Immunochemical Studies on Tobacco Mosaic Virus Protein. V. The Solid-Phase Synthesis of Peptides of an Antigenically Active Decapeptide of Tobacco Mosaic Virus Protein and the Reaction of These Peptides with Antibodies to the Whole Protein*

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ABSTRACT: It has been previously shown that tobacco mosaic virus protein (TMVP) tryptic peptide 8 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 and the synthetic C-terminal decapeptide of peptide 8 having the sequence Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg possess immunological activity related to that of the whole protein (TMVP). The present paper reports the solid-phase synthesis and the immunological activity of the C-terminal di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, and nonapeptides of the decapeptide. The peptides were acetylated with [^{14}C]acetic anhydride and the resulting [^{14}C]acetyl peptides were tested for specific binding with anti-TMVP. The pentapeptide Leu-Asp-Ala-Thr-Arg and all larger peptides were found to possess this immunological activity, while the shorter peptides lacked significant activity.

Tryptic peptide 8 is an eicosapeptide of tobacco mosaic virus protein (TMVP) containing residues 93-112 of 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 (Gish, 1961; Funatsu *et al.*, 1964; Anderer *et al.*, 1965). This peptide has been shown to possess immunological activity related to TMVP and was demonstrated to bind specifically with rabbit antibodies to the whole protein (Young *et al.*, 1963; Benjamini *et al.*, 1964, 1965). Degradation studies revealed that the carboxyl area of the eicosapeptide was more important for immunological activity than its N-terminal part since the removal of merely the arginine residue from the C terminus of the peptide resulted in a more pronounced decrease in activity than the removal of five residues from the N-terminal end (Young *et al.*, 1966). In further studies, the C-terminal decapeptide of peptide 8 having the amino acid sequence Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg was synthesized and was shown to bind specifically with anti-TMVP (Stewart *et al.*, 1966). It was, therefore, of interest to ascertain the smallest peptide of the decapeptide which exhibits specific binding with antibodies. For this purpose, the C-terminal di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, and nonapeptides were synthesized and the binding of each peptide with anti-TMVP was measured. These peptides were prepared by removing a portion of the peptide-resin prior to the addition of the next amino acid in the Merrifield nonautomated solid-phase synthesis of the decapeptide. The peptides were purified by ion-exchange chromatography and characterized by amino acid analysis, paper chromatography, and paper electrophoresis.

Experimental Section

Solid-Phase Synthesis of Peptides. The synthesis was similar to that described for the automated synthesis of the decapeptide (Stewart *et al.*, 1966), except that the reagents were added and removed manually. (A detailed description of automated synthesis of peptides has recently been published, Merrifield *et al.*, 1966.) The *t*-butyloxycarbonylamino acids (Cyclo Chemical Corp., Los Angeles, Calif.) were of high purity as ascertained by thin layer chromatography (tlc) (Merrifield, 1964). After each of the coupling reactions a portion of the peptide-resin was removed for isolation of the peptide. A 10.84-g batch of *t*-butyloxycarbonylnitro-L-arginine-resin[1] (0.48 mequiv of Arg/g of resin) which was obtained by esterification of *t*-BOC-nitro-L-arginine to the chloromethylpolystyrene–2% divinylbenzene copolymer resin was loaded into a reaction vessel (48 × 130 mm, 200-ml capacity, Berkeley Glass Laboratory, Oakland, Calif.), and the amino acid-resin was swollen with 60 ml of methylene chloride by shaking the vessel on a mechanical shaker (Chipco Manufacturing Co., San Bruno, Calif.).

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[1] Abbreviation use: *t*-BOC, *t*-butyloxycarbonyl; DCCI, dicyclohexylcarbodiimide.

TABLE I: Sequence of Steps for One Cycle of Solid-Phase Peptide Synthesis.

Step	Reagent	No. of Applications[a]	Vol. (ml)	Shaking Time (min)
1	1 M HCl in HOAc	1	30	30
2	HOAc	3	30	3
3	EtOH	3	30	3
4	$CHCl_3$	3	30	3
5	$CHCl_3$–Et_3N (9:1)	1	30	10
6	$CHCl_3$	3	30	3
7	CH_2Cl_2	3	30	3
8	*t*-BOC-amino acid in CH_2Cl_2	1	25[b]	5
9	DCCI in CH_2Cl_2	1	*c*	120
10	CH_2Cl_2[d]	3	30	3
11	HOAc	3	30	3
12	Remove a portion of peptide-resin	—	*b*	

[a] Steps 2, 3, 4, 6, 7, 10, and 11 were shaken manually between each of the three solvent washes. [b] The amount of *t*-BOC-amino acid used and the amount of resin removed are given in Table II. The volume of solvent used in the coupling step was reduced in proportion as amounts of peptide-resin were reduced. [c] The quantity of DCCI (1 g made to 2 ml with CH_2Cl_2) used was 1.05 moles/mole of *t*-BOC-amino acid added at step 8. [d] When left overnight the peptide-resin was left in CH_2Cl_2.

