Rocket Immunoelectrophoresis Assay for Cauliflower Mosaic Virus

Thomas J. Hagen, Delia Barnes Taylor, and Richard B. Meagher

First and third authors, Department of Botany; and second author, Department of Molecular and Population Genetics, University of Georgia, Athens 30602.
Accepted for publication 1 June 1981.

ABSTRACT

Hagen, T. J., Taylor, D. B., and Meagher, R. B. 1982. Rocket immunoelectrophoresis assay for cauliflower mosaic virus. Phytopathology 72:239-242.

A rocket immunoelectrophoresis assay is described for cauliflower mosaic virus (CaMV), a DNA-containing plant virus. The assay is efficient in terms of reagents, can accommodate a large number of samples, is quantitative over a wide range of virus concentration (from 25 ng to 10 μg) and requires less than 8 hr to complete. Virus was detected in as little as 25 mg of plant leaf tissue. The assay is suitable for a number of physiological studies on the caulimoviruses. A small-scale virus enrichment designed to screen quantitatively a large number of independent infected plants by using the rocket assay is described. An improved large-scale virus purification procedure, and the assay and production of serum antibody against CaMV are discussed. Initial studies on the callus culture of CaMV-infected leaves are reported.

Additional key words: diamidino phenylindole (DAPI) immunology.

Recent interest in the application of genetic engineering technology to higher plants has focused attention on DNA-containing plant viruses as potential vehicles for molecular cloning in plants (5,8,9). Cauliflower mosaic virus (CaMV) is the most intensively studied of the plant DNA viruses (12,13); however, very little is known about the cellular or molecular physiology of virus-infected plants. It is likely that this lack of general knowledge about the caulimoviruses will hamper further molecular studies. The lack of simple quantitative assays for the virus has been a limiting factor in analysis of the virus. Although sensitive radioimmune precipitation assays for the virus are available (4,10,13), they require a high level of technical competence.

This manuscript describes a simple quantitative rocket immunoelectrophoresis assay for CaMV. The assay can detect from 25 ng to 10 μg of virus contained in milligram quantities of leaf tissue. The rocket assay was used to measure virus in turnip leaves and roots and in callus tissues from infected plants. A microscaled virus purification procedure suitable for screening a large number of infected plant samples with the rocket assay technique is also described.

MATERIALS AND METHODS

Virus and plant strains. Cauliflower mosaic virus strains Cabbage B and CM4-184 (12) were propagated in turnip (*Brassica rapa* L. 'Just Right' Everett Seed Company, Atlanta, GA 30344). Inoculum was prepared by grinding (with a mortar and pestle) 2 g of infected leaves and 0.1 g Celite in 10 ml of 10 mM potassium phosphate buffer, pH = 7.0. Plants were inoculated by gently rubbing a drop of this slurry of virus-infected leaf tissue and Celite on the first two true leaves of 4-wk-old plants. After 2 wk the procedure was repeated, resulting in 100% infectivity. After 2 wk leaves could be harvested continuously for at least 2 mo from the same plants. Leaves ~20 cm long and 8 cm wide yielded the most virus.

Virus purification. Cauliflower mosaic virus strains Cabbage B and CM4-184 were purified by a modification of the procedure of Hull et al (6). Grinding buffer (A) contained 200 mM potassium phosphate, pH 7.2, 50 μg/ml phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, MO 63178), and 50 μg/ml *N*-ethylmaleimide (Calbiochem, Behring Corp., LaJolla, CA 46250). Gradient buffer (B) contained 10 mM tris-HCl pH 7.5, 2 mM disodium EDTA (J. T. Baker Chemical Co., Phillipsburg, NJ 08865), 2 mM EGTA (Sigma Chemical Co.), and 25 μg/ml PMSF. Sucrose gradients were prepared in buffer B as follows: 9 ml 40% (w/v) sucrose, 9 ml 30% sucrose, 7.5 ml 20% sucrose, and 5 ml 10% sucrose were layered in a Beckman SW27 polyallomer tube. Gradients were allowed to become linear by sitting 4–8 hr at 4 C or by a few seconds of gentle mixing. Virus storage buffer was the same as the gradient buffer with the addition of 50 mM *N*-ethylmaleimide.

