Antibody Combining Sites to a Series of Peptide Determinants of Increasing Size and Defined Structure*  

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SUMMARY  

Proteins to which a series of peptides of defined length and structure have been attached were used in this study as immunogens and as cross-reacting antigens in order to evaluate to what extent the hapten inhibition method reflects the size and nature of the antigenic determinant to which the animal was exposed. Peptides of the structure (DAla)n-Gly (n = 1 to 4) were coupled to RNase and rabbit serum albumin in a one-step synthesis. Peptides of the structures (DAla)n (n = 2 to 5), (DAla)n-Gly (n = 1 to 4), and (DAla)n-Gly-ε-aminocaproic acid (n = 1 to 3) were used as inhibitors of the immunological reactions. From cross-precipitation and inhibition experiments it was concluded that the size of the combining sites was in all cases such as to accommodate 4 amino acid residues, and thus the antigenic determinant is a tetrapeptide. Direct evidence is presented that the lysine residue in the protein carrier participates in the antigenic determinant only when the hapten attached is smaller than a tetrapeptide. The most exposed portion of the determinant (NH2-terminal amino acid residue) plays an immunodominant role. An increase in the inhibitory capacity in a series of related hapten's does not necessarily reflect the properties of the combining site. Nevertheless, the inhibition technique can be used as a tool for estimating the size and nature of the combining sites of antibodies, provided that the structure of the antigenic determinants is known.

The capacity to inhibit antigen-antibody reactions by means of oligosaccharides (2), oligonucleotides (3), and oligopeptides (4–8) has led to considerable insight into the size and nature of the combining sites in the antibody molecule. Previous studies of the capacity of alanine peptides to inhibit the poly-L-alanyl and poly-D-alanyl immune systems led to the conclusion that the size of the combining region of the antibodies is such as to accommodate up to 3 to 4 alanine residues (6). Moreover, from the extent of inhibition of the stereospecific antigen-antibody reactions with alanine peptides containing both L and D residues at defined positions, we reached the conclusion that the region of the antigenic determinant furthest removed from the protein carrier is of paramount importance in determining the specificities of the antibodies formed.

The polypeptidyl proteins used in the above studies as immunogens and as cross-reacting antigens were prepared by polymerization techniques (reaction of the protein with N-carboxy-α-amino acid anhydrides) and, thus, the peptides attached to the protein are of somewhat different sizes. This heterogeneity is relatively restricted, because of the nature of the polymerization reaction (9, 10). Such antigenic determinants, differing in their size, may lead to a more heterogeneous population of antibodies. In order to circumvent this, and to find out to what extent the hapten inhibition data reflect the size and nature of the antigenic determinants to which the animal was exposed, we have now used as immunogens and as cross-reacting antigens proteins to which peptides of defined length and structure have been attached.

A series of peptides of the structure (DAla)n-Gly (n = 1 to 4) were coupled to ribonuclease in a one-step synthesis. The conjugates obtained elicited in rabbits peptide-specific precipitating antibodies. Results of inhibitions of the precipitin reactions with various chemically related peptides showed that the size of the combining sites of antibodies accommodates 4 amino acid residues, irrespective of the size of the conjugated hapten. Thus, when the attached peptide was composed of 4 (or more) amino acid residues, the antipeptidyl antibodies did not show any carrier1 specificity. On the other hand, when the hapten attached was smaller than a tetrapeptide the antipeptidyl antibodies exhibited definite carrier specificity. This study confirms also that the inhibition technique can be used as a tool for estimating the size and nature of the combining site of antibodies, provided that the structure of the antigenic determinants is known. Otherwise, it is possible to reach erroneous conclusions.

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**Materials and Methods**

Ribonuclease A, five times recrystallized, was obtained from Sigma. Rabbit serum albumin (crystallized), d-alanine (Mann- assayed grade, [α]$_D^{28}$ = −14.5° in 6 N HCl), and e-amino-n-caproic acid (chemically pure grade) were purchased from Mann. N-Benzoyloxycarbonyl-L-tryptophan was obtained from Yeda, Rehovot. N$_2$N'-Dicyclohexyl carbodiimide was purchased from Fluka AG, Switzerland. Palladium on powdered charcoal (10% catalyst) was obtained from Matheson, Coleman and Bell. Complete Freund's adjuvant was obtained from Difco. All solvents used were of analytical grade.

Poly-n-allyl RSA, prepared as described previously (6). To each molecule 25 chains of polyalanine were attached, on the average. Each polymeric side chain was composed of 8.2 n-alanine residues, on the average. The (nAla)$_n$ (n = 2 to 5) peptides used were already described (12).

Benzoyloxycarbonylglycine (m.p. 120.5°) and benzoyloxycarbonyl-d-alanine (m.p. 85.5°; [α]$_D^{28}$ = +4.4° in dioxane; [α]$_D^{28}$ = −14.7°, in acetic acid) were prepared according to Bergmann and Zervas (13). 2-Nitrophenylsulfenyl-d-alanine dicyclohexylammonium salt was prepared according to Zervas, Borovas, and Gazis (14). After recrystallization from methanol, m.p. 176–177.5°, [α]$_D$ = +58.4°, in methanol (reported for the Rehovot, N, N'-Dicyclohexyl carbodiimide was purchased from Borovas, and Gazis (14). After recrystallization from methanol, the (nAla)$_n$ (m.p. 122°, reported for the 13°) was obtained from Fluka, Switzerland. Palladium on powdered charcoal (10% catalyst) was obtained from Matheson, Coleman and Bell. Complete Freund's adjuvant was obtained from Difco. All solvents used were of analytical grade.

