Immunochemical Studies on the Tobacco Mosaic Virus Protein. VIII. Solid-Phase Synthesis and Immunological Activity of Peptides Related to an Antigenic Area of Tobacco Mosaic Virus Protein*

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ABSTRACT: A pentapeptide (I) having the amino acid sequence, Leu-Asp-Ala-Thr-Arg, representing residues 108–112 of the tobacco mosaic virus protein has previously been found to bind to antibodies from several rabbits following immunization with the whole protein, tobacco mosaic virus protein. The synthesis of 18 peptides related to I is reported here and the binding of these peptides with antitobacco mosaic virus protein from a single rabbit is compared with that of I. Twelve pentapeptides were synthesized in which only one amino acid residue was different from I. All of these peptides had either no immunological activity or had less activity than I suggesting that the antibodies which bind to I exhibit a specific recognition of the residues in I. The binding of these peptide analogs also revealed that the hydrophobic nature of leucine is probably important to its binding and the shape of the aspartic acid residue in I is more important than its negative charge since this residue could be replaced by asparagine but not by glutamic acid.

The reverse-sequence pentapeptide, Arg-Thr-Ala-Asp-Leu, was inactive. Several peptides containing a residue on either side of I were synthesized. Thr-Leu-Asp-Ala-Thr was inactive but Asp-Ala-Thr-Arg-Arg (II) was active. This suggests that the C-terminal arginine in I is more important for binding in the antisera than the N-terminal leucine in I. The C-terminal arginine in II which represents tobacco mosaic virus protein residue 113 may be a part of the area of the protein which binds to I specific antibodies.

A pentapeptide having the amino acid sequence, Leu-Asp-Ala-Thr-Arg,[1] representing residues 108–112 of the TMVP, has been characterized as an antigenic determinant of this protein (Young *et al.*, 1967a). This peptide, after acetylation with [^{14}C]acetic anhydride, was found to bind specifically with antibodies obtained from several rabbits following immunization with the whole-protein TMVP. In the search for the factors which affect the specific binding of the peptide with antibodies, 18 peptides related to this pentapeptide area of TMVP were synthesized by the Merrifield (1964) solid-phase synthesis method and their binding with anti-TMVP was compared with that of the native pentapeptide, Leu-Asp-Ala-Thr-Arg. The present communication reports the results of these studies.

Experimental Section

Solid-Phase Synthesis of Peptides. The peptides were synthesized by the solid-phase method of Merrifield essentially as described by Young *et al.* (1967a). All of the amino acid derivatives were obtained from either Cyclo Chemical Corp., Los Angeles, or from Schwarz BioResearch, Inc., Orangeburg, N. Y., and were found to be of good purity as judged by thin-layer chromatography. The amino acid derivatives were: *N*-Boc-L-aspartic acid β-benzyl ester, *N*-Boc-L-nitroarginine, *N*-Boc-*O*-benzyl-L-tyrosine, *N*-benzyloxycarbonyl-D-leucine, *N*-Boc-L-glutamic acid γ-benzyl ester, *N*-Boc-L-asparagine-α-*p*-nitrophenyl ester, *N*-Boc-*O*-benzyl-L-serine, *N*-Boc-L-leucine, *N*-Boc-L-alanine, *N*-Boc-L-threonine, *N*-Boc-L-isoleucine, and *N*-Boc-glycine. The Boc-amino acid-resins were prepared as described by Merrifield (1964) using reflux times for Boc-L-nitroarginine, Boc-L-leucine, and Boc-L-threonine of 72, 50, and 72 hr, respectively. The amount of amino acid-resin or peptide-resin used in each synthesis is given in Table I. The reaction vessel was 1.5 × 11 cm (Laboratory Glass Apparatus, Berkeley, Calif.) and 5-ml portions of reagents were used. In cycles where *p*-nitrophenyl esters or nitroarginine were added, the resin was washed with dimethylformamide instead of CH_2Cl_2 and the amino acid derivative, dissolved in dimethylformamide, was allowed to react with the peptide-resin for 4 hr. A 2 molar ratio of amino acid derivative and a 2.1 molar ratio of *N*,*N*′-dicyclohexylcarbodiimide to amino acid-resin or peptide-resin were used in each coupling reaction. Dicyclohexylcarbodiimide was not used when the *p*-nitrophenyl ester derivative was used for coupling. The peptides were cleaved from the resin with anhydrous HBr in trifluoroacetic acid (Merrifield, 1964). After

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[1] All amino acid residues are in the L configuration unless designated as D.

