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Inhibition of Bacterial Growth by β-Chloro-D-Alanine

(cell wall/alanine racemase/D-amino acids)

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ABSTRACT **The D- and L-isomers of β-chloroalanine inhibit the growth of *Diplococcus pneumoniae, Streptococcus pyogenes, Bacillus subtilis,* and *Escherichia coli.* With pneumococcus the inhibition by β-chloro-D-alanine is completely prevented by either D-alanine or D-alanyl-D-alanine, while L-alanine is not effective in preventing the inhibition. The inhibition of growth by β-chloro-L-alanine is not affected by D-alanine and is only partially prevented by high concentrations of L-alanine. The intracellular free alanine in untreated *E. coli* and *B. subtilis* is about 95% in the D-configuration while the free intracellular alanine in both organisms after treatment with β-chloro-D-alanine is predominantly the L-isomer. These results suggested that the β-chloroamino acid inactivates alanine racemase (EC 5.1.1.1). Indeed, when extracts of *E. coli* or *B. subtilis* were treated with β-chloro-D-alanine, the activities of alanine racemase and of D-glutamate-D-alanine transaminase were found to be 90–95% inhibited. Studies with mice have shown that β-chloro-D-alanine is an effective antibacterial agent *in vivo* againt *D. pneumoniae, S. pyogenes,* and *E. coli.***

One of the marked differences between bacterial and mammalian cells is that bacterial cell walls are characterized by their high content of D-amino acids (1), especially D-alanine (2), while the proteins of mammalian cell constituents are comprised of amino acids exclusively of the L-configuration. Several antibiotics in common usage owe their efficacy to the inhibition of pathways involved in the incorporation of D-amino acids into the bacterial cell wall (3), and hence are relatively innocuous to mammalian cell metabolism. Thus, D-cycloserine (4) and *O*-carbamyl-D-serine (5) are competitive inhibitors of alanine racemase (EC 5.1.1.1), a catalyst for the formation of D-alanine in bacterial cells, while penicillin is an efficient inhibitor of D-alanyl-D-alanine carboxypeptidase, an enzyme involved in the latter stages of bacterial cell-wall synthesis (6).

β-Chloroamino acids have been shown recently to bind efficiently to transaminases and decarboxylases and to undergo β-elimination reactions *in situ* (7, 8). In some cases the enzyme catalyzing the β-elimination reaction is irreversibly inactivated presumably by reaction of the enzyme-bound aminoacrylic acid with some functional group of the protein (8). Accordingly, it has occurred to us that β-chloro-D-alanine might inhibit irreversibly the pyridoxal phosphate enzymes responsible for the formation of D-alanine in bacterial cells and thereby preclude the synthesis of a cell-wall constituent necessary for bacterial growth.

MATERIALS

The D- and L-isomers of β-chloroalanine were purchased from Cyclo as the hydrochloride salts. Elemental analysis of β-chloro-D-alanine gave: C, 22.80; H, 4.49; N, 8.66 (calculated: C, 22.50; H, 4.49; N, 8.75). At pH 7.4 and 37° in potassium phosphate, β-chloroalanine has a half-life of 30 hr. D-alanine, pyridoxal 5′-phosphate, and crystalline suspensions of lactate dehydrogenase (EC 1.1.1.27; L-lactate:NAD^+ oxidoreductase) and of glutamate dehydrogenase [EC 1.4.1.2; L-glutamate:NAD^+ oxidoreductase(deaminating)] in saturated $(NH_4)_2SO_4$ were obtained from Sigma. D-Alanyl-D-alanine was a product of Fox Chemical Co. The strain of *Bacillus subtilis* (no. 168) used in these experiments was a generous gift of Dr. Z. Borowska. Crystalline D-amino acid oxidase [EC 1.4.3.3; D-amino-acid:oxygen oxidoreductase(deaminating)] from hog kidney, flavin adenine dinucleotide, and catalase (EC 1.11.1.6; hydrogen-peroxide:hydrogen-peroxide oxidoreductase) were kindly provided by Dr. D. Wellner.

