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Immunochemistry of a Synthetic Peptidoglycan–Precursor Pentapeptide†

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ABSTRACT: A protected pentapeptide with the sequence found in several precursors to a major class of bacterial peptidoglycans, Ala-γ-DGlu-Lys-DAla-DAla, has been synthesized by classical methods. After selective deprotection of the α-amino group, the pentapeptide was covalently linked to the random polypeptide $(Glu^{60}Ala^{40})_n$. The fully deprotected conjugate evoked an antibody response in each of two rabbits immunized. The specificity of the antibodies obtained from a single bleeding was studied by radioimmunoassay, using related polypeptides and haptens as inhibitors. The immunodominant region comprised the carboxyl terminus of the pentapeptide, *X*-Lys-DAla-DAla. The tripeptide with the *tert*-butoxy-protecting group in position *X* was almost as effective an inhibitor as the precursor pentapeptide with Ala-DisoGlu in position *X*. The antibody was absorbed specifically by a natural peptidoglycan from group C *Streptococcus*.

A major class of bacterial peptidoglycans is synthesized from several glycopeptide precursors containing the pentapeptide, Ala-γ-DGlu-Lys-DAla-DAla. This peptide is attached by the α-amino group to the glycan, which consists of alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid (for recent reviews, see Osborn, 1969; Schleifer and Kandler, 1972). Several modifications of this peptide can occur prior to the final transpeptidation step, including amidation of D-glutamic acid and peptidation of the ε-amino group of lysine to form the so-called "peptide bridge." In the transpeptidation, a bond is formed between the penultimate D-alanine of one chain (with the release of the terminal D-alanine) and the ε-amino group of lysine, or the α-amino group of the peptide bridge of a second chain. This process can be repeated with the formation of polymers of the tetrapeptide, (Ala-γ-DGlu-Lys-DAla), plus the modifications which had previously occurred. In *Staphylococcus aureus* at least, this polymerization may not be complete. Tipper and Strominger (1965) have shown the peptidoglycan to consist of 7% of the material, *N*-acetylglucosaminyl-*N*-acetylmuramyl(Ala-γ-DGln-Lys-DAla-DAla)$(Gly)_5$.

Schleifer and Krause (1971) have studied the immune response of rabbits to group A variant *Streptococcus*. Inhibition of the precipitin reaction of this antiserum with solubilized peptidoglycan from *Staphylococcus epidermidis* was far greater with the pentapeptide, Ala-γ-DGlu-Lys-DAla-DAla, than with the tetrapeptide, Ala-γ-DGlu-Lys-DAla. By inhibition studies with smaller haptens, they could show that the dominant antibody specificity was directed against the carboxyl terminal, D-alanyl-D-alanine. This implied that the unpolymerized peptide portion of the peptidoglycan is immunodominant to the polymerized peptide portion.

We wish to study the immune response of rabbits to this peptidoglycan–precursor pentapeptide in the form of a branched copolymer and compare the antibody specificity with the results obtained by Schleifer and Krause from group A-variant *Streptococcus*. Group A streptococci have been shown to have materials with endotoxic activity (Stetson, 1956; Rotta *et al.*, 1965), as well as materials with determinants whose antibodies cross-react with several mammalian cell types (Kaplan and Meyeserian, 1962; Zabriskie and Freimer, 1966; Rotta and Bednar, 1970; Kingston and Glynn, 1971; Wagner and Weppe, 1972). Presumably, these materials will be absent from the synthetic immunogen. The present report includes the synthesis and immunochemistry of the peptidoglycan–precursor pentapeptide (Ala-γ-DGlu-Lys-DAla-DAla).

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Materials and Methods

All melting points are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter at the 589-nm sodium line. Elemental analyses were performed by the Alfred Bernhardt Microanalytical Laboratories, Engelskirchen, West Germany. Amino acid analyses were performed in a Phoenix amino acid analyzer, Model K5000A, on samples which had been hydrolyzed for 16 hr in evacuated, sealed tubes at 110°. The following peptide moieties were detected by thin-layer chromatography using silica gel plates (Brinkmann): free amine, 0.1% ninhydrin spray followed by heating; *tert*-butoxycarbonylamine, hydrochloride vapor for 15 min followed by the procedure to detect free amine; benzyloxycarbonylamine, iodine vapor; peptide bond, 1% sodium hypochlorite spray followed by 1% potassium iodide–1% soluble starch spray. R_F values on these plates refer to the following solvent systems: (1) methanol–ethyl acetate (1:2, v/v); (2) *n*-butyl alcohol–acetic acid–pyridine–water (4:2:1:1, v/v); (3) *n*-butyl alcohol–acetic acid–water (4:1:5, v/v). Protected amino acid monomers were purchased from Fox Chemical Co. (Los Angeles, Calif.) and were homogeneous by thin-layer chromatography.

Synthesis of Pentapeptide. *tert*-BUTOXYCARBONYL-D-ALANYL-D-ALANINE BENZYL ESTER (I). To the *p*-tosylate salt of D-alanine benzyl ester (7.0 g, 20 mmol) suspended in 100 ml of ethyl acetate was added 2.8 ml (20 mmol) of triethylamine and 50 ml of water. The aqueous solution was removed and the organic layer was reextracted with water. The ethyl acetate layer was dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in 100 ml of methylene chloride to which was added successively at 4° 3.78 g (20 mmol) of *tert*-butoxycarbonyl-D-alanine and 4.12 g (20 mmol) of dicyclohexylcarbodiimide. After 16 hr at 4°, the reaction mixture was filtered and evaporated to dryness. The residue was brought up in 100 ml of ethyl acetate, refiltered, and extracted with 0.5 M citric acid (3 × 50 ml), water, 1.0 M sodium bicarbonate (3 × 50 ml), and water. The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to yield 6.0 g (86%) of an oil (R_F 0.75[1]).