Poly-n-allyl RSA, was prepared analogously. Dicyclohexylamine was released from the Nps-n-Ala dicyclohexylammonium salt by heating, if necessary. Water (10% by volume) and acetic acid (10 to 100 ml per g of peptide, according to solubility) were added, and the hydro- genation was carried out at room temperature and pressure during 12 hours. The catalyst was filtered off, and the solution was concentrated to a few milliliters in a rotary evaporator at 40° (peptide (nAla)$_n$-Gly came out of solution). Water was added, and the volume was again reduced to a few milliliters. On the addition of ethanol and ether (the peptides precipitated out, were collected on a sintered glass filter, washed with ethanol and ether, and dried under reduced pressure. The yields were 90 to 100%. The peptides nAla-Gly, (nAla)$_n$-Gly, and (nAla)$_n$-Gly were recrystallized from water-ethanol mixture with 86 to 90% yields; nAla-Gly-e-AC was recrystallized from water-ethanol-acetone mixture with 50% yield. The peptide Gly-e-AC precipitated as a brown substance; it was dissolved in hot 80% ethanol treated with activated charcoal (Darco G60, Fluka), the charcoal was filtered off, and crystals came out from the clear filtrate (70% yield).

Microanalysis indicated that the free peptides contained of 85 to 90%. In the case of Z-dAla-Gly, upon acidification of the aqueous solution an oily precipitate was formed. This was extracted into ethyl acetate; the extract was dried over Na$_2$SO$_4$ and concentrated. The blocked peptide was precipitated by adding absolute ether, collected on a sintered glass filter, and dried under reduced pressure. Yield of Z-dAla-Gly, 55%. Analytical data of the peptides are given in Table I.

The purity of benzoyloxycarbonyl peptides was determined in three ways: (a) lack of reaction of the blocked peptides (20 to 50 μmole) with ninhydrin; (b) determination of neutral equivalents; (c) nitrogen analysis (Table I).

**Free Peptides**—Benzoyloxycarbonyl peptides were dissolved in acetic acid (10 to 100 ml per g of peptide, according to solubility) with heating, if necessary. Water (10% by volume) and palladium on charcoal (10%, 200 mg) were added, and the hydrogenation was carried out at room temperature and pressure during 12 hours. The catalyst was filtered off, and the solution was concentrated to a few milliliters in a rotary evaporator at 40° (peptide (nAla)$_n$-Gly came out of solution). Water was added, and the volume was again reduced to a few milliliters. On the addition of ethanol and ether (the peptides precipitated out, were collected on a sintered glass filter, washed with ethanol and ether, and dried under reduced pressure. The yields were 90 to 100%. The peptides nAla-Gly, (nAla)$_n$-Gly, and (nAla)$_n$-Gly were recrystallized from water-ethanol mixture with 86 to 90% yields; nAla-Gly-e-AC was recrystallized from water-ethanol-acetone mixture with 50% yield. The peptide Gly-e-AC precipitated as a brown substance; it was dissolved in hot 80% ethanol treated with activated charcoal (Darco G60, Fluka), the charcoal was filtered off, and crystals came out from the clear filtrate (70% yield).

Microanalysis indicated that the free peptides contained

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**Table I. Analytical Data of Benzoyloxycarbonyl Peptides**

| Peptide          | Molecular weight | Neutral equivalent | Nitrogen Calculated | Found |
|------------------|------------------|--------------------|---------------------|-------|
| Z-dAla-Gly       | 280              | 279                | 10.0                | 10.0  |
| Z-((nAla)$_n$-Gly) | 351              | 353                | 12.0                | 11.9  |
| Z-((nAla)$_n$-Gly) | 422              | 420                | 13.3                | 13.3  |
| Z-((nAla)$_n$-Gly) | 493              | 490                | 14.2                | 14.0  |
| Z-Gly-e-AC       | 322              | 322                | 8.7                 | 8.7   |
| Z-dAla-Gly-e-AC  | 393              | 393                | 10.7                | 10.6  |
| Z-((nAla)$_n$-Gly)-e-AC | 464 | 464                | 12.1                | 11.8  |
| Z-((nAla)$_n$-Gly)-e-AC | 535 | 530                | 13.1                | 13.0  |

*a* Anhydrous titration with sodium methoxide in dimethylformamide.

*b* Dumas analysis.

*c* Insoluble in dimethylformamide. Therefore, dissolved in aqueous sodium hydroxide and back titrated with hydrochloric acid.
varying amounts (0 to 6% w/w) of bound water. No effort was made to remove it by drying at elevated temperatures. The purity of the peptides was determined in three ways. (a) One was by high voltage paper electrophoresis at pH 1.4, 3000 volts for 4 hours, where the peptides are separated according to their size (12). Thus contamination by the reactants in the peptide synthesis would show up as additional spots. With ninhydrin reagent only one spot was detected on the electrophoresis sheet at the appropriate distance from the application point (300 mmoles of peptide load). (b) By chromatography in n-butyl alcohol-acetic acid-water, one spot was detected. (c) It was also done by determining the ratio of total nitrogen (micro-Kjeldahl) to amino nitrogen (Van Slyke), which should be equal to \( n \), where \( n \) = number of amino acid residues in the peptide. The data for \( c \) as well as the specific rotations of the peptides are given in Table II.

