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Immunochemical Studies on the Tobacco Mosaic Virus Protein. VI. Characterization of Antibody Populations Following Immunization with Tobacco Mosaic Virus Protein*

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ABSTRACT: Studies were undertaken to elucidate the area or areas on an antigenic tryptic eicosapeptide, representing residues 93–112 of the tobacco mosaic virus protein (TMVP), which bind with antibodies derived from different rabbits following immunization with the whole protein. Experiments were performed on the binding of antibodies obtained from various rabbits with [^{14}C]acetylated peptides, representing various areas on the eicosapeptide, and on the inhibition of this binding by various nonacetylated peptides. These experiments revealed that all antisera tested contained antibodies which reacted with the eicosapeptide; however different antisera contained antibodies which bind with one or more different areas on the eicosapeptide. Moreover, it appears that populations of antibodies produced by a single rabbit at various time intervals following the initial immunization may change with respect to binding with various antigenic areas, the binding with smaller antigenic areas increasing with time and/or quantity of injected antigen.

Antigens which have been used for characterization of antibodies have consisted of oligosaccharides, synthetic polypeptides, and hapten conjugates. These investigations have been the subject of several reviews (Karush, 1962; Singer, 1965; Kabat, 1966). Since the characterization of antibodies to protein antigens suffered from the lack of information on the antigenic determinant or determinants of proteins, no parallel work on the characterization of antibodies to proteins has been performed.

Recently, several antigenic determinants of protein antigens have been elucidated (reviewed by Crumpton, 1967). One such protein is that of the tobacco mosaic virus, which it is felt affords a tool for the characterization of antibodies to a defined antigenic area of a protein.

It has been reported from this laboratory that a tryptic eicosapeptide representing residues 93–112 of the tobacco mosaic virus protein (TMVP) and having the amino acid sequence Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg exhibited immunological activity related to the whole protein (Benjamini *et al.*, 1964, 1965). It was further reported that the synthetic C-terminal decapeptide and C-terminal pentapeptide of the above tryptic peptide exhibited specific binding with anti-TMVP whereas shorter C-terminal peptides did not (Stewart *et al.*, 1966; Young *et al.*, 1967).

It was observed in our laboratory that not all anti-TMVP sera obtained from different rabbits exhibited binding with the C-terminal decapeptide or pentapeptide, although strong binding was always observed with the eicosapeptide. It was therefore of interest to ascertain the area or areas on the eicosapeptide which bind with antibodies obtained from different rabbits and with antibodies obtained from an individual rabbit during the course of immunization with TMVP. The present communication reports the results of these studies.

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Materials and Methods

Tobacco mosaic virus protein (TMVP) was prepared from tobacco mosaic virus[1] by 67% acetic acid treatment (Fraenkel-Conrat, 1957).

Preparation of Peptides. Tryptic eicosapeptide (tryptic peptide 8 of TMVP[2]) was prepared by tryptic digestion of TMVP as described by Funatsu (1964) and by Young *et al.* (1966). The purity of the peptide was ascertained by electrophoresis and by amino acid analysis (Young *et al.*, 1966).

The desarginated C-terminal decapeptide of TMVP tryptic peptide 8 was prepared by carboxypeptidase B treatment of the synthetic [^{14}C]acetyl decapeptide, in a manner similar to that described for the preparation of desarginated peptide 8 (Young *et al.*, 1966). The amino acid analysis of the [^{14}C]acetyl desarginated decapeptide presented in Table I shows that the molar ratios of the amino acids are close to the theoretically expected values.

The C-terminal deca- and the pentapeptide portions of the above tryptic peptide 8, having the amino acid sequences Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg[3] and Leu-Asp-Ala-Thr-Arg,[3] respectively, and a decapeptide having the sequence Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg[3] were synthesized by the Merrifield solid-phase peptide synthesis method (Merrifield, 1964), as described by Stewart *et al.* (1966) and Young *et al.* (1967). Only L-amino acids were used. Following cleavage from the resin and reduction, each peptide was purified by chromatography on a 1 × 150 cm Dowex 1-X2 ion-exchange column, equilibrated, and eluted as previously described (Young *et al.*, 1966). The purity of the peptides was ascertained by paper electrophoresis at pH 6.4 (Benjamini *et al.*, 1965) and by amino acid analysis of 15-hr hydrolysates (6 N HCl under open reflux). The amino acid analyses of the peptides used for the present experiments are shown in Table I.

