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On the Mechanism of Action of the Antibiotic *O*-Carbamyl-D-Serine in *Streptococcus faecalis*[1]

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ABSTRACT

LYNCH, JUDITH L. (Northwestern University, Evanston, Ill.), AND FRANCIS C. NEUHAUS. On the mechanism of action of the antibiotic *O*-carbamyl-D-serine in *Streptococcus faecalis*. J. Bacteriol. **91**:449–460. 1966.—The antibiotic *O*-carbamyl-D-serine, an analogue of D-alanine, is an inhibitor of bacterial cell-wall biosynthesis. Growth of *Streptococcus faecalis* R in the presence of *O*-carbamyl-D-serine resulted in the accumulation of the cell-wall precursor uridine diphosphate-NAc-muramyl-L-alanyl-D-glutamyl-L-lysine (UDP-NAc-muramyl-L-ala-D-glu-L-lys). The incorporation of D-alanine from L-alanine into peptidoglycan is catalyzed by the sequential action of the following enzymes: (i) alanine racemase; (ii) D-alanine: D-alanine ligase [adenosine diphosphate (ADP)]; (iii) UDP-NAc-muramyl-L-ala-D-glu-L-lys: D-ala-D-ala ligase (ADP); (iv) phospho-NAc-muramyl-pentapeptide translocase [uridine monophosphate (UMP)]. *O*-carbamyl-D-serine is an effective inhibitor of the alanine racemase ($K_i = 4.8 \times 10^{-4}$ M, K_m of L-alanine $= 6.8 \times 10^{-3}$ M). In addition, D-ala-*O*-carbamyl-D-ser was formed when D-alanine and *O*-carbamyl-D-serine were incubated with D-alanine: D-alanine ligase (ADP). This dipeptide was utilized by the UDP-NAc-muramyl-L-ala-D-glu-L-lys: D-ala-D-ala ligase (ADP) with the formation of UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-*O*-carbamyl-D-ser. From a consideration of the following results, i.e., (i) accumulation of UDP-NAc-muramyl-L-ala-D-glu-L-lys; (ii) absence of D-ala-*O*-carbamyl-D-ser accumulation in bacterial cultures grown in the presence of *O*-carbamyl-D-serine; and (iii) effective inhibition of the racemase, it was concluded that the first enzyme, the racemase, is the primary site of antibiotic action.

The antibiotic *O*-carbamyl-D-serine, an analogue of D-alanine, is an inhibitor of bacterial cell-wall biosynthesis (30). It was demonstrated that the antibacterial activity could be reversed by D-alanine, and that an intracellular accumulation of *N*-acylamino sugar resulted when *Bacillus subtilis* was grown in the presence of the antibiotic. In addition, *O*-carbamyl-D-serine inhibited the incorporation of C^{14}-glutamic acid into the cell-wall fraction of *B. subtilis* and *Staphylococcus aureus*. Tanaka and Umezawa (31) observed a pronounced synergism between *O*-carbamyl-D-serine and D-cycloserine (D-4-amino-3-isoxazolidone) and proposed that the two antibiotics may inhibit sequential enzymatic reactions in the biosynthesis of the cell wall.

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The incorporation of D-alanine from L-alanine into the peptidoglycan (mucopeptide) portion of the wall is catalyzed by the sequential action of the following enzymes: (i) alanine racemase (5.1.1.1) (35); (ii) D-alanine: D-alanine ligase [adenosine diphosphate (ADP)] (6.3.2.4) [D-alanyl-D-alanine (D-ala-D-ala) synthetase] (7, 12); (iii) UDP-NAc-muramyl-L-ala-D-gly-L-lys: D-ala-D-ala ligase (ADP) (D-ala-D-ala adding enzyme) (4, 7, 14); (iv) phospho-NAc-muramyl-pentapeptide translocase [uridine monophosphate (UMP)] (1, 29, Struve et al., Biochemistry, *in press*); (v) transfer of NAc-glucosamine from UDP-NAc-glucosamine to acceptor-phospho-NAc-muramyl-pentapeptide with the formation of acceptor (lipid)-phosphodisaccharide (1); (vi) peptidoglycan synthetase (1). Peptidoglycan synthetase catalyzes the polymerization of the acceptor (lipid)-phosphodisaccharide, with the formation of peptidoglycan which is not cross-linked (1, 3).

When bacteria are grown in the presence of penicillin, the predominant nucleotide which

accumulates is UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala (15, 16, 17, 19, 24). In contrast, if *S. aureus* is grown in the presence of D-cycloserine, the major nucleotide that accumulates is UDP-NAc-muramyl-L-ala-D-glu-L-lys (28). Strominger et al. (26) observed that D-cycloserine inhibits the D-ala-D-ala synthetase and the alanine racemase. Inhibitor studies (13) indicated that both D-alanine binding sites of the synthetase are sensitive to D-cycloserine. It was suggested that one of the primary sites of antibiotic action may be the donor site of the synthetase.

It is the purpose of this communication to define the mode of action of *O*-carbamyl-D-serine in the sequence of enzymes responsible for the introduction of alanine into the peptidoglycan portion of the wall. The major nucleotide which accumulates in the presence of *O*-carbamyl-D-serine is identified as UDP-NAc-muramyl-L-ala-D-glu-L-lys. Of the four potential sites of action which were examined, it was concluded that a primary site of action is the alanine racemase.

MATERIALS AND METHODS

Materials. We are indebted to P. H. Hidy and M. C. Bachman of Commercial Solvents Corp., Terre Haute, Ind., for samples of *O*-carbamyl-D-serine; to C. G. Skinner for *O*-carbamyl-L-serine; to J. T. Park for muramic acid; to O. K. Behrens for D-cyclosyrine and β-aminoxy-D-alanine. D-Alanine-*l*-C^{14}, L-alanine-*l*-C^{14}, and L-cycloserine were purchased from Calbiochem. Glutamic-pyruvic transaminase (2.6.1.2) (27 units per mg) and pyruvate kinase (2.7.1.40) (125 units per mg) were the products of Boehringer Mannheim, New York, N. Y. D-Amino acid oxidase (1.4.3.3), 4.5 units per mg (electrophoretically purified), and catalase (CTR) were purchased from Worthington Biochemical Corp., Freehold, N. J. Lactic dehydrogenase (1.1.1.27) (58 units per mg) was purchased from Sigma Chemical Co., St. Louis, Mo. All other intermediates, amino acids, and nucleotides were the products of Sigma Chemical Co., Calbiochem, and Nutritional Biochemicals Corp., Cleveland, Ohio.

