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Enzymatic Synthesis of Analogs of the Cell-Wall Precursor. I. Kinetics and Specificity of Uridine Diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysine:D-Alanyl-D-alanine Ligase (Adenosine Diphosphate) from *Streptococcus faecalis* R*

Francis C. Neuhaus and William G. Struve

ABSTRACT: A specificity profile of the enzyme uridine diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysine:D-alanyl-D-alanine ligase (ADP) has been established. The addition of substituents to the N-terminal residue of the dipeptide enhances the binding of dipeptide in two cases. In contrast, the addition of substituents to the C-terminal residue decreases the binding of the dipeptide in every case. For example, the following pattern is observed ($K_{m_t} \times 10^4$): D-α-amino-*n*-butyryl-D-alanine (0.9), D-norvalyl-D-alanine (1.2), D-alanyl-D-alanine (1.6), D-alanyl-D-α-amino-*n*-butyryic acid (7.6), D-alanyl-D-norvaline (>50). The kinetic studies are consistent with a uridine nucleotide and dipeptide binding site which are independent of each other. A comparison of the specificity profile of this enzyme with that of D-alanine–D-alanine ligase (ADP) has established a cooperative specificity pattern between two sequential enzyme reactions which are involved in the incorporation of D-alanine into the bacterial cell-wall precursor, UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala.

The basal structure of many bacterial cell walls includes a peptide containing L-alanine, D-glutamic acid, L-lysine (or diaminopimelic acid), and D-alanine covalently linked to muramic acid (3-*O*-lactyl-NAc-glucosamine) (Salton, 1964). The precursor of the NAc-muramyl-peptide moiety is believed to be UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (Strominger *et al.*, 1959a). This is the predominant nucleotide which accumulates when many bacteria are exposed to the antibiotic penicillin (Park, 1952a,b,c; Strominger, 1957; Saito *et al.*, 1963). It is formed by the addition of amino acids to UDP-NAc-muramic acid, catalyzed by the sequential action of four enzymes (Ito and Strominger, 1962a,b, 1964; Nathenson *et al.*, 1964). They are as follows: (1) UDP-NAc-muramic acid:L-alanine ligase (ADP), (2) UDP-NAc-muramyl-L-Ala:D-glutamic acid ligase (ADP), (3) UDP-NAc-muramyl-L-Ala-D-Glu:L-lysine ligase (ADP) (6.3.2.7), (4) UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys:D-Ala-D-Ala ligase (ADP) (Comb, 1962). The synthesis of the dipeptide D-Ala-D-Ala is catalyzed by D-alanine:D-alanine ligase (ADP) (6.3.2.4) (Neuhaus, 1962a,b; Ito and Strominger, 1962b).

The amino acid sequence of the uridine nucleotide is determined by the specificity of these enzymes. There are a number of growth experiments with *Streptococcus faecalis* R which suggest that critical substitutions may occur in the pentapeptide when the bacteria are grown in the presence of certain analogs of D-alanine (Snell and Guirard, 1943; Snell *et al.*, 1955).

It is the purpose of this communication to report the specificity profile of the D-Ala-D-Ala adding enzyme (UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys:D-Ala-D-Ala ligase [ADP]) from *S. faecalis* R with respect to the terminal dipeptide moiety and compare it with the

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TABLE I: Optical Activity of Dipeptides.

	$[\alpha]_D^{25}$°[a]		
D-Ala-D-Ala	+38.8[b]	L-Ala-L-Ala	−38.9[b]
D-Butyr-D-Ala	+17.4	L-Butyr-L-Ala	−16.3
D-Ala-D-Butyr	+21.0	L-Ala-L-Butyr	−20.6
D-Butyr-D-Butyr	+ 6.0	L-Butyr-L-Butyr	− 6.0
D-Ala-D-Norval*	+18.0	L-Ala-L-Norval*	−18.9
D-Ala-D-Val	+ 5.0	L-Ala-L-Val	− 5.2
D-Val-D-Ala*	+ 6.5	L-Val-L-Ala*	− 6.7
D-Norval-D-Ala*	+ 6.2	L-Norval-L-Ala*	− 4.8
D-Ser-D-Ala	+30.6	L-Ser-L-Ala	−27.2[b]
D-Ala-D-Ser	− 6.5		
D-Ala-D-Thr	− 4.4		

[a] The rotations were measured in a Rudolph Model 80 photoelectric polarimeter at $c = 1$ except for * where $c = 2$ in 0.5 N HCl at 25° ($l = 1$). [b] The $[\alpha]_D^{24}$° for D-Ala-D-Ala = +37.9°, $c = 2$ in 0.5 N HCl; for L-Ala-L-Ala $[\alpha]_D^{24}$° = −37.3° (Erlanger and Brand, 1951). For L-Ser-L-Ala $[\alpha]_D^{26}$° = −30.4°, $c = 6$ in 1 N HCl (Fruton, 1942).

product specificity pattern of the D-Ala-D-Ala synthetase (D-alanine:D-alanine ligase [ADP]) (Neuhaus, 1962a).

Experimental Procedure

Materials. D-[1-^{14}C]Alanine and DL-α-amino-*n*-[1-^{14}C]butyric acid were purchased from California Corp. for Biochemical Research. We are indebted to Dr. T. Park and Dr. H. Plaut for samples of muramic acid, to Dr. O. K. Behrens of Eli Lilly Co. for D-cycloserine, and to Dr. E. Snell and Dr. M. Ikawa for samples of D-Ala-L-Ala, L-Ala-D-Ala, and D-Ala-Gly. Sephadex G-25 and DEAE-Sephadex (A-50) were the products of Pharmacia Fine Chemicals, Inc. D-,D-Dipeptides were synthesized by Dr. H. Plaut of Cyclo Chemical Corp. These compounds have been further characterized by ion-exchange chromatography on the Beckman-Spinco amino acid analyzer (Model 120B) with methods outlined previously (Neuhaus, 1962a) and by total hydrolysis followed by analysis of the amino acids. The dipeptides in a few cases were recrystallized until a single peak was observed on ion-exchange chromatography. In Table I the specific rotations are compared with those obtained with L-,L-dipeptides.

Chromatography. The uridine nucleotides were chromatographed on Whatman 3MM paper in the following descending solvent systems: (A) isobutyric acid–concd NH_4OH–H_2O (66:1:33) (Pabst, 1961); (B) ethanol–1 M ammonium acetate (7.5:3) (Ito and Strominger, 1962a). For the initial preparative steps unwashed paper was used, while for final chromatography paper which had been washed overnight with 1.0 M acetic acid was used. Photographs were made according to the procedure described by Markham and Smith (1949). These prints were used as contact negatives for making the positives that are shown.

