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Immunochemical Studies on the Tobacco Mosaic Virus Protein. I. The Immunological Relationship of the Tryptic Peptides of Tobacco Mosaic Virus Protein to the Whole Protein*

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The immunochemical activity of the tryptic peptides of tobacco mosaic virus protein was studied in a system composed of the viral protein and antiprotein, using complement fixation and complement-fixation inhibition. A low-molecular-weight fraction ($G_{50}S_2$) of a tryptic digest of the protein was obtained by fractionation on G-50 Sephadex. Peptide mapping revealed that this fraction contained all the characteristic tryptic peptides of tobacco mosaic virus protein except the N-terminal peptide (peptide 1). (The nomenclature of the tryptic peptides of tobacco mosaic virus protein is according to that proposed by A. Tsugita, D. T. Gish, J. Young, H. Fraenkel-Conrat, C. A. Knight, and W. M. Stanley [1960, *Proc. Natl. Acad. Sci. U. S. 46*, 1463].) Peptide 1 was isolated by repeated isoelectric precipitation at *p*H 4.7. Neither the $G_{50}S_2$ nor peptide 1 fixed complement with anti-tobacco mosaic virus protein serum. However, the $G_{50}S_2$ completely inhibited the fixation of complement by tobacco mosaic virus protein and anti-tobacco mosaic virus protein, whereas peptide 1 was inactive. The peptides in the $G_{50}S_2$ fraction were isolated by Dowex 1 × 2 ion-exchange chromatography followed by paper chromatography. The immunochemical activity of $G_{50}S_2$ in the tobacco mosaic virus protein and anti-tobacco mosaic virus protein system could be attributed to tryptic peptide 8 having the amino acid sequence: Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg.

Immunological activity related to that of the whole protein has been obtained from fragments of several protein antigens (Cebra, 1961; Porter, 1959; Goodman, 1963; LaPresle and Durieux, 1957; Press and Porter, 1962). A report from our laboratory demonstrated inhibition of systemic anaphylaxis in egg albumin–sensitive guinea pigs by fragments of egg albumin (Benjamini *et al.*, 1962); however, proteins of known amino acid sequence greatly facilitate the study of antigenic determinants, as exemplified by the study of

* A preliminary report on this study has been presented (Young *et al.*, 1964).

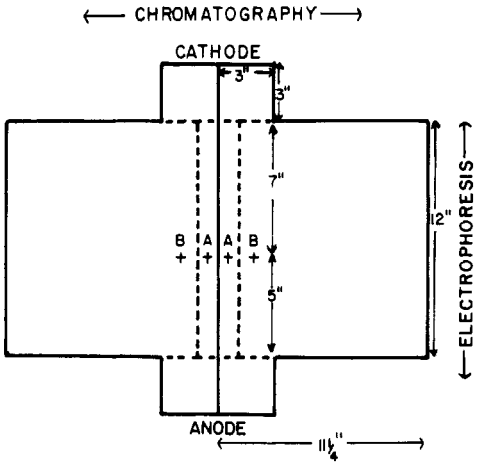

FIG. 1.—Peptide-map dimensions. Two papers are cut from Whatman 3 MM paper. The 76-mm (3-in.) tabs are immersed in buffer wells. Paper is wet with buffer, *p*H 6.4; the sample is spotted with marker (0.3 mg TMVP tryptic digest) at (+), A, and material to be mapped (equivalent to 1–2 mg TMVP) at (+), B. Broken lines (– – – –) indicate where sheets are cut prior to paper chromatography.