α-*tert*-BUTOXYCARBONYL-ε-BENZYLOXYCARBONYLLYSYL-D-ALANYL-D-ALANINE BENZYL ESTER (II). Compound I (1.51 g, 4.34 mmol) was allowed to react with 15 ml of 4 N HCl in dioxane for 15 min. Precipitation with ether yielded 0.99 g (80%) of the hygroscopic dipeptide; R_F 0.38[1] with a ninhydrin-positive trace at the origin. To 0.99 g of D-alanyl-D-alanine benzyl ester hydrochloride (3.5 mmol) in 100 ml of methylene chloride was added at 4° 1.6 g (4.2 mmol) of α-*tert*-butoxycarbonyl-ε-benzyloxycarbonyllysine, 490 μl (3.5 mmol) of triethylamine, and 730 mg (3.5 mmol) of dicyclohexylcarbodiimide. After 16 hr at 4°, the solution was filtered and evaporated to dryness. The residue was dissolved in 100 ml of methylene chloride and extracted with 0.5 M citric acid, water, 1.0 M sodium bicarbonate, and water. The ethyl acetate layer was dried with anhydrous sodium sulfate, filtered, and concentrated to a small volume. The product precipitated with ether in two crops having similar properties: yield, 1.49 g (70%); mp 110–112°; $[\alpha]_D^{20}$ +32.1° (*c* 1.35, methanol); R_F 0.89[1], 0.92[2], 0.88[3].

Anal. Calcd for $C_{32}H_{44}N_4O_8$: C, 62.73; H, 7.24; N, 9.14. Found: C, 62.89; H, 7.32; N, 9.29.

ε-BENZYLOXYCARBONYLLYSYL-D-ALANYL-D-ALANINE BENZYL ESTER HYDROCHLORIDE (III). Compound II (734 mg, 1.2 mmol) was dissolved in 8 ml of 5.25 N HCl in dioxane for 12 min. Precipitation with ether yielded 560 mg (85%) of a sticky precipitate: R_F 0.47[1], 0.79[2], 0.59[3]. There was a ninhydrin-positive trace at the origin[1], 0.42[2], 0.20[3] and a ninhydrin-negative, strach–iodide-positive trace at 0.85[1], 0.95[2], 0.84[3], which was probably II.

tert-BUTOXYCARBONYLALANYL-D-GLUTAMIC ACID α-BENZYL ESTER (IV). The hydrochloride salt of D-glutamic acid α-benzyl ester (1.65 g, 6.0 mmol) was dissolved in 1.18 g (14 mmol) of sodium bicarbonate in 15 ml of water. To this was added at room temperature a solution of 2.0 g (7.0 mmol) of *tert*-butoxycarbonylalanine *N*-hydroxysuccinimide ester in 100 ml of dioxane. After 40 hr, the solution was evaporated to dryness. The residue was dissolved in a mixture of 30 ml of ethyl acetate and 20 ml of 1 M sodium bicarbonate. The aqueous layer was acidified to pH 3.5 with 6 N HCl and extracted four times with 30 ml of ethyl acetate. The four ethyl acetate fractions were pooled, dried with anhydrous sodium sulfate and evaporated to dryness: yield, 1.96 g (80%); R_F 0.53[1], 0.80[2], 0.85[3]. The γ-benzyl ester of *tert*-butoxycarbonylalanyl-D-glutamic acid had R_F 0.35[1], 0.73[2], 0.71[3].

tert-BUTOXYCARBONYLALANYL-α-BENZYL-D-GLUTAMYL-ε-BENZYLOXYCARBONYLLYSYL-D-ALANYL-D-ALANINE BENZYL ESTER (V). Compound III (560 mg, 1.0 mmol) and 0.14 ml (1.0 mmol) of triethylamine were dissolved in a mixture of 50 ml of ethyl acetate and 50 ml of water. The ethyl acetate layer was dried with anhydrous sodium sulfate, filtered, and concentrated to dryness. The residue was dissolved in 100 ml of methylene chloride. To this was added at room temperature, 430 mg (1.0 mmol) of IV and 206 mg (1.0 mmol) of dicyclohexylcarbodiimide. After 16 hr, the solution was evaporated and the residue was brought up in 10 ml of distilled *N*,*N*-dimethylformamide and filtered. The solvent was removed *in vacuo* and the product was obtained in two crops from refluxing ethyl acetate: yield, 605 mg (66%); mp 154–156°; $[\alpha]_D^{20}$ +18.1° (*c* 0.8, methanol); R_F 0.82[1], 0.93[2], 0.84[3].

Anal. Calcd for $C_{47}H_{62}N_6O_{12}$: C, 62.51; H, 6.92; N, 9.31. Found: C, 62.69; H, 7.11; N, 9.42.

ALANYL-α-BENZYL-D-GLUTAMYL-ε-BENZYLOXYCARBONYLLYSYL-D-ALANYL-D-ALANINE BENZYL ESTER HYDROCHLORIDE (VI). Compound V (125 mg, 0.14 mmol) was dissolved in 5 ml of 5.25 N HCl in dioxane for 12 min. The product was precipitated with ether: yield, 100 mg (86%); R_F 0.33[1], 0.75[2], 0.52[3].

Polymers. Random polypeptides were polymerized from *N*-carboxy-α-amino acid anhydrides as previously described (Katchalski and Sela, 1958) using an anhydride:initiator ratio of 200–300:1. The random polymers were deprotected with HBr in benzene and the molecular weights estimated by intrinsic viscosity in dichloroacetic acid (Doty *et al.*, 1956). The polymers were dissolved in (0.15 M) phosphate-buffered NaCl, pH 7.0 (PBS),[1] and dialyzed against distilled water, changed twice a day for 1 week. The solutions were then lyophilized and stored at −20°. A list of the polymers used in this study and their estimated molecular weights is presented in Table I.