Analytical Procedures. Amino acids were determined by ion-exchange chromatography on the amino acid analyzer according to the methods of Spackman *et al.* (1958). The normal system (150-cm column, 30 ml/hr) was used for the maximum resolution of glutamic acid and muramic acid. The accelerated system (50-cm column, 40 ml/hr) of analysis was used in situations where this resolution was not necessary. All hydrolyses were performed with 5.7 N HCl in vials under N_2 at 110° for the appropriate time. Measurements of radioactivity were made in polyethylene vials using the Packard Tri-Carb liquid scintillation spectrometer (Model 314-EX). The scintillation fluid (15 ml) contained: naphthalene (60 g), 2,5-diphenyloxazole (4 g), 1,4-bis-2-(5-phenyloxazolyl)benzene (200 mg), methanol (100 ml), ethylene glycol (20 ml), and *p*-dioxane to 1 liter (Bray, 1960). The counting efficiency with this scintillation mixture was 56% when 1.6 ml of 0.2 N sodium citrate buffer, *p*H 2.2, was added. All measurements of radioactivity for the kinetic runs were made for 100 minutes or for 10^5 counts. Protein was determined by the method of Warburg and Christian (1941) and by the method of Lowry *et al.* (1951). Inorganic phosphate was determined by the method of Marsh (1959) and reducing sugar was measured by the method of Park and Johnson (1949). *N*-Acetylamino sugar was determined according to the procedure of Reissig *et al.* (1956).

Preparation of ^{14}C-Labeled Dipeptides. The labeled dipeptides were prepared with the D-Ala-D-Ala synthetase (Neuhaus, 1962a). For D-[^{14}C]Ala-D-[^{14}C]Ala the incubation contained: 20 μmoles of D-[1-^{14}C]alanine (10 μc); 28 μmoles of ATP neutralized with NaOH; 40 μmoles of $MgCl_2$; 40 μmoles of KCl; 40 μmoles of Tris-HCl buffer, *p*H 7.8; 0.88 mg of synthetase preparation (specific activity = 78 μmoles D-Ala-D-Ala/hr/mg) (Neuhaus, 1962a) in a total volume of 1 ml. The tube was incubated for 2 hours at 37° and the reaction was terminated by placing the tube in a boiling-water bath for 2 minutes. The dipeptide was isolated by descending chromatography on Whatman 3MM paper with 1-

butanol–acetic acid–water (4:1:5) (organic phase) (Moore and Baker, 1958) as the solvent. The dipeptide was eluted and then concentrated at 30° on a rotary evaporator. The following procedure was performed in order to separate radioactive decomposition products from the labeled D-Ala-D-Ala. The sample was applied to a Dowex 50(H^+) (200–400 mesh) (5 × 25 mm) column after being made 0.2 N with respect to HCl. The column was washed successively with 1 ml of 0.2 N HCl, 2 ml of water, and 1 ml of 1 N NH_4OH. The dipeptide was eluted with 1 ml of concd NH_4OH and the eluate was immediately taken to dryness *in vacuo*. The dipeptide was dissolved in water and its concentration was determined by comparison with an authentic sample on the amino acid analyzer. D-[^{14}C]-Ala-D-Butyr[1] was prepared in a similar manner. Although D-[^{14}C]Ala-D-Ser and D-[^{14}C]Ala-D-Thr were prepared using similar incubations, preparative ion-exchange chromatography on Amberlite IR-120 (Na^+) (200–400 mesh) (150 × 2.7 cm) was performed in order to isolate the labeled mixed dipeptide from contaminating D-alanine and D-Ala-D-Ala. The preparative column was first eluted with 0.2 N Na citrate buffer, *p*H 3.25, and then 0.2 N Na citrate buffer, *p*H 4.25. The mixed dipeptide fractions were pooled and the sodium citrate was replaced with ammonium formate by column chromatography on Dowex 50 (NH_4^+) according to Hirs *et al.* (1956). The ammonium formate was removed by lyophilization and the dipeptide was dissolved in water. The concentration of the dipeptide was determined by comparison with an authentic sample on the amino acid analyzer.

Preparation of UDP-NAc-muramyl-(Ala, Glu, Lys). *S. faecalis* R (ATCC 8043) was grown at 37° for 4.3 hours in a medium (8 liters) containing 0.5% glucose, 0.5% K_2HPO_4, 1% yeast extract, and 1% tryptone. The yield of bacteria (wet wt) was 39 g. The bacteria were suspended in 8 liters of fresh medium which contained 20% sucrose in addition to the above components. According to Mandelstam *et al.* (1962) the yield of cell-wall nucleotides from *S. faecalis* grown in the presence of penicillin could be doubled when the medium contained 20% sucrose. After 1 hour of growth, the medium was made 2.0×10^{-4} M with respect to D-cycloserine. In growth studies, $1–2 \times 10^{-4}$ M D-cycloserine was demonstrated to be the minimum concentration necessary to observe lysis (Neuhaus and Lynch, 1964). When the cells had grown in the presence of the antibiotic for 40 minutes, they were harvested. The cells were suspended in water (50 ml) and subjected to three cycles of freezing and thawing. The suspension was centrifuged at 17,000 × *g* for 15 minutes, and the supernatant solution was made 5% with respect to trichloroacetic acid. The solution was maintained at 5° for 30 minutes and the protein was removed by centrifugation at 17,000 × *g* for 30 minutes. The trichloroacetic acid was removed by ether extraction and the aqueous layer was concentrated *in vacuo* (fraction I). In addition, a second extraction of the residue with water at 100° was performed (fraction II).

The purification of the nucleotide containing alanine, glutamic acid, and lysine was accomplished by preparative paper chromatography in three solvent systems. Fraction I was chromatographed in solvent B and the major nucleotide which contained the above amino acids was eluted with water. The eluate was chromatographed in solvent A and the nucleotide which contained these amino acids was eluted and rechromatographed on Whatman 3MM paper with 70% ethanol. Ammonium butyrate and other contaminants derived from the unwashed paper were separated from the nucleotide in the last solvent system.

The yield from fractions I and II was 33 and 18 μ-moles of UDP-NAc-muramyl-(Ala, Glu, Lys), respectively. The analytical data are shown in Table II.

The presence of uridine in this nucleotide is indicated by the agreement of the 280:260 and 250:260 ratio with those of UDP (Table II). Mild acid hydrolysis (0.1 N HCl, 100°, 3 minutes) resulted in the formation of a nucleotide with a R_{UMP} in solvent B equal to that of UDP. Strong acid hydrolysis (1 N HCl, 100°, 15 minutes) resulted in the formation of an ultraviolet-absorbing compound with a R_F equal to UMP. The ratio of labile phosphate (1 N HCl, 100°, 7 minutes) to glutamic acid was 1.06 and the ratio of labile reducing sugar (0.1 N HCl, 100°, 5 minutes) was 1.04. The ratio of acid-labile *N*-acetylamino sugar (0.13 N HCl, 100°, 3 minutes) with *N*-acetylmuramic acid as the standard was 1.00. The similarities in amino acid composition, pyrimidine and muramic acid content, and properties in acid provide evidence that the nucleotide which has been isolated from *S. faecalis* R is similar to the class of compounds isolated by Park (1952a,b,c) and Strominger (1957, 1959). It is presumed to be identical to that isolated by Strominger *et al.* (1959b) from *Staphylococcus aureus* Copenhagen grown in the presence of D-cycloserine.

Preparation of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-Ala-D-Ala and Analogs. Each incubation contained: 5 μmoles of Tris-HCl buffer, *p*H 7.8; 20 μmoles of $MgCl_2$; 2 μmoles of ATP neutralized with NaOH; 5 μmoles of dipeptide; 40 μg of enzyme preparation; and 0.5 μmole of UDP-NAc-muramyl-(Ala, Glu, Lys) in a volume of 0.45 ml. The incubations were performed at 37° for 1 hour and the reactions were terminated by heating in a boiling-water bath for 2 minutes. The uridine nucleotide containing the pentapeptide was separated from ADP, ATP, peptide, and UDP-NAc-muramyl-(Ala, Glu, Lys) by chromatography on Whatman 3MM with solvent B for 48–64 hours. This nucleotide was eluted with water and the absorbance of an aliquot was measured at 250, 260, and 280 mμ at *p*H 7.0. Another aliquot was taken to dryness and hydrolyzed with 5.7 N HCl under N_2 for either 12 or 50 hours at 110°. After removal of the HCl, the hydrolysate was analyzed on the amino acid analyzer.

