Comparison of the Passive Haemagglutination and Bentonite Flocculation Tests for Serological Work With Plant Viruses

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SUMMARY

The passive haemagglutination and bentonite flocculation tests were compared for detecting plant viruses, using antibody-sensitized tanned red cells or bentonite particles. Parsnip yellow fleck, raspberry ringspot, turnip yellow mosaic, narcissus mosaic, potato X and tobacco rattle viruses were detected by both tests in purified preparations and, where studied, in crude plant extracts. The highest antigen titres were obtained only when the red cells or bentonite particles were sensitized with optimal amounts of antibody; these had to be found experimentally because they differed between antisera and between the two tests. In comparative experiments the bentonite flocculation and passive haemagglutination tests were respectively about 2 to 5 and 100 to 125 times more sensitive than tube-precipitin tests for detecting the elongated viruses and about 10 to 20 and 500 to 600 times more sensitive for detecting the isometric viruses. The minimum concentrations of virus detected were 1.8 to 2.6 µg./ml. with the bentonite flocculation test and 0.04 to 0.24 µg./ml. with the passive haemagglutination test. Red cells treated with formalin before they were tanned and sensitized with antibody could be preserved at −14°C for up to 8 weeks without loss of activity.

In experiments with turnip yellow mosaic virus, extracts from healthy plants of eight species tested had little or no effect on either test but concentrated extracts from Chenopodium amaranticolor and Spinacia oleracea plants caused non-specific agglutination of unformalinized red cells, and dilute extracts from Petunia hybrida plants decreased eightfold the sensitivity of the passive haemagglutination test. These effects did not occur with formalinized red cells.

Antibody-sensitized red cells were used in haemagglutination-inhibition tests to determine antiserum titres; antisera to turnip yellow mosaic and raspberry ringspot viruses gave titres 32 times higher than those obtained in tube-precipitin tests.

INTRODUCTION

The serological methods usually employed for detecting plant viruses are various forms of precipitin test. Of these, the Ouchterlony agar gel-diffusion test has found wide application in recent years, partly because it can be done with crude plant saps; for most other tests, plant extracts must first be clarified in some way. However, many viruses do not reach sufficiently high concentrations in plants to give satisfactory results in gel-diffusion tests, or do so for only brief periods. Moreover, many elongated viruses react poorly unless they are first disrupted, for example with ultrasound (Tomlinson et al. 1965). Serological tests that detect very small quantities of virus in crude plant extracts are therefore of interest.
Bozicevich, Scott & Vincent (1963) detected 0.15 μg./ml of tobacco mosaic virus, 0.3 μg./ml of southern bean mosaic virus and 0.5 μg./ml of tobacco ringspot virus, using the bentonite flocculation (BF) test, in which the antigens were allowed to react with antibody-coated bentonite particles. Saito & Iwata (1964) detected as little as 0.01 μg./ml of barley stripe mosaic virus with the passive haemagglutination (PHA) test, using tanned erythrocytes coated with the purified γ-globulin fraction of the homologous antiserum. These workers were the first to show that the PHA test could be used with plant viruses, although the technique was first described by Boyden (1951) for soluble proteins and has since been used in medical virology. Similar results were obtained by Cunningham, Tinsley & Walker (1966), using the related haemagglutination-inhibition (HI) test with antigen-coated particles; the minimum quantities of virus they detected were 0.015 μg./ml of tobacco mosaic virus and 0.024 μg./ml of carnation latent virus. Both BF and PHA tests detected viruses in crude plant extracts (Bozicevich et al. 1963; Saito, Takanashi & Iwata, 1964). The BF test has been used to detect potato viruses X, M and S in single eyes of dormant potato tubers (Scott et al. 1964, Kahn et al. 1967).

Other carrier materials which have been tested, mainly in medical pathology, include latex (Singer & Plotz, 1956; Fleck & Evenchik, 1962), collodion (Cannon & Marshall, 1940; Cavelti, 1947), bismuth tannate and barium sulphate (Gilboa-Garber & Nelken, 1963). Jermoljev & Albrechtova (1965) used the latex test to detect potato virus X in tobacco and potato leaves but they obtained only an eightfold increase in titre compared with droplet precipitation tests.