TABLE II: Data on Synthesis of Peptides.

Cycle	*t*-BOC-amino Acid Added	*t*-BOC-amino Acid Used (mequiv)	Peptide-Resin Removed (g)
1	L-Threonine	10.4	0.5
2	L-Alanine	6.6	1.17
3	β-Benzyl-L-aspartic acid	5.9	0.79
4	L-Leucine	5.3	2.32
5	L-Threonine	4.22	1.54
6	γ-Benzyl-L-glutamic acid	3.3	1.56
7	L-Alanine	2.4	1.76
8	L-Threonine	1.61	2.15
9	L-Threonine	0.74	2.33 (total remaining)

The sequence of steps used for the coupling of each amino acid to the resin is outlined in Table I. The methylene chloride was removed and the *t*-BOC-nitro-L-arginine-resin was washed with acetic acid (step 11, Table I). The *t*-BOC group of the amino acid was removed by treatment with 1 M HCl in acetic acid (step 1, Table I), and the amino acid-resin was washed successively with acetic acid, ethanol, and chloroform (steps 2–4). It was then treated with triethylamine (step 5) and washed with chloroform and methylene chloride (steps 6, 7); *t*-BOC-L-threonine in methylene chloride was added to the amino acid-resin (step 8) followed by the addition of DCCI (step 9). Following the reaction, the peptide-resin was washed with methylene chloride and acetic acid (steps 10 and 11). A portion of the peptide-resin was removed for isolation of the dipeptide threonyl-arginine. The remainder of the peptide-resin was left in the reaction vessel, and the rest of the amino acids given in Table II (cycles 2–9) were successively attached to the peptide-resin as described above utilizing the steps 1–12 (Table I) for the addition of each amino acid. The amount of *t*-BOC-amino acid used in each cycle (*ca.* 2 moles/mole of peptide) and the weight of peptide-resin removed after each coupling step are given in Table II. The number of moles of dicyclohexylcarbodiimide added was 5% more than the moles of *t*-BOC-amino acid used. Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer using the method of Spackman *et al.* (1958). For amino acid analysis, peptides were hydrolyzed by open reflux for 15 hr in 6 N HCl using 50-ml 24/40 ⅋ flasks fitted with ⅋ 50-cm air condensers. Peptide-resins were first hydrolyzed by open reflux for 15 hr using 2 ml of peroxide-free dioxane and 2 ml of 12 N HCl, and following filtration and rotary evaporation, were rehydrolyzed by open reflux in 4 ml of 6 N HCl. An amino acid analysis of each of the hydrolyzed peptide-resins expressed in mole ratios is given in Table III (top analysis). The yield of peptide per gram of peptide-resin (calculated from the amino acid analysis of the resin-peptide) was 0.2–0.3 mequiv/g of peptide-resin.

A portion of each peptide-resin sample was cleaved with anhydrous HBr in trifluoroacetic acid (Merrifield, 1964). The crude peptides were dissolved in methanol containing 10% acetic acid and 10% water and were reduced with hydrogen using 5% palladium on barium sulfate (Engelhard Industries, Newark, N. J.) as the catalyst. The reduced peptides were electrophoresed in a pH 6.4 pyridine–acetic acid–water buffer (10:0.4:90) (Stewart *et al.*, 1966), and the mobilities obtained for each of the ninhydrin and Sakaguchi-positive materials are given in Table IV (column 1). Each of the reduced peptides was chromatographed on a 1 × 150 cm Dowex 1-X2 ion-exchange column equilibrated with a pH 8.8 collidine–pyridine–acetic acid–water buffer (40:40:0.25 in 4 l.). The Thr-Arg and Ala-Thr-Arg peptides were eluted with a pH 8.1 collidine–

TABLE III: Amino Acid Ratios of Peptides.