A portion of each peptide was acetylated with [^{14}C]-acetic anhydride and the [^{14}C]acetyl peptide was purified by chromatography on Sephadex G-10 as previously described (Stewart *et al.*, 1966). Hydrolysates of the [^{14}C]acetyl peptides (15 hr in 6 N HCl at 110° under open reflux) were subjected to amino acid analyses. Results of the analyses presented in Table I show that the molar ratios of the amino acids agree with the theoretical values. The purity of each of the [^{14}C]acetyl peptides was further ascertained by paper electrophoresis at pH 6.4 (Benjamini *et al.*, 1965). The presence of a single radioactive peak which coincided with a single peptide peak after spraying for the presence of peptides (Nitecki and Goodman, 1966) and with a single Sakaguchi positive spot indicated the purity of each acetylated peptide. The specific activities of the [^{14}C]acetyl peptides were approximately 2×10^6 cpm/μmole.

In order to exclude the possibility of aggregation, the size of some of the peptides was estimated by gel filtration using Bio-Gel P-2 (obtained from Bio-Rad Laboratories, Richmond, Calif.) columns equilibrated and eluted with borate-buffered saline (pH 8.0). It was thus established that 10 μmoles of the decapeptide, 65 mμmoles of [^{14}C]acetyl decapeptide, and 65 mμmoles of [^{14}C]acetyl pentapeptide eluted after the void volume of the column, indicating a molecular weight less than 2000. Previous work done in this laboratory (Benjamini *et al.*, 1965) showed that [^{14}C]acetyl eicosapeptide was of a molecular weight less than 6000. It can therefore be concluded that at most, if at all, the pentapeptide and the decapeptide may exist as dimers, and the eicosapeptide as a trimer.

Other peptides used for some of the reported experiments were the [^{14}C]*N*-acetyl di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, and nona- C-terminal peptides of the decapeptide Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg. The synthesis and analysis of these peptides have been described (Young *et al.*, 1967).

Immunological Assays. All of the rabbits were immunized by repeated intramuscular injections of the same preparation of 10 mg of TMVP in 1 ml of saline emulsified with an equal volume of Freund's complete adjuvant. The rabbits were injected at 10–14-day intervals for several months. Antisera were obtained periodically (10–14 days after the last injection) by bleeding from the ear vein. Rabbit antiacetylcholinesterase serum was used as control. Preparation of globulins and measurements of binding of ^{14}C-acetylated peptides with globulins were performed as previously described (Benjamini *et al.*, 1965). These assays were performed using 0.75 or 7.5 mμmoles of the [^{14}C]acetyl peptide and globulins as specified, in a total volume of 1 ml made up with borate-buffered saline. Following three precipitations at 50% saturation of ammonium sulfate, the precipitates were dissolved in 1 ml of saline and radioactivity was determined on aliquots. Each assay was performed in duplicate with measurements of radioactivity on at least two aliquots. Inhibition of binding between [^{14}C]acetyl peptides and globulins by unlabeled peptides was determined in a similar way except that the antibody was mixed with the nonradioactive peptide prior to the introduction of the labeled peptide.

Results and Discussion

Globulins obtained from a single bleeding of each rabbit approximately 30–35 weeks following the initial injection were titrated with [^{14}C]acetyl eicosa-, [^{14}C]-acetyl deca-, and [^{14}C]acetyl pentapeptide in order to ascertain the amounts of globulins and peptide to be employed in the inhibition experiments. On the basis of these titrations the amounts of ^{14}C peptide and globulins which were chosen are presented in Table II. The proportions of labeled antigens to antibodies were all at antibody deficiency.

Rabbit 24300. It is evident from data presented in

[1] The tobacco mosaic virus was generously supplied by Dr. C. A. Knight of the Virus Laboratory, University of California, Berkeley, Calif.

[2] According to the nomenclature of Tsugita *et al.* (1960).

[3] Henceforth designated as decapeptide, pentapeptide, and $(Ala)_6$-pentapeptide conjugate, respectively.

TABLE I: Amino Acid Analyses of Peptides.

