Structural Requirements for Binding to Antibody of Sequence Variants of Bradykinin*

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ABSTRACT: Antibody to bradykinin was elicited in rabbits by the injection of a branch-chain copolymer of the peptide and poly-L-lysine. Binding of bradykinin and of various sequence analogs to this antibody was examined by a radioimmunoassay technique. It was apparent that changes in amino acid side chains which altered charge or hydrophobic character had little effect on binding to antibody. Change in length of the peptide chain was of greater importance. Alterations in residues which had an obligatory effect on conformation, such as changes in glycine or proline, exhibited the most profound effect. Variable relationships between changes in biologic activity and immunologic activity were observed.

The structural requirements of antigenic sites on polypeptides have been examined utilizing proteins (LaPresle and Webb, 1965; Crumpton and Wilkinson, 1965) and random copolymers of amino acids (Maurer, 1964; Sela, 1966; Gill *et al.*, 1967). While the former studies have been valuable in defining the size of the antigenic site and its relation to conformation, and the latter, the importance of individual amino acid residues, little information is available concerning specific amino acid sequence requirements.

The production of antibody directed specifically against the nonapeptide, bradykinin, and the availability of many sequence variants of this peptide have permitted an examination of the capacity of the variants to bind with antibody directed specifically against the native molecule. This allows for definition of structural requirements of a peptide antigen of defined sequence, which is not possible with the use of random amino acid copolymers. The antibody binding data obtained yield information concerning the antigenic role of different regions of the bradykinin molecule.

Materials

Preparation of Antibradykinin Antibody. The preparation of the antigen used to elicit antibradykinin antibody, the immunization of rabbits, and the demonstration of specificity of the antibody have been reported (Spragg *et al.*, 1966). The branch-chain copolymer antigen, in which bradykinin residues were coupled *via* their amino-terminal amino groups to the ϵ-amino groups of poly-L-lysine (BK-(*N*-Arg)-PL),[1] is shown in Figure 1. This antigen has been shown by amino acid analysis to contain one bradykinin molecule per five lysine residues.

BK-(*N*-Arg)-PL was prepared in an emulsion with complete Freund's adjuvant and injected into the toepads of Australian white rabbits from which preimmunization sera had been obtained. Each animal received 2.5 mg of antigen. Three and six months later, emulsified antigen was administered subcutaneously using, respectively, 3.5 and 2.0 mg of antigen. Four and seven months after the initial immunization, all animals were boosted intravenously on alternate days during a 1-week period, each animal receiving a total of 200 μg of antigen during each period. Eight days after the termination of intravenous injection of antigen, arterial blood samples were obtained.

The antibody elicited by BK-(*N*-Arg)-PL has been shown by complement fixation and radioimmunoassay to be directed specifically against the bradykinin portion of the immunizing antigen. In the present study, antiserum from a single bleeding from one rabbit was used. This antibody was not purified because of its low serum concentration (25 μg/ml). The serum concentration was estimated by binding to intrinsically labeled [^{14}C]bradykinin present in excess and measuring the antibody-bound counts.

Isotopically Labeled Bradykinin. Tritiated bradykinin was prepared following the method described previously (Spragg *et al.*, 1966). Bradykinin triacetate (1 mg), dissolved in 1.0 ml of freshly distilled dimethyl sulfoxide, was added with mixing to 25 μmoles of [^{3}H]acetic anhydride (Nuclear-Chicago; sp act. 4370 mc/mmole). After

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[1] Abbreviations used: The usual notation for peptides has been used in which, for example, Ala^{3}-bradykinin indicates that the proline in position 3 has been replaced by an alanine residue. BK-(*N*-Arg)-PL denotes polylysine carrying bradykinin residues coupled *via* their amino-terminal amino groups. Tlys, ϵ-tosyllysine; OMT, tyrosine methyl ether.