Peptide	Sequence	Mole Ratio of Amino Acids[a]					
		Arg	Thr	Ala	Asp	Leu	Glu[b]
Dipeptide		0.90	1.10				
	Thr-Arg	1.04	0.96				
		1.06	0.94				
Tripeptide		0.88	1.01	1.09			
	Ala-Thr-Arg	1.02	0.96	1.02			
		1.05	0.98	0.96			
Tetrapeptide		0.94	0.99	0.99	1.07		
	Asp-Ala-Thr-Arg	1.00	0.96	1.00	1.05		
		0.99	0.98	1.00	1.02		
Pentapeptide		0.92	0.98	0.98	1.09	1.03	
	Leu-Asp-Ala-Thr-Arg	0.95	1.05	1.00	1.02	0.98	
		1.01	1.00	1.03	0.98	1.00	
Hexapeptide		0.85	2.00	1.06	1.13	0.94	
	Thr-Leu-Asp-Ala-Thr-Arg	0.98	2.06	0.99	1.01	0.96	
		1.08	1.82	1.01	1.00	0.99	
Heptapeptide		0.90	2.21	0.96	1.00	1.04	0.26
	Glu-Thr-Leu-Asp-Ala-Thr-Arg	0.98	1.99	1.02	1.03	1.01	0.98
		1.10	1.95	1.00	1.05	1.05	0.95
Octapeptide		0.81	2.03	1.92	1.11	1.11	0.74
	Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	0.99	2.01	1.95	1.04	1.01	0.99
		1.21	2.01	1.93	1.05	0.95	1.02
Nonapeptide		0.90	3.03	1.89	1.11	1.04	0.76
	Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	1.07	2.73	2.07	1.08	1.04	1.03
		1.09	2.80	2.07	1.09	1.00	1.02
Decapeptide		0.94	3.62	2.04	1.20	1.11	0.82
	Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	1.04	3.24	1.97	1.02	1.00	0.99
		1.04	3.38	1.99	1.04	0.97	0.99

[a] The amino acid mole ratios given for each peptide are the peptide-resin (top), peptide after column purification (middle), and the [^{14}C]acetyl peptide which was subsequently used for the immunological assay (bottom). [b] Values for glutamic acid are characteristically low in peptide-resin hydrolysates. Recently (J. Stewart, unpublished) it was found that 10 ml instead of 4 ml for hydrolysis increased the Glu yield.

pyridine–acetic acid–water buffer (40:40:1.5 ml in 4 l.); all other peptides were eluted from the ion-exchange resin with the acetic acid gradient described previously (Young *et al.*, 1966). The peptides were located in aliquots of the eluate either by the Folin–Lowry reaction (Lowry *et al.*, 1951) or by the Sakaguchi reaction for arginine (Weber, 1930). The dipeptide and tripeptide (both basic peptides) eluted from the column at about 65 ml; the tetrapeptide through the hexapeptide (neutral peptides) eluted from the column at about 175 ml; and the heptapeptide through the decapeptide (acidic peptides) gave two peaks, one minor peak which eluted at 175 ml (indicating a neutral peptide) and a major peak which eluted at about 255 ml. The main peak obtained from each column was lyophilized and characterized. The amino acid analysis of each of the peptides after ion-exchange chromatography is shown in Table III (middle values). Results of paper electrophoresis at pH 2.7 (1 M acetic acid) and 6.4 as well as paper chromatography of the peptides in 1-butanol–acetic acid–water (3:1:1) and in 1-butanol–acetic acid–water–pyridine (15:3:12:10) are compiled in Table IV (columns 2–5).

A portion of each peptide was acetylated with [^{14}C]acetic anhydride and separated from [^{14}C]acetate by chromatography on Sephadex G-10 as previously described (Stewart *et al.*, 1966). Amino acid analyses of the acetylated peptides are included in Table III (bottom values). The specific activities were between 1.2 and 2.4 $\times$ 10^6 cpm/μmole.

Immunological Assay of Peptides. The anti-TMVP was obtained from a single rabbit. Antiacetylcholinesterase was used as a control serum. The sensitization, preparation of globulins, and measurements of binding of [^{14}C]acetyl peptides with anti-TMVP were performed as described previously by Benjamini *et al.* (1965). The assay for immunological activity was performed using 7.5 mμmoles of the [^{14}C]acetyl peptide or [^{14}C]-arginine (Calbiochem lot 880374, 16.42 mc/mmoles) and 0.5 ml of globulins in a total volume of 1 ml. Following

TABLE IV: Chromatographic, Electrophoretic, and Binding Properties of Synthetic Peptides and Several Reference Compounds.