Sequence	Mole Ratio of Amino Acids					
	Arg	Thr	Ala	Asp	Leu	Glu
Leu-Asp-Ala-Thr-Arg	0.97	1.00	1.03	1.05	0.95	
[^{14}C]Acetyl-Leu-Asp-Ala-Thr-Arg	1.00	1.00	1.06	1.03	1.00	
Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	1.02	3.89	2.03	1.02	1.01	1.02
[^{14}C]Acetyl-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	1.00	3.82	2.00	1.03	1.00	1.05
Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg	1.01	0.93	6.19	0.96	0.93	
[^{14}C]Acetyl-Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg	1.04	1.01	5.91	1.04	0.99	
[^{14}C]Acetyl-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr	*a*	3.81	1.99	1.04	0.99	1.18

[a] The arginine of the carboxypeptidase B treated peptide was not analyzed; however, analysis of arginine following carboxypeptidase B digestion showed 89% arginine released.

TABLE II: The Binding of [^{14}C]Acetyl Peptides with Anti-TMVP Globulins and Inhibition by Nonacetylated Peptides.

Globulins		[^{14}C]Acetyl Peptide (mμmole)	Cpm Bound (no inhibitor)	% Inhibition of Binding between [^{14}C]Acetyl Peptides and Anti-TMVP Globulins by Unlabeled Peptides Molar Ratio of Inhibitor to [^{14}C]Acetyl Peptide													
				Eicosapeptide			Decapeptide				Pentapeptide				$(Ala)_5$-Pentapeptide Conjugate			
Rabbit	ml			1:1	10:1	100:1	1:1	10:1	100:1	1000:1	1:1	10:1	100:1	1000:1	1:1	10:1	100:1	1000:1
24300	0.1	0.7 eicosa-	465	49	80	82	4	33	71		4	0	10	32	0	13	35	
24300	0.1	0.7 deca-	350	83	97	94	35	82	95		0	5	24	44	11	45	66	75
24300	0.1	7.5 penta-	306				75	98	100		20	32	75					
31500	0.07	0.7 eicosa-	1290	31	47	71	0	0	0	8	1	6	5	2	0	3	12	
31500	0.5	7.5 deca-	340					80	90			34	81			73		
31500	0.5	7.5 penta-	235					79	87			42	83[a]			75		
32600	0.1	0.7 eicosa-	1161	47	82	96	5	4	6	18	9	8	11	9[b]	2	6	4	9
32600	0.2	7.5 deca-	627	97	98		47	85	95		0	0	0		0	0	0	
32600	0.5	7.5 penta-	10															
31400	0.3	0.7 eicosa-	702	51	90	96	0	0	18	28			0	0[c]		0	0	
31400	0.5	7.5 deca-	410				37	76	88		0	0	0		0	0	0	
31400	0.5	7.5 penta-	8															

[a] At inhibitor to ^{14}C-antigen ratio of 20:1. [b] At inhibitor to ^{14}C-antigen ratio of 500:1. [c] At inhibitor to ^{14}C-antigen ratio of 5000:1.

Table II that the average association constant (which is reflected by the number of counts bound to globulins) of [^{14}C]acetyl pentapeptide with globulins obtained from this rabbit is lower than that of [^{14}C]-acetyl deca- or [^{14}C]acetyl eicosapeptide, especially in view of the fact that [^{14}C]acetyl pentapeptide was used at a concentration ten times higher than the other labeled peptides. The differences in average association constants of [^{14}C]acetyl pentapeptide, of [^{14}C]acetyl $(Ala)_5$-pentapeptide conjugate, of [^{14}C]acetyl decapeptide, and of [^{14}C]acetyl eicosapeptide with globulins derived from this rabbit are reflected in the inhibition data presented in Table II. Thus, when [^{14}C]acetyl eicosapeptide was used as the antigen, 49% inhibition was achieved by an equimolar amount of the nonacetylated, nonradioactive eicosapeptide. In contrast, 33% inhibition could be achieved only with over tenfold excess of decapeptide, 35% inhibition with 100-fold excess of $(Ala)_5$-pentapeptide conjugate, and 32% inhibition with 1000-fold excess of pentapeptide. When [^{14}C]acetyl decapeptide was used as the antigen, 83% inhibition was achieved by an equimolar amount of eicosapeptide whereas 35, 11, and 0% inhibition was achieved by equimolar amounts of the decapeptide, $(Ala)_5$-pentapeptide conjugate, and pentapeptide, respectively. In fact, in order to achieve a degree of inhibition similar to that caused by the eicosapeptide, the decapeptide and the $(Ala)_5$-pentapeptide conjugate had to be used at 10- and 1000-fold excess respectively; the pentapeptide, even at 1000-fold excess, inhibited to the extent of only 44%. When [^{14}C]acetyl pentapeptide was used as the antigen, 75% inhibition was achieved with an equimolar amount of the decapeptide but only with 100-fold excess of the pentapeptide; when the latter was used at an equimolar amount and at 10-fold excess only 20 and 32% inhibition, respectively, was achieved.