D-Ala-*O*-carbamyl-D-ser was synthesized by Herman Plaut of Cyclo Chemical Corp., Los Angeles, Calif., with the procedure described by Skinner et al. (21) for *O*-carbamyl-D-serine. The starting material was *N*-benzyloxycarbonyl-D-ala-D-ser-*p*-nitrobenzyl ester. The product was recrystallized from water-ethyl alcohol. Analysis: $C_7H_{13}N_3O_5$; calculated: C, 38.35; H, 5.97; N, 19.17; found: C, 38.9; H, 6.31; N, 18.7. Specific rotation: $[\alpha]_D^{20} = -28°$ (C = 2 in 1 N HCl; 1 = 1). The R_F was 0.21 and 0.44 in solvents B and C, respectively. The dipeptide was eluted at 300 ml on an Amberlite IR-120 column (0.9 by 150 cm) on a Beckman Spinco model 120B amino acid analyzer (23). Elution was initiated at *p*H 3.28 with 0.2 N sodium citrate and changed to *p*H 4.25 at 8 hr and 40 min. Under these conditions, the following elution volumes were observed: *O*-carbamyl-D-serine, 128 ml; D-serine, 169 ml; D-alanine, 283 ml; D-ala-D-ser, 354 ml; D-ala-D-ala, 398 ml. No alanine, serine, or *O*-carbamyl-D-serine was detected in the dipeptide. The routine separations were performed on the accelerated system (40 ml/hr, 0.9 by 50 cm column) described by Spackman (Federation Proc., **22**:244, 1963). No resolution of D-ala-*O*-carbamyl-D-ser and alanine was achieved with the Beckman spherical research resin (type AA-15).

UDP-NAc-muramyl-L-ala-D-glu-L-lys and UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala were prepared as previously described (14). D-Ala[C^{14}]-D-ala[C^{14}] was prepared with D-ala-D-ala synthetase (12).

Growth of bacteria. Streptococcus faecalis R (ATCC 8043) was grown in the following medium: peptone-T, 1%; yeast extract, 1%; K_2HPO_4, 0.5%; glucose, 0.5%. Growth was arrested in the log phase by cooling, and the cells were harvested by centrifugation. A portion of a suspension in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-HCl (*p*H 7.8) was used to inoculate tubes (10 ml) of medium containing inhibitor to an initial optical density of 0.1. The tubes were maintained at 37 C, and the turbidity was measured at 670 mμ.

Enzyme preparations. Except for the phospho-NAc-muramyl-pentapeptide translocase, all extracts were prepared from *S. faecalis* R (ATCC 8043) according to procedures previously described (12). A unit of enzyme is that amount which will catalyze the formation of 1 μmole of product per min at 37 C. A sonic extract was used as a source of the alanine racemase (0.056 units per mg, "L-alanine to D-alanine assay"). D-Ala-D-ala synthetase (1.3 units per mg) was purified through the acetone fractionation (12). For the "nucleotide-tripeptide assay" a 55 to 70% ammonium sulfate fraction (0.12 units per mg) was used (14) as a source of the D-ala-D-ala adding enzyme. For the experiments described in Fig. 9, a diethylaminoethyl (DEAE)-Sephadex fraction (0.49 units per mg) was employed (14). Phospho-NAc-muramyl-pentapeptide translocase (UMP) was prepared from *S. aureus* Copenhagen (Struve et al., *in press*).

Alanine racemase assay. The alanine racemase was assayed in both directions by converting the product to pyruvic acid. In the forward direction ("L-alanine to D-alanine assay"), D-alanine was deaminated with D-amino acid oxidase, and in the reverse direction ("D-alanine to L-alanine assay"), the L-alanine was deaminated with the glutamic-pyruvic acid transaminase. The C^{14}-pyruvic acid was separated from the substrate by ion-exchange chromatography.

A standard reaction mixture (first stage) contained: 0.05 M potassium phosphate buffer, *p*H 8.0; 0.005 M D-alanine-C^{14} (9.3×10^4 counts per min per μmole) or 0.005 M L-alanine-C^{14} (9.8×10^4 counts per min per μmole); and 84 μg of protein in a total volume of 0.1 ml. The enzyme was added to the reaction mixture at 37 C and incubated for 10 min. The reaction was terminated by placing the tube in a boiling-water bath for 2 min.

In the second stage for the deamination of D-alanine in the forward reaction ("L-alanine to D-alanine assay"), 0.01 ml of a solution containing 16.7 μg of D-amino acid oxidase, 40 units of catalase, 0.1 nmole

of flavin adenine dinucleotide (FAD), and 0.2 μmole of sodium pyrophosphate buffer (*p*H 8.3) were added to the reaction mixture (0.1 ml, first stage) and incubated at 37 C for 1 hr. The reaction was terminated with 0.5 ml of 0.2 M sodium citrate buffer (*p*H 2.2).

For the deamination of L-alanine in the reverse reaction ("D-alanine to L-alanine assay"), 0.02 ml of a solution containing 36 μmoles of α-ketoglutarate adjusted to *p*H 8.0 with NaOH and 4 μg of glutamic-pyruvic acid transaminase was added to the reaction mixture (0.1 ml, first stage) and incubated at 37 C for 45 min. The reaction was terminated by the addition of 0.1 ml of 1 N HCl and 0.4 ml of 0.2 N sodium citrate (*p*H 2.2).

The labeled pyruvic acid in both assays was separated from C^{14}-alanine on a Dowex 50(Na^+)X-8 (200 to 400 mesh) column (5 by 20 mm). The reaction mixture from the second stage was quantitatively applied to the column and eluted with two portions (0.7 ml each) of 0.2 N sodium citrate (*p*H 2.2). The eluate (2.0 ml) was collected in a polyethylene vial for the determination of radioactivity. Under these conditions, C^{14}-alanine was eluted between 6 and 10 ml. Appropriate controls were performed in each series to correct for the slow decomposition of the C^{14}-alanine to compounds not retained by the column. The amount of D-amino acid oxidase and glutamic-pyruvic acid transaminase was sufficient to deaminate 100 nmoles of D- or L-alanine. The usual amount of D- or L-alanine converted was between 5 and 50 nmoles. Not more than 10% of the alanine was converted to product.

Assay of UDP-NAc-muramyl-L-ala-D-glu-L-lys ("nucleotide-tripeptide assay"). The UDP-NAc-muramyl-L-ala-D-glu-L-lys was quantified by the conversion of the nucleotide-tripeptide to the nucleotide-pentapeptide according to the following reaction:

$$\text{ATP} + \text{UDP-NAc-muramyl-tripeptide} + \text{D-ala}[C^{14}]\text{-D-ala}[C^{14}] \xrightarrow{Mg^{2+}} \text{UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala}[C^{14}]\text{-D-ala}[C^{14}] + \text{ADP} + P_i \quad (I)$$

The D-ala-D-ala adding enzyme from *S. aureus* Copenhagen is specific for UDP-NAc-muramyl-tripeptide (7). The reaction mixture contained: 10 μmoles of Tris-HCl buffer (*p*H 7.8), 0.1 μmole of disodium ATP neutralized with NaOH, 10 μmoles of $MgCl_2$, 0.1 μmole of D-ala[C^{14}]-D-ala[C^{14}] (1,280 counts per min per nmole), 0.16 units of the adding enzyme preparation, a sample of the extract to be tested, and water to a final volume of 0.1 ml. The tubes were incubated at 37 C for 20 min, and the reaction was terminated by the addition of 0.5 ml of 0.2 M sodium citrate (*p*H 2.2).

The UDP-NAc-muramyl-[C^{14}]pentapeptide was separated from the D-ala[C^{14}]-D-ala[C^{14}] on Dowex 50(Na^+) X-8 columns (5 by 20 mm) as described by Neuhaus and Struve (14). The UDP-NAc-muramyl-[C^{14}]pentapeptide was collected in a polyethylene vial for determination of the radioactivity. When the medium was assayed, a 5 by 45 mm column was used. The standard curve for this assay is illustrated in Fig. 1.