Compound	Electrophoretic Mobility, R_{Arg}[a] pH 6.4 Column 1[b]	pH 6.4 Column 2[c]	pH 2.7 Column 3[c]	Chromatographic R_F Values[d] Solvent A Column 4[c]	Solvent B Column 5[c]	Binding to 0.5 ml of anti-TMVP[e] Column 6
Arginine	*−1.0*	**−1.0** (S, N)	**−1.0** (A, N)			0
Dipeptide	*−0.77*, −0.13	**−0.70**	**−0.99**	**0.23**	**0.37**	0
Tripeptide	*−0.72*, −0.13	**−0.67**, −0.13 (P)	**−0.99**, −0.52 (S)	**0.23**	**0.37**, 0.51 (P)	42 ± 42
Tetrapeptide	*−0.24*	**−0.16**	**−0.72**, −0.52 (S, P)	**0.17**	**0.27**	0
Pentapeptide	*−0.21*	**−0.28**	**−0.72**, −0.55 (N, P)[f] −0.43 (N)	**0.50**, 0.39 (S, N)[f]	**0.43**, 0.22 (N)[f] 0.24 (N), 0.82 (N)	1750 ± 35
Hexapeptide	*−0.19*, −0.14	**−0.22**	**−0.71**, −0.38 (N)	**0.44**	**0.44**, 0.39 (N)	1665 ± 166
Heptapeptide	*−0.20*, *−0.15*, +0.12	**+0.24**	**−0.66**	**0.42**	**0.36**, 0.42 (N)	1825 ± 91
Octapeptide	*−.10*	**+.13**	**−0.60**	**0.41**, 0.38 (P)	**0.37**, 0.42 (N)	2050 ± 40
Nonapeptide	*−0.14*, −0.06, −0.09	**+0.13**	**−0.54**	**0.45**, 0.37 (P)	**0.40**	2260 ± 66
Decapeptide	*−0.08*	**+0.13**	**−0.53**	**0.44**	**0.41**	2865 ± 27
Aspartic Acid	+0.9	+0.86 (N)	−0.21 (N)			
Threonine	−0.10 to −0.18	−0.13 (N)	−0.37 (N)			
Picric acid	+0.8	+0.75	+0.66			

[a] Electrophoretic mobility (R_{Arg}) is expressed as centimeters travelled from origin divided by centimeters travelled by arginine; (+) indicates migration to the anode; (−) indicates migration to the cathode; pH 2.7, 1 M acetic acid; pH 6.4, pyridine–acetic acid–water (10.0:0.4:90). [b] Column 1 represents R_{Arg} values of peptides prior to ion-exchange chromatography. The R_{Arg} of spots reacting to the arginine stain and the ninhydrin stain are in italics; other R_{Arg} values given reacted only to the ninhydrin stain. [c] The R_{Arg} and R_F values in columns 2–5 were obtained for the peptides after ion-exchange chromatography. The major spots in columns 2–5 are in boldface type; the stains used were: ninhydrin (N), Sakaguchi (S), and peptide (P). (The peptide stain is described by Nitecki and Goodman, 1966.) All major spots in columns 2–5 reacted to all three stains; other spots reacted to the stains as indicated in the table. [d] Solvent A,1-butanol–acetic acid–water (3:1:1); solvent B, 1-butanol–acetic acid–water–pyridine (15:3:12:10). [e] Binding of the compounds to anti-TMVP globulins was determined by reacting 0.5 ml of anti-TMVP globulins with 7.5 mμmoles of [^{14}C]acetyl peptides or [^{14}C]arginine; the counts per minute were not corrected for variation in specific activity of the peptides; the values represent averages of three identical assays. The assay of each compound with anti-acetylcholinesterase globulins was performed and revealed that the compounds did not bind with these globulins (see text). [f] Subsequent resynthesis of the pentapeptide has yielded a single spot in the electrophoretic and chromatographic systems given above.

precipitation at 50% saturation of ammonium sulfate and two washings, the precipitates were dissolved in saline and their radioactivity was measured. Results are given in Table IV (column 6). Each ^{14}C peptide was also assayed for its binding with antiacetylcholinesterase globulins; no radioactivity was found to be associated with these globulins.