From data on the inhibition of the reaction between [^{14}C]acetyl peptides with anti-TMVP globulins obtained from this rabbit a rough comparison of the average association constants of these peptides may be made. Thus, the average association constant of the eicosapeptide is approximately ten times higher than that of the decapeptide, approximately 100–1000 times higher than that of the $(Ala)_5$-pentapeptide conjugate, and approximately 1000–10,000 times higher than that of the pentapeptide.

The inability of the pentapeptide, even when used at large excess, to significantly inhibit the reaction between [^{14}C]acetyl eicosapeptide and anti-TMVP may be due to the drastic difference between the average association constant of the eicosapeptide and that of the pentapeptide. It may also be due to the presence of antibodies which bind with other areas on the eicosapeptide in addition to antibodies which bind with the C-terminal pentapeptide. However, the findings that the decapeptide can largely inhibit the reaction between [^{14}C]acetyl eicosapeptide and antibodies and that (Ala)-pentapeptide conjugate can largely inhibit the reaction between [^{14}C]acetyl decapeptide and antibodies indicate that most if not all of the antibodies contained in the globulins derived from this rabbit bind with the C-terminal pentapeptide portion or a portion which contains the C-terminal pentapeptide area of the eicosapeptide.

The apparent increase in average association constant in the order eicosapeptide > decapeptide > $(Ala)_5$-pentapeptide conjugate > pentapeptide must be attributed to the increase in peptide size N terminally to the C-terminal pentapeptide, an increase which in some manner yet unclear enhances binding with antibodies. The finding that the $(Ala)_5$-pentapeptide conjugate exhibits an average association constant one to two orders of magnitude higher than that of the pentapeptide indicates that the increase must be attributed to the increase in peptide size by five N-terminal alanine residues which are found neither in the decapeptide nor in the native protein. Although conclusive evidence to this end is lacking, it appears that the alanine residues contribute to the binding with anti-TMVP in some nonspecific manner, perhaps by conferring hydrophobicity upon the pentapeptide, perhaps by promoting a helical structure for some or most of the peptide. Whether or not the addition of N-terminal alanines will result in enhanced binding of antigenic peptides of other proteins with corresponding antibodies remains to be investigated. It should be pointed out in this connection that in several instances it was indicated (Table II) that the *N*-acetyl derivative of the pentapeptide exhibited an increased average association constant when compared to the nonacetylated form. Furthermore, the *N*-octanoyl derivative of the C-terminal tetrapeptide exhibited a drastic increase in the average association constant when compared to that of *N*-acetyl pentapeptide, that of *N*-acetyl-$(Ala)_5$-pentapeptide conjugate, or that of *N*-acetyl decapeptide (Benjamini *et al.*, 1968). These latter findings indicate that the increase in average association constant is due to increase in hydrophobicity which in turn may or may not confer structural changes to the peptide, rather than to neutralization of the N-terminal charge or to the increase in the number of peptide bonds.

In summary, from the data presented in Table II dealing with the globulins derived from rabbit 24300 it appears that most if not all of the antibodies to the whole protein which bind with the eicosapeptide bind with the C-terminal decapeptide portion of the eicosapeptide, and that in this area, the antibodies bind with the C-terminal pentapeptide portion.

Rabbit 31500. Data on the binding of [^{14}C]acetyl eicosa-, deca-, and pentapeptides with globulins derived from this rabbit (Table II) show that the average association constants of the peptides are in the order eicosa- $\gg$ deca- > pentapeptide. This order is the same as that found for the binding of these peptides with globulins derived from rabbit 24300.

Data on the inhibition of the reaction between [^{14}C]-acetyl eicosapeptide and anti-TMVP (Table II) indicate that the average association constant of [^{14}C]acetyl eicosapeptide is higher than that of the nonacetylated eicosapeptide. The data also show the inability of the decapeptide, pentapeptide, or $(Ala)_5$-pentapeptide conjugate, even when used at large excesses, to significantly inhibit the reaction between [^{14}C]acetyl eicosapeptide

TABLE III: The Binding between Anti-TMVP Globulins Obtained from Different Rabbits with [^{14}C]Acetyl Decapeptide and [^{14}C]Acetyl Desarginated Decapeptide.