Synthesis of Immunogen and Antigen. The polymers $(Glu^{60}Ala^{40})_n$ and $(DGlu^{60}DAla^{40})_n$, which had been lyophilized, were acidified to pH 1–2. The residues were filtered and dried

[1] Abbreviations used are: PBS, 0.15 M phosphate-buffered saline; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyl ester; OMe, methyl ester; *p*-Tos, *p*-tosylate salt; ONSu, hydroxysuccinimide ester; R-1357, antiserum obtained from rabbit 1357; LL copolymer, $(Glu^{60}Ala^{40})_n(Ala\text{-}\gamma\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}DAla)^{5.1}$; DD copolymer, $(DGlu^{60}DAla^{40})_n(Ala\text{-}\gamma\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}DAla)^{5.7}$; protected DD copolymer, $(DGlu^{60}DAla^{40})_n(Ala\text{-}\alpha\text{-}OBzl\text{-}DGlu\text{-}\epsilon\text{-}Z\text{-}Lys\text{-}DAla\text{-}DAla\text{-}OBzl)^{5.7}$; PAMEG, (γ-2-*N*-morpholinylethylglutamic acid)$_n$.

TABLE I: Polymers of α-Amino Acids Employed in Present Study.

Nomenclature of Polymer[a]	Mol Wt
$(Glu^{60}Ala^{40})_n$	43,000[b]
$(DGlu^{60}DAla^{40})_n$	50,000[b]
$(DGlu^{16}Lys^{16}DLAla^{68})_n$	5,000[b]
$(Glu^{42}Lys^{28}DAla^{30})_n$	12,000[b]
$(Ala\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}Gly)_n$	15,000[c]
$\overline{(Glu^{60}Ala^{40})_n}(Ala\text{-}\gamma\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}DAla)^{\cdot 1}$	56,000[d]
$\overline{(DGlu^{60}DAla^{40})_n}(Ala\text{-}\gamma\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}DAla)^{5\cdot 7}$	68,000[d]
$\overline{(DGlu^{60}DAla^{40})_n}(Ala\text{-}\alpha\text{-}OBzl\text{-}DGlu\text{-}\epsilon\text{-}Z\text{-}Lys\text{-}DAla\text{-}DAla\text{-}OBzl)^{5\cdot 7}$	80,000[d]

[a] As recommended by the IUPAC–IUB Commission on Biochemical Nomenclature, Abbreviated Nomenclature of Synthetic Polypeptides (1972). [b] Determined by viscometric measurements in dichloroacetic acid. [c] Determined by the Scheraga–Mandelkern treatment (see Zeiger *et al.*, 1973). [d] Determined by viscometric measurements and amino acid analysis.

TABLE II: Amino Acid Analyses of Inhibitors.

Inhibitor	Ala	Lys	Glu
Ala-γ-DGlu-Lys-DAla-DAla	3.01[a]	1.03	0.96
Ala-DGlu-Lys-DAla-Gly[c]	2.00[a]	1.01	1.04
Ala-DGlu-Lys-DAla	1.99[a]	1.00	1.01
Lys-DAla-DAla	2.01[a]	0.99	
$(Glu^{60}Ala^{40})_n$	38.7[b]		61.3
$\overline{(Glu^{60}Ala^{40})_n}(Ala\text{-}\gamma\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}DAla)^{5\cdot 1}$	45.5[b]	4.9	49.9
$(DGlu^{60}DAla^{40})_n$	40.3[b]		59.7
$\overline{(DGlu^{60}DAla^{40})_n}(Ala\text{-}\gamma\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}DAla)^{5\cdot 7}$	46.1[b]	5.6	48.2

[a] Molar ratio. [b] Percentage. [c] Contains 0.96 molar ratio of glycine.

over P_2O_5. Compound VI (80 mg, 90 μmol) was added to 125 mg of $(Glu^{60}Ala^{40})_n$ or $(DGlu^{60}DAla^{40})_n$ in 25 ml of *N,N*-dimethylformamide at room temperature followed by 31 mg (150 μmol) of dicyclohexylcarbodiimide, 23 mg (200 μmol) of *N*-hydroxysuccinimide and 15 μl (107 μmol) of triethylamine. After 2 hr at room temperature the reaction mixture was kept at 50° overnight. The solvent was then evaporated to dryness and brought up in 5 ml of water. Several drops of glacial acetic acid were added, followed by 10 ml of 1.0 M sodium bicarbonate. After filtration of the mixture, HCl was added to pH 2 and the mixture was filtered. The yield was 76% of the protected LL copolymer and 69% of the protected DD copolymer. The ratio of pentapeptide to random polymer was determined by amino acid analysis.

To 125 mg of a suspension of protected LL copolymer, in benzene–anisole (5:1, v/v), was bubbled hydrogen bromide gas through a benzene–anisole trap. After 70 min at room temperature, the system was sealed and the solution was stirred overnight. The suspension was allowed to settle and the supernatant was decanted. This procedure was repeated twice with ether as solvent. The deprotected peptide salt was filtered and dried over P_2O_5 to yield 91.5 mg (100%). The residue was dissolved in 25 ml of PBS (pH 7.0) and dialyzed against distilled water for 4 days with repeated changes of water. The solution was lyophilized to yield 70 mg and stored at −20°. Six rabbits injected with 1.0 mg of this material failed to develop a significant febrile response within 6 hr.

The protected DD copolymer (40 mg) was deprotected in the same fashion to yield 33.6 mg of lyophilized material.

Hapten Inhibitors. All inhibitors were synthesized by classical methods. Deprotection was performed by hydrogenation in methanol with 10% Pd on charcoal at 38 psi of hydrogen in a Parr hydrogenator (Parr Instrument Co., Moline, Ill.) or by HBr in benzene as described above. The haptens, DAla-DAla, DAla-DAla-OBzl, Boc-DAla-DAla, Boc-Lys-DAla-DAla, Lys-DAla-DAla, and Ala-γ-DGlu-Lys-DAla-DAla, were derived from intermediates in the synthesis of the peptidoglycan–precursor pentapeptide. The pentapeptide, Ala-DGlu-Lys-DAla-Gly, and the tripeptide, Boc-Lys-DAla-Gly, were derived from intermediates previously synthesized (Zeiger *et al.*, 1973). Synthesis of the tetrapeptide, Ala-DGlu-Lys-DAla, and the hexapeptide, $(Gly)_4$-DAla-DAla, will be presented elsewhere. The latter was contaminated with an equimolar amount of $(Gly)_4$. Concentrations of inhibitors were determined by amino acid analysis of stock solutions of PBS of approximately 10 mg/ml. The amino acid ratios are presented in Table II.