METHODS

Conditions for Growth of Bacteria. For periodic measurement of the effect of β-chloro-D-alanine on the growth of pneumococcus, portions of cultures in the logarithmic phase of growth were added under sterile conditions to 9 volumes of a minimal medium devoid of alanine (9) in 18 × 150-mm tubes. The compounds to be tested (the L- and D-isomers of β-chloroalanine, D- and L-alanine, and D-alanyl-D-alanine) were then added, and incubations were carried out at 37°. In these experiments bacterial growth was measured by nephelometry on a Coleman instrument periodically for 3–6 hr.

For determination of enzyme activities and of free intracellular alanine, cultures of either *E. coli* or *B. subtilis* were grown aerobically at 37° in 25-ml Erlenmeyer flasks in yeast extract-supplemented medium [0.5% dialyzed Difco yeast extract added to the minimal medium of Frantz (10)] and nutrient broth, respectively. The cultures were treated with 3–4 mM β-chloro-D-alanine while in the logarithmic phase of growth, and incubated for an additional 3–4 hr. The cells were collected by centrifugation at 23,000 × *g* at 4° for 10 min. After being washed in 5–10 ml of 0.85% NaCl, they were resuspended in 10 ml 0.1 M potassium phosphate, pH 7.0. The cells were ruptured by sonication for 1–2 min at 0° in a Branson 750-watt sonicator at a setting of 3; the sonicate was then centrifuged for 10 min at 27,000 × *g* and 4°. The concentration of protein in the extract was estimated by the absorbancies at 280 and 260 nm (11).

Determination of the Amounts and Configurations of β-Chloroalanine and Alanine. The amounts of β-chloroalanine and alanine in the culture supernatants and extracts of *E. coli* and *B. subtilis* were determined by amino-acid analysis (12, 13). Under our experimental conditions, β-chloroalanine eluted at 58 ml and alanine at 122 ml from the 0.9 × 50-cm

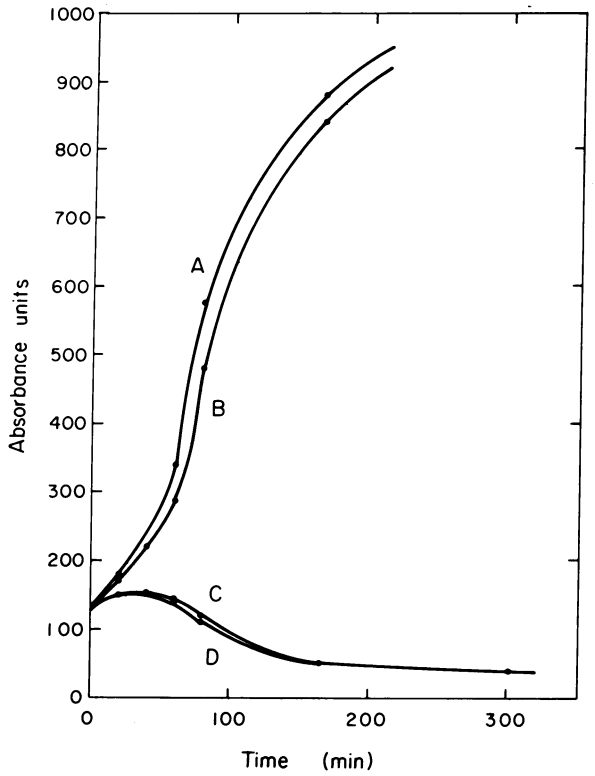

FIG. 1. Inhibition of the growth of pneumococcus by β-chloro-D-alanine. At the beginning of the incubation the following compounds were added to a fresh inoculum of pneumococcus R6 in alanine-free medium: (*A*) no additions; (*B*) β-chloro-D-alanine (500 μg/ml) and D-alanine (500 μg/ml); (*C*) β-chloro-D-alanine (500 μg/ml) and L-alanine (500 μg/ml); (*D*) β-chloro-D-alanine (500 μg/ml). The numbers in parentheses are the final concentrations of each compound. Incubations were carried out under sterile conditions at 37°. The inhibition by β-chloro-L-alanine (500 μg/ml) was similar to that found for the D-isomer. In the presence of D-alanyl-D-alanine (500 μg/ml) the growth pattern was the same as that described in curve *B*.

column of the amino-acid analyzer operated at 52° with 0.2 N sodium citrate, pH 3.25, as the eluant. The ninhydrin constant for β-chloroalanine was 0.75 that of alanine.

