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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## 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 $\mu$mole 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

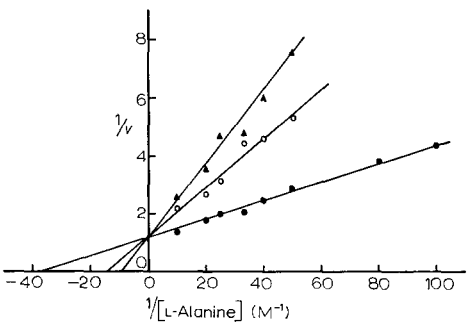
reaction between the inhibitor and cofactor. L-Alanine at pH 8.9 was used as substrate. D-Alanine formed during the reactions was determined by a modification of the method of DIVEN, SCHOLZ AND JOHNSTON[4], in which D-alanine is quantitatively converted to pyruvate by incubation with D-amino-acid oxidase (EC 1.4.3.3). Pyruvate was determined by reaction with 2,4-dinitrophenylhydrazine.

Aminoxyacetic acid hemihydrochloride was synthesized according to the method of ANKER AND CLARKE[5].

In Fig. 1 is shown a double-reciprocal plot for a series of aminoxyacetate inhibition experiments with alanine racemase. The Michaelis constant ($K_m$) for L-alanine calculated from the data is 0.03 M. The plots obtained from the two concentrations of aminoxyacetate indicate that the inhibition is competitive. The inhibition constants ($K_i$) calculated from aminoxyacetate concentrations of $0.69 \cdot 10^{-6}$ M and $1.4 \cdot 10^{-6}$ M are $4 \cdot 10^{-7}$ M and $5 \cdot 10^{-7}$ M, respectively.

For purposes of comparison the inhibitory potency of hydroxylamine against alanine racemase was determined from the data plotted in Fig. 2. The $K_i$ calculated at each of the three inhibitor concentrations is $1 \cdot 10^{-5}$ M. As in the experiments with aminoxyacetate, the inhibition is competitive. ROZE AND STROMINGER[6] previously have determined a $K_i$ of $1.2 \cdot 10^{-5}$ M for hydroxylamine against alanine racemase obtained from *Staphylococcus aureus*. In the same study, a $K_i$ of $5 \cdot 10^{-5}$ M was reported for D-cycloserine, a well-known competitive inhibitor of the enzyme.

Aminoxyacetic acid, like hydroxylamine, readily forms oxime derivatives with carbonyl compounds[7]. Previous investigations have shown that the inhibitory actions of aminoxyacetate on alanine aminotransferase[2] and of hydroxylamine on aspartate



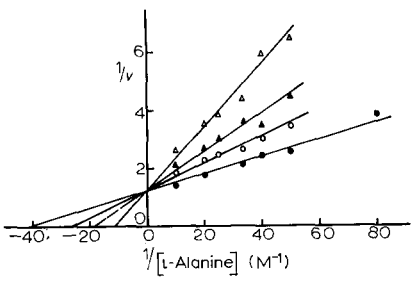




Fig. 1. Lineweaver–Burk plot of velocities against L-alanine concentration at fixed concentrations of aminoxyacetate. Assay mixtures contained 0.10 M Tris–HCl, pH 8.9, 50 $\mu$M pyridoxal phosphate, 0.05 unit alanine racemase per ml and inhibitor concentrations as indicated. The mixtures were preincubated for 3 min at 37°, after which reactions were initiated by substrate addition. Linear reaction rates were determined from 1-ml aliquots withdrawn at 5, 10 and 15 min following substrate addition. Although a brief initial lag in the reaction velocity has been observed in the mixtures containing aminoxyacetate, the reaction becomes zero order prior to the first sampling at 5 min. ●—●, reactions conducted in the absence of inhibitor; ○—○, reactions in 0.69 $\mu$M aminoxyacetate; ▲—▲, reactions in 1.4 $\mu$M aminoxyacetate.

Fig. 2. Lineweaver–Burk plot of velocities against L-alanine concentration at fixed concentrations of hydroxylamine. Assay mixtures contained 0.10 M Tris–HCl, pH 8.9, 0.085 unit alanine racemase per ml and inhibitor concentrations as indicated. The mixtures were preincubated for 2 min at 37°, after which reactions were initiated by substrate addition. Linear reaction rates were determined from 1-ml aliquots withdrawn at 4, 7 and 10 min following substrate addition. ●—●, reactions conducted in the absence of inhibitor; ○—○, reactions in 7.2 $\mu$M hydroxylamine; ▲—▲, reactions in 14.4 $\mu$M hydroxylamine; △—△, reactions in 21.6 $\mu$M hydroxylamine.

aminotransferase (EC 2.6.1.1) (ref. 8) are competitive only with the amino acid substrates. In the latter study, moreover, hydroxylamine reacted only with the phosphopyridoxal, and not the phosphopyridoxamine, form of the enzyme. These results suggest that inhibition by aminoxyacetate and hydroxylamine may occur *via* reversible formation of oximes with enzyme-bound pyridoxal phosphate. To test the possibility of aminoxyacetate inhibition of alanine racemase *via* formation of the oxime with free pyridoxal phosphate in solution rather than on the enzyme surface, the carboxymethoxime of pyridoxal phosphate was prepared and found inactive as an inhibitor at a concentration of 0.002 M.

Alanine racemase has a 20-fold greater affinity for aminoxyacetate than for hydroxylamine, as indicated by comparison of the respective $K_i$ values. Because aminoxyacetate is active against a racemase in addition to at least two aminotransferases and a decarboxylase, the possibility of inhibitory activity of the compound against other pyridoxal phosphate enzymes is indicated.

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