Characteristics of the Microplate Method of Enzyme-Linked Immunosorbent Assay for the Detection of Plant Viruses

By M. F. CLARK AND A. N. ADAMS

East Malling Research Station, Maidstone, Kent

SUMMARY

Some characteristics of a microplate method for the detection and assay of plant viruses using enzyme-labelled antibodies are described. The method enabled the highly sensitive detection of a number of morphologically different viruses in purified preparations and in unclarified extracts of herbaceous hosts and of infected crop plants. Virus concentrations were estimated by photometric measurement of the colour intensity of the hydrolysed substrate. The suitability of the technique for various field and research applications is considered.

INTRODUCTION

Of the possible ways to detect plant viruses, serological techniques are frequently favoured because of their specificity, speed, and the scope they provide for standardization. However, for many important viruses conventional serological techniques cannot be used because of limitations such as low virus concentrations, unsuitable particle morphology, or the presence in plant extracts of virus inactivators or inhibitors. These limitations can largely be overcome by use of the microplate method of enzyme-linked immunosorbent assay (ELISA). The application of this method to the detection and quantitative assay of plant viruses is described in this paper.

Enzyme-labelled antibodies have been used for some years for the detection of virus antigens in tissue sections (Nakane & Pierce, 1966; Wicker & Avrameas, 1969), but their use in quantitative procedures is relatively recent (Engvall & Perlmann, 1971, 1972; van Weemen & Schuurs, 1971, 1972). In various forms, enzyme immunoassays are being used increasingly in clinical pathology and immunology (Feldmann et al. 1976) where they are reported to have sensitivities comparable with radio-immunoassay techniques (Engvall & Perlmann, 1971, 1972). For plant viruses, the 'double antibody sandwich' form of ELISA (Voller, Bidwell & Bartlett, 1976b) has been found to be suitable. In this method (Fig. 1) virus in the test sample is selectively trapped and immobilized by specific antibody adsorbed on a solid surface (polystyrene microtitre plates, Voller et al. 1974). Trapped virus is then reacted with further specific antibody to which an enzyme has been linked. After washing, enzyme-labelled antibody that has complexed with the trapped virus is detected colorimetrically by adding a suitable enzyme substrate. A preliminary report on the application of the method to two plant viruses has been made (Voller et al. 1976a).
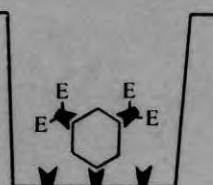
1. Specific antibody adsorbed to plate
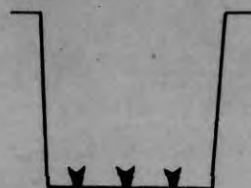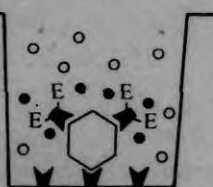
Wash

2. Add test sample containing virus
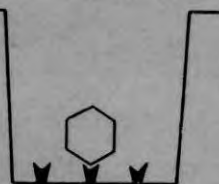
Wash

3. Add enzyme-labelled specific antibody

Wash

4. Add enzyme substrate

Colour intensity ∝ virus concentration

Fig. 1. Principle of the ELISA technique for plant viruses.

METHODS

Reagents and equipment. The following buffers were used: coating buffer – 0·05 M-sodium carbonate at pH 9·6; substrate buffer – 10% diethanolamine adjusted to pH 9·8 with HCl; phosphate-buffered saline (PBS) – 0·02 M-phosphate plus 0·15 M-NaCl at pH 7·4; PBS with 0·05% Tween 20 (PBS-Tween). All buffers contained 0·02% sodium azide as preservative.

Polystyrene microtitre plates (Cooke M29AR) were obtained from Dynatech Ltd. The enzyme was alkaline phosphatase EC. 3.1.3.1 (Type VII, Sigma) and the substrate was 2-nitrophenyl phosphate (Sigma) used at 0·67 or 1·0 mg/ml in substrate buffer. Hydrolysed enzyme substrate was determined by measuring the extinction at 405 nm with either a Model DB-GD spectrophotometer (Beckman-RIIC Ltd.) or a Vitatron MPS photometer (MSE Instruments Ltd.).

Viruses. Most tests were made with arabis mosaic virus (AMV) or plum pox virus (PPV) which were selected for their importance as pathogens of fruit crops, and as representing isometric and filamentous viruses, respectively. Other viruses which were tested included

raspberry ringspot virus (RRV), the apple mosaic serotype of prunus necrotic ringspot virus (ApMV), hop mosaic virus (HMV), apple stem grooving virus (ASGV) and apple chlorotic leafspot virus (CLSV). For convenience and reproducibility of results, tests were generally made with virus preparations and extracts from herbaceous hosts, rather than with infected crop plants.