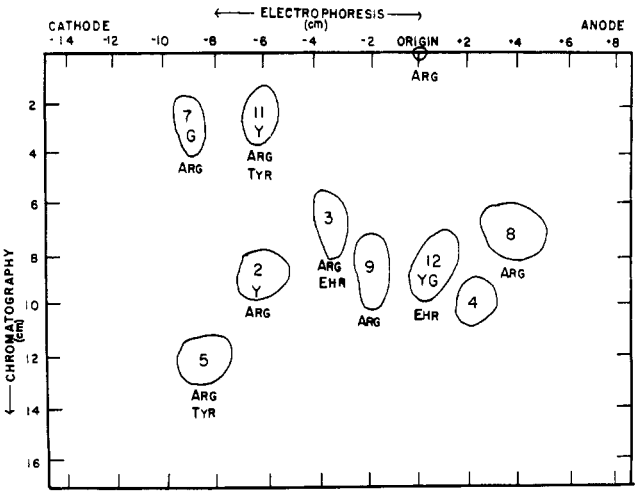

FIG. 2.—Peptide map of tryptic peptides of TMVP. First direction: *p*H 6.4 pyridine–acetic acid buffer; second direction: 1-butanol–acetic acid–water (3:1:1). Numbers refer to the tryptic peptides. Abbreviations used: Ninhydrin-positive (NIN); arginine-positive (ARG); tyrosine-positive (TYR); Ehrlich-positive (EHR); yellow (Y); yellow-gray (YG); gray (G). Colors refer to ninhydrin-staining color of spots that are not the characteristic purple.

Brown (1962) on the antigenic structure of oxidized ribonuclease.

The tobacco mosaic virus and its protein are particularly well suited for immunochemical studies (Anderer, 1963a,b,c; Knight, 1961; Takahashi and Gold, 1960; Aach, 1959; Kleczkowski, 1961; Starlinger, 1955). The virus particle contains 2200 protein subunits (Franklin *et al.*, 1959) and the sequence of the 158 amino acids of each protein subunit, as well as amino acid sequences of proteins of several virus mutants, are fairly well established (Anderer and Handschuh, 1962; Tsugita *et al.*, 1960; Funatsu *et al.*, 1964). Immunochemical studies on the protein would therefore be expected to yield information on the role which the primary, secondary, and tertiary structures of the protein play in its antigenicity; comparative studies with the whole virus should illuminate the role of the quaternary structure in viral antigenicity.

A preliminary report from our laboratory showed that the tryptic peptides of the tobacco mosaic virus protein exhibited immunological activity related to the protein and antiprotein system (Young *et al.*, 1963). This was demonstrated by passive cutaneous anaphylaxis and by studies utilizing isolated ileum strips. Further studies on the immunochemical activity of the tryptic peptides of TMVP[1] are reported in the present communication. The outstanding finding is that tryptic peptide 8 inhibits complement fixation by TMVP and its homologous antiserum.

Experimental Procedures

Peptide Mapping of the Tryptic Peptides of TMVP.—The glass-sandwich technique of Ingram (1958) was utilized in peptide mapping: two papers were cut from a single sheet of Whatman 3 MM paper 46 × 57 cm (18¼ × 22½) in., and marked as indicated in Figure 1. A reference marker of TMVP tryptic digest (0.2 mg) was applied at the (+) point designated A, and the material to be mapped (equivalent to 1–2 mg of TMVP) was applied at the (+) point designated B. The buffer was composed of pyridine–acetic acid–water (100:4:900), *p*H 6.4. The voltage was adjusted from 1000 v initially to that required to maintain 47 ma. At the end of 2.5–3 hours of electrophoresis, the sheets were laid horizontally on glass plates and dried in an oven at 70° for approximately 30 minutes. The side marker and tabs were then cut off (as indicated in Fig. 1 by the broken lines) and the sheets were subjected overnight to ascending chromatography in 1-butanol–acetic acid–water (3:1:1). The peptides were located and characterized by ninhydrin staining followed by the Ehrlich stain, the arginine stain, or the tyrosine stain (Smith, 1960). The peptide map of the TMVP tryptic peptides obtained by this method (Fig. 2) was similar to that described by Woody and Knight (1959) except that peptides 7 and 11 were in exchanged positions.