The peptide Gly-\( \epsilon \)-AC fulfilled the criteria a and b for purity, but in Van Slyke's analysis the amount of amino nitrogen found was 30% higher than calculated. The same value was obtained after repeated recrystallisations from water-ethanol. However, photometric titration gave a satisfactory result. Thus, 20.5 \( \mu \)moles of peptide (41 \( \mu \)moles of nitrogen, by Kjeldahl analysis) contained: carboxyl group, 19.9 \( \mu \)moles; amino group, 21 \( \mu \)moles (pK\( a = 4.5 \); pKb = 8.2). Apparently, amino-terminal glycine in this peptide is not determined accurately by the Van Slyke nitrous acid method.

\( N\)-o-Nitrophenylsulfonyl-(o-alanyl)\( \epsilon \)-glycine—These peptides were prepared by coupling the succinimide ester of N-o-Nitrophenylsulfenyl-(o-alanyl),-Gly-OSu (n = 1 to 3) in 40 ml of dimethylformamide after treatment with HCl and precipitation with ether, the dicyclohexylurea formed was filtered off and washed with ether, dried under reduced pressure, the peptide was obtained. The yields were between 60 to 80%.

The average number of peptides attached to a protein molecule was determined in three ways: (a) by amino acid analysis of the peptides after separation by high voltage paper electrophoresis at pH 1.4, 3000 volts for 4 hours, where the peptides are separated according to their size (12). Thus contamination by the reactants in the peptide synthesis would show up as additional spots. With ninhydrin reagent only one spot was detected on the electrophoresis sheet at the appropriate distance from the application point (300 mmoles of peptide load). (b) By chromatography in n-butyl alcohol-acetic acid-water, one spot was detected. (c) It was also done by determining the ratio of total nitrogen (micro-Kjeldahl) to amino nitrogen (Van Slyke), which should be equal to \( n \), where \( n \) = number of amino acid residues in the peptide. The data for \( c \) as well as the specific rotations of the peptides are given in Table II.

| Peptide | \( N_{\text{total}}/m\text{nmol} \) | Specific rotation in 0.2M HCl | a (cp) | c (cg) |
| --- | --- | --- | --- | --- |
| dAla-Gly\( \epsilon \) | 0.97 | -20.0 | 1.0 |
| dAla-oAla-Gly\( \epsilon \) | 1.01 | +57.4 | 1.0 |
| dAla-oAla-dAla-Gly\( \epsilon \) | 1.00 | +104.1 | 1.0 |
| dAla-oAla-dAla-Gly\( \epsilon \) | 0.97 | +138.8 | 0.7 |
| dAla-Gly-\( \epsilon \)-AC\( d \) | 0.95 | -22.2 | 0.8 |
| dAla-oAla-Gly-\( \epsilon \)-AC \( d \) | 1.04 | +29.1 | 0.9 |
| dAla-oAla-dAla-Gly-\( \epsilon \)-AC | 1.00 | +63.1 | 0.9 |

a Micro-Kjeldahl.  
b Van Slyke; \( n \), number of amino acid residues in the peptide.  
c Recrystallized from water-ethanol.  
d Recrystallized from water-ethanol-acetone.
[(nAla)$_z$-Gly$_y$]-protein; (b) by the desamination method used for polyalanyl ribonuclease (10), where lysine residues to which a peptide was coupled are not desaminated. Therefore, the moles of lysine residues per mole [(nAla)$_z$-Gly$_y$]-protein found should be equal to the moles of peptide attached; (c) by measuring the absorbance of the [Nps-(nAla)$_z$-Gly$_y$]-RSA at 390 m$\mu$, in a solution of 0.14 M NaCl-0.01 M Na$_2$HPO$_4$-0.0034 M HCl, pH 7.2 (with the value of $\varepsilon = 3660$ at 390 m$\mu$ for the molar absorbance of Nps-$\alpha$-alanine dicyclohexylammonium salt in the same solution). The [Nps-(nAla)$_z$-Gly$_y$]-RNase derivatives are not soluble in the above medium, and their absorbance could not be measured. The analytical data for the protein derivatives are given in Table III.

**Amino Acid Analysis**—The protein samples were subjected to hydrolysis with 6 M hydrochloric acid in sealed tubes at 110$\circ$C for 24 hours. The amino acids were then determined by quantitative analysis (16), with the Beckman-Spinco automatic amino acid analyzer, model 120 B.

**High Voltage Electrophoresis**—Electrophoretic separation of peptides was carried out as described before for alamine peptides (12).

**Spectrophotometric Measurements**—Absorbances of proteins were measured in a Zeiss spectrophotometer, model PM Q11.

**Immunochemistry Procedure**—Randomly bred rabbits (2.5 to 3.5 kg) of both sexes were used. The antigens were incorporated in a water in oil adjuvant mixture (17). Equal parts of 2% antigen solution in aqueous 0.9% sodium chloride and complete Freund's adjuvant were homogenized by repeated filling and forcible ejection from a syringe. After bleeding prior to immunization, 1 ml of the adjuvant-antigen mixture was administered intramuscularly into the thighs of the hind legs of the animal. Four injections were given at 10-day intervals. Animals were bled 5 days after the third and fourth injections, and then at weekly intervals for another 2 months. Studies were performed on sera of individual rabbits, and for this purpose the sera from each rabbit were pooled separately.