Immunization Methods. The schedules of immunization of rabbits with $\overline{(Glu^{60}Ala^{40})_n}(Ala\text{-}\gamma\text{-}DGlu\text{-}Lys\text{-}DAla\text{-}DAla)^{5\cdot 1}$ in complete Freund's adjuvant will be described elsewhere. The vaccine carrier used to produce the antiserum used in this study was obtained from a rough strain of *Pneumococcus*, R36A. Growth conditions for this organism as well as the coating of the pneumococcal cells with polymer have been described (McDonald *et al.*, 1972). The immunogen (5 mg in 20 ml of PBS) was absorbed to heat-shocked pneumococci (4000 μg of bacterial nitrogen) and stirred slowly overnight. The suspension was centrifuged at 3000 rpm for 20 min and the supernatant was removed. The suspension was washed three times with 20 ml of PBS and suspended in formalized saline (0.9% NaCl–0.2% formaldehyde) to a concentration of 200 μg of N/ml. Two rabbits were injected in the ear vein three times a week for 2 weeks with 0.5 ml of vaccine. Antibody of similar titer was obtained from both rabbits. The results reported in this study were obtained using antiserum from a single bleeding of rabbit 1357 after 2 weeks of the above schedule. Antisera obtained from later bleedings of both animals were inhibited to a similar extent by the hapten, Boc-Lys-DAla-DAla, as the antiserum which has been studied here.

Radioimmunoassay. To 10 mg of $\overline{(DGlu^{60}DAla^{40})_n}(Ala\text{-}\alpha\text{-}OBzl\text{-}DGlu\text{-}\epsilon\text{-}Z\text{-}Lys\text{-}DAla\text{-}DAla\text{-}OBz)^{5\cdot 7}$ in 1 ml of distilled *N,N*-dimethylformamide were added successively 1 μmol of tyrosine methyl ester hydrochloride, 1.2 μmol of triethylamine, 1.2 μmol of *N*-hydroxysuccinimide, and 1.2 μmol of dicyclohexylcarbodiimide, each in 100 μl of *N,N*-dimethylformamide. After 16 hr at room temperature, the product was precipitated with ether, filtered, and washed with ether. The polymer was deprotected in HBr and benzene as above, dialyzed, and lyophilized to dryness; yield, 3.5 mg.

This polymer was then iodinated with ^{125}I using the Chloramine-T procedure (Hunter, 1969). The labeled polymer was dialyzed until no ^{125}I could be detected in the dialysate. The specific activity was about 0.25 mCi/mg of polymer.

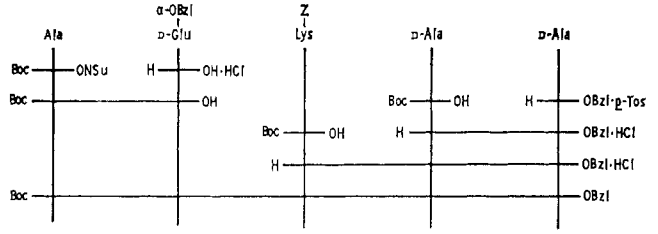

FIGURE 1: Synthetic route to the protected pentapeptide, *tert*-butoxycarbonylalanyl-α-benzyl-D-glutamyl-ε-benzyloxycarbonyllysyl-D-alanyl-D-alanine benzyl ester.

As the polymer did not precipitate appreciably in 50% saturated ammonium sulfate, a modification of the ammonium sulfate precipitation technique of Farr (1971) was used to monitor antigen binding. To 25 μl of immune serum or immune serum diluted with nonimmune rabbit serum was added, with mixing, 25 μl of freshly diluted antigen (10 ng). After 60 min at 37°, the solution was cooled to 4° and 150 μl of 66% saturated ammonium sulfate was added with mixing. After incubation at 4° for 30 min, the mixture was centrifuged for 30 min at 4°. An aliquot consisting of 100 μl of the supernatant was counted in a well-type gamma scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Determinations were always done in duplicate. Control A included 150 μl of PBS instead of 66% saturated ammonium sulfate and control B included 25 μl of nonimmune serum in place of the immune serum. Nonspecific precipitation, (control B − background)/(control A − background) × 100, was 7.0 ± 3.2%. The maximum precipitation of labeled antigen in undiluted serum was only 66%. To test whether inorganic ^{125}I was present in the sample, an aliquot of the labeled polymer was redialyzed for several days. The specific activity of the recovered material was unchanged. In later studies with R1357 serum having a 25-fold greater titer, maximal precipitation was still only 70% (A. R. Zeiger, unpublished results). Using lower concentrations of antigen was not feasible because of the specific activity of the labeled antigen.

Inhibition of binding was done as follows. To 25 μl of serum, which had been freshly diluted (1:10) with nonimmune serum, was added 10 μl of inhibitor (ranging up to 10 mg/ml). After incubation at 37° for 1 hr, 20 μl of freshly diluted radioactive antigen (8 ng) was added. After incubation for an additional hour at 37°, the mixture was cooled to 4° and 150 μl of 66% saturated ammonium sulfate was added with mixing. The assay then proceeded identically as above.

Two additional controls were included routinely. Control C contained 10 μl of PBS instead of inhibitor. Binding of the diluted serum, (control C − background)/(control B − background) × 100, was 33.9 ± 6.6%. Control D contained 25 μl of nonimmune serum to which 10 μl of the highest concentration of inhibitor was added. In no instance was the precipitation of antigen significantly different from control B.