Preparation of the Tryptic Peptides of TMVP.—Tobacco mosaic virus protein was obtained from tobacco mosaic virus (TMV) by treatment with 67% acetic acid (Fraenkel-Conrat, 1957).[2] The trypsin used was a twice-recrystallized salt-free preparation (Worthington Biochemical Corp., lot 6118). TMVP, 100 mg in 20 ml water, was digested with 2 mg trypsin at 40°. Additional trypsin, 2 mg, was added after 1 hour of digestion. The *p*H was maintained at 8.0 with 0.2 N NaOH by means of a *p*H-stat (Model J, International Instrument Co., Canyon, Calif.). After 2 hours the digest was made 1 N with acetic acid, resulting in the formation of a precipitate. The precipitate was removed and the soluble material was lyophilized. The lyophilized digest was taken up in 5 ml 1 N acetic acid and insoluble material was removed by centrifugation. The solution was passed through a 2 × 30–cm Sephadex G-50 column which was equilibrated and eluted with 1 N acetic acid. Fractions (5 ml each) were collected and their absorbancies were read at 280 mμ (Fig. 3). Peptides eluting in the low-molecular-weight region[3] were combined and lyophilized. This low-molecular-weight fraction is designated as $G_{50}S_2$. The $G_{50}S_2$ fraction was chromatographed on a 1 × 150–cm Dowex 1 × 2 column which was developed with a polygradient buffer system ranging from a pyridine-

[1] Abbreviation used in this work: TMVP, tobacco mosaic virus protein.

[2] The TMV was generously supplied by Dr. C. A. Knight of the Virus Laboratory, University of California, Berkeley.

[3] The low- and high-molecular-weight regions were determined by passing a mixture of NaCl and γ-globulin through the column prior to fractionation of the digest.

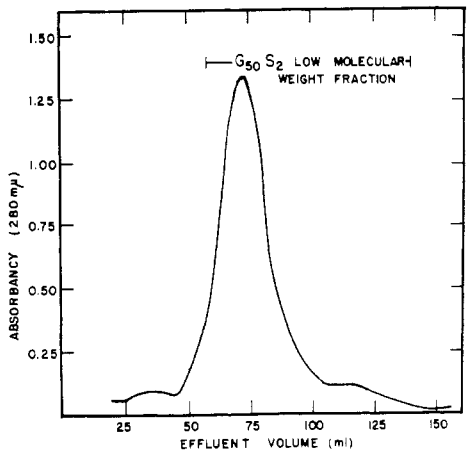

FIG. 3.—Sephadex G-50 elution pattern of tryptic digest of TMVP soluble in 1 N acetic acid. Column eluted with 1 N acetic acid.

collidine buffer of *p*H 8.8 to 50% acetic acid (Funatsu *et al.*, 1964). The elution pattern, which was obtained by Folin analysis, and the fractions which were taken are shown in Figure 4. Peptide 1 was obtained by repeated (4 times) isoelectric precipitation (*p*H 4.7) of a tryptic digest of TMVP, as described by Funatsu *et al.* (1964).

The presence of the characteristic tryptic peptides of TMVP was ascertained by comparing our elution pattern on Dowex 1 × 2 with that obtained by Tsung *et al.* (1964), and by peptide mapping and paper chromatography. Peptide mapping (Fig. 2) revealed with certainty the presence of tryptic peptides 2, 3, 4, 5, 7, 8, 9, 11, and 12. Paper chromatography of the ion-exchange fraction expected to contain peptides 12 and 6 revealed ninhydrin- and peptide-staining areas (Pan and Dutcher, 1956) with the expected R_F values (0.4 and 0.8, respectively) for these peptides, thus confirming their identity. The solvent system for the above chromatography was 1-butanol–acetic acid–water–pyridine (30:6:24:20) (Waley and Watson, 1953). Peptide mapping of the fraction obtained from the Dowex 1 × 2 fractionation which was expected to contain peptide 10 revealed a ninhydrin-negative but arginine-positive reaction at the point of application. The N-terminal amino acid for this fraction was, as expected, serine. Peptide 4 was isolated by paper chromatography using the 1-butanol–acetic acid–water–pyridine solvent (R_F 0.46). Peptide 8 was isolated by isoelectric precipitation (at *p*H 3.8) of the ion-exchange fraction containing peptides 4 and 8 (Funatsu *et al.*, 1964). The identity of peptides 4 and 8 was confirmed by quantitative amino acid analysis, using the DNP method (Levy, 1955). Peptide 8 was also obtained from Dr. C. M. Tsung of the University of California Virus Laboratory. The amino acid analysis of this material on the Spinco amino acid analyzer agreed with that of peptide 8 with less than 10% amino acid impurities. Throughout this report, the concentrations of each peptide fraction are expressed in terms of TMVP equivalents, i.e., the weight of TMVP from which the peptide fraction was obtained.