TABLE I: Data on Synthesis and Electrophoretic and Chromatographic Properties of the Peptides.

No.	Peptide or Ref Compd	Starting Amino Acid or Peptide-resin	Starting mmoles/g of Resin	Resin Used (g)	Electrophoretic Mobility, R_{Arg}[a] pH 6.4	R_{Arg}[a] pH 2.7	R_F[b] Solvent A	R_F[b] Solvent B
1	Leu-Asp-Ala-Thr-Arg				−0.14	−0.69	0.51	0.43
2	*Ala*-Asp-Ala-Thr-Arg	Asp-Ala-Thr-Arg-resin	0.285	0.1	−0.13	−0.73	0.24	0.25
3	*Ile*-Asp-Ala-Thr-Arg	Arg-resin	0.284	0.5	−0.09	−0.59	0.45	0.45
4	*Tyr*-Asp-Ala-Thr-Arg	Asp-Ala-Thr-Arg-resin	0.285	0.1	−0.13	−0.68	0.34	0.42
5	D-*Leu*-Asp-Ala-Thr-Arg	Arg-resin	0.297	0.5	−0.09	−0.72	0.54	0.52
6	Leu-*Glu*-Ala-Thr-Arg	Ala-Thr-Arg-resin	0.333	0.2	−0.13	−0.73	0.54	0.49
7	Leu-*Asn*-Ala-Thr-Arg	Ala-Thr-Arg-resin	0.333	0.2	−0.49	−0.73	0.56	0.52
8	Leu-Asp-*Leu*-Thr-Arg	Arg-resin	0.284	0.5	−0.09	−0.67	0.63	0.74
9	Leu-Asp-*Gly*-Thr-Arg	Arg-resin	0.284	0.5	−0.09	−0.70	0.43	0.43
10	Leu-Asp-Ala-*Leu*-Arg	Arg-resin	0.284	0.5	−0.08	−0.67	0.68	0.81
11	Leu-Asp-Ala-*Gly*-Arg	Arg-resin	0.284	0.5	−0.09	−0.66	0.45	0.44
12	Leu-Asp-Ala-*Ser*-Arg	Arg-resin	0.288	0.5	−0.09	−0.64	0.33	0.43
13	Arg-Thr-Ala-Asp-Leu	Leu-resin	0.570	1.0	−0.05	−0.73	0.58	0.52
14	Ala-Arg-Thr-Ala-Asp-Leu	Arg-Thr-Ala-Asp-Leu-resin	0.370	0.7	−0.06	−0.70	0.53	0.48
15	Leu-Asp-Ala-Thr	Thr-resin	0.200	1.0	+0.39	−0.40	0.66	0.49
16	Thr-Leu-Asp-Ala-Thr	Leu-Asp-Ala-Thr-resin	0.170	0.5	+0.33	−0.33	0.66	0.57
17	Ala-Thr-Arg-Arg	Arg-resin	0.480	0.9	−0.92	−1.04	0.14	0.24
18	Asp-Ala-Thr-Arg-Arg	Ala-Thr-Arg-Arg-resin	0.260	0.7	−0.46	−0.81	0.14	0.19
19	Leu-Asp-Ala-Thr-Arg-Arg	Asp-Ala-Thr-Arg-Arg-resin	0.260	0.4	−0.45	−0.79	0.33	0.33
	Threonine				−0.03	−0.19		
	Aspartic acid				+1.10	−0.32		
	Picric acid				+0.80	+0.65		

[a] Electrophoretic mobility, R_{Arg}, is expressed as centimeters traveled from origin divided by centimeters traveled by arginine; (+) indicates migration to anode; (−) indicates migration to cathode; pH 6.4, pyridine–acetic acid–water (10:0.4:90); pH 2.7, 1 M acetic acid. [b] Solvent A, 1-butanol–acetic acid–water (3:1:1), solvent B, 1-butanol–acetic acid–water–pyridine (15:3:12:10).