The amounts of D-isomers present were determined by treatment of a portion of either the supernatant or the extract fractions with partially purified D-amino acid oxidase (0.2–0.8 mg/ml), flavin adenine dinucleotide (0.1 mM), and a few grains of penicillin for 16 hr at 25°. After acidification with 1 drop of 6 N HCl, 0.2 N sodium citrate, pH 2.2, was added to the desired volume and the precipitated protein was removed by centrifugation at 800 × *g* in a clinical centrifuge. Amino-acid analysis was carried out as described above. The amount of D-isomer was calculated by the difference in amino-acid concentration before and after treatment with D-amino acid oxidase. Walsh *et al.* (14) have shown that β-chloroalanine and alanine are equally good substrates for D-amino acid oxidase. Since, under our experimental conditions, β-chloro-D-alanine is completely removed by the oxidase (Table 1), we have assumed that the alanine remaining after the oxidase treatment represents only the L-isomer.

Assays for Enzymes. Alanine racemase activity was determined as follows: L-alanine (15 mM) was incubated with either an untreated extract or with an extract that had been previously treated with β-chloro-D-alanine and then extensively dialyzed. The incubation with L-alanine was carried out for 2 hr at 37° in 0.1 M potassium phosphate, pH 8.0. The reaction was terminated by heating the solution for 2.5 min at 100°. After centrifugation for 5 min at 800 × *g* for removal of precipitated protein, the D-alanine formed by the racemase was quantitatively oxidized to pyruvate by D-amino acid oxidase, and the pyruvate was reduced to lactate by NADH and lactate dehydrogenase (15). The assay was carried out in the following manner: a portion of the reaction mixture was added to a cuvette containing crystalline D-amino acid oxidase (0.3 mg), flavin adenine dinucleotide (0.1 mM), catalase (400 units), and NADH (0.1 mM) in a final volume of 2.5 ml of 60 mM potassium phosphate, pH 8.0, at 37°. The absorbance at 340 nm was monitored on an Aminco DW2 spectrophotometer. When the absorbance was constant, lactate dehydrogenase (10 μl, 100 μg) was added to the cuvette and the decrease in A_{340} was measured. Separate experiments had shown that the decrease in the absorbance at 340 nm was directly proportional to the amount of pyruvate present.

D-Glutamate-D-alanine transaminase activity was measured by incubation of dialyzed extracts with D-glutamic acid (10 μmol) and pyruvate (5 μmol) in a final volume of 1.0 ml of 0.1 M potassium phosphate, pH 8.0, for 2–3 hr at 37°. After the solution was heated at 100° for 2.5 min, the amount of 2-oxoglutarate formed by the transaminase was measured by addition of a portion of the solution to a cuvette containing NADH (0.1 mM) and 60 mM potassium phosphate, pH 8.0, at 37°. When the absorbance at 340 nm was constant (usually after 1–2 min), glutamate dehydrogenase (50 μl, 1 mg) was added to the cuvette. The decrease in the absorbance at 340 nm was a direct measure of the amount of 2-oxoglutarate present.