Viruses were purified from glasshouse-grown herbaceous plants using standard procedures. The final step for antiserum production was either rate zonal sucrose density gradient centrifugation, or sucrose density gradient zonal electrophoresis in an apparatus similar to that used by van Regenmortel (1964).

Antiserum production. With the exception of the antiserum to ApMV (kindly provided by Professor R. W. Fulton) antisera were produced by injecting rabbits at 3 week intervals with approx. 1 mg purified virus mixed with an equal volume of Freund's incomplete adjuvant (Freund's complete adjuvant was used for some PPV injections). Rabbits were immunized by a combination of subcutaneous and intramuscular injections and were bled at 1 week intervals after 2 to 4 injections. Antisera were stored at −18 °C, either frozen or mixed with 50 % glycerol. Titres were determined by ring interface precipitin tests.

γ-globulin purification. Samples of each antiserum (1 ml) were diluted to 10 ml with distilled water and 10 ml saturated solution of ammonium sulphate added. After standing, the precipitate was collected by centrifugation and dissolved in 2·0 ml half-strength PBS. This was then either dialysed against half-strength PBS (at least three changes of 500 ml) or desalted by exclusion chromatography on Sephadex G 25 (Pharmacia Ltd.) in half-strength PBS. The γ-globulin was further purified by passage through a column of DE 22 cellulose (Whatman Ltd.), collecting the unadsorbed fraction which showed an $E_{278}:E_{250}$ ratio of 2·5 to 2·7. The γ-globulin was adjusted to approx. 1 mg/ml (E_{278} = 1·4) and stored frozen in 1·0 ml portions in silicone-treated glass tubes.

Conjugation of alkaline phosphatase with γ-globulin. Two mg alkaline phosphatase were dissolved in 1·0 ml of the above γ-globulin preparation and dialysed extensively against PBS at 4 °C. Glutaraldehyde was added to 0·05 % final concentration (Avrameas, 1969) and the mixture incubated at 22 °C for 4 h. Glutaraldehyde was then removed by dialysis against several changes of PBS and the conjugate stored with approx. 1 % bovine serum albumin at 4 °C. Because of volume changes and possible γ-globulin losses during the conjugation procedure all references to the use of the conjugates are in terms of dilutions of the conjugate rather than as absolute concentrations.

RESULTS

The procedure outlined in Fig. 2 was adopted as a compromise between operational convenience and maximum reaction efficiency. Some effects of varying the concentrations of reactants and the reaction conditions are described below. It is advisable to determine experimentally the best conditions for each virus-antiserum system.

Coating the plate

The concentration of γ-globulin used for optimum coating of the wells was generally 1 to 10 μg/ml, despite the differences among antiserum titres (Table 1). Concentrations exceeding 10 μg/ml reduced the strength of virus-specific reactions but increased that of non-specific reactions, presumably due to the presence of a few antibodies to normal plant proteins (Fig. 3). Antisera with lower titres than those tested here may require higher concentrations of γ-globulin for maximum coating efficiency.

Table 1. *Characteristics of antisera used for ELISA tests*

Antiserum	Titre* (reciprocal)	Coating concentration (μg/ml)
AMV	16000	2
RRV	32000	2
ApMV†	(500)	4
PPV	4000	1
HMV	1000	1
ASGV	500	4
CLSV	1000	4

* Determined by ring interface precipitin tests.
† Californian apple mosaic isolate, titre determined by gel diffusion.