TABLE II: Amino Acid Ratios of Peptides.

No.	Peptide	Leu	Asp	Ala	Thr	Arg	Other
1	Leu-Asp-Ala-Thr-Arg	1.0	1.0	1.0	1.0	1.0	
2	*Ala*-Asp-Ala-Thr-Arg		1.0	2.0	1.0	1.0	
3	*Ile*-Asp-Ala-Thr-Arg	0.9	1.0	1.1	1.0	1.0	
4	*Tyr*-Asp-Ala-Thr-Arg		1.1	1.1	0.9	0.9	Tyr, 0.6
5	*D-Leu*-Asp-Ala-Thr-Arg	1.0	1.0	1.0	1.0	1.0	
6	Leu-*Glu*-Ala-Thr-Arg	1.0		1.2	0.9	0.9	Glu, 1.0
7	Leu-*Asn*-Ala-Thr-Arg	1.0	1.1	1.1	1.0	1.0	
8	Leu-Asp-*Leu*-Thr-Arg	2.0	1.2		1.0	1.0	
9	Leu-Asp-*Gly*-Thr-Arg	1.0	1.0		1.0	0.9	Gly, 1.0
10	Leu-Asp-Ala-*Leu*-Arg	2.0	1.0	1.0		1.0	
11	Leu-Asp-Ala-*Gly*-Arg	1.0	1.0	1.0		0.9	Gly, 1.0
12	Leu-Asp-Ala-*Ser*-Arg	1.0	1.0	1.0		1.0	Ser, 0.9
13	Arg-Thr-Ala-Asp-Leu	1.0	1.0	1.0	1.0	0.9	
14	Ala-Arg-Thr-Ala-Asp-Leu	1.0	1.0	2.1	1.0	1.0	
15	Leu-Asp-Ala-Thr	1.0	1.0	0.9	1.1		
16	Thr-Leu-Asp-Ala-Thr	0.9	1.1	1.0	2.0		
17a	Ala-Thr-Arg-Arg			1.0	1.0	2.5	
17b	[^{14}C]Acetyl-Ala-Thr-Arg-Arg[a]			1.0	1.0	1.9	
18	Asp-Ala-Thr-Arg-Arg		1.0	1.0	0.9	1.9	
19	Leu-Asp-Ala-Thr-Arg-Arg	1.0	1.0	1.1	1.1	1.8	

[a] The amino acid analysis of ^{14}C-acetylated peptide 17 is included since peptide 17a was high in arginine but had the expected value after acetylation.

evaporation of the trifluoroacetic acid the peptides were dissolved in HOAc–H_2O (9:1) and reduced by bubbling hydrogen through the solution at atmospheric pressure, overnight, using 5% palladium on barium sulfate as the catalyst. Most of the peptides were purified by column chromatography using Dowex 1-X2 as previously described (Young *et al.*, 1966). The neutral peptides containing C-terminal threonine (peptides 15 and 16, Table I) eluted from the Dowex 1 column much later than neutral peptides containing arginine or lysine. This behavior corroborates a similar observation of Funatsu (1964). D-Leu-Asp-Ala-Thr-Arg (peptide 5, Table I) and Leu-Asn-Ala-Thr-Arg (peptide 7) required paper chromatography after Dowex 1-X2 chromatography, and Ala-Asp-Ala-Thr-Arg (peptide 2) and Tyr-Asp-Ala-Thr-Arg (peptide 4) were purified solely by paper chromatography since very little peptide was synthesized. Solvent A (1-butanol–acetic acid–water (3:1:1)) was used for chromatography of peptides 2, 4, and 7 and solvent B (1-butanol–acetic acid–water–puridine (15:3:12:10)) was used for peptide 5. Leu-Asp-Leu-Thr-Arg (peptide 8) and Leu-Asp-Ala-Leu-Arg (peptide 10) were purified further after Dowex 1-X2 chromatography by Sephadex G-10 chromatography (Stewart *et al.*, 1966).

