STUDIES ON THE INHIBITION OF D-ALANYL-D-ALANINE SYNTHETASE BY THE ANTIBIOTIC D-CYCLOSERINE[1]

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The inhibition of bacterial growth by D-cycloserine (D-4-amino-3-isoxazolidone) can be reversed by D-alanine (Bondi et al., 1957; Shockman, 1959; Morrison, 1962) and to a lesser extent by D-α-amino-n-butyric acid. Moreover, the antibiotic was shown to inhibit the incorporation of DL-alanine-1-C^{14} into the cell wall and total protein fraction of Escherichia coli (Barbieri et al., 1960a). When Staphylococcus aureus is grown in the presence of D-cycloserine, an accumulation of the uridine mucopeptide precursor which lacks D-ala-D-ala occurs (Ciak and Hahn, 1959; Strominger et al., 1959). It was subsequently found that the antibiotic inhibits D-ala-D-ala synthetase and D-alanine racemase from S. aureus (K_I = 2-4 x 10^{-5}M and 1.0 x 10^{-4}M respectively) (Strominger et al., 1960; Strominger, 1962). The D-alanine activating enzyme (Baddiley and Neuhaus, 1960), which may be involved in the introduction of D-alanine into teichoic acid, is not inhibited by D-cycloserine.

A number of transaminases (Azarkh et al., 1960; Barbieri et al., 1960b) have been found which are sensitive to both D- and

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L-cycloserine (e.g. L-glutamic-pyruvic transaminase from pig heart, $K_I \cong 10^{-3}$M D- and 8 x 10^{-6}M L-cycloserine)(Braunstein et. al., 1961). The inhibition of the alanine transaminase appears to involve specific binding of the L-cycloserine to the enzyme, Schiff base followed by oxime formation with the pyridoxal phosphate, and acylation of a cationic group in the active site (Braunstein et al., 1961; Khomutov et al., 1961; Polyanovskii and Torchinskii, 1961; Vyshepan et al., 1961).

D-ala-D-ala synthetase, purified 250-fold from sonic extracts of Streptococcus faecalis, was shown to catalyze a reaction with the stoichiometry (Neuhaus, 1960; Neuhaus, 1962a):

$$2\ \text{D-alanine} + \text{ATP} \xrightarrow{Mg^{++}\quad K^{+}} \text{D-ala-D-ala} + \text{ADP} + P_i$$

Kinetic studies in the absence of inhibitor are consistent with two successive reactions each of which is first-order with respect to D-alanine (Neuhaus, 1962b), i.e.

$$E + A \rightleftharpoons EA \qquad K_A \qquad (1)$$

$$EA + A \rightleftharpoons EAA \qquad K_{AA} \qquad (2)$$

$$EAA \longrightarrow EA\text{-}A \rightleftharpoons E + A\text{-}A$$

where EA and EAA are binary and ternary complexes of enzyme (E) and D-alanine (A)[2]. K_A is 6.6 x 10^{-4}M while K_{AA} is 0.01M (Mg^{++} and K^+). The inhibition by D-cycloserine and β-aminoxy-D-alanine methyl ester has been studied in an attempt to characterize further the two D-alanine binding sites. The specificity of the inhibition is described, and an attempt is made to distinguish between the two inhibitors.

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[2] The proposed sequence of reactions does not include a consideration of Mg^{++}, K^+, or ATP.

As shown in Table I only one analogue of D-cycloserine is active as an inhibitor. These results indicate that, in addition to the amino group in the D-configuration, the R-O-NH_2 or R-O-N = C(O-)-R of the 3-isoxazolidone ring are necessary for inhibition. In the absence of this latter group, e.g. D-serine methyl ester and D-serine amide, only a small inhibition is observed at higher concentrations.

TABLE I

Inhibitor Specificity

Compound	Inhibition	
	K_I[a]	%
1. D-4-Amino-3-isoxazolidone	9.0×10^{-5}	-
2. β-Aminoxy-D-alanine methyl ester[b]	3.1×10^{-4}	-
3. DL-Serine amide (0.02M D-)		28
4. D-Serine methyl ester (0.02M)		16
5. D-Serine (0.02M)[c]		3
6. 3-Isoxazolidone (0.02M)		+ 12 (stimulation)
7. 4-Iminoisoxazolidone (0.02M)		0
8. β-Aminoxy-propionic acid (0.02M)		29
9. L-4-Amino-3-isoxazolidone (0.001M)		0

Assay contained 0.01M $MgCl_2$; 0.05M Tris-HCl, pH 7.8; 0.01M ATP; 2.5mM glutathione; 0.01M D-alanine; 0.2M choline chloride; 0.05M KCl; inhibitor as specified; and 52 μg enzyme preparation/ml. Aliquots removed at 0, 5, 10, 15 min. and assayed for P_i by the method of Marsh (1959).

a. Apparent K_I determined from Dixon plots at three D-alanine concentrations (0.01, 0.04, and 0.10M).
b. β-Aminoxy-D-alanine ethyl ester (0.001M), 26% inhibition. No cycloserine formation from the methyl ester was detected during the incubation (cf. Vyshepan *et al.*, 1961).
c. In the presence of D-alanine and D-serine, the dipeptide D-ala-D-ser is formed in addition to D-ala-D-ala (Neuhaus, 1962a).