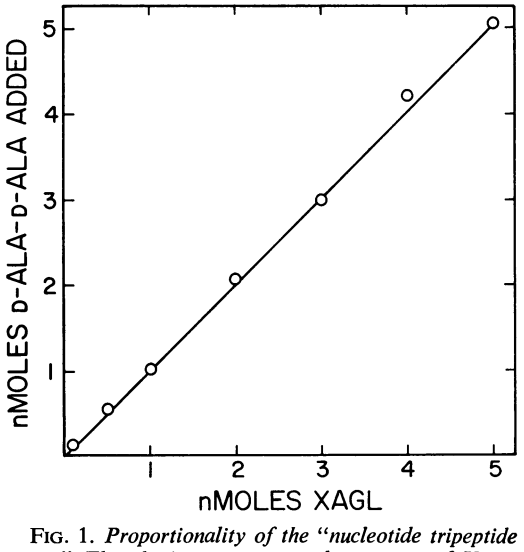

FIG. 1. *Proportionality of the "nucleotide tripeptide assay." The abscissa represents the amount of UDP-NAc-muramyl-tripeptide (XAGL) in the reaction mixture, and the ordinate represents the amount of D-ala[C^{14}]-D-ala[C^{14}] transferred in the "nucleotide tripeptide assay."*

TABLE 1. *Comparison of extraction procedures**

Extraction procedure	UDP-NAc-muramyl-L-ala-D-glu-L-lys	*N*-Acyl-amino sugar
	nmoles	*nmoles*
1. Standard procedure	13.4	641
2. a. Four cycles of freezing and thawing (fraction A)	11.7	619
b. Extraction of precipitate from 2a with water at 100 C for 3 min (fraction B)	1.8	78
Sum	13.5	697
3. Perchloric acid (7%) at 0 to 4 C for 60 min	11.0	425
4. Trichloroacetic acid (10%) at 0 to 4 C for 60 min	12.5	505

* The above extraction procedures were performed on samples of late-log phase *Streptococcus faecalis* R [83 mg (dry weight) of bacteria]. In each procedure, the total extraction volume was 3 ml. The "nucleotide-tripeptide assay" was used to establish the amount of UDP-NAc-muramyl-L-ala-D-glu-L-lys, and the *N*-acylamino sugar was determined by the method of Reissig et al. (18).

Extraction methods. The standard extraction was performed according to the following procedure. The cell suspension [e.g. 25 mg (dry weight) in 1 ml of water] was subjected to four cycles of freezing and thawing. The suspension was then placed in a boiling-water bath for 3 min. The precipitate was removed by centrifuga-

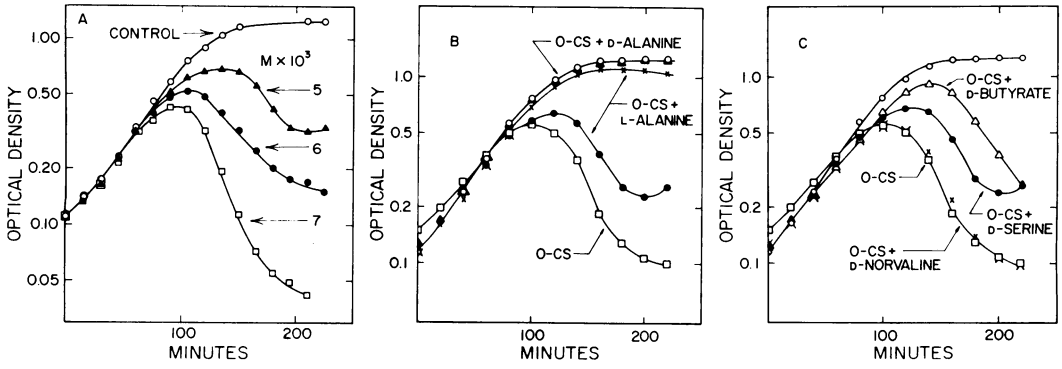

FIG. 2. *Growth studies with Streptococcus faecalis R. Effect of increasing concentrations of O-carbamyl-D-serine (A), reversal by alanine (B), and specificity of reversal (C). In (A), (B), and (C), ○ represents the control culture. (A) Concentrations of O-carbamyl-D-serine are: ▲, 5.0 × 10^{-3} M; ●, 6.0 × 10^{-3} M; □, 7.0 × 10^{-3} M. All cultures in (B) and (C), except the controls, contain 7.0 × 10^{-3} M O-carbamyl-D-serine (O-CS). (B) Concentration of L-alanine in ● and × is 3.0 × 10^{-3} M and 3.0 × 10^{-2} M, respectively, and, in ▲, the concentration of D-alanine is 3.0 × 10^{-3} M. (C) Concentrations of D-α-amino-n-butyric acid (D-butyrate) (△), D-serine (●), and D-norvaline (×) are 3.0 × 10^{-3} M.*

tion at 23,000 × *g*, and the supernatant fraction was assayed for UDP-NAc-muramyl-tripeptide and *N*-acylamino sugar. A number of different extraction procedures were considered (Table 1). The one described above is similar to that used by Strominger (25). In Table 1, four samples of late-log phase *S. faecalis* R (ATCC 8043) were subjected to the following procedures for extraction: (i) standard extraction procedure; (ii) four cycles of freezing and thawing (fraction A), followed by extraction with water at 100 C (fraction B); (iii) extraction with 7% perchloric acid at 0 to 4 C according to the procedure of Saukkonen and Virkola (20); (iv) extraction with 10% trichloroacetic acid according to the method described by Strominger (24). From the data in Table 1, it was apparent that each method allowed for the effective extraction of the UDP-NAc-muramyl-tripeptide. There was a lower yield of *N*-acylamino sugar in those procedures which utilized acid.

The "nucleotide-tripeptide assay" was evaluated with regard to the following. (i) Was there an additional intermediate which utilized D-ala-D-ala and was eluted with UDP-NAc-muramyl-pentapeptide? (ii) Was there an inhibitor of the D-ala-D-ala adding enzyme in the extract? (iii) Was there a significant amount of D-ala-D-ala in the extract to dilute the added D-ala[C^{14}]-D-ala[C^{14}] and, thus, give an apparent inhibition? Water extracts ("standard extraction procedure") and UDP-NAc-muramyl-tripeptide were chromatographed in solvent A, and the bands corresponding to the UDP-NAc-muramyl-tripeptide were eluted with water. With standard nucleotide, the recovery by use of the "nucleotide-tripeptide assay" was 80 ± 2%. With a water extract, 78% was recovered. With the addition of a sample of UDP-NAc-muramyl-tripeptide (1 nmole) to the water extract, 80% was recovered. On the basis of these results, it was concluded that the major nucleotide which utilized D-ala-D-ala was the UDP-NAc-muramyl-tripeptide. To correct for endogenous D-ala-D-ala and