Assay of the D-Ala-D-Ala Adding Enzyme ("Adding Assay"). The enzyme was assayed by the addition of labeled D-Ala-D-Ala to UDP-NAc-muramyl-(Ala, Glu,

[1] Abbreviations used in this work: Butyr, α-amino-*n*-butyric acid (aminobutyrate); Norval, norvaline.

TABLE II: Analytical Data on the Analogs of the Complete Nucleotide from *S. faecalis* R.[a]

Dipeptide Added	Absorbance Ratio	Amino Acids (Ratio to Glutamic)					
		Glutamic	Lysine	Alanine	Amino-butyric	Serine	Valine
None	1.02	1.00	0.93	1.00			
(1) D-Ala-D-Ala	0.99	1.00	1.01	2.95			
(2) D-Ala-D-Butyr	0.99	1.00	0.90	1.88	1.01		
(3) D-Butyr-D-Ala	1.02	1.00	0.97	2.11	0.98		
(4) D-Butyr-D-Butyr	1.02	1.00	0.94	0.75	1.77		
(5) D-Ala-D-Ser	0.98	1.00	0.92	1.98		0.97	
(6) D-Ser-D-Ala	0.98	1.00	0.97	2.02		0.99	
(7) D-Ala-D-Val	0.92	1.00	0.91	2.02			1.05
(8) D-Val-D-Ala	0.90	1.00	0.80	1.80			0.87

[a] The ratio 280:260 = 0.38 ± 0.01, 250:260 = 0.71 ± 0.03. The ratios for UDP are 0.38 and 0.74, respectively (Pabst, 1961). The ratio of muramic acid was 0.73–0.89 (12-hour hydrolysis). The molar ratio of dipeptide incorporated for 1 and 2 was 1.07 and 1.14, respectively.

Lys) with the formation of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala. The assay is similar to that described by Ito and Strominger (1962b) and Comb (1962). Columns of Dowex 50 (Na^+) were used for the separation of the dipeptide and the labeled nucleotide, instead of charcoal. One unit of enzyme is that amount which will catalyze the formation of 1 μmole of the UDP-NAc-muramyl-(Ala, Glu, Lys)-D-Ala-D-Ala per minute under the conditions of the assay. Specific activity is defined as the number of units per mg of protein. Unless stated, not more than 20% of either the nucleotide or labeled dipeptide was converted to product.

The standard assay mixture contained: 10 μmoles of Tris-HCl buffer, *p*H 7.8; 1 μmole of $MgCl_2$; 10 mμmoles of UDP-NAc-muramyl-(Ala, Glu, Lys); 0.1 μmole of ATP neutralized with NaOH; 45 mμmoles of D-Ala-D-Ala (1300 cpm/mμmole); and enzyme preparation in a total volume of 0.10 ml. The *p*H of the incubation mixture at 37° was 7.6. The enzyme preparation was added to the assay mixture at 37° and incubated for 5 minutes at this temperature. The reaction was terminated by the addition of 0.5 ml of 0.2 N Na citrate buffer, *p*H 2.2. The solution was quantitatively applied to a Dowex 50 (Na^+) X8 column (200–400 mesh) (5 × 20 mm). Two aliquots (0.5 ml) of 0.2 N citrate buffer, *p*H 2.2, were added and the effluents (1.6 ml) were collected in a polyethylene vial for determination of the radioactivity. A new column was used for each sample. Appropriate controls were performed in each series to correct for the slow decomposition of the radioactive dipeptide into material which was not retained by the column. The separation of the labeled dipeptide from the nucleotide is illustrated in Figure 1. With these assay conditions the amount of product formed is linear for 30 minutes (Figure 2). In addition, under these conditions the rate of product formation is proportional to the enzyme concentration. The amount

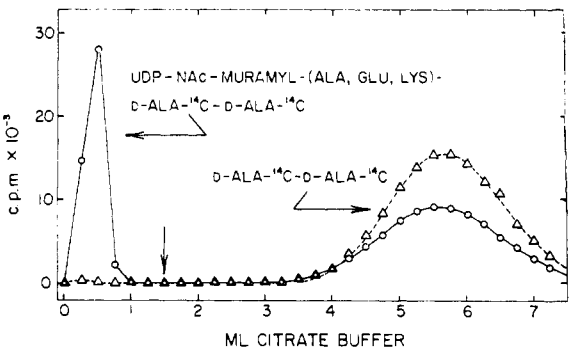


FIGURE 1: Elution profile in the "adding assay." The "adding assay" was used with 3.8 μg of enzyme preparation and 3 × 10^{-4} M D-[^{14}C]Ala-D-[^{14}C]Ala. The complete system (○—○) and the control (△—△) (minus UDP-NAc-muramyl-[Ala, Glu, Lys]) were incubated for 5 minutes at 37°. The incubation was terminated and applied to the ion-exchange column as described in the text. Fractions (0.25 ml) were collected and assayed for radioactivity. The arrow shows that portion of the eluate (1.6 ml) which was collected and assayed for radioactivity in the "adding assay."

of UDP-NAc-muramyl-(Ala, Glu, Lys) which was available for this project precluded the assay of P_i, ADP, or H^+.

Purification of the Adding Enzyme. Sonic extracts of *S. faecalis* R (ATCC 8043) were prepared as previously described (Neuhaus, 1962a). To the extract (400 ml), an equal volume of a 2% protamine sulfate solution was added over 1 hour at 4°. The precipitate was removed by centrifuging at 13,200 × *g* for 20 minutes. The supernatant solution was brought to 0.55 saturation by the slow addition of 884 ml of saturated ammonium sulfate, *p*H 7.0. The precipitate was removed by centrifugation and the supernatant fraction was brought to

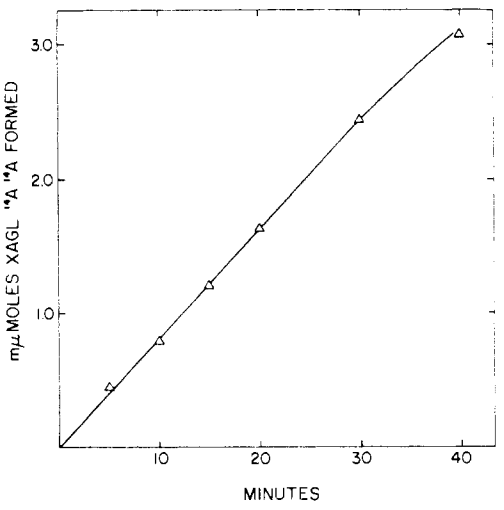


FIGURE 2: Formation of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala as a function of time. The abbreviation is: XAGL^{14}A^{14}A, UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala.