**Quantitative Precipitin Studies**—To a constant volume of antiserum (0.2 to 0.5 ml) increasing amounts of the precipitant dissolved in 0.9% sodium chloride were added (the amount of precipitant was determined by Kjeldahl nitrogen analysis, with the factor of 6.25 for conversion from micrograms of nitrogen to micrograms of protein), and the final volume was brought to 1.7 ml with 0.9% sodium chloride. The reaction mixture was incubated at 37$\circ$C for 45 min and then kept at 4$\circ$C for 18 hours. The precipitates formed were separated by centrifugation, washed treated as described above for quantitative precipitin.

**RESULTS**

**Synthesis and Characterization of Peptidyl Proteins**

For the purpose of the present study a series of peptidyl proteins were prepared, to be used as immunogens or cross-reacting antigens (precipitins). The scheme of a representative synthesis is given below:

\[
\text{Nps-}[\text{DAla}]_z\text{-Gly-CONH} \xrightarrow{N\text{-hydroxysuccinimide, } 0^\circ} N',N'-\text{dicyclohexyl carbodiimide} \]

(1) \[ \text{Nps-}[\text{DAla}]_z\text{-Gly-OSu} \xrightarrow{\text{dioxane, } H_2O} \text{NaHCO}_3 \]

(2) \[ \text{Nps-}[\text{DAla}]_z\text{-Gly-COOH} \xrightarrow{N\text{-hydroxysuccinimide, } 0^\circ} N',N'-\text{dicyclohexyl carbodiimide} \]

(3) \[ \text{Nps-}[\text{DAla}]_z\text{-Gly-OSu} \xrightarrow{\text{dimethylformamide}} \text{Nps-}[\text{DAla}]_z\text{-Gly-OH} \]

(4) \[ \text{Nps-}[\text{DAla}]_z\text{-Gly-RNase} \xrightarrow{\text{HCl, acetic acid}} \text{Z-Try} \]

(5)

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| Peptidyl protein | Excess $\alpha$ | Excess $\alpha$/excess Gly | From amino acid analysis | From desamination analysis | From spectral analysis |
|-----------------|---------------|-------------------------|------------------------|---------------------------|----------------------|
| Nps-[(nAla)$_z$-Gly$_y$]-RNase | 11.0 | 11.2 | 1.0 | 11.1 | 11.0 |
| (nAla)$_z$-Gly-RNase | 20.5 | 10.2 | 2.0 | 10.2 | 10.5 |
| (nAla)$_z$-Gly-RSA | 29.6 | 25.5 | 3.0 | 2.9 | 9.0 |
| (nAla)$_z$-Gly-RSA | 10.0 | 4.4 | 3.8 | 4.3 | 5.0 |
| Nps-[(nAla)$_z$-Gly$_y$]-RNase | 35.0 | 36.4 | 1.0 | 35.7 | 36.4 |
| (nAla)$_z$-Gly-RSA | 77.8 | 42.0 | 1.9 | 40.5 | 42.5 |
| (nAla)$_z$-Gly-RSA | 107.0 | 36.0 | 3.0 | 35.8 | 38.0 |
| (nAla)$_z$-Gly-RSA | 82.5 | 20.3 | 1.1 | 20.5 | 10.5 |

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* Determined by quantitative amino acid analysis, and calculated on the basis of 12 moles of alanine residues and 3 moles of glycine residues per mole of RNase, and of 54 moles of alanine residues and 15 moles of glycine residues per mole of RSA.

* Obtained by determining the lysine content after desamination of the precipitin protein with nitrous acid (0).

* Calculated from the molar absorbance of the Nps-group at 390 m$\mu$ ($\varepsilon = 3660$).

additional 45 min at 37$\circ$C and then at 4$\circ$C for 18 hours. The precipitates formed were separated by centrifugation and were treated as described above for quantitative precipitin.
The agreement between the results obtained by three independent measurements at 390 nm of the Nps-peptidyl RSA derivatives molecule. The results (Table III) are in reasonable agreement with those obtained by two other methods, namely, desamination. Analytical data the average number of peptide chains per protein (Table III). Moreover, it is possible to calculate from the amino acid shows that the number of amino acid residues in excess of those expected from the composition of the peptides attached (Table II). The peptides were prepared by stepwise synthesis. We used D-alanine in the synthesis of the peptides as such determinants would be more resistant to alteration in the organism, and in order to prevent their proteolytic digestion by serum (6). The COOH-terminal residue of the peptide attached to proteins was always glycine, in order to avoid possible racemization during activation of the carboxyl function. The carboxyl function was activated in the form of succinimide ester, as such peptide derivatives are known to react well in aqueous solutions (15). Benzoxycarbonyl-L-tryptophan was added to the reaction mixture at the stage of the Nps-removal, as the Nps-activated in the form of succinimide ester, as such peptide inhibitors were performed.

