Synthetic Polypeptide Antigens of Defined Geometry[1]

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Abstract: The protected octadecapeptide Bpoc-Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_5$-OPh was synthesized by a series of fragment condensations using the tripeptides N^{α}-2-(4-biphenylyl)isopropoxycarbonyl-γ-*tert*-butylglutamyl-*O*-*tert*-butyltyrosyl-N^{ϵ}-benzyloxycarbonyllysine phenyl ester and N^{α}-benzyloxycarbonyl-γ-*tert*-butylglutamyl-*O*-*tert*-butyltyrosylalanine phenyl ester. After removal of the terminal protecting groups the octadecapeptide was polymerized using dicyclohexylcarbodiimide plus 1-hydroxybenzotriazole in dimethylformamide. Subsequent removal of side-chain protecting groups and fractionation on Sephadex G-50 yielded the polyoctadecapeptide of average molecular weight 11 000. Reaction of the lysine amino groups with 2,4,6-trinitrobenzenesulfonic acid afforded a polypeptide antigen bearing the trinitrophenyl substituent on each 18th amino acid in the peptide chain. Circular dichroism studies indicated a partially helical conformation under physiological conditions. Immune response studies showed that the product was immunogenic in mice of strains Balb/c and DBA/2J, both of which bear the *H-2*^{d} haplotype. The particular combination of protecting groups employed in the synthesis of the octadecapeptide enables this product to be used as the starting point for the fragment condensation synthesis of a series of large polypeptide antigens of regular conformation possessing a known spatial separation between haptenic groups. Antigens of this type are required for studies of the molecular mechanism of immune induction.

Progress in the understanding of the mechanism by which antigen-specific lymphocytes are triggered, to generate an immune response, is largely dependent on the availability of antigenic derivatives having structures susceptible to systematic variation by chemical means. The hapten–protein conjugates[2] provided the first antigens of this type and these have led to major advances in the understanding of cell cooperation events in immune induction.[3,4] However, the lack of information concerning the chemical environment and spatial arrangement of the haptenic groups causes problems when these conjugates are employed to study the role of hapten spacing in the initiation of the immune response and also in studies requiring the production of antibodies of restricted heterogeneity. More recently, sequential peptide polymers have been introduced as antigens.[5,6] These have provided a simple system for the investigation of the relationship between amino acid composition and immunogenicity[7] and have allowed the detailed study of the genetic factors controlling the immune response.[8,9] However, the application of the polymeric antigens in hapten-spacing studies is limited because the maximal regular spatial separation which can be achieved between haptenic groups is determined by the size of the peptide monomeric unit, which generally has not been more than seven amino acids. The ideal route for the production of synthetic antigens containing a defined arrangement of haptenic groups appears to be via the methods of controlled peptide synthesis but this approach has not so far been exploited because the antigen size normally required to achieve satisfactory immune responses lies in the protein molecular weight range. Although the synthesis of enzymes and enzyme analogues in this size range has recently been tackled by several groups,[10-12] there is no doubt that such syntheses still present formidable technical difficulties.

In order to obtain a series of macromolecular antigens of defined structure, we are developing a fragment condensation route for the preparation of relatively simple molecules in which large portions of the structure are built from a repetitive tripeptide sequence. The antigens are similar in some respects to the sequential peptide polymers,[13-18] but the use of controlled synthetic procedures allows us to incorporate lysine residues at selected positions during the total synthesis, thereby providing attachment sites for the haptenic group at known positions in the primary structure. Polymers of the chosen tripeptide sequence have been previously found to assume an α-helical conformation under physiological conditions. Therefore, the peptide backbone in the completed molecule can act as a matrix of known conformation to hold the haptenic groups in a defined spatial arrangement determined by the coordinates of the helix and the positions at which the lysine residues are built into the primary sequence. The helical conformation of the backbone also ensures that the chemical environment of the haptenic group is identical at every position at which the group occurs in the molecule. Thus, the antigens are designed to be useful both in hapten-spacing studies and in studies requiring the clonal restriction of the immune response. Here we provide details of the synthesis of the protected octadecapeptide which is required for the production of the macromolecular antigens by the fragment condensation route. We also describe the polymerization of the octadecapeptide to obtain a polymer of molecular weight 11 000, together with data on the conformation and immunogenicity of this synthetic antigen.

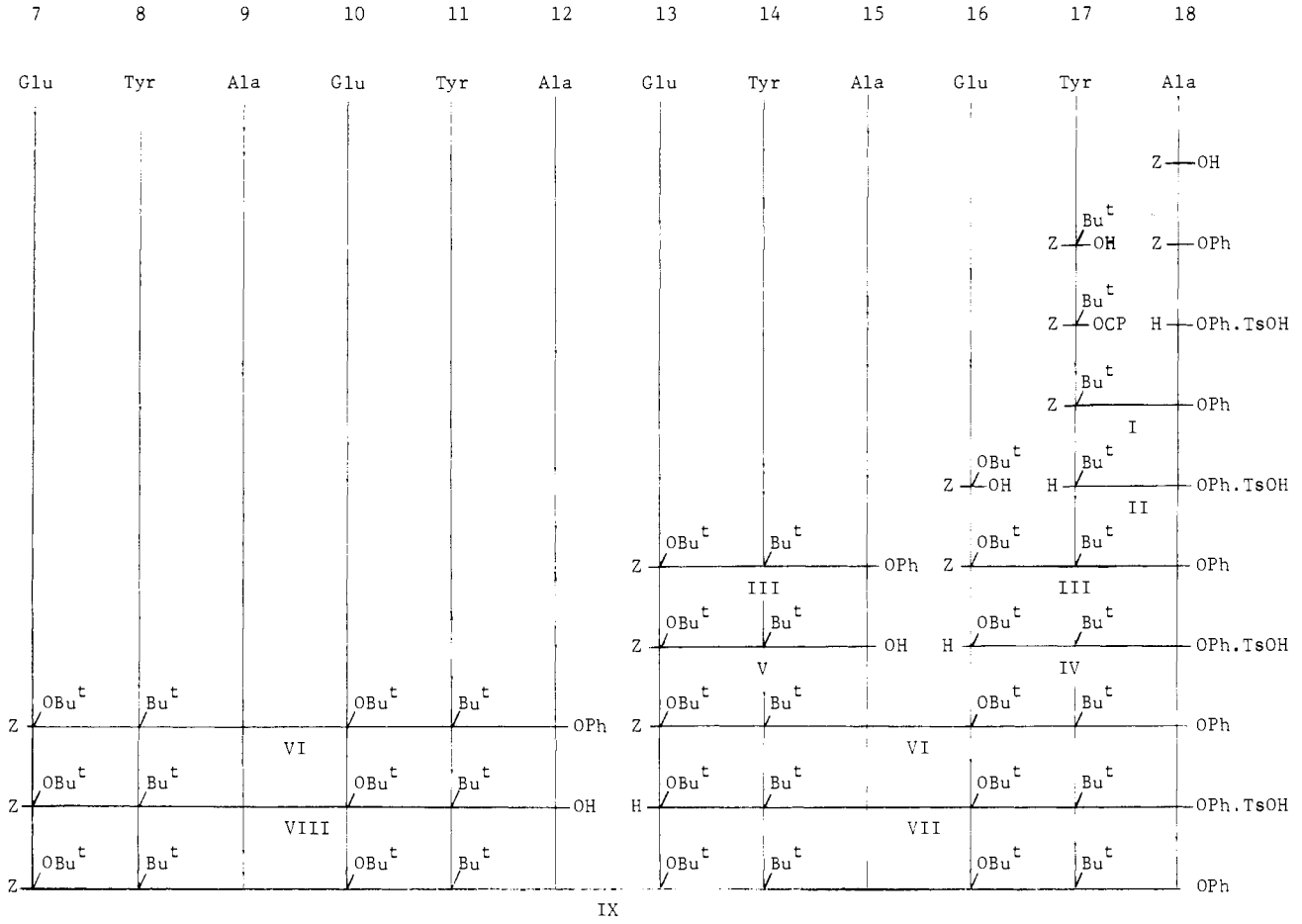
Discussion

The plan for the construction of the synthetic antigens was based on the use of the repeating tripeptide sequence Tyr-Ala-Glu as the α-helical polypeptide backbone. The conformation of this sequential polymer was previously studied by Ramachandran et al.[19] and evidence was obtained by Schechter et al.[14,20,21] for the retention of the α-helical conformation under in vivo conditions. In the present work, a site for the attachment of the antigenic group was incorporated at each 18th position in the primary sequence. It was calculated that this arrangement would give a spatial separation of 27 Å (i.e., 5 turns of helix) between haptenic groups in the α-helical conformation, as shown in Figure 1.