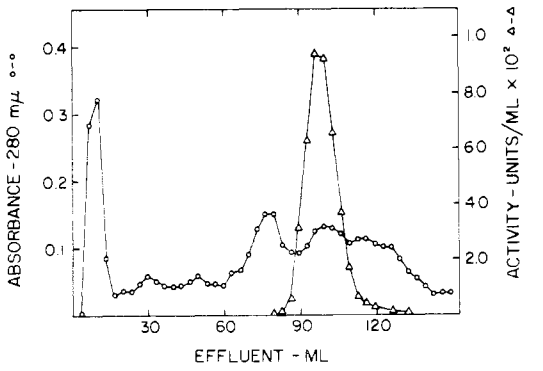


FIGURE 3: Elution profile of the D-Ala-D-Ala adding enzyme. An aliquot of fraction 3 (Table III) which had been equilibrated against the initial buffer was applied to the column. The column was then eluted as described in the text. The tubes which contained 40% of the activity were used for the results in this paper.

0.70 saturation by the further addition of 1320 ml of saturated ammonium sulfate. The precipitate was collected and dissolved in 0.01 M Tris-HCl buffer, *p*H 7.0, to a final volume of 20 ml. This fraction was stored at −20° for 1 year without appreciable loss in activity.

The protein in a 1-ml aliquot of the 55–70% ammonium sulfate fraction was equilibrated with 0.01 M Tris-HCl buffer, *p*H 7.8, containing 0.10 M KCl (buffer A) by filtration over a Sephadex G-25 column (1.1 × 27 cm). The protein solution was applied to a DEAE-Sephadex column (1.1 × 22 cm) which was prepared according to the instructions from the manufacturer and which had been equilibrated with buffer A. A linear gradient was established with 80 ml of buffer A in the mixing chamber and 80 ml of 0.4 M KCl in 0.01 M Tris-HCl buffer, *p*H 7.8, in the reservoir. A flow rate of 24 ml/hr was maintained. In Figure 3 an elution profile is shown. The 280:260 ratio of the active fraction was 1.60. The enzyme lost activity rapidly when stored at −20°; however, the addition of bovine serum albumin (2% final concentration) stabilized this fraction. A summary of the results from a purification is presented in Table III.

TABLE III: Partial Purification of the D-Ala-D-Ala Adding Enzyme.

Purification Step	Activity (units)	Specific Activity (milli-units/mg)	Yield (%)
(1) Sonic extract	132	20.6	
(2) Protamine sulfate treatment	41.6	24.0	31
(3) Ammonium sulfate (55–70%)	31	119	23.5
(4) DEAE-Sephadex eluate[a]	11.4	490	8.7

[a] The results for step 4 are based on the chromatographic separation shown in Figure 3. The yield is calculated on the assumption that the whole ammonium sulfate fraction was chromatographed.

Results

Synthesis of UDP-NAc-muramyl-pentapeptides. A series of analogs of the dipeptide D-Ala-D-Ala were incubated in the presence of UDP-NAc-muramyl-(Ala, Glu, Lys), D-Ala-D-Ala adding enzyme, Mg^{2+}, and ATP. In Figure 4 two chromatograms are shown to illustrate the separation of the uridine nucleotides that contain analogs of the pentapeptide. Preparative incubations were performed and the uridine nucleotides which contained the pentapeptide were isolated. As shown in Table II the analytical data are consistent with pentapeptides that contain the analog of the dipeptide D-Ala-D-Ala in the penultimate and C-terminal residue. The introduction of valine, norvaline, and aminobutyrate into the penultimate residue resulted in a pronounced stability of the pentapeptide to complete acid hydrolysis. For this reason two hydrolysis times were used, 12 and 50 hours. The 12-hour sample gave a higher ratio of muramic acid while the 50-hour sample gave better ratios of the amino acids in the analog of the pentapeptide.

Evaluation of Michaelis Constants for the Analogs of D-Ala-D-Ala. Two methods were used to establish the Michaelis constants[2] and specificity profile of the di-

[2] The values for pK_{a_2}' of the dipeptides studied in this paper range from 8.10 to 8.25 (unpublished observations). Thus, the changes in K_m are not attributed to differences in pK_{a_2}'.

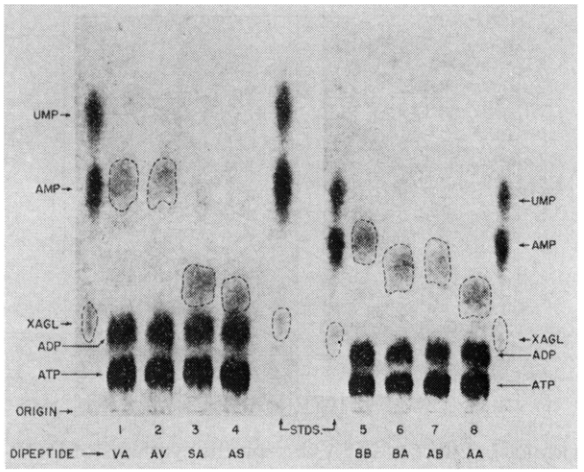


FIGURE 4: The synthesis of analogs of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-Ala-D-Ala. The incubations contained: 1 μmole of Tris-HCl buffer, *p*H 7.8; 4 μmoles of $MgCl_2$; 0.2 μmole of ATP neutralized with NaOH; 0.5 μmole of dipeptide; 0.05 μmole of UDP-NAc-muramyl-(Ala, Glu, Lys); and 3.8 μg of enzyme preparation in a total volume of 0.044 ml. The reaction mixtures were incubated for 1 hour at 37° and the reaction was terminated by placing the tube in a boiling-water bath for 2 minutes. The uridine analogs of the NAc-muramyl-pentapeptide were separated from the dipeptide, ATP, ADP, and UDP-NAc-muramyl-(Ala, Glu, Lys) by descending chromatography in solvent B. The abbreviations are VA, D-Val-D-Ala; AV, D-Ala-D-Val; SA, D-Ser-D-Ala; AS, D-Ala-D-Ser; BB, D-Butyr-D-Butyr; BA, D-Butyr-D-Ala; AB, D-Ala-D-Butyr; AA, D-Ala-D-Ala; XAGL, UDP-NAc-muramyl-(Ala, Glu, Lys).

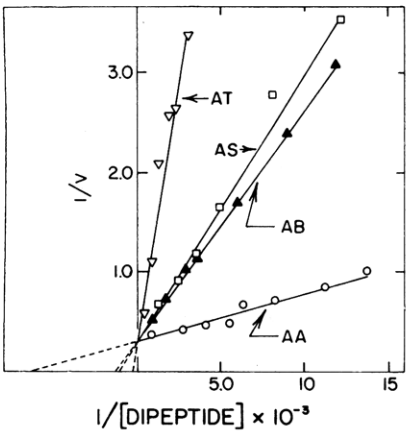


FIGURE 5: Effect of D-Ala-D-Ala, D-Ala-D-Butyr, D-Ala-D-Ser, and D-Ala-D-Thr on the velocity of UDP-NAc-muramyl-(Ala, Glu, Lys)-dipeptide formation. The "adding assay" was used with 0.47 μg of enzyme preparation. In Figures 5–10 the velocity (v) is product (mμmoles) formed in 5 minutes. Abbreviations as in Figure 4.

peptide analogs in the reaction catalyzed by the adding enzyme. The single-substrate method involves the addition of labeled dipeptides to UDP-NAc-muramyl-(Ala, Glu, Lys). The two-substrate method involves the inhibition of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala synthesis by unlabeled analogs of the dipeptide D-Ala-D-Ala. In this system the analog competes with labeled D-Ala-D-Ala in two simultaneous reactions. In the single-substrate method it is possible to evaluate both the K_m and V_{max} for each analog whereas in the two-substrate method it is only possible to evaluate K_{m_i} (see The Two-Substrate System).