Peptide	mμmoles Used	Rabbit[a]			
		31500	32600	31400	Control
[^{14}C]Acetyl decapeptide	7.0	417	2220	530	75
[^{14}C]Acetyl desarginated decapeptide	7.0	95	2090	380	100

[a] Counts per minute bound to 0.5 ml of globulins.

and anti-TMVP, although binding of these peptides with antibodies is unequivocal. This latter finding strongly suggests that there is present in the antiserum a large population of antibodies binding with the eicosapeptide which bind with the N-terminal decapeptide region (which may be wholly or partially outside the C-terminal decapeptide area), *in addition* to a population of antibodies binding with the C-terminal portion of the eicosapeptide.

Data on the inhibition of the reaction between [^{14}C]-acetyl decapeptide and anti-TMVP globulins by the deca- and pentapeptides and by $(Ala)_5$-pentapeptide conjugate (Table II) show that, as expected, the reaction can be almost completely inhibited by the decapeptide. Furthermore, the reaction can also be greatly inhibited by the pentapeptide and by $(Ala)_5$-pentapeptide conjugate, indicating that most of the antibodies which bind with the decapeptide bind with a portion containing its C-terminal pentapeptide. This conclusion is also supported by experiments in which the binding of [^{14}C]acetyl decapeptide and of [^{14}C]acetyl desarginated decapeptide were compared. Results (Table III) show that the removal of arginine from the decapeptide caused the abolition of binding with globulins derived from this rabbit.

From data on the inhibition of the reaction between [^{14}C]acetyl decapeptide with anti-TMVP globulins by the deca- and pentapeptides, and by $(Ala)_5$-pentapeptide conjugate, it appears that the average association constant of the decapeptide is at least ten times higher than that of the pentapeptide, and approximately the same as that of $(Ala)_5$-pentapeptide conjugate. These ratios are different from those exhibited by these peptides with globulins derived from rabbit 24300. These findings point out the possibility that although antibodies of the two rabbits bind with the same pentapeptide area, there may exist differences in binding affinities between these antibodies and the peptides.

Data on the inhibition of the reaction between [^{14}C]-acetyl pentapeptide and globulins from rabbit 31500 by the pentapeptide and by $(Ala)_5$-pentapeptide conjugate again indicate the increased binding of the acetylated peptide over that of the nonacetylated peptide, and the increased average association constant of $(Ala)_5$-pentapeptide conjugate over that of the pentapeptide.

Rabbit 32600. Data on the binding of [^{14}C]acetyl eicosa-, deca-, and pentapeptides with globulins derived from this rabbit indicate that the average association constants of the peptides are in the order of eicosapeptide > decapeptide, and that the globulins of this rabbit do not contain antibodies which bind with the C-terminal pentapeptide area of the eicosapeptide. As with globulins derived from rabbit 31500, it appears that the globulins derived from rabbit 32600 contain at least two distinct antibody populations, those binding with an area on the C-terminal decapeptide and those binding with an area on the N-terminal decapeptide portion of the eicosapeptide (which may include some residues of the C-terminal decapeptide). This conclusion is based on data on the inhibition of the reaction between [^{14}C]acetyl peptides by nonacetylated peptides (Table II) which show that the reaction between [^{14}C]acetyl eicosapeptide and anti-TMVP is nearly completely inhibited by the nonacetylated eicosapeptide, but is inhibited only to the extent of 18% by 1000-fold excess of the decapeptide. Also, the reaction cannot be inhibited to any appreciable extent by the pentapeptide or by $(Ala)_5$-pentapeptide conjugate. Excluding drastic differences (over five to six orders of magnitude) between the average association constant of the eicosapeptide and that of the decapeptide with antibodies produced by this rabbit (a situation which does not lend itself to experimental testing because of the amounts of inhibitior to be used) there remains the alternative, namely, that at least two distinct antibody populations exist in this serum, one population which binds with the C-terminal decapeptide and another which binds with the N-terminal decapeptide portion (which may include some residues of the C-terminal decapeptide) of the eicosapeptide.