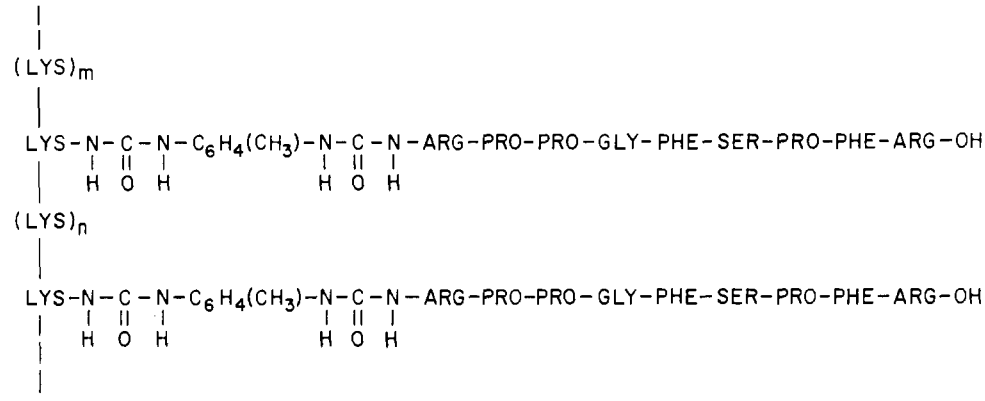

FIGURE 1: A schematic representation of the BK-(*N*-Arg)-PL branch-chain copolymer antigen. The poly-L-lysine backbone, of which only a small portion is shown, extends vertically on the left-hand side of the figure. The aromatic rings introduced between the ε-amino groups of bradykinin are derived from the coupling agent, toluene-2,4-diisocyanate (from Spragg *et al.*, 1966).

24 hr at room temperature, the mixture was repeatedly lyophilized from 0.1 N acetic acid to remove the dimethyl sulfoxide and [^{3}H]acetic acid. The specific activity of the [^{3}H]acetylbradykinin recovered was calculated to be 2185 μc/μmole.

Unlabeled acetylated bradykinin, prepared by the above method, was shown by dinitrophenylation (Sanger, 1945) and amino acid analysis (Spackman *et al.*, 1958) to be completely acetylated at the N-terminal α-amino position, which is the only position available for acetylation under the conditions used. Unlabeled acetylbradykinin prepared as described above bound to antibradykinin antibody somewhat better than did native bradykinin (Figure 2), which was confirmed in later studies on acetylbradykinin produced by solid-phase peptide synthesis.

Bradykinin Analogs. Singly substituted alanine analogs of bradykinin were prepared by classical methods (Schröder and Lübke, 1966). The other kinin analogs tested were synthesized by the solid-phase method of Merrifield (Stewart and Woolley, 1966). All weights given are acetate salt weights; correction to free-peptide weight does not significantly alter the binding data.

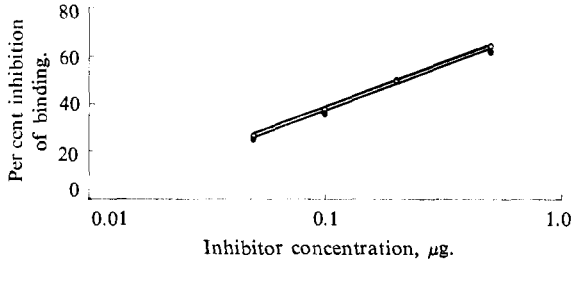

FIGURE 2: Binding inhibition curves of bradykinin and of acetylated bradykinin prepared in dimethyl sulfoxide. From a preliminary experiment in which 100 μl of antiserum was used in the incubations. (●) Bradykinin; (○) Arg(Ac)^1-bradykinin.

Method of Radioimmunoassay

Measurement of Radioactive Binding by Antibradykinin Antibody. [^{3}H]Acetylbradykinin (20 mμg) was incubated for 7.5 min at 4° with 50 μl of antiserum in a total volume adjusted to 160 μl by the addition of 0.1 M Tris-acetate (pH 7.5). After the incubation

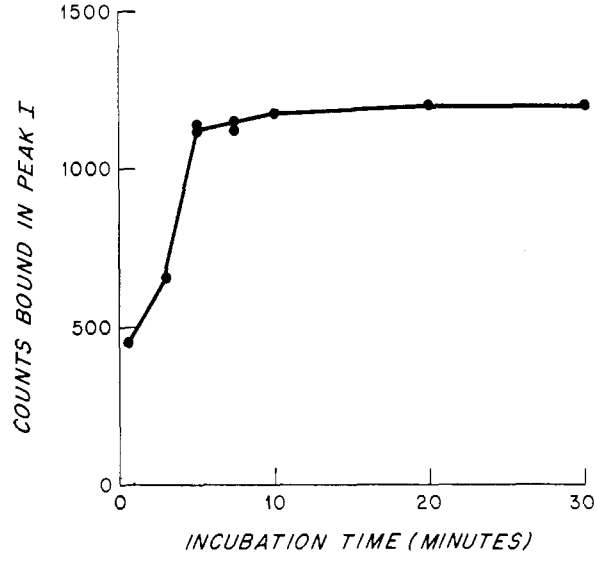

FIGURE 3: The amount of tritiated bradykinin bound by antibody plotted as a function of incubation time. Antibody-bound labeled bradykinin is expressed as the counts contained in the void volume peak (peak I) eluted from Sephadex G-25. The number of counts bound increases rapidly between 0 and 5 min, and then much more slowly between 5 and 30 min.