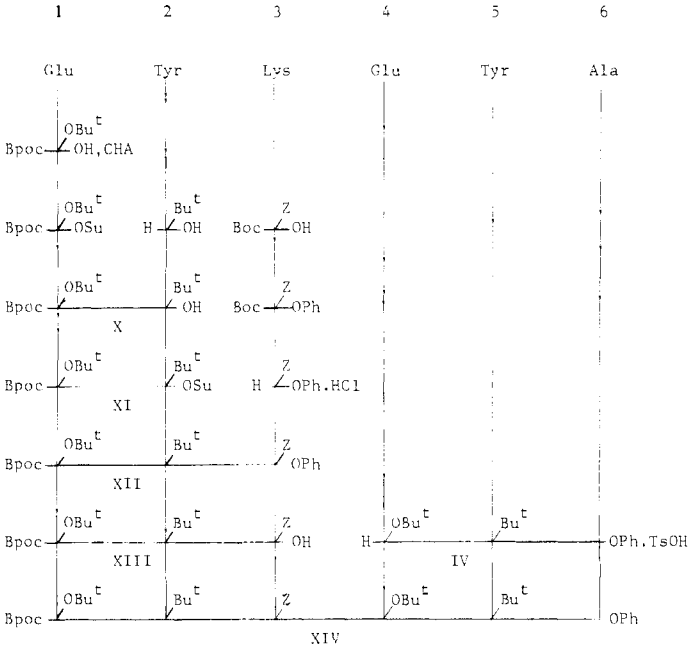
Synthetic Strategy. The initial synthetic objective was a protected 18 amino acid peptide (peptide XVII, Scheme III), a derivative of the sequential polypeptide (Glu-Tyr-Ala)$_6$ in which the alanine residue at position 3 was replaced by lysine. The lysine side chain provided the site for the attachment of the antigenic group after the construction of the high molecular weight polypeptides from XVII. The repeating sequence Glu-Tyr-Ala was chosen, rather than Tyr-Ala-Glu as used by Ramachandran et al.,[19] because this allowed the synthesis to be carried out via a series of fragment condensations involving the sterically favorable C-terminal alanine residue, while not significantly altering the amino acid sequence of the completed polypeptide.

The protected octadecapeptide XVII was obtained by the fragment condensation route shown in Schemes I–III, utilizing the tripeptides III and XII. Glutamic acid and tyrosine side

Scheme I



Scheme II



chains were protected throughout by the use of *tert*-butyl ester and *tert*-butyl ether groups, respectively, and the lysine side chain was protected by the benzyloxycarbonyl group. The 2-(4-biphenylyl)isopropoxycarbonyl group was used for amino-terminal protection of the octadecapeptide and the carboxyl terminus was protected by the phenyl ester function.[22]

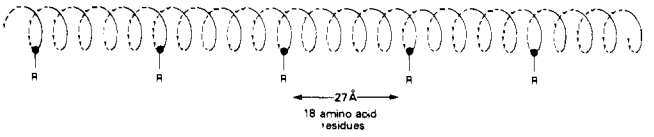


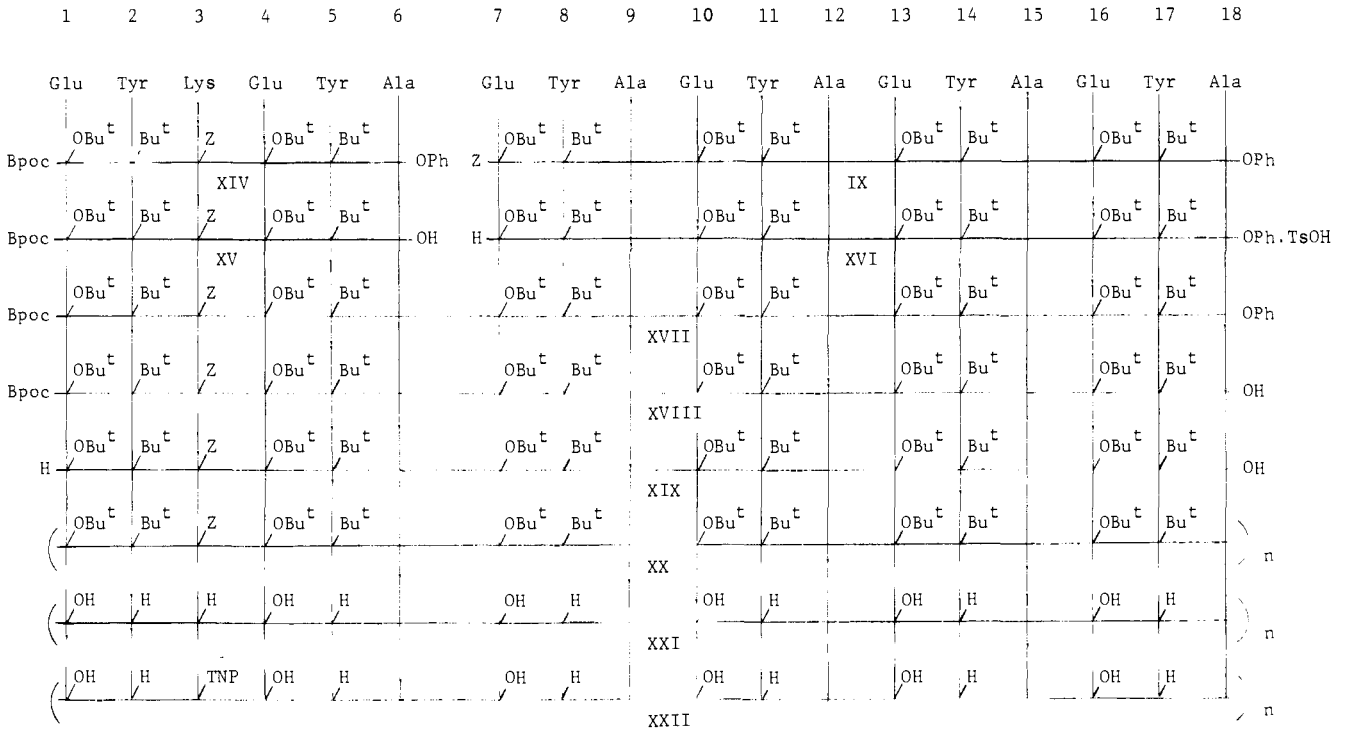
Figure 1. α-Helical polypeptide antigen bearing a haptenic group (R) on each 18th amino acid residue.

This pattern of protection allowed for the selective removal of either the amino-terminal or carboxyl-terminal group under mild conditions and was modelled on that presently in use by Kenner et al.[12] in the synthesis of lysozyme-related proteins. Thus, the route was applicable for the extension of the synthesis by either a polymerization procedure, as described here, or by a fragment condensation scheme using selectively deprotected amino and carboxyl components.

Fragment condensations in Schemes I-III were carried out by the dicyclohexylcarbodiimide–hydroxybenzotriazole method,[23] which is reported to cause little racemization of the C-terminal residue. In order to confirm that racemization levels in the coupling and phenyl ester hydrolysis steps were acceptably low, in our hands, a study was made of the coupling of the model dipeptide *tert*-butyloxycarbonylglycyl-L-alanine with L-leucine phenyl ester. After deprotection, the diastereoisomeric tripeptides were quantitated[24,25] using an amino acid analyzer. The extent of racemization was found to be 0.2% for the complete reaction series, under our standard conditions. It is noteworthy that careful pH control is essential during the cleavage of the phenyl ester group, as considerable racemization (14%) occurred on one occasion when inadequate mixing allowed a momentary increase in pH during this reaction.

During the synthesis of XVII, intermediates were purified

Scheme III



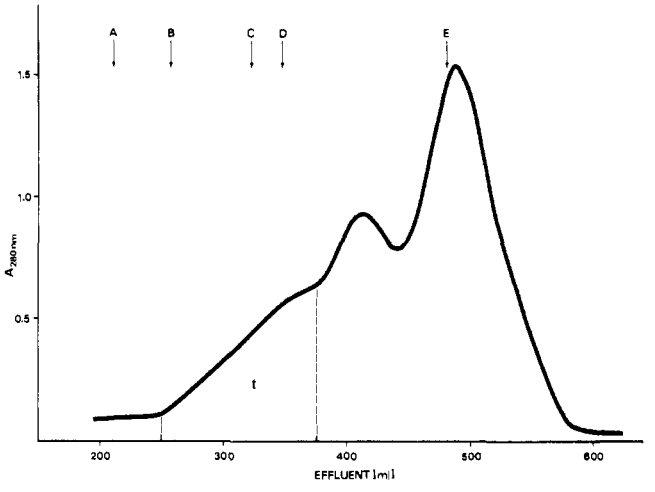


Figure 2. Fractionation of the deprotected polyoctadecapeptide XXI on a calibrated column of Sephadex G-50 (4 × 46 cm) in 0.04 M sodium phosphate (pH 9): (A) bovine serum albumin; (B) chymotrypsinogen; (C) ribonuclease; (D) insulin dimer; (E) octadecapeptide XVII after deprotection.