From the published information, the PHA, BF and collodion tests seemed the most sensitive methods. We know of no report of the use of the collodion test with plant viruses and the work described in this paper was done to compare the PHA and BF tests and to assess their general usefulness as routine techniques. When this work was almost completed, Bercks (1967) published results showing that, for work with plant viruses, the BF and latex tests seemed to be more sensitive than the barium sulphate test.

METHODS

Tests were made with the following viruses, propagated in the host plants indicated.


Preparation of antigens. Tests were made both on crude infective sap and on purified virus preparations. Crude saps were obtained by grinding leaves in a mortar and expressing the sap through cheesecloth. Turnip yellow mosaic virus was purified by the method of Markham & Smith (1949), potato virus X by the method of Loring & Wyckoff (1937) and raspberry ringspot and parsnip yellow fleck viruses by the butanol/chloroform method of Steere (1956). Partially purified preparations of narcissus mosaic and tobacco rattle viruses were supplied by Mr W. P. Mowat and Dr R. M. Lister respectively.

Preparation of antisera. Antisera to turnip yellow mosaic, raspberry ringspot and
Sensitive serological tests for plant viruses

Parsnip yellow fleck viruses were prepared by injecting rabbits intramuscularly with emulsions consisting of equal parts of partially purified virus preparations and Freund's complete adjuvant. The rabbits were bled 6 weeks after injection. A preliminary intravenous injection, 2 weeks before the intramuscular one, was used to produce raspberry ringspot virus antiserum I. With parsnip yellow fleck virus, a second intramuscular injection was necessary to produce antiserum of adequate titre. Antisera to narcissus mosaic, potato X and tobacco rattle viruses were supplied by Drs A. A. Brunt, D. A. Govier and R. M. Lister respectively.

Passive haemagglutination (PHA) test. The procedures were based on those of Saito & Iwata (1964) and Cunningham et al. (1966). Sheep red blood cells were obtained fresh from a slaughterhouse or supplied in Alsever's solution by Oxoid Ltd. They were washed three times by alternately centrifuging the cell suspension for 5 min. at 750 g, and resuspending the sedimented cells in saline (0.85 % NaCl). They were finally resuspended to give a 3.5 % (v/v) suspension in phosphate-buffered saline (0.02 M-sodium phosphate buffer, pH 7.3, containing 0.85 % NaCl). One volume of this suspension was added to one volume of 0.005 % tannic acid (Analar) in saline, and incubated in a water bath at 37° for 10 min. The tanned red cells were washed three times in phosphate-buffered saline, and finally resuspended in one volume of saline.

Antiserum (0.5 ml.) was diluted to 10 ml. with distilled water, and an equal volume of saturated ammonium sulphate solution added. After centrifugation at 750 g for 15 min., the precipitate was resuspended in distilled water and the process repeated. The final precipitate was resuspended in 3 ml. saline. After dialysis against saline (two changes of 2 hr each), the preparation was diluted with saline to 5 ml., giving a concentration of 10 % (v/v) with respect to the original antiserum. All serum globulin concentrations are expressed in this way. Gel-diffusion tests showed that this procedure gave full recovery of the antibody globulins.

To 4 ml. phosphate-buffered saline (pH 5.2), 1 ml. serum globulin preparation (suitably diluted) and 1 ml. tanned red cell suspension were added in that order, and the mixture kept at room temperature for 20 min. The sensitized cells were centrifuged and washed once with 1 % normal rabbit serum (NRS) in saline. After a further centrifugation, the cells were finally resuspended in 1 ml. 1 % NRS. Control cells were treated in a similar manner, except that saline was used in place of the serum globulin preparation.