Supporting evidence for at least two antibody populations against the eicosapeptide in globulins derived from rabbit 32600 may be drawn from data on the characterization of antibodies at different intervals following the initial sensitization (Figure 1). In this experiment globulins were obtained at different time intervals during the course of sensitization with TMVP and were assayed for binding with [^{14}C]acetyl eicosapeptide and with [^{14}C]acetyl decapeptide. The assay was performed on all the globulins on the same day using the same solutions of peptides. As may be realized from data presented in Figure 1, 2.5 and 5 weeks following sensitization the antibody titer to [^{14}C]acetyl eicosapeptide (as expressed by number of counts bound

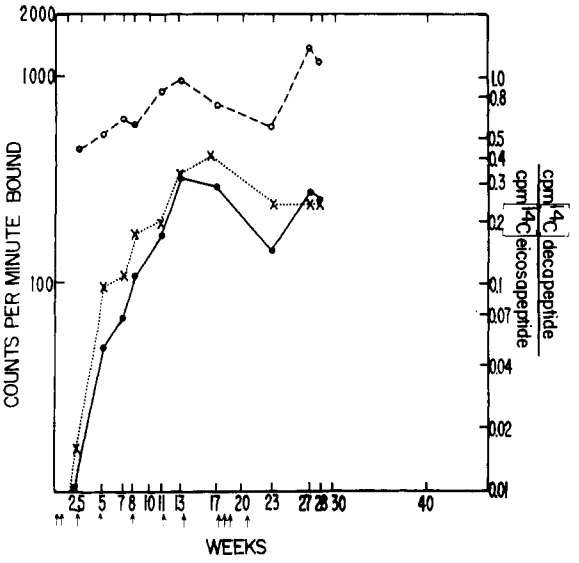

FIGURE 1: The binding of [^{14}C]acetyl eicosapeptide and of [^{14}C]acetyl decapeptide with 0.075 ml of globulin derived from rabbit 32600 at various time intervals following initial injection with TMVP. Globulins (0.5 ml) were treated with 7.5 mμmoles of [^{14}C]acetyl eicosapeptide (○--○), and with 7.5 mμmoles of [^{14}C]acetyl decapeptide (●—●). The ratio of counts per minute of [^{14}C]acetyl decapeptide bound over counts per minute of [^{14}C]acetyl eicosapeptide bound (×·····×). Injections of antigen are indicated by arrows.

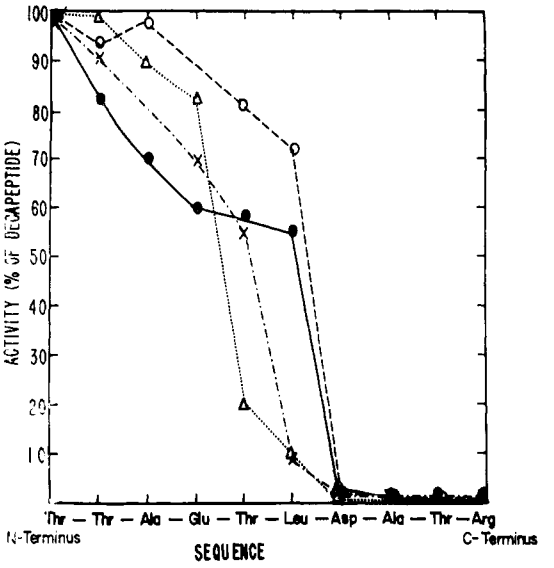

FIGURE 2: The binding of [^{14}C]acetyl decapeptide, of [^{14}C]-arginine, and of [^{14}C]acetyl di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, and nona- C-terminal peptides of the decapeptide with globulins derived from various rabbits immunized with TMVP. Rabbit 24300 (●—●), rabbit 31500 (○--○), rabbit 32600 (△·····△), and rabbit 31400 (×-·-·×). Globulins of each rabbit (0.5 ml) were treated with 7.5 mμmoles of each peptide.

to globulins) was by far higher than that to [^{14}C]acetyl decapeptide. However, as the interval following the first sensitization increased (with booster injections at intervals as specified in Figure 1) the titer to [^{14}C]-acetyl decapeptide increased significantly whereas the titer to [^{14}C]acetyl eicosapeptide did not appreciably change. The ratio of titer to [^{14}C]acetyl decapeptide over that to [^{14}C]acetyl eicosapeptide (expressed as a ratio of radioactivity bound to globulins) increased for approximately 13 weeks, as evident from Figure 1. If the eicosa- and decapeptides were recognized by the same antibodies, a constant ratio in the titer would be expected. As is evident from Figure 1 this is not the case.