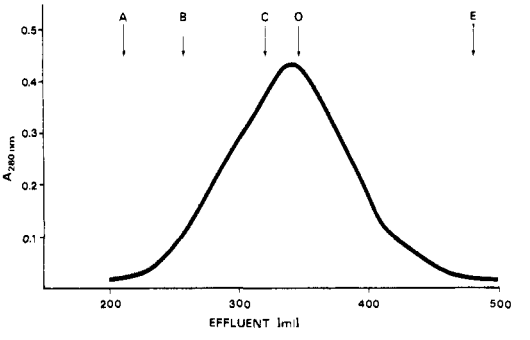


Figure 3. Rechromatography of polyoctadecapeptide XXI (fraction 1; Figure 2) in the system shown in Figure 2.

and characterized at each fully protected stage. In instances where amino acid analysis was of no diagnostic significance (e.g., preparation of dodecapeptide IX, Scheme I), the products were further characterized by chromatography on Sephadex LH-20 and by high-voltage paper electrophoresis or polyacrylamide gel electrophoresis[26] of a totally deprotected sample. The optical homogeneity of the octadecapeptide was confirmed by enzymic digestion of the deprotected peptide, using aminopeptidase M, when complete degradation to the constituent amino acids was obtained.

Polymerization of Octadecapeptide (XVII). The polymerization of the octadecapeptide was carried out by the dicyclohexylcarbodiimide-hydroxybenzotriazole method after removal of the 2-(4-biphenylyl)isopropoxycarbonyl group and the phenyl ester group. Dimethylformamide was used as solvent and the reaction was allowed to proceed for 2 h at 0 °C followed by 10 days at room temperature. The product was treated with hydrogen bromide in acetic acid to remove all side-chain protecting groups and was then fractionated by chromatography in aqueous solution on a column of Sephadex G-50 (Figure 2). The highest molecular weight fractions were pooled and rechromatographed on a column of Sephadex G-50 calibrated according to molecular weight (Figure 3). A symmetrical peak was obtained corresponding to a molecular weight of 11 000 (Figure 4). The relatively low degree of polymerization was attributed to the large size of the peptide monomer. However, the product was of a sufficient size for the purpose of the immunogenicity studies, as described below, and therefore the polymerization reaction was not investigated more extensively. We favor the adoption of a fragment condensation scheme for the controlled synthesis of high molecular polypeptides.

Trinitrophenylation of Polyoctadecapeptide XXI. The final step in the preparation of the polymeric antigen was the attachment of the haptenic group onto the lysine side chains. In the present investigation the 2,4,6-trinitrophenyl function was utilized as the haptenic group but other chemical groupings could be employed equally well, provided a suitable reactive site was available for coupling with the lysine amino function. Attachment of the 2,4,6-trinitrophenyl group was accom-

Table I. Percentage of Radiolabeled TNP-BSA Precipitated by Various Mouse Sera

Antigen	CBA/J $H\text{-}2^k$	CBA/CaJ $H\text{-}2^k$	C3H/HeJ $H\text{-}2^k$	Balb/c $H\text{-}2^d$	DBA/2J $H\text{-}2^d$	C57Bl/6J $H\text{-}2^b$	C3H/SWSN $H\text{-}2^b$
None	2.0	<1.0	3.0	5.0	3.0	2.5	3.0
TNP_{19}-RGG[a]	54.5	54.3	51.7	49.7	49.3	56.5	53.8
TNP-polyoctadecapeptide XXII	<1.0	<1.0	<1.0	35.6	49.7	5.5	7.5

[a] 2,4,6-Trinitrophenylated rabbit γ-globulin prepared by the picroylsulfonic acid method (T. Okuyama and K. Satake, *J. Biochem. (Tokyo)*, **47**, 454 (1960)).

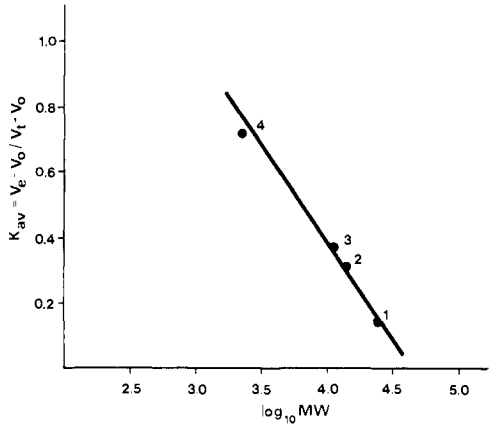


Figure 4. Calibration of Sephadex G-50 column (Figure 2): (1) chymotrypsinogen; (2) ribonuclease; (3) insulin dimer; (4) octadecapeptide XVII after deprotection; V_0, 210 mL; V_t, 578 mL.

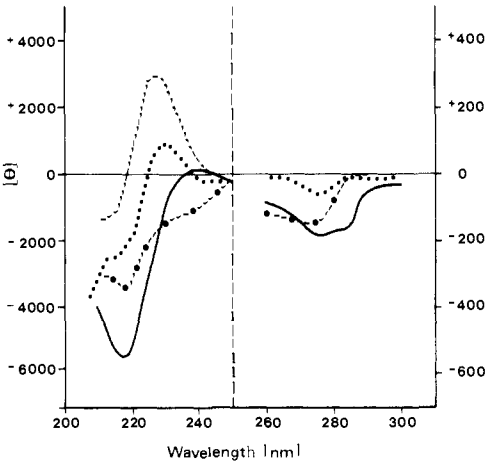


Figure 5. Circular dichroic spectra in 0.15 M sodium chloride-0.02 M sodium phosphate (pH 7.4): (- - -) $(Glu\text{-}Tyr\text{-}Ala)_4$, ··· polyoctadecapeptide before trinitrophenylation; (- · - · -) polyoctadecapeptide after trinitrophenylation; (—) $(Glu\text{-}Tyr\text{-}Ala)_n$. Mean residue ellipticity $[\theta]$ is defined as $M\theta/10cl$, where θ is observed ellipticity in degrees, M is mean residue weight (121), l is path length in cm, and c is concentration in g/mL.

plished by nucleophilic attack of the lysyl ε-amino group on 2,4,6-trinitrobenzenesulfonic acid. The product was characterized by UV absorption at 348 nm, which indicated complete substitution of the lysine residues.

Circular Dichroism Studies. The tertiary structure of the polymeric antigen was investigated by circular dichroism spectroscopy both before and after trinitrophenylation and the spectra were compared with those of the dodecapeptide $(Glu\text{-}Tyr\text{-}Ala)_4$ and the polymerized tripeptide $(Glu\text{-}Tyr\text{-}Ala)_n$, the latter obtained by the method of Ramachandran et al.[19] The spectra were recorded at pH 7.4 in 0.15 M sodium chloride-0.02 M sodium phosphate buffer. As shown in Figure 5, the tripeptide polymer showed a negative ellipticity band at 218 nm ($[\theta]_{max} = 5400$) plus a broad negative band at 275 nm ($[\theta]_{max} = 190$). The spectrum was similar to that reported by Schechter et al.[27] for the closely related polymer $(Tyr\text{-}Ala\text{-}Glu)_n$ (220 nm, $[\theta]_{max} = -8700$; 273 nm, $[\theta]_{max} = -360$), although our ellipticity values were somewhat reduced. Following the interpretation of Schechter et al.,[27] the bands at 218 and 275 nm were attributed to the helical conformation of the polypeptide. In contrast, the spectrum of the dodecapeptide showed only a positive band at 227 nm ($[\theta]_{max} = 2900$), with no evidence of helical structure. A similar positive band at 230 nm ($[\theta]_{max} = 1000$) was observed in the spectrum of the polyoctadecapeptide before trinitrophenylation; however, this spectrum also showed negative bands at 215 nm ($[\theta]_{max} = -2400$) and at 275 nm ($[\theta]_{max} = -46$), indicative of a low level of helix formation. After trinitrophenylation, the positive band at 227 nm disappeared completely and the two negative bands increased in intensity (λ_{max} 218 nm, $[\theta]_{max} = -3400$; λ_{max} 275 nm, $[\theta]_{max} = -135$) indicating an increase in the helical content of the molecule. It is likely that the presence of the positively charged lysine amino groups destabilized the helix in the case of the underivatized polyoctadecapeptide. When the positive charges were removed via trinitrophenylation, the extent of helix formation was significantly increased.