After Dowex 1-X2 chromatography of the cleaved and reduced peptide, the stereoconfiguration of the leucine in D-Leu-Asp-Ala-Thr-Arg was checked by digestion with the L-amino acid specific enzyme, leucine aminopeptidase. The digestion was performed on 7.2 μmoles of peptide using 0.05 mg of leucine aminopeptidase (LAP–DFP 113 obtained from Worthington Biochemical Corp., Freehold, N. J.) in a total volume of 2.5 ml, pH 8.5, at 40° for 1 hr. The conditions given in the Worthington Enzyme Manual (1967) were used. An aliquot of 1.31 μmoles of peptide was diluted to 1.1 ml with the amino acid analyzer buffer, and 1 ml was subjected to amino acid analysis on the Spinco 120B analyzer using the neutral and acidic column. The analysis revealed that no leucine was released by the digestion. Digestion and analysis, under identical conditions, of 0.85 μmole of the reference peptide L-Leu-Asp-Ala-Thr-Arg yielded a quantitative release of the leucine from the peptide.

Results of paper electrophoresis and paper chromatography of the peptides are given in Table I. Portions of each of the peptides were acetylated with [^{14}C]acetic anhydride and separated from [^{14}C]acetate by chromatography on Sephadex G-10 columns (Stewart *et al.*, 1966). Amino acid analyses of the nonacetylated peptides are given in Table II. The mole ratios of the acetylated peptides were essentially the same as the corresponding nonacetylated peptides and are not included in Table II except for [^{14}C]acetyl-Ala-Thr-Arg-Arg (peptide 17b). The G-10 Sephadex column routinely used to separate the [^{14}C]acetyl peptide from [^{14}C]acetate probably removed some free arginine from peptide17.

The counts per minute per micromole of each peptide were determined from the average of three 0.5-ml aliquots of the peptide (7.5 mμmoles/ml) dissolved in 1 M acetic acid. The specific activities of each of the peptides are given in Table III. All radioactivity measure-

TABLE III: Immunological Activity of Peptides.

No.	Peptide: TMVP Residue Number[c] 107 108 109 110 111 112 113	cpm/μmole × 10^6	cpm Obtained in Experiment[a] I C	I T	II C	II T	III C	III T	IV C	IV T	V C	V T	Net cpm/0.5 ml of anti-TMVP[b]	% of [^{14}C]-Asp-Ala-Thr Arg Act.
1	Leu-Asp-Ala-Thr-Arg	2.01	17	159	12	127	6	123	7	101	14	70	473 (I); 383 (II); 390 (III); 314 (IV); 187 (V)[a]	100
2	*Ala*-Asp-Ala-Thr-Arg	2.39			19	39							56	15
3	*Ile*-Asp-Ala-Thr-Arg	1.89					17	54					130	33
4	*Tyr*-Asp-Ala-Thr-Arg	1.76	23	68									170	36
5	D-*Leu*-Asp-Ala-Thr-Arg	2.01			11	62							178	44
6	Leu-*Glu*-Ala-Thr-Arg	1.87					16	13					0	0
7	Leu-*Asn*-Ala-Thr-Arg	2.20									18	52	104	56
8	Leu-Asp-*Leu*-Thr-Arg	2.96			23	29							14	4
9	Leu-Asp-*Gly*-Thr-Arg	2.53			15	17							5	1
10	Leu-Asp-Ala-*Leu*-Arg	2.24			13	19							18	5
11	Leu-Asp-Ala-*Gly*-Arg	2.14			23	21							0	0
12	Leu-Asp-Ala-*Ser*-Arg	1.27					4	27					121	31
13	*Arg-Thr-Ala-Asp-Leu*	2.21	35	37									6	1
14	*Ala-Arg-Thr-Ala-Asp-Leu*	2.28	37	39									6	1
15	Leu-Asp-Ala-Thr	1.28					14	15					5	1
16	Thr-Leu-Asp-Ala-Thr	2.32			17	24							20	5
17	Ala-Thr-Arg-Arg	2.15			18	26							25	7
18	Asp-Ala-Thr-Arg-Arg	2.17			17	143							390	102
19	Leu-Asp-Ala-Thr-Arg-Arg	1.99					12	221					700	180
20	Thr-Leu-Asp-Ala-Thr-Arg	2.23							11	124			340	108

