Differentiation of Tobacco Mosaic Virus Strains by Complement Fixation.* (19294)

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Strains of tobacco mosaic virus (TMV) have been differentiated serologically by applying a precipitin test to antiserum previously absorbed with heterologous antigen (1,2) and by determining the amount of virus required to reach equivalence with antiserum(3). Results of the present study show that strains of TMV can also be differentiated by complement fixation in three different ways; first, by determining the amount of virus that reacts optimally with homologous and heterologous antisera; second, by differences in antiserum dilution end-points in homologous and heterologous reactions, and, third, by complement fixation after absorption of antiserum with heterologous antigen.

Materials and methods. Viruses. The type and rosette(4) strains of tobacco mosaic virus and 5 laboratory strains derived by mutation from the type were used. Isolation and symptomatology of the latter will be described elsewhere. Tomato ringspot(5) and Southern bean mosaic (SBM) viruses were included in some tests as useful controls. SBMV was propagated in the Bountiful variety of garden bean. The other viruses were propagated in Turkish type tobacco. *Preparation of antigens.* Virus antigens were obtained by 2 alternating cycles of high (14,000 RPM for 1½ hours) and low (5,000 RPM for ½ hour) speed centrifugation of sap from diseased plants after preliminary mincing and freezing at —20°C. Purified preparations were suspended in fresh physiological saline and stored in small antigen bottles at —20°C. Since the antisera produced by injection with these preparations failed to react with normal tobacco sap, it can be concluded that the preparations contained an insignificant amount of normal tobacco protein. Virus content of the samples was determined by micro-Kjeldahl analysis.† *Preparation of antisera.* Normal serum was taken aseptically before each of 6 rabbits was injected with varying amounts of antigen on the 1st, 10th, 20th, 35th, 52nd, 60th, 78th, and 85th days and bled on the 20th, 35th, 60th, and 100th days. Total amount of virus injected varied from 8.6 mg for the Y24Y1 strain to 134.7 mg for the type strain. Intravenous injections were given exclusively after the 10th day. All sera were inactivated at 56°C for ½ hour. The 10-day antisera had no detectable titer. Hemolysin and complement titrations were carried out as previously described(5), commercial preparations of hemolysin, complement, and sheep red blood cells being used. *Antigen and antiserum titrations.* The optimum antigenic unit is here defined as that quantity of antigen (purified virus) that reacts optimally with a minimal concentration of homologous antibody. The antiserum dilution end-point is defined as the highest dilution of antiserum that completely fixes complement in the presence of an optimum antigenic unit. The optimum antigenic unit and the antiserum dilution end-point were determined by reacting a series of antigen dilutions with 2-fold serial dilutions of antiserum plus 2 full units of complement in all possible combinations for 15 to 18 hours at 4°C, adding sensitized red blood cells and incubating at 37°C for 30 minutes. A typical grid is in Table I. *Cross absorption.* 0.25 ml of heterologous antigen was added to 1 ml of antiserum and incubated at 37°C for ½ hour. The tube was then centrifuged, the supernatant removed, 0.25 ml of antigen again added, and the mixture incubated overnight at 4°C. The tube was again centrifuged to re-

* Based on thesis submitted by senior author in partial fulfillment of requirements for Master of Science degree. This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service. Contribution from the Plant Virus Laboratory of the University of Pittsburgh.

† The writers are indebted to Mr. David Trkula for carrying out the micro-Kjeldahl analyses.

TABLE I. Data Obtained in Titrating Homologous Antigen and 20-Day Antiserum for the Type Strain of TMV.

Serum dilution	Antigen dilution 1:1000	1:2000	1:3000	1:4000	1:5000*	1:10000	1:20000	Serum control
1:10	4	4	4	4	4	4	3	0
20	4	4	4	4	4	4	2	0
40	3	3	4	4	4	4	2	0
80†	1	2	3	4	4	3	1	0
160	+	+	+	1	2	+	0	0
320	0	0	0	0	+	0	0	0
Antigen control	0	0	0	0	0	0	0	

* Optimum antigenic unit. † Antiserum dilution end-point.

move precipitate, and more antigen added. The procedure was continued until no further precipitate was obtained on addition of antigen. The absorbed serum was then inactivated by heating at 56°C for ½ hour and subsequently used in complement fixation tests.

Experimental. The optimum antigenic unit. It is apparent from Table I that the 1:5000 dilution of type strain antigen is the highest dilution that reacted maximally (4 plus) with the greatest range of antiserum dilutions. The amount of virus present in this dilution, based on micro-Kjeldahl determinations, was 2.8 μg and is, by definition, the optimum antigenic unit. When the type and 5 laboratory strains of TMV were reacted with homologous antiserum from rabbits bled on the 20th, 35th, and 60th day following the initial injection, the optimum antigenic unit in each case was independent of the antiserum used, even though the antiserum dilution end-point increased with increasing length of the immunization period, and of the total amount of virus injected into the rabbit. Moreover, in every instance in which a virus strain was reacted with heterologous antiserum, the optimum antigenic unit was the same as that determined from the homologous reaction.