Immunological Response to Antigen XXII. The polymeric antigen XXII was injected into mice of various strains and the generation of antibodies specific for the trinitrophenyl group was measured by radioimmunoassay. As shown in Table I, it was found that mice of strains Balb/c and DBA/2J showed good responses while strains C3H/HeJ, CBA/J, CBA/CaJ, and C57BL/6J failed to respond. The nonresponder strains all possess either the $H\text{-}2^k$ or $H\text{-}2^b$ gene in the histocompatibility complex while both responder strains are of $H\text{-}2^d$ haplotype. Thus, it appears that the capacity to respond to antigen XXII is genetically controlled, with mice of $H\text{-}2^k$ and $H\text{-}2^b$ haplotypes being incapable of mounting a response. All these mouse strains gave a normal response to trinitrophenylated rabbit γ-globulin. Further experiments dealing with the genetics of the response to the synthetic antigen will be reported elsewhere.

Experimental Section

All amino acids were of the L configuration unless otherwise stated. Melting points are uncorrected. Thin-layer chromatograms were run on glass plates precoated with silica gel 60F-254, thickness 0.25 mm (Brinkman), using the following solvent systems: TLC-1, chloroform-methanol (95:5); TLC-2, chloroform-methanol (90:10); TLC-3, 1-butanol-acetic acid-water (3:1:1). Spots were revealed by spraying with ninhydrin or with the chlorine peptide spray.[28] All evaporations were performed under reduced pressure using a Buchi evaporator fitted with a dry ice-acetone condenser. Amino acid analyses were performed using a Beckman amino acid analyzer, Model 120C. Samples for analysis were hydrolyzed for 24 h in vacuo at 110 °C in 6 N hydrochloric acid. Acetyl biphenyl (Aldrich) was converted to biphenylbimethylcarbinol by the method of Mowry et al.;[29] the product was treated with phenyl chloroformate as described by Sieber and Iselin[30] to give the phenyl carbonate which was then converted to the corresponding hydrazide.[30] The product was stored as the hydrazide and was converted to the azide when required for the preparation of 2-(4-biphenylyl)isopropoxycarbonyl (Bpoc) derivatives.[30]

Benzyloxycarbonylalanine Phenyl Ester. Benzyloxycarbonylalan-

ine[31] (129 g; 580 mmol) was dissolved in ethyl acetate (600 mL) and the solution was cooled to −5 °C. Phenol (54.6 g; 580 mmol) and pyridine (46.8 mL; 580 mmol) were added followed by dicyclohexylcarbodiimide (DCC) (131 g; 635 mmol) in ethyl acetate (100 mL). The solution was stirred at 0 °C for 2 h followed by overnight at room temperature. Acetic acid (3.5 mL) was added and after stirring for 1 h at room temperature the reaction mixture was filtered. The filtrate was washed with water and sodium bicarbonate (5%) and finally with water until neutral. The organic phase was dried ($MgSO_4$) and evaporated. Recrystallization from ethyl acetate/petroleum ether gave the product (149 g; 86%) having mp 94–95 °C. Thin-layer chromatography showed a single component (TLC-1, R_f 0.76): $[\alpha]^{25}_D$ −18.8° (*c* 2, chloroform).

Anal. ($C_{17}H_{17}NO_4$) C, H, N.

Alanine Phenyl Ester *p*-Toluenesulfonate. Benzyloxycarbonylalanine phenyl ester (149 g; 0.5 mol) was dissolved in dimethylformamide (DMF) (300 mL) together with *p*-toluenesulfonic acid monohydrate (95.1 g; 0.5 mol) and hydrogenation was carried out at room temperature and atmospheric pressure using 5% palladium-charcoal catalyst (5 g). When evolution of carbon dioxide ceased, the reaction mixture was filtered through Celite and evaporated. Recrystallization from 2-propanol/ether gave the product (116 g; 74%) having mp 151–154 °C. Thin-layer chromatography showed one major spot (TLC-3, R_f 0.64): $[\alpha]^{25}_D$ +7.5° (*c* 1.74, H_2O), −4.3° (*c* 2.22, DMF).

Anal. ($C_{16}H_{19}O_5NS$) C, H, N, S.

N^{ϵ}-Benzyloxycarbonyllysine Phenyl Ester Hydrochloride. N^{α}-*tert*-Butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyllysine (8 g; 21 mmol) was dissolved in ethyl acetate (30 mL) and cooled in an ice-salt bath. Phenol (1.9 g; 21 mmol) and pyridine (1.7 mL; 21 mmol) were added followed by DCC. The reaction mixture was stirred for 2 h at 0 °C and for a further 20 h at room temperature. The mixture was filtered and the filtrate was washed with 10% citric acid, aqueous sodium bicarbonate, and water, dried ($MgSO_4$), and evaporated to give N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyllysine phenyl ester, as an oil. The ester was treated with a trifluoroacetic acid (F_3AcOH)–water mixture (9:1; 20 mL), under an atmosphere of N_2, for 1 h at room temperature. The solution was then evaporated and the residue was dissolved in ethyl acetate (30 mL). A solution of HCl in ethyl acetate (12 mL; 4 N) was added and the mixture was evaporated. Addition of ether gave the crystalline N^{ϵ}-benzyloxycarbonyllysine phenyl ester hydrochloride (6.1 g; 74%), having mp 126–128 °C. Thin-layer chromatography showed a single spot (TLC-2, R_f 0.35): $[\alpha]^{25}_D$ +21.0° (*c* 1.58, 2-propanol).

Anal. ($C_{20}H_{25}O_4N_2Cl$) C, H, N, Cl.

Leucine Phenyl Ester Hydrochloride. N^{α}-*tert*-Butyloxycarbonylleucine (2.1 g; 9.2 mmol) in ethyl acetate (10 mL) was treated with phenol (880 mg; 9.3 mmol), pyridine (742 mg; 9.3 mmol), and DCC (2.0 g; 9.7 mmol) as in the foregoing experiment. Crystallization from petroleum ether gave the phenyl ester (1.99 g; 65%), having mp 74 °C. A portion of the product (700 mg; 2.1 mmol) was treated with hydrogen chloride in acetic acid (1 N; 3 mL) for 15 min at room temperature. Evaporation and washing with ether gave leucine phenyl ester hydrochloride (520 mg; 99%): mp 190 °C; $[\alpha]^{25}_D$ +22.0° (*c* 2.03, 2-propanol).

Anal. ($C_{12}H_{18}O_2NCl$) C, H, N.

Extent of Racemization Occurring during Coupling and Phenyl Ester Hydrolysis. N^{α}-*tert*-Butyloxycarbonylglycylalanine[32] (104 mg; 0.42 mmol) was dissolved in DMF (4 mL) together with leucine phenyl ester hydrochloride (105 mg; 0.42 mmol), 1-hydroxybenzotriazole monohydrate (114 mg; 0.84 mmol), *N*-methylmorpholine (50 μL; 0.45 mmol), and DCC (101 mg; 0.49 mmol) were added and the mixture was stirred for 2 h at 4 °C followed by 40 h at room temperature. The crude product was obtained, as described for peptide VI, and the phenyl ester group was removed at pH 10.5 as described for peptide V. Final deprotection was accomplished by treatment with an anhydrous solution of hydrogen chloride in acetic acid (1 N) for 15 min at room temperature. The product was dissolved in sodium citrate buffer (pH 2.2; 0.2 N) and an aliquot (3.6 μmol) was chromatographed at pH 4.30 using a Beckman amino acid analyzer, as described by Stewart.[25] The major product, Gly-L-Ala-L-Leu (elution volume 104 mL), was separated from the racemization products, Gly-D-Ala-L-Leu and Gly-L-Ala-D-leu which eluted together at 125 mL. The extent of racemization was 0.2%.

Z-Tyr(Bu^t)-Ala-OPh (I). N^{α}-Benzyloxycarbonyl-*O*-*tert*-butyltyrosine[33] (118 mmol) in ethyl acetate (200 mL) was treated with 2,4,5-trichlorophenol (24.7 g; 125 mmol) and DCC (24.5 g; 119 mmol) for 2 h at 0 °C, followed by 20 h at room temperature. After filtration, the solution was evaporated and the ester was crystallized (52.6 g; 80%) from cyclohexane, mp 95–97 °C. The product (52.6 g; 95.7 mmol) was dissolved in DMF (200 mL) and alanine phenyl ester *p*-toluenesulfonate (29.8 g; 93.8 mmol) was added, followed by *N*-methylmorpholine (10.3 mL; 93.8 mmol). The mixture was stirred overnight at room temperature, and was then evaporated and dissolved in ethyl acetate (300 mL). The solution was washed with water, dried ($MgSO_4$), and evaporated. Crystallization from ethyl acetate–ether gave the dipeptide (37.5 g; 77%) having mp 128 °C; $[\alpha]^{25}_D$ −20.39° (*c* 2.6, chloroform). Thin-layer chromatography (TLC-1) showed a single spot, R_f 0.68.

Anal. ($C_{30}H_{34}O_6N_2$) C, H, N.