The final results indicate, as expected (6), that the combining sites of the antibodies investigated are complementary to a peptide length greater than 3 amino acid residues. In these experiments the peptide reacting with the antibody was attached to a protein carrier, whose vicinity may have affected the results. For a more precise analysis, experiments with peptides as inhibitors were performed.

Immune Response to Peptidyl Proteins

In view of the possible differences in the combining sites of antibodies of the same specificity elicited in different rabbits, the present study was carried out in each case with sera from individual rabbits. Sera collected before immunization did not precipitate with any of the immunogen or the cross-reacting antigens.

Groups of six rabbits were immunized with the following peptidyl derivatives of RNase: [(D-Ala)n-Gly]-RNase, [(n-Ala)n-Gly]-RNase, [(n-Ala)n-Gly]-RNase, and [(n-Ala)n-Gly]-RNase. The presence of antibodies to the peptidyl moiety was followed by the precipitation reaction of the sera with the respective peptidyl derivatives of RSA. Typical precipitin reactions are shown in Fig. 1. In the same figure results are also given of cross-precipitin reactions with poly-D-alanyl-RSA (the poly-D-alanyl chains contained, on the average, 8.2 D-alanine residues). The absolute amount of antibodies precipitated from sera of individual rabbits by the respective peptidyl derivatives of RSA varied between 0.35 mg and 2.1 mg of antibody per ml of serum (most animals had antipeptidyl antibodies in the range of 0.8 to 1.2 mg per ml of serum), but the ratio of the amount of antibodies precipitated by the poly-D-alanyl-RSA and by the peptidyl-RSA was in each case very similar.

Poly-D-alanyl-RSA cross-reacted only to a limited extent with anti-D-Ala–Gly antibodies, whereas it cross-reacted completely with anti-D-Ala–Gly antibodies. As seen in Fig. 1, the extent of cross-reaction increased in parallel with the increment in the length of the alanyl peptide in the immunogen. Similarly, antibodies to poly-D-alanyl-RNase (6) reacted much better with (n-Ala)n–Gly–RSA (75% of the reaction with poly-D-alanyl-RSA) than with (n-Ala)n–Gly–RSA (67%) or with n-Ala–Gly–RSA (47%).

The above results indicate, as expected (6), that the combining sites of the antibodies investigated are complementary to a peptide length greater than 3 amino acid residues. In these experiments the peptide reacting with the antibody was attached to a protein carrier, whose vicinity may have affected the results. For a more precise analysis, experiments with peptides as inhibitors were performed.

Inhibition Studies

None of the peptides used in inhibition studies was degraded when incubated with rabbit sera under the conditions of inhibition experiments, as followed by high voltage electrophoresis (6). Peptides used in the inhibition studies did not cause any precipitation with either sera from before immunization or with immune sera.

The peptides used for the inhibition of the immunospecific peptidyl reactions were of three categories, possessing the general formulae (D-Ala)n (n = 2 to 5), (n-Ala)n–Gly (n = 1 to 4), and (n-Ala)n–Gly–κ-AC (n = 1 to 3). The alanine peptides are representative of the NH2-terminal part of the antigenic determinant. The peptides with COOH-terminal glycine are of the same structure as the peptides attached to the protein carrier in
the immunogen. Peptides with COOH-terminal ε-aminocaproic acid should reflect any carrier specificity, as ε-AC is chemically related to the side chain of lysine to which the peptides are coupled in the immunogen.

**Inhibition of nAla-Gly System**—Results of the inhibition of the precipitin reaction between nAla-Gly-RSA and an anti-nAla-Gly-RNase serum are shown in Fig. 2. While the data given in Fig. 2 were obtained with the serum of an individual rabbit, closely similar findings were observed when antisera of three other animals were tested. In all cases nAla-Gly-ε-AC was the most efficient inhibitor (50% inhibition at 0.10 to 0.12 mM peptide). The peptide attached to the protein, nAla-Gly, was far less inhibitory (50% inhibition at 2.5 to 4.0 mM peptide). All of the other peptides were either similar in their inhibitory capacity to nAla-Gly, or less efficient.

In the (nAla)ₙ series, an increase in n (from 2 to 4) did not improve inhibitory efficiency, in agreement with the structure of the peptide attached, which had only 1 d-alanine residue at its NH₂ terminus. The efficiency of inhibition of the nAla-Gly peptide was considerably increased only when ε-AC was attached to its COOH terminus (25- to 40-fold increase). This “specific” effect indicates that the protein moiety (side chain of lysine to which nAla-Gly is attached) contributed in this system to the specificity of the antibodies formed (19). Apparently in this case the peptide nAla-Gly-ε-AC represents the complete antigenic determinant.

The backbone of ε-AC is slightly bigger than the backbone of a dipeptide

![Dipeptide](http://www.jbc.org/)

Thus, the size of the combining site in this case corresponds to that of 4 amino acid residues.

Elongation of the nAla-Gly at the NH₂ terminus did not improve the inhibitory efficiency, and, in the case of the best inhibitor, nAla-Gly-ε-AC, the attachment of additional d-alanine residues at the NH₂ terminus decreased dramatically the capacity of the peptides to inhibit. These data indicate that the interaction of the peptide inhibitor with the combining site “starts” from the NH₂-terminal portion of the peptide.