Infected leaves were homogenized at 4 C in 1 L of grinding buffer in a 3.77-L (1-gal) commercial Waring Blendor at maximum speed for 10 min. The homogenate was squeezed through two layers of cheesecloth and kept at 4 C. In order to deaggregate the virus, 500-ml aliquots of this homogenate were made 2.5% in Triton X-100 and 1 M in urea and stirred for 2 hr. Debris was removed by a low-speed centrifugation at 4 C at 4,000 *g* for 20 min. The supernatant was sedimented at 95,000 *g* for 90 min at 4 C in a Beckman Type 45 rotor. The pellets from 1 kg of leaf tissue were resuspended in a total of 30 ml storage buffer using a Polytron homogenizer and given another low-speed spin to remove debris. With preparations of CM4-184, 5 ml of virus sample were mixed with 35 μl of a 1 mg/ml stock of the fluorescent DNA strain 4,6-diamidino-2-phenylindole (DAPI) (Boehringer-Mannheim Co., Indianapolis, IN 46250) (15) and layered on the 30.5 ml sucrose step gradient described above. DAPI fluoresces only when bound to double-stranded DNA and shows no fluorescence with RNA. Gradients were centrifuged at 95,000 *g* for 3 hr at 4 C in an SW 27 rotor. The blue fluorescent virus band in the sucrose gradient was visualized with a UV lamp, removed, mixed with three volumes of storage buffer, and repelleted in a type 45 rotor as described above. Virus pellets were resuspended in a few milliliters of storage buffer with gentle agitation for 2 hr at 4 C. Rebanding the pooled preparation from 1 kg of leaves in another sucrose gradient yielded a virus preparation of about 99% purity, as judged by electrophoresis on a 12% acrylamide SDS gel (7).

Virus used in preparation of antiserum was further purified by overnight banding in CsCl. Following the second sucrose gradient run, the repelleted virus was resuspended in 5 ml of storage buffer and mixed with 2.5 g CsCl, 50 μl of the DAPI stock and spun at 85,000 *g* for 24 hr in a 50 Ti rotor at 20 C. The fluorescent virus band was collected, dialyzed exhaustively against storage buffer, and concentrated again by high-speed centrifugation, if required. Virus was stored at −20 C.

Preparation of antisera. Two young white male New Zealand rabbits were injected in the foot pads with 50–125 μg of CaMV in 0.1 ml of 10 mM tris-HCl, pH 7.5, 0.15 M NaCl mixed with an equal volume of Freund's complete adjuvant (Difco Labs., Detroit,

0031-949X/82/02023904/$03.00/0

MI 48232). Ten days later the rabbits were given a second foot pad injection with 20–60 μg of virus prepared as above with Freund's incomplete adjuvant instead of Freund's complete adjuvant. After 30 days the rabbits were bled weekly by heart puncture (8). Booster injections of 20 μg were given every 2 mo using incomplete adjuvant.

The gamma globulin fraction was precipitated three times with 33% ammonium sulfate by the method of Garvey et al (3). The purified antisera were stored at −20 C. Both crude and purified antisera were used in the rocket immunoelectrophoresis method described below. The use of purified antisera resulted in only slightly sharper rockets.

Rocket immunoelectrophoresis. The rocket immunoelectrophoresis assay is a modification of the method of Oxford and Schild (11). A fine review of immunoelectrophoretic methods is given in Verbruggen (14). Gel plates were poured on varied lengths of 10-cm-wide strips of transparent gel bond film (Marine Colloids Division, EMC Corporation, Rockland, ME 20760). One milliliter of agarose was poured per 5 cm^2 gel area. Running buffer and gel buffer were prepared by titrating 50 mM barbital (Sigma) and 1 mM sodium azide to exactly pH 8.6 with glacial acetic acid.

Agarose (1%) (SeaKem, Marine Colloids, Rockland, ME 20760) was melted in gel buffer in a microwave oven. The liquid agarose was cooled to 60 C and 0.67 μl of purified antisera per milliliter of gel was added with swirling. With crude serum, about 1 μl was mixed per milliliter of gel. The gel was immediately poured onto the hydrophilic side of the gel bond film. For a typical gel, 25 ml of 1% agarose at 60 C were mixed with 25 μl of crude serum and poured onto a 10 × 15-cm piece of gel bond. The gel hardened in a few minutes and sample wells were made using a 3-mm-diameter thin-walled stainless steel tube and gentle suction to remove agarose plugs. Wells were spaced every 5 mm. The gel was set in a water-cooled, flat bed electrophoresis apparatus similar to the LKB model 2117 Multiphor. Both sides of the gel were connected to the buffer reservoirs with two layers of Whatman 3M filter paper or Handy Wipes saturated with running buffer. The gel was cooled to 16 C with tap water.