Discussion

Synthesis. After cleavage from the resin and reduction, some of the peptides were heterogeneous, as judged by their electrophoretic behavior at pH 6.4 (Table IV, column 1). This heterogeneity was not reflected in the amino acid analyses of the peptide-

resin in that the mole ratios were very close to the expected values, except for the glutamic acid value which is characteristically low in peptide-resin hydrolysates. Electrophoresis of the peptides after ion-exchange chromatography revealed that they were more homogeneous than prior to chromatography (Table IV, columns 2–5). The impurities still present, as revealed by electrophoresis and paper chromatography, gave much weaker reactions to the ninhydrin, Sakaguchi, and peptide stains than did the major spot of each peptide, and only this spot gave a reaction to all three stains. Subsequent to this work we have observed that impurities detected by electrophoresis in 1 M acetic acid could be largely removed by Sephadex G-10 chromatography (using a pyridine–collidine pH 8 buffer), indicating that the impurities are of lower molecular weight than the desired peptides. If this is the case in the preparation of peptides reported in this study, then these peptides after ^{14}C acetylation were more homogeneous than the peptides prior to acetylation, since the [^{14}C]acetyl peptides were subjected to the G-10 Sephadex chromatography.

In the present study, the di-, tri-, tetra-, penta-, and hexapeptides, after cleavage and reduction, had the expected electrophoretic mobility. Unexpected electrophoretic mobility of the peptides was observed in each case after the addition of the glutamic acid residue. As is seen in Table IV (column 1), the hepta-, octa-, nona-, and decapeptides, rather than possessing the expected negative charge at pH 6.4 were found to be neutral. In order to keep the carboxyl group of the glutamic acid protected during the cleavage step, the decapeptide was recently synthesized in our laboratory (by M. Shimizu), using *N-t*-BOC-L-glutamic acid–γ-*p*-nitrobenzyl ester–α-*p*-nitrophenyl ester instead of the N-*t*-BOC-L-glutamic acid–γ-benzyl ester. Electrophoresis of the resulting crude decapeptide, after cleavage and reduction, revealed that although some neutral peptide was still present, the major peptide was acidic. These data, as well as other studies to be subsequently reported, indicate that the charge anomaly in the peptides is due to a masking of the glutamic acid carboxyl group in a way that is labile to either the basic resin or the buffer used in the chromatographic purification. The most likely such masking would be the intramolecular esterification of the glutamic carboxyl to one of the threonine hydroxyl groups in the anhydrous acidic medium used for the cleavage of the peptides from the resin. When the *p*-nitrobenzyl ester is used, the carboxyl group of glutamic acid remains protected during this cleavage step. In the hydrogenation step where the *p*-nitrobenzyl ester is removed, the medium is one that does not favor esterification. In any event, as was the case in the preparation of the decapeptide by Stewart *et al.* (1966), the peptides prepared in this study lost their anomalous electrophoretic behavior after chromatography on a Dowex 1 column equilibrated with a pH 8.8 collidine–pyridine buffer using an acetic acid gradient (Table IV, column 2).

Immunological Activity. The bindings of the synthesized peptides with anti-TMVP which are given in Table IV (column 6) demonstrate that significant binding with antibodies is exhibited by the pentapeptide representing residues 108–112 of the TMVP subunit and having the sequence Leu-Asp-Ala-Thr-Arg and by all larger peptides. No binding of the pentapeptide and larger peptides could be detected where anti-acetylcholinesterase was used instead of anti-TMVP. Bindings of the smaller peptides with anti-TMVP were not demonstrable utilizing the experimental procedure described in this communication, nor were they demonstrable by subsequent equilibrium dialysis studies using the same antiserum. Quantitative studies to determine binding constants and relative bindings of the various peptides are underway and preliminary results indicate that the binding increases with peptide size.

The pentapeptide possesses a number of functional groups: a large bulky aliphatic group (leucine), a carboxyl group (aspartic acid), a hydroxyl group (threonine), and a basic group (arginine). We are now synthesizing various analogs of this pentapeptide in order to ascertain the exact structural requirements in a peptide for binding with anti-TMVP.

Although the binding experiments reported here were performed on anti-TMVP from a single rabbit, the binding of these peptides with anti-TMVP globulins from a number of other rabbits has been tested. Anti-TMVP sera from some rabbits showed no binding to the decapeptide or lower peptides while sera from other rabbits showed binding; all anti-TMVP sera, however, showed binding with the eicosapeptide, *i.e.*, TMVP tryptic peptide 8. This phenomenon is now under study.

