Biochemistry VOLUME 3, NUMBER 4

April 10, 1964

The Enzymatic Synthesis of D-Alanyl-D-alanine. III. On the Inhibition of D-Alanyl-D-alanine Synthetase by the Antibiotic D-Cycloserine*

FRANCIS C. NEUHAUS AND JUDY L. LYNCH

Department of Chemistry, Northwestern University, Evanston, Illinois

Received November 4, 1963

Inhibitor studies indicate that both D-alanine(A) binding sites of D-alanyl-D-alanine synthetase are sensitive to D-4-amino-3-isoxazolidone (D-cycloserine [I]). The K_I for the reaction E + I $\rightleftharpoons$ EI is 2.2×10^{-5} M, whereas K_{AI} for the reaction EA + I $\rightleftharpoons$ EAI is 1.4×10^{-4} M. The inhibition is competitive with respect to D-alanine, instantaneous, and completely reversible. The zwitterion of D-cycloserine is the active form of the inhibitor. Specificity studies with analogs of D-cycloserine have demonstrated the features of the molecule which contribute to the inhibitory activity and some of the modifications which can be made in the molecule and which still retain this activity. For example, *cis*-5-methyl substitution (*cis*-5-methyl-D-4-amino-3-isoxazolidone) affects the binding of antibiotic at the donor site ($K_I = 1.2 \times 10^{-4}$ M), but it has little effect at the acceptor site ($K_{AI} = 1.9 \times 10^{-4}$ M). For *trans*-5-methyl substitution (*trans*-5-methyl-D-4-amino-3-isoxazolidone) K_I is 5.4×10^{-4} M and K_{AI} is 5.6×10^{-4} M. In addition to D-alanyl-D-alanine, D-alanyl-D-threonine and D-alanyl-D-allothreonine can be isolated when D-alanine and the corresponding amino acid are incubated with the synthetase, ATP, Mg^{2+}, and K^+. A correlation is observed between the effectiveness of D-allothreonine as an acceptor and *cis*-5-methyl-D-4-amino-3-isoxazolidone as an inhibitor, and of D-threonine as an acceptor and *trans*-5-methyl-D-4-amino-3-isoxazolidone as an inhibitor.

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). It was suggested by Park (1958) that the antibiotic is a structural analog of D-alanine and might "prevent the normal incorporation of D-alanine into the wall." Barbieri *et al.* (1960) demonstrated that D-cycloserine inhibits the incorporation of DL-alanine-1-^{14}C into the cell wall and protein fractions of *Escherichia coli*.

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). The biosynthesis of the cell wall mucopeptide precursor proceeds by the stepwise addition of amino acids to uridine diphosphate *N*-acetylglucosamine-3-*O*-lactic acid ether (Ito and Strominger, 1962a). However, the terminal dipeptide moiety D-ala-D-ala is added as a unit (Ito and Strominger, 1962b; Strominger, 1962b; Comb, 1962). The dipeptide intermediate was shown to accumulate as a major product in *Streptococcus faecalis* which was grown in a medium deficient in vitamin B_6 supplemented with D-alanine-1-^{14}C (Ikawa and Snell, 1958).

The biosynthesis of D-ala-D-ala is catalyzed by the enzyme D-ala-D-ala synthetase (Strominger, 1962a; Neuhaus, 1962a) as shown in the reaction:

$$2\ \text{D-alanine} + \text{ATP} \xrightarrow[K^+]{Mg^{2+}} \text{D-ala-D-ala} + \text{ADP} + P_i \quad (1)$$

This enzyme has been found in *S. faecalis* (Neuhaus, 1962a), *E. coli* (Comb, 1962), and *S. aureus* (Strominger, 1962a), and has been extensively purified from *S. faecalis* strain R 8043 (Neuhaus, 1962a). Strominger and co-workers found that the antibiotic inhibits the D-ala-D-ala synthetase and D-alanine racemase from *S. aureus* (Strominger *et al.*, 1960). In *E. coli* D-ala-D-ala synthetase appears to be a major site of the inhibitory action of D-cycloserine (Chambers *et al.*, 1963).

Kinetic and specificity studies on the D-ala-D-ala synthetase from *S. faecalis* provided evidence for two D-alanine-binding sites which have different specificity patterns and Michaelis constants (Neuhaus, 1962a,b). It is the purpose of this communication to examine some of the requirements for antibiotic inhibition on each binding site, to evaluate the inhibitor-binding constants, and to compare the specificity of growth inhibition with the enzyme-specificity studies. It is suggested that one of the primary sites of antibiotic action may be the donor site of the synthetase.

* Supported in part by a grant (AI-4615) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by a Public Health Service training grant (5 TI GM-626) from the Division of General Medical Sciences, U. S. Public Health Service, and by grants from the Graduate School Research Fund of Northwestern University. Preliminary reports have been presented (Neuhaus and Lynch, 1962, 1963).

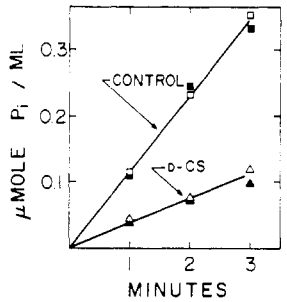


FIG. 1.—Effect of preincubation on the inhibition by D-cycloserine (D-CS). The "P_i assay" contained 110 μg of enzyme preparation per ml. The control (□—□) and the preincubated control tests (■—■) were initiated with 0.04 M D-alanine. In the experimental tests (▲—▲) the enzyme and all components were preincubated at 37° with 1×10^{-3} M D-cycloserine for 10 minutes before the addition of D-alanine (0.04 M). For Δ—Δ the D-cycloserine and D-alanine were added at zero time.

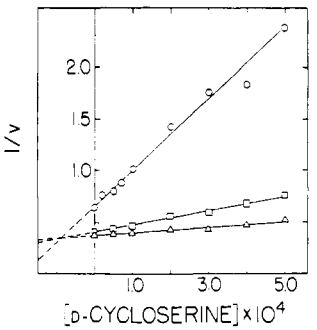


FIG. 2.—Evaluation of K_i from Dixon plots (Dixon, 1953b). The "P_i assay" with 52 μg of enzyme preparation per ml was used. The concentrations of D-alanine are: O—O, 0.01 M; □—□, 0.04 M; and Δ—Δ, 0.10 M.

EXPERIMENTAL PROCEDURE

Materials.—D-ala-D-ala synthetase[1] was purified through the acetone fractionation as previously described (Neuhaus, 1962a). A second acetone fractionation was performed with identical conditions to remove most of the adenosine triphosphatase. The specific activity of the enzyme preparations averaged 55 units/mg protein. A unit is the amount of enzyme that will catalyze the formation of 1.0 μmole of dipeptide per hour. The sources of amino acids, nucleotides, and dipeptides have been previously described (Neuhaus 1962a).

We are indebted to Dr. O. K. Behrens and Dr. A. Pohland of the Eli Lilly Co. for generous samples of L-cycloserine, D-cycloserine, β-aminoxy-D-alanine methyl ester (ethyl ester), 3-iminoisoxazolidine, 3-isoxazolidone, and β-aminoxy-propionic acid; to Dr. K. Folkers and Dr. C. H. Stammer of Merck, Sharp and Dohme Research Laboratories for β-aminoxy-D-alanine and initial samples of L- and D-cycloserine; to Dr. E. B. Hodge of Commercial Solvents Corp. for a sample of 2,5-(diaminoxymethyl)-3,6-diketopiperazine; to Dr. Pl. A. Plattner of Hoffmann-La Roche Laboratories for generous samples of *cis*-DL-cyclothreonine (*cis*-5-methyl-DL-4-amino-3-isoxazolidone) and *trans*-DL-cyclothreonine; and to Dr. W. Shive and Dr. C. G. Skinner for a sample of *trans*-5-isopropyl-DL-4-amino-3-isoxazolidone.

A sample of 2,5-(diaminoxymethyl)-3,6-diketopiperazine was prepared by a modification of the procedures used by Bretschneider and Vetter (1959) and Hodge.[2] D-Cycloserine (0.5 g) was suspended in 25 ml of absolute ethanol. Glacial acetic acid (1 g) was added, and the suspension was refluxed for 30 minutes. The mixture was cooled and the product was collected by filtration. The yield was 0.38 g (77%). The product was recrystallized from a water-ethanol solution. This sample was identical with that provided by Hodge. No ninhydrin-reactive material was observed on chromatography of this compound in solvent 3 (Table I).