RESULTS

Effects of β-Chloroalanine on Bacterial Growth. The growth of pneumococcus R6 is completely inhibited when β-chloro-D-alanine is present in the alanine-free media used in this experiment (Fig. 1*D*), and under these experimental conditions there is some autolysis of the cells. However, when D-alanine is added to the incubation mixture together with β-chloro-D-alanine (Fig. 1*B*), the rate of bacterial growth is normal; the dipeptide, D-alanyl-D-alanine, which is essential in the synthesis of the bacterial cell wall, is also able to overcome the inhibition characteristic of β-chloro-D-alanine. Conversely, L-alanine did not prevent the inhibition of the growth of pneumococcus by β-chloro-D-alanine (Fig. 1*C*), a finding that suggests that the chloro-D-amino acid had inhibited a function peculiar to the synthesis of D-alanine. β-Chloro-L-alanine also effectively inhibits the growth of pneumococcus but the inhibition is not prevented by D-alanine (500 μg/ml). High concentrations of L-alanine (800 μg/ml) partially reversed the inhibition by β-chloro-L-alanine (100 μg/ml).

The extent of inhibition of the growth of pneumococcus R6 by β-chloro-D-alanine is related to the amount of the compound present in the incubation medium; complete inhibition of growth was observed at a β-chloroalanine concentration of 100 μg/ml.

β-Chloro-D-alanine, when added to cultures during the exponential phase of growth at a concentration of 100 μg/ml, also inhibits the growth of Group A streptococci (S43) as well as that of pneumococci (SVI) when grown in Todd–Hewitt medium (16). Bacterial counts at the time of addition of the inhibitor and at intervals thereafter for a period of 3 hr in-

TABLE 1. *Amounts (μM) of intracellular and extracellular* D- *and* L-*alanine after treatment with β-chloro-*D-*alanine*

Amino acid	Untreated cells		β-Chloro-D-alanine-treated cells	
	D-Isomer	L-Isomer	D-Isomer	L-Isomer
E. coli				
Intracellular				
β-Chloroalanine	0	0	980	0
Alanine	530	24	0	1090
Extracellular				
β-Chloroalanine	0	0	4000	0
Alanine	0	830	0	1070
B. subtilis				
Intracellular				
β-Chloroalanine	0	0	—*	0
Alanine	260	12	40	140
Extracellular				
β-Chloroalanine	0	0	3000	0
Alanine	0	52	0	265

The intracellular and extracellular fractions were prepared as described in *Methods*. The amounts of β-chloroalanine and alanine were determined by amino-acid analysis on a 0.9 × 50-cm column of AA-15 resin at 52° and a flow rate of 50 ml/hr. The concentrations of the amino acids are those in the 10 ml of extract and in the supernatant after centrifugation of the culture.

* Not determined because of the large amount of aspartic acid, which is not completely resolved from β-chloroalanine in this chromatographic system.

dicate that the cells continue to divide for only about one generation before growth ceases; there was no observable cell lysis or decrease in viable units in the enriched medium used for this experiment.

Content and Configuration of Intracellular and Extracellular Alanine after β-Chloroalanine Treatment. For comparison of the cellular content of amino acids and their configurations, β-chloro-D-alanine (final concentration: 3–4 mM) was added to cultures of *E. coli* E-4 or *B. subtilis* in the logarithmic phase of growth. The incubations were continued for 3–4 hr. While the suppression of growth in a minimal medium (9) under similar conditions (see Fig. 1) was complete, the growth of *E. coli* in yeast extract-supplemented medium was reduced about 40% while that of *B. subtilis* in nutrient broth was reduced about 20% compared with untreated cultures; cells were harvested from the medium for analysis of the intracellular and extracellular alanine.

Extracts of the treated and untreated cells after centrifugation were prepared from each organism by sonication as described in *Methods*. Portions of the cellular supernatants and the extracts were analyzed for the amounts and configurations of β-chloro-alanine and alanine by amino-acid analysis. The absence of β-chloro-D-alanine in samples treated with D-amino acid oxidase (Table 1) is an indication that the reaction with the oxidase had proceeded to completion. The most notable difference between the untreated and the β-chloro-alanine-treated cells is in the configuration of the intracellular alanine; about 95% of the free intracellular alanine content of the untreated cultures of both *E. coli* and *B. subtilis* is in the D-configuration, while the free intracellular alanine in the β-chloro-D-alanine-treated cultures is predominantly the L-isomer. These results strongly suggest that alanine racemase, which catalyzes the conversion of L-alanine to D-alanine, had been inactivated by β-chloro-D-alanine.