TosOH·H-Tyr(Bu^t)-Ala-OPh (II). The protected dipeptide I (37.5 g; 72 mmol) was dissolved in DMF (200 mL) together with *p*-toluenesulfonic acid monohydrate (13.7 g; 72 mmol) and hydrogenation was carried out at room temperature and atmospheric pressure using 5% palladium-charcoal catalyst (7 g). When evolution of carbon dioxide ceased, the reaction mixture was filtered through Celite and evaporated. The product (39.6 g; 99%) crystallized on addition of ether and was used directly in the next stage without further characterization.

Z-Glu(OBu^t)-Tyr(Bu^t)-Ala-OPh (III). γ-*tert*-Butyl benzyloxycarbonylglutamate dicyclohexylammonium salt[34] (37.3 g; 72 mmol) was suspended in ethyl acetate (500 mL) and 1 N HCl (75 mL) was added at 4 °C. The mixture was shaken for 70 min and the organic phase was separated. The aqueous phase was washed with ethyl acetate and all ethyl acetate extracts were then combined, dried ($MgSO_4$), and evaporated. The product was dissolved in methylene chloride (350 mL), together with peptide II (39.6 g; 71 mmol), and the mixture was cooled to 0 °C. DCC (147 g; 71 mmol) and *N*-methylmorpholine (78 mL; 71 mmol) were added and the mixture was stirred for 2 h at 0 °C, followed by overnight at room temperature. The reaction mixture was filtered and evaporated to give a residue which was dissolved in ethyl acetate. The solution was washed with 10% citric acid, aqueous sodium bicarbonate and water, dried ($MgSO_4$), and evaporated. Trituration of the residue with ethyl acetate–petroleum ether gave a solid which was dissolved in ethyl acetate, filtered through Celite, and evaporated to small volume. Addition of petroleum ether gave the crystalline product (44.6 g; 89%) having mp 130–132 °C. $[\alpha]^{25}_D$ +11.26 (*c* 2.95, chloroform). Thin-layer chromatography (TLC-1) showed a single spot, R_f 0.49. Amino acid analysis: Glu, 1.00, Tyr 1.00, Ala, 0.99.

Anal. ($C_{39}H_{49}O_9N_3$) C, H, N.

TosOH·H-Glu(OBu^t)-Tyr(Bu^t)-Ala-OPh (IV). The protected tripeptide III (18.6 g; 26.5 mmol) was dissolved in DMF (150 mL) together with *p*-toluenesulfonic acid monohydrate (5 g; 26.5 mmol). Hydrogenation was carried out as described for I above, yielding a crystalline product which was used directly in the syntheses of hexapeptides VI and XIV.

Z-Glu(OBu^t)-Tyr(Bu^t)-Ala-OH (V). The protected tripeptide phenyl ester III (18.6 g; 26.5 mmol) was dissolved in DMF (90 mL) and water (20 mL) was added. A trace of hydrogen peroxide was added and the solution was titrated at pH 10.5 by addition of 1 N sodium hydroxide solution using a pH stat. Uptake of base was complete in 5 min. The solution was then brought to pH 7 by addition of 10% citric acid and was evaporated to remove DMF. The residue was diluted with water and acidified to pH 3 using 10% citric acid. The solution was extracted with ethyl acetate and the extracts were washed with water, dried ($MgSO_4$), and evaporated. The residue was washed with warm water and with petroleum ether to give a glasslike solid (18 g) which was used directly in the synthesis of the hexapeptide VI.

Z-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_2$-OPh (VI). The protected tripeptide acid V (26.5 mmol) and the tripeptide hydrogenation product IV (26.5 mmol) were combined and dissolved in DMF (200 mL). 1-Hydroxybenzotriazole monohydrate (7.2 g; 53 mmol), *N*-methylmorpholine (2.9 mL; 26.5 mmol), and DCC (6.38 g; 30.9 mmol) were added and the mixture was stirred overnight at 4 °C, and then for 48 h at room temperature. The solution was filtered and the filtrate was evaporated. The residue was dissolved in ethyl acetate and washed with 5% citric acid, 5% sodium bicarbonate, and water. The organic phase was dried ($MgSO_4$) and evaporated to small volume. Addition of petroleum ether gave a solid which was filtered and washed with petroleum ether (29 g; 94%). After reprecipitation from ethyl acetate–petroleum ether, the product (21 g; 67%) showed one main spot on thin-layer chromatography (TLC-1), R_f 0.47, together with a minor impurity. It was

found that the impurity was more readily removed at the dodecapeptide stage; therefore the peptide was not further characterized at this point. Amino acid analysis: Glu, 1.08; Tyr, 0.92; Ala, 0.99.

TosOH·H-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_2$-OPh (VII). The protected hexapeptide VI (10.1 g; 9 mmol) was dissolved in DMF (175 mL) together with *p*-toluenesulfonic acid monohydrate (1.71 g; 9 mmol) and hydrogenation was carried out as described for I above, yielding a white solid product (9.87 g; 90%) which was used directly in the preparation of the dodecapeptide IX.

Z-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_2$-OH (VIII). The protected hexapeptide phenyl ester VI (10.1 g; 9 mmol) was dissolved in DMF/water (180 mL, 4:1) and hydrolysis was carried out at pH 10.5 as described for peptide V. The product was used directly in the preparation of dodecapeptide IX.

Z-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_4$-OPh (IX). The protected hexapeptide acid VIII (8.93 g; 8.1 mmol) and the hexapeptide hydrogenation product VII (9.85 g; 8.1 mmol) were combined, dissolved in DMF, and cooled to −20 °C. 1-Hydroxybenzotriazole monohydrate (2.14 g; 16.2 mmol), DCC (1.84 g; 16.2 mmol), and *N*-methylmorpholine (0.89 mL; 8.1 mmol) were added and the mixture was stirred at 0 °C for 6 h and at room temperature for a further 70 h. The reaction mixture was filtered and the gelatinous precipitate was washed with warm DMF, to remove dicyclohexylurea. The product (9.87 g; 56%) showed one spot on thin-layer chromatography (TLC-1, R_f 0.25; TLC-2, R_f 0.57) and gave a single symmetrical peak on LH-20 Sephadex chromatography (V_e/V_t = 0.36, DMF): amino acid analysis, Glu, 1.01; Tyr, 0.93; Ala, 0.98; $[\alpha]^{25}_D$ −12.4° (*c* 1.63, hexamethylphosphoramide).

Anal. ($C_{114}H_{160}O_{27}N_{12}$) C, H, N.

The product was further characterized by electrophoretic examination of a totally deprotected sample, as described below.

Bpoc-Glu(OBu^t)-Tyr(Bu^t)-OH (X). γ-*tert*-Butyl 2-(4-biphenylyl)-isopropoxycarbonylglutamic acid cyclohexylammonium salt[30] (37.5 g; 68.5 mmol) was suspended in ethyl acetate and shaken with 10% citric acid. The organic layer was washed with water, dried ($MgSO_4$), and evaporated. The residue was dissolved in a mixture of dioxane and ethyl acetate (150 mL; 1/1). The solution was cooled to 0 °C and *N*-hydroxysuccinimide (7.9 g; 68.5 mmol) was added, followed by DCC (15.5 g; 75 mmol). The mixture was stirred overnight at 4 °C and was then filtered, and evaporated in vacuo. The residue was redissolved in ethyl acetate, filtered, washed with water, dried ($MgSO_4$), and evaporated in vacuo to give the hydroxysuccinimide ester. The residue was dissolved in DMF (240 mL) and tyrosine-*tert*-butyl ether (17.9 g; 75.5 mmol) was added followed by *N*-methylmorpholine (7.5 mL; 68.5 mmol) and water (90 mL). The reaction mixture was stirred for 2 days at room temperature, filtered, and evaporated. The residue was dissolved in ethyl acetate and washed with 10% citric acid and water, dried ($MgSO_4$), and evaporated to a foam (44 g; 97%): amino acid analysis: Glu, 1.03; Tyr, 0.96; thin-layer chromatography (TLC-1), R_f 0.45; $[\alpha]^{25}_D$ +17.3° (*c* 1.44, chloroform).

Anal. ($C_{38}H_{48}O_8N_2$) C, H, N.

Bpoc-Glu(OBu^t)-Tyr-(Bu^t)-OSu (XI). The protected dipeptide acid X (44 g; 67 mmol) was dissolved in ethyl acetate–dioxane (280 mL 1:1) and *N*-hydroxysuccinimide (14 g; 122 mmol) was added. The mixture was cooled in ice and DCC (15.4 g; 73 mmol) was added. The mixture was stirred at 4 °C for 3 days, followed by 3 h at room temperature, and was then filtered and evaporated. The residue was dissolved in ethyl acetate and the solution was washed with water, dried ($MgSO_4$), and evaporated to give a crystalline product which was removed by filtration and washed with ethyl acetate–petroleum ether (1:1). The product (33 g; 63%; mp 112–115 °C) showed a single spot on thin-layer chromatography (TLC-2, R_f 0.69).