[a] The binding of the peptides to anti-TMVP globulins in the various experiments which are indicated by roman numerals was determined by reaction 0.5 ml of globulins with 7.5 mμmoles of [^{14}C]acetyl peptides; the counts per minute given were obtained from 0.3 ml/1 ml of total (see text for assay procedure). The anti-TMVP globulins were all from rabbit 31,500. The same pooled globulin preparation was used for expt II–IV. Different globulin preparations were used for expt I and V. Counts bound to control globulins are indicated by C; counts bound to anti-TMVP are indicated by T. [b] The net cpm/0.5 ml of anti-TMVP has been corrected for the variation in specific activity exhibited by the peptides (see text). [c] Amino acid residues differing from those in the TMVP sequence are in italic type.

ments were performed using a Nuclear-Chicago Model D-47 gas-flow detector.

Immunological Assay of Peptides. The anti-TMVP was obtained from pooled bleedings of a single rabbit (rabbit 31500). Antiacetylcholinesterase was used as the control serum. The sensitization, preparations of globulins, and measurements of the binding of the [^{14}C]-acetyl peptides with anti-TMVP were performed as previously described by Benjamini *et al.* (1965). The assay for immunological activity was performed using 7.5 mμmoles of the [^{14}C]acetyl peptide and 0.5 ml of globulins in a total volume of 0.6 ml. Following precipitation at 50% saturation of ammonium sulfate and two additional precipitations with ammonium sulfate in a total volume of 1 ml, the precipitates were dissolved in 1 ml of saline and radioactivity was measured on three 0.3-ml aliquots. Each [^{14}C]acetyl peptide was also assayed for binding with antiacetylcholinesterase globulins. The net number of counts of each peptide bound with anti-TMVP was derived by subtracting the number of counts bound with antiacetylcholinesterase from that bound with anti-TMVP. This number was adjusted with the value that would have been obtained if the peptide had the same counts per minute per micromole as [^{14}C]acetyl-Leu-Asp-Ala-Thr-Arg (2.0×10^6 cpm/μmole). Results of the immunological assays are given in Table III.

Discussion

Eighteen peptides were synthesized for the purpose of comparing their binding with anti-TMVP with that of the pentapeptide, Leu-Asp-Ala-Thr-Arg, which represents residues 108–112 of TMVP. This peptide will be referred to as the reference pentapeptide (peptide 1, Tables I–III). Amino acid analyses (Table II), electrophoretic mobilities (Table I), and paper chromatographic R_F values in two solvents (Table I) of the peptides are given. Some of the peptides contained minor impurities but were considered of adequate purity for the immunological assay. The estimated tyrosine content of the peptide, Tyr-Asp-Ala-Thr-Arg (peptide 4, Table II) was low. However, since paper chromatography of the peptide revealed no residual tetrapeptide, Asp-Ala-Thr-Arg, the low value of tyrosine was likely due to the open reflux hydrolysis technique involved. The low yield of the peptide prevented repeating the amino acid analysis using *in vacuo* acid hydrolysis. A large amount of Asp-Ala-Thr-Arg was present in the unpurified preparation of D-Leu-Asp-Ala-Thr-Arg (peptide 5) but this was effectively removed by paper chromatography. The leucine in this peptide was found to be all in the D configuration as judged by its complete resistance to digestion by the L-stereospecific enzyme, leucine aminopeptidase. As little as 5% release of leucine from the peptide could have easily been detected. Under identical conditions of digestion, the leucine of the reference pentapeptide was quantitatively released, thus demonstrating that the leucine of the reference peptide was all in the L configuration. There was no [^{14}C]acetyl reference pentapeptide in the [^{14}C]acetyl-Leu-Asn-Ala-Thr-Arg (peptide 7) preparation as judged by a radioelectrophoretogram of the [^{14}C]acetyl peptide 7. As little as 5% of the nonamidated [^{14}C]acetyl reference pentapeptide would have easily been detected.