Virus preparations were made up in 25 mM barbital-acetate, pH 8.6, with 1% Triton X-100 and 3 μl each of 0.1% bromphenol blue and 0.1% cyanol blue. Samples were prepared to final volume of 20 μl, and 15 μl was placed in each well. Some samples were heated to 65 C prior to loading. Electrophoretic runs were conducted for 2–3 hr at 120 ma or until the slower moving dye, the cyanol blue, had migrated off the gel. The gel was then processed to remove unprecipitated proteins and stained as described by Oxford and Schild (11).

Rapid miniscreen for CaMV in infected leaf tissue. One gram of plant tissue was homogenized in 2 ml of grinding buffer with urea and Triton (as described above) in a Polytron Tissue Mizer at 80% of full speed for about 2 min or until 90% of the plant cells appeared to be broken. About 1.5 ml of this homogenate was allowed to stand at room temperature for 30 min and then spun for 30 min at 9,800 *g* in an Eppendorf-Brinkman tabletop centrifuge at 4 C. A 175-μl sample of the supernatant was centrifuged in a Beckman Airfuge at 110,000 *g* for 15 min. Although virus was easily detected by our rocket assay, a more quantitative estimate of CM4-184 was made by combining and sedimenting several aliquots in the same tube. Virus pellets were resuspended in 75 μl of grinding buffer and layered on 100 μl of 20% w/v sucrose in 0.1 M tris-HCl, pH 7.3, and 0.2 M NaCl in a 200-μl Beckman Airfuge centrifuge tube. The virus was pelleted through the sucrose pad for 1 hr at 110,000 *g*. Virus pellets were resuspended in 40 μl of sample buffer and a series of twofold dilutions was loaded to give quantitative rockets.

Callus culture. Young leaves (10 cm long) from infected turnips were picked and allowed to wilt for 45 min. To insure aseptic conditions, leaves were dipped for 1 min in fresh 5% calcium hypochlorite, followed by several quick dips in 95% ethanol, and this process was repeated a second time. Plugs of leaf tissue (6-mm diameter) were removed with a sterile corkborer and placed in 1 ml of B5 medium or on agar plates containing this medium. B5 medium was prepared by the method of Gamborg et al (2). The cytokinin isopentenyladenine (N^6 dimethylallyladenine; Calbiochem) was added at a final concentration of 0.3 μg/ml. Concentrations of 2',4'-dichloroindophenol (2,4-D; Sigma) from 0.1 μg to 20 μg/ml were examined, with optimum callus growth for infected tissues occurring with 1.5 and 2 μg of 2,4-D per milliliter. This range of concentrations was used in all cultures discussed in this paper.

RESULTS

Virus purification. Although sucrose gradient centrifugation of the wild type CaMV-B strain always resulted in visible virus bands, bands of strain CM4-184 were often at the limit of detection. However, the addition of DAPI resulted in fluorescent virus bands, which were obvious and greatly simplified preparation of CM4-184 (Fig. 1). No loss of infectivity was observed for virus purified with DAPI. Furthermore, the use of DAPI made other forms of the virus visible in all sucrose gradient runs. For example, along with a native 220S monomeric virus particle band, three other virus bands were often visible. The two faster migrating bands probably correspond to dimer and trimer aggregates of the virus. Repeated pelleting, resuspending, and rebanding the 220S component resulted in detectable levels of these other forms. A band that migrates more slowly than the native virus monomers, at about 65S, probably represented damaged virus particles (1).

In our hands virus strain CM4-184 was unstable in CsCl. We seldom recovered more than 20% of the virus in an overnight CsCl banding. If gradients were run for longer periods, the fluorescent virus band continued to fade. CM4-184 purified by this method possessed the appearance of a homogeneous preparation based on SDS acrylamide protein patterns (12).