TABLE I
IDENTIFICATION OF CYCLOSERINE AND ANALOGS

Compound	R_F[a] 1[b]	2[b]	3[b]	4[b]
D-Cycloserine	0.31	0.43	0.37[c]	0.17
cis-DL-Cyclothreonine	0.39	0.57	0.54[c]	0.21
trans-DL-Cyclothreonine	0.41	0.61	0.60[c]	0.23
β-Aminoxy-D-alanine methyl ester	[d]	0.66	0.88[e]	[f]
β-Aminoxy-D-alanine	[d]	0.22	0.55[e]	0.08[e]
D-Serine	0.18	0.27	0.18	0.12
D-Alanine	0.28	0.40	0.24	0.20

[a] Descending chromatography on Whatman No. 3MM paper. [b] Solvent 1, 1-butanol–acetic acid–water (4:1:5) (organic phase) (Moore and Baker, 1958); solvent 2, 77% ethanol (Moore and Baker, 1958); solvent 3, methyl ethyl ketone–pyridine–water (20:5:8) (Stammer, 1962); solvent 4, isopropanol–concd NH_4OH–water (80:2:18) (Plattner *et al.*, 1957). [c] Major spot but some streaking present. [d] Poor solvent system as indicated by extensive decomposition. [e] Chromatographs as the oxime. [f] Possible cycloserine formation.

Assay.—The "P_i assay" contained 0.05 M KCl; 0.2 M choline chloride (Neuhaus, 1962b); 0.01 M $MgCl_2$; 0.05 M Tris-HCl buffer, *p*H 7.8; 0.01 M disodium ATP neutralized with NaOH; 2.5 mM glutathione; and enzyme, substrate, and additions as specified. All solutions of cycloserine and derivatives were made fresh daily. The ester derivatives were titrated to *p*H 7.8 with NaOH at 2–4°. The assay tubes were incubated at 37°, and aliquots were removed at 0, 5, 10, and 15 minutes and added to 4.3 ml of 0.1 N HCl for subsequent P_i analyses by the method of Marsh (1959). Unless specified, all velocities are reported as μmoles P_i per ml liberated in 1 hour of incubation.

RESULTS

Inhibitor Specificity.—As shown in Table II, a number of cycloserine analogs have been tested for their action on the D-ala-D-ala synthetase. Although many of the compounds inhibit the enzyme at high concentrations, only *cis*- and *trans*-DL-cyclothreonine and β-aminoxy-D-alanine methyl ester are effective at low concentrations. The apparent inhibition by β-aminoxy-D-alanine methyl ester, however, is complicated by a secondary reaction (cf. *Inhibition by β-Aminoxy-D-alanine Methyl Ester*). 3-Isoxazolidone and 3-iminoisoxazolidine which lack the 4-amino group have no apparent inhibitory activity. Cleavage of the isoxazolidone ring gives analogs, D-serine amide, D-serine

[1] Two types of D-ala-D-ala synthetase have been observed. Type *A*, which has been observed in *S. faecalis*, *Lactobacillus casei*, and *E. coli*, has no requirement for a cofactor and does not require a preincubation in the presence of ATP at *p*H 7.2 for maximum activity (Neuhaus, 1962a; and unpublished observations). Type *B* requires a cofactor and preincubation at *p*H 7.2 in the presence of ATP for maximal activity. This type has been observed in *S. aureus* Copenhagen (Ito and Strominger, 1962b).

[2] E. B. Hodge, personal communication.

TABLE II
INHIBITOR SPECIFICITY

Compound	Inhibition[a] K_i (moles/liter)	(%)
1. D-Cycloserine	0.9×10^{-4}	
2. *cis*-DL-Cyclothreonine	1.2×10^{-4}[b]	
3. *trans*-DL-Cyclothreonine	4.8×10^{-4}[b]	
4. β-Aminoxy-D-alanine methyl ester	3.1×10^{-4}	
5. *trans*-5-Isopropyl-DL-cycloserine (0.0025 M D-)		0
6. DL-Serine amide (0.02 M D-)		28
7. D-Serine methyl ester (0.02 M)		16
8. D-Serine (0.02 M)		3
9. 3-Isoxazolidone (0.02 M)	(stimulation)	+12
10. 3-Iminoisoxazolidine (0.02 M)		0
11. β-Aminoxy-propionic acid (0.02 M)		29
12. L-Cycloserine (0.001 M)		0
13. β-Aminoxy-D-alanine (0.02 M)		0
14. 2,5-(Diaminoxymethyl)-3,6-diketopiperazine (0.02 M)		0

[a] The values for K_i of inhibitors 1–4 were calculated from Dixon plots illustrated in Fig. 2 and Fig. 4, A and B. For inhibitors 5–14, the "P_i assay" was used with a fixed concentration of inhibitor and 0.01 M D-alanine. [b] Molarity on basis of the D-isomer.

EA and EAA are binary and ternary complexes of enzyme (E) and D-alanine (A), respectively. These reactions are characterized by their respective Michaelis constants, K_A (6.6×10^{-4} M) and K_{AA} (0.01 M). The results are consistent with the proposal that the enzyme has two binding sites for D-alanine.

As shown by Strominger *et al.* (1960), D-cycloserine is a competitive inhibitor of D-ala-D-ala formation. The results presented in Figure 3A confirm this observation. In Table III a number of reactions are considered in the interpretation of the data for a competitive inhibitor of the synthetase. The rearranged reciprocal rate equations for the ordered sequence of substrate binding, i.e., (a) donor, (b) acceptor (cf. Discussion, Neuhaus, 1962b), and inhibitor binding are presented for each case. Thus, K_I is the inhibitor constant for the binding of I to the donor site while K_{AI} is the inhibitor constant for the binding of I to the acceptor site when the donor site binds a molecule of D-alanine. It is not possible to establish K_I for the acceptor site (Ac) in the absence of D-alanine bound to the donor site, i.e., $E + I \rightleftharpoons EI_{Ac}$, for the same reason that it is not possible to establish K_A for the reaction, $E + A \rightleftharpoons EA_{Ac}$.

In Figure 3B $[A](1/v - 1/V_{max})$ against $1/[A]$ is plotted for each level of D-cycloserine. It is apparent from these results that an intercept and slope change

TABLE III
ANALYSIS OF RECIPROCAL RATE EXPRESSIONS FOR THE INHIBITED SYSTEM

System[a]	Rearranged Reciprocal Equations	Comments
Uninhibited	$[A]\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}} + \frac{K_A K_{AA}}{[A]V_{max}}$	
1. $E + I \rightleftharpoons EI$ (K_I)	$[A]\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}} + \frac{K_A K_{AA}}{V_{max}[A]}\left[1 + \frac{[I]}{K_I}\right]$	Common intercept, slope change
2. $EA + I \rightleftharpoons EAI$ (K_{AI})	$[A]\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}}\left[1 + \frac{[I]}{K_{AI}}\right] + \frac{K_A K_{AA}}{V_{max}[A]}$	Intercept change, no slope change
3. $E + I \rightleftharpoons EI$ (K_I) $EA + I \rightleftharpoons EAI$ (K_{AI})	$[A]\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}}\left[1 + \frac{[I]}{K_{AI}}\right] + \frac{K_A K_{AA}}{V_{max}[A]}\left[1 + \frac{[I]}{K_I}\right]$	Intercept change, slope change
4. $E + I \rightleftharpoons EI$ (K_I) $EA + I \rightleftharpoons EAI$ (K_{AI}) $EI + I \rightleftharpoons EII$ (K_{II}) $EI + A \rightleftharpoons EIA$ (K_{IA})	$[A]\left[\frac{1}{v} - \frac{1}{V_{max}}\right] = \frac{K_{AA}}{V_{max}}\left[1 + \frac{K_A[I]}{K_I K_{IA}} + \frac{[I]}{K_{AI}}\right] + \frac{K_A K_{AA}}{[A]V_{max}}\left[1 + \frac{[I]}{K_I} + \frac{[I]^2}{K_I K_{II}}\right]$	Intercept change, slope change

[a] The rate-limiting step is assumed to be dipeptide-bond formation. Product inhibition studies, however, give evidence for product dissociation as the rate-limiting step in the reaction catalyzed by the synthetase (cf. footnote 2, Neuhaus, 1962b). This assumption does not invalidate the interpretation of the rearranged plots.

methyl ester, β-aminoxy-D-alanine, and D-serine, with little or no inhibitory activity.