TABLE 2. *Inhibition of alanine racemase from E. coli by β-chloro-*D-*alanine*
(nmoles)

	D-Alanine formation from L-alanine	
	Without pyridoxal 5′-phosphate	With pyridoxal 5′-phosphate
Untreated	255	248
β-Chloro-D-alanine-treated	9	25

The alanine racemase of the extracts from the β-chloroalanine-treated and the untreated cells was measured as described in the *text*. Pyridoxal 5′-phosphate at a final concentration 0.5 mM was added where indicated. The protein concentration was 2.3 mg/ml.

The free alanine in the extracellular fractions (i.e., culture supernatants) of both the β-chloroalanine-treated and the untreated cultures is almost all of the L-configuration. Separate analysis of the yeast-extract dialysate used for the experiments showed that all of the free alanine present was also of the L-configuration.

The amounts of aspartic acid, glycine, valine, isoleucine, and leucine were similar in both the extracellular and intracellular fractions of the β-chloroalanine-treated and the untreated cultures. There was, however, a 3- to 4-fold increase in the intracellular concentration of glutamic acid in the β-chloroalanine-treated cells.

Inhibition of E. coli Alanine Racemase by β-Chloroalanine. An extract of *E. coli* E-4 was dialyzed against 400 volumes of 10 mM potassium phosphate, pH 7.8, for 16 hr at 4°. A portion of the extract was treated with 40 mM β-chloro-D-alanine for 3 hr at 37° and subjected to extensive dialysis as described above. As shown in Table 2, the activity of alanine racemase after β-chloro-D-alanine treatment was only about 4% compared with that of the racemase in an extract that had been subjected to identical conditions of incubation and dialysis but without β-chloro-D-alanine. Addition of pyridoxal 5′-phosphate to a portion of the untreated extract did not increase the activity of alanine racemase, a finding that indicates that sufficient cofactor is already present in the extract. The inhibition of alanine racemase by β-chloro-D-alanine was not due to dissociation of pyridoxal 5′-phosphate from the enzyme since addition of excess pyridoxal 5′-phosphate resulted in only a slight increase in alanine racemase activity. It appears that the inhibition by β-chloro-D-alanine is irreversible since the extract was thoroughly dialyzed after incubation with β-chloro-D-alanine. We have found that both alanine racemase activity and bacterial growth are inhibited to the same extent by the D- and L-isomers of β-chloroalanine.

*Inhibition of Alanine Racemase and D-Glutamate-*D*-alanine Transaminase of B. subtilis.* Both alanine racemase (17) and D-glutamate-D-alanine transaminase (18) have been reported to be present in *B. subtilis*. A dialyzed extract of *B. subtilis* was treated with 40 mM β-chloro-D-alanine for 3 hr at 37° followed by extensive dialysis against 10 mM potassium phos-

TABLE 3. *Inhibition by β-chloro-D-alanine of bacterial growth in vivo in mice*

Strain	Inoculum (no. of cells)	Mortality in mice (%) Untreated	Treated
D. pneumoniae			
SVI	1.2×10^2	80	0
A66	1.2×10^2	100	0*
	1.2×10^3	100	0*
	1.2×10^4	100	0*
S. pyogenes			
S43	1.2×10^2	0	0
	1.2×10^3	80	0
	1.2×10^4	100	0
T23	1.0×10^2	80	0
	1.0×10^3	100	0
	1.0×10^4	100	0
E. coli			
E4	2.3×10^3	40	0
	2.3×10^4	100	20
	2.3×10^5	100	60
	2.3×10^6	100	80
	2.3×10^7	100	100

Each group, consisting of female mice (15–20 g each), was injected intraperitoneally with the indicated number of cells. The treated groups were also injected subcutaneously twice each day with 2 mg of β-chloro-D-alanine. Mortality in each group was assessed 72 hr after the initial infection.