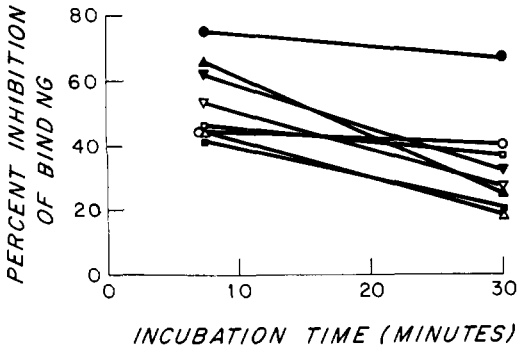

FIGURE 4: Rate of decrease in binding inhibition of the kinin analogs whose inhibition curves are shown in Figure 7. The indicated amount of each analog was incubated with tritiated bradykinin and antibody for either 7.5 or 30 min. The following represent peptide concentrations in micrograms: (●) bradykinin, 0.20; (△) Arg(Ac)^1-bradykinin, 0.03; (▼) Arg$(NO_2)^1$-Arg$(NO_2)^9$-bradykinin, 0.10; (▽) D-Arg^9-bradykinin, 0.10; (▲) Tlys^6-bradykinin, 0.25; (■) Arg^{10}-bradykinin, 0.25; (○) Phe^6-bradykinin, 0.50; (△) Des-(Arg^9)-bradykinin, 1.00.

period, the mixture was diluted to 1.0 ml and applied to a Sephadex G-25 fine bead column (1.0 × 70 cm) equilibrated with 0.1 M Tris-acetate (pH 7.5) (Haber *et al.*, 1965). The column was developed with the same buffer at 4° at a flow rate of 1.0 ml/min. The radioactivity in the excluded volume protein peak, which contained antibody-bound material, was determined in a Nuclear-Chicago liquid scintillation counter using Bray's (1960) solution.

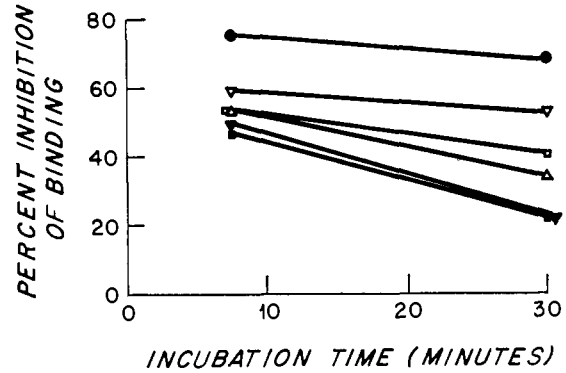

FIGURE 5: Rate of decrease in binding inhibition of the kinin analogs whose inhibition curves are shown in Figure 8. The indicated amount of each analog was incubated with tritiated bradykinin and antibody for either 7.5 or 30 min. The following represent peptide concentrations in micrograms: (●) bradykinin, 0.20; (△) Gly^6-OMT^8-bradykinin, 0.25; (▼) D-Pro^2-bradykinin, 1.00; (□) D-Pro^7-bradykinin, 1.00; (▽) OMT^5-Gly^6-OMT^8-bradykinin, 10.00; (■) D-Pro^3-bradykinin, 5.00.

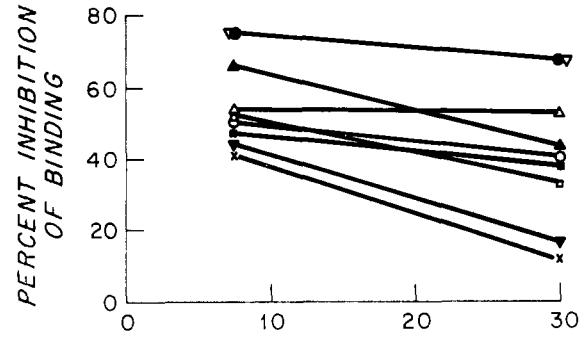

FIGURE 6: Rate of decrease in binding inhibition of the singly substituted alanine analogs of bradykinin whose inhibition curves are shown in Figure 9. The indicated amount of each analog was incubated with tritiated bradykinin and antibody for either 7.5 or 30 min. The following represent peptide concentrations in micrograms: (●) bradykinin, 0.20; (■) Ala^2-bradykinin, 0.75; (×) Ala^3-bradykinin, 10.00; (▼) Ala^4-bradykinin, 3.00; (▲) Ala^5-bradykinin, 10.00; (□) Ala^6-bradykinin, 0.10; (▽) Ala^7-bradykinin, 1.00; (○) Ala^8-bradykinin, 0.10; (△) Ala^9-bradykinin, 0.05.

Quantification of Analog Binding by Antibradykinin Antibody. Varying known concentrations of unlabeled bradykinin or of bradykinin analogs were incubated with 20 mμg of labeled acetylbradykinin and 50 μl of antiserum in a total volume of 160 μl. After 7.5 min, the mixture was diluted to 1.0 ml, applied to Sephadex G-25, eluted, and counted as above.