**Inhibition of (nAla)₂-Gly System**—Results of the inhibition of the precipitin reaction between (nAla)₂-Gly-RSA and an anti-(nAla)₂-Gly-RNase serum are shown in Fig. 3. As seen in Fig. 3, (nAla)₂-Gly-ε-AC was the most efficient inhibitor. The values for 50% inhibition varied for antisera of four different rabbits between 0.22 and 0.34 mM peptide. (nAla)₂-Gly-ε-AC was 4 to 13 times more inhibitory than (nAla)₂-Gly, indicating that also in this system the protein moiety contributed to the specificity of antibodies formed.

In contrast to the nAla-Gly system, in the (nAla)₂-Gly system the efficiency of the inhibition by means of (nAla)ₙ peptides increased with n. (nAla)ₙ was in one animal less inhibitory than (nAla)₂-Gly (Fig. 3); however, in three other animals it was 2 to 3 times more inhibitory. Attachment of 1 alanine residue at the NH₂ terminus of (nAla)₂-Gly resulted in an inhibitor which was less efficient than (nAla)₂-Gly in one rabbit (Fig. 3), but 2 to 3 times more efficient in three other animals. Nevertheless, in all cases the most efficient inhibitor was (nAla)₂-Gly-ε-AC (see Table IV). It seems that this peptide contains all of the components of the complete antigenic determinant in the correct order. The attachment of an additional alanine residue at the NH₂ terminus of (nAla)₂-Gly-ε-AC resulted in a peptide, (nAla)₂-Gly-ε-AC, which was less inhibitory than (nAla)₂-Gly-ε-AC with the sera of all four animals tested. This is in agreement...
TABLE IV
Concentration of peptide causing 50% inhibition
Average values from antisera of four rabbits.

| Peptide inhibitor | Immune systema |
|-------------------|----------------|
|                   | (nAla)3-Gly | (oAla)4-Gly | (DAla)5-Gly | (nAla)2-Gly | (DAla)3-Gly | (nAla)3-Gly | (nAla)2-Gly |
| (nAla)2-Gly        | >4          | >4           | >4           | >4           | 2.9         | 0.44         | 0.52        |
| (nAla)3-Gly        | >4          | 1.3          | 0.19         | 0.16         | 1.2         | 0.19         | 0.07        |
| (DAla)5-Gly        | >4          | >4           | >4           | >4           | 3.1         | 1.2          | 1.4         |
| (DAla)3-Gly        | >4          | 1.2          | 0.17         | 0.20         | 1.2         | 0.19         | 0.07        |
| (nAla)2-Gly-AC     | >4          | >4           | >4           | >4           | 3.6         | 0.37         | 0.34        |
| (nAla)2-Gly-AC     | >4          | >4           | >4           | >4           | >4          | >4           | >4          |

a Antisera were obtained against the peptidyl-RNase, and were reacted with the respective peptidyl-RSA.

b Italicized numbers are related to peptide representing the complete antigenic determinant or to its closest neighbor.

Fig. 4. Inhibition of the precipitin reaction between anti-(nAla)3-Gly-RNase and (nAla)3-Gly-RSA. The inhibitors are: Δ, nAla-Gly; +, (nAla)3-Gly; △, (nAla)5-Gly; ●, (nAla)2-Gly-AC; ■, (nAla)5-Gly-e-AC; ●, (nAla)3-Gly-e-AC; ○, (nAla)5-Gly-ε-AC; □, (nAla)3; □, (nAla)5; ▽, (nAla)3; ◊, (nAla)5.

The data given in Fig. 4 permit an evaluation of the capacity of the antibody to distinguish between glycine and alanine residues. Although (nAla)3 is a poor inhibitor, it is better than DAla-Gly. (nAla)5 is a better inhibitor than (nAla)5-Gly. On the other hand, when glycine is in the fourth and fifth positions from the NH₂ terminus ((nAla)5-Gly and (nAla)5-Gly), its replacement by alanine ((nAla)5 and (nAla)5) is not reflected in significant changes in inhibitory efficiency.

Inhibition of (nAla)5-Gly System—Results of the inhibition of the precipitin reaction between (nAla)5-Gly-RSA and an anti-(nAla)5-Gly-RNase serum are shown in Fig. 5. In this system protein in the immunogen shows that (nAla)3 was less inhibitory than (nAla)5-Gly, and (nAla)5 was a very poor inhibitor. Thus, it seems that the antibody active site is complementary in this case to the tetrapeptide (nAla)5-Gly.

The data given in Fig. 4 permit an evaluation of the capacity of the antibody to distinguish between glycine and alanine residues. Although (nAla)5 is a poor inhibitor, it is better than DAla-Gly. (nAla)5 is a better inhibitor than (nAla)5-Gly. On the other hand, when glycine is in the fourth and fifth positions from the NH₂ terminus ((nAla)5-Gly and (nAla)5), Gly, its replacement by alanine ((nAla)5 and (nAla)5) is not reflected in significant changes in inhibitory efficiency.