The inability of the pentapeptide or $(Ala)_5$-pentapeptide conjugate, even when used at large excesses, to inhibit the reaction between [^{14}C]acetyl decapeptide and anti-TMVP globulins derived from this rabbit indicates that, unlike the situation with the two previous rabbits (24300 and 31500), the C-terminal pentapeptide by itself is not the portion which is responsible for the binding of the decapeptide, although it (or part of it) may constitute part of an antigenic area. This is supported by data on the binding of [^{14}C]acetyl peptides with globulins derived from this rabbit (Figure 2). These data show that [^{14}C]acetyl pentapeptide does not exhibit binding with globulins derived from this rabbit, and that significant binding with globulins begins with the [^{14}C]acetyl C-terminal heptapeptide portion of the decapeptide. Whether or not the C-terminal portion of this heptapeptide is required for binding is partially elucidated by experiments comparing the binding of [^{14}C]acetyl decapeptide and of [^{14}C]-acetyl desarginated decapeptide with globulins obtained from this rabbit. These experiments (Table III) indicate that at least the C-terminal arginine of the decapeptide was not essential for binding. It can therefore be postulated that the antibody population produced by this rabbit which is directed against the C-terminal decapeptide area of the antigenic eicosapeptide of TMVP binds with the sequence or a portion of the sequence Glu-Thr-Leu-Asp-Ala-Thr representing residues 106–111 of the tobacco mosaic virus protein. Work is now in progress to ascertain this postulate.

Rabbit 31400. Data on the binding of [^{14}C]acetyl eicosa-, deca-, and pentapeptides by globulins derived from this rabbit show that, as in the case of globulins derived from rabbit 32600, the average association constants of the peptides are in the order of eicosa- > decapeptide and there is essentially no binding with the [^{14}C]acetyl pentapeptide (Table II). From data on the inhibition of the reaction between [^{14}C]acetyl eicosapeptide and anti-TMVP globulins by nonlabeled, nonacetylated peptides it appears that the decapeptide, even when used at 100- or 1000-fold excess, was inhibitory only to a slight extent. On the basis of the same arguments as those given earlier in the case of rabbit 32600, it can be proposed that in the globulins derived from rabbit 31400 there exist at least two distinct antibody populations, one population binding with an area or areas of the C-terminal decapeptide portion of the eicosapeptide and another population binding with the N-terminal decapeptide portion of the eicosapeptide (which may include residues of the C-terminal decapeptide).

The inability of the pentapeptide or of $(Ala)_5$-pentapeptide conjugate to inhibit the reaction between

[^{14}C]acetyl decapeptide and globulins derived from this rabbit (Table II) indicates that the antibody population which binds with the C-terminal decapeptide portion of the eicosapeptide binds with an area other than just the C-terminal pentapeptide portion but which may contain the pentapeptide or a portion of the pentapeptide area. This is corroborated by data presented in Figure 2 which show that substantial binding between [^{14}C]acetyl peptides with globulins begins only with the C-terminal hexapeptide portion of the decapeptide. Comparison of the binding of [^{14}C]acetyl decapeptide and that of [^{14}C]acetyl desarginated decapeptide with globulins derived from this rabbit (Table III) show 530 and 380 cpm of [^{14}C]-acetyl deca- and of [^{14}C]acetyl desarginated decapeptides, respectively, bound to the same quantity of globulins (0.5 ml). On the basis of these experiments it can be postulated that the antibodies which bind with the C-terminal decapeptide portion of the eicosapeptide bind with the sequence or part of the sequence Thr-Leu-Asp-Ala-Thr representing residues 107–111 of the tobacco mosaic virus protein. Work is now in progress to ascertain this postulate.

From the foregoing results and discussion, several general conclusions may be drawn regarding antibody populations elicited by immunization of different rabbits with the protein antigen TMVP and regarding the area or areas on the antigenic eicosapeptide with which these antibodies bind. It appears that all of the animals which were immunized with TMVP and which were tested (these include numerous rabbits in addition to those described in this communication, and also guinea pigs, rats, and mice) produce antibodies which bind with the tryptic eicosapeptide (peptide 8) of TMVP, representing residues 93–112 of the protein. However, it also appears that the antibodies produced by the different rabbits do not necessarily bind with the same area or areas on the eicosapeptide and that the antibody response of a given rabbit may depend on the length of the immunization period and/or the amount of antigen injected. In fact, it appears that three of the four tested rabbits elicited antibodies which bind with the N-terminal as well as with the C-terminal decapeptide regions of the eicosapeptide whereas one rabbit produced antibodies which bind primarily with the C-terminal decapeptide portion. Two of the rabbits produced antibodies which bind with the C-terminal pentapeptide portion of the eicosapeptide while the other two rabbits did not. These data demonstrate that although antigens could lead to an immune response in all animals, the specificity of the immune response of a given animal (which is measured by the ability of the antibody to bind with antigen) may be due to different determinants on the antigenic molecule. Although the rabbits which were used in the reported experiments were randomly bred, it is possible that the different responses which were witnessed are under genetic control as is indicated by experiments on the immune response of guinea pigs to insulin (Arquilla and Finn, 1965), and of mice and guinea pigs to hapten conjugates and to synthetic polypeptides (summaried by Lennox, 1966; Ben-Efraim *et al.*, 1967). Utilizing inbred strains of mice, experiments are now in progress to ascertain whether or not responses to different areas on the eicosapeptide are indeed under genetic control.