The per cent inhibition of binding produced by each concentration of the analogs used was calculated in the following manner:

$$\% \text{ inhibition of binding} = \frac{\text{cpm bound without analog} - \text{cpm with analog}}{\text{cpm bound without analog}} \times 100$$

The per cent inhibition of binding was plotted against the analog concentration, and the 50% inhibitory concentration was determined graphically.

Each analog was compared to native bradykinin in its capacity to compete with [^{3}H]acetylbradykinin for antibody. This was expressed in the following manner:

$$\text{analog binding activity} = \frac{50\% \text{ inhibitory concentration of bradykinin}}{50\% \text{ inhibitory concentration of analog}}$$

In the case of bradykinin analogs which poorly inhibited radioactive binding by antibody, inhibitory concentrations were used which showed that these analogs have less than $^1/_{1400}$ the binding activity of bradykinin.

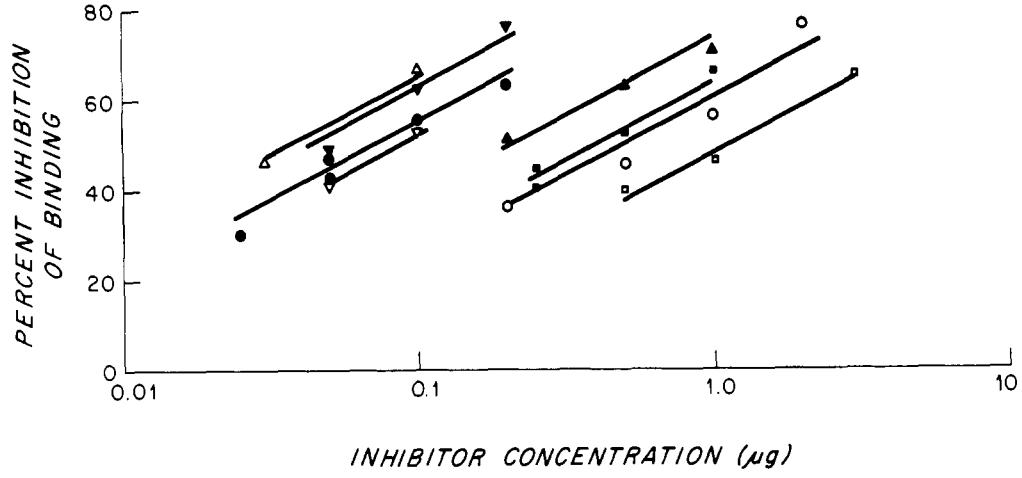

FIGURE 7: Binding inhibition produced by analogs of bradykinin altered at either end of the molecule or at position 6. The inhibition produced by bradykinin is shown for comparison. These data are summarized in Table I. (●) Bradykinin; (△) Arg(Ac)^{1}-bradykinin; (▼) Arg(NO_2)^{1}-Arg(NO_2)^{9}-bradykinin; (▽) D-Arg^{9}-bradykinin; (▲) Tlys^{6}-bradykinin; (■) Arg^{10}-bradykinin; (○) Phe^{6}-bradykinin; (□) Des-(Arg^{9})-bradykinin.

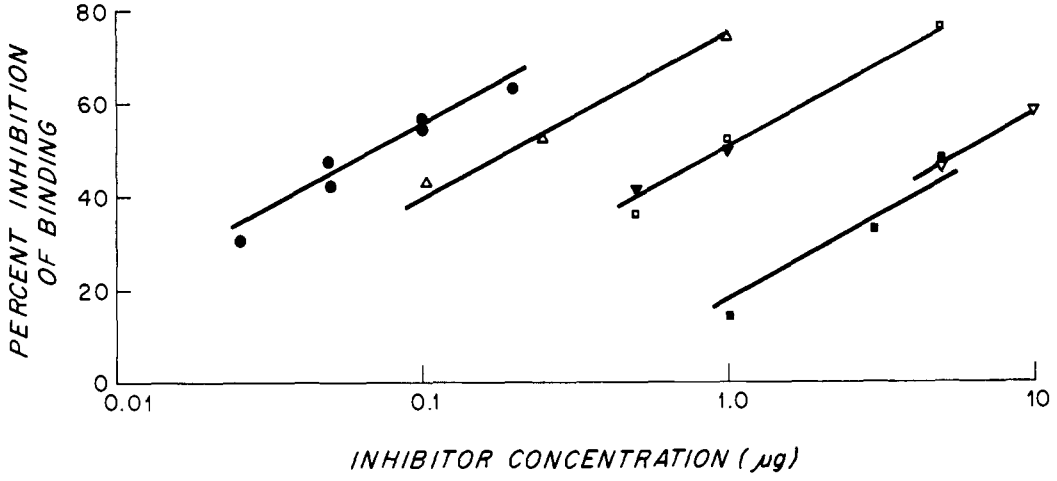

FIGURE 8: Binding inhibition produced by analogs of bradykinin altered either at the serine and phenylalanine positions or at the proline positions. The inhibition produced by bradykinin is shown for comparison. These data are summarized in Table I. (●) Bradykinin; (△) Gly^{6}-OMT^{8}-bradykinin; (▼) D-Pro^{2}-bradykinin; (□) D-Pro^{7}-bradykinin; (▽) OMT^{5}-Gly^{6}-OMT^{8}-bradykinin; (■) D-Pro^{3}-bradykinin.