Serial twofold dilutions of purified virus preparations or infective saps were made with 1 % NRS, and 0.4 ml. portions placed in the wells of a standard Perspex haemagglutination plate. To each of these was added 0.05 ml. antibody-sensitized tanned red cell suspension. Controls were (a) 1 % NRS + unsensitized tanned red cells, (b) 1 % NRS + sensitized tanned red cells, (c) dilutions of purified virus or infective sap + unsensitized tanned red cells, (d) dilutions of preparations or sap from healthy plants + unsensitized tanned red cells, and (e) dilutions of preparations or sap from healthy plants + sensitized tanned red cells. Readings were usually made after 1 to 3 hr at room temperature, but the results were completely stable and if necessary the tests could be left overnight. When haemagglutination occurred the red blood cells formed a uniform layer covering the bottom of the well; when it did not they formed a closely packed 'button' in the centre of the well, or sometimes a ring. The end point was taken to be the greatest dilution of antigen forming a uniform layer with no trace of a ring.
Haemagglutination-inhibition (HI) test. Twofold serial dilutions of the antisera (0.2 ml.) were made with 1% NRS and placed in tubes, together with 0.2 ml. antigen preparation containing four PHA units (i.e. four times the minimum concentration needed to react in PHA tests). Controls were (a) antiserum dilutions + 1% NRS, (b) antigen + 1% NRS, and (c) 1% NRS. All the tubes were incubated in a water bath for 3 hr at 37°. The contents of each tube were then transferred to a well in the Perspex haemagglutination plate, and 0.05 ml. antibody-sensitized tanned red cells added to each well. Readings were made in the same way as for PHA tests. The highest dilution of antiserum in which no haemagglutination occurred was taken as the end point of the titration.

Bentonite flocculation (BF) test. The bentonite stock suspension was prepared and sensitized with antibody using the methods of Bozicevich et al. (1963), except that the serum globulin preparations were made as described above and used at various concentrations. The bentonite powder used was no. 200 standard Volclay (Wyoming bentonite; American Colloid Co., Chicago, Illinois), kindly supplied by Dr H. A. Scott.

Dilutions of purified virus preparations or infective saps were made in saline and 0.2 ml. samples placed in the Perspex haemagglutination plate, together with 0.1 ml. antibody-sensitized bentonite suspension. The haemagglutination plate was shaken in a Bara laboratory shaker (Baird and Tatlock, Ltd.) for 20 min. at 120 oscillations per min., and readings taken 10 to 20 min. afterwards. In a positive reaction all the bentonite particles formed small clumps. In a negative reaction they were fully dispersed. Results were as clearly visible with the naked eye as with a microscope. Controls were (a) dilutions in saline of preparations or sap from healthy plants + antibody-sensitized bentonite, and (b) saline + antibody-sensitized bentonite.

Tube-precipitin tests. Dilutions of purified virus preparations and antiserum were made in saline and 1 ml. of each dilution of antigen was placed in a tube together with 1 ml. antiserum diluted to half its titre. The tubes were incubated in a water bath at 37° and readings made for 5 hr. Control tubes containing antigen + saline or antiserum + saline were included in all tests.

Agar gel-diffusion tests. Agar gel-diffusion tests were done in 9 cm. Petri dishes filled with 15 ml. of 1.0% Oxoid Ionagar no. 2 containing 0.85% sodium chloride and 0.02% sodium azide. Wells 9 mm. in diameter were spaced 15 mm. apart between centres and filled with 0.12 ml. of reactant. The Petri dishes were placed in polythene bags and incubated at 22°. Readings were made after 48 hr.

RESULTS

Tests with antigen-sensitized particles*

In preliminary experiments to determine antiserum titres, the optimum quantities of purified turnip yellow mosaic virus needed to sensitize 1 ml. bentonite particles or tanned red cells were 1.0 and 0.4 mg. respectively. When these quantities were halved the sensitivities of both tests were much decreased. Bozicevich et al. (1963) and Cunningham et al. (1966) have quoted similar figures for other viruses. Such quantities of purified virus preparations are uneconomical to use with viruses which reach low

* Unless qualified, the term 'particle' will be used throughout to refer equally to tanned red cells and bentonite particles.
concentrations in plants or are unstable in vitro. Accordingly, all further work was done using antibody-sensitized particles. Although the BF test has been frequently performed in this way, most workers (except Saito & Iwata, 1964) seem to have sensitized red cells with antigen, using them either in PHA tests to determine antiserum titres or in HI tests to detect antigen concentrations.