Binding to Anti-Pneumococcus Antiserum. To test the immunogenicity of the *Pneumococcus* R36A in our system, we examined the direct binding of our antigen with antiserum obtained from an innoculum of *Pneumococcus R36A* alone or with antiserum obtained from an innoculum of *Pneumococcus R36A* which had been coated with (γ-2-*N*-morpholinylethylglutamic acid)$_n$ (PAMEG) as follows. To 25 μl of *Pneumococcus* R36A antiserum or the antiserum against *Pneumococcus* R36A coated with PAMEG was added 25 μl of freshly diluted labeled antigen (10 ng). The procedure was then identical with that used above to detect antigen binding.

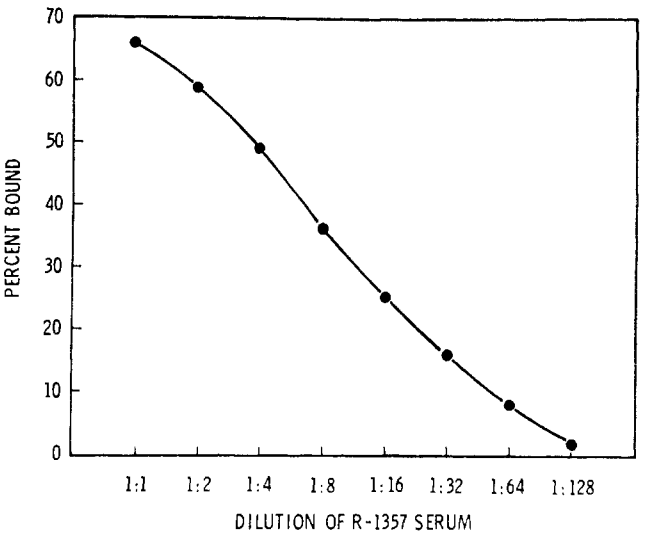

FIGURE 2: Titration of R-1357 serum. Serial dilutions of R-1357 serum with nonimmune rabbit serum (25 μl) were incubated with 10 ng of radioactive antigen (25 μl) at 37° for 1 hr, followed by precipitation at 4° for 30 min with 66% saturated ammonium sulfate (150 μl).

Inhibition of these antisera was the same as that for R-1357 antiserum.

Absorption to Peptidoglycan. To 10 μg of insoluble peptidoglycan in 10 μl of PBS was added 100 μl of diluted R-1357 antiserum. The mixture was shaken every 5 min at 37° for 30 min, and centrifuged. A 25-μl aliquot of the supernatant was added to 20 μl of labeled antigen (8 ng). Thereafter, the procedure was the same as for a direct binding assay. As a control for the specificity of this absorption, an antiserum from a rabbit against the polymer, (Ala-DGlu-Lys-DAla-Gly)$_n$, was diluted to approximately the same titer as R-1357 antiserum and substituted for it. The polymer, (Ala-DGlu-Lys-DAla-Gly)$_n$, which had been tyrosinated lightly and labeled with ^{251}I, was substituted for the DD copolymer. About 16 ng of the antigen was used per assay.

Results

Synthesis of the Immunogen and Antigen. The scheme for the synthesis of the pentapeptide, Ala-γ-DGlu-Lys-DAla-DAla, is presented in Figure 1. The condensation of two peptides was chosen to reduce the number of synthetic steps for the D-alanine derivatives as well as to facilitate the purification of the final product. The dipeptides, Boc-DAla-DAla and Boc-Ala-α-OBzl-DGlu, were oils. In addition, the hydrochloride salts of the intermediates were somewhat hygroscopic. Characterizable crystals were obtained only for the tripeptide III and the pentapeptide V. Nevertheless, the product appeared to be homogeneous by melting point, thin-layer chromatography, elemental analysis, and amino acid analysis. Coupling of the pentapeptide by dicyclohexylcarbodiimide to (Glu^{60}Ala^{40})$_n$ and (DGlu^{60}DAla^{40})$_n$ proceeded routinely and quantitatively. The former had the pentapeptide joined to approximately 5.1% of its residues, whereas the latter was modified to the extent of 5.7% of its residues.

Titration of Serum. The binding of labeled antigen was followed by serial dilutions of the antiserum with nonimmune rabbit serum (Figure 2). The maximum precipitation of labeled antigen was only 66%. A 1:10 dilution of R-1357 antiserum was chosen for the inhibition studies because it is near the inflection point of the titration curve.

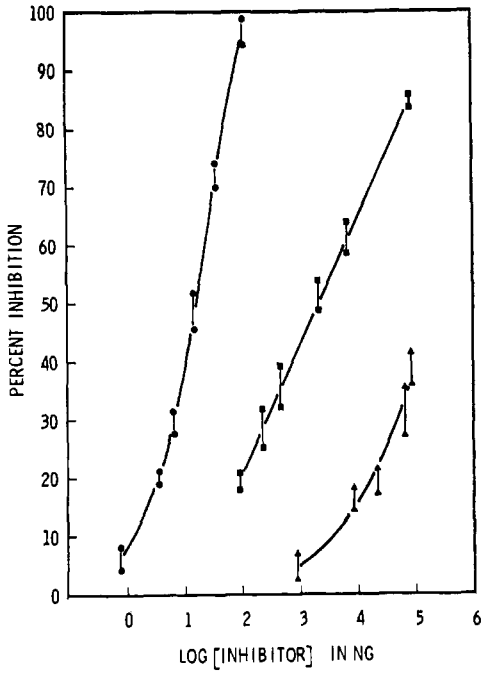

FIGURE 3: Inhibition of R-1357 serum with polypeptides. Dilutions of the polypeptide (10 μl) were added to R-1357 serum (25 μl) which had been diluted 1:10 with nonimmune rabbit serum at 37° for 1 hr, followed by 8 ng of radioactive antigen (20 μl). The solution was incubated again for 1 hr at 37°, followed by precipitation at 4° for 30 min in 66% saturated ammonium sulfate (150 μl): (●) DD copolymer; (■) $(\text{DGlu}^{60}\text{DAla}^{40})_n$(Ala-$\alpha$-OBzl-DGlu-$\epsilon$-Z-Lys-DAla-DAla-OBzl)$^{5.7}$; (▲) $(\text{DGlu}^{16}\text{Lys}^{16}\text{DLAla}^{68})_n$.