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References

Anderer, F. A., Wittmann-Liebold, B., and Wittmann, H. G. (1965), *Z. Naturforsch. 20b*, 1203.

Benjamini, E., Young, J. D., Peterson, W. J., Leung, C. Y., and Shimizu, M. (1965), *Biochemistry 4*, 2081.

Benjamini, E., Young, J. D., Shimizu, M., Leung, C. Y. (1964), *Biochemistry 3*, 1115.

Funatsu, G., Tsugita, A., and Fraenkel-Conrat, H. (1964), *Arch. Biochem. Biophys. 105*, 25.

Gish, D. T. (1961), *J. Am. Chem. Soc. 83*, 3303.
Lowry, O. H., Rosebrough, N. H., Farr, A. L., and Randall, R. N. (1951), *J. Biol. Chem. 193*, 265.
Merrifield, R. B. (1964), *Biochemistry 3*, 1385.
Merrifield, R. B., Stewart, J. M., and Jernberg, N. (1966), *Anal. Chem. 38*, 1905.
Nitecki, D. E., and Goodman, J. W. (1966), *Biochemistry 5*, 665.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stewart, J. M., Young, J. D., Benjamini, E., Shimizu, M., and Leung, C. Y. (1966), *Biochemistry 5*, 3396.
Weber, C. J. (1930), *J. Biol. Chem. 86*, 217.
Young, J. D., Benjamini, E., Shimizu, M., and Leung, C. Y. (1966), *Biochemistry 5*, 1481.
Young, J. D., Benjamini, E., Shimizu, M., Leung, C. Y., and Feingold, B. F. (1963), *Nature 199*, 831.

The Effect of Chaotropic Ions on the Dissociation of Antigen–Antibody Complexes*

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ABSTRACT: Hydrophobic, ionic, and hydrogen bonds are probably responsible for the interaction of antigens and antibodies. Certain ions such as thiocyanate, perchlorate, and iodide (chaotropic ions), known to unfold or dissociate macromolecular structures by disrupting these bonds, have now been found to dissociate primary antigen–antibody complexes without destroying the immunospecific activity of antibody. The order of decreasing effectiveness is thiocyanate > perchlorate > iodide. Demonstrations that chaotropic ions can dissociate primary antigen–antibody complexes are provided by the recovery of functionally active antibody from influenza virus–antiinfluenza complexes, and from complexes of antibody with solid immunoadsorbents in which the antigen is covalently bonded to an inert matrix. Other experimental approaches include inhibition of specific precipitation and dissolution of specific precipitates.

The three-dimensional structure of protein molecules is maintained by numerous, relatively weak bonds formed between different parts of the molecule. Evidence accumulated during the past 10 years indicates that the nature of these bonds, their relative positions, and the conformation of the resulting structure are a direct consequence of the sequence of amino acid residues in the molecular chain (Kauzmann, 1959; Singer, 1965). In "simple" or unconjugated proteins, the possible types of such bonds are limited, and include hydrogen bonds, "hydrophobic" bonds, electrostatic attractions, and London dispersion forces. One structural role of disulfide bonds seems to be that of locking into place a single, relatively stable conformation.

A line of evidence substantiating the importance of these weak bonds in influencing the tertiary structure stems from the unfolding or disruption of macromolecules by urea, guanidine, and more recently by certain ions (chaotropic ions) (Hamaguchi and Geiduschek, 1962; von Hippel and Wong, 1964; Robinson and Jencks, 1965a,b; Nagy and Jencks, 1965; Warren and Cheatum, 1966; Warren and Peterson, 1966). Under appropriate conditions, these materials presumably break a sufficient fraction of the bonds responsible for the tertiary structure, and the molecule unfolds under the influence of thermal forces.

Antibodies and antigens also owe their conformational stability to the bond types enumerated above. The unfolding of antibody in guanidine and the refolding with recovery of antibody activity upon the removal of guanidine have been demonstrated (Whitney and Tanford, 1965), but there exists a possibility (Singer and Doolittle, 1966), that even though most of the molecule certainly is unfolded, the active site may remain intact during the guanidine treatment.

The various weak bonds mentioned above are responsible not only for the conformation of the antigen and antibody molecules themselves, but also probably furnish the binding free energy for the formation of the antigen–antibody complex (Karush, 1962). Accordingly, it may be expected that chaotropic ions would dissociate antigen–antibody complexes, possibly under conditions innocuous to the antigen and antibody molecules themselves.

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