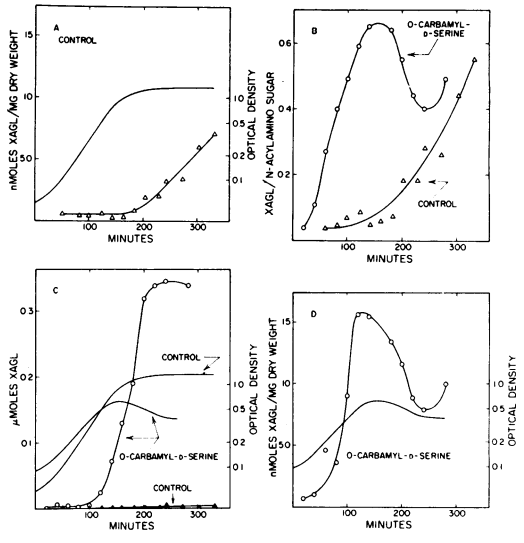

FIG. 3. *Accumulation of UDP-NAc-muramyl-L-ala-D-glu-L-lys. (A) Accumulation of UDP-NAc-muramyl-tripeptide in a control culture; (B) fraction of N-acyl-amino sugar that is UDP-NAc-muramyl-tripeptide; (C) accumulation of UDP-NAc-muramyl-tripeptide in the medium; (D) accumulation of UDP-NAc-muramyl tripeptide in a culture grown in the presence of 5.0 × 10^{-3} M O-carbamyl-D-serine. Samples (25 ml) were removed from a culture at the appropriate time and chilled in an acetone-Dry Ice bath. The cells were harvested by centrifugation at 23,000 × g. Extracts were prepared and assayed for UDP-NAc-muramyl-tripeptide and N-acylamino sugar.*

possible inhibitors, all assays were performed in the presence and absence of 1 nmole of UDP-NAc-muramyl-tripeptide. The per cent recovery of the

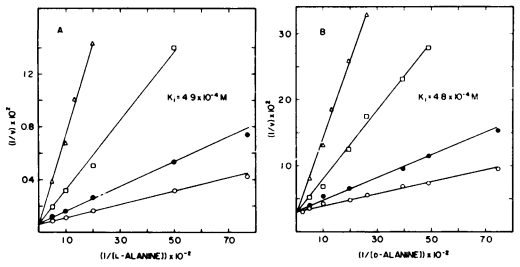

FIG. 4. *Effect of O-carbamyl-D-serine on the racemase. (A) The "L-alanine to D-alanine assay" was used; (B) the "D-alanine to L-alanine assay" was used. Concentrations of O-carbamyl-D-serine are:* ○, *none;* ●, *5.0 × 10⁻⁴ M;* □, *2 × 10⁻³ M;* △, *5.0 × 10⁻³ M.*

standard was used to correct the amount of UDP-NAc-muramyl-tripeptide that was present in the extract. The largest correction was 17%, with the average around 5%.

Analytical procedures. *N*-acylamino sugar was determined by the semimicro method of Reissig et al. (18). When the water extracts were used, the samples were hydrolyzed in 0.13 M HCl by placing the tube in a boiling-water bath for 3 min. The reaction mixtures were neutralized before assaying. The standard muramic acid was acetylated with acetic anhydride according to the procedure described by Strominger et al. (27). The ϵ_{585} for *N*-acetyl-muramic acid and UDP-NAc-muramyl-tripeptide was 23,200. Measurements of radioactivity were made in polyethylene vials with a Packard Tri-Carb scintillation spectrometer (model 314-EX). The scintillation fluid was prepared according to Bray (2). All measurements were made for 10^5 counts.

Chromatography. The nucleotides were chromatographed on Whatman 3MM paper in the following descending solvent system: (A) isobutyric acid-concentrated NH_4OH-water (66:1:33) (Circular OR-17, Pabst Brewing Co., Milwaukee, Wis.). The dipeptides were chromatographed in the following descending solvent systems: (B) 1-butanol-acetic acid-water (4:1:5; organic phase) (11); (C) *t*-butyl alcohol-88% formic acid-water (70:15:15) (11).

RESULTS

Effect of O-carbamyl-D-serine on growth. The effect of increasing concentrations of antibiotic on growth is illustrated in Fig. 2A. The minimal concentration that resulted in lysis was 5×10^{-3} M. The initial growth rate in each case was identical. In agreement with the results observed with D-cycloserine (13), the time necessary to observe lysis decreased as the concentration of *O*-carbamyl-D-serine increased. In Fig. 2B, the reversal by alanine of the effects by *O*-carbamyl-D-serine is demonstrated. D-Alanine (3×10^{-3} M) and L-alanine (3×10^{-2} M) completely reversed the effect of *O*-carbamyl-D-serine (7.0×10^{-3} M). The specificity of the reversal is shown in Fig. 2C. D-α-Amino-*n*-butyric acid and D-serine partially reversed the effects of *O*-carbamyl-D-serine. The same concentration of D-norvaline had no effect.

TABLE 2. *Inhibitor specificity of alanine racemase*[a]

Addition	Per cent inhibition	
	"L-Alanine to D-alanine assay"	"D-Alanine to L-alanine assay"
O-Carbamyl-D-serine	53	38
O-Carbamyl-L-serine	0	0
D-Cycloserine	87	79
L-Cycloserine	8	—[b]
D-Serine	10	9
L-Serine	0	0
D-Threonine	0	0
L-Threonine	0	0
D-α-NH_2-*n*-butyric acid	12	6
L-α-NH_2-*n*-butyric acid	0	0
D-Norvaline	0	0
L-Norvaline	0	0
β-Aminoxy-D-alanine	—[c]	84[d]

[a] The "L-alanine to D-alanine assay" and the "D-alanine to L-alanine assay" were used with 84 μg of protein. The concentration of alanine and inhibitor were 5×10^{-3} M and 10^{-3} M, respectively. In the "L-alanine to D-alanine assay" and the "D-alanine to L-alanine assay," the velocities were 46 and 28 nmoles converted per min, respectively. In the "L-alanine to D-alanine assay," 20 μliters of the D-amino-acid oxidase mixture was added to the second stage. Controls were performed to show that 100 nmoles of alanine was deaminated in the second stage of the assays.

[b] L-Cycloserine is an effective inhibitor of the glutamic-pyruvic transaminase.

[c] β-Aminoxy-D-alanine inhibits D-amino-acid oxidase.

[d] Additional glutamic-pyruvic transaminase (40 μg) was added to the second stage.

Accumulation of UDP-NAc-muramyl-L-ala-D-glu-L-lys as a function of growth phase. A control culture was examined for the accumulation of UDP-NAc-muramyl-tripeptide (Fig. 3A). The amount of this nucleotide per milligram (dry weight) remained constant until the stationary phase of growth was attained. An increase in nucleotide was then observed. Thus, after 2 hr in the stationary phase, UDP-NAc-muramyl-tripeptide constituted over 50% of the total *N*-acylamino sugar (Fig. 3B). In the medium, the amount of UDP-NAc-muramyl-tripeptide was undetectable until after the stationary phase was attained (Fig. 3C).