Inhibition with Polymers. The per cent inhibition of binding by increasing amounts of unlabeled homologous antigen is presented in Figure 3. Inhibition reached 100% at a concentration of 190 ng. The ratio of unlabeled to labeled antigen required to give 25 and 50% inhibition was 0.65 and 2.0, respectively (Table III). The protected antigen, $(\text{DGlu}^{60}\text{DAla}^{40})_n$(Ala-$\alpha$-OBzl-DGlu-$\epsilon$-Z-Lys-DAla-DAla-OBzl)$^{5.7}$, also was able to inhibit binding of the antigen (Figure 3), but was approximately two orders of magnitude less effective than the homologous antigen at 50% inhibition. At the highest ratio of inhibitor to antigen used (12,500 w/w), inhibition was approximately 85%. A random polymer, $(\text{DGlu}^{16}\text{Lys}^{16}\text{DLAla}^{68})_n$, whose amino acid composition is similar to that found in cell walls and which was able to cross-react with a natural peptidoglycan from *Staphylococcus* (Karakawa *et al.*, 1970), was an even less effective inhibitor, producing less than 50% inhibition at the highest concentration (Figure 3). (Ala-DGlu-Lys-DAla-Gly)$_n$, a known sequence polymer (Zeiger *et al.*, 1973), and the random polymer, $(\text{Glu}^{42}\text{Lys}^{28}\text{DAla}^{30})_n$, did not inhibit significantly at the concentrations used. Also showing no significant inhibition were $(\text{Glu}^{60}\text{Ala}^{40})_n$ and $(\text{DGlu}^{60}\text{DAla}^{40})_n$ which served as the carriers of the immunogen and antigen, respectively. These results are tabulated in Table III.

Inhibition with Haptens. A linear plot of percent inhibition by concentrations of peptides of different length is shown in Figure 4. More information, such as the maximum inhibition, relative equilibrium and homogeneity, is shown in Figure 5, where the fractional inhibition of each hapten is plotted against the log of the concentration of inhibitor (Pauling *et al.*, 1944). The pentapeptide, Ala-γ-DGlu-Lys-DAla-DAla (A), and the tripeptide, Boc-Lys-DAla-DAla (B), both reached 100% inhibition of the homologous polymer. Boc-DAla-DAla (C) and Boc-DAla (D) inhibited much less. The slopes of the lines indicate that an array of antibodies was present which had large differences in their binding constants to hapten inhibitors of different size and composition. Table IV summarizes the data from these haptens, including the concentrations necessary for 25 and 50% inhibition, K_{rel} at 25%, and $\Delta(\Delta F°)$ at 25%. It is apparent that the negative free energy of binding was increased markedly up to the protected tripeptide. The pentapeptide was only marginally more effective than Boc-Lys-DAla-DAla.

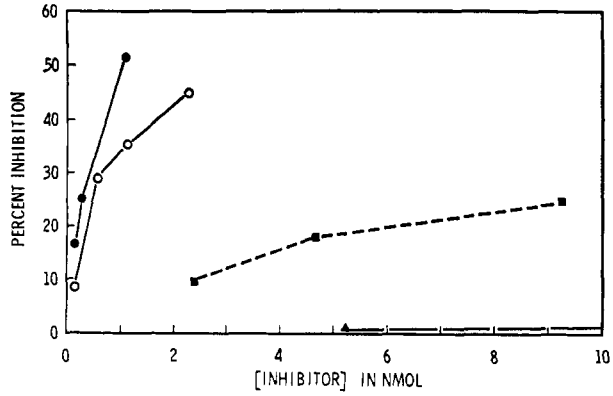

FIGURE 4: Inhibition of R-1357 serum with haptens. The conditions of inhibition were the same as in Figure 3: (●) Ala-γ-DGlu-Lys-DAla-DAla; (○) Boc-Lys-DAla-DAla; (■) Boc-DAla-DAla; (▲) Boc-DAla.

The inhibitory effect of other haptens structurally related to the precursor pentapeptide is summarized in Table V. Elimination of the terminal amino acid, D-alanine, or replacement with glycine resulted in inhibitors which were more than two orders of magnitude less effective than the tripeptide, Boc-Lys-DAla-DAla. Both DAla-OBzl and DAla-DAla-OBzl were poor inhibitors compared to Boc-DAla and Boc-DAla-DAla. The magnitude of the difference in inhibition in the case of the dipeptide is difficult to ascertain in view of the tendency of free dipeptide esters to form diketopiperizines (Fischer, 1906).

TABLE III: Concentration of Polypeptides Necessary to Inhibit Binding of Labeled Antigen (8 ng) by R-1357 Antiserum (1:10 Dilution).[a]

Polymer	Concn in ng for 25% Inhbn	Concn in ng for 50% Inhbn
DD copolymer	5.2	16.2
Protected DD copolymer	178	2140
$(\text{DGlu}^{16}\text{Lys}^{16}\text{DLAla}^{68})_n$	25,000	*b*
$(\text{Glu}^{42}\text{Lys}^{28}\text{DAla}^{30})_n$	*b*	*b*
$(\text{Glu}^{60}\text{Ala}^{40})_n$	*b*	*b*
$(\text{DGlu}^{60}\text{DAla}^{40})_n$	*b*	*b*
(Ala-DGlu-Lys-DAla-Gly)$_n$	*b*	*b*

[a] Dilutions of the polypeptide in 10 μl were added to 25 μl of R-1357 antiserum which had been diluted 1:10 with nonimmune rabbit serum. After standing at 37° for 1 hr, 8 ng of the labeled DD copolymer was added in 20 μl. The solution was again incubated at 37° for 1 hr, followed by precipitation with the addition of 150 μl of 66% saturated ammonium sulfate for 30 min at 4°. [b] Less than the required amount of inhibition at 25,000 ng of polypeptide.

