RELATIONSHIPS OF LEGUME VIRUSES IN AUSTRALIA

II. SEROLOGICAL RELATIONSHIPS OF BEAN YELLOW MOSAIC VIRUS AND PEA MOSAIC VIRUS

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Summary

Antisera were prepared to one strain of bean yellow mosaic virus (B.Y.M.V.) and two strains of pea mosaic virus (P.M.V. strain 1 and P.M.V. strain 2). By utilizing the agglutination reaction, the serological activity of both strains of P.M.V. is demonstrated for the first time. The results of cross-agglutination experiments show the strains of P.M.V. and B.Y.M.V. to be serologically related. Cross-protection tests support the hypothesis that these are strains of one and the same virus.

Serological relationships within the legume virus group are discussed.

I. INTRODUCTION

The demonstration of serological activity for bean yellow mosaic virus (B.Y.M.V.) and pea mosaic virus (P.M.V.) has presented difficulties. Bawden (1948) discusses the probable reasons for this, and considers low concentration of virus within the host to be the most serious limiting factor. Increasing attention is now being directed towards techniques of antiserum production and the use of suitable adjuvants may well play an important role in plant virus serology. New techniques associated with demonstrating antigen-antibody reactions are being developed (van Slogteren 1955) and this should also help to increase the number of known serologically active plant viruses.

It has been considered desirable to purify partially the plant virus antigen before inoculating the animal to be used in antiserum production. Beemster and van der Want (1951) used massive doses of dialysed infected sap, injected intravenously, as antigen for antibody production. They were able to demonstrate serological activity for bean mosaic virus and B.Y.M.V. and laid the foundations for antiserum preparation to many viruses considered to be serologically inactive. In achieving this, use was made of the agglutination reaction for demonstrating antigen-antibody reactions, first described for plant viruses by Chester (1937). Precipitin reactions could not be demonstrated, but sufficiently reproducible results were obtained by the agglutination technique for it to be utilized in determining serological relationships within the legume virus group. Beemster and van der Want showed bean mosaic virus and B.Y.M.V. to be serologically similar, and the work reported here shows the relationships between the strains of B.Y.M.V. and P.M.V. reported by Goodchild (1956).

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II. Materials and Methods

Antiserum was prepared to strain 1 of B.Y.M.V. (Grogan and Walker 1948) and to the two strains of P.M.V. (Murphy and Pierce 1937; Stubbs 1937) reported in a previous paper (Goodchild 1956). Antigen was obtained from plants of *Vicia faba* L., var. Windsor Long Pod, which had been showing symptoms for at least 2 weeks but not longer than 4 weeks. Sufficient antigen for one inoculation was usually obtained from a single plant. Antigen was prepared by crushing plants in a mortar with a pestle, expressing the sap, and filtering first through a cotton wool pad and then through a Whatman No. 541 filter paper, using a Buchner funnel.

The filtered sap, which was dialysed against running tap water for 3 hr in a "Cellophane" bag, constituted the antigen. Rabbits were used to produce antiserum and each received 12 intravenous injections of 4 ml, four each week for 3 weeks with a 3-day interval at weekends. In the case of P.M.V. strain 1, however, sufficient antigen for 10 injections only was available. Several millilitres of normal serum were obtained from the marginal ear vein of each rabbit before injections commenced, and each serum sample was retained separately.

Eight days after the final injection blood was taken from the jugular vein of each rabbit and allowed to clot at 37°C for 1 hr and then at 3°C for a further 2 hr. Antiserum was separated by decantation and clear-centrifuged (10 min at 3000 r.p.m.). Normal serum was separated in a similar manner and all sera were stored in small ampules at −20°C until required. This procedure was adopted as Beemster and van der Want (1951) considered that the antiserum lost activity if retained under normal storage conditions. Two rabbits died, but anaphylaxis was not the cause in either case as shown by post-mortem examination.

The micro-technique developed by van Slogteren and others was used by Beemster and van der Want to demonstrate agglutination reactions, but due to technical difficulties it was decided to adapt the agglutination tube technique for these reactions. Dreyer-type tubes, with a capacity of approximately 1 ml, were used and in each case 0·5 ml of antigen was added to 0·5 ml of antiserum. All dilutions of antigen and antiserum were made with 0·85 per cent. saline, and for each antigen-antiserum test, controls of healthy, dialysed sap-antiserum, antigen-normal serum, and healthy, dialysed sap-normal serum were run. All tubes were incubated in racks in a water-bath.

III. Results

(*a*) *Agglutination Technique*

(i) *Optimum Agglutinating Temperature.*—As the antigen in all agglutination tests consisted of filtered infective sap, containing a large proportion of cell components, this would be expected to precipitate spontaneously, particularly at high temperatures. At low temperatures, however, the antigen-antibody reaction would proceed slowly so that effective agglutination may not be possible. Therefore, the temperature desired was one at which spontaneous precipitation did not take place at such a rapid rate that the antigen-antibody reaction could not be separated from it. Normal bacterial agglutinations take from 1–3 hr, so that 3 hr was considered to be the minimum time before which spontaneous precipitation should occur.