* These mice were injected twice a day with 10 mg of β-chloro-D-alanine. Mice infected with the same number of pneumococci strain A66 and treated with 2 mg twice a day showed 100% mortality.

phate, pH 7.3, at 4°. Alanine racemase, assayed as described in *Methods*, was inhibited 75% by β-chloro-D-alanine treatment; D-glutamate-D-alanine transaminase was completely inhibited by the chloroamino acid, but about 75% of the transaminase activity was restored by addition of 0.5 mM pyridoxal 5′-phosphate. Thus, most of the inhibition of the transaminase by β-chloro-D-alanine appears to be due to removal of the cofactor for the reaction after Schiff base formation with the β-chloroamino acid.

Inhibition of Bacterial Growth In Vivo by β-Chloro-D-alanine. Mice were infected intraperitoneally with *Diplococcus pneumoniae* Type I (SVI) and Type III (A66), *Streptococcus pyogenes* Type 6 (S43) and Type 23 (T23), or *E. coli* K1 (E4). The inocula used for infection, the amounts and frequency of administration of β-chloro-D-alanine, and the mortality observed 72 hr after infection are given in Table 3. The animals were observed further for several days but the results after that time were difficult to interpret for several reasons. In some instances upon cessation of treatment, delayed death due to infection occurred. In other instances, some of the animals died upon further treatment with 20 mg of β-chloro-D-alanine per day. We do not know whether the compound exhibits cumulative toxicity or whether toxic degradation products occur upon storage of the solution of the chloroamino acid.

DISCUSSION

The inhibition of bacterial growth by β-chloro-D-alanine appears to be the result of the inactivation of the enzyme systems that produce D-alanine. However, it is possible that β-chloroalanine also affects some of the later steps in cell-wall synthesis as well, and could itself be incorporated into the cell wall. The lability of β-chloroalanine towards the usual conditions for the acid hydrolysis of proteins precluded studies into these questions; experiments with radioactive β-chloro-D-alanine should reveal any other sites of incorporation of the chloroamino acid.

Our results with the L-isomer of β-chloroalanine are similar to those reported by Arfin and Koziell (19) on the inhibition of growth of *Salmonella typhimurium* with this compound. In our studies, comparison of the two enantiomers of chloroalanine, however, show that whereas the L-isomer is as effective in the inhibition of bacterial growth as the D-isomer, the prevention of inhibition by D-alanine is much less effective for β-chloro-L-alanine than for β-chloro-D-alanine. Based on these studies and on the reported inactivation of decarboxylases and transaminases for L-amino acids by β-chloro-L-alanine (8, 20), we expect that β-chloro-L-alanine would be less specific in its spectrum of reactivity and would probably lead to loss of viability of bacterial and mammalian cells by blocking the production of essential L-amino acids.

The finding that the intracellular, free alanine content of untreated *E. coli* and *B. subtilis* is about 95% in the D-configuration under our conditions of growth may be due to the induction of L-alanine dehydrogenase by D-alanine, as described by Berberich *et al.* (21). Excess D-alanine would activate the dehydrogenase, which catalyzes the deamination of L-alanine, thus serving as an effective control system for the intracellular concentration of L-alanine. In the β-chloro-D-alanine-treated cells, there is probably insufficient D-alanine formed to induce synthesis of L-alanine dehydrogenase and L-alanine accumulates. However, alternative mechanisms for the accumulation of intracellular L-alanine are also possible.

While these studies were in progress, Kollonitsch *et al.* (22) reported that β-fluoro-D-alanine was an effective inhibitor of bacterial growth. A mechanism for the inhibition by β-fluoro-D-alanine was not suggested by these workers, but in view of the similarity of the halo amino acids and of the reported β-elimination undergone by β-fluoroaspartic acid (23), it would seem that fluoroalanine may also act by inhibition of pyridoxal phosphate-dependent transaminases and racemases.

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