Add 200 μl purified γ-globulin in
coating buffer to each well

Incubate 2 to 6 h at 37 °C

Wash

Add 200 μl test sample
in PBS-Tween

Incubate overnight at 6 °C

Wash

Add 200 μl enzyme-labelled
γ-globulin in PBS-Tween

Incubate 3 to 4 h at 37 °C

Wash

Add 300 μl p-nitrophenyl phosphate substrate
in diethanolamine buffer

Incubate 0·5 to 2 h at room temperature

Stop reaction with 50 μl 3·0 M-NaOH

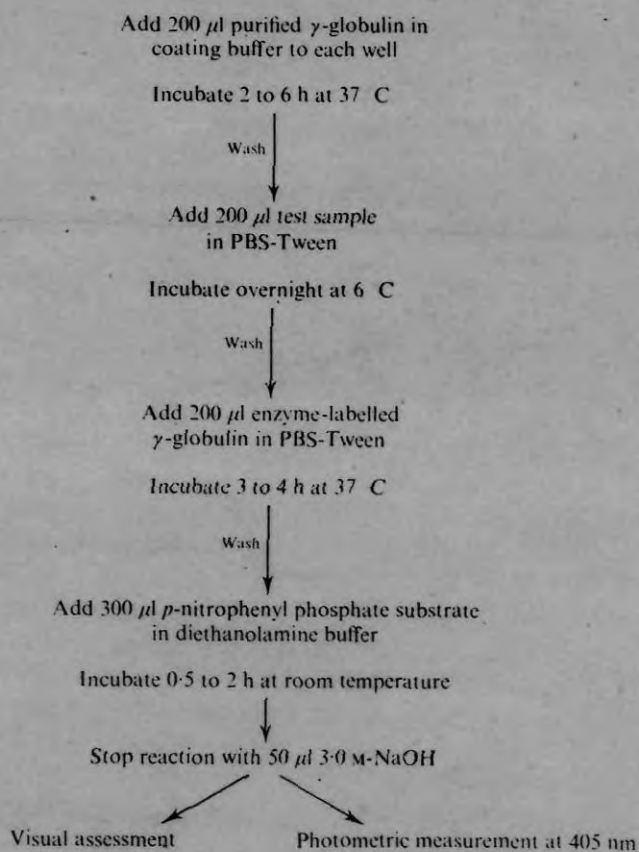Visual assessment   Photometric measurement at 405 nm

Fig. 2. Standard procedure for carrying out microplate ELISA technique for virus detection.

Adsorption of γ-globulin to the well surface was rapid. There was little difference in extinction values obtained from wells coated for 2 or 4 h at 37 °C or for 18 h at 6 °C (Fig. 4). Coated plates could be stored at −18 °C for several weeks or dried over $CaCl_2$ for several days without significant loss of efficiency.
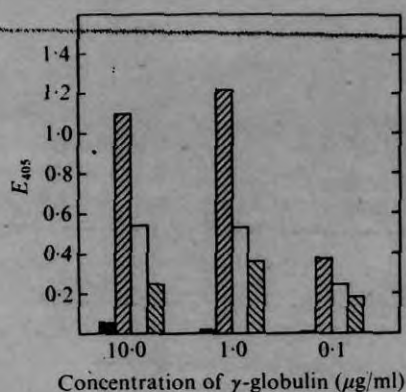
Fig. 3

Fig. 4

Fig. 3. Effect of different concentrations of coating γ-globulin on the detection of PPV in extracts of infected *N. clevelandii* leaves. Healthy: ■, 1:100. PPV-infected: ▨, 1:100; □, 1:1000; ◪, 1:5000.
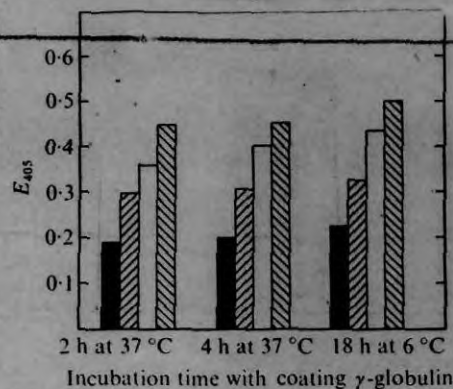
Fig. 4. Effect of different incubation times with coating γ-globulin and with the test sample on the detection of PPV in an extract of infected *N. clevelandii* diluted 1:1000 with PBS-Tween. Incubation time with virus: ■, 1 h at 37 °C; ▨, 2 h at 37 °C; □, 4 h at 37 °C; ◪ 18 h at 6 °C.

Washing the plate

The plate must be thoroughly washed between each reaction stage to remove traces of soluble reactants that could cause non-specific reactions. Plates were washed at least three times by flooding them with PBS-Tween and leaving for several minutes between washings. The Tween 20 was included to prevent post-coating adsorption of protein to the well surface.

Nature of the test sample

Virus could be detected both in purified preparations and in unclarified plant homogenates. Sap constituents of herbaceous hosts had little apparent effect on the test. Thus, there was no difference in extinction values for PPV in a purified preparation diluted in PBS-Tween and the same virus diluted in a 1:100 (w/v) extract of healthy *Nicotiana clevelandii* in PBS-Tween (Fig. 5). Similarly, comparable dilution curves were obtained for AMV in a purified preparation and in an extract of infected *Chenopodium quinoa* (Fig. 6).