Michaelis Constants for D-*Ala*-D-*Ser*, D-*Ala*-D-*Butyr*, D-*Ala*-D-*Ala, and* D-*Ala*-D-*Thr*. The rates of formation of uridine nucleotide-containing analogs of the pentapeptide were measured at varying concentrations of dipeptide (analog) at a fixed concentration of UDP-NAc-muramyl-(Ala, Glu, Lys). From the Lineweaver-Burk plots (Lineweaver and Burk, 1934) shown in Figure 5, the Michaelis constants (K_m) and V_{max} for each dipeptide were established. The values for K_m are summarized in Table IV. It is apparent from these plots that V_{max} is identical for each dipeptide. In a simple kinetic treatment of the reaction:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \qquad (1)$$

where E is enzyme, S is total peptide, and P is product, K_m is $(k_{-1} + k_{+2})/k_{+1}$, and V_{max} is $k_{+2}[E_t]$. Since V_{max} is identical for each dipeptide, k_{+2} is independent of structural changes.

This treatment, however, does not describe adequately the reaction catalyzed by the adding enzyme. This enzyme must have binding sites for the uridine nucleotide, $MgATP^{2-}$, and the dipeptide. Thus changes in the Michaelis constants may reflect interaction of each substrate on its specific binding site.

TABLE IV: Summary of Constants.

Dipeptide	K_m[a]	K_{m_i}[b]	K_{m_i}'[c]
	(moles/liter × 10^4)		
(1) D-Ala-D-Ala	1.6	1.6[d]	1.1[d] (1.4)[e]
(2) D-Ala-D-Butyr	7.6	8.8	8.9
(3) D-Ala-D-Ser	9.1	9.0	9.1
(4) D-Ala-D-Thr	33	27	36

[a] Lineweaver-Burk plots (Figure 5). [b] Dixon plots (Figure 8, 9A, B, C). [c] Calculated with equation (6). In (1) $K_m^{AB} = 7.6 \times 10^{-4}$ M while in (2–4) $K_m^{AA} = 1.6 \times 10^{-4}$ M. [d] The labeled substrate is D-[^{14}C]Ala-D-Butyr. [e] Calculated with equation (6) but using K_{m_i} for D-Ala-D-Butyr = 8.8×10^{-4} M.

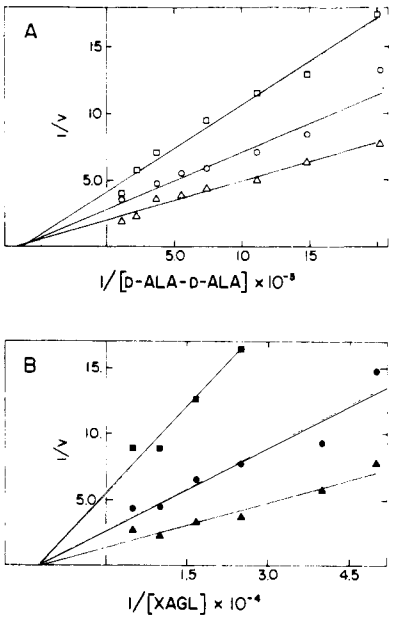


FIGURE 6: Effect of D-Ala-D-Ala (A) and UDP-NAc-muramyl-(Ala, Glu, Lys) (B) on the velocity of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala formation. The "adding assay" was used with 0.19 μg and 0.38 μg of enzyme preparation in (A) and (B), respectively. The concentrations for UDP-NAc-muramyl-(Ala, Glu, Lys) in (A) are: △—△, 1×10^{-4} M; ○—○, 4×10^{-5} M; □—□, 2×10^{-5} M. The concentrations for D-Ala-D-Ala in (B) are: ▲—▲, 4.5×10^{-4}; ●—●, 9.0×10^{-5} M; ■—■, 3.0×10^{-5} M.

Effect of D-*Ala*-D-*Ala and UDP-NAc-muramyl-(Ala, Glu, Lys) on the Rate of UDP-NAc-muramyl-(Ala, Glu, Lys)*-D-[^{14}C]*Ala*-D-[^{14}C]*Ala Formation.* The rates of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-Ala-D-Ala formation were determined at varying concentrations of D-Ala-D-Ala at three concentrations of uridine nucleotide (Figure 6A) and at varying concentrations of uridine nucleotide at three concentrations of D-Ala-D-Ala (Figure 6B). From these plots the K_m of D-Ala-D-Ala and the K_m of UDP-NAc-muramyl-(Ala, Glu, Lys) were established. The K_m for D-Ala-D-Ala is 1.6×10^{-4} M and the K_m for the uridine nucleotide is 8.0×10^{-5} M. These results indicate that the K_m of the dipeptide is independent of the uridine nucleotide concentration and that the K_m of the uridine nucleotide is independent of the dipeptide concentration. If we assume that $MgATP^{2-}$ also binds to an independent site, a random series of reactions can be formulated which are consistent with the data in Figure 6A,B:

$$A + B + C + E \rightleftharpoons \begin{matrix} EA \rightleftharpoons EAB \\ EB \rightleftharpoons EAC \rightleftharpoons EABC \rightarrow \text{products} \\ EC \rightleftharpoons EBC \end{matrix} \qquad (2)$$

where A is the uridine nucleotide, B is the dipeptide, and C is the $MgATP^{2-}$. The reciprocal rate expression for this series of reactions is:

$$\frac{1}{v} = \frac{1}{V_{max}}\left[1 + \frac{K_A}{[A]}\right]\left[1 + \frac{K_B}{[B]}\right]\left[1 + \frac{K_C}{[C]}\right] \qquad (3)$$

where K_A, K_B, K_C, are the Michaelis constants for uridine nucleotide, dipeptide, and $MgATP^{2-}$, respectively. If uridine nucleotide is varied at constant B and C, the intercept at $1/v = 0$ is $-1/K_A$. This is the same formulation which has been considered in the interpretation of the kinetics for triose phosphate dehydrogenase (Velick and Furfine, 1963), phosphoenolpyruvate kinase (Reynard *et al.*, 1961), and yeast hexokinase (Fromm and Zewe, 1962). Since the binding site for the uridine nucleotide is independent of the dipeptide-binding site, it is possible to establish the specificity profile at concentrations of uridine nucleotide which are less than saturating. In order to conserve uridine nucleotide, the routine concentration was 1×10^{-4} M. Furthermore, these results indicate that changes in K_m with the analogs of D-Ala-D-Ala probably do not reflect interaction between the dipeptide-binding site and the binding site for the uridine nucleotide.

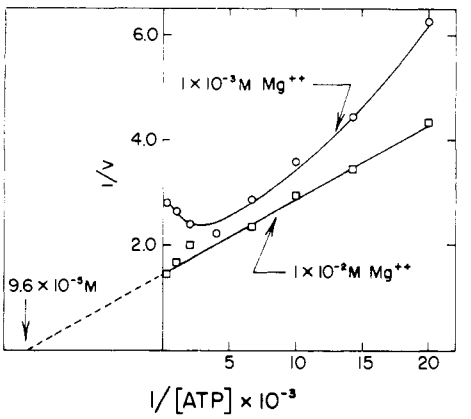


FIGURE 7: Effect of $MgATP^{2-}$ on the synthesis of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala. The "adding assay" was used with 0.63 μg of enzyme preparation. The concentrations of $MgCl_2$ are: ○—○, 1×10^{-3} M; □—□, 1×10^{-2} M. The concentrations of D-Ala-D-Ala and UDP-NAc-muramyl-(Ala, Glu, Lys) are 1.8×10^{-4} M and 1.0×10^{-4} M, respectively.