The pattern of accumulation, as well as the amount of nucleotide measured, was different in

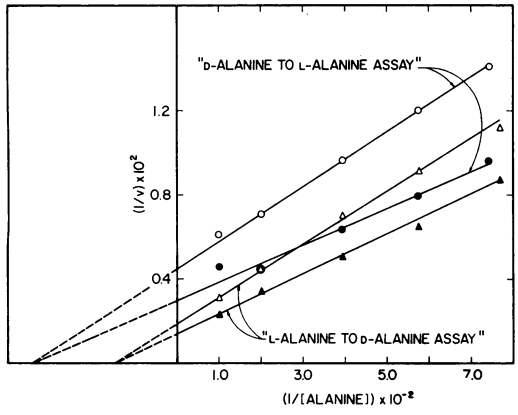

FIG. 5. *Effect of alanine on the forward and reverse reactions in the presence and absence of added pyridoxal phosphate. For ▲ and △ the "L-alanine to D-alanine assay" was used; for ● and ○ the "D-alanine to L-alanine assay" was used. The assays contained 80 μg of protein. With the solid symbols the reaction mixtures contained 4 × 10⁻⁵ M pyridoxal phosphate.*

TABLE 3. *Summary of constants for alanine racemase*

Assay	K_m	V_{max}	K_i
	moles/liter	*nmoles /min*	*moles/liter*
"L-Alanine to D-alanine"...	6.8×10^{-3}	5.4	4.9×10^{-4}
"D-Alanine to L-alanine"...	3.0×10^{-3}	2.2	4.8×10^{-4}

TABLE 4. *Inhibition of the D-ala-D-ala synthetase by D-ala-D-ala and D-ala-O-carbamyl-D-ser**

Dipeptide	Inhibition
	%
D-Ala-D-ala	80
D-Ala-*O*-carbamyl-D-ser	14
D-Ala-D-ser	68
D-Ala-D-norvaline	90
D-Ala-D-α-amino-*n*-butyric acid	90
D-Ala-D-val	43

* D-Ala-D-ala synthetase was measured by the "assay for ADP formation" described by Neuhaus (12). The D-alanine and dipeptide concentrations were 10^{-3} M and 3×10^{-3} M, respectively. In addition the reaction mixture contained: 0.05 M Tris-HCl buffer (*p*H 7.8), 0.01 M $MgCl_2$, 5 mM ATP neutralized with NaOH, 0.05 M KCl, 0.2 M choline chloride, 10^{-4} M reduced nicotinamide adenine dinucleotide ($NADH_2$), 1.2 mM phosphoenolpyruvate (tricyclohexylammonium salt), 40 μg of lactic dehydrogenase, 12.5 μg of pyruvate kinase, and 2.7 units of purified D-ala-D-ala synthetase preparation in a volume of 0.5 ml. The oxidation of $NADH_2$ was followed at 340 mμ at 25 C.

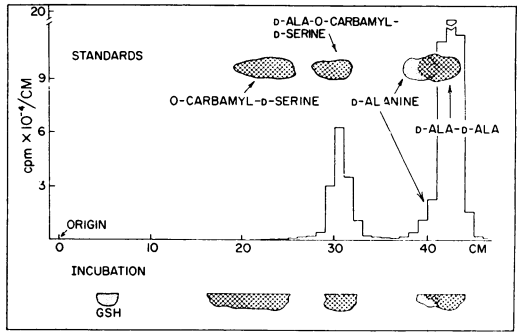

FIG. 6. *Synthesis of D-ala-O-carbamyl-D-ser. The reaction mixture contained: 0.05 M Tris-HCl, pH 7.8; 0.01 M ATP neutralized with NaOH; 0.01 M $MgCl_2$; 0.05 M KCl; 5 × 10^{-3} M D-alanine-C^{14} (1 μc/μmole); 0.12 M O-carbamyl-D-serine; and 1.1 mg of protein (synthetase preparation) in a total volume of 1.0 ml. After 2 hr at 37 C, the reaction was terminated by placing the mixture in a boiling-water bath. A sample (0.2 ml) was chromatographed on Whatman 3 MM paper with solvent C in a descending system. Guide strips were developed with ninhydrin (lower row), and sections (bar graph) (2 by 1 cm) were counted in the scintillation spectrometer. The standards are shown in the upper row.*

cells grown in the presence of *O*-carbamyl-D-serine (Fig. 3D). UDP-NAc-muramyl-tripeptide accumulated during the exponential phase of growth. The fraction of this nucleotide in the cytoplasm reached 0.6 at 150 min (Fig. 3B). At 120 min, the cells grown in the presence of *O*-carbamyl-D-serine contained 15.6 nmoles of UDP-NAc-muramyl-tripeptide per mg (dry weight) of cells, and the control culture contained 0.55 nmoles/mg (dry weight). In contrast to the control culture, there was a rapid accumulation of UDP-NAc-muramyl-tripeptide in the medium (Fig. 3C).

Effect of O-carbamyl-D-serine on the enzymes responsible for the incorporation of alanine into the complete nucleotide UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala. Alanine racemase. The rates of alanine racemization were measured at varying concentrations of alanine at three concentrations of *O*-carbamyl-D-serine (Fig. 4A and B). In Fig. 4A the "L-alanine to D-alanine assay" was used, and in Fig. 4B the "D-alanine to L-alanine assay" was used. In both assays, *O*-carbamyl-D-serine was a competitive inhibitor of the racemization. From these results, values for K_i were established (4.8×10^{-4} M and 4.9×10^{-4} M). A number of analogues were tested for their action on the racemase (Table 2). At the concentration (1.0×10^{-3} M) that was tested, *O*-carbamyl-D-serine and D-cycloserine were effective inhibitors. D-Serine and D-α-amino-*n*-butyric acid were poor inhibitors. In contrast to the

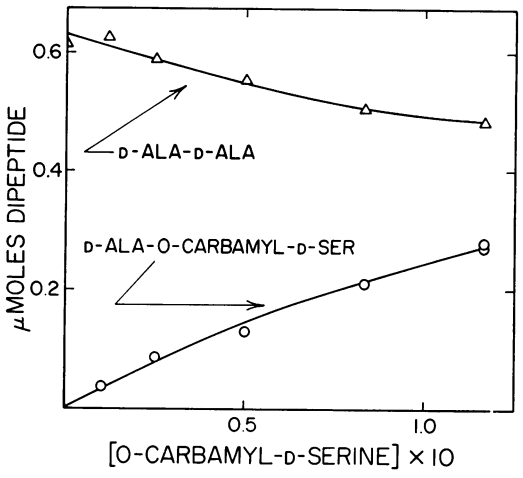

FIG. 7. *Effect of O-carbamyl-D-serine on the synthesis of D-ala-D-ala and D-ala-O-carbamyl-D-ser. The reaction mixtures contained: 0.05 M Tris-HCl, pH 7.8; 0.05 M KCl; 0.01 M $MgCl_2$; 0.01 M ATP neutralized with NaOH; 5.0 × 10^{-3} M D-alanine; O-carbamyl-D-serine; and 0.44 mg of protein (synthetase preparation) in a total volume of 0.3 ml. After 30 min at 37 C, the reaction was terminated by placing the tube in a boiling-water bath for 2 min. The dipeptides, D-ala-D-ala (△) and D-ala-O-carbamyl-D-ser (○), were quantified by the procedure described in Materials and Methods.*

results with the D-isomers, *O*-carbamyl-L-serine L-serine, and L-α-amino-*n*-butyric acid had no, effect on the racemase, and L-cycloserine (10^{-3} M) inhibited to a small extent (8%). β-Aminoxy-D-alanine was an effective inhibitor of the racemase.