Results

Optimal Incubation Time. When constant amounts of [^{3}H]acetylbradykinin and antibody were incubated for varying lengths of time and developed on Sephadex, the counts bound to antibody increased rapidly between 0.5 and 5 min, and then much more slowly between 5 and 30 min (Figure 3). For all inhibition experiments, a point near the beginning of the flat portion of the curve, 7.5 min, was chosen as the incubation time in order to minimize formation of endogenous kinin and enzymatic degradation of the kinin analogs.

Binding Decay Rate. In duplicate experiments, a single concentration of each analog was incubated with [^{3}H]acetylbradykinin and antibody for either 7.5 or 30 min, eluted from Sephadex, and the first effluent peak was counted. The ability of each analog to inhibit binding of radioactive bradykinin by the antibody was plotted as a function of time (Figures 4–6). Inhibition of binding decreased at a rate between 0.1 and 0.5% inhibition per min of incubation time for bradykinin, Des-(Arg^{9})-bradykinin, Ala^{2}-bradykinin, Ala^{7}-bradykinin, Ala^{8}-bradykinin, and Ala^{9}-bradykinin. Also in this group were Phe^{6}-bradykinin and OMT^{5}-Gly^{6}-OMT^{8}-bradykinin. All other analogs studied were in the range of 0.7–1.3 decrease in per cent inhibition per minute of incubation with the exception of Tlys^{6}-bradykinin, for which this figure was 1.8%

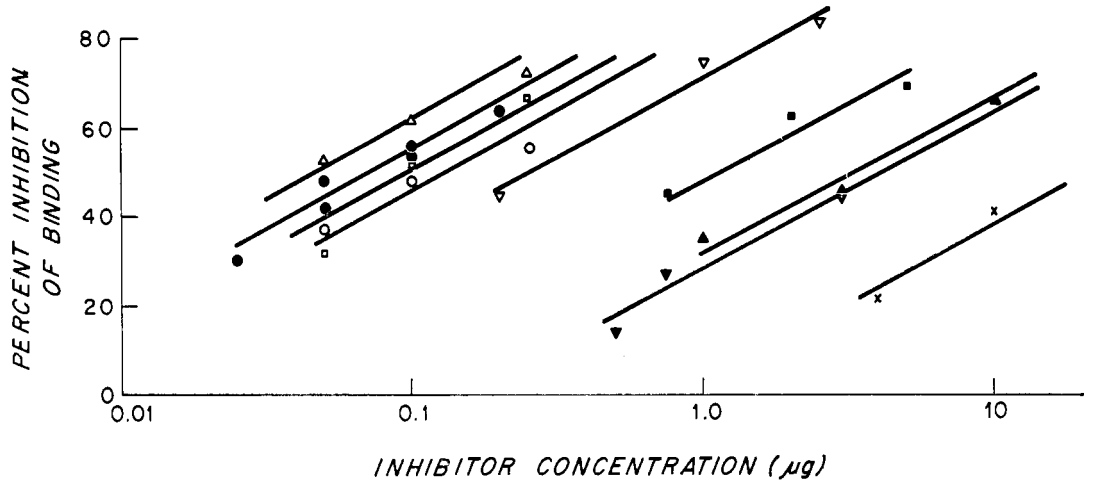

FIGURE 9: Binding inhibition produced by a series of singly substituted alanine analogs of bradykinin. The inhibition produced by bradykinin is shown for comparison. These data are summarized in Table II. (●) Bradykinin; (■) Ala^{2}-bradykinin; (×) Ala^{3}-bradykinin; (▼) Ala^{4}-bradykinin; (▲) Ala^{5}-bradykinin; (□) Ala^{6}-bradykinin; (▽) Ala^{7}-bradykinin; (○) Ala^{8}-bradykinin; (△) Ala^{9}-bradykinin.

inhibition/min. If the assumption is made that this rate can be linearly extrapolated to zero time, the resulting correction does not significantly alter the binding activity.

