# *ANTIBODIES TO A UNIQUE REGION IN LYSOZYME PROVOKED BY A SYNTHETIC ANTIGEN CONJUGATE**

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*Abstract and Summary.*—A synthetic conjugate, prepared by covalent binding of a lysozyme fragment (sequence 64–83, denoted "loop" peptide) to a synthetic branched polypeptide, elicited in rabbits the formation of antibodies with specificity directed against a unique region in native lysozyme. These anti-"loop" antibodies were isolated immunospecifically on a lysozyme-cellulose immunoadsorbent. Antibodies with a similar specificity were isolated from antilysozyme sera with an immunoadsorbent prepared from the same "loop" peptide. The capacity of the anti-"loop" antibodies to distinguish between the "loop" peptide, containing a disulfide bridge, and the open-chain peptide derived from it suggests that they are directed against a conformation-dependent determinant.

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Antiprotein sera usually contain mixtures of antibodies against different determinants on the protein.[1, 2] As the determinants of globular proteins are mostly conformation-dependent, their elucidation clearly requires a detailed knowledge of both the amino acid sequence and the three-dimensional structure of the protein. Indeed, recent immunological studies of myoglobin[3–5] and of lysozyme[6, 7] have pinpointed some immunopotent regions in these molecules.

We have recently investigated the role of steric conformation in the immunogenicity and the antigenic specificity of some synthetic polypeptides[8] and have shown, for example, that the collagenlike triple-stranded helical polymer of the tripeptide ProGlyPro led to the formation of antibodies cross-reacting with collagens of several species.[2] It was also shown that the attachment of various small molecules, such as nucleosides[9] and glycolipids,[10] to synthetic polypeptides resulted in conjugates that are capable of provoking antibodies which react specifically, for example, with single-stranded DNA[9] and with the naturally occurring cytolipin H.[10] We report here another instance of a synthetic polypeptide conjugate, which leads to antibodies cross-reacting with a natural protein, egg-white lysozyme.

A peptide prepared by the action of pepsin on lysozyme[11] is its major antigenic determinant.[6, 7] Recently, Arnon[12] used an immunoadsorbent prepared from this peptide[11] and bromoacetylcellulose[13] to fractionate antilysozyme antibodies. We have now separated this pepsin-produced lysozyme peptide, after reductive cleavage and reoxidation, into two peptide chains. One of these chains, denoted the "loop" peptide and containing one disulfide bridge (Fig. 1), was attached chemically to multichain poly-DL-alanine.[14] The immunization of rabbits with the "loop" polyalanine conjugate led to the formation of antibodies capable of binding lysozyme. These antibodies were isolated immunospecifically on a lysozyme-cellulose immunoadsorbent. Antibodies of a similar specificity were

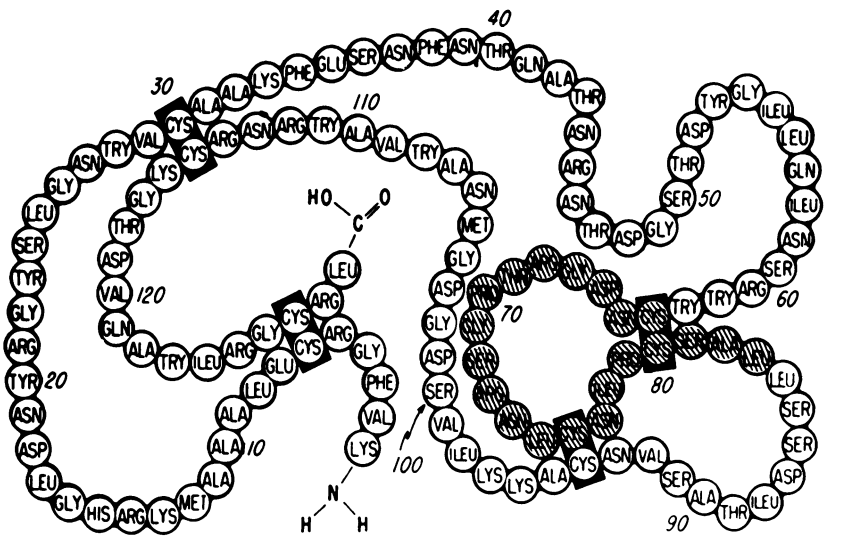

FIG. 1.—Amino acid sequence of hen egg-white lysozyme (reproduced from Canfield and Liu[11]). The region of the "loop" peptide is shaded.

isolated from antilysozyme sera, making use of an immunoadsorbent prepared from the "loop" peptide and bromoacetylcellulose.