A close analysis of the area on the C-terminal decapeptide portion of the eicosapeptide reveals that antibodies derived from the different rabbits do not necessarily bind with the same area on the peptide. Binding with the C-terminal pentapeptide was exhibited by antibodies from two of the four rabbits whereas antibodies produced by the two other rabbits required the C-terminal hexa- and heptapeptides, respectively, for binding. Data on the binding of the desarginated C-terminal decapeptide portion of the eicosapeptide indicate that the C-terminal arginine is not required for binding with antibodies produced by the latter two rabbits. This suggests that although the antibodies produced by all four rabbits bind with sequences part of which is the common sequence Leu-Asp-Ala-Thr, C-terminal arginine is critical for binding with antibodies produced by two rabbits, N-terminal threonine is critical for binding with antibodies produced by another rabbit, and N-terminal glutamylthreonine is essential for binding with antibodies from still another rabbit. It can therefore be postulated that the sequence Leu-Asp-Ala-Thr (or part of it) constitutes a portion of an area which is antigenic to the four rabbits. Indeed, very recent experiments (E. Benjamini, unpublished) showed that globulins obtained from all these rabbits bind with [^{14}C]octanoyl-Ala-Thr-Arg. It can therefore be suggested that part of the sites of antibodies produced by these rabbits is directed against the sequence Leu-Asp-Ala-Thr or Ala-Thr-Arg, but that the entire antibody reactive site is probably not the same.

The phenomenon of antibody heterogeneity is well known and, as previously mentioned, has been the subject of numerous reviews. As stated by Kabat (1966) all studies to date indicate that even toward a single antigenic determinant the antibody formed is not homogeneous. This is exemplified by work with the dextran–human antidextran system carried out by Kabat and his collaborators (summarized by Kabat, 1966), and by the work of Eisen and Siskind (1964) on anti-2,4-dinitrophenyl (DNP) antibodies. It has been shown by Torii *et al.* (1966) that, although two antidextran sera obtained from two human subjects possessed specificity to the α-1$\rightarrow$3-D-glucopyranosyl-D-glucose linkage, the inhibition of the precipitin reaction between the individual antisera and dextran by maltose and by isomaltose was better by isomaltose in the case of one serum and better by maltose in the case of another serum. With anti-DNP, it was shown by Eisen and Siskind that all the populations of rabbit anti-DNP examined, whether isolated from pooled sera or from single bleedings of individual rabbits, were heterogeneous with respect to affinity for dinitrobenzenes. It is therefore not surprising that data presented in the present communication indicate that the combining sites of antibodies obtained from the two rabbits, which exhibit binding with the C-terminal pentapeptide portion of the eicosapeptide, are different. This is

suggested by the finding that although both antisera contain antibodies which bind with the C-terminal pentapeptide portion of the eicosapeptide, the reaction between [^{14}C]acetyl decapeptide and antibodies produced by rabbit 31500 is inhibited to about the same extent by $(Ala)_5$-pentapeptide conjugate as by the native decapeptide whereas the reaction between the [^{14}C]acetyl decapeptide and antibodies produced by rabbit 24300 is inhibited better by the native decapeptide than by $(Ala)_5$-pentapeptide conjugate. The question as to whether or not the antibodies produced by a given rabbit to a given sequence with which they exhibit binding are still heterogeneous remains to be answered.

It thus appears that the definition of an antigenic area or areas of a protein antigen and of the corresponding complementary antibody-reactive site should be derived cautiously not only because generalizations derived from randomly bred animals are difficult but also because the populations of antibodies derived from the same animal, at various time intervals following immunization, may change with respect to their quantity and areas with which they bind. However, from the foregoing it is apparent that characterization of antibodies can be afforded by investigations utilizing protein antigens with characterized antigenic determinants.

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