Nature of the Inhibition.—As shown in Figure 1, the inhibition is instantaneous and is not affected by preincubation with D-cycloserine. Furthermore, when the enzyme is incubated at 37° with 0.01 M D-cycloserine for 20 minutes in the presence of all reaction components except D-alanine, it can be completely reactivated by passage over Sephadex G-25.

Kinetic Studies on D-Cycloserine Inhibition.—An apparent value for K_i of 9×10^{-5} M was established from the intersection of Dixon plots (Dixon, 1953b) (Fig. 2). This value was in agreement with that determined from the limiting slopes of the Lineweaver-Burk plots presented in Figure 3A.

Previous studies (Neuhaus, 1962b) have shown that the kinetics of the synthetase are consistent with two reactions, (2) and (3), each of which is first order with respect to D-alanine.

$$E + A \rightleftharpoons EA \quad (2)$$

$$EA + A \rightleftharpoons EAA \quad (3)$$

$$EAA \longrightarrow EA - A \rightleftharpoons E + A - A \quad (4)$$

are characteristic of the D-cycloserine inhibition. Thus, cases 1 and 2 are eliminated. In case 3 linear secondary plots of slope and intercept are predicted. On the other hand, case 4 predicts secondary plots in which the slope is nonlinear and the intercept is linear with respect to the inhibitor concentration. The secondary plots of slope and intercept are presented in Figure 3C. Since the data are not sufficient to decide between case 3 and case 4, the more general case (4) will be used to evaluate inhibitor constants. The equation for the slope from case 4 is:

$$\text{slope} = \frac{K_A K_{AA}}{V_{max}}\left[1 + \frac{[I]}{K_I} + \frac{[I]^2}{K_I K_{II}}\right] \quad (5)$$

At low [I] the slope reduces to $K_A K_{AA}/V_{max}\,[1 + ([I]/K_I)]$, and if the slope = 0, then $K_I = -[I]$ From the secondary plot of slope (Fig. 3C), the value for $K_I = 2.2 \times 10^{-5}$ M. The equation for the intercept is:

$$\text{intercept} = \frac{K_{AA}}{V_{max}}\left[1 + \frac{K_A[I]}{K_I K_{IA}} + \frac{[I]}{K_{AI}}\right] \quad (6)$$

Two constants, K_{IA} and K_{AI} are unknown in the

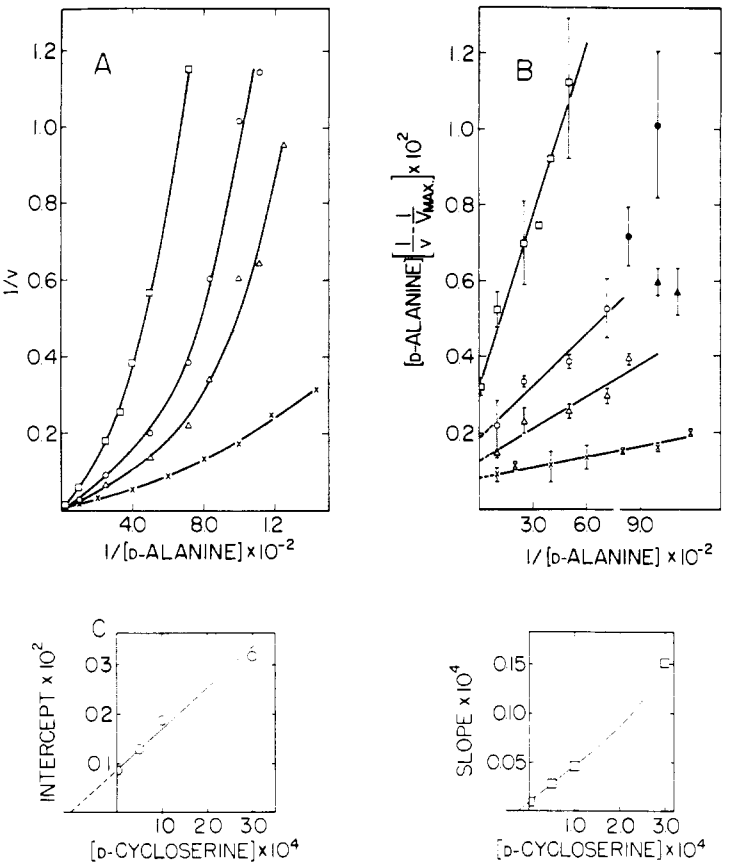


FIG. 3.—Analysis of D-cycloserine inhibition. The "P_i assay" with 260 μg of enzyme preparation per ml was used. The concentrations are: ×—×, 0; Δ—Δ, 5×10^{-5} M; ○—○, 1×10^{-4} M; and □—□, 3×10^{-4} M. The Lineweaver-Burk plots (A) and rearranged plots (B) are presented. In B the average deviations are given for points where two or more determinations were performed. The lines in B are drawn according to a least squares treatment (open points only). In C the secondary plots for the evaluation of K_I and K_{AI} are presented. The slopes and intercepts from B are used.

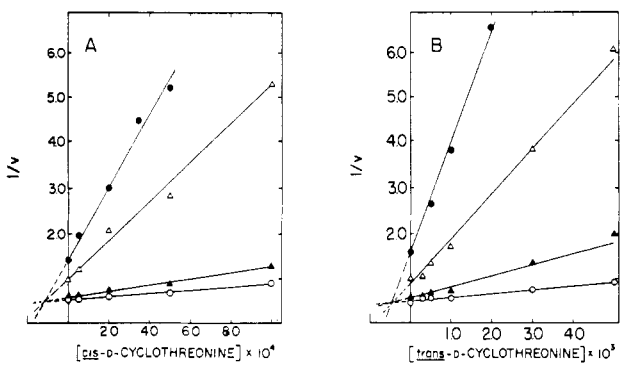
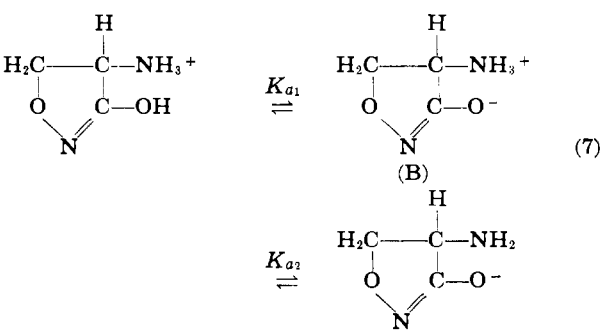


FIG. 4.—Dixon plots of *cis*-D-cyclothreonine (A) and *trans*-D-cyclothreonine (B). The "P_i assay" with 33 μg of enzyme preparation per ml was used. The concentrations are: ○—○, 0.1 M; ▲—▲, 0.04 M; Δ—Δ, 0.01 M; ●—●, 0.005 M (molarity on the basis of the D isomer).

intercept equation. However, if $K_{IA} = K_{AA}$, then a value for K_{AI} of 1.4×10^{-4} M can be calculated. Since this result is in good agreement with that established from the Dixon plot (Dixon, 1953b), it is suggested that the K_i from the Dixon plot is a good estimation of K_{AI}.

5-Methyl Substitution.—As bactericidal agents *cis*-DL-cyclothreonine and *trans*-DL-cyclothreonine are not as effective as D-cycloserine (cf. *Growth Studies with S. faecalis R*). Apparent values for K_i were determined for the *cis*-isomer (1.2×10^{-4} M) (Fig. 4A) and the *trans*-isomer (4.8×10^{-4} M) (Fig. 4B) from Dixon plots (Dixon, 1953b). An analysis of the inhibition (Fig. 5) was performed in a manner similar to that for D-cycloserine. From the secondary plots K_I and K_{AI} (Fig. 5C) were evaluated. The values are summarized in Table IV and compared with those obtained with D-cycloserine.

Variation of K_i', K_m, and V_{max} with pH.[3]—D-Cycloserine has two ionizable groups with pK_{a_1} equal to 4.4–4.5 and pK_{a_2} equal to 7.4 (Neilands, 1956; Hidy *et al.*, 1955). The pK_{a_2} of *cis*- and *trans*-cyclothreonine does not differ significantly from that of cycloserine. These results have been interpreted in terms of the zwitterion intermediate (B) as follows:



(7)

[3] For this section the apparent inhibitor constant will be represented by K_i' and the true inhibitor constant by K_i to conform with the presentation by Webb (1963b).