*Materials and Methods.*—Twice-crystallized egg-white lysozyme was obtained from Worthington. "Peptide 1" was prepared from a peptic digest of lysozyme.[11, 12] The "loop" peptide was prepared by reduction of "peptide 1" (20 mg/ml) with dithiothreitol (final conc. 0.025 mM), in 0.05 *M* Tris-HCl buffer, pH 8.2. After 1 hr at 37°, the reaction mixture was diluted fivefold with 0.05 *M* $NH_4OH$, and the products were separated on a Sephadex G-25 (fine) column, equilibrated with 0.05 *M* $NH_4OH$ (Fig. 2). The third peak eluted off the column contained the "loop" peptide. Reduced and carboxymethylated "loop" peptide (RCM "loop") was prepared by reduction of the "loop" peptide in 0.05 *M* Tris-HCl buffer, pH 8.2, with 0.1 *M* 2-mercaptoethanol at 37° for 1 hr, and by alkylation with a molar excess of $C^{14}$-labeled iodoacetic acid (Amersham). (Concentrated Tris solution was added to adjust the pH to 8.2.) RCM "loop" was purified by gel filtration on Sephadex G-25 in 0.05 *M* $NH_4OH$. The extent of carboxymethylation was determined by monitoring the radioactivity and by amino acid analysis.

The multichain polymer, multi-poly-DL-alanyl-poly-L-lysine (A--L),[14] was prepared from *N*-carboxy-DL-alanine anhydride and poly-L-lysine, in a residue molar ratio of Ala:Lys, 16.6:1. To a mixture of 250 mg A--L and 70 mg "loop" peptide in 23 ml $H_2O$, 50 mg of 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide hydrochloride (Ott Chemicals) was added. After 20 hr at room temperature, the slight precipitate formed was centrifuged off, and the mixture was applied to a Sephadex G-100 column (75 × 2.5 cm) and eluted with water. The material under the main peak was freeze-dried. H contained 4.85% (w/w) of the "loop" peptide, as determined by amino acid analysis. It had a sedimentation coefficient, 3.88*S*, in 0.9% NaCl solution, similar to that of the parent A--L. Its electrophoretic mobility on cellulose acetate at pH 8.2 was distinctly different from that of A--L. This material is designated here as "loop"-peptide-multi-poly-DL-alanyl--poly-L-lysine ("loop" A--L).

Lysozyme immunoadsorbent was prepared from lysozyme and bromoacetylcellulose.[13] Attachment of the "loop" peptide to bromoacetylcellulose was carried out analogously.

*Immunological procedures:* Four rabbits were immunized by the injection of 10 mg of "loop" A--L, in complete Freund's adjuvant (Difco), into multiple intradermal sites, fol-

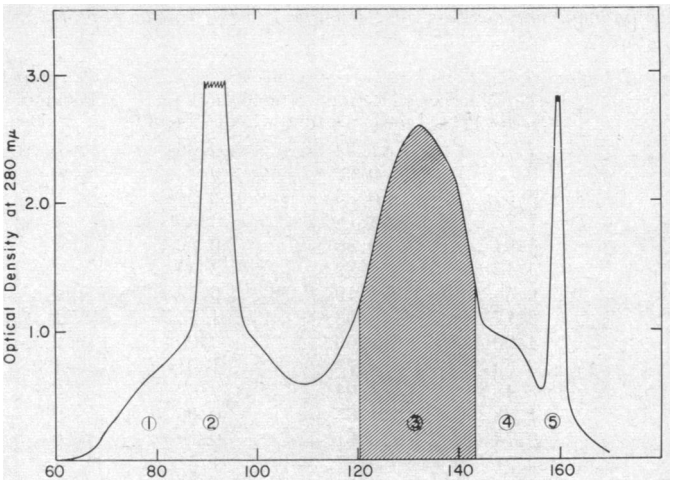


FIG. 2.—Chromatographic separation on a Sephadex G-25 (fine) column (270 × 3 cm) equilibrated with 0.05 *M* $NH_4OH$ of the reduced and reoxidized "peptide 1." The shaded zone contains the "loop" peptide.