Inhibition of (nAla)5-Gly System—Results of the inhibition of the precipitin reaction between (nAla)5-Gly-RSA and an anti-(nAla)5-Gly-RNase serum are shown in Fig. 5. In this system
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**TABLE VI**

| Peptide inhibitor | Anti-(nAla) Gly-RNase (animal 35) with | Anti-(nAla) Gly-RSA with |
|-------------------|-------------------------------------|-------------------------|
|                   | (nAla) Gly-RNase | (nAla) Gly-RSA | (nAla) Gly-RNAse | (nAla) Gly-RSA |
| (nAla) Gly        | >4 4 | >4 4 | >4 4 | >4 4 |
| (nAla) Gly        | 0.006 0.12 | >4 4 | >4 4 | >4 4 |
| (nAla) Gly        | 0.06 0.12 | >4 4 | >4 4 | >4 4 |
| (nAla) Gly        | 0.65 1.05 | >4 4 | >4 4 | >4 4 |
| (nAla) Gly        | 0.45 0.76 | >4 4 | >4 4 | >4 4 |
| (nAla) Gly-AC     | 0.038 0.075 | >4 4 | >4 4 | >4 4 |

**Fig. 6.** Inhibition of the precipitin reactions of anti-(nAla) Gly-RNase (animal 35) with (nAla) Gly-RSA (A) or with (nAla) Gly-RNase (B). The inhibitors are: Δ, nAla-Gly; ▲, (nAla) Gly; ●, nAla-Gly-AC; X, (nAla) Gly-AC.

**Fig. 7.** Inhibition of the precipitin reactions of anti-nAla-Gly-RNase (animal 57) with nAla-Gly-RSA (A) or with nAla-Gly-RNase (B). The inhibitors are: Δ, nAla-Gly; ▲, (nAla) Gly; ●, nAla-Gly-AC; X, (nAla) Gly-AC.

dervative was used as precipitant was 50 to 65% of that found when the RSA derivative was used for precipitation. However, the relative potencies of the different peptides within each experiment were quite similar, and thus led to the same conclusions concerning the size and nature of the combining sites.

The participation of the protein lysine residues in the antigenic determinant is reflected to the same extent, independently of whether the homologous or the cross-reacting antigens are used (Figs. 6 and 7).

**DISCUSSION**

Both the immunogens and the cross-reacting precipitants used in this study were prepared by covalent attachment of peptides to proteins. The attachment was carried out via N-blocked peptide succinimide ester. The first account of chemical attachment of a peptide to a protein was given in an immunochemical study by Landsteiner and Van der Scheer (23-25), who have coupled the peptides via azo links to tyrosine residues in the protein. Recently, several reports have appeared in which the attachment of peptides to proteins was performed making use of water-soluble carbodiimide derivatives (26-28).

The main purpose of the present investigation was to elucidate to what extent inhibition studies may reflect the structure of the hapten to which the animal was exposed and the size and nature of the combining sites of the antibodies formed. For a more precise evaluation of the results we attached to RNase as haptens a homologous series of peptides of defined amino acid sequences. The reactions of antisera against the various immunogens with cross-reacting precipitants, in which the same haptens were attached to RSA, were inhibited by different related peptides, and the main results are summarized, for the sake of comparison, in Table IV. The difference in inhibitory capacity between two peptides which was less than a factor of 2 was not considered significant, because of thermodynamic considerations (6).

It must be remembered that the exploration of the combining site is based on the strength of the interaction between a series of haptens and the antibody combining site; the precipitating antigen serves only as a tool for estimating its degree of saturation with the hapten. In fact, any molecule which binds to the antibody combining site would serve this purpose, provided that its degree of binding can be measured. The properties of the combining site are deduced from the correlation between the extent of antibody-hapten interaction and structural features of the hapten.

At least 70 to 90% of the antipeptidyl antibodies formed by
injecting peptidyl-RNase derivatives were precipitated by the corresponding peptidyl-RSA derivatives (Table V). Moreover, the relative efficiency of inhibition with different peptides was similar in each case tested, whether the homologous or the cross-precipitating system was used (Figs. 6 and 7; Table VI).

The efficiency of inhibition increases in each system with the increment in the length of the peptide resembling the hapten in the immunogen, starting from the NH₂ terminus. As seen in Table IV, a bigger peptide of related composition is not necessarily a better inhibitor. Thus, e.g., in the DAla-Gly system (DAla)₉-Gly is a less efficient inhibitor than (DAla)₄-Gly, and (DAla)₈-Gly-e-AC is much less inhibitory than DAla-Gly-e-AC (see also Fig. 2). In contradistinction, in a homologous series of peptides of composition and amino acid sequence resembling the hapten, e.g., in the system (DAla)ᵦ-Gly, the inhibitory efficiency increases in the order (DAla)ᵦ, (DAla)₄, (DAla)₈, and (DAla)₉-Gly (Fig. 5; Table IV). When peptides of the same size are compared, the peptide possessing the amino acid sequence identical with that starting from the NH₂ terminus of the hapten attached to the immunogen is in each case the most efficient inhibitor. Thus, e.g., in the DAla-Gly system, the peptide DAla-Gly-e-AC (e-AC corresponding in size to a dipeptide) is a better inhibitor than (DAla)₉-Gly, whereas in the (DAla)₉-Gly system, the peptide (DAla)₈-Gly inhibits better than DAla-Gly-e-AC. Similarly, in the (DAla)₈-Gly system, the peptide (DAla)₇-Gly-e-AC is a more efficient inhibitor than (DAla)₈-Gly, whereas in the (DAla)₉-Gly system, (DAla)₈-Gly inhibits better than (DAla)₉-Gly-e-AC.