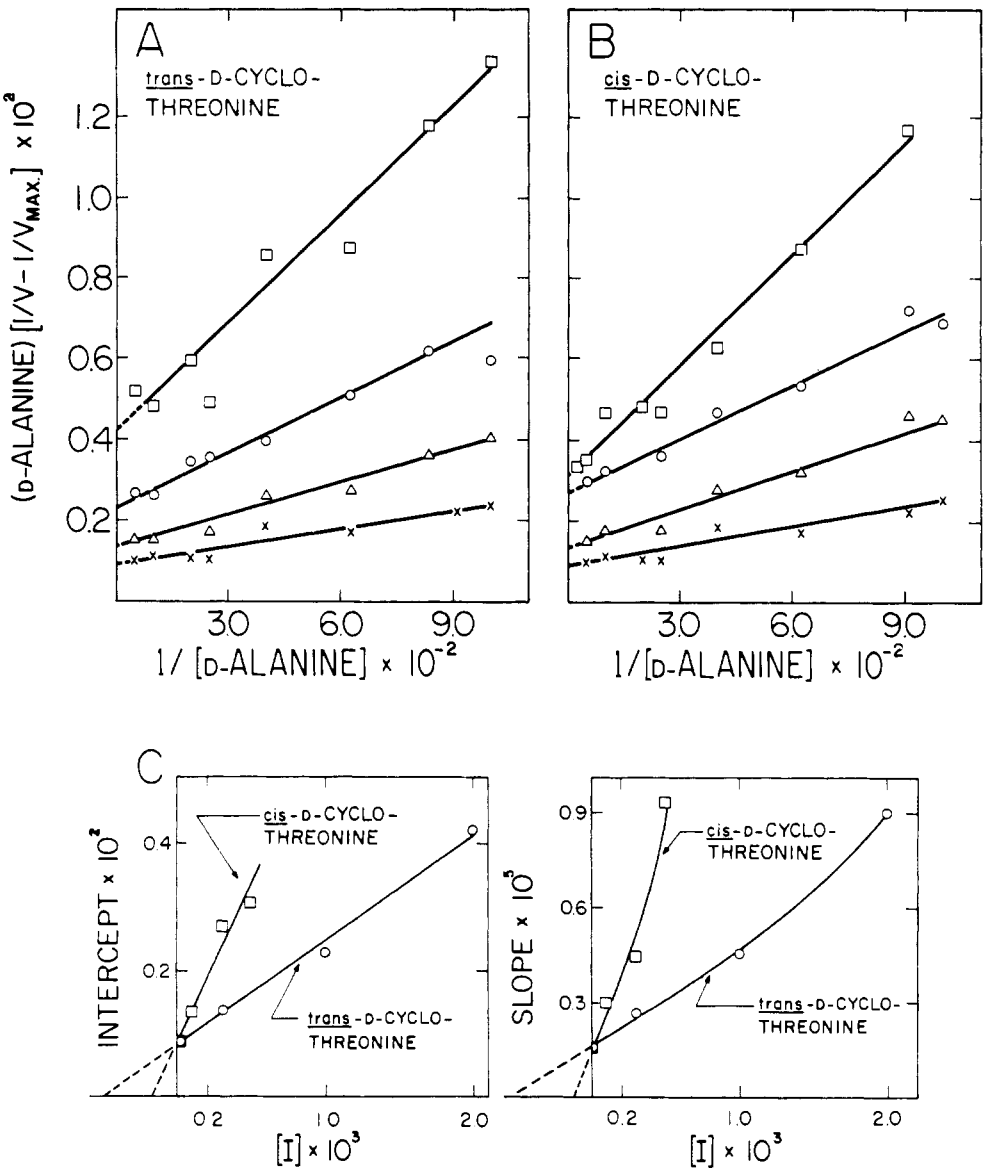


FIG. 5.—Inhibition analysis of *trans*-D-cyclothreonine (A) and *cis*-D-cyclothreonine (B). The "P_i assay" with 165 μg of enzyme preparation was used. The concentrations for *cis*-D-cyclothreonine are: Δ—Δ, 1×10^{-4} M; O—O, 3×10^{-4} M; □—□, 5×10^{-4} M; for *trans*-D-cyclothreonine: Δ—Δ, 3×10^{-4} M; O—O, 1×10^{-3} M; □—□, 2×10^{-3} M (molarity on the basis of the D isomer). The values are averages of several determinations and the lines are drawn according to the method of least squares. In C, the secondary plots for the evaluation of K_I and K_{AI} for *cis*- and *trans*-D-cyclothreonine are presented. The slopes and intercepts from A and B are used.

Since the pK_{a_2} of the amino group is near the *p*H optimum of the enzyme reaction (*p*H 8–9), it was necessary to determine the effect of ionization on the inhibition. As shown in Figure 6, V_{max} and K_{AA} (K_m) are essentially independent of *p*H in the range 7.7–9.3. In contrast to these results the K_i' is markedly dependent on *p*H. The effect of *p*H on K_i' was analyzed by the Dixon treatment (Dixon, 1953a). In a system where the inhibitor ionizes according to the equation $I^{\pm} \rightleftharpoons I^- + H^+$ and $I^{\pm}$ is the form of active inhibitor, the apparent pK_i' is related to the true pK_i by the following expression (Webb, 1963a):

$$pK_i' = pK_i - \log \left[1 + \frac{K_a}{[H^+]}\right] \qquad (8)$$

In Figure 6B when pK_i' is plotted as a function of *p*H, an inflection at *p*H 8.0 is observed. In the low *p*H range the slope is zero while in the high *p*H range the slope is −1. These results are consistent with equation (8) and indicate that the zwitterion (B) is the active inhibitor.

TABLE IV
SUMMARY OF INHIBITOR CONSTANTS

Compound	K_I (moles/liter)	K_{AI} (moles/liter)
D-Cycloserine	2.2×10^{-5}	1.4×10^{-4}
cis-DL-Cyclothreonine[a]	1.2×10^{-4}	1.9×10^{-4}
trans-DL-Cyclothreonine[a]	5.4×10^{-4}	5.6×10^{-4}

[a] Molarity on the basis of the D isomer.

Biosynthesis of D-*Ala-β-aminoxy*-D-*ala*.—In a number of cases, a significant antibacterial activity has been observed with β-aminoxy-D-alanine (Stammer, 1962; Breger, 1961). The resemblance in structure between the antibiotic and this analog suggested a similar mechanism of action. However, as shown in Table II,

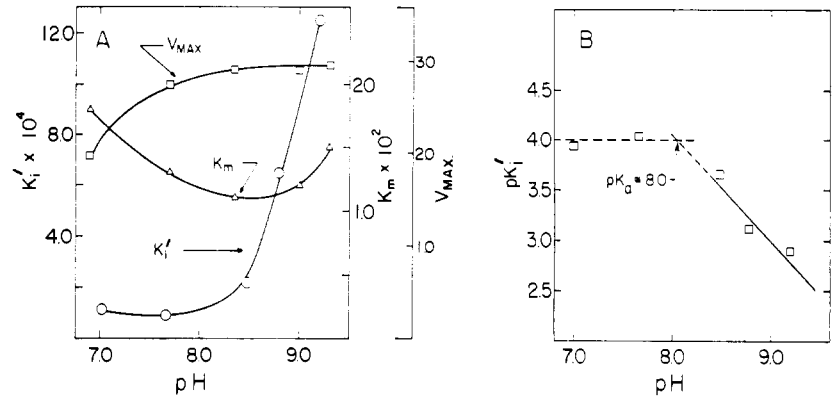


FIG. 6.—Variation of K_m (K_{AA}), V_{max}, and K_i' with *p*H (A). For the evaluation of K_m and V_{max} the "P_i assay" was used with 33 μg of protein per ml while for the determination of K_i' 42 μg of protein per ml was used. K_m was determined by extrapolating the tangent to $1/V_{max}$ with the abscissa (i.e., $1/v = 0$). These values represent K_{AA} (Neuhaus, 1962b). Values for K_i' were determined from Dixon plots (Dixon, 1953b) as described in Fig. 2. All buffers were 0.05 M Tris-HCl and the final *p*H of the complete incubation minus enzyme was measured at 37°. In B, a Dixon treatment (Dixon, 1953a) of the K_i' values for the evaluation of pK_{a_2} is presented.