Effect of $MgATP^{2-}$ *on the Synthesis of UDP-NAc-muramyl-(Ala, Glu, Lys)*-D-[^{14}C]*Ala*-D-[^{14}C]*Ala.* The initial rates of product formation were measured at varying ATP concentrations at two concentrations of $MgCl_2$. As shown in Figure 7, with 1×10^{-3} M Mg^{2+} a nonlinear reciprocal plot was observed whereas at 1×10^{-2} M Mg^{2+} a linear reciprocal plot was observed. The K_m for ATP (9.6×10^{-5} M) was established from the latter plot. All results in this paper were performed with 1×10^{-2} M $MgCl_2$ and 1×10^{-3} M ATP. This ratio ensured that essentially all of the ATP was present as the $MgATP^{2-}$ complex at *p*H 7.8 (Bock, 1960).

Inhibition of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala Synthesis by Analogs of D-Ala-D-Ala. A series of potential dipeptide analogs were tested for their ability to inhibit the incorporation of D-[^{14}C]Ala-D-[^{14}C]Ala into a complete nucleotide. From the data presented in Table V it is apparent that the inhibition is specific for dipeptides of the D-,D-configuration. No significant inhibition is observed with L-Ala-D-Ala, D-Ala-L-Ala, and L-Ala-L-Ala. These results confirm those reported by Ito and Strominger (1962b). The data also demonstrate that the inhibition is specific for the dipeptide. D-Alanine has no effect and the tripeptide D-Ala-D-Ala-D-Ala (1×10^{-3} M) inhibits to only a small extent. These studies show that a wide variety of dipeptides compete with D-[^{14}C]Ala-D-[^{14}C]Ala for the transfer to the uridine nucleotide. The two-substrate method has been used to establish the specificity profile of these analogs.

TABLE V: Inhibition of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala Synthesis by Unlabeled Analogs of the Dipeptide.

Addition[a]	UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala (mμmoles/5 minutes)
None	4.15
(1) D-Alanine	4.14
(2) D-Ala-D-Ala-D-Ala	3.80
(3) L-Ala-L-Ala	4.14
(4) L-Ala-D-Ala	3.94
(5) D-Ala-L-Ala	4.18
(6) D-Ala-Gly	3.87
(7) D-Ala-D-Butyr	2.87
(8) D-Ala-D-Norval	4.08
(9) D-Ala-D-Val	3.75
(10) D-Ala-D-Ser	2.78
(11) D-Ala-D-Thr	3.68
(12) D-Butyr-D-Butyr	2.92
(13) D-Butyr-D-Ala	0.94
(14) D-Norval-D-Ala	1.33
(15) D-Val-D-Ala	2.57
(16) D-Ser-D-Ala	2.28
(17) Gly-D-Ala[b]	3.93

[a] The "adding assay" was used with 1×10^{-3} M analog. [b] Although the DL-dipeptide was added, the final concentration is on the basis of the D- isomer.

The Two-Substrate System. In the two-substrate system two dipeptides are competing for the enzyme in two simultaneous reactions as follows:

$$E + S_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES_1 \xrightarrow{k_{+2}} E + P_1 \qquad (4)$$

$$E + S_2 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} ES_2 \xrightarrow{k_{+4}} E + P_2 \qquad (5)$$

where $K_m^{S_1}$ is the Michaelis constant for the substrate S_1 in the first reaction (4) and $K_m^{S_2}$ is the Michaelis constant for the substrate S_2 in the second reaction (5). In an equilibrium treatment it is valid to consider S_2 as a competitive inhibitor of P_1 synthesis; and thus $K_i^{S_2} = K_s^{S_2}$. In the steady-state treatment, however, $K_i^{S_2} = (k_{-3} + k_{+4})/k_{+3}$ which also equals $K_m^{S_2}$. Thus values for K_i established from kinetic experiments are not true dissociation constants as in the case of a dead-end inhibitor. This situation has been discussed by Dixon and Webb (1958) and Reiner (1959). The velocity of P_1 synthesis in the presence of S_2 is given by:

$$v_{P_1} = \frac{V_{max}}{1 + \frac{K_m^{S_1}}{[S_1]}\left[1 + \frac{[S_2]}{K_m^{S_2}}\right]} \qquad (6)$$

Two methods have been used to evaluate $K_m^{S_2}$ (K_{m_i}) of the nonlabeled substrate analogs (S_2).

(a) In the Dixon method (Dixon, 1953) equation (6) is rearranged as follows:

$$\frac{1}{v_{P_1}} = \frac{1}{V_{max}} + \frac{K_m^{S_1}}{V_{max}[S_1]} + \frac{K_m^{S_1}[S_2]}{V_{max}[S_1]K_m^{S_2}} \qquad (7)$$

If $1/v_{P_1}$ is plotted against S_2 at two (or more) concentrations of S_1, the plots will intersect at $(-K_m^{S_2}, 1/v)$.

(b) In the second method, $K_m^{S_2}$ (K_{m_i}') is established by calculation using $K_m^{S_1}$, V_{max}, $[S_1]$, and $[S_2]$.

Determination of K_{m_i} for D-Ala-D-Ala by the Dixon Method with D-[^{14}C]Ala-D-Butyr as the Substrate. According to the Dixon treatment the coordinates of the intersection of the plots are independent of the substrate (S_1). If D-[^{14}C]Ala-D-Butyr is used as the labeled substrate, it is possible to evaluate the K_{m_i} for D-Ala-D-Ala. A value of 1.6×10^{-4} M for the K_{m_i} of D-Ala-D-Ala was established from the Dixon plots in Figure 8. This value is identical with that determined by the method of Lineweaver and Burk (1934) (Table IV). Thus the Michaelis constant (K_{m_i}) obtained with the two-substrate method is in good agreement with the Michaelis constant obtained with the single-substrate method.

Determination of K_{m_i} for Analogs of D-Ala-D-Ala by the Dixon Method. In Figure 9, Dixon plots with four analogs of D-Ala-D-Ala are shown. From these plots the values for K_{m_i} of the analogs have been established. They are summarized in Tables IV and VI and are in good agreement with values for K_m established from Lineweaver-Burk plots. From the foregoing discussion it is apparent that these constants are not necessarily dissociation constants and may be kinetic constants.