Bpoc-Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-OPh (XII). The dipeptide active ester XI (6.86 g; 9.06 mmol) was dissolved in DMF (50 mL) and N^ϵ-benzyloxycarbonyllysine phenyl ester hydrochloride (3.6 g; 9.06 mmol) was added followed by triethylamine (1.3 mL; 9.5 mmol). The reaction mixture was stirred for 2 days at room temperature and was then evaporated. The residue was dissolved in ethyl acetate and the solution was washed with 10% citric acid, aqueous sodium bicarbonate, and water, dried ($MgSO_4$), and evaporated. The product solidified on addition of ether: yield 8.0 g; 88%; mp 112 °C, thin-layer chromatography (TLC-2) showed one major spot, R_f 0.66; amino acid analysis: Glu, 1.01; Tyr, 0.96; Lys, 1.03; $[\alpha]^{25}_D$ −14.8° (*c* 2.37, chloroform).

Anal. ($C_{58}H_{70}O_{11}N_4$) C, H, N.

Bpoc-Glu(OBu^t)-Tyr-(Bu^t)-Lys(Z)-OH (XIII). The protected tripeptide XII (2.9 g; 2.9 mmol) was dissolved in DMF (8 mL) plus water (2 mL) and hydrolysis was carried out at pH 10.5 as described for V above. The product was used directly in the preparation of hexapeptide XIV.

Bpoc-Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-Glu(OBu^t)-Tyr(Bu^t)Ala-OPh (XIV). The protected tripeptide acid XIII (2.9 mmol) was dissolved in DMF (25 mL) and the solution was cooled to −15 °C. The tripeptide *p*-toluenesulfonic acid salt (IV) (2.15 g; 2.9 mmol) was added followed by 1-hydroxybenzotriazole monohydrate (1.0 g; 6.0 mmol), DCC (0.75 g; 3.6 mmol), and triethylamine (0.4 mL; 2.9 mmol). The reaction mixture was stirred for 2 h at 0 °C, followed by 48 h at room temperature. The mixture was then filtered and the filtrate was evaporated. The residue was dissolved in ethyl acetate and refiltered to remove dicyclohexylurea. The solution was washed with 5% citric acid, saturated NaCl solution, and water, dried ($MgSO_4$), and evaporated. The product precipitated on addition of ether: yield, 3 g, 70%; thin-layer chromatography (TLC-2) showed one major spot, R_f 0.64; amino acid analysis: Glu, 2.00; Tyr, 1.82; Lys, 1.03; Ala, 0.97; $[\alpha]^{D}_{25}$ −10.8° (*c* 2.00, hexamethylphosphoramide).

Anal. ($C_{83}H_{107}O_{17}N_7$) C, H, N.

Bpoc-Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-Glu(OBu^t)-Tyr(Bu^t)-Ala-OH (XV). The protected hexapeptide XIV (500 mg; 0.32 mmol) was dissolved in DMF (21 mL) plus water (3 mL) and hydrolysis was carried out at pH 10.5 as described for V above. The product was used directly in the preparation of the octadecapeptide XVII.

TosOH·H-[Gly(OBu^t)-Tyr(Bu^t)-Ala]$_4$-OPh (XVI). The protected dodecapeptide IX (1.29 g; 0.6 mmol) was dissolved in DMF (235 mL) together with *p*-toluenesulfonic acid monohydrate (114 mg; 0.6 mmol) and hydrogenation was carried out as described for I above. After filtration the solution was evaporated to a volume of 100 mL. Thin-layer chromatography (TLC-2) showed a single spot, R_f 0.29, with no trace of starting material. The dissolved product was used directly in the next stage without further characterization.

Bpoc-Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_5$-OPh (XVII). The protected hexapeptide acid XV (960 mg; 0.69 mmol) was dissolved in DMF (5 mL) and the dodecapeptide *p*-toluenesulfonic acid salt (XVI) (0.6 mmol) in DMF (100 mL) was added. The mixture was cooled to 0 °C and 1-hydroxybenzotriazole monohydrate (185 mg; 1.37 mmol), DCC (282 mg; 1.37 mmol), and *N*-methylmorpholine (66 μL; 0.6 mmol) were added. The reaction mixture was stirred for 2 h at 0 °C, followed by 10 days at room temperature, and evaporated. The residue was dissolved in hexamethylphosphormaide (68 mL) and DMF was added to a total volume of 120 mL. A portion (12 mL) of the solution was utilized in investigations of purification procedures. The remainder (108 mL) was purified in two batches by chromatography on a column (9.5 × 77 cm) of LH-20 Sephadex in DMF (fraction size 21 mL; fraction time 12 min) monitored by UV absorption at 280 nm. Fractions comprising the first peak (fractions 87-95; V_e/V_t = 0.34) were pooled and evaporated to yield the octadecapeptide (0.36 mmol; 67%) having the following amino acid analysis: Glu, 6.1; Ala, 4.9; Tyr, 5.4; Lys, 1.0; $[\alpha]^{25}_D$ −6.07° (*c* 1.96, hexamethylphosphoramide). Thin-layer chromatography (TLC-1) showed a single spot, R_f 0.36. The product was further characterized by polyacrylamide gel electrophoresis, Sephadex G-25 chromatography, and enzymic digestion, as described below, after removal of protecting groups.

Bpoc-Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_5$-OH (XVIII). The protected octadecapeptide XVII (735 mg; 218 μmol) was dissolved in hexamethylphosphoramide (10 mL) plus water (1.5 mL) and hydrolysis was carried out at pH 10.5 as described for V above. The product was used directly in the next stage.

HCl·H-Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-[Glu(OBu^t)-Tyr(Bu^t)-Ala]$_5$-OH (XIX). The protected octadecapeptide acid XVIII (813 mg; 246 μmol) was dissolved in trifluoroethanol (4.5 mL), hydrogen chloride in ethyl acetate (4 N; 0.5 mL) was added, and the reaction mixture was stirred for 30 min at room temperature. The precipitated product was collected by filtration and washed with ether: yield, 584 mg, 76%. The product was used directly in the preparation of the polyoctadecapeptide XX.

[Glu(OBu^t)-Tyr(Bu^t)-Lys(Z)-Glu(OBu^t)-Tyr(Bu^t)-Ala-Glu-(OBu^t)-Tyr(Bu^t)-Ala-Glu(OBu^t)-Tyr(Bu^t)-Ala-Glu(OBu^t)-Tyr(Bu^t)-Ala]$_n$ (XX). The octadecapeptide hydrochloride XIX (584 mg; 188 μmol) was dissolved in hexamethylphosphoramide (5 mL). The solution was cooled to 0 °C and 1-hydroxybenzotriazole monohydrate (60 mg; 376 μmol), DCC (80 mg; 376 μmol), and *N*-methylmorpholine (50 μL; 376 μmol) were added. The

reaction mixture was stirred for 2 h at 0 °C followed by 4 days at room temperature. 1-Hydroxybenzotriazole monohydrate, DCC, and *N*-methylmorpholine were then added in the same amounts as above and the reaction was allowed to continue for a further 6 days at room temperature. The reaction mixture was then poured into ice-water and the supernatant was acidified to pH 3 in order to convert the excess DCC to the urea. The precipitate was collected by centrifugation and was washed with water, dried, and washed with ether: yield, 740 mg (containing dicyclohexylurea). The product was converted to the fully deprotected polymer (XXI) before characterization.

[Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$]$_n$ (XXI). The partially protected polymer XX (from 188 μmol of octadecapeptide) was dissolved in trifluoroacetic acid (80 mL) containing anisole (8 mL). The flask was flushed with nitrogen for 10 min and anhydrous hydrogen bromide was then passed into the reaction mixture for 20 min at room temperature. The solution was evaporated and the residue was washed with ether: yield, 676 mg, including dicyclohexylurea from a previous reaction. The product was fractionated by chromatography in batches (100 mg) on a column (4 × 46 cm) of Sephadex G-50 in 0.04 M sodium phosphate (pH 9) (fraction size 5.2 mL; fraction time 10 min). For each chromatogram, the sample was dissolved in the phosphate buffer (5 mL) and filtered to remove dicyclohexylurea before application to the column. Fractions comprising the first peak (V_e/V_t = 0.595, Figure 2) were pooled, evaporated to small volume, and acidified to pH 3 by addition 1 N hydrochloric acid. The product was obtained as a precipitate which was collected by centrifugation, washed with water, and dried: yield, 84 mg; amino acid analysis: Glu, 6.32; Tyr, 5.80; Ala, 4.90; Lys, 1.0. Chromatography on a calibrated column of Sephadex G-50 (Figures 3 and 4) indicated an average molecular weight of 11 000.