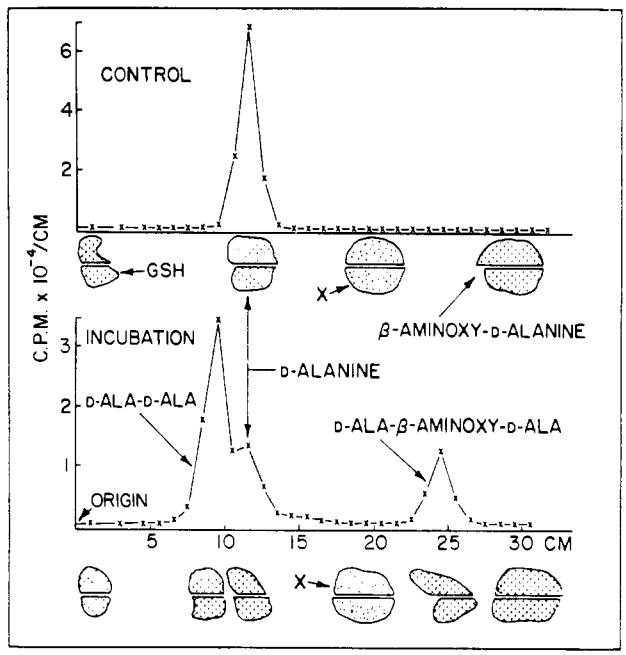


FIG. 7.—Biosynthesis of D-ala-β-aminoxy-D-ala. Each incubation contained 2 μmoles of ATP neutralized with NaOH, 2 μmoles of $MgCl_2$, 2 μmoles of D-alanine (0.2 μc of D-alanine-1-^{14}C), 10 μmoles of KCl, 4 μmoles of β-aminoxy-D-alanine neutralized with NaOH, 212 μg of protein, and 10 μmoles of Tris-HCl, *p*H 7.8, in a volume of 0.2 ml. The incubation was performed at 37° for 30 minutes and then was terminated by placing it in a boiling water bath for 2 minutes. The contents were chromatographed on Whatman 3MM (4 cm band) with solvent 3 for 12 hours in a descending system. Guide strips were developed with ninhydrin and sections (1 × 2.5 cm) were counted in a Packard Tri-Carb liquid scintillation spectrometer with 15 ml of scintillation mixture (0.3% 2,5-diphenyloxazole in toluene). $R_{ala\text{-}ala}$ (solvent 3) for ala-ser is 0.52. The band labeled X is a minor impurity in the β-aminoxy-D-alanine.

β-aminoxy-D-alanine has no inhibitory effect on the synthetase.

In incubations containing D-alanine and D-serine, it was shown that D-ala-D-ser was formed in addition to D-ala-D-ala (Neuhaus, 1962a). When β-aminoxy-D-alanine and D-alanine-1-^{14}C were incubated together with ATP, Mg^{2+}, K^+, and enzyme, a new peptide was detected with ninhydrin and radioactivity scanning on paper chromatograms (Fig. 7). The ^{14}C-labeled mixed dipeptide was isolated by preparative paper chromatography (solvent 3, Table I). On hydrolysis with 5.7 N HCl (12 hours) only serine and alanine-^{14}C were detected. The 2,4-dinitrophenyl derivative was prepared, hydrolyzed, and chromatographed as previously described (Neuhaus, 1962a). Only 2,4-dinitrophenylalanine-^{14}C was detected. On the basis of these results, the substrates, and the analogy with D-ala-D-ser formation, it was tentatively concluded that the mixed dipeptide is D-ala-β-aminoxy-D-ala.

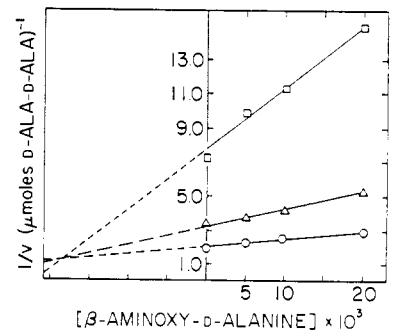


FIG. 8.—Evaluation of K_i for β-aminoxy-D-alanine. Each incubation (0.5 ml) contained 0.05 M Tris-HCl buffer, *p*H 7.8; 0.2 M choline chloride; 0.01 M $MgCl_2$; 0.05 M KCl; 0.01 M disodium ATP neutralized with NaOH; 24 μg of enzyme preparation; D-alanine; and β-aminoxy-D-alanine. Three concentrations of D-alanine are shown: O—O, 0.01 M; Δ—Δ, 0.005 M; and □—□, 0.002 M. The incubations were performed at 37° for 30 minutes, and the reactions were terminated by placing the tubes in a boiling water bath for 2 minutes. The amount of D-ala-D-ala was determined by chromatography on the Amberlite IR-120 column (50 cm) of the amino acid analyzer according to previously described methods (Neuhaus, 1962a). The results are presented as $1/\mu$moles of D-ala-D-ala formed per 30 minutes per 0.5 ml.

Evaluation of K_i for β-Aminoxy-D-alanine.—Since the mixed dipeptide of D-alanine and β-aminoxy-D-alanine is formed in addition to D-ala-D-ala, it is not possible to evaluate K_i for the analog by measurements with the "P_i assay." D-Ala-D-ala formation was assayed in the presence of β-aminoxy-D-alanine by ion-exchange chromatography (Fig. 8). A value for K_i of 0.019 M was established from the Dixon plot (Dixon, 1953b).

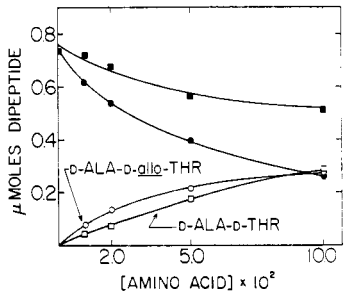
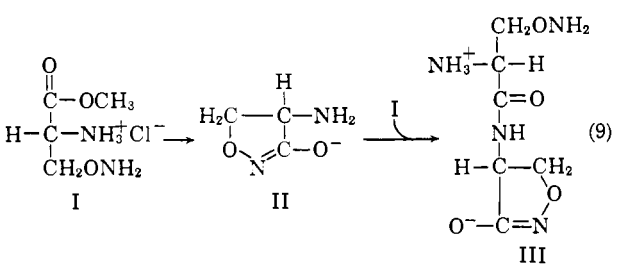


FIG. 9.—Effect of D-threonine and D-allothreonine on the synthesis of D-ala-D-ala and the mixed dipeptides, D-ala-D-allothr and D-ala-D-thr. Each incubation contained 5 μmoles of Tris-HCl, *p*H 7.8, 5 μmoles of KCl, 1 μmole of $MgCl_2$, 1 μmole of ATP neutralized with NaOH, 2 μmoles of D-alanine, D-threonine or D-allothreonine, and 165 μg of enzyme preparation. The components were incubated for 30 minutes at 37° in a total volume of 0.1 ml. The dipeptides were separated on the Amberlite IR-120 column (150 cm) as previously described (Neuhaus, 1962a). The conversion constant (C_{HW}) (Spackman, 1960) for D-ala-D-allothreonine was assumed to be the same as D-ala-D-thr(26.6). The C_{HW} for D-ala-D-ala was 33.8. D-Ala-D-allothr preceded D-ala-D-thr by 2.7 ± 0.7 ml. D-Ala-D-ala formation in the presence of D-allothreonine ●—● and D-threonine ■—■ is shown. In the lower curves mixed dipeptide formation in the presence of D-threonine □—□ and D-allothreonine ○—○ is shown. Although DL-allothreonine was added, the concentration is based on the D isomer. The results are presented as μmoles dipeptide formed in 30 minutes per 0.1 ml of incubation.

Formation of D-*Ala*-D-*thr and* D-*Ala*-D-*allothr.*—D-Allothreonine was observed to be both a more effective acceptor and a better inhibitor than D-threonine (Fig. 9). For example, when 0.02 M D-alanine and 0.02 M acceptor were incubated together, 73 mμmoles of D-ala-D-thr were found using D-threonine, and 132 mμmoles of D-ala-D-allothr were found using D-allothreonine.