Antisera.—Antisera to TMVP were obtained from three rabbits which had received three weekly intramuscular injections of 10 mg antigen in 1 ml saline combined with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). The animals were bled approximately 10 days after the last injection. The sera were heated to 56° for 30 minutes to deactivate the complement. Antisera were divided into 1-ml aliquots and stored at −20°.

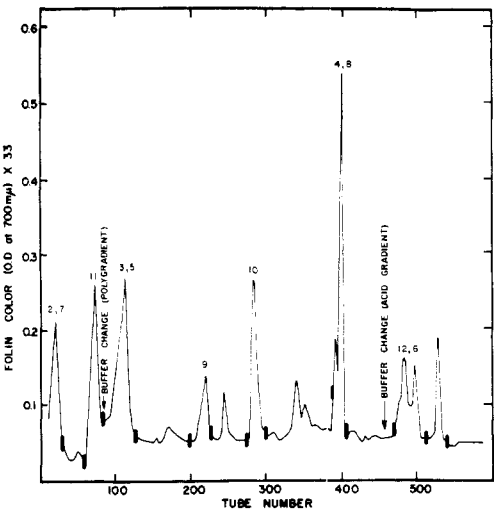

FIG. 4.—Dowex 1 × 2 ion-exchange separation of the TMVP tryptic peptides. Folin analyses were performed on 0.1-ml aliquots from alternate tubes. Aliquots were evaporated to dryness prior to assay; temperature, 40°, flow rate, 42 ml/hr; fractions, 3 ml. Fractions were pooled as indicated by the heavy bars (|) on the curve. Numbers refer to the tryptic peptides found in the fractions.

Complement Fixation and Inhibition of Complement Fixation.—The diluent used throughout these experiments consisted of a barbital buffer containing NaCl, Mg^{2+}, and Ca^{2+}, as described by Kabat and Mayer (1961). Complement-fixation tests were performed as follows: The antigen, antiserum, 1-ml complement, and diluent to total 2 ml were incubated at 37° for 30 minutes, after which 0.5 ml of hemolysin and 0.5 ml of 2% sheep erythrocytes were added. (Sheep hemolysin, obtained from Colorado Serum Co., Denver, Colo., was used at a dilution of 1:4000 as determined by titration. Guinea pig complement was used at a dilution of 1:43 as determined by complement titration.) The mixture in a total volume of 3 ml was incubated at 37° for 15 minutes (during which time controls consisting of identical mixtures, but lacking either antigen or antibody, hemolyzed completely). Following centrifugation for 15 minutes at 1500 rpm at 4°, optical densities of the supernatants were determined spectrophotometrically at 541 mμ. When complement-fixation inhibition by the peptides was studied, the peptides were incubated with antibodies for 30 minutes at 37° prior to addition of antigen and complement. Here again, the total volume of the reacting mixture was 3 ml.