The optimum antigenic units found were: type strain, 2.8 μg; M1 strain, 7.0 μg; 7A strain, 6.4 μg; R1Y1 strain, 5.6 μg; Y21R1 strain, 3.6 μg; and Y24Y1 strain, 3.5 μg. These figures are subject to errors estimated at less than 50%.‡ Thus, there is reason to believe that some of the virus preparations were differentiated from one another by differences in their optimum antigenic units. The question of whether differences observed represent merely differences between virus preparations or real differences between TMV strains has been partly answered by the finding that the weights of virus in the optimum antigenic unit for two additional preparations of the type strain were 2.5 μg and 2.8 μg, respectively.

Antiserum dilution end-points. The 60-day antiserum for the type and 5 laboratory strains of TMV varied in titer from 1:256 to 1:640, the differences being attributable to variation in individual rabbits and not necessarily characteristic of the strains. In some cases, 2-fold differences were found between heterologous and homologous reactions of the same antiserum, but since they were not reproducible, the strains could not be differentiated on this basis. On the other hand, when the rosette strain of TMV was used as antigen to react with antiserum for the type strain, the antiserum dilution end-point was 1:80 as compared with 1:640 for the homologous reaction(4). This 8-fold difference was reproducible and provides a means of differentiating these two strains.

Antiserum for the type strain failed to react with Southern bean mosaic or tomato ringspot viruses. Antiserum for Southern bean mosaic virus reacted with the homologous virus, with an antiserum dilution end-point of 1:20, but failed to react with the type or rosette strains of TMV, or with tomato ringspot virus. The results provide additional evidence for specificity of the reaction.

‡ Duplicate micro-Kjeldahl analyses of the same samples revealed errors of less than 20% while duplicate or triplicate complement fixation tests revealed errors of less than 30% in determining the antigen dilution that contained the optimum antigenic unit.

Cross absorption. Tests were carried out in which samples of antiserum for the type strain were completely absorbed with a laboratory strain to see whether or not antibodies capable of reacting with the homologous antigen would remain. The absorbed serum itself failed to fix complement, showing that no residual antigen-antibody complex could be responsible for the fixation observed when the absorbed serum was titrated with its homologous antigen. Absence of complement-fixing antibodies against the virus strain used for absorption showed that the absorption had been complete in each case. After complete absorption with strains Y21R1, R1Y1, and M1, the type antiserum still fixed complement when mixed with type antigen with antiserum dilution end-points of 1:10, 1:40, and 1:5 respectively, indicating again that these strains differ antigenically from the type. Type antiserum absorbed with strain 7A fixed complement only when antiserum at a dilution of less than 1:5 was mixed with type antigen, indicating that such antigenic differences as exist between the type and 7A strains could not be detected by means of the cross absorption test used.

Discussion. Differentiation of the type and rosette strains of TMV by differences in antiserum dilution end-points in homologous and heterologous reactions and failure to differentiate between mutant strains on the same basis suggest a correlation between genetic and serological relationships. A parallel situation is found in the observation by Knight(6) that strains of TMV can be differentiated on the basis of the kinds and amounts of amino acids they contain, provided the strains are widely separated genetically but not if they are closely related genetically. Whether or not the antiserum dilution end-point method can be used to differentiate between strains only when they differ in amino acid content, or whether the method will always differentiate strains that differ in amino acid content, remains to be determined.

In quantitative precipitin tests reported by Kleczkowski(3), less of the aucuba mosaic strain of TMV than of the type strain was required to reach equivalence with antiserum prepared against the type. This quantitative difference between strains would seem to parallel the difference in optimum antigenic units, and perhaps may be a related phenomenon. In any case, data from the present study indicate that strains differ in the optimum concentration at which they react with antisera, whether the antisera are homologous or heterologous, and that the optimum antigenic unit is a characteristic property of the strain.

If the optimum antigenic unit is independent of the antiserum, then it follows that complement fixation can be adapted for measuring TMV concentration. The reaction is more sensitive than the precipitin reaction previously employed for this purpose and thus should permit studies involving lower virus concentrations. It is suggested that the method might be applied to viruses for which at present there are no adequate biological methods of measuring concentration.

Summary. Tests with purified preparations of 6 strains of tobacco mosaic virus and antisera produced in rabbits with each of these strains show that the complement fixation reaction can be used for differentiating mutant strains of this virus. In some cases, antibody remaining after absorption of specific antiserum with heterologous antigen could be detected by complement fixation in the presence of homologous antigen. In one case, a significantly lower antiserum dilution end-point was attained in the heterologous reaction than in the homologous reaction. Finally, strains were found to differ in optimum antigenic units, in the weight of antigen required to react optimally with specific immune serum. The suggestion is made that complement fixation can be used to measure tobacco mosaic virus concentration.

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Received November 26, 1951. P.S.E.B.M., 1952, v79.