An arbitrary saturation (see Section III (*a*) (ii)) of 1 : 30 with normal serum was made and this then diluted 1 : 1, 1 : 2, 1 : 4, and 1 : 8. Healthy, dialysed sap was diluted 1 : 2 and 1 : 4 and added to separate series of tubes which were incubated at 30, 32, 35, 37, and 52°C. Only slight precipitation after 3 hr resulted at 30, 32, and 35°C, but bulky precipitates were evident 1 hr after incubation at 37 and 52°C, so that 35°C was used for all subsequent reactions.

(ii) *Saturation of Antiserum.*—Antibodies are developed to plant proteins, as well as virus protein, when crude infective sap is used as an antigen. These are removed by saturating the antiserum with crude, uninfected sap and centrifuging, when the clear supernatant antiserum is used for agglutination or precipitin reactions. For each antiserum the optimum saturation point was determined by saturating the antisera at 25°C for 3 hr and attempting agglutinations with healthy antigen. Saturation was achieved with healthy, broad bean sap diluted 1 : 1 with saline.

TABLE 1

DETERMINATION OF ANTISERA TITRE

+ = Agglutination; ± = slight agglutination; − = no agglutination

Antigen	Homologous Saturated Antiserum Dilutions						
	1:1	1:2	1:4	1:8	1:16	1:32	1:64
Bean yellow mosaic virus	+	+	+	+	±	−	−
Pea mosaic virus strain 1	+	+	+	+	+	±	−
Pea mosaic virus strain 2	+	+	+	+	+	±	−

For B.Y.M.V. the optimum saturation ratio was found to be 1 : 24, while for P.M.V. strain 1 this was 1 : 18 and 1 : 22 for P.M.V. strain 2. At saturations below the optimum, bulky precipitates were obtained in the agglutination tubes, while at higher saturations anomalous precipitates occurred in the antigen-antiserum and antigen-normal serum tubes.

(iii) *Optimum Antigen Dilution.*—Clearest results were obtained at an antigen dilution of 1 : 4 with normal saline.

(iv) *Optimum Dialysis Time.*—Dialysis of antigen against tap water is considered to result in a loss of toxic constituents. When undialysed infective sap was used as antigen in the agglutination reaction, no precipitate could be observed, and, when dialysis was allowed to proceed for $2\frac{1}{2}$, 3, or $3\frac{1}{4}$ hr, clearest results were obtained after $3\frac{1}{4}$ hr. No precipitate resulted after $2\frac{1}{2}$ hr dialysis. It is evident that a substance which inhibits agglutination is present in undialysed sap, and such a substance may inhibit antibody production if undialysed sap is used as antigen for inoculation.

(v) *Antiserum Stability.*—Beemster and van der Want (1951) emphasize that antiserum to B.Y.M.V. is unstable and that serological activity is lost after 2 or 3 days storage at 1°C. This is suggestive of complement exerting a non-specific effect, but when antisera to both strains of P.M.V. were heated to 56°C for 30 min, following saturation, no loss in activity could be demonstrated.

(vi) *Determination of Titre.*—Antisera were diluted as shown in Table 1, and 1 : 4 dilution of antigen was used in each case. A positive reaction was characterized by the presence of a bulky precipitate in the bottom of each tube, and a clear solution above, in which a few white floccules could usually be observed (Fig. 1). This reaction was apparent 1–1½ hr after incubation.

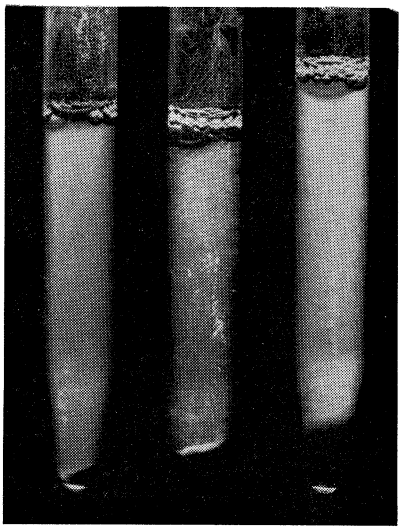

Fig. 1.—A positive agglutination test is indicated by the centre antigen-antiserum tube, which shows a clearing above the bulky precipitate and the presence of a few floccules, compared with the tube containing healthy sap–antiserum on the left, and with antigen–normal serum tube on the right. The healthy sap–normal serum tube is not shown but is similar in appearance to the tube on the left.

Thus the antiserum to B.Y.M.V. has an effective titre of 1 : 384, and effective titres of 1 : 576 and 1 : 704 to P.M.V. strain 1 and P.M.V. strain 2 respectively.