Tests with antibody-sensitized particles

Effect of quantity of antibody used to sensitize the particles

In experiments with partially purified preparations (Fig. 1), all the plant viruses tested gave positive results in both PHA and BF tests, provided that the particles were sensitized with optimal amounts of antibody. Previous workers have shown that in both tests the antibody titre obtained using antigen-sensitized particles is determined by the quantity of antigen used to sensitize them (Stavitsky, 1954; Wolff, Ward & Landy, 1963). Similarly, in our experiments with antibody-sensitized particles, there was an optimum concentration of each serum globulin preparation for sensitizing each kind of particle. These concentrations bore only approximate relationships to the titres of the antisera in tube-precipitin tests and had to be found experimentally. Thus, turnip yellow mosaic virus antiserum 1 and 2 both had titres of 1/1024 in tube-precipitin tests, but for sensitizing tanned red cells serum globulin preparations made from serum 1 were effective over a wider range of the concentrations tested and had lower optimum concentrations than preparations made from serum 2. The behaviour of these two antisera was reversed in the BF test. Turnip yellow mosaic virus antiserum 3 had a titre of only 1/256 in the tube-precipitin tests and, although serum globulin preparations made from it sensitized red cells and bentonite particles at several of the concentrations tested, these particles did not detect antigen as efficiently as did those sensitized with serum globulins prepared from antisera 1 and 2, possibly because none of the concentrations used was optimal. The two antisera to raspberry ringspot virus behaved similarly. Results with one antiserum to narcissus mosaic virus (titre in tube-precipitin tests = 1/4096) are given in Fig. 1. Serum globulin preparations from a second antiserum with a titre of 1/256 gave no results in BF or PHA tests, even at 10% (v/v), the highest concentration tested.

The optimum concentration of the serum globulin preparations for sensitizing tanned red cells tended to lie between 2 and 10% (v/v) for antisera with titres (in tube-precipitin tests) between 1/1024 and 1/4096. The optimum concentrations for sensitizing bentonite particles were less predictable and ranged from 0.5% for the antiserum to potato virus X (titre = 1/1024) to 10% (v/v) for a higher-titred antiserum to parsnip yellow fleck virus (titre = 1/4096). Thus, the optimum concentration of the serum globulin preparations for sensitizing red cells or bentonite particles must be determined for each antiserum and for each kind of particle. Antisera with titres less than 1/1024 seem unsatisfactory for these tests because of the high concentrations of serum globulins necessary to sensitize the particles optimally.

With turnip yellow mosaic and raspberry ringspot viruses, tanned red cells or bentonite particles sensitized with suboptimal concentrations of the serum globulin preparations sometimes failed to agglutinate or flocculate in the presence of excess antigen. Over a 1000-fold range of antigen concentrations (Fig. 1) this effect did not occur when the particles were optimally sensitized.
Fig. 1. Minimum quantities of antigen detected with tanned red cells or bentonite particles sensitized with different concentrations of the serum globulin preparations. PYFV = parsnip yellow fleck virus, RRV = raspberry ringspot virus, TYMV = turnip yellow mosaic virus, NMV = narcissus mosaic virus, PVX = potato virus X, TRV = tobacco rattle virus. Figures in parentheses are the titres of the antisera in tube-precipitin tests. Where more than one antiserum to a single virus was used, each was tested against the same virus preparation, so that the sensitivities shown may be directly compared.
Relative sensitivity of the PHA and BF tests

In tests made with partially purified virus preparations using optimally sensitized particles, the PHA test was between 20 and 80 times more sensitive than the BF test (Fig. 1, Table 1). Compared with standard tube-precipitin or gel-diffusion tests, the BF and PHA tests were respectively about 2 to 5 and 100 to 125 times more sensitive for detecting the elongated viruses and 10 to 20 and 500 to 600 times more sensitive for detecting the isometric viruses. In these experiments, tube-precipitin tests were terminated after an incubation period of 5 hr, although, using purified virus preparations, approximately fourfold increases in sensitivity could be obtained by prolonging the tests for 20 hr. The period of 5 hr was chosen because non-specific precipitation usually occurs after this time when tests are made with clarified plant saps, the most usual laboratory procedure.

Table 1. Relative sensitivities of tube-precipitin, gel-diffusion, bentonite flocculation (BF) and passive haemagglutination (PHA) tests

<table>
<thead>
<tr>
<th>Virus</th>
<th>Gel-precipitin test</th>
<th>BF test</th>
<th>PHA test</th>
<th>Tube-precipitin test</th>
<th>BF test</th>
<th>PHA test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parsnip yellow fleck</td>
<td>1</td>
<td>10</td>
<td>400</td>
<td>95</td>
<td>—</td>
<td>0.24</td>
</tr>
<tr>
<td>Raspberry ringspot</td>
<td>1</td>
<td>20</td>
<td>640</td>
<td>20</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Turnip yellow mosaic</td>
<td>1</td>
<td>8</td>
<td>512</td>
<td>21</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Narcissus mosaic</td>
<td>—</td>
<td>1.5</td>
<td>125</td>
<td>12</td>
<td>—</td>
<td>0.12</td>
</tr>
<tr>
<td>Potato X</td>
<td>—</td>
<td>5</td>
<td>100</td>
<td>8</td>
<td>1.8</td>
<td>0.09</td>
</tr>
</tbody>
</table>