From the Lineweaver-Burk plots (10) in Fig. 4A and B, Michaelis constants (K_m) were established for comparison with the values for K_i of *O*-carbamyl-D-serine. The values for K_m were 6.8 × 10^{-3} M for L-alanine and 3.0 × 10^{-3} M for D-alanine.

In Fig. 5, the assays for the forward and reverse directions are compared. For the conversion of L-alanine to D-alanine and D-alanine to L-alanine, V_{max} was 5.4 and 2.2 nmoles converted per min, respectively. The addition of pyridoxal phosphate (4 × 10^{-5} M) increased the V_{max} in the "L-alanine to D-alanine assay" to 7.3 and in the "D-alanine to L-alanine assay" to 3.4 (Fig. 5). The values for K_m were identical to those observed in the absence of additional pyridoxal phosphate. Since the K_{eq} for this reaction is 1, the validity of these constants was examined in the Haldane relationship (5).

$$K_{eq} = \frac{(K_{m\ D\text{-alanine}})(V_{max\ L\to D})}{(K_{m\ L\text{-alanine}})(V_{max\ D\to L})} \qquad (II)$$

From the above results, K_{eq} was calculated to be 0.95 and 1.1. The values for K_m, K_i for *O*-carbamyl-D-serine, and V_{max} are summarized in Table 3.

D-Ala-D-ala-synthetase. With the synthetase, two effects have been examined. The first involved the synthesis of the mixed dipeptide D-ala-*O*-carbamyl-D-ser, and the second involved the inhibition by this dipeptide of the synthesis of D-ala-D-ala. Previous studies (12, 13) have shown that the mixed dipeptides, D-ala-D-butyr, D-ala-D-ser, D-ala-D-thr, D-ala-D-norvaline, and D-ala-β-aminoxy-D-ala, were formed in addition to D-ala-D-ala when D-alanine and the analogue of D-alanine were incubated in the presence of enzyme, ATP, and Mg^{2+}. When D-alanine-C^{14} and *O*-carbamyl-D-serine were incubated under these conditions, a new ninhydrin-reactive C^{14}-labeled compound (Fig. 6) was observed with an R_F identical to that of authentic D-ala-*O*-carbamyl-D-ser. The C^{14}-labeled compound was isolated

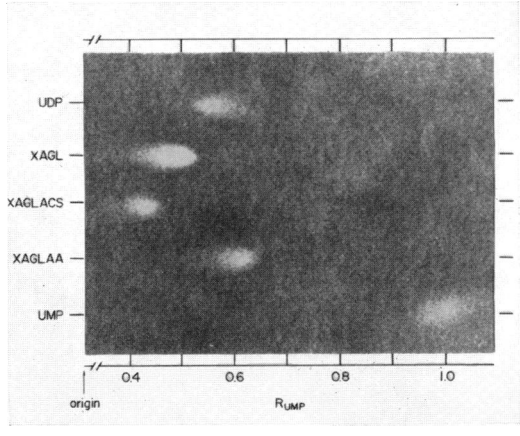

FIG. 8. *Synthesis of UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-O-carbamyl-D-ser. The reaction mixture contained: 0.01 M Tris-HCl, pH 7.8; 0.02 M ATP neutralized with NaOH; 0.05 M D-ala-O-carbamyl-D-ser; 4.3 × 10^{-3} M UDP-NAc-muramyl-L-ala-D-glu-L-lys; 2.6 mg of enzyme preparation (adding enzyme, 0.12 units per mg) in a total volume of 1 ml. The reaction mixture was incubated for 2 hr at 37 C. The reaction was terminated by placing the tube in a boiling-water bath for 3 min. The UDP-NAc-muramyl-peptide fraction was isolated by gel filtration on a column of Sephadex G-25 (200 by 1.9 cm) with 0.02 M NH_4HCO_3 (pH 7.8) as the eluant. The NH_4HCO_3 was removed by lyophilization, and a sample was chromatographed along with standards on Whatman 3 MM paper with solvent A for 66 hr. The abbreviations are: XAGL, UDP-NAc-muramyl-tripeptide; XAGLAA, UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala; XAGLACS, UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-O-carbamyl-D-ser. A portion of the UDP-NAc-muramyl-peptide-analogue was hydrolyzed in 5.7 N HCl for 12 hr at 110 C. The amino acid analysis gave: glutamic acid–lysine–alanine–serine–muramic acid (1.0:0.94:1.93:0.95:0.59). The ratio 280:260 = 0.39, and the ratio 250:260 = 0.74.*

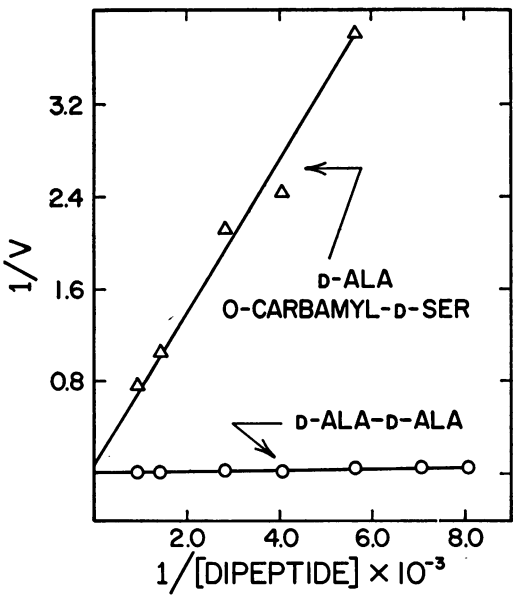

FIG. 9. *Effect of D-ala-O-carbamyl-D-ser on the D-ala-D-ala adding enzyme. The reaction mixture contained: 0.10 M Tris-HCl, pH 7.8; 0.01 M $MgCl_2$; 10^{-4} M UDP-NAc-muramyl-L-ala-D-glu-L-lys; 10^{-3} M ATP neutralized with NaOH; D-ala[C^{14}]-D-ala[C^{14}] (1,200 counts per min per nmole) in (○) or D-ala[C^{14}]-O-carbamyl-D-ser (1,180 counts per min per nmole) in (△); and 4 μg of enzyme preparation (0.49 units per mg) in a total volume of 0.10 ml. The enzyme preparation was added to the assay mixture at 37 C and incubated for 5 min at this temperature. The reaction was terminated by the addition of 0.5 ml of 0.2 N sodium citrate buffer, pH 2.2. The UDP-NAc-muramyl-pentapeptide was separated and assayed for radioactivity as previously described (14). The velocity is reported in nanomoles per minute.*

by preparative paper chromatography with solvent C. Hydrolysis with 5.7 N HCl for 18 hr at 110 C gave alanine and serine in a ratio of 1.0:0.99. Treatment of a sample with 0.1 N NaOH for 10 min at 100 C converted the dipeptide to ala-ser. In addition, the new dipeptide was eluted at the same position as the authentic sample of D-ala-*O*-carbamyl-D-ser on the Amberlite IR 120 column (150 by 0.9 cm) of the amino acid analyzer. A series of incubations were performed with increasing concentrations of *O*-carbamyl-D-serine (Fig. 7). At 0.05 M *O*-carbamyl-D-serine and 0.005 M D-alanine, 0.13 μmole of D-ala-*O*-carbamyl-D-ser and 0.56 μmole of D-ala-D-ala were formed.