(*b*) *Serological Relationships*

(i) *Cross-agglutination Relations of the Viruses.*—Results of duplicate determinations are shown in Table 2. For each, a dilution series of saturated antisera from 1 : 1 to 1 : 16 (as shown in Table 1) was used.

Other field isolates whose host ranges corresponded to those of the antigens used for producing the antisera were also tested, viz. two of B.Y.M.V., one of P.M.V. strain 1 and two of P.M.V. strain 2. In each case the reactions were similar to those shown. Another strain of B.Y.M.V. (reported by Hutton and Peak 1954) also gave positive cross-agglutination reactions with all antisera.

(ii) *Cross-absorption Experiments.*—When B.Y.M.V. antiserum was saturated with P.M.V. strain 1 and P.M.V. strain 2 antigens, respectively, and the saturated

antiserum then tested against B.Y.M.V. antigen, no positive agglutinations could be observed. Similarly, when P.M.V. strain 1 and P.M.V. strain 2 were saturated with their heterologous antigens, no agglutinations resulted when the homologous antigens were used to test serological activity.

(c) *Cross-protection Experiments*

Ideally, the challenging virus used in demonstrating mutual immunization should be one capable of producing local lesions in the host chosen (Bennett 1953). With P.M.V. and B.Y.M.V. this is not possible but the two may be separated by the inability of P.M.V. to infect beans (Goodchild 1956). Broad bean plants were inocu-

TABLE 2

SUMMARIZED RESULTS OF CROSS-AGGLUTINATION EXPERIMENTS

+ = Positive agglutination reaction

Antigen	Sera		
	Bean Yellow Mosaic Virus	Pea Mosaic Virus Strain 1	Pea Mosaic Virus Strain 2
Bean yellow mosaic virus	+	+	+
Pea mosaic virus strain 1	+	+	+
Pea mosaic virus strain 2	+	+	+

lated with P.M.V. strains 1 and 2, the immunizing virus. When a basal shoot had developed two leaves with mottle symptoms, the main shoot was removed and these leaves inoculated with the challenging virus B.Y.M.V. Three weeks later the shoots were removed and the extracted sap from each used to inoculate six bean, var. Canadian Wonder, and six broad bean, var. Windsor Long Pod, plants. Of 24 plants with P.M.V. strain 1 and 36 plants with P.M.V. strain 2 treated in this way, in no case could the challenging virus be isolated, indicating an immunizing effect.

IV. Discussion

By the use of massive doses of P.M.V. antigen for inoculating the marginal ear vein of a rabbit and the appropriate conditions conducive to agglutination, it has been possible to demonstrate serological activity for this virus. The chief weakness in the technique lies in the preparation of antigen and the inability to provide sufficiently aseptic conditions, so that pathogenic organisms may enter, resulting in death of the animal. In practice, however, provided reasonable care is taken the chances of this occurring are relatively small. Anaphylaxis is also a danger, but is overcome to some extent by successive massive doses of antigen at relatively short time intervals, although in using crude plant sap there is a danger of the animal already having become sensitized through its feeding ration.

The methods used in demonstrating serological activity by agglutination have been described in as much detail as is possible, for conditions must be controlled to

ensure the success of the technique. The results of the agglutination tests between B.Y.M.V. and P.M.V. strains 1 and 2 and their heterologous antisera indicate the two viruses to be serologically related. The degree of the relationship seems to be absolute, as no agglutinations could be effected after antisera had been saturated with heterologous antigens. However, as the virus appears to be present in low concentration in the host, the titre of such antisera, with respect to the homologous antigen after absorption by the heterologous antigen, may be so low that the relatively crude agglutination technique could not detect its presence.

The serological similarity of B.Y.M.V. and P.M.V. is supported by the results of cross-protection tests, which show a mutual immunizing effect between the two strains of P.M.V. and B.Y.M.V. strain 1.

Beemster and van der Want (1951) have shown bean mosaic and B.Y.M.V. to be serologically similar, and Grogan and Walker (1948) were able to show that the two viruses have a certain immunizing effect in french beans. The results reported here show a similar relationship between B.Y.M.V. and P.M.V. Van der Want (1954) has reported alfalfa mosaic, B.Y.M.V., and white clover mosaic as being serologically distinct and results of cross-protection tests show that these viruses do not exert an immunizing effect for each other.

Thus it is now possible to group the legume viruses with respect to their sero-serological properties, but the large group consisting of strains of bean mosaic virus, B.Y.M.V., and P.M.V. are shown to be serologically related. It is likely, however, that some strains of B.Y.M.V. and, in particular, the necrotic lesion-producing strain of Zaumeyer and Fisher (1953) may not be serologically related to the strain reported here and by Beemster and van der Want (1951) by virtue of their reactions on hosts outside the family Leguminosae and other divergent properties. To determine the presence of strains of viruses belonging to this broad group, then, it is still necessary to consider reactions on differential hosts (Goodchild 1956) as the main criteria for separation.

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