From the relatively small number of analogs tested it appears that the antibodies which bind to the reference pentapeptide exhibit a specific recognition, or fit to the shape of the residues in the reference pentapeptide. All of the 11 peptides (peptides 2–12, Table III) which differed from the reference peptide, Leu-Asp-Ala-Thr-Arg, by a single amino acid exhibited less binding (as reflected by the amount of radioactivity bound to globulins) with anti-TMVP than did the reference peptide. Compared with the reference pentapeptide, five of these peptides (peptides 3, 4, 5, 7, and 12) exhibited 31–56% activity, the remaining peptides exhibited either no activity or very marginal activity. In general the active peptide analogs had residue replacements similar to the residue in that position in the reference peptide. For example D-leucine in peptide 5 and isoleucine in peptide 3 are similar to the replaced leucine, asparagine in peptide 7 is similar to aspartic acid, and serine in peptide 12 is similar to threonine. Substituting the aspartic acid residue for glutamic acid resulted in the inactive peptide, Leu-Glu-Ala-Thr-Arg (peptide 6). This result along with the finding that the asparagin-containing peptide is active indicates that the shape of the aspartic acid residue is more important than the negative charge in this residue for binding. The pictures (Figure 1) of the Corey–Pauling models of the reference pentapeptide and the peptides containing asparagine or glutamic acid instead of aspartic acid illustrate the relative shapes of the residues in this area of the reference pentapeptide. As can be seen in the figure, the asparagine side group is very similar to that of the aspartic acid side group but the additional CH_2 group in glutamic acid changes the shape of the residue considerably.

Substitution of the N-terminal leucine by isoleucine, by D-leucine, or by tyrosine resulted in active peptides (peptides 3–5), whereas alanine in this position resulted in a peptide, Ala-Asp-Ala-Thr-Arg (peptide 2), which exhibited marginal activity. From this data it appears that the role of the N-terminal leucine in the binding of the reference pentapeptide with antibodies is that of rendering this portion of the molecule a certain amount of hydrophobicity and that the shape of the leucine, though important, is not as critical for binding as it is for the other residues in the reference pentapeptide. In this connection it was recently shown by Benjamini *et al.* (1968b) that substituting the leucine with octanoic acid yielded the octanoylated tetrapeptide, octanoyl-Asp-Ala-Thr-Arg, which exhibited much higher binding with anti-TMVP than did the reference pentapeptide.

The reverse sequence of the reference pentapeptide, Arg-Thr-Ala-Asp-Leu (peptide 13), and the reverse sequence with an alanine residue added N terminally (peptide 14) were inactive.

Peptides 15–20 contain residues on either side of the reference pentapeptide (TMVP residues 107–113). These peptides were tested for their binding with anti-TMVP in order to characterize which area in these sequences is most important for binding. The active peptides were Asp-Ala-Thr-Arg-Arg (peptide 18), Leu-Asp-Ala-Thr-