RESULTS

Activity of the Tryptic Digest of TMVP.—Results of the inhibition, by various concentrations of the $G_{50}S_2$ fraction, of complement fixation by anti-TMVP at a dilution of 1:100 and TMVP at 0.1–0.8 μg are shown in Figure 5. The concentration of antibodies used in these experiments was chosen to ensure a slight antibody deficiency, so that the competition between the antigen and the tryptic peptides for antibody-reactive sites would be more pronounced. As may be realized from Figure 5, the degree of inhibition depends on the amount of peptides in the reacting mixture. When 50 μg of the $G_{50}S_2$ was used the inhibition was complete within experimental error. Comparable results were obtained using different antisera from three rabbits.

In order to eliminate the possibility that the inhibition by the tryptic peptides was nonspecific, experi-

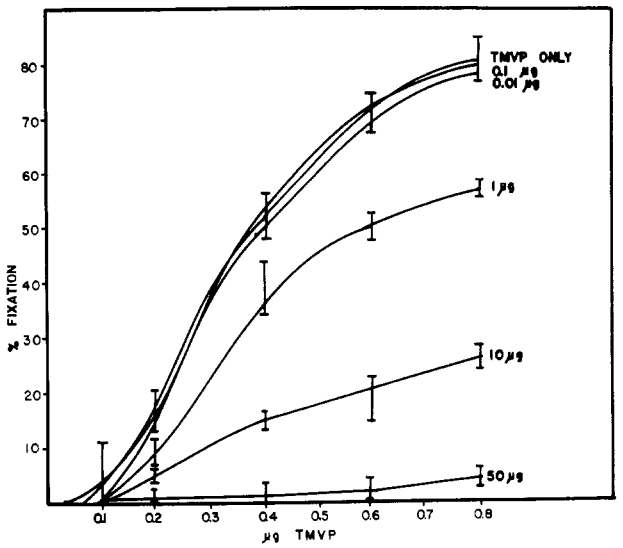

FIG. 5.—Complement fixation by TMVP and 1:100 anti-TMVP and its inhibition by the tryptic peptides of TMVP. The tryptic peptides of TMVP were obtained by fractionation on G-50 Sephadex. The weights of the peptides, indicated by a figure at the end of each curve, are given in terms of the weight of TMVP from which the peptides were obtained.

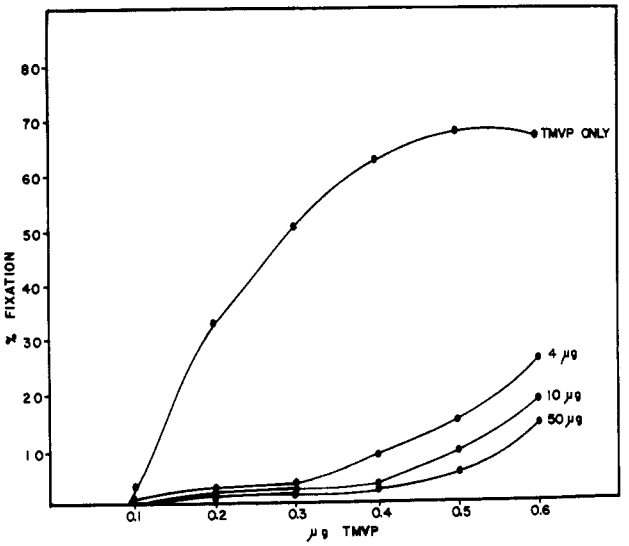

FIG. 6.—Complement fixation by TMVP and 1:300 anti-TMVP and its inhibition by TMVP tryptic peptide 8. The weight indicated for peptide 8 corresponds to the weight of TMVP from which the peptide was obtained.

ments were performed on the effect of the preparation on the complement fixation by a system consisting of acetylcholinesterase as an antigen and homologous antiserum (also in slight antibody deficiency). The $G_{50}S_2$ fraction of the tryptic peptides of TMVP had no effect on this system.

The fixation of complement by the peptides with anti-TMVP was tested. The peptides at concentration ranges equivalent to 0.01–200 μg TMVP were tested with a range of 1:5 to 1:100 anti-TMVP. In no case was complement fixed, indicating that the inhibition by the peptides of complement fixation by TMVP and anti-TMVP was not caused by antigen excess due to traces of TMVP in the peptide mixture.