By contrast, some extracts from woody plants reduced the strength of virus-induced reactions or gave rise to non-specific reactions. Extracts from AMV-infected blackcurrant buds inhibited the reaction at a dilution of 1:10 but not at a dilution of 1:100. Extracts of *Prunus* leaves collected in midsummer and extracts of leaves of some other woody perennials gave occasional non-specific reactions which were sometimes strong enough to mask those due to virus. The intensity of these non-specific reactions was reduced or eliminated by including polyvinyl pyrrolidone, mol. wt. 25000 or 44000, at 1 to 2 % (w/v) in the extraction buffer. This allowed continued testing of *Prunus* extracts throughout the growing season, although there was some reduction in sensitivity for virus detection. Post-coating plates with high concentrations of bovine serum albumin or ovalbumin (< 0·5 %) to block any unadsorbed sites was not sufficiently effective against non-specific reactions to justify routine use.

The effect of varying the test sample incubation period was examined with an extract of PPV-infected *N. clevelandii*. Leaf extracts, diluted 1:1000 with PBS-Tween and left in the wells for 18 h at 6 °C gave higher extinction values than those left for 1, 2 or 4 h at 37 °C (Fig. 4).
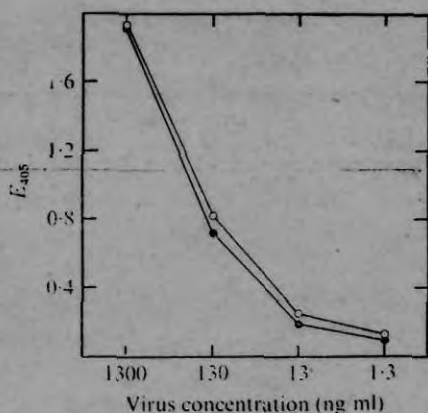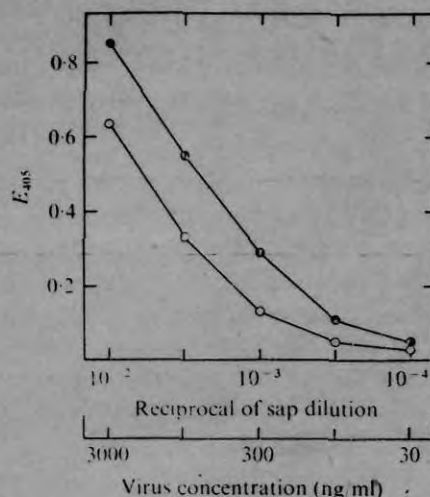
31

Fig. 5

Fig. 6

Fig. 5. ELISA absorbance values for a purified preparation of PPV diluted in PBS-Tween (○—○) and diluted in a 1:100 (w/v) extract of *N. clevelandii* in PBS-Tween (●—●).

Fig. 6. ELISA absorbance values for AMV in a purified preparation (—○) and in a leaf extract of infected *C. quinoa* (●—●).
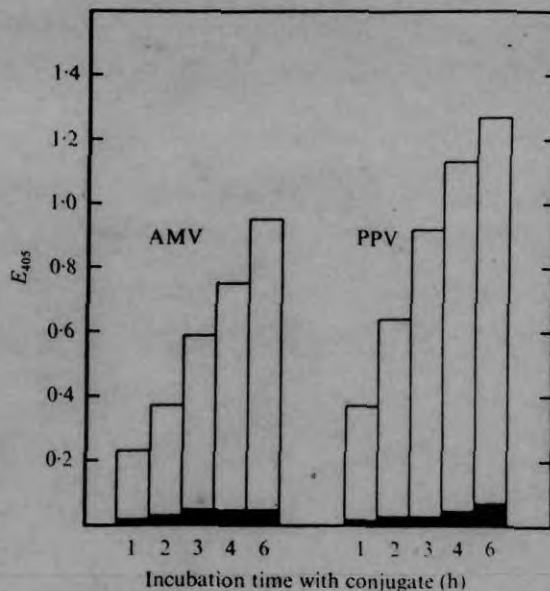

Fig. 7. Effect of different incubation times with conjugate on the detection of AMV and of PPV in extracts of *C. quinoa* and *N. clevelandii*, respectively. Extracts were diluted 1:200 in PBS-Tween. ■, Healthy; □, infected.