lowed 20 and 50 days later by two additional injections of 10 mg each. The rabbits were bled weekly from the marginal ear vein, and the sera collected were used for precipitin inhibition tests.[15] Antigen-binding capacity experiments were carried out with $I^{125}$ (Amersham)-labeled lysozyme or with $C^{14}$-labeled RCM "loop," with the use of goat anti-(rabbit IgG) serum.[12] Antigen-binding experiments with purified antibodies were carried out also by adding excess labeled lysozyme to the antibody preparations and passing the complex through a Sephadex G-100 column. The extent of binding was calculated from the radioactivity and the optical density of the globulin peak. Passive cutaneous anaphylaxis (PCA) reactions and assays of their inhibition were carried out by the methods described by Ovary.[16]

The antibodies were tested for their capacity to inhibit[12] the activity of lysozyme either on *M. lysodeikticus* or on penta-*N*-acetylglucosamine.[17]

*Results.—Preparation and characterization of the "loop" peptide:* The "peptide 1" of Canfield and Liu[11] consisted of the sequences 64–83 and 91–107 of lysozyme, linked by a disulfide bridge, whereas a peptide prepared by an analogous procedure by Shinka *et al.*[7] contained the residues 57–63 in addition. The amino acid composition of "peptide 1" used in this study showed the presence of both types of peptides, as indicated by the presence of less than equimolar amounts of glutamic acid and of tryptophan (Table 1). Efforts to separate the two component peptides of "peptide 1," after reduction and carboxymethylation by gel filtration, were unsuccessful. The separation was successful, however, when the reduction of "peptide 1" was followed by reoxidation in dilute solution and gel filtration in an alkaline medium (Fig. 2). Fraction 1 had the amino acid composition of the linear peptide sequence 91–107, fraction 2 was a mixture (or aggregate) of both the linear and the "loop" peptide, whereas fraction 3 had the amino acid composition of the "loop" peptide (Table 1). Fraction 4 had the same amino acid composition as fraction 3, but as it was somewhat turbid, it was not

TABLE 1. *Amino acid composition of "peptide 1," the linear peptide, and the "loop" peptide.**

| Amino acid | Number of Residues Found† in: "Peptide 1" | Linear peptide | "Loop" peptide | Calculated Number of Residues in Sequences 64–83 | 57–83 |
|---|---|---|---|---|---|
| Lys | 2.22 | 1.82 | 0.06 | 0 | 0 |
| His | 0 | 0.02 | 0 | 0 | 0 |
| Arg | 2.54 | 0.25 | 2.59 | 2 | 3 |
| Asp | 7.72 | 3.78 | 4.35 | 4 | 5 |
| Thr | 0.94 | 0.20 | 0.92 | 1 | 1 |
| Ser | 4.17 | 1.82 | 2.35 | 2 | 3 |
| Glu | 0.54 | 0.10 | 0.57 | 0 | 1 |
| Pro | 2.17 | 0.03 | 1.74 | 2 | 2 |
| Gly | 4.00 | 2.00 | 2.00 | 2 | 2 |
| Ala | 2.89 | 1.78 | 1.01 | 1 | 1 |
| ½ Cys‡ | 3.48 | 1.04 | 2.62 | 3 | 3 |
| Val | 1.88 | 1.68 | 0.08 | 0 | 0 |
| Met | 0.74 | 0.61 | 0 | 0 | 0 |
| Ileu | 2.21 | 0.87 | 1.51 | 1 | 2 |
| Leu | 2.22 | 0.26 | 1.88 | 2 | 2 |
| Tyr | 0 | 0 | 0 | 0 | 0 |
| Phe | 0 | 0 | 0 | 0 | 0 |
| Try§ | 1.38 | 0 | 1.32 | 0 | 2 |

* The results were calculated from the average recoveries of duplicate analyses of three different preparations.

† The number of residues per molecule of peptide was calculated assuming that "peptide 1" contains four residues of glycine, the linear peptide contains two residues of glycine, and the "loop" peptide contains two residues of glycine.

‡ The values in the table represent the average between the results obtained in amino acid analysis after reduction and alkylation and after performic acid oxidation.