Previous studies with the synthetase (12, Neuhaus et al., *unpublished data*) have demonstrated that the product of the synthetase was an effective inhibitor of the reaction. The results (Table 4) with D-ala-*O*-carbamyl-D-ser were compared with other dipeptides that have been studied. In comparison with D-ala-D-ala, D-ala-*O*-carbamyl-D-ser was a poor inhibitor of the synthetase.

D-Ala-D-ala adding enzyme. When D-ala-*O*-carbamyl-D-ser was incubated in the presence of the D-ala-D-ala adding enzyme, UDP-NAc-muramyl-tripeptide, ATP, and Mg^{2+}, a new nucleotide was observed (Fig. 8). This nucleotide was identified as UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-*O*-carbamyl-D-ser. The rates of formation of this nucleotide were measured at varying concentrations of D-ala-*O*-carbamyl-D-ser at a fixed concentration of UDP-NAc-muramyl-tripeptide. For comparison, the rates of formation of UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala at varying concentrations of D-ala-D-ala were measured. From the Lineweaver-Burk plots (10), illustrated in Fig. 9, the K_m values for D-ala-*O*-carbamyl-D-ser (3.6×10^{-3} M) and D-ala-D-ala (1.6×10^{-4} M) were established. It is apparent that V_{max} for each dipeptide is identical. The ratio for the K_m of D-ala-*O*-carbamyl-D-ser to the K_m of D-ala-D-ala is 22. *O*-carbamyl-D-

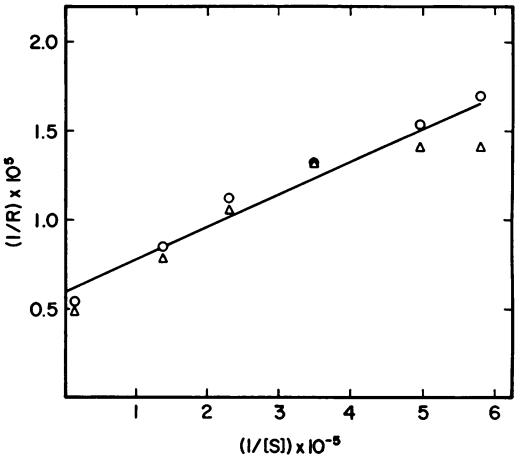

FIG. 10. *Effect of UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-O-carbamyl-D-ser* (△) *and UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala* (○) *on the reaction catalyzed by phospho-NAc-muramyl-pentapeptide translocase (UMP). The reaction mixture contained: 0.05 M Tris-HCl, pH 7.8; 0.01 M $MgCl_2$; UDP-NAc-muramyl-pentapeptide; 4.5×10^{-6} M H^3-UMP (4.5×10^4 counts per min per nmole); and 0.81 mg of enzyme preparation in a total volume of 0.10 ml. The enzyme preparation was added to the assay mixture at 25 C and incubated for 10 min at this temperature. The reaction was terminated by the addition of 0.005 ml of 12 M $HClO_4$. The H^3-UDP-NAc-muramyl-pentapeptide was separated from H^3-UMP by chromatography on Sephadex G-25 as described by Struve et al. (in press). The rate of exchange (R) is presented in moles exchanged per liter per minute.*

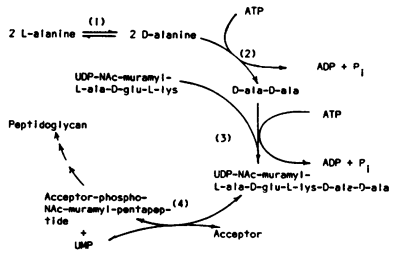

FIG. 11. *Incorporation of D-alanine from L-alanine into peptidoglycan.*

serine (0.05 M) has no effect on the D-ala-D-ala adding enzyme.

Phospho-NAc-muramyl-pentapeptide translocase (UMP). The translocase catalyzes the transfer of phospho-NAc-muramyl-pentapeptide from UDP-NAc-muramyl-pentapeptide to an acceptor with the following stoichiometry.

$$\text{UDP-NAc-muramyl-pentapeptide} + \text{Acceptor} \xrightleftharpoons{Mg^{2+}} \text{Acceptor-phospho-NAc-muramyl-pentapeptide} + \text{UMP} \qquad \text{(III)}$$

In addition, the enzyme catalyzes the exchange of H^3-UMP with the UMP moiety of the UDP-NAc-muramyl-pentapeptide (Struve et al., *in press*). UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-*O*-carbamyl-D-ser and UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala were compared in the "exchange assay" with the enzyme preparation from *S. aureus* Copenhagen. From the Lineweaver-Burk plots (10) shown in Fig. 10, it is apparent that the K_m values are identical (2.9×10^{-6} M).

DISCUSSION

The incorporation of D-alanine from L-alanine into peptidoglycan is summarized in Fig. 11. Four potential sites of action of *O*-carbamyl-D-serine have been considered: alanine racemase, D-ala-D-ala synthetase, D-ala-D-ala adding enzyme, and phospho-NAc-muramyl-pentapeptide translocase. Two approaches have been used to establish the site of action of *O*-carbamyl-D-serine. The first has been to determine the effects on intact cells, and the second, to study the effects of the antibiotic on the enzymes described above. The correlation of these approaches may provide a definition of the mechanism by which *O*-carbamyl-D-serine affects the bacterial cell.

The studies with *S. faecalis* R confirmed the observations made by Tanaka et al. (30) that growth in the presence of the antibiotic resulted in lysis and that its action was reversed by D-alanine. Skinner et al. (21) reported that the D-isomer had no effect on *S. lactis*, *Lactobacillus arabinosus*, and *Escherichia coli* at 200 μg/ml (1.3×10^{-3} M). Since 5×10^{-3} M was required for growth inhibition, these results were also in agreement with those reported by Skinner et al. (21). In addition, the medium used by Skinner et al. (21) contained a significant concentration of D-alanine (2.3×10^{-4} M), which would reverse the effect of *O*-carbamyl-D-serine. These results also confirmed that growth in the presence of the antibiotic caused the accumulation of *N*-acyl-amino sugar (30). In this paper, it was demonstrated that the accumulation of *N*-acylamino sugar was the result of UDP-NAc-muramyl-L-ala-D-glu-L-lys. It is recognized that other nucleotides may have accumulated in small amounts which were not quantified by the "nucleotide-tripeptide assay."