Immunoassay of Bradykinin Analogs. Antibody binding of various analogs was examined. Sequence variants were chosen in which alterations occurred in chain length, in identity of residues at the C terminus, in charge, in the hydrophobic or hydrophilic nature of the side chains, and in residues which have an effect on backbone conformation. The results are detailed in Figures 7–9 and are summarized in Tables I and II.

Discussion

Exclusion of Nonspecific Effects in the Radioimmunoassay

In assessing the ability of bradykinin analogs to bind to antibradykinin antibody by the radioimmunoassay method, it was desirable to have a radiolabeled hapten with binding properties similar to native bradykinin. It is apparent from the results that displacement by acetylated bradykinin is similar to, although somewhat better, than that of bradykinin, indicating that acetylation does not markedly alter the binding properties of bradykinin.

It was necessary to exclude several sources of error: nonspecific binding of the labeled hapten, enzymatic degradation of the peptides, and a high degree of antibody heterogeneity. It has been shown that tritiated acetylbradykinin was not bound by normal rabbit serum (Spragg *et al.*, 1966). Binding to surfaces was minimized by carrying out all incubations in either polypropylene or polycarbonate tubes.

To minimize differential rates of destruction of the analogs by serum enzymes, incubations were carried out at 4° for a 7.5-min period, by which time equilibrium has been reached. The rates of enzymatic degradation as determined by timed incubation studies indicate that differential rates of hydrolysis of the various peptides at 7.5 min only minimally affect the binding data.

The inhibition curves (Figures 7–9) suggest relative homogeneity of antibody binding. The curves are parallel for all analogs and may be extrapolated to full inhibition, suggesting that the same antibody population recognizes each of the analogs.

Analysis of Analog Inhibition Data

The inhibition data summarized in Tables I and II define many of the features of the sequence of bradykinin important for antibody recognition.

Nonapeptide Backbone Length. While the identity of the C-terminal residue is of little consequence, addition or removal of a residue at this end of the molecule considerably affects binding. Analogs with either alanine (Table II) or D-arginine (Table I) in this position are very well recognized by the antibradykinin antibody. The addition of an arginine (Table I) at this end of the molecule decreases binding to one-sixth that of bradykinin. The removal of the arginine in position 9 (Table I) decreases binding to $^{1}/_{16}$ that of bradykinin. Thus, the peptide group in position 9 contributes to the immunologic recognition of bradykinin while the side chain of this amino acid can be altered without significantly affecting binding. A possible interpretation of this requirement for optimal length is that the peptide exists in a cyclic form, and alterations in the length of the chain are incompatible with the stability of the ring.

Charge. Decreasing the positive charge by 1 either at the N terminus (Arg(Ac)^{1}-bradykinin, Table I) or at the C terminus (Ala^{9}-bradykinin, Table II), or by 2 (Arg(NO$_2$)^{1}-Arg(NO$_2$)^{9}-bradykinin, Table I)

TABLE I: Immunologic and Biologic Activity of Several Bradykinin Sequence Variants.

Peptide	Amino Acid Sequence 1	2	3	4	5	6	7	8	9	50% Inhibitory Concn (μg)	Binding Act.	Biol. Act. (rat uterus)[a]
Bradykinin	H-Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg-OH	0.070	1	1
Arg(Ac)^1-bradykinin	Ac-Arg									0.045	1.6	$^1/_2$
Arg(NO$_2$)^1-Arg(NO$_2$)^9-bradykinin	Arg(NO$_2$)								Arg(NO$_2$)	0.052	1.3	$^1/_{100}$[b]
D-Arg^9-bradykinin									D-Arg	0.088	$^1/_{1.3}$	$^1/_{1,000}$
Gly^6-OMT^8-bradykinin						Gly		OMT		0.200	$^1/_3$	1.2
Tlys^6-bradykinin						Tlys				0.210	$^1/_3$	$^1/_{2,000}$
Arg^{10}-bradykinin									-Arg	0.400	$^1/_6$	$^1/_{25}$
Phe^6-bradykinin						Phe				0.500	$^1/_7$	$^1/_{1,000}$
D-Pro^2-bradykinin		D-Pro								1.00	$^1/_{14}$	$^1/_{500}$
D-Pro^7-bradykinin							D-Pro			1.00	$^1/_{14}$	$^1/_{75}$
Des-(Arg^9)-bradykinin									□	1.15	$^1/_{16}$	Inactive[c]
OMT^5-Gly^6-OMT^8-bradykinin					OMT	Gly		OMT		6.40	$^1/_{91}$	$^1/_{15}$
D-Pro^3-bradykinin			D-Pro							8.00	$^1/_{110}$	$^1/_{10,000}$
D-Arg^1-D-Phe^5-D-Ser^6-D-Phe^8-D-Arg^9-bradykinin	D-Arg				D-Phe	D-Ser		D-Phe	D-Arg	>100	$<^1/_{1,400}$	$^1/_{10,000}$
D-Pro^2-D-Pro^3-D-Pro^7-bradykinin		D-Pro	D-Pro				D-Pro			>100	$<^1/_{1,400}$	$^1/_{200,000}$
"All D-bradykinin"	D-Arg	D-Pro	D-Pro		D-Phe	D-Ser	D-Pro	D-Phe	D-Arg	>100	$<^1/_{1,400}$	$^1/_{200,000}$