§ Tryptophan contents were calculated from the absorbency at 280 mμ and by the colorimetric determination with Koshland's reagent.[18]

combined with fraction 3. Fraction 5 contained the reducing agent. The molar recoveries of the linear (fraction 1) and the "loop" peptide (fraction 3) were very similar, although this is not reflected in Figure 2 due to the absence of tryptophan and tyrosine in the linear peptide.

The "loop" peptide had an average molecular weight of 2500 ± 20 per cent, as determined by the approach to equilibrium method in the analytical ultracentrifuge[19] (molecular weight calculated for sequence 57–83 is 2930, and for sequence 64–83 is 2116). Half-cystine residues in the "loop" peptide were determined either after reduction and alkylation (2.66 carboxymethylcysteine residues per peptide) or after performic acid oxidation (2.57 cysteic acid residues per peptide). It can therefore be concluded that each mole of the "loop" peptide contains 3 moles of half-cystine, as expected. However, no free cysteine could be detected in the "loop" peptide either after alkylation or upon reaction with *p*-mercuribenzoate.

*Anti-"loop" peptide antibodies obtained with a synthetic antigen conjugate:* The "loop" peptide was attached, with the aid of a water-soluble carbodiimide, to multichain poly-DL-alanine, and the resulting synthetic conjugate was used for immunization of rabbits.

The homologous precipitin reaction of the system "loop" A--L–anti-"loop" A--L is shown in Figure 3. Multichain polyalanine precipitated some antibodies, but

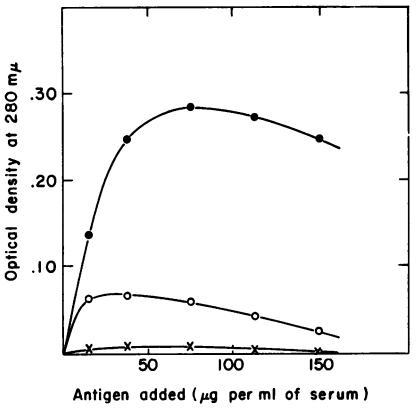


FIG. 3.—Precipitin reaction of anti-"loop" A–L serum with "loop" A–L (●), A–L (○), and lysozyme (×).

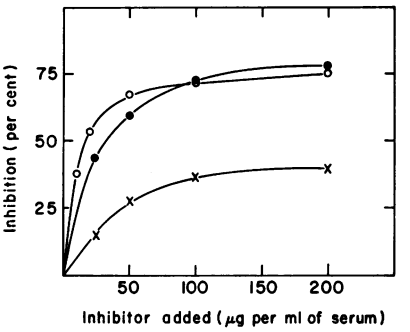


FIG. 4.—Inhibition of the "loop" A–L-anti-"loop" A–L precipitin reaction by the "loop" peptide (●), RCM "loop" (○), and lysozyme (×).

no precipitate was formed with lysozyme. Similarly, the PCA reaction was positive for the homologous system, but negative for lysozyme.

The specificity of the antibodies obtained is apparent from inhibition studies. The "loop" peptide inhibited up to 75 per cent of the homologous precipitin reaction, and the RCM "loop" was almost as efficient an inhibitor (Fig. 4). The difference between their inhibitory efficiency was much more noticeable when the inhibition of the "loop" A–L-anti-"loop" A–L system was followed by the PCA technique (Table 2). Neither peptide caused any inhibition of the unrelated precipitating system ovalbumin-antiovalbumin.

The relationship of lysozyme to the anti-"loop" A–L antibodies is demonstrated by its capacity to inhibit both the precipitin (Fig. 4) and the PCA (Table 2) reactions, whereas nonrelated proteins, such as ribonuclease, had no inhibitory effect. The anti-"loop" A–L antibodies capable of reacting with lysozyme were purified on a lysozyme immunoadsorbent. Out of 200 ml antiserum containing 60 mg antibodies precipitable with the homologous antigen, 50 mg antibodies were isolated on 1 gm immunoadsorbent. The purified antibodies did not precipitate with lysozyme but were capable of specifically binding it, as shown in Table 3. The relative amount of lysozyme bound to these antibodies is lower than that bound to specific antilysozyme antibodies, probably due to differences in their affinities.

In contrast to purified antilysozyme antibodies, which inhibit efficiently the enzyme activity of lysozyme,[12] the isolated anti-"loop" antibodies had almost no inactivating effect when *M. lysodeikticus* was the substrate (Fig. 5), and there was no effect at all when the activity of lysozyme was assayed on penta-*N*-acetylglucosamine.