There are two distinct cell-wall phenomena which could result in lysis or inhibition of growth. One is the inhibition of cell-wall biosynthesis, and the other is the synthesis of defective wall material. If the bacterium is deprived of the cell-wall precursor UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala, lysis or inhibition of growth results because of inadequate wall synthesis. Toennies et al. (33) suggested that lysis occurs as a consequence of the imbalance between degradative and synthetic processes involved in wall formation. On the other hand, defective wall structure as a consequence of analogue incorporation may result in growth inhibition or lysis, or both. The defective structure may not withstand the osmotic pressure differential, or it may not be able to serve as a primer for further synthesis of wall material. Lark and Lark (9) and Lark et al. (8) reported that the growth of *Alcaligenes faecalis* in the presence of D-methionine induced the formation of spheroplasts, and that C^{14}-methionine was incorporated into the wall. Growth of *Micrococcus lysodeikticus* in the presence of D-serine resulted in a replacement of glycine residues with D-serine (34). As with *O*-carbamyl-D-serine and D-cycloserine, the growth inhibition by D-serine observed with *S. faecalis* (22) and *M. lysodeikticus* (34) was reversed with D-alanine. *O*-carbamyl-D-serine and D-cycloserine caused the accumulation of UDP-NAc-muramyl-tripeptide; however, cells grown in the presence of D-serine contained a subnormal level of this nucleotide (Hopkins and Neuhaus, *unpublished data*). Although the effects of these compounds were reversed by D-alanine, the general mode of action of D-serine appears to be completely different from *O*-carbamyl-D-serine and D-cycloserine. If a compound is incorporated into the wall without a concomitant inhibition at one of the biosynthetic steps, it seems reasonable that

there should not be an accumulation of wall precursor.

The accumulation of UDP-NAc-muramyl-tripeptide in stationary-phase cultures can be correlated with the observation by Ito and Strominger (7) that the specific activity of the D-ala-D-ala adding enzyme in *S. aureus* Copenhagen markedly decreased as the culture entered the postexponential phase of growth. On the other hand, the specific activities of the D-ala-D-ala synthetase, the L-alanine adding enzyme, D-glutamic acid adding enzyme, and L-lysine adding enzyme remained essentially constant from the early-log phase to the postexponential phase of growth (6). Previously, Strominger (24) reported that resting cultures showed a small accumulation of nucleotides, and Shockman et al. (20*a*) found that a stationary-phase culture accumulated ultraviolet-absorbing intermediates. Thus, the marked decrease in specific activity of the D-ala-D-ala adding enzyme in the postexponential phase could explain the observed accumulation of the UDP-NAc-muramyl-tripeptide.

The accumulation of UDP-NAc-muramyl-tripeptide in the presence of the antibiotic suggests that the bacterium is unable to synthesize D-ala-D-ala, the intermediate that is necessary for UDP-NAc-muramyl-pentapeptide formation. This may result by inhibition of the alanine racemase or by formation of D-ala-*O*-carbamyl-D-serine, which could inhibit the D-ala-D-ala synthetase or the D-ala-D-ala adding enzyme.

O-carbamyl-D-serine is an effective inhibitor of the alanine racemase. The K_m for L-alanine is 6.8×10^{-3} M, and the K_i for *O*-carbamyl-D-serine is 4.9×10^{-4} M. The value for the K_m of L-alanine is in agreement with the value of 8.5×10^{-3} M reported by Wood and Gunsalus (35) and the value of 6.5×10^{-3} M reported by Strominger et al. (26). In substrate specificity studies, Wood and Gunsalus (35) showed that the alanine racemase from *S. faecalis* is specific for alanine. The results in this paper describe a unique specificity profile of inhibitors for the reaction catalyzed by the racemase. *O*-carbamyl-D-serine and D-cycloserine are effective inhibitors of the racemase. The result with D-cycloserine confirmed the observation made by Strominger et al. (26) with the racemase from *S. aureus* Copenhagen. D-Serine and D-α-amino-*n*-butyric acid were poor inhibitors. In contrast to the results with the D-isomers, *O*-carbamyl-L-serine did not inhibit, and L-cycloserine inhibited to only a small extent at 10^{-3} M.

Thorne et al. (32) reported that the velocity of the racemase in the conversion of L-alanine to D-alanine was faster than the conversion of D-alanine to L-alanine. In agreement with this observation, it was observed that V_{max} for the conversion of L-alanine to D-alanine was greater than the V_{max} for the conversion of D-alanine to L-alanine. The K_m for L-alanine is 6.8×10^{-3} M, and the K_m for D-alanine is 3.0×10^{-3} M. These results may have implications in the control of the D-alanine level for peptidoglycan synthesis.

When *O*-carbamyl-D-serine was incubated with D-alanine, Mg^{2+}, ATP, and D-ala-D-ala synthetase, D-ala-*O*-carbamyl-D-ser was formed in addition to D-ala-D-ala. Although *O*-carbamyl-D-serine was not an effective acceptor in this reaction, the mixed dipeptide may have significant effects on the synthetase or the adding enzyme. D-Cycloserine was an effective competitive inhibitor of the D-ala-D-ala synthetase (13, 26). Incubation of D-cycloserine with D-alanine, Mg^{2+}, ATP, and the synthetase did not result in the formation of the corresponding dipeptide D-ala-D-cycloserine. Thus, with the D-ala-D-ala synthetase, we have a distinction between the effects of D-cycloserine and *O*-carbamyl-D-serine. The formation of D-ala-*O*-carbamyl-D-ser was comparable to the formation of D-ala-β-aminoxy-D-ala (13).

Previous work with the D-ala-D-ala adding enzyme established a specificity profile in which the addition of substituents in the N-terminal residue of the dipeptide enhanced binding, whereas the addition of substituents in the C-terminal residue decreased the binding (14). The results with D-ala-*O*-carbamyl-D-ser were comparable to the dipeptide D-ala-D-thr. The ratio of the K_m for D-ala-*O*-carbamyl-D-ser to the K_m for D-ala-D-ala is 22. Thus, D-ala-D-ala is preferentially utilized in the reaction catalyzed by the D-ala-D-ala adding enzyme. With the phospho-NAc-muramyl-pentapeptide translocase (UMP), the K_m and V_{max} for UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-*O*-carbamyl-D-ser were identical to those observed for UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala.

D-Ala-D-ala is an effective inhibitor ($K_i = 1.4 \times 10^{-3}$ M) of the D-ala-D-ala synthetase (12, Neuhaus and Lynch, *unpublished data*). If D-ala-*O*-carbamyl-D-ser should accumulate during growth, the inhibition of the synthetase may be a site of action. At the concentrations tested, D-ala-*O*-carbamyl-D-ser was less effective than D-ala-D-ala in the inhibition of the synthetase. However, a high concentration of D-ala-*O*-carbamyl-D-ser could inhibit the D-ala-D-ala synthetase. This would result in the inhibition of D-ala-D-ala synthesis and would lead to an accumulation of UDP-NAc-muramyl-L-ala-D-glu-L-lys. Cultures of *S. faecalis* R were grown in the presence of 7×10^{-3} M *O*-carbamyl-D-serine. Samples were removed at various phases of the

growth cycle and analyzed for the accumulation of dipeptides. No D-ala-O-carbamyl-D-ser was observed under the conditions used (Lynch and Neuhaus, *unpublished data*).

From a consideration of the results, it is proposed that the primary site of action of O-carbamyl-D-serine is the inhibition of the alanine racemase. This conclusion is based on a comparison of the antibiotic effects on the four potential enzyme sites, on the observed accumulation of UDP-NAc-muramyl-L-ala-D-glu-L-lys, and on the absence of D-ala-O-carbamyl-D-ser accumulation. The inhibition of the racemase deprives the bacterium of the D-alanine necessary for synthesis of D-ala-D-ala; thus, UDP-NAc-muramyl-L-ala-D-glu-L-lys accumulates.

ACKNOWLEDGMENTS

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