Activity of Isolated Peptides.—In order to ascertain that the activity of the $G_{50}S_2$ was retained after fractionation by ion-exchange chromatography, equal portions of each fraction obtained from the ion-exchange chromatography were pooled and assayed. The activity of this preparation was the same as that of the $G_{50}S_2$. Experiments on the inhibition of the complement-binding capacity of TMVP and anti-TMVP by each of the fractions obtained from the Dowex ion-exchange column revealed that only the fraction containing peptides 4 and 8 and the fraction eluting before this fraction were inhibitory. The fraction containing peptides 4 and 8 was approximately 175 times more active immunologically than was the other active fraction. When peptide 4 and peptide 8 were tested separately for activity, only peptide 8 had activity. The total inhibition by peptide 8 of complement fixation by TMVP and anti-TMVP was found to occur when different antisera from three rabbits were used. Peptide 8 had no effect on complement fixation by acetylcholinesterase and antiacetylcholinesterase. The ability of this peptide to inhibit completely the fixation of complement by TMVP and anti-TMVP is evident from Figure 6.

The fraction eluting prior to that containing peptides 8 and 4 from the Dowex column was not expected to contain any of the tryptic peptides of TMVP. It was therefore suspected that this fraction contained small amounts of peptide 8, which would account for its activity. Eluates obtained from paper chromatography of this fraction confirmed this hypothesis: the area where peptide 8 was found to chromatograph contained all the activity.

Since peptide 1 was not subjected to G-50 Sephadex gel filtration, the possibility existed that it might be contaminated with trace amounts of undigested TMVP. However, when tested for complement-binding capacity with anti-TMVP, no fixation of complement occurred at combinations ranging from 0.1 to 100 μg TMVP equivalents of peptide 1 and of 1:5 to 1:100 dilutions of anti-TMVP. When peptide 1 was tested for inhibition of complement fixation by the TMVP and anti-TMVP system, it was found inactive, even when used at a concentration equivalent to 100 μg TMVP.

DISCUSSION

The immunological activity of the tryptic digest of TMVP excluding peptide 1 has been demonstrated by its specific inhibition of complement fixation by TMVP and anti-TMVP. The fractionation of the digest on Sephadex G-50 was performed to eliminate the presence of any possible undigested protein. That the $G_{50}S_2$ fraction does not contain undigested protein was deduced from the fact that in all ranges tested it did not give a precipitin reaction nor did it fix complement with anti-TMVP. The specificity of activity of the $G_{50}S_2$ fraction to the TMVP and anti-TMVP interaction is supported by the finding that this fraction did not interfere in the complement fixation by acetylcholinesterase and its homologous antiserum. At the concentrations of antigen and antibodies used in the present studies approximately 100-fold excess of $G_{50}S_2$ (in terms of protein equivalents) was required for the complete inhibition of complement fixation by TMVP and anti-TMVP; however, substantial degrees of inhibition were achieved by as little as a 2-fold excess (Fig. 5). While no attempt has been made to quantitate the inhibition by the $G_{50}S_2$ fraction, one would expect that the degree of inhibition will depend on the relative amounts of the antigen, peptides, and antibodies present.

The findings that total inhibition of complement fixation could be achieved by the $G_{50}S_2$ fraction of the tryptic peptides of TMVP using three different antisera from three different rabbits may mean that all the

antigenic determinants of the protein were present in the $G_{50}S_2$ fraction, and that no antigenic determinants were lost during the tryptic cleavage of the susceptible peptide bonds of the protein. This total inhibition may mean that antibodies to TMVP are directed against parts of the primary sequence of the protein, since one would not expect the retention of much of the original secondary or tertiary structure following tryptic cleavage. Furthermore, any possible secondary or tertiary structure of the tryptic peptides would not be expected to be identical with that formed by these same areas in the undegraded protein. However, it is also possible that the antibody to TMVP is directed against a larger area on the protein which includes peptide 8 and that the size of peptide 8 is sufficient to sterically hinder the combination of the complement-fixing antibody with such an antigenic area. Furthermore, the findings that no activity was detected in any of the other tryptic peptides does not necessarily mean that these sequences do not contribute to the antigenicity of an area as proposed; the antigenic activity of such sequences may have been lost during the tryptic cleavage. If this is the case, the participation of the secondary or tertiary structure in antigenicity cannot be excluded.