*Anti-"loop" antibodies obtained after immunization with lysozyme:* A small fraction of antilysozyme antibodies is specific to the "loop" peptide. This is apparent both from the capacity of the "loop" peptide to inhibit the precipitin reaction of lysozyme with rabbit antilysozyme sera (Fig. 6) and from its ability

TABLE 2. *Inhibition of passive cutaneous anaphylaxis reactions.**

| | Homologous Reaction | | | | |
|---|---|---|---|---|---|
| | "Loop" A–L–Anti-"Loop" A–L (serum dilution 1:128) | | | Lysozyme-Antilysozyme (serum dilution 1:1024) | |
| | Inhibitor | | | | |
| Concentration (mg/ml) | "Loop" | RCM "Loop" | Lysozyme | "Loop" | RCM "Loop" |
| | Diameter of Lesions (mm) | | | | |
| 0 | 12; 10 | 14; 16 | 13; 12 | 12; 13 | 14; 11 |
| 0.031 | | | | 12; 15 | 13; 12 |
| 0.062 | 0; 0 | 13; 13 | 10; ± | 12; 12 | 13; 12 |
| 0.125 | 0; 0 | 10; 10 | 10; ± | 9; 9 | 14; 11 |
| 0.25 | 0; 0 | 0; 0 | 10; 0 | 0; 6 | 14; 10 |
| 0.5 | 0; 0 | 0; 0 | 0; 0 | 0; 0 | 15; 12 |
| 1 | 0; 0 | 0; 0 | 0; 0 | 0; 0 | 15; 12 |

* Preliminary experiments were carried out with each of the two antisera to determine the lowest concentration giving a positive reaction. All inhibition experiments were performed at that antiserum concentration by mixing with different concentrations of inhibitor. The mixtures (0.1 ml) were injected intradermally into guinea pigs, which were challenged after 6 hr intracardially by a mixture of the homologous antigen (1 mg/ml) and Evans blue (0.7%).

TABLE 3. *Binding of lysozyme to antibodies.**

| Antibody sample | Antibody in complex (mg) | Lysozyme in complex (μg) | Ab/Ag ratio (M/M) |
|---|---|---|---|
| Anti-"loop" A–L | 1.4 | 37 | 4.0 |
| " | 3.1 | 73 | 4.2 |
| " | 1.05 | 29 | 3.6 |
| Antilysozyme | 2.75 | 165 | 1.67 |
| Normal rabbit IgG | 4.5 | 5 | 90. |

* A sample of purified antibodies (or normal rabbit IgG) was incubated with an excess of $I^{125}$-labeled lysozyme. The mixture was passed through a Sephadex G-100 (equilibrated with 0.05 *M* Tris buffer, pH 7.5) column (50 × 1 cm), and both the optical density and the radioactivity of the first eluted peak were monitored for the quantitation of the lysozyme and the antibody in the complex.

to inhibit the homologous lysozyme PCA reaction (Table 2). Neither the precipitin nor the PCA reaction is inhibited at all by the RCM "loop" (Fig. 6 and Table 2). Thus, the anti-"loop" antibodies obtained with lysozyme discriminate between the peptide still containing a disulfide bridge and the same peptide after the bridge was opened, to a much greater extent than do the anti-"loop" antibodies obtained with the synthetic polypeptide conjugate.

The fraction of the antilysozyme antibodies which is capable of interacting with the "loop" peptide was isolated on an immunoadsorbent prepared from the "loop" peptide and bromoacetylcellulose. Out of 500 ml antilysozyme serum, containing 1 gm antibodies, 70 mg anti-"loop" antibodies were isolated on 1 gm immunoadsorbent.

*Discussion.*—The chemical formula of the hen egg-white lysozyme has been elucidated by Jollès *et al.*[20, 21] and by Canfield,[22, 11] and its crystallographic structure has been established by Phillips and his colleagues.[23, 24] The present investigation shows that it is possible to prepare antibodies against a unique region in lysozyme, corresponding to the sequence 57–83, still containing one disulfide bridge. These antibodies may be obtained either by their selective isolation from antilysozyme sera or by immunization with a synthetic polypeptide conjugate, followed by isolation on a lysozyme immunoadsorbent. In both cases the antibodies are due to conformation-dependent determinants.