Enzyme-antibody conjugate

ELISA extinction values were affected both by the concentration of the conjugate and by the length of the incubation period. Twofold dilutions of conjugates in the range 1:100 to 1:2000 usually halved the extinction values over a wide range of antigen concentrations. This effect could be partially compensated for by increasing the incubation period (Fig. 7).

Table 2. *Sensitivity of the microplate ELISA technique for detecting plant viruses*

Virus	Concentration	
	Purified preparation (ng/ml)	Plant sap*
AMV	< 30	10000 (CQ)
RRV	< 10	10000 (NC)
ApMV†	–	10000 (C)
PPV	< 1	100000 (NC)
HMV	< 10	50000 (NC)
ASGV	–	1000 (CQ)

* Reciprocal of dilution; extracts of systemically infected leaves were diluted in PBS-Tween. CQ, *C. quinoa*; NC, *N. clevelandii*; C, cucumber.

Table 3. *Comparison of the sensitivity of different methods for detecting PPV*

Method	Virus titre (ng/ml)
ELISA	1
Radial diffusion	1000
Tube precipitin	500
Electron microscopy	100
Infectivity*	100

* Less than 1 lesion per inoculated leaf of *Chenopodium foetidum*.

There was a concomitant increase in non-specific reactions, but over the periods used this was not sufficient to affect the test. In contrast to the responses obtained to variations in the coating conditions, there was no clear optimum for the concentration of conjugate. Selection of an appropriate conjugate concentration was dependent upon the degree of detection sensitivity required.

Substrate

Extinction values increased linearly with incubation time over a period of 1 h, the longest time tested. A substrate concentration of 1 mg/ml was generally used although concentrations of 0·67 and 0·5 mg/ml gave only slightly lower values. The volume of substrate solution used for each well was 300 μl, the minimum volume necessary to fill the photometer cuvette.

Other factors

A number of other factors affected the sensitivity and specificity of the microplate ELISA test. Polystyrene plates from different sources varied considerably in reaction strength and the reproducibility of extinction values. The most significant variations were the irregular, non-specific reactions which occurred in some batches of plates, particularly in the outer row of wells. To avoid this 'plate effect' we found it advisable not to use the outside row of wells in plates of a suspect batch, and to test all samples in duplicate using pairs of wells. PVC plates also exhibited this 'plate effect'. Tissue culture grade plates were unsuitable due to lack of adsorption of γ-globulin to the well surface.

The limits of visual detection of colour corresponded approximately to an extinction value $E_{405} = 0·1$. Photometric measurement enabled the detection of virus at a dilution up to 10-fold compared with visual detection. However, for many purposes, e.g. qualitative field screening tests, visual observation was adequate.

Table 4. *Distribution of PPV in plum flowers*

Flower part	Average sample wt. (mg)·	Dilution	No. of samples with virus*	Mean E_{405}†
Calyx	10	1:100	8	0·28
Petals	10	1:100	6	0·04
Stamens	5	1:100	8	0·13
Gynoecium	2	1:300	2	0·07

* 10 flowers examined.
† Average for samples with $E_{405} \geqslant$ 0·02.

Virus detection

Several viruses with particles of widely different morphology and stability were successfully detected by the ELISA technique (Table 2). Of the viruses so far tested, only CLSV has proved unsuited to this technique although preparations treated with glutaraldehyde before testing did respond normally.

The high sensitivity of the microplate ELISA method compared with other methods of detecting virus is evident from results (Table 3) of tests with PPV, a member of the potyvirus group for which few suitable screening methods are available. A comparison with the latex flocculation test (Jermoljev & Albrechtova, 1965; Bercks, 1967) was not made as we were unable to obtain reliable results with this method.

The quantitative potential of the ELISA technique is illustrated by results obtained for the distribution of PPV among the parts of a plum flower (Table 4). Of ten flowers examined individually, eight contained detectable virus, with highest concentrations of virus in the calyx and lowest in the petals.

DISCUSSION

The particular advantages of the ELISA technique over other labelled-antibody methods, i.e. those employing latex particles, fluorescent compounds, ferritin, etc., are its combination of economy of reactants, extreme sensitivity and potential for measurement. It is also highly versatile, detecting isometric and filamentous viruses in both purified preparations and untreated extracts of herbaceous hosts and infected crop plants.