*Inhibition by β-Aminoxy-*D-*alanine Methyl Ester.*—As shown in Table II, β-aminoxy-D-alanine methyl ester is an effective inhibitor of the synthetase. An analysis of the inhibition with this analog gave results (Neuhaus and Lynch, 1962) consistent with the binding of one inhibitor molecule per catalytic area (i.e., E + I ⇌ EI). Subsequent experiments, however, showed a marked enhancement of the inhibition during preincubation of the ester at 37° (Fig. 10A). Cycloserine formation from the ester was observed under identical conditions by the appearance of the absorption band at 226 mμ (ϵ = 3940 M^{-1} cm^{-1}; Kuehl *et al.*, 1955). It is apparent from these results that cycloserine formation (Fig. 10B) does not parallel the inhibition enhancement. In Figure 10A at the arrow (30 minutes), 85–90% of the inhibition enhancement is complete; in Figure 10B at the same time, only 45% of the cyclization of β-aminoxy-D-alanine methyl ester is observed. A series of reactions which are consistent with these results is proposed:



(III) 2(II) (10)

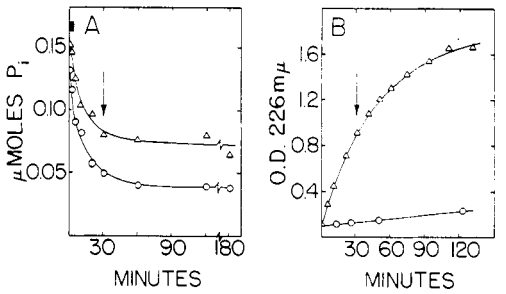


FIG. 10.—Enhancement of inhibition by β-aminoxy-D-alanine methyl ester (A). The ester was adjusted to *p*H 7.8 at 4° with 1 N NaOH on a *p*H meter with efficient stirring. The preincubation was conducted at 37° in the presence of the complete assay mixture as described for the "P_i assay" except for enzyme. The concentrations are: Δ—Δ, 3 × 10^{-4} M and ○—○, 8 × 10^{-4} M. At the specified time 212 μg of enzyme preparation was added and aliquots for P_i analyses were removed at 1-minute intervals for 4 minutes. The 1-minute time sample was used as the blank and the rate was determined from plots of P_i extrapolated to the 1-minute time point. The control is indicated by (■). The results are presented as μmoles P_i formed in 1 minute per ml. Formation of cycloserine from β-aminoxy-alanine methyl ester (B). The complete system (Δ—Δ) in a cuvet (3 ml) contained 5 × 10^{-4} M β-aminoxy-D-alanine methyl ester, 0.05 M Tris-HCl buffer, *p*H 7.8, 0.05 M KCl, 0.2 M choline chloride, and 0.01 M $MgCl_2$ at 37°. The stock ester solution was prepared in the same manner as described in part A. The control (○—○) was performed by taking aliquots at the indicated time intervals from the stock solution maintained at 4° (final concentration 5 × 10^{-4} M). The reaction was followed in a spectrophotometer at 226 mμ.

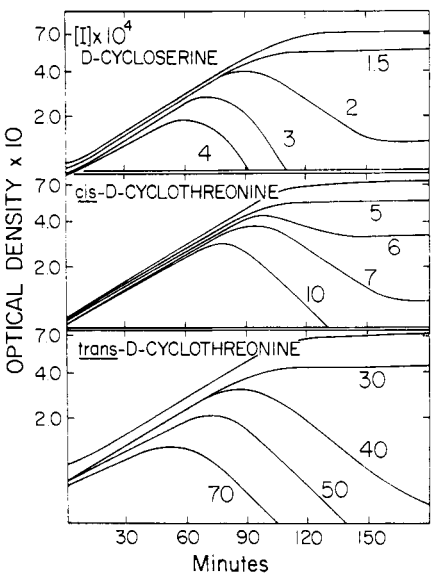


FIG. 11.—Growth studies with *S. faecalis* R. *S. faecalis* R was grown in medium as previously described (Neuhaus, 1962a). Growth was arrested in the log phase by cooling, and the cells were harvested by centrifugation. An aliquot of a suspension was used to inoculate tubes (8 ml) of medium containing 0.1% glucose and inhibitor to an initial optical density of 0.1. The tubes were incubated at 37° and the turbidity was measured at 550 mμ. The molarity of cyclothreonine is on the basis of the D isomer.

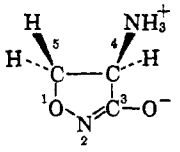
In this scheme the dimer (III) inhibits by the formation of an EI complex. The presence of inhibitor (III) is consistent with the rates of cycloserine formation and inhibition enhancement. Intermediate dimer (III) has been suggested on the basis of a series of

reactions which are proposed to explain the formation of 2,5-(diaminoxymethyl)-3,6-diketopiperazine from cycloserine (Khomutov *et al.*, 1961). In a second reaction (equation 10) intermediate III undergoes elimination of cycloserine by nucleophilic attack of the aminoxy nitrogen on the carbonyl carbon.

Growth Studies with S. faecalis R.—A series of growth experiments were performed to determine the minimal concentration of antibiotic or analog necessary to observe lysis. As shown in Figure 11 the growth rate is identical regardless of the antibiotic concentration. The growth plateau in the controls is a result of glucose depletion. The time necessary to observe lysis decreases as the concentration of inhibitor increases. For D-cycloserine, *cis*-D-cyclothreonine, and *trans*-D-cyclothreonine the minimal concentration for lysis is 1–2 $\times 10^{-4}$ M, 5–6 $\times 10^{-4}$ M, and 3–4 $\times 10^{-3}$ M, respectively.

Discussion

Specificity studies with analogs of D-cycloserine have demonstrated the structural features which contribute to the inhibitory activity and some of the modifications which can be made in the molecule and which still retain this activity. Three critical points have been defined: (1) C-5 substitution, (2) C-4 substitution, (3) 1-2 or 2-3 cleavage.



The K_I for D-cycloserine is 2.2 $\times 10^{-5}$ M, whereas K_I for *cis*-D-cyclothreonine is 1.2 $\times 10^{-4}$ M. K_{AI} for D-cycloserine and *cis*-D-cyclothreonine is 1.4 $\times 10^{-4}$ M and 1.9 $\times 10^{-4}$ M, respectively. On the basis of these results it is suggested that *cis*-5-methyl substitution affects the binding of antibiotic at the donor site while it appears to have almost no effect on binding at the acceptor site. It is difficult to estimate the effect of structure on the binding in the interpretation of K_{AI}. If interaction between the alanine on the donor site and inhibitor on the acceptor site occurs, the K_{AI} may be a function of steric and electrostatic effects between D-alanine and inhibitor in addition to the inherent affinity of the site for the inhibitor. For *trans*-D-cyclothreonine K_I and K_{AI} are 5.4 $\times 10^{-4}$ M and 5.6 $\times 10^{-4}$ M, respectively. Thus, *trans*-5-methyl substitution hinders binding at both the donor and acceptor sites of the synthetase. The results for *cis*-5-methyl substitution are consistent with those reported for the substrate specificity studies (Neuhaus, 1962b), i.e., the addition of a methyl group hinders the binding to the donor site but has little effect on the acceptor site. Omission of the amino group, replacement with an L-4 amino group, or proton removal ($RNH_3^+ \rightarrow RNH_2$) give analogs with no inhibitory activity (C-4 substitution). Cleavage of the ring at the 1-2 or 2-3 position results in a marked decrease of inhibitory activity. β-Aminoxy-D-alanine, D-serine amide, D-serine methyl ester, and D-serine are either poor inhibitors or show no inhibitory activity.

Michalský *et al.* (1962a) have proposed that the active *in vivo* form of D-cycloserine is the 2,5-(diaminoxymethyl)-3,6-diketopiperazine. In addition, it was found that this dimer had the same antibacterial activity as DL-cycloserine in *Mycobacterium tuberculosis* (Michalský *et al.*, 1962b). In the present work[4] this compound had no effect on the synthetase, and bacterial lysis in *S. faecalis* was only observed in the presence of high concentrations of the diketopiperazine (0.01 M) in contrast to the lysis observed with 2 $\times 10^{-4}$ M D-cycloserine. With L-dopadecarboxylase the dimer is 20 times more effective as an inhibitor than either D- or L-cycloserine (Dengler, 1962). In addition, the dimer is a very effective inhibitor of the glutamic-aspartic transaminase (Karpeiskii *et al.*, 1963).