The fact that the entire TMVP and anti-TMVP immunological activity of the tryptic peptides resided in the $G_{50}S_2$ fraction was corroborated by the experiments using antisera from three different rabbits in which the total activity could be attributed to peptide 8 (Fig. 6). The inactivity of peptide 1 was somewhat surprising since it comprises approximately one-fourth of the protein. However, the length of a peptide does not necessarily determine antigenicity.

Although the amount of each of the other tryptic peptides which were assayed for complement-fixation inhibition of TMVP and anti-TMVP was not known, all the peptides were inactive when assayed at a concentration at which peptide 8 showed high activity. Peptide 8 is the third largest of the tryptic peptides having the following sequence of 20 amino acids: Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg. It is the most acidic and one of the least soluble of the tryptic peptides of TMVP, and has a fairly high proportion of nonpolar amino acids. The possible contribution of peptides rich in nonpolar groups to immunological activity of peptides obtained from oxidized ribonuclease has been pointed out by Brown (1962). It should be noted however, that although the active tryptic peptide of TMVP contains 5 nonpolar residues from the total 20 residues, two other tryptic peptides of TMVP, which have a higher proportion of nonpolar residues, were found to be inactive.

Although immunological activity of $G_{50}S_2$ was also demonstrated by passive cutaneous anaphylaxis and activity on isolated ileum strips (Young *et al.*, 1963), it should be emphasized that the criterion employed for detection of the immunological activity of the individual tryptic peptides throughout the present studies and the finding that the activity is attributed to peptide 8 was based on their ability to inhibit the fixation of complement by TMVP and anti-TMVP.

Analogous to our work, Anderer (1963b) has recently reported a study on the immunological activity of peptides of TMVP in a system consisting of the virus and antivirus. Results of these studies showed that several peptides, including the C-terminal peptide, peptide 12, but excluding peptide 8, partially inhibited precipitin reactions between the virus (TMV) and anti-TMV. In addition to significant differences in techniques employed for detection of activity, which were used by us and by Anderer, the differences between our findings and those of Anderer may stem chiefly from the difference in systems used. Our studies have been on the protein and antiprotein system, whereas those of Anderer utilized the virus-antivirus system. Although we have confirmed the observations of other workers (Knight, 1961) that there is an immunological cross-reactivity between TMVP[4] and TMV (although not complete, as shown by absorption tests), we have found in preliminary experiments that there is a marked difference between the two systems with respect to their complement-fixation inhibition by a mixture of the tryptic peptides of TMVP. In contrast to the protein-antiprotein system we found that there was no inhibition of complement fixation by TMV and anti-TMV by the $G_{50}S_2$ fraction at ratios of hapten to antigen as high as 50:1. This finding is in agreement with that of Anderer, where no inhibition of the TMV and anti-TMV precipitin reaction was achieved by digests of TMVP, using hapten to antigen ratios as high as 60:1. Only when very high hapten to antigen ratios were used (1000:1 to 4000:1) was Anderer able to show partial inhibition.

Considering our finding that peptide 8 is an antigenic determinant of the viral protein, and assuming from Anderer's work that peptides other than 8 are implicated as antigenic determinants of the virus, then these areas of the protein subunits play different roles in the antigenicity of the viral protein and the intact virus. This possibility is highly speculative and awaits further clarification.