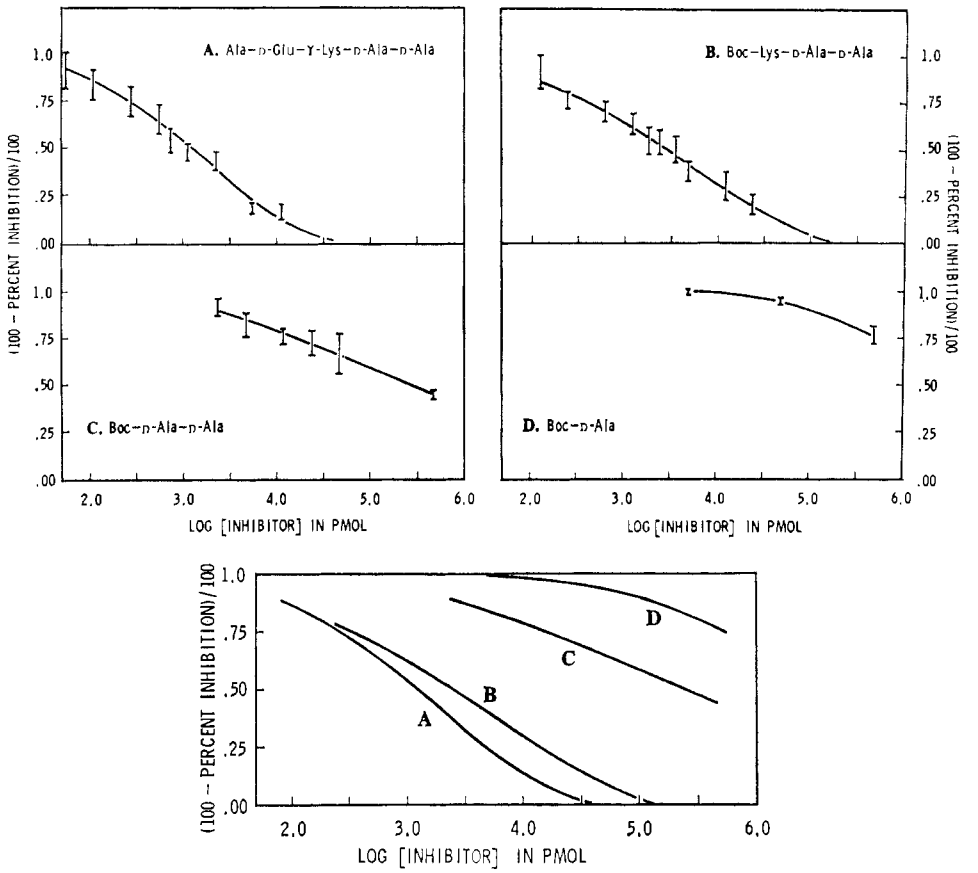

FIGURE 5: Inhibition of R-1357 serum with haptens. A plot similar to that of Pauling *et al.* (1944) in which (100 minus the per cent inhibition)/100 is plotted against the log (inhibitor) for the haptens shown in Figure 4: (A) Ala-γ-DGlu-Lys-DAla-DAla; (B) Boc-Lys-DAla-DAla; (C) Boc-D-Ala-DAla; (D) Boc-DAla.

On the other hand, it is apparent that protection of the α-amines enhanced the inhibitory capacity of these peptides. The dipeptide, Boc-DAla-DAla, was a much more effective inhibitor than the free dipeptide DAla-DAla. In addition, the tripeptide, Boc-Lys-DAla-DAla, produced greater inhibition than the free tripeptide, Lys-DAla-DAla. The fact that inhibition with $(Gly)_4$-DAla-DAla, yielded inhibitory results more consistent with Boc-DAla-DAla than with the free or protected tripeptide could indicate that the size of the group attached to the α-amine is less important than the nature of that group.

Effect of Pneumococcus R36A on the Immune Response. In view on the results of Schleifer and Krause (1971) demonstrating the cross-reactivity of antiserum against group A-variant *Streptococcus* with its peptidoglycan–precursor pentapeptide, we have tested the ability of antisera of rabbits immunized with uncoated *Pneumococcus* R36A or with *Pneumococcus* R36A coated with PAMEG (McDonald *et al.*, 1972) to bind the radioactive pentapeptide antigen. Undiluted antiserum against *Pneumococcus* R36A alone bound labeled antigen 34.8%. On the other hand, undiluted antiserum against *Pneumococcus* R36A coated with PAMEG bound labeled antigen 8.9%. This was only slightly greater than the value of 6.5% found in this experiment for nonimmune rabbit serum. The binding to anti *Pneumococcus* R36A serum was specific. It was inhibited by the cold homologous DD copolymer. In addition, Boc-Lys-DAla-DAla gave almost two orders of magnitude better inhibition than Boc-Lys-DAla-Gly. Finally, preliminary experiments showed that antibodies from R-1357 serum, which had been purified by means of an immunoabsorbent of the synthetic precursor pentapeptide covalently bound to Sepharose, were able to agglutinate *Pneumococcus* R36A. These results indicate that *Pneumococcus* R36A does

TABLE IV: Concentration of Haptens Necessary to Inhibit the Binding of Labeled Antigen (8 ng) by R-1357 Antiserum (1:10 Dilution).[a]

Peptide	Concn in pmol at 25% Inhbn	K_{rel} 25%	$\Delta(\Delta F°)$ 25%[b] in kcal/mol	Concn in pmol at 50% Inhbn
Ala-γ-DGlu-Lys-DAla-DAla	263	*c*	*c*	1,250
Boc-Lys-DAla-DAla	398	1.5	0.26	2,880
Boc-DAla-DAla	17,000	65	2.70	224,000
Boc-DAla	562,000	2140	4.75	

[a] The conditions were the same as in Table III. [b] At 37°. [c] The pentapeptide was the basis for K_{rel} and $\Delta(\Delta F°)$.