The inhibition of the glutamic-alanine transaminase by L-cycloserine has been studied in detail (Karpeiskii *et al.*, 1963; Braunstein *et al.*, 1962; Polyanovskii, 1961; Vyshepan *et al.*, 1961). The degree of inhibition increased with the time of incubation and required a preincubation period for maximum effect. Although the inhibition is competitive with respect to L-alanine, it is reactivated to only a slight extent by dialysis or passage over Sephadex G-25. The inhibition of the alanine transaminase appears to involve specific binding of L-cycloserine to the enzyme, Schiff base followed by oxime formation with the pyridoxal phosphate, and acylation of a group in the active site. In contrast to these results, the inhibition of the synthetase by D-cycloserine was instantaneous and completely reversible. There is no evidence to indicate that the inhibition of the synthetase involves the formation of a covalent bond (i.e., oxime formation).

Modification of the antibiotic molecule causes in every case a decrease in bactericidal activity (Breger, 1961; Kotschetkow, 1961). A good correlation exists between the enzyme-specificity data and the growth-inhibitor studies.[5] If the primary site of antibiotic action were the acceptor site of the synthetase, one might predict that *cis*-D-cyclothreonine and D-cycloserine would have the same inhibitory activity in bacterial growth experiments. It was observed, however, that a 4-fold greater concentration of *cis*-D-cyclothreonine than D-cycloserine was required to observe lysis. Since these results correlate with the specificity of the donor site rather than the acceptor site, it is proposed that the donor site is a primary site of antibiotic action. This proposal assumes that *S. faecalis* R 8043 is equally permeable to D-cyclothreonine and D-cycloserine.

The hypothesis has been advanced by Strominger (1962b) that the substrate on the enzyme surface has the conformation of the D-cycloserine. Experimental support for this hypothesis has been found with the observation that D-allothreonine is a better acceptor for mixed dipeptide synthesis than D-threonine. A possible explanation for these results is suggested by comparing D-allothreonine with *cis*-D-cyclothreonine and D-threonine with *trans*-D-cyclothreonine (Fig. 12). Intramolecular hydrogen bonding between the hydrogen of the hydroxyl and the carboxyl oxygen on the binding site can give a planar conformation in the case of D-allothreonine and a folded conformation in the case of D-threonine. These structures compare with the fixed planar conformation of the *cis*-cyclothreonine and the fixed folded conformation of the *trans*-cyclothreonine.

[4] Several groups (Hidy *et al.*, 1955; Khomutov *et al.*, 1961; Michalský *et al.*, 1962a) have shown that cycloserine dimerizes to the 2,5-(diaminoxymethyl)-3,6-diketopiperazine at neutral *p*H by the proposed intermediate III, reaction 9. The amount of diketopiperazine formation in the "P_i assay" is not significant at *p*H 7.8. The assay is based on the α-β elimination of H_2NOH in alkali to give 2,5-dimethylene-3,6-diketopiperazine. Standard conditions: 0.05 N NaOH, 10 minutes at room temperature, λ_{max} = 284 mμ, ϵ = 15,200 M^{-1} cm.$^{-1}$

[5] F. C. Neuhaus and J. L. Lynch, unpublished results.

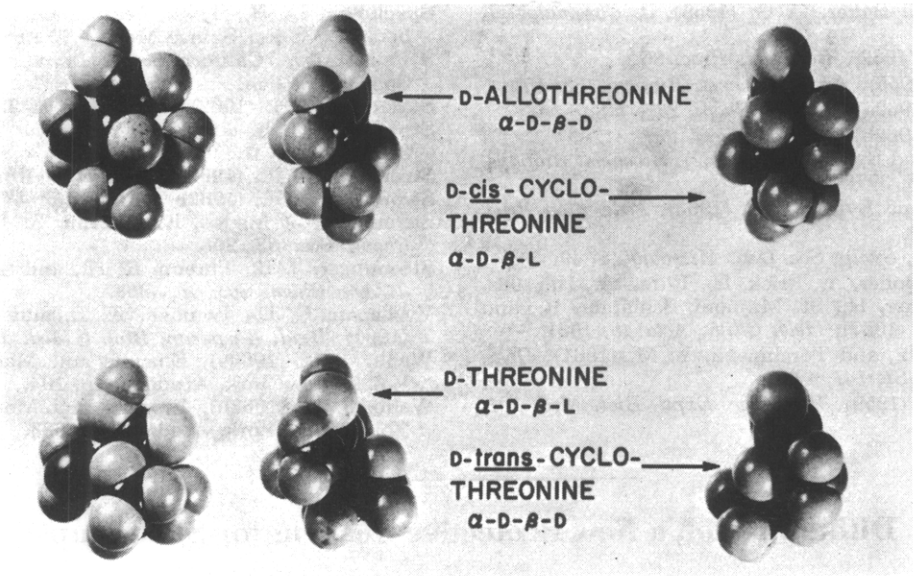


FIG. 12.—Molecular models for the comparison of D-allothreonine with *cis*-D-cyclothreonine and D-threonine with *trans*-D-cyclothreonine.

From a comparison of the Michaelis constants and inhibitor constants it is apparent that the enzyme binds the inhibitor more effectively than the substrate. The ratio K_A/K_I for D-alanine and D-cycloserine is 30 while for D-α-amino-*n*-butyric acid and *cis*-D-cyclothreonine the ratio is 92. On the basis of a steady-state derivation (Neuhaus, 1962b) K_A and K_B are dissociation constants while K_{AA} and K_{BB} are a complex ratio of kinetic constants.[6] The ratio of K_A/K_I may be interpreted on the basis that the effective conformer concentration of the substrate is given by the concentration of inhibitor. Additional support for this interpretation is given by the high value of K_i (0.019 M) observed for β-aminoxy-D-alanine. The major difference between this analog and D-cycloserine is the fixed conformation.

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 (Ito and Strominger, 1962b).[5] On the basis of this observation it is suggested that a different conformation of D-alanine from that bound to the synthetase is bound to the activating enzyme.

ACKNOWLEDGMENTS

The authors thank Mrs. Pi-yu Cheng for some of the "P_i assays," Mr. K. A. Thompson for the dipeptide analyses on the amino acid analyzer, and Mr. Robert Stickgold for enzyme preparations.

[6] The ratio K_m/K_i for the D-ala-D-ala synthetase has been discussed by Strominger (1961, 1962a,b; Strominger *et al.*, 1960). However, since K_m equals K_{AA} and K_{AA} is a ratio of kinetic constants (Neuhaus, 1962b), the ratio K_m/K_i is not valid until it can be shown that K_m is a dissociation constant (Webb, 1963a). Since K_A is a dissociation constant, the present work with the ratio K_A/K_I gives a valid comparison (K_I is not corrected for *p*H). A comparison of K_{AA}/K_{AI} (72, I is D-cycloserine) and K_{BB}/K_{AI} (174, I is *cis*-D-cyclothreonine) with the values for K_A/K_I and K_B/K_I shows that the Michaelis constants (K_m) (i.e., K_{AA} and K_{BB}) are approximations of the dissociation constants (K_s). Therefore, on the basis of these results, the original interpretation by Strominger of the ratio K_m/K_i is valid.

REFERENCES

Baddiley, J., and Neuhaus, F. C. (1960), *Biochem. J. 75*, 579.

Barbieri, P., Di Marco, A., Fuoco, L., and Rusconi, A. (1960), *Biochem. Pharmacol. 3*, 101.

Bondi, A., Kornblum, J., and Forte, C. (1957), *Proc. Soc. Exptl. Biol. Med. 96*, 270.

Braunstein, A. E., Azarkh, R. M., and Syui Tin-sen (1961), *Biokhimiya 26*, 882.

Breger, M. A. (1961), *Antibiotiki 6*, 26, cited from *Chem. Abstracts 56*, 9222g (1962).

Bretschneider, H., and Vetter, W. (1959), *Monatsh. Chem. 90*, 799.

Chambers, P., Bing, J., Lynch, J., Neuhaus, F. C., and Brockman, R. W. (1963), *Bacteriol. Proc.* 119.

Ciak, J., and Hahn, F. E. (1959), *Antibiot. Chemotherapy 9*, 47.

Comb, D. G. (1962), *J. Biol. Chem. 237*, 1601.

Dengler, H. J. (1962), *Arch. Exptl. Pathol. Pharmakol. 243*, 366.

Dixon, M. (1953a), *Biochem. J. 55*, 161.

Dixon, M. (1953b), *Biochem. J. 55*, 170.

Hidy, P. H., Hodge, E. B., Harned, R. L., Brewer, G. A., Phillips, W. F., Runge, W. F., Stavely, H. E., Pohland, A., Boaz, H., and Sullivan, H. R. (1955), *J. Am. Chem. Soc. 77*, 2345.