[Glu-Tyr-Lys(TNP)-(Glu-Tyr-Ala)$_5$]$_n$ (XXII). Poly[Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$] (XXI) (6.6 mg) was dissolved in water (3 mL) containing sodium bicarbonate (15 mg) and sodium chloride (27 mg). 2,4,6-Trinitrobenzenesulfonic acid (8.5 mg) was added and the solution was stirred overnight at room temperature. The solution was applied to a Sephadex G-10 column (1 × 30 cm) in 0.04 M sodium phosphate (pH 7.4) and the trinitrophenylated (TNP) product was eluted with the same buffer. The TNP derivative was precipitated at pH 2.5 and collected by centrifugation. Measurement of absorption at 348 nm indicated a substitution ratio of 1.2 TNP groups per octadecapeptide unit (molar extinction of TNP-Lys, 15 400; λ_{max} 348 nm, pH 7.4).

Deprotection of Peptides for Electrophoresis. (1) Octadecapeptide XVII. The peptide (8.8 μmol) was dissolved in hexamethylphosphoramide (1 mL) plus DMF (1 mL). Water (100 μL) was added followed by 0.1 N sodium hydroxide (100 μL) and 30% hydrogen peroxide (25 μL). After 2 h at room temperature 5% citric acid (1 mL) was added and the precipitate was filtered, washed with water, and dried in vacuo (P_2O_5). The product was dissolved in a F_3AcOH-water-anisole mixture (9:1:2, 2 mL), the reaction mixture was kept 3 h at room temperature and evaporated, and the residue was washed with ether. The product, which retained the benzyloxycarbonyl group, was examined by paper electrophoresis and polyacrylamide gel electrophoresis.

(2) Dodecapeptide IX. The peptide (100 mg; 47 μmol) was treated with base to remove the phenyl ester as described for XVII above. The product was dissolved in DMF (5 mL) containing 5% palladium-charcoal catalyst (30 mg) and hydrogen gas was bubbled through the suspension for 20 h at room temperature. The catalyst was removed by filtration (Celite) and the solution was evaporated. The residue was dissolved in F_3AcOH-water-anisole mixture (9:1:2) and after 2 h at room temperature the solution was evaporated. The residue was washed with ether, dried, and examined by paper electrophoresis.

(3) Hexapeptide VIII. The hexapeptide acid VIII was hydrogenated as described for IX above, using a mixture of DMF plus methanol (1:1) as solvent. The product was treated with a F_3AcOH-water-anisole mixture, as described for IX, to remove *tert*-butyl ether and *tert*-butyl ester protecting groups and was examined by paper electrophoresis.

High-Voltage Paper Electrophoresis. The deprotected samples of peptides VIII, IX, and XVII were applied to Whatman No. 1 paper in 15% acetic acid (pH 2.1) and electrophoresis (4000 V; 40 mA; 30 min) was carried out at room temperature in the same buffer. The paper was dried and spots were detected with ninhydrin and with the chlorine peptide spray.[28] Mobilities relative to glutamic acid were as follows: hexapeptide, single spot, 1.0; dodecapeptide, single spot, 0.65; octadecapeptide, single spot at origin.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out using a polyacrylamide slab (20 × 20 × 0.2 cm; acrylamide concentration, 12.5%; ratio of acrylamide to methylenebis(acrylamide), 10:1) containing sodium dodecyl sulfate and urea. The gel was prepared as described by Weber and Osborn[26] with the exception that the pH of the gel and reservoir buffer was adjusted to 7.2. After polymerization of the gel the samples were injected into holes punched in the gel surface and a current of 45 mA (200 V) was applied for 5 h with cooling to 10 °C, using a Desaga/Brinkman thin-layer electrophoresis double chamber. The sample spots were identified both by transference to a print paper and by precipitation on the gel (12% trichloroacetic acid; 1 h) followed by staining with a 0.25% solution of Coomassie blue in water. The excess stain was removed by washing the gel overnight with 3% acetic acid. The print paper was developed by spraying with the Pauly reagent.[35] The deprotected octadecapeptide showed a single spot which was positive to both the Coomassie blue stain and the Pauly reagent (mobility relative to bromophenol blue, 0.70).

Deprotection of Octadecapeptide XVII for Sephadex G-25 Chromatography and Enzymic Digestion. The protected peptide XVII (8.8 μmol) was treated with base to remove the phenyl ester, as described above. The remaining protecting groups, including the benzyloxycarbonyl function, were then removed by treatment of a solution of the peptide, in acetic acid (2 mL) containing anisole (0.2 mL), with anhydrous hydrogen bromide gas for 20 min at room temperature. The solution was evaporated and the residue was dissolved in acetic acid-water (2:1) and reevaporated. The product was washed with ether, dried, and examined by Sephadex G-25 chromatography.

Gel Chromatography of the Deprotected Octadecapeptide. A fully deprotected sample of peptide XVII (7 mg; 3 μmol) was dissolved in ammonium acetate buffer (0.05 M; pH 8.0; 1 mL) and was applied to a column of Sephadex G-25 (92 × 3.8 cm) prepared in the same buffer. The column was eluted with the same buffer at a flow rate of 45 mL/h and the effluent was monitored by absorption at 280 nm using a Pharmacia Duomonitor, Model 200. A single symmetrical peak was obtained having an elution volume of 546 mL. Samples of bovine serum albumin and tyrosine, chromatographed on the same column, eluted at 397 and 1004 mL, respectively.

Enzymic Digestion of the Deprotected Octadecapeptide Using Aminopeptidase M. A fully deprotected sample of peptide XVII (0.22 mg) was dissolved in ammonium bicarbonate buffer (0.2 M; 0.2 mL). Aminopeptidase M (Sigma; 0.74 mg) was diluted with water (1 mL) plus manganous chloride (0.025 M; 0.1 mL). The peptide solution was incubated at 37 °C with addition of aliquots (0.1 mL) of the enzyme solution at times 0, 1, 2, 3.5, and 5 h. The mixture was kept overnight at 37 °C and then was acidified with glacial acetic acid, evaporated, and examined by amino acid analysis: found, Glu, 6.1; Tyr, 5.9; Ala, 5.1; Lys, 1.0; yield, 0.0935 μmol of octadecapeptide. An identical yield was obtained by acid hydrolysis, indicating that enzymic digestion proceeded to completion.

Circular Dichroic Spectra. The deprotected sample of dodecapeptide XVI was dissolved in a 0.15 M NaCl-0.02 M sodium phosphate (pH 7.4) at a concentration of 0.325 mg/mL. The deprotected polyoctadecapeptide XXI, the trinitrophenylated polyoctadecapeptide XXII, and the polytripeptide (Glu-Tyr-Ala)$_n$[19] were dissolved in the same buffer at concentrations of 0.365, 0.250, and 0.290 mg/mL, respectively. Circular dichroic spectra (Figure 5) were measured in the wavelength range 200-300 nm using a Cary 60 spectropolarimeter equipped with a Model 60001 CD attachment.

Immune Response to [Glu-Tyr-Lys(TNP)-(Glu-Tyr-Ala)$_5$]$_n$ in Various Mouse Strains. (i) Immunization. Mice of strains CBA/J, CBA/CaJ, C3H/HeJ, Balb/c, DBA/2J, and C57Bl/6J (animals aged 10-12 weeks; 5 mice per group) were injected in the right hind foot pad with an emulsion containing the antigen (50 μg) in saline (25 μL) plus Freund's complete adjuvant (25 μL; Difco Laboratories). After 10 days the mice were injected in the left hind foot pad with an emulsion containing the same antigen (50 μg) in saline (25 μL) plus Freund's incomplete adjuvant (25 μL). One week after the second injection the mice were bled from the retro-orbital plexus. The blood was allowed to clot at room temperature for 1 h and then overnight at 4 °C. Sera were collected and antibody levels were determined by radioimmunoassay.

(ii) Preparation of ^{125}I-Labeled 2,4,6-Trinitrophenolated Bovine Serum Albumin (TNP-BSA). Bovine serum albumin was treated with trinitrobenzenesulfonic acid according to the method of Okuyama

et al.[36] to give TNP-BSA. The product (100 μg) was dissolved in 0.1 M sodium phosphate (pH 7.4) (1 mL) and carrier-free $Na^{125}I$ (1 mCi) was added followed by chloramine T (250 μg) as described by Greenwood et al.[37] The solution was thoroughly mixed and after 3 min sodium metabisulfite (625 μg) was added to terminate the reaction and the solution was dialyzed against saline.

(iii) **Measurement of Antibody Levels by Radioimmunoassay.** ^{125}I-Labeled TNP-BSA (20 μL in saline; 30 000 cpm) was added to the mouse serum (20 μL) and the mixture was diluted with normal rabbit serum (0.5 mL) and 0.2 M Tris buffer (pH 7.4) (0.5 mL). The solution was stirred and was left at 4 °C overnight and then 7.5% poly(ethylene glycol)-6000 (1 mL) in 0.2 M Tris buffer (pH 7.4) was added, as described by Desbuquois and Aurbach.[38] The mixture was centrifuged at 2000 rpm for 45 min and the supernatant and precipitate were separated. The precipitate was washed once with 0.2 M Tris buffer (pH 7.4) (1 mL) and the washings were added to the supernatant. The activity (cpm) in the precipitate and supernatant was determined using a Packard γ-counter (Model 3002). The percentage of activity precipitated gave a measure of the anti-TNP antibody level in the serum. The results for the various mouse strains are given in Table I.