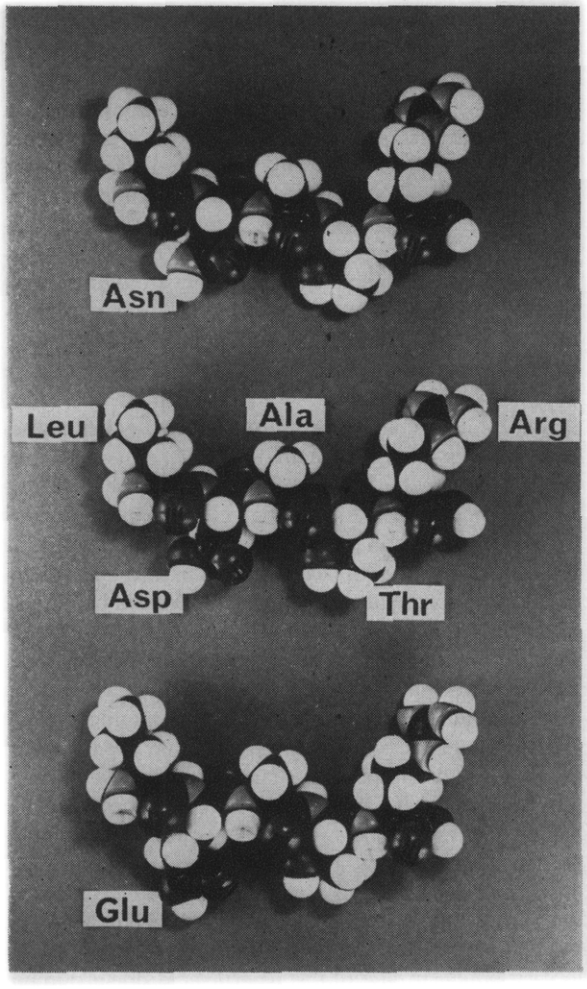

FIGURE 1: Corey–Pauling–Koltun atomic models of Leu-Asn-Ala-Thr-Arg (top), Leu-Asp-Ala-Thr-Arg (middle), and Leu-Glu-Ala-Thr-Arg (bottom).

Arg-Arg (peptide 19), and Thr-Leu-Asp-Ala-Thr-Arg (peptide 20). It is interesting that the peptides which exhibit binding contain the tetrapeptide sequence Asp-Ala-Thr-Arg, even though the tetrapeptide itself exhibited no binding with antibodies produced by this rabbit or by other rabbits tested (Benjamini *et al.*, 1968a). The peptide, Thr-Leu-Asp-Ala-Thr (peptide 16), was inactive indicating that the C-terminal arginine is critical for binding with antibodies produced by this rabbit. This is in agreement with an earlier report in which the desarginated C-terminal decapeptide portion of TMVP peptide 8[2] (Benjamini *et al.*, 1968a) when tested with this antiserum was inactive. The N-terminal leucine of the reference pentapeptide, therefore, contributes, but not as much as the arginine (residue 112), to the binding with antibodies.

Residues other than arginine in position 113 have not been tested for their binding with this antiserum. One would expect that any other residue here would result in a peptide of lower binding with this antiserum since this arginine residue exhibited as much toward its binding as did the leucine in the reference peptide and any leucine substitution caused a reduction in binding. One also would not expect that the antibody recognition area would end precisely at a tryptic cleavage bond (between residues 112 and 113).

As already mentioned the activity exhibited by the pentapeptide, Asp-Ala-Thr-Arg-Arg (peptide 18), which contains the N-terminal arginine of tryptic peptide 9 is the same as that of the reference pentapeptide. The activity of the hexapeptide, Leu-Asp-Ala-Thr-Arg-Arg (peptide 19), is about two times that of the reference pentapeptide whereas the activity of the hexapeptide, Thr-Leu-Asp-Ala-Thr-Arg (peptide 20), is only 1.1 times that of the reference pentapeptide. The activities of the peptides containing residue 113 may be due to the presence in the globulins of this rabbit of additional antibodies which are directed against an area of the protein composed of the C-terminal area of tryptic peptide 8[2] and the N-terminal portion of tryptic peptide 9,[2] as well as the population directed only against the reference pentapeptide. On the other hand, it may be that residue 113 in Asp-Ala-Thr-Arg-Arg (peptide 18) and Leu-Asp-Ala-Thr-Arg-Arg (peptide 19) may have enhanced the binding of the same antibodies which bind to the reference pentapeptide.