Ikawa, M., and Snell, E. E. (1958), *Arch. Biochem. Biophys. 78*, 338.

Ito, E., and Strominger, J. L. (1962a), *J. Biol. Chem. 237*, 2689.

Ito, E., and Strominger, J. L. (1962b), *J. Biol. Chem. 237*, 2696.

Karpeiskii, M. Y., Breussov, Y. N., Khomutov, R. M., Severin, E. S., and Polyanovsky, O. L. (1963), *Biokhimiya 28*, 345.

Khomutov, R. M., Karpeiskii, M. Y., and Severin, E. S. (1961), *Biokhimiya 26*, 772.

Kotschetkow, N. K. (1961), *Oesterr. Chemiker-Ztg. 62*, 276.

Kuehl, F. A., Wolf, F. J., Trenner, R. N., Peck, R. L., Howe, E., Hunnewell, B. D., Downing, G., Newstead, E., Buhs, R. P., Putter, I., Ormond, R., Lyons, J. E., Chaiet, L., and Folkers, K. (1955), *J. Am. Chem. Soc. 77*, 2344.

Marsh, B. B. (1959), *Biochem. Biophys. Acta 32*, 357.

Michalský, J., Čtvrtnik, J., Horakova, Z., and Bydzovsky, V. (1962b), *Experientia 18*, 217.

Michalský, J., Opichal, J., and Ctvrtnik, J. (1962a), *Monatsh. Chem. 93*, 618

Moore, T. B., and Baker, C. G. (1958), *J. Chromatog. 1*, 513.
Morrison, N. E. (1962), *Bacteriol. Proc.* 86.
Neilands, J. B. (1956), *Arch. Biochem. Biophys. 62*, 151.
Neuhaus, F. C. (1962a), *J. Biol. Chem. 237*, 778.
Neuhaus, F. C. (1962b), *J. Biol. Chem. 237*, 3128.
Neuhaus, F. C., and Lynch, J. L. (1962), *Biochem. Biophys. Res. Commun. 8*, 377.
Neuhaus, F. C., and Lynch, J. L. (1963), *Federation Proc. Abstracts 22*, 423.
Park, J. T. (1958), *Symp. Soc. Gen. Microbiol. 8*, 49.
Plattner, Pl. A., Boller, A., Frick, H., Fürst, A., Hegedüs, B., Kirchensteiner, H., St. Majnoni, Schläpfer, R., and Spiegelberg, H. (1957), *Helv. Chim. Acta 40*, 1531.
Polyanovskii, O. L., and Torchinskii, Y. M. (1961), *Dokl. Akad. Nauk. SSSR 141*, 488.
Shockman, G. D. (1959), *Proc. Soc. Exptl. Biol. Med. 101*, 693.
Spackman, D. H. (1960), Instruction Manual and Handbook, Beckman/Spinco Model 120 Amino Acid Analyzer, Palo Alto, California, Beckman Instruments Inc., Spinco Division.
Stammer, C. H. (1962), *J. Org. Chem. 27*, 2957.
Strominger, J. L. (1961), *Antimicrobial Agents Ann. 1960*, 328.
Strominger, J. L. (1962a), *Federation Proc. 21*, 134.
Strominger, J. L. (1962b), *Bacteria 3*, 413.
Strominger, J. L., Ito, E., Threnn, R. H. (1960), *J. Am. Chem. Soc. 82*, 998.
Strominger, J. L., Threnn, R. H., and Scott, S. S. (1959), *J. Am. Chem. Soc. 81*, 3803.
Vyshepan, E. D., Ivanova, K. I., and Chernukh, A. M. (1961), *Byul. Eksperim. Biol. i Med. 52*, 76.
Webb, J. L. (1963a), Enzyme and Metabolic Inhibitors, Vol. I, New York, Academic, p. 104.
Webb, J. L. (1963b), Enzyme and Metabolic Inhibitors, Vol. I, New York, Academic, p. 683.

Dithiothreitol, a New Protective Reagent for SH Groups*

W. W. CLELAND

From the Department of Biochemistry, University of Wisconsin, Madison

Received November 4, 1963

Because of its low redox potential (−0.33 volts at *p*H 7), dithiothreitol (and its isomer, dithioerythritol) is capable of maintaining monothiols completely in the reduced state and of reducing disulfides quantitatively. Since this compound is a highly water-soluble solid with little odor and little tendency to be oxidized directly by air, it should prove much superior to the thiols now used as protective reagents for sulfhydryl groups.

Thiol groups such as those of coenzyme A and of some enzymes are readily oxidized in air to disulfides. To maintain these groups in the reduced state, another thiol such as cysteine, glutathione, mercaptoethanol, 2,3-dimercaptopropanol, or thioglycolate is often added so that interchange takes place according to reactions (1) and (2):

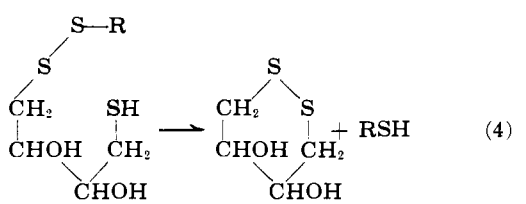
$$\text{CoA—SS—CoA} + \text{RSH} \rightleftharpoons \text{CoA—SH} + \text{R—SS—CoA} \quad (1)$$

$$\text{R—SS—CoA} + \text{RSH} \rightleftharpoons \text{R—SS—R} + \text{CoA—SH} \quad (2)$$

However, the equilibrium constants of these reactions are near unity, so that a sizable excess of the second thiol must be used. It occurred to this author that if reaction (2) were intramolecular and RSSR were a sterically favorable cyclic disulfide, there would be two products produced from one reactant, so that the equilibrium should be displaced to the right, particularly in dilute solutions. It appeared that a 1,4-dithiolbutane structure would produce the most sterically favorable cyclic disulfide, and that addition of hydroxy groups on the middle carbons should make the compound water soluble and reduce the stench of the thiol groups.

Dithiothreitol (DTT)[1] and dithioerythritol (DTE), the threo and erythro isomers of 2,3-dihydroxy-1,4-dithiolbutane, were therefore prepared as described by Evans *et al.* (1949) and found to have the desired properties. Reaction with a disulfide takes place according to reactions (3) and (4), and is complete in several minutes at *p*H 8.

$$\text{R—SS—R} + \text{HS—CH}_2(\text{CHOH})_2\text{CH}_2\text{—SH} \rightleftharpoons \text{RSH} + \text{R—SS—CH}_2(\text{CHOH})_2\text{CH}_2\text{—SH} \quad (3)$$



(4)

Attempts were made to determine the over-all equilibrium constant for reactions (3) and (4) by following the reduction of cystine by DTT or DTE, which can be conveniently measured because the thiol groups of DTT and DTE give only 4% as much color as cysteine in the nitroprusside assay of Grunert and Phillips (1951). Within experimental error, reaction between cystine and DTT or DTE went to completion, even when concentrations of the cyclic oxidized form of DTT or DTE (prepared by ferricyanide oxidation of DTT or DTE) ten times those of DTT or DTE were added.

The actual redox potential of DTT was measured by equilibrating the DTT-oxidized DTT system with the DPN^+-DPNH system in the presence of lipoamide and dihydrolipoic dehydrogenase, and measuring the amount of DPNH at equilibrium at 340 mμ (making suitable corrections for the absorption of lipoamide and oxidized DTT at this wavelength). The equilibrium constant for reaction of DTT with DPN^+ to give oxidized DTT and DPNH was about 2.5 at *p*H 7.0 and 35 at *p*H 8.1. Assuming the redox potential of DPN^+ to be −0.330 v at *p*H 7.0 (Burton and Wilson, 1953), the redox potential of DTT is −0.332 v at *p*H 7.0, and −0.366 v at *p*H 8.1. This is about 0.044 v more negative than the potential of lipoamide (Massey, 1960), corresponding to an equilibrium constant for reaction of lipoamide and DTT of 31.

* Supported in part by a grant (HE-05095) from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used in this work: DTT, dithiothreitol; DTE, dithioerythritol; oxidized DTT, cyclic disulfide of DTT (*trans*-4,5-dihydroxy-*o*-dithiane); oxidized DTE, cyclic disulfide of DTE (*cis*-4,5-dihydroxy-*o*-dithiane).