TABLE V: Concentration of Haptens Necessary to Inhibit the Binding of Labeled Antigen (8 ng) by R-1357 Antiserum (1:10 Dilution).[a]

Peptide	Concn in pmol at 25% Inhibn
Lys-DAla-DAla	3,600
$(Gly)_4$-DAla-DAla	22,000
Ala-DGlu-Lys-DAla-Gly	78,000
Ala-DGlu-Lys-DAla	*c*
DAla-DAla-OBzl[b]	*c*
DAla-OBzl	*c*
DAla-DAla	*c*

[a] The conditions were the same as in Table III. [b] Possibly inaccurate due to formation of DAla-DAla-diketopiperizine. [c] Less than the required amount of inhibition at 25,000 ng of peptide.

TABLE VI: Dilution of R-1357 Serum Absorbed by Group C Peptidoglycan.[a]

Dilution of R-1357 Serum	% Antigen Bound by Serum after Incubn with Buffer alone	% Antigen Bound by Serum after Incubn with Peptidoglycan
1:2	58.9	38.6
1:4	47.8	27.8
1:8	34.6	15.8
Control[b]	5.3	5.7
Dilution of Anti-(Ala-DGlu-Lys-DAla-Gly)$_n$ Serum		
1:16	47.1	47.7
1:32	30.7	27.0
1:64	9.2	9.6
Control[b]	−0.7	1.3

[a] Dilutions of the antiserum in 100 μl were added to 10 μl of a 1-mg/ml suspension of peptidoglycan from group C *Streptococcus*. After 30 min at 37°, a 25-μl aliquot of the supernatant was added to 25 μl of the labeled antigen, either 10 ng of the DD copolymer or 16 ng of (Ala-DGlu-Lys-DAla-Gly)$_n$. After 1 hr at 37°, the solution was cooled to 4° and 150 μl of 66% saturated ammonium sulfate was added. After 30 min at 4°, the solutions were centrifuged and the supernatants (100 μl) were counted. [b] In the control, 100 μl of nonimmune rabbit serum was used instead of the antiserum.

possess determinants similar to those of the immunogen used in this study but that these determinants could be hidden by coating this rough strain of *Pneumococcus* with a synthetic immunogen.

Cross-Reactivity with Peptidoglycan from Group C Streptococcus. Varying amounts of R-1357 antiserum were incubated with insoluble peptidoglycan from group C *Streptococcus*. The mixture was centrifuged and an aliquot of the supernatant was added to the radioactive antigen. Table VI shows that the antiserum was partially depleted of antibody by adsorption to the cell walls. An approximately equal titer of antibody made against another immunogen, (Ala-DGlu-Lys-DAla-Gly)$_n$, was not absorbed, thus implying that there was some specificity to this absorption.

Discussion

A pentapeptide related to several peptidoglycan precursors has been carefully synthesized and used as immunogen in the form of a branched polymer. Previous studies on branched polymers have shown the specificity of the antibody response to be directed against the unattached terminus of the peptide side chain of the branched polymer, either amino terminus (Sela and Arnon, 1960; Sela *et al.*, 1962; Fuchs and Sela, 1963, 1964; Schechter *et al.*, 1971) or carboxyl terminus (Schleifer and Krause, 1971). The results reported here are consistent with those studies. The immunodominant region of the pentapeptide appears to extend from the free carboxyl to the lysine residue.

The size of the antibody combining site against peptide determinants has also been probed by several investigators (Arnon *et al.*, 1965; Schechter *et al.*, 1966; Schechter, 1970; Van Vunakis *et al.*, 1966; Goodman *et al.*, 1968; Schleifer and Krause, 1971), using immunogens of different homogeneity and, possibly, conformation. Generally, these studies indicated a binding site comparable to three to five amino acids. The size of the antibody combining site studied here was complementary to Boc-Lys-DAla-DAla, which is consistent with the studies cited above.

The presence of a free α-carboxyl group on D-alanine appears to be an important part of the antibody specificity in view of the poor inhibitory capacity of the polymer (DGlu^{16}-Lys^{16}DLAla^{68})$_n$, which has a similar amino acid composition to the pentapeptide. The poor inhibitory capacities of the protected DD copolymer, DAla-OBzl and DAla-DAla-OBzl, may also be due at least partially to esterification of the carboxyl-terminal D-alanine.

Protection of the α-amino group of the small hapten inhibitors, on the other hand, caused a significant enhancement of the inhibition. Similar systems have been found by Benjamini *et al.* (1968) who showed that anti-tobacco mosaic virus serum would bind the carboxyl-terminal tripeptide Ala-Thr-Arg only when the α-amino group is octanoylated, and by Schechter (1970) who showed that amidation of small alanine oligomers aided the binding to anti-(alanyl)$_n$-(human serum albumin serum).

Despite small differences, the specificity of the antibodies directed against the peptidoglycan precursor pentapeptide is quite similar to that found by Schleifer and Krause (1971) for *Streptococcus* peptidoglycan. In addition, we have found that the specificity of R-1357 serum is similar to that of anti-*Pneumococcus* R36A serum.

Prior to further biological studies, it is important to demonstrate that the antiserum against the synthetic immunogen studied here could cross-react with bacterial peptidoglycan. Preliminary experiments indicated that R-1357 antiserum could be absorbed by formamide-extracted cell walls from a group C *Streptococcus*. This is consistent with the finding of precursor pentapeptide in the cell walls of *Staphylococcus aureus* (Tipper and Strominger, 1965).

In addition, it is important to demonstrate that the antibody response is due to the LL copolymer immunogen rather than the bacterial carrier. The fact that antiserum against *Pneumococcus* R36A coated with PAMEG was unable to bind the labeled antigen in contrast to antiserum against uncoated *Pneumococ-*

cus R36A indicates that coating the bacteria carrier with immunogen can cover up at least some of the determinants.

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