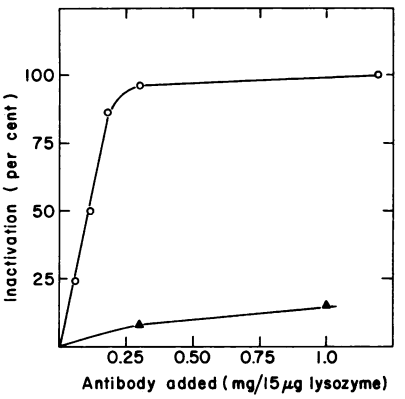


FIG. 5.—Inhibition of lysozyme activity on *M. lysodeikticus* by antilysozyme antibodies (○) and by anti-"loop" antibodies (▲), isolated from anti-"loop" A--L sera.

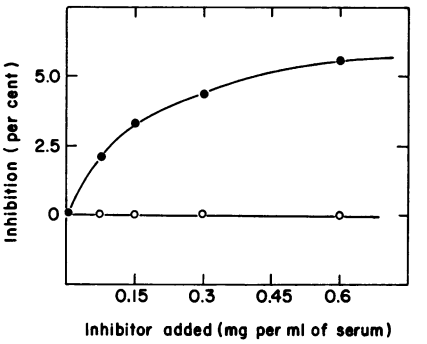


FIG. 6.—Inhibition of lysozyme–antilysozyme precipitin reaction by the "loop" peptide (●) and by RCM "loop" (○).

The main finding of this paper is that it is possible to prepare a synthetic conjugate from a branched polypeptide and a unique peptide isolated from a protein, and that such a conjugate provokes in rabbits the formation of antibodies cross-reacting with the protein. The chemical binding of the "loop" peptide of lysozyme with multichain poly-DL-alanine, by means of a water-soluble carbodiimide, occurred between the $\alpha$-amino termini of the poly-DL-alanine side chains and either the $\alpha$-carboxyl of the carboxy-terminal leucine (position 83) or the $\beta$-carboxyl of the aspartyl residue in position 66.

Most of the antibodies formed against "loop" A--L were reactive with lysozyme, as is apparent both from the capacity of lysozyme to inhibit precipitin (Fig. 4) and PCA (Table 2) reactions, and from the successful isolation of the anti-"loop" A--L antibodies on a lysozyme immunoadsorbent. These anti-"loop" antibodies did not give visible precipitates with lysozyme, in accordance with the assumption that only one unique region in lysozyme is responsible for the interaction. This does not necessarily imply a unique determinant, as it is still possible that the "loop" contains several overlapping determinants. In the latter case, the "loop" would not be able to combine simultaneously with more than one antibody.

The antibodies obtained by immunization with the synthetic conjugate and directed against the lysozyme "loop" reacted also with RCM "loop," with the peptide resulting from the opening of the disulfide bridge in the "loop" peptide (Fig. 4). As shown in Table 2, RCM "loop" was definitely less efficient in the inhibition of the PCA reaction than the original "loop." The anti-"loop" antibodies obtained by immunization with lysozyme did not react at all with RCM "loop" (Fig. 6 and Table 2). Thus, it seems that the anti-"loop" antibodies are directed against a conformation-dependent determinant. The "loop" must have been of a more rigid conformation within the native lysozyme than when attached to the branched polyalanine, since the antibodies obtained with lysozyme did not cross-react with the open-chain "loop," whereas those obtained with the syn-

thetic conjugate did. The capacity of the latter antibodies to react with the RCM "loop" may be due to the ease with which, in this case, the open-chain peptide may transconform to fit the antibody cavity.

Previous studies have shown that antilysozyme antibodies against antigenic determinants on "peptide 1," which contains the "loop," were very poor lysozyme inhibitors.[12] As described in this paper, the anti-"loop" antibodies, indeed, had almost no inactivating effect on lysozyme. These results are consistent with the known crystallographic structure of the enzyme.[23, 24]

Antibodies to single protein determinants should be useful in efforts to obtain a better understanding of antigenic determinants of native proteins. Moreover, the availability of two populations of antibodies, directed most probably against the same unique region within a protein, provides a good opportunity to compare them and to learn the role of the antigenic "carrier"[25] in the homogeneity of antibodies to a unique determinant.

The following abbreviations were used: RCM, reduced and carboxymethylated peptide; and PCA, passive cutaneous anaphylaxis.

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