In contrast to the L-glutamic-pyruvic transaminase (Vyshepan et al., 1961; Polyanovskii and Torchinskii, 1961), 3-isoxazolidone. β-aminoxy-propionate (compare with γ-aminoxybutyrate) and H_2NOH (Neuhaus, 1962a) give no significant inhibition when tested at comparable concentrations. The degree of inhibition of the transaminases with cycloserine increased with the time of incubation (Braunstein et al., 1961) whereas with the D-ala-D-ala synthetase the inhibition with cycloserine and β-aminoxy-D-alanine methyl ester was instantaneous (i.e. within 30 seconds).

A Lineweaver-Burk treatment of the inhibition results with D-cycloserine and β-aminoxy-D-alanine methyl ester give reciprocal plots with common intercepts. A further analysis[3] of these data, however, reveals a significant difference between the two inhibitors. As shown in Fig. 1, A when [D-alanine] x $[1/v - 1/V_{max}]$ is plotted against 1/[D-alanine] at varying levels of D-cycloserine, an intercept-slope change is observed while with β-aminoxy-D-alanine methyl ester (Fig. 1, B) a slope change with common inter-

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[3]An equilibrium treatment of the reaction sequence (1, 2), which assumes ordered binding with Michaelis constants characteristic for each reaction, gives the following reciprocal velocity expression (Neuhaus, 1962b):

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_{AA}}{[A]V_{max}} + \frac{K_A K_{AA}}{[A]^2 V_{max}} \qquad 1$$

Rearrangement of this equation gives:

$$[A]\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}} + \frac{K_A K_{AA}}{[A]V_{max}} \qquad 2$$

Addition of inhibitor(I) to both sites, i.e.,

$$E + I \rightleftharpoons EI, \quad EA + I \rightleftharpoons EAI$$

results in a predicted intercept-slope change in Equation 2 whereas if only $E + I \rightleftharpoons EI$ occurs, then a slope change is predicted with a constant intercept. A complete derivation of the equations and a consideration of other possible reactions, e.g.,

$$EI + I \rightleftharpoons EII, \quad EI + A \rightleftharpoons EIA$$

will be published elsewhere.

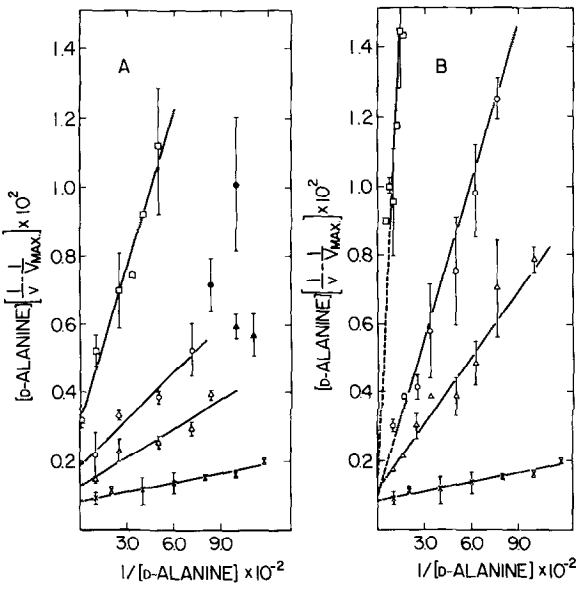

Fig. 1. Inhibition analysis of D-cycloserine(A) and β-aminoxy-D-alanine methyl ester(B).

Assay (260 μg protein/ml) is described in Table I, x-x control. For A-: Δ-Δ, 5 x 10^{-5}M; o-o, 1 x 10^{-4}M; □-□, 3 x 10^{-4}M. For B: Δ-Δ, 3 x 10^{-4}M; o-o, 8 x 10^{-4}M; □-□, 3 x 10^{-3}M. Average deviations calculated for points where two or more determinations were performed. Lines drawn according to least squares treatment (open points only).

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cept is found. These observations are consistent with the suggestion that both D-alanine sites bind D-cycloserine whereas with β-aminoxy-D-alanine methyl ester only one site binds inhibitor. Inhibition by the product D-ala-D-ala in the presence of Mg^{++} gives results similar to those observed with β-aminoxy-D-alanine methyl ester.

The present studies provide a preliminary definition of the inhibition specificity of the D-ala-D-ala synthetase by the antibiotic D-cycloserine and distinguish between the inhibition by D-cycloserine and β-aminoxy-D-alanine methyl ester.

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