To find the minimum quantities of virus detectable by BF and PHA tests, some of the viruses were further purified by density gradient centrifugation and the amount of virus in each preparation calculated from the extinction of the solution at 260 m\(\mu\), using the following extinction coefficients (\(E_{260}^{\text{abs}}\)): turnip yellow mosaic virus (bottom component), 9.5 (Markham, 1959); potato virus X, 2.7 (Reichmann, 1959). Extinction coefficients of raspberry ringspot virus (bottom component) and parsnip yellow fleck virus (\(E_{260}/280 \approx 1.7\)) were assumed to be 10, and that of narcissus mosaic virus (\(E_{260}/280 \approx 1.3\)) was assumed to be 3.0. In titrations made on these preparations, the minimum quantities of virus detected in BF and PHA tests (Table 1) were, with the exception of parsnip yellow fleck virus, similar to those given for other viruses by previous workers. The reasons for the low sensitivity of all the serological tests for detecting parsnip yellow fleck virus are not understood. Nevertheless, the results of seven experiments showed that PHA tests were only between 6 and 16 times less sensitive than infectivity assays (to inoculated leaves of Chenopodium quinoa) for detecting this virus. For the other viruses the sensitivity of PHA tests relative to infectivity assay may well be greater.

The reproducibility of the tests was studied by assaying a standard preparation of turnip yellow mosaic virus with bentonite suspensions or red cells prepared and sensitized with optimal amounts of antibody on different occasions. No differences in sensitivity were observed amongst any of eight bentonite or twelve red cell preparations.
Preservation of red cells by treatment with formalin

Sensitized red cells haemolyse when kept longer than 2 or 3 days. This is potentially a major disadvantage of the PHA test. In contrast, sensitized or unsensitized bentonite suspensions can be stored for several months without losing their efficiency. Some time can be saved in performing the PHA test by storing serum globulin preparations in small samples at \(-14^\circ\) and using them as required to sensitize fresh batches of red cells. In earlier experiments this method proved successful and no loss of serological activity was detected over a period of 4 months. However, treating the red cells with formalin was later found to be more convenient. The following method, similar to that of Csizmas (1960), was used. Fresh sheep red cells (3 ml.) were washed nine times in 20 ml. portions of saline and finally suspended in 28.5 ml. saline. A dialysis bag containing 1.5 ml. neutral formalin was immersed in a beaker containing the red cell suspension, which was stirred overnight at room temperature. The dialysis bag was then broken to release the contents into the beaker, giving a final formalin concentration of 5\% (v/v). The formalinized cells were washed twice, resuspended in 3 ml. saline, and then tanned and sensitized with antibody globulins in the usual way. In tests with a standard preparation of turnip yellow mosaic virus, the formalinized cells were slightly less sensitive than unformalinized cells for detecting antigen, but they could be stored in 1 ml. samples at \(-14^\circ\) for up to 8 weeks with no change in serological activity. After this time the cells, although not haemolysed, ceased to react.

Detection of viruses in crude plant extracts

With all the viruses tested, dilutions of infective saps reacted positively in PHA and BF tests. In earlier experiments, the saps were first absorbed with sheep red cells (1 vol. red cells + 100 vol. plant sap) before use in PHA tests, but this was later found unnecessary. The optimum concentrations of the serum globulin preparations for sensitizing the particles were the same as for purified virus preparations. The relative sensitivities of BF, PHA and gel-diffusion tests were much the same as with purified virus preparations (Table 2).