In all of the four immune systems investigated, the size of the antigenic determinant to which the antibody site is complementary seems to consist of 4 amino acid residues. Thus in the (DAla)₇-Gly system, (DAla)₈-Gly is not significantly better as an inhibitor than (DAla)₉, whereas (DAla)₈ is much more inhibitory than (DAla)₉ (Fig. 5; Table IV). In the (DAla)₇-Gly system, (DAla)₉-Gly is slightly better as inhibitor than (DAla)₈, whereas (DAla)₈-Gly-e-AC does not inhibit significantly better than (DAla)₉-Gly (Fig. 5; Table IV). In the last case ε-aminocaproic acid serves as a model for the participation of the protein carrier in antigenic specificity, as it resembles the lysine residue of protein to which the peptides were attached. Indeed, in the (DAla)₇-Gly system, the peptide (DAla)₈-Gly-e-AC inhibits better than (DAla)₇-Gly by one order of magnitude. The contribution of the protein carrier is even more remarkable in the DAla-Gly-Gly system (compare the efficiency of inhibition with DAla-Gly-e-AC and with DAla-Gly; Fig. 2 and Table IV).

We may conclude that the protein carrier contributes to the antigenic determinant only when the peptide attached contains less than 4 amino acid residues. In other words, antibodies would show carrier specificity only when the hapten attached is less than a tetrapeptide. Many haptens commonly used in immunochromical studies, such as the 2, 4-dinitrophenyl group and the p-nitrobenzenesulfonate group, are smaller in size than a tetrapeptide, and antibodies produced against them would be expected to reflect partially also the protein area to which the hapten was attached. Indeed, Eisen and Siskind (19) showed that in the case of dinitrophenylated proteins lysine contributed to the antigenic dinitrophenyl determinant. More recently Parker, Gott, and Johnson (24) have reported, using as immunogen a protein to which a dinitrophenyl-tetrapeptide was attached, that antibody specificity can extend beyond the dinitrophenyl group. In both cases the investigators did not establish limits as to the size of the dinitrophenyl-peptide antigenic site. It is pertinent to remark here that, in the poly-D-alanyl system, the combining site of antibodies of the immunoglobulin M class is also complementary to a tetrapeptide, similarly to that of antibodies of the immunoglobulin G class (26).

As seen in Table IV, even though the determinant in the (DAla)ᵦ-Gly system, is (DAla)₉-Gly-e-AC, peptides of the structure (DAla)ᵦₖ and (DAla)ᵦₔ are better inhibitors than (DAla)ᵦ-Gly. If the antigenic determinant was not known, such results would lead to false conclusions about its nature. It seems that, in this particular case, the presence of additional alanine residues enhances in a non-specific way the binding to the antibody, similarly to the increased efficiency of binding by anti-(tobacco mosaic virus protein) of a decapetide in which (Ala)ₚ was attached to the specific pentapeptide, as compared to the pentapeptide alone (27). Findings of a similar nature were reported by Arakatsu, Ashwell, and Kabat (28), who have shown that, with some rabbit antibodies to an isomaltotrioseic acid derivative of bovine serum albumin, the efficiency of inhibition increased up to isomaltotrioseic. Nevertheless, the authors drew the conclusion that the general approach of elucidating sizes of the combining sites of antibodies is valid, although possible difficulties in the interpretation of the data may be encountered. The contribution of non-specific hydrophobic interactions to the binding energy of haptens to antibodies has also been reported by Metzger, Wofsey, and Singer (29) and by Benjamini, et al. (30).

Conclusions concerning the size and nature of the combining sites of antibodies were first drawn from inhibition studies by Kabat (2, 31, 32). The present study confirms that this technique is useful for estimation of the dimensions and complementarity of the active sites of antibodies, provided that the structure of the antigenic determinants is known.

In connection with the present work it should be mentioned that in a recent study of the immunological specificity of the bradykinin system, which made use of an immunogen prepared by binding covalently bradykinin to polylgsine, replacement of glycine with alanine had a profound effect on binding capacity, and this was interpreted as an obligatory effect on conformation (38). Similarly, in a study of a major antigenic determinant of tobacco mosaic virus protein, Young, Benjamini, and Leung (34) found that replacement of alanine with glycine decreased dramatically the capacity of the antibody to bind the peptide determinant. In the last case it is not known which amino acid residues composing the antigenic determinant are on its periphery.

In contrast to enzyesc, which represent a homogeneous population of molecules possessing one type of active site, antibody combining sites to a unique determinant are known to be heterogeneous. Nevertheless, by comparing pairs of peptides in which only 1 residue was replaced by another, or the effect of elongation of a peptide on its binding efficiency, it seems possible to evaluate the contribution of the various parts of the inhibitory molecule.

For example, in the present study the NH₂-terminal d-alanine residue plays always an immunodominant role. The necessity for a good interaction to occur at the NH₂ terminus is also evident from the previous findings that, in the poly-D-alanine system, the tetra-L-alanine peptide, which is an excellent inhibitor (6),
loses completely its inhibitory capacity when the NH₂-terminal α-amino group is acetylated (35).

The use in the present study of a series of determinants, whose size and structure were changed systematically, indicates that detailed features of the antigenic determinants are reflected in the combining sites of the antibodies formed.

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