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References and Notes

(1) This investigation was supported by the Medical Research Council of Canada (Grant No. MA-4638).
(2) K. Landsteiner, "The Specificity of Serological Reactions", Harvard University Press, Cambridge, Mass., 1945.
(3) (a) N. A. Mitchison, *Cold Spring Harbor Symp. Quant. Biol.*, **32**, 431 (1967); (b) N. A. Mitchison, K. Rajewsky, and R. B. Taylor, *Czech. Acad. Sci.*, 547 (1970).
(4) D. H. Katz and B. Benacerraf, *Adv. Immunol.*, **15**, 1 (1972).
(5) M. Sela, *Science*, **166**, 1365 (1969).
(6) For a review on polymeric antigens, cf. E. Rüde, *Angew. Chem., Int. Ed. Engl.*, **9**, 206 (1970).
(7) M. Sela, *Adv. Immunol.*, **5**, 29 (1966).
(8) B. Benacerraf and H. O. McDevitt, *Science*, **175**, 273 (1972).
(9) B. Benacerraf and M. Dorf, in "The Role of Products of the Histocompatibility Gene Complex in Immune Responses", D. H. Katz and B. Benacerraf, Ed., Academic Press, New York, N.Y., 1976, p 225.
(10) (a) R. G. Denkewalter, D. F. Veber, F. W. Holly, and R. Hirschmann, *J. Am. Chem. Soc.*, **91**, 502 (1969); (b) B. Gutte and R. B. Merrifield, *ibid.*, **91**, 501 (1969); (c) H. T. Storey, J. Beacham, S. F. Cemosek, F. M. Finn, C. Yanaihara, and K. Hofmann, *ibid.*, **94**, 6170 (1972); (d) L. Moroder, G. Borin, F. Marchiori, and E. Scoffone, *Biopolymers*, **12**, 477 (1973); (e) W. S. Hancock, D. J. Prescott, G. R. Marshall, and P. R. Vagelos, *J. Biol. Chem.*, **247**, 6224 (1972); (f) I. M. Chaiken and C. B. Anfinsen *ibid.*, **246**, 2285 (1971); (g) A. R. Zeiger and C. B. Anfinsen, *J. Am. Chem. Soc.*, **95**, 880 (1973).
(11) P. Sieber, B. Kamber, A. Hartmann, A. Johl, R. Riniker, and W. Rittel, *Helv. Chim. Acta*, **67**, 2617 (1974).
(12) G. W. Kenner, *Proc. R. Soc. London, Ser. A*, **353**, 441 (1977).
(13) F. F. Richards, J. H. Pincus, K. J. Bloch, W. T. Barnes, and E. Haber, *Biochemistry*, **8**, 1377 (1969).
(14) B. Schechter, I. Schechter, J. Ramachandran, A. Conway-Jacobs, M. Sela, E. Benjamini, and M. Shimizu, *Eur. J. Biochem.*, **20**, 309 (1971).
(15) A. R. Zeiger and P. H. Maurer, *Immunochemistry*, **11**, 555 (1974).
(16) A. R. Zeiger, C. H. Lai, and P. H. Maurer, *Biopolymers*, **14**, 2281 (1975).
(17) A. A. Luderer, P. H. Maurer, and R. L. Woodland, *J. Immunol.*, **117**, 1079 (1976).
(18) S. A. St-Pierre and R. S. Hodges, *Biochem. Biophys. Res. Commun.*, **72**, 581 (1976).
(19) J. Ramachandran, A. Berger, and E. Katchalski, *Biopolymers*, **10**, 1829 (1971).
(20) B. Schechter, A. Conway-Jacobs, and M. Sela, *Eur. J. Biochem.*, **20**, 321 (1971).
(21) A. Conway-Jacobs, B. Schechter, and M. Sela, *Eur. J. Biochem.*, **20**, 325 (1971).
(22) (a) G. W. Kenner and J. H. Seely, *J. Am. Chem. Soc.*, **94**, 3259 (1972); (b) D. Hudson, G. W. Kenner, B. Mason, B. Morgan, R. Ramage, B. Singh, and R. Tyson, "Peptides 1972", H. Hanson and H. D. Jakubke, Ed., American Elsevier, New York, N.Y., 1973.
(23) W. Konig and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
(24) N. Izumiya and M. Muroata, *J. Am. Chem. Soc.*, **91**, 2391 (1969).
(25) F. H. C. Stewart, *Aust. J. Chem.*, **23**, 1073 (1970).
(26) K. Weber and M. Osborn, *Proteins, 3rd Ed.*, **1**, 194, (1975).
(27) B. Schechter, I. Schechter, J. Ramachandran, A. Conway-Jacobs, and M. Sela, *Eur. J. Biochem.*, **20**, 301 (1971).
(28) R. H. Mazur, B. W. Ellis, and F. S. Cammarata, *J. Biol. Chem.*, **237**, 1619 (1962).
(29) D. T. Mowry, J. Dazzi, M. Renoll, and R. W. Shortridge, *J. Am. Chem. Soc.*, **70**, 1916 (1948).
(30) P. Sieber and B. Iselin, *Helv. Chim. Acta*, **51**, 622 (1968).
(31) J. P. Greenstein and M. Winitz, in "Chemistry of the Amino Acids", Vol. 2, Wiley, New York, N.Y., 1961, p 887.
(32) M. A. Barton, R. U. Lemieux, and J. Y. Savoie, *J. Am. Chem. Soc.*, **95**, 4501 (1973).
(33) E. Wünsch and A. Zwick, *Chem. Ber.*, **99**, 105 (1966).
(34) D. I. Marlborough and H. N. Rydon, *J. Chem. Soc., Perkin Trans. 1*, 1 (1972).
(35) J. M. Stewart and J. D. Young, in "Solid Phase Synthesis", W. H. Freeman, San Francisco, Calif., 1969, p 62.
(36) T. Okuyama and K. Satake, *J. Biochem. (Tokyo)*, **47**, 454 (1960).
(37) F. C. Greenwood, W. M. Hunter, and J. S. Glover, *Biochem. J.*, **89**, 114 (1963).
(38) B. Desbuquois and G. D. Aurbach, *J. Clin. Endocrinol. Metab.*, **33**, 732 (1971).

Communications to the Editor

Theoretical Study of Binding and Proton-Labilizing Properties of Zn^{2+}

Sir:

In the active site of carbonic anhydrase,[1] which catalyzes the hydration of CO_2, x-ray crystallography shows[2] that a Zn^{2+} ion is coordinated to three imidazoles from histidine residues and to a water molecule in a distorted tetrahedral geometry. To account for the fact that the enzyme activity is controlled by ionization of a group having a $pK_a = 7$, close to the Zn^{2+} ion,[1] various mechanisms have been proposed, assuming either ionization of an imidazole (bound[3] or not[4] to Zn^{2+}) and nucleophilic attack on CO_2 by the coordinated imidazolate anion (directly[5] or through water[6]), or ionization of the Zn^{2+}-coordinated water and nucleophilic attack of CO_2 by OH^-.[7-9] Furthermore, it was proposed by analogy to the imidazole inhibitor that "CO_2 binds weakly to the fifth coordination site of the metal ion in the hydrophobic region of the active site".[8] Others have proposed that CO_2 binds to Zn^{2+} in a transient five-coordinate intermediate.[10]

As a first step in a theoretical study of the problems raised by these proposals, we have performed ab initio molecular orbital calculations, with high quality basis sets, on the binding of Zn^{2+} with the molecules imidazole (ImH), water (OH_2), and carbon dioxide (CO_2), as well as with the anions imidazolate (Im^-) and hydroxyl (OH^-). Leaving aside other factors present in the enzyme, this allows us to ascertain (i) the nature and *intrinsic* characteristics of Zn^{2+} binding with each ligand, (ii) the aptitude of Zn^{2+} to bind CO_2, (iii) the effect of Zn^{2+} binding on the ease of deprotonation of ImH and OH_2. Gaussian basis sets taken from the literature[11] were contracted single ζ for the core and double ζ for the valence shell.[12] We added a diffuse p and a diffuse d set to the zinc basis ($\zeta = 0.2539$ and 0.2313), and a diffuse p set on oxygen ($\zeta = 0.08$, optimized for OH^-) and nitrogen ($\zeta = 0.067$).[13] Diffuse p functions on carbon ($\zeta = 0.047$) were found to have little in-