Experiments (Benjamini *et al.*, 1968a) have indicated that antibodies from different rabbits bind to different areas of tryptic peptide 8 of TMVP (residues 93–112). Furthermore it was shown that the ratio of binding of the eicosapeptide with anti-TMVP to that of the binding of the decapeptide with anti-TMVP varied depending upon the time interval following initial immunization. It is not known therefore to what extent the relative bindings of the peptides to the globulins of rabbit 31,500 given in Table III reflect general properties of all antibodies which bind with this area of TMVP. However, the binding data reported here do not pertain exclusively to rabbit 31,500 since the globulins from another rabbit (24,300) were tested with many of the peptides (peptides 2–4, 6–11, 13–16, 18, and 20) and their activities were qualitatively the same for every peptide as those obtained using rabbit 31,500. For example, of the peptide analogs of the reference pentapeptide, peptides with isoleucine or tyrosine for leucine, or asparagine for aspartic acid were active, and substitutions of alanine for leucine, glutamic acid for aspartic acid, glycine for either alanine or threonine, and leucine for either the alanine or threonine residue, were inactive.

Studies with purified antibodies prepared from different rabbits and isolated by their specific binding to the reference pentapeptide should help to determine whether there are some general aspects of the peptide which must be kept unchanged for binding. Studies with such purified antibodies would also help to answer the question as to whether the same antibodies which bind to Leu-Asp-Ala-Thr-Arg (peptide 1) also bind to Asp-Ala-Thr-Arg-Arg (peptide 18) or whether the observed equal activity of these two peptides involves different populations of antibodies.

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

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Glycopeptides from Fibrinogen and Fibrin*

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ABSTRACT: Four major glycopeptides fractions were isolated from bovine fibrinogen and from fibrin. Two of them are characterized by the presence of lysine and the other two by the presence of arginine, glutamic acid, glycine, and valine. One of the lysine-containing glycopeptides present in both fibrinogen and fibrin contained 1.4–2.0 moles of sialic acid. This glycopeptide has not been reported previously. (When this manuscript was in preparation, we read the report of Mester *et al.* (Mester, L., Moczar, E., and Szabados, L. (1967), *Compt. Rend. Acad. Sci. Paris 265*, 877) who have now obtained as many as seven glycopeptides by high-voltage electrophoresis, one of which has lysine and 24% sialic acid.) These data and the facts that in the glycopeptide mixtures arginine and lysine are equal on a molar basis, and that glutamic acid is present to the extent of only 3 moles/mole of fibrinogen indicate the chains of fibrinogen are not all paired with respect to their glycopeptides. Degradation of one of the glycopeptides with sialidase followed by crude β-glucosidase indicated the sequence from the nonreducing end of sialic acid, galactose, *N*-acetylglucosamine, and mannose.

In each glycopeptide, both *N*-acetyl- and *N*-glycolylsialic acids were present and could be removed by purified *Clostridium perfringens* sialidase. The glycopeptides from cross-bonded fibrin contained 1 mole less of aspartic acid than those from fibrinogen. Also, one of the glycopeptides from fibrin was held more strongly on Dowex 1 (formate column) than was its counterpart from fibrinogen.

The carbohydrate moiety of fibrinogen (Laki, 1951a) may be necessary to the function of fibrinogen as a substrate for thrombin since periodate oxidation of bovine fibrinogen results in a 50% loss of clottability by thrombin when 30% of the hexose and 40% of the sialic acid were oxidized (Laki and Mester, 1962). There is a controversy as to whether the carbohydrate is involved when fibrin is stabilized by the enzyme variously called Laki–Lorand factor, fibrinase, or factor XIII. Enzymatic removal of the sialic acid resulted in a fibrinogen which was not capable of stabilization by the enzyme (Laki and Chandrasekhar, 1963), but on the question of loss of sialic acid under the action of the Laki–Lorand factor, there are reports pro (Chandrasekhar *et al.*, 1962, 1964; Chandrasekhar and Laki, 1964) and con (Blombäck, 1958; Raisys *et al.*, 1966). The same confusion exists regarding the possibility that stabilized fibrin contains 10–20% less hexose, there being reports pro (Laki, 1951a; Blombäck, 1958; Brown, 1963; Szara and Bagdy, 1953; Bagdy and Szara, 1955) and con

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