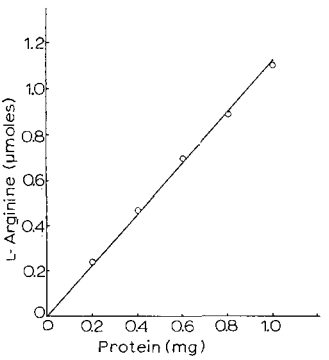
Occurrence of arginine racemase in bacterial extract

Since WOOD AND GUNSALUS[1] discovered and isolated an enzyme racemizing alanine from *Streptococcus faecalis*, several amino acid racemases including glutamate racemase[2–4] and lysine racemase[5,6] have been found in bacteria. Neither the natural occurrence of D-arginine nor the existence of arginine racemase has, however, been demonstrated. The experiments reported here are concerned with evidence for the occurrence of a bacterial enzyme catalyzing the conversion of either D- or L-arginine to the racemate.

Pseudomonas graveolens IFO 3460 was grown at 30° in a medium containing 0.5% glucose, 0.2% D-arginine·HCl, 0.01% $MgSO_4 \cdot 7\,H_2O$, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.1% NaCl and 0.01% yeast extract (pH 7.2, D-arginine medium). For the production of large quantities of cells, they were cultivated in a medium composed of 1.0% peptone, 0.2% yeast extract, 0.5% NaCl and 0.1% K_2HPO_4. The pH was adjusted to 7.2. The cultures were grown at 30° under aeration for about 24 h. The cells were harvested by centrifugation and washed twice with 0.85% NaCl solution. The washed cells were suspended in 0.01 M Tris–HCl buffer (pH 8.0) and subjected to sonication in a 19 Kc Kaijo Denki oscillator. The supernatant, dialyzed against 4 mM Tris–HCl buffer (pH 8.0), was used as a cell-free extract. The racemase activity was assayed by determining L-arginine formed from the D isomer with arginase.

Approximately 15% of the D-arginine added to the D-arginine medium was



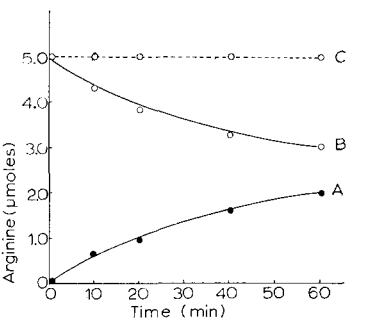


Fig. 1. L-Arginine formation as a function of amount of enzyme. The reaction mixture contained 10 μmoles D-arginine·HCl, 50 μmoles Tris–HCl buffer (pH 8.0) and enzyme in a final vol. of 1.0 ml. Incubation was carried out at 37° for 20 min. The reaction was stopped by immersing the tubes in boiling water for 3 min. After cooling, 250 μmoles glycine–NaOH buffer (pH 10.2) and 2.5 mg of crude arginase prepared from beef liver were added to a 1-ml aliquot of the reaction mixture in a total vol. of 2.5 ml. After incubation at 37° for 40 min, 2.5 ml of 10% trichloroacetic acid was added to the mixture. A 1-ml aliquot of the deproteinized supernatant was employed to determine urea formed from L-arginine with diacetylmonoxime according to the modified method of ARCHIBALD[7].

Fig. 2. Relationship between incubation time and racemization of arginine. Curve A: The reaction mixture consisted of 5.0 μmoles D-arginine, 50 μmoles Tris–HCl buffer (pH 8.0) and 1.3 mg protein in a final vol. of 1.0 ml. After incubation at 37°, L-arginine converted from the substrate was determined as described in Fig. 1. Curve B: D-Arginine was replaced by the L isomer in the reaction mixture of Curve A. Curve C: Total arginine was determined colorimetrically according to the method of ROSENBERG, ENNOR AND MORRISON[8] in both experiments.

converted to the L enantiomorph during growth of the bacteria for 30 h. When D-arginine was incubated with the cell-free extract at pH 8.0, the formation of L-arginine from the substrate proceeded as a function of amount of enzyme and incubation time (Fig. 1 and Curve A in Fig. 2). The total amount of arginine remained constant and neither ornithine nor citrulline was formed under the conditions employed, although L-arginine was also converted to the D isomer at the same rate (Curves B and C in Fig. 2). The boiled enzyme showed no activity. The enzymatic conversion ratio of either isomer never exceeded 50%. These findings indicate that the conversion of an optically active arginine to its enantiomorph is due essentially not to an isomerase, but to a racemase. It is known, however, that amino acids may be racemized indirectly by the combined actions of D- and L-amino-acid transaminases and alanine racemase (or another amino acid racemase) as follows:

D-Arginine + pyruvate $\rightleftharpoons$ D-alanine + α-keto-δ-guanidinovalerate
D-Alanine $\rightleftharpoons$ L-alanine
L-Alanine + α-keto-δ-guanidinovalerate $\rightleftharpoons$ L-arginine + pyruvate