The best inhibitor in the series shown in Table V is D-Butyr-D-Ala. It was not possible to synthesize sufficient amounts of labeled D-Butyr-D-Ala with the D-Ala-D-Ala synthetase for substrate studies (see Neuhaus, 1962b). Since there is a good correlation

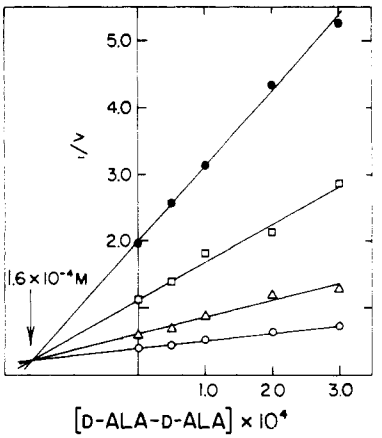


FIGURE 8: Determination of K_{mi} for D-Ala-D-Ala by the Dixon method with D-[^{14}C]Ala-D-Butyr as the substrate. The "adding assay" was used with 1.9 μg of enzyme preparation. The concentrations of D-[^{14}C]Ala-D-Butyr are: O—O, 4.5×10^{-4} M; △—△, 2.3×10^{-4} M; □—□, 1.1×10^{-4} M; ●—●, 5.6×10^{-5} M.

between K_m and K_{mi} (Table IV), a Dixon plot was used to evaluate the K_{mi} for D-Butyr-D-Ala (9×10^{-5} M). These results, shown in Figure 10, demonstrate that the enzyme has a lower K_{mi} for D-Butyr-D-Ala than for D-Ala-D-Ala.

K_{mi}' Values for Analogs of the Dipeptide D-*Ala*-D-*Ala.* Since the values for K_{mi}' agree with those established from the Dixon plots and Lineweaver-Burk plots (Table IV), an additional series of dipeptides from Table V were further tested in order to establish values for their K_{mi}'. The analogs were tested in the "adding assay" at two concentrations and at least four determinations were averaged to calculate the $K_m^{S_2}$ (K_{mi}') by equation (6). These values are summarized in Table VI.

Discussion

A specificity profile of the D-Ala-D-Ala adding enzyme has been established and is summarized in Table VII.

TABLE VI: Summary of K_{mi} and K_{mi}' Values for Analogs of D-Ala-D-Ala.

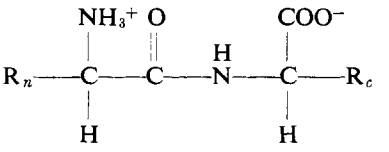
Dipeptide	K_{mi}[a] (moles/liter × 10⁴)	K_{mi}'[b] (moles/liter × 10⁴)
(1) D-Butyr-D-Ala	0.9	
(2) D-Butyr-D-Butyr	7.0	
(3) D-Norval-D-Ala		1.2
(4) D-Val-D-Ala		3.9
(5) D-Ser-D-Ala		2.6
(6) D-Ala-D-Val		26
(7) D-Ala-D-Norval		>50

[a] Dixon plots (Figures 9D and 10). [b] Calculated values.

TABLE VII: Specificity Profile of the D-Ala-D-Ala Adding Enzyme.

R_n	R_c	K_{mi} (moles/liter × 10⁴)
CH_3CH_2—	—CH_3	0.9
$CH_3CH_2CH_2$—	—CH_3	1.2
CH_3—	—CH_3	1.6
$HOCH_2$—	—CH_3	2.6
$(CH_3)_2CH$—	—CH_3	3.9
CH_3CH_2—	—CH_2CH_3	7.0
CH_3—	—CH_2CH_3	7.6
CH_3—	—CH_2OH	9.1
CH_3—	—$CH(CH_3)_2$	26.
CH_3—	—$CH(OH)CH_3$	33.
CH_3—	—$CH_2CH_2CH_3$	>50.

Two sites of substitution have been studied: R_n and R_c.



The addition of substituents in the R_n position enhances the binding[3] in the case of D-aminobutyrate and D-norvaline. Isopropyl substitution in the R_n position, on the other hand, decreases the binding of the dipeptide. In contrast to the results with R_n substitution, R_c substitution in every case results in a decreased binding of the dipeptide. The conclusions are evident when the values for K_{mi} of the following pairs are considered: D-Butyr-D-Ala (0.9×10^{-4} M) and D-Ala-D-Butyr (8.8×10^{-4} M), and D-Norval-D-Ala (1.2×10^{-4} M) and D-Ala-D-Norval ($>50 \times 10^{-4}$ M).

In contrast to these results, specificity studies with D-Ala-D-Ala synthetase give a pattern different from that observed with the adding enzyme (Neuhaus, 1962a,b). The addition of a methyl group, i.e., D-aminobutyrate, hinders the binding of substrate to donor site (N-terminal) but has only a minor effect on the binding at the acceptor site (C-terminal). In addition, D-serine, D-threonine, and D-norvaline are almost entirely excluded from the N-terminal site whereas these amino acids are good acceptors resulting in the formation of D-Ala-D-Norval, D-Ala-D-Ser, and D-Ala-D-Thr when incubated in the presence of D-alanine. The two enzymes, D-Ala-D-Ala synthetase and D-Ala-D-Ala adding enzyme, have complementary specificity profiles; as a result D-Ala-D-Ala is the major dipeptide which is

[3] The assumption is made that $k_{+2} << k_{-1}$.