[a] From Stewart and Woolley (1966), except where noted. [b] From Bodanzsky *et al.* (1963). [c] From Schröder and Hempel (1964).

slightly enhances binding. The addition of a charge (Arg^{10}-bradykinin, Table I) somewhat decreases binding, but this may well be because of the increase in the length of the polypeptide chain as discussed above. Thus, the over-all molecular charge does not seem to contribute to the immunologic recognition of bradykinin.

Hydrophobic and Hydrophilic Groups. In general, changes from hydrophilic to hydrophobic groups and *vice versa* have little effect on antibody binding. For example, when the hydrophobic nature of the side chains is reduced, as by substituting alanine for phenylalanine in position 8 (Table II), or the hydrophilic nature is modified by substituting phenylalanine (Table I) or alanine (Table II) for the serine in position 6, there is little alteration in binding. However, the identity of phenylalanine in position 5 is important, for any substitution of this residue, such as by alanine (Table II) or OMT (Table I) substantially reduces binding.

It is of considerable interest that the addition of a bulky side-chain residue, such as Tlys or phenylalanine, instead of serine in position 6 (Table I) has little effect on binding. These results indicate that the serine hydroxyl group is present in the peptide in a location that is not sterically confined, so that a large substituent group such as an aromatic ring can be introduced in this location without altering the conformation of the entire peptide.

Peptide-Backbone Alterations. The substitution of glycine by alanine (Table II) decreases binding to $^1/_{57}$ that of bradykinin, which represents a major alteration in binding by a single residue substitution. A possible explanation is that the addition of a β-carbon limits rotation about the N–α-C and α-C–C bonds (Ramachandran *et al.*, 1963), and may exclude the conformation of the native molecule.

Changes in bradykinin analogs which have the greatest effect on antigenicity are alterations of the proline in position 3. Substitution of D-proline in this position decreases binding by a factor of 110 (Table I), and substitution of alanine, by a factor of 270 (Table II). When the proline in position 2 was replaced by either alanine (Table II) or D-proline (Table I), binding was decreased to $^1/_{14}$ that of bradykinin. Replacement of the proline in position 7 by alanine decreased binding to $^1/_4$ (Table II), and replacement of this proline by D-proline, to $^1/_{14}$ that of bradykinin (Table I). When all three prolines were in the D configuration, binding was decreased by a factor of 1400 (Table I). The presence of proline limits rotation about the peptide bond and essentially fixes backbone structure in the region of the molecule where it is present (Ramachandran *et al.*, 1963). Replacement by alanine increases the possible conformations; replacement by D-proline fixes the conformation in the opposite sense. The results indicate that the proline in position 3 is of particular importance in determining the structure of bradykinin, for its substitution by alanine or D-proline has a tenfold greater effect on binding than do changes made in the prolines in positions 2 or 7.

TABLE II: Immunologic and Biologic Activity of Alanine-Substituted Bradykinin Variants.

Peptide	Amino Acid Sequence									50% Inhibitory Concn (μg)	Binding Act.	Biol Act. (rat uterus)[a]
	1	2	3	4	5	6	7	8	9			
Bradykinin	H-Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg-OH	0.070	1	1
Ala^9-bradykinin									Ala	0.045	1.6	$^1/_{500}$
Ala^6-bradykinin						Ala				0.100	$^1/_{1.4}$	$^1/_{10}$
Ala^8-bradykinin								Ala		0.160	$^1/_2$	$^1/_{1500}$
Ala^7-bradykinin							Ala			0.250	$^1/_4$	$^1/_{80}$
Ala^2-bradykinin		Ala								1.00	$^1/_{14}$	$^1/_{300}$
Ala^5-bradykinin					Ala					3.10	$^1/_{44}$	$^1/_{1000}$
Ala^4-bradykinin				Ala						4.00	$^1/_{57}$	$^1/_{1500}$
Ala^3-bradykinin			Ala							19.00	$^1/_{270}$	1

[a] From Schröder and Hempel (1964).