Sum: D-Arginine $\rightleftharpoons$ L-arginine

Alanine and glutamate racemases were assayed by incubating 40 μmoles L-alanine or sodium D-glutamate, respectively, 100 μmoles Tris–HCl buffer (pH 8.0) with 1.3 mg protein in a total vol. of 2.0 ml. After incubation at 37° for 20 min, D-alanine or L-glutamate was determined manometrically with D-amino-acid oxidase or L-glutamate decarboxylase, respectively. No L-glutamate was detected and

TABLE I

EFFECT OF PYRUVATE AND α-KETOGLUTARATE ON THE ENZYMATIC RACEMIZATION OF ARGININE

Standard additions included 10 μmoles D- or L-arginine, 8 μmoles pyruvate or α-ketoglutarate, 40 μmoles Tris–HCl buffer (pH 8.0) and 1.2 mg protein to a final vol. of 1.0 ml. Glutamate and alanine were determined by reaction with ninhydrin, after separation with circular paper chromatography[9].

Addition	*Glutamate (μmoles)*	*Alanine (μmoles)*	L-*Arginine (μmoles)*
L-Arginine, α-ketoglutarate	0	—	—
D-Arginine, α-ketoglutarate	0	—	1.39
L-Arginine, pyruvate	—	0	—
D-Arginine, pyruvate	—	0	1.40
D-Arginine	—	—	1.40

4.3 μmoles of D-alanine was formed. However, none of the activities of L-arginine:α-ketoglutarate transaminase, L-arginine:pyruvate transaminase, D-arginine:α-ketoglutarate transaminase and D-arginine:pyruvate transaminase was found and the arginine racemization was not stimulated by the addition of either pyruvate or α-ketoglutarate as shown in Table I. These results exclude the possibility of racemization *via* the combined reactions, and present good evidence that this reaction may be ascribed to arginine racemase. The addition of pyridoxal phosphate to the reaction

system caused a slight increase in the activity and stability of the enzyme, but further work is needed to elucidate the role of the cofactor.

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1 W. A. WOOD AND I. C. GUNSALUS, *J. Biol. Chem.*, 190 (1951) 403.
2 P. AYENGAR AND E. ROBERTS, *J. Biol. Chem.*, 197 (1952) 453.
3 S. A. NARROD AND W. A. WOOD, *Arch. Biochem. Biophys.*, 35 (1952) 462.
4 M. TANAKA, Y. KATO AND S. KINOSHITA, *Biochem. Biophys. Res. Commun.*, 4 (1961) 114.
5 H. T. FUANG, D. A. KITA AND J. W. DAVISSON, *J. Am. Chem. Soc.*, 80 (1958) 1006.
6 A. ICHIHARA, S. FURIYA AND M. SUDA, *J. Biochem.*, 48 (1960) 277.
7 R. M. ARCHIBALD, *J. Biol. Chem.*, 156 (1944) 121.
8 H. ROSENBERG, A. H. ENNOR AND J. F. MORRISON, *Biochem. J.*, 63 (1956) 153.
9 H. KATAGIRI, K. SODA AND T. TOCHIKURA, *J. Agr. Chem. Soc. Japan*, 34 (1960) 814.

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Inhibition of alanine racemase by aminoxyacetic acid

During comparative investigations of the inhibitory potency of various substrate analogs against the bacterial enzyme alanine racemase (EC 5.1.1.1) the compound aminoxyacetic acid has demonstrated unusually high activity relative to that of all other inhibitors tested. The compound previously has been shown to act as a potent competitive inhibitor of the enzymes aminobutyrate aminotransferase (EC 2.6.1.19) (ref. 1) and alanine aminotransferase (EC 2.6.1.2) (ref. 2). It also strongly inhibits glutamate decarboxylase (EC 4.1.1.15) (ref. 3). The mechanism of inhibition of alanine racemase by aminoxyacetate is of particular interest inasmuch as each of the above enzymes utilizes pyridoxal phosphate as a cofactor.

Alanine racemase was obtained from a Pseudomonas sp., Squibb Culture 3550, grown in the presence of 0.5% L-alanine. The enzyme was purified approx. 150-fold from a pH 8.1 extract of an acetone powder of the cells. The pH optimum of the enzyme in 0.10 M Tris–HCl, 0.10 M L-alanine buffer is from pH 8.9 to 9.5 (25°). The specific activity of the purified enzyme was approx. 2500 units/mg, the unit being defined as that amount of enzyme required to produce 1 μmole of D-alanine per min at 37° in 0.10 M L-alanine at pH 8.9. Although the purified enzyme does not require addition of pyridoxal phosphate for activity, low levels of the cofactor improve the stability of the enzyme in assay mixtures.

Kinetic assays were performed at 37° in 0.10 M Tris–HCl, pH 8.9 (25°), containing $5 \cdot 10^{-5}$ M pyridoxal phosphate. In inhibition experiments involving hydroxylamine, the pyridoxal phosphate was omitted from the assay mixtures due to a rapid