The results of BF and PHA tests were difficult to read when the saps were diluted less than about 1/5. Extracts from healthy or infected spinach plants at dilutions of 1/10 or less sometimes caused non-specific precipitation of unformalinized red cells, both sensitized and unsensitized, but this did not occur with extracts from the other species listed in Table 2. No such effects were observed in BF tests. To examine more thoroughly the effect of plant extracts on the PHA test, five volumes of a standard preparation of turnip yellow mosaic virus were added to one volume of crude sap and the mixture then diluted with 1\% NRS and titrated with unformalinized red cells sensitized with homologous antibodies. Extracts from 11 species were used (Table 3); those from Spinacia oleracea and Chenopodium amaranticolor interfered with the HA test at high concentrations (greater than 1/10 and 1/20 respectively). The interference occurred with both sensitized and unsensitized cells and seemed to be some kind of partial non-specific agglutination—some of the cells settled out into a ‘button’, others were distributed evenly over the bottom of the well. The presence of extracts from these two species did not affect antigen titre, but extracts from Chenopodium quinoa and Pastinaca sativa decreased it slightly and Petunia hybrida extracts caused eightfold decreases in each of three experiments. Losses of titre were smaller when
the proportion of *P. hybrida* sap to antigen was either increased or decreased. These effects of some plant extracts on red cells or on the antigen titres obtained in PHA tests could often be avoided by choice of a suitable dilution of the sap and were rarely serious enough to invalidate the test. Moreover, they were never observed when formalinized red cells were used, a feature which enhances the value of this treatment.

Table 2. *End points of crude extracts from infected plants, as determined by gel-diffusion, bentonite flocculation (BF) and passive haemagglutination (PHA) tests*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host species</th>
<th>Gel-diffusion</th>
<th>BF</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parsnip yellow f</td>
<td><em>Spinacia oleracea</em></td>
<td>2</td>
<td>20</td>
<td>2,000</td>
</tr>
<tr>
<td>Raspberry ringspot</td>
<td><em>Nicotiana clevelandii</em></td>
<td>&lt; 10</td>
<td>50</td>
<td>1,600</td>
</tr>
<tr>
<td>Turnip yellow mosaic</td>
<td><em>Brassica pekinensis</em></td>
<td>160</td>
<td>1,600</td>
<td>102,400</td>
</tr>
<tr>
<td>Narcissus mosaic</td>
<td><em>Chenopodium quinoa</em></td>
<td>--</td>
<td>40</td>
<td>1,600</td>
</tr>
<tr>
<td>Potato X</td>
<td><em>Nicotiana tabacum</em></td>
<td>--</td>
<td>320</td>
<td>5,120</td>
</tr>
</tbody>
</table>

* Figures are the reciprocals of the maximum reacting dilutions of the crude sap.

Table 3. *Effect of various plant extracts on detection of a standard purified preparation of turnip yellow mosaic virus in PHA tests*

<table>
<thead>
<tr>
<th>Plant extract added</th>
<th>Reciprocal of dilution of virus + sap mixture*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>Brassica pekinensis</td>
<td>+</td>
</tr>
<tr>
<td>Chenopodium amaranticolor</td>
<td>N</td>
</tr>
<tr>
<td>C. quinoa</td>
<td>+</td>
</tr>
<tr>
<td>Cucumis sativa</td>
<td>+</td>
</tr>
<tr>
<td>Datura stramonium</td>
<td>+</td>
</tr>
<tr>
<td>Nicotiana clevelandii</td>
<td>+</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>+</td>
</tr>
<tr>
<td>Pastinaca sativa</td>
<td>+</td>
</tr>
<tr>
<td>Petunia hybrida</td>
<td>+</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>+</td>
</tr>
<tr>
<td>Spinacia oleracea</td>
<td>N</td>
</tr>
</tbody>
</table>

* Five volumes of purified turnip yellow mosaic virus preparation were mixed with one volume of crude sap and the mixture diluted with 1% NRS. Dilutions are expressed as the reciprocals of the dilutions of the crude sap.

N = Non-specific agglutination.

In none of these experiments have specific reactions occurred with extracts or preparations from healthy plants. None of the antisera used had titres to healthy plant extracts greater than 1/8. By analogy with the results for virus antigens it seems probable that much higher concentrations of the serum globulin preparations than were used in these experiments would have been necessary to sensitize the particles to healthy plant antigens.

**Haemagglutination-inhibition (HI) tests with antibody-sensitized red cells**

In the same way that red cells sensitized with antigen may be used in HI-type tests to determine antigen end points, our experiments have shown that antibody-sensitized
cells may be used to determine antiserum titres. Thus, antisera to turnip yellow mosaic and raspberry ringspot viruses with titres of 1/1024 and 1/2048 respectively in gel-diffusion tests reacted at dilutions up to 1/32,000 and 1/64,000 respectively in HI tests. No similar experiments were done with antibody-sensitized bentonite particles.