Unlike nearly all other serological techniques in plant virology which are based on the formation and detection of immune precipitates, the ELISA technique relies upon the sensitive detection of non-precipitating reactions, made possible by the use of enzyme-labelled antibodies. There are two main consequences of this difference in reaction principle. First, the efficiency of the ELISA technique is, for practical purposes, independent of the ratio of antibody to antigen. Thus, once the appropriate concentrations for the antibody preparations are ascertained these are applicable for subsequent tests for detecting virus at all concentrations. Second, because retention of enzyme-labelled antibody is a function of, and proportional to, virus concentration, the technique has high quantitative potential.

The second point is illustrated by the results obtained for the distribution of PPV in plum flowers (Table 4). Although for this example the actual virus concentration in each part of the flower was not determined, such a calculation may be made by direct comparison of extinction values of the test sample with those for dilutions of a virus standard run in parallel. We have used this approach to compare virus concentrations in different plants and plant species, and to assay virus in plants subjected to changes in environmental conditions (unpublished results). There are few practicable methods which could elicit such information so easily, particularly for large numbers of samples or for very small amounts

The microplate procedure is particularly suited to the large scale testing of field samples, such as might be required, for example, in surveys of virus disease or for epidemiological investigations. Its sensitivity for the direct detection of virus in plant extracts should facilitate field studies of those viruses for which adequate and practical methods of detection have not been available. Even for viruses detectable by the traditional gel diffusion methods, the greater sensitivity of ELISA should permit the batch testing of samples; a procedure likely to be of particular value where the incidence of virus infection in crops is low. These applications are currently being investigated and will form the subject of future reports.

As with all new and relatively sophisticated techniques some perseverance and practice will undoubtedly be necessary before the full advantages of the ELISA technique can be exploited. The ease with which the microplate procedure has been adapted to plant virus serology from its origin in medical immunology is indicative of its versatility for a wide range of situations and applications. Undoubtedly, experience will modify and refine the procedures according to specific applications, but we hope that sufficient detail has been presented to be of immediate practical use to those concerned with the detection or quantitative assay of plant viruses.

REFERENCES

AVRAMEAS, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. *Immunochemistry* 6, 43–52.

BERCKS, R. (1967). Methodische Untersuchungen über den serologischen Nachweis pflanzenpathologener Viren mit dem Bentonit-Flockungstest, dem Latex-Test und dem Bariumsulfat-Test. *Phytopathologische Zeitschrift* 58, 1–17.

ENGVALL, E. & PERLMANN, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 8, 871–874.

ENGVALL, E. & PERLMANN, P. (1972). Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *Journal of Immunology* 109, 129–135.

FELDMANN, G., DRUET, P., BIGNON, J. & AVRAMEAS, S. (1976). Immunoenzymatic techniques. In *Proceedings of the First International Symposium on Immunoenzymatic Techniques*, p. 512. Amsterdam: North-Holland Publishing Co.

JERMOLJEV, E. & ALBRECHTOVA, L. (1965). Aplikace suspenze latex v sérologické diagnóze virových chorob rostlin. *Ochrana Rostlin* 2, 51–56.

NAKANE, P. K. & PIERCE, G. B. (1966). Enzyme-labelled antibodies: preparation and application for the localization of antigens. *The Journal of Histochemistry and Cytochemistry* 14, 929–931.

van REGENMORTEL, M. V. H. (1964). Purification of plant viruses by zone electrophoresis. *Virology* 23, 495–502.

van WEEMEN, B. K. & SCHUURS, A. H. W. M. (1971). Immunoassay using antigen-enzyme conjugates. *FEBS Letters* 15, 232–235.

van WEEMEN, B. K. & SCHUURS, A. H. W. M. (1972). Immunoassay using hapten-enzyme conjugates. *FEBS Letters* 24, 77–81.

VOLLER, A., BARTLETT, A., BIDWELL, D. E., CLARK, M. F. & ADAMS, A. N. (1976a). The detection of viruses by enzyme-linked immunosorbent assay (ELISA). *Journal of General Virology* 33, 165–167.

VOLLER, A., BIDWELL, D. E. & BARTLETT, A. (1976b). Enzyme immunoassays in diagnostic medicine: theory and practice. *Bulletin of the World Health Organization* 53, 55–65.

VOLLER, A., BIDWELL, D., HULDT, G. & ENGVALL, E. (1974). A microplate method of enzyme-linked immunosorbent assay and its application to malaria. *Bulletin of the World Health Organization* 51, 209–211.

WICKER, R. & AVRAMEAS, S. (1969). Localization of virus antigens by enzyme-labelled antibodies. *Journal of General Virology* 4, 465–471.