The results indicate that those substitutions which necessarily change the over-all shape of the peptide have major effects on binding. This suggests that the entire bradykinin molecule comprises the antigenic site. Unlike the peptide azoproteins of Landsteiner (1945) or the branch-chain copolymers of Fuchs and Sela (1963), alterations in the amino acid residues at the distal end of the bradykinin branch chains of the immunizing antigen do not have a significant effect on antigenicity, as seen with D-Arg^9-bradykinin, Arg^{10}-bradykinin, Ala^9-bradykinin, Ala^8-bradykinin, and Arg$(NO_2)^1$-Arg$(NO_2)^9$-bradykinin, for example. Conversely, a single amino acid change made in a residue, that is, for example, six (Ala^4-bradykinin) or seven (Ala^3-bradykinin or D-Pro^3-bradykinin) residues away from the distal end of the immunizing antigen has a profound effect on antigenicity. Further support of the antigenic importance of the entire peptide comes from the requirement of optimal chain length for binding.

The points made above regarding the conformation of the bradykinin molecule as inferred from the immunologic studies have been supported by building a hypothetical space-filling model incorporating accurate van der Waals' radii. This model, described by Haber *et al.* (1967), is a tightly packed, globular structure with a diameter of approximately 16 A. Models of bradykinin analogs indicate that the greatest changes in the antigenicity of the peptide are correlated with substitutions that have an obligatory effect on the over-all shape of the model.

Relationship between Immunologic and Biologic Activity

The relationship between binding to antibody and biologic activity on the estrus rat uterus is shown in Tables I and II. This bioassay system was chosen because it is the only one in which data are available for all of the analogs discussed here. Where data in other assay systems are available, the biologic activity relative to bradykinin generally correlates well with the relative biologic activities measured on the rat uterus (Schröder and Hempel, 1964; Stewart, 1967; Donaldson and Ratnoff, 1967).

In most instances, any change in the carboxy-terminal end of bradykinin results in a great loss in biologic activity, as in D-Arg^9-bradykinin (Table I) or Ala^9-bradykinin (Table II). Alterations of the residue in this position did not greatly alter binding in the immunoassay.

Backbone length is important for immunologic binding (Table I) and for biologic activity as indicated by the work of Stewart and Woolley (1966). They measured the uterotonic activity of a series of nonapeptide bradykinin analogs and the corresponding octapeptides which lacked the amino-terminal residue. For each pair of analogs, the octapeptide was at least one order of magnitude less active than the corresponding nonapeptide. The metabolic breakdown product of bradykinin, Des-(Arg^9)-bradykinin is biologically inactive, as are most other octapeptide analogs (Schröder and Hempel, 1964).

While any charge alteration has a slight enhancing effect on immunologic binding, the importance of charge in reducing biologic activity depends upon the location of the charged group in the molecule. Neutralization of the N-terminal amino group has very little effect on biologic activity, while neutralization of the guanidino group charges in Arg$(NO_2)^1$-Arg$(NO_2)^9$-bradykinin reduces biologic activity 100-fold. Neutralization of the guanidino charge in position 9 alone, by replacement of that arginine residue with alanine, reduces biologic activity 500-fold.

Alterations of the phenylalanines have a mixed effect depending upon the specific change. Replacement of either the phenylalanine in position 8 or both phenylalanines with OMT produces a slight enhancement or a moderate decrease, respectively, in biologic activity. This contrasts to the considerable importance in the immunologic system of the phenylalanine in position 5. Loss of both aromatic rings reduces biologic activity to $^1/_{30,000}$ that of bradykinin (Stewart and Woolley, 1966), which is consistent with major immunologic effects.

Unlike the immunologic system, substitution of the serine hydroxyl group by Tlys or by phenylalanine greatly reduces biologic activity, suggesting that this side chain is important for biologic activity or that the residues spatially close to the serine residue are necessary for biologic activity and that the introduction of a bulky aromatic group in this region of the molecule sterically interferes with interaction of bradykinin with the biologic receptor site. The latter hypothesis is strengthened by the relatively lesser effect of substituting alanine for serine.

As in the immunoassay, alterations in the peptide backbone have a profound effect upon biologic activity, with the exception of Ala^3-bradykinin, which has full biologic activity. D-Pro^3-bradykinin has $^1/_{10,0000}$ the biologic activity of bradykinin. Replacement of the proline in position 7 by alanine or D-proline reduces biologic activity 80- or 75-fold, and the same replacements in position 2 decrease biologic activity 300- or 500-fold. The substitution of alanine for glycine in position 4 results in $^1/_{1500}$ the biologic activity of bradykinin.

The relationship between immunologic and biologic activity of bradykinin analogs indicates both common and unrelated structural requirements. Whether the differences between the two reflect a difference in conformation between the free peptide and the peptide covalently linked to polylysine or bound by antibody, or a difference in the regions of the bradykinin molecule that are important for immunologic or pharmacologic activity is unknown.

The data presented here show that bradykinin, as a haptenic determinant coupled to a homopolymer backbone, or as a free hapten bound by antibody, exists as a structured, possibly globular, peptide, the over-all conformation of which is of primary importance in determining antigenic identity.

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