**DISCUSSION**

The results show that the PHA test, like the BF test, can be used to detect isometric and elongated plant viruses, and can be done with antibody-sensitized particles. Saito & Iwata (1964) also sensitized erythrocytes with unfractionated serum globulin preparations but this technique does not otherwise seem to have been much studied. With different antisera the optimum concentrations of the serum globulin preparations for sensitizing red cells or bentonite particles bore no constant relation either to each other or to the titres (in tube-precipitin tests) of the parent antisera. This suggests perhaps that the various antibody globulin fractions, which differ in their relative proportions from one antiserum to another, may also differ in their relative importance for BF, PHA and tube-precipitin tests.

Variation between antisera in their suitability for use in the BF, latex and barium sulphate tests was observed by Bercks (1967), working with a large number of antisera to several plant viruses. Sensitizing the particles with serum globulin preparations diluted to half the titre (in droplet precipitation tests) of the parent antiserum, he found that the minimum amount of antigen detected varied with the antiserum, some of which gave negative results. Our studies with both BF and PHA tests suggest that positive results can be obtained with any antiserum when it is used at its optimum concentration, although the tests may require excessive amounts of some low-titred antisera. In fact, none of the antisera used in our experiments with BF tests could be diluted as much as those used by Bercks. Moreover, although many of the antisera he used in BF tests gave increases in antigen titre similar to those we found, others were much more effective, giving increases comparable to those we obtained in PHA tests. In view of our results it would be interesting to know if the antisera which were highly effective in Bercks's BF tests would give even higher sensitivities in PHA tests. Bercks gave no data for the minimum quantities of virus detected in his experiments, but our figures are of the same order as those reported by previous workers for BF and PHA tests (Bozicevich et al. 1963; Saito & Iwata, 1964; Cunningham et al. 1966). With each test, the isometric viruses were mostly detected as efficiently as the elongated ones. However, relative to tube-precipitin tests, the increase in sensitivity was greater for the isometric viruses, presumably because these are detected less efficiently than elongated viruses in tube-precipitin tests.

The PHA test was in every instance more sensitive than the BF test, by factors of 20 to 80. Both tests could be used with crude plant extracts, but it was necessary to dilute the saps at least 1/5 to make the results readable. The BF test gave only approximately tenfold increases in antigen titre compared with the gel-diffusion test and this increase was thus largely cancelled out by dilution of the sap. The PHA test was therefore preferred, both because of its greater sensitivity, approaching that of infectivity tests, and also because the results are somewhat easier to read. Its main disadvantage, the short storage life of sensitized red cells, was overcome by formalinizing them before they were tanned and sensitized with antibody. They could then be stored for
up to 8 weeks at \(-14^\circ\) without loss of sensitivity. Formalinized cells were also apparently unaffected by extracts from some plant species which had adverse effects on unformalinized cells. Other workers (Butler, 1963; Csizmas, 1960; Fulthorpe et al. 1961; Ingraham, 1958), using various procedures for formalinizing and sensitizing red cells, have preserved them frozen for periods up to 15 months.

Large amounts of antibody or antigen are necessary to sensitize tanned red cells and the choice of which to use will depend on which is the more readily available. Red cells sensitized either with antigen or antibody can be used to titrate antisera or virus preparations by an appropriate choice of PHA or HI tests. If the cells are to be sensitized with antibody, a number of antisera should be tested, if possible, to find one which can be used most economically. Another way in which economies can be made was reported by Stavitsky (1964), who was able to re-use antigen preparations for sensitizing further batches of erythrocytes. In preliminary experiments we have successfully re-used turnip yellow mosaic virus antiserum globulin preparations five times with little loss of sensitivity of the erythrocytes.

We have studied only the two tests which seemed most likely to be of value. The recent results of Bercks (1967) suggest that a comparison of the PHA test with the latex test would also be worthwhile. For many purposes, tests using sensitized carrier particles are too laborious to replace those commonly used in plant virology, but there are some types of investigation for which they seem likely to be useful. Among these are the detection of viruses in field material, the investigation of distant serological relationships between viruses, and the study of virus multiplication in plants. In preliminary experiments we have found the PHA test useful in studies on the accumulation of parsnip yellow fleck virus, which reaches only low concentrations in plants.

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