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Resemblance of the Gross Arrangement of Polypeptide Chains in Reconstituted and Native γ-Globulins*

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A study has been made of the conditions of reconstitution of 7S γ-globulin molecules from separated polypeptide chains and of the arrangement of chains in the reconstituted molecules. The molar ratios of H and L chains in the reconstituted molecules approached those of native γ-globulin when a molar excess of L chains was mixed with H chains. Hydrolysis of reconstituted molecules with papain produced S and F fragments in yields similar to those obtained with native γ-globulins. The contribution of the chains to the fragments was assessed by the use of reconstituted molecules formed from L chains and H chains labeled with different iodine isotopes (^{125}I and ^{131}I). The S fragment contained material from H and L chains; F fragments consisted largely of H-chain material. The results indicate that the gross arrangement of chains is the same in reconstituted and native γ-globulin molecules, and support the notion that the 7S γ-globulin molecule consists of two L chains and two H chains arranged in two interacting L·H pairs.

It has recently been shown that the separated H and L polypeptide chains of 7S γ-globulin will form reconstituted 7S molecules (Edelman *et al.*, 1963b; Olins and Edelman, 1964; Roholt *et al.*, 1964; Gally and Edelman, 1964; Fougereau *et al.*, 1964). This was achieved by mixing the chains in a dissociating solvent (0.5 N propionic acid) and dialyzing the mixture against neutral aqueous buffers. Interchain disulfide bonds could also be re-formed, if partially reduced chains which had not been alkylated were used as starting materials (Olins and Edelman, 1964). Reconstituted γ-globulins closely resembled native 7S γ-globulin in their electrophoretic, antigenic, and physicochemical properties. In addition it was found that the phage-neutralizing activity recovered (Edelman *et al.*, 1963b) after reassociation of chains from antibodies to f1 phage was present mainly in the 7S reconstituted fraction (Olins and Edelman, 1964).

The present communication provides additional information both on the conditions of reconstitution of H and L chains of human γ-globulin and on the similarity of the product to native 7S γ-globulin. Fragments obtained from reconstituted 7S material and native 7S γ-globulin after hydrolysis with papain have been found to be similar in their antigenic and electrophoretic properties. The data are compatible with models of the molecule consisting of two H chains and two L chains (Edelman *et al.*, 1963b; Fleischman *et al.*, 1963; Edelman and Gally, 1964) and suggest that the gross arrangement of the chains is the same in native and reconstituted γ-globulin.

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Materials and Methods

Human γ-globulin, lyophilized Cohn fraction II (lot C780) from Lederle Laboratories (Pearl River, N. Y.) was used.

Labeling of γ-Globulin with Radioactive Iodine.—Isotopic labeling of H and L chains with ^{131}I and ^{125}I was accomplished as previously described (Olins and Edelman, 1964). The iodination with carrier-free solutions of sodium [^{125}I]iodide (Volk Radiochemical Co., Chicago, Ill.) and sodium [^{131}I]iodide (Oak Ridge National Laboratory, Tennessee), followed the procedure of McFarlane (1963) except that the protein was dissolved in 0.15 M NaCl brought to *p*H 8.0 with 0.2 M sodium borate buffer as suggested by Helmkamp *et al.* (1960). Unreacted iodide was removed by passage of the protein solutions through 5 × 1.0-cm columns of Amberlite ion-exchange resin IRA-401 (Mallinckrodt Chemical Works, St. Louis, Mo.) equilibrated with the borate buffer. The specific activity of the labeled proteins ranged between 5×10^5 and 3×10^6 cpm per unit absorbancy at 280 mμ.

Reduction of Labeled γ-Globulin and Separation of H and L Polypeptide Chains.—The ^{125}I- and ^{131}I-labeled γ-globulins to be used in the same sequence of experiments were treated simultaneously. After labeling with isotope, the γ-globulin solutions were concentrated by ultrafiltration and then made 0.1 N in mercaptoethanol. Reduction proceeded in the absence of urea (Edelman and Poulik, 1961) for 2 hours and was stopped by addition of iodoacetamide to a final concen-