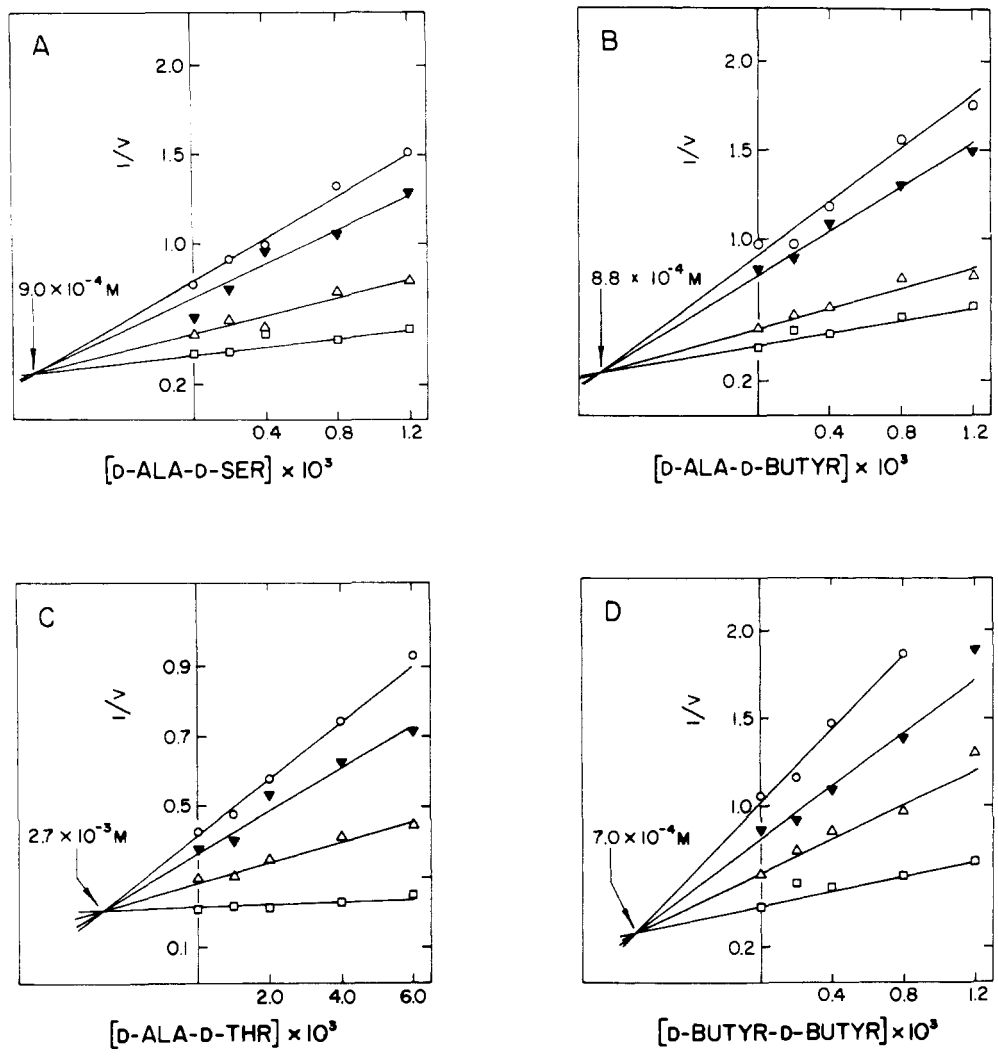


FIGURE 9: Determination of K_{m_t} for D-Ala-D-Ser (A), D-Ala-D-Butyr (B), D-Ala-D-Thr (C), and D-Butyr-D-Butyr (D) by the Dixon method. The "adding assay" was used with the following concentrations of D-[^{14}C]Ala-D-[^{14}C]Ala: □—□, 4.5×10^{-4} M; △—△, 1.8×10^{-4} M; ▼—▼, 9.0×10^{-5} M; ○—○, 6.8×10^{-5} M.

added to the nucleotide precursor UDP-NAc-muramyl-(Ala, Glu, Lys). Thus these studies have established a cooperative specificity pattern between sequential enzyme reactions involved in the biosynthesis of the cell-wall precursor.

The results presented in this paper and those previously reported provide a rational basis for explaining a number of growth studies with *S. faecalis* R by Snell and others. With *S. faecalis* R grown on vitamin B_6-deficient medium, Snell *et al.* (1955) observed that D-aminobutyrate was the only amino acid which would replace the D-alanine requirement. In addition, Bondi *et al.* (1957) found that the only amino acid besides D-alanine which would overcome to a small extent the inhibition of growth resulting from D-cycloserine was D-aminobutyrate (1%). In the enzyme-specificity studies it is shown that D-Butyr-D-Butyr is the only analog of D-Ala-D-Ala in the single substrate series that is formed by the synthetase and then utilized by the adding enzyme. Thus, UDP-NAc-muramyl-(Ala, Glu, Lys)-D-Butyr-D-Butyr appears to be a cell-wall precursor in vitamin B_6-deficient cells grown in the presence of D-aminobutyrate.

Snell and Guirard (1943) reported that DL-serine and DL-threonine inhibited the growth of *S. faecalis* R. DL-Alanine was the only amino acid which was effective in reversing the growth inhibition by these compounds. The specificity profile of the adding enzyme permits certain mixed dipeptide analogs of D-Ala-D-Ala, such as D-Ala-D-Ser and D-Ala-D-Thr, to be substrates with the formation of the pentapeptide analog of the uridine nucleotide. The effect of D-serine on bacterial growth has been studied in several bacteria. Growth of *Micrococcus lysodeikticus* in the presence of D-serine results in the replacement of glycine in the mucopeptide fraction (Whitney and Grula, 1964). The incorporation of D-serine is decreased in the presence of D-alanine, L-alanine, L-serine, or glycine. The replacement of glycine

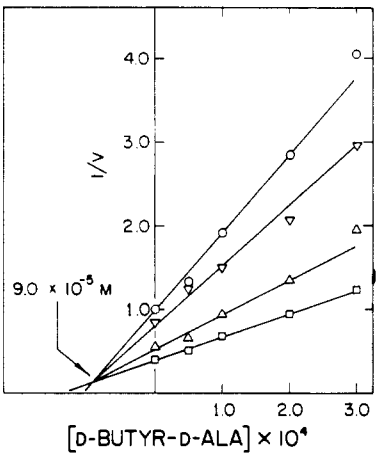


FIGURE 10: Determination of K_{m_i} for D-Butyr-D-Ala by the Dixon method. The "adding assay" was used with 1.9 μg of enzyme preparation and the following concentrations of D-[^{14}C]Ala-D-[^{14}C]Ala: □—□, 4.5×10^{-4} M; △—△, 1.8×10^{-4} M; ▽—▽, 9.0×10^{-5} M; O—O, 6.8×10^{-5} M.

by D-serine is not readily explained by the specificity studies reported in this paper. Czerkawski *et al.* (1963) reported that the C-terminal amino acid in the mucopeptide fraction from *M. lysodeikticus* is glycine and proposed that the sequence is probably Ala-Glu-Lys-Ala-Gly. On the basis of this sequence it was proposed that D-Ala-D-Ser might replace D-Ala-Gly. A series of experiments[4] were performed with sonic extracts of *M. lysodeikticus* ATCC 4698. Under conditions which result in D-Ala-D-Ala synthesis, no D-Ala-Gly was observed when equimolar concentrations of D-alanine and glycine were incubated with extract, ATP, and Mg^{2+}. In addition, D-Ala-Gly (2×10^{-2} M) is not an inhibitor of UDP-NAc-muramyl-(Ala, Glu, Lys)-D-[^{14}C]Ala-D-[^{14}C]Ala synthesis in experiments with 2×10^{-4} M D-[^{14}C]Ala-D-[^{14}C]Ala (see Table IV). In this respect the adding enzyme from this organism appears to be the same as that observed in *S. faecalis* R. Thus these experiments cannot be correlated with those of Whitney and Grula (1964).

D-Serine has another site of action which is not related to mucopeptide formation. Maas and Davis (1950) showed that D-serine inhibits the synthesis of pantothenate in *E. coli*. This inhibition could be overcome by the addition of β-alanine in a *Flavobacterium* sp. (Durham and Milligan, 1962). Grula and Grula (1963) observed that D-serine inhibits the α-decarboxylation of aspartic acid in *Erwinia* sp. It has not been established whether this site of inhibition occurs in *S. faecalis* R.

Ghuysen *et al.* (1964) find that 90% of the peptide fragments in the mucopeptide fraction of *S. aureus* Copenhagen have the sequence L-Ala-D-Glu-L-Lys-D-Ala, whereas only 10% of the peptide fragments have the sequence L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. Since the major fragment is a tetrapeptide, the penultimate amino acid of the nucleotide is probably the precursor of the C-terminal amino acid in the mucopeptide. The C-terminal amino acid of the nucleotide must be eliminated or transferred to another component. Perkins (1963) proposed the enzymatic scission of the terminal D-Ala-D-Ala moiety with the C-terminal D-alanine remaining attached to teichoic acid. A similar proposal has also been considered by Mandelstam and Strominger (1961). The availability of precursor analogs which are labeled in the penultimate and (or) C-terminal residues may allow one to define some of the stages in the polymerization reactions which have been discovered by Chatterjee and Park (1964) and Meadow *et al.* (1964). These systems utilize particulate preparations of *S. aureus* to polymerize UDP-NAc-glucosamine and UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala with the formation of cell-wall mucopeptide.

The growth of *Flavobacterium* sp. in the presence of D-serine results in an increased fragility to sonic oscillation (Durham, 1963). With *S. faecalis* R a marked change in permeability was observed when the bacteria were grown with D-serine in the medium.[5] Thus incorporation of analogs of D-alanine into the wall mucopeptide via the analog of the nucleotide-pentapeptide, UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-analog, may result in the formation of altered or defective walls.

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