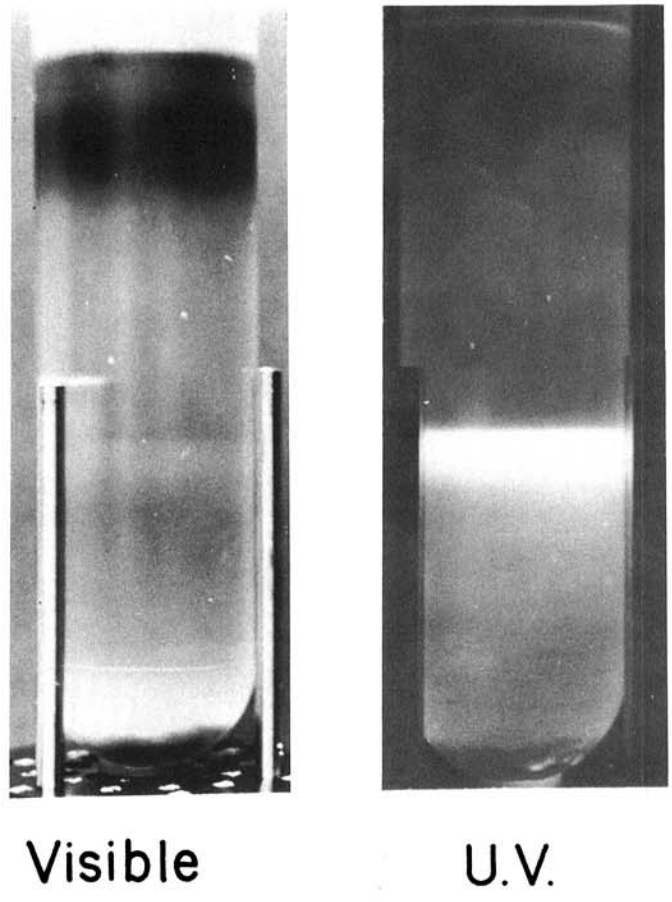

Fig. 1. DNA fluorescence of DAPI-labeled virus. The first high-speed pellet of CM4-184 virus from 100 g of cells was spun in a SW27 sucrose gradient containing a total of 25 μg of DAPI (see Methods). The photographs contrast the visibility of virus gradient band illuminated by indirect visible light vs long-wave ultraviolet.

Rocket immunoelectrophoresis. The rocket immunoelectrophoresis system developed for CaMV was quantitative (Fig. 2-I). The area under each rocket pattern was directly proportional to a wide range of virus concentrations tested (Fig. 3). Rocket height alone did not give a linear relationship to virus concentration as in the influenza system of Oxford and Schild (11). Independently prepared rocket gels gave essentially the same assay results (Fig. 2-I and 2-II). The assay was also reproducible for a large number of samples across a rocket gel (Fig. 2-III).

To obtain quantitative rockets, notable modifications of other published rocket systems were necessary. Due to the slow migration of the CaMV antigens through the gel, a slower marker dye, cyanol blue, was substituted for bromophenol blue. Viral antigens migrated immediately behind the dye, unless intercepted by virus antibody. The slow migration rate of these antigens in the pH 8.6 rocket buffer system was not surprising considering the basic pKi of the major virus proteins (8). The presence of Triton in the sample buffer enhanced sharpness of the rockets and allowed better quantitation, particularly at high virus concentrations of 1–10 μg. Other detergents such as sarcosyl, sodium lauryl sulfate, and NP40 were examined either in the sample buffer, running gel, or both, but these only resulted in nonquantitative or less sensitive assays. The addition of sarcosyl, for example, which was critical to the influenza rocket system (11), or calcium lactate resulted in stunted rockets for CaMV. Several other running and gel buffers, such as Tricine (BioRad, Richmond, CA 94804), yielded interpretable rockets, but they were never as satisfactory as those run with the barbital-acetate buffer.

Electrophoresis in parallel rocket strips 10 cm long × 2 cm wide, each containing a different antiserum sample, facilitated the titration of sera from different rabbits and from successive bleedings from the same rabbit (Fig. 4-II). We found that higher titer antisera were obtained from 2 to 10 wk after each booster injection, and that 3 mo after each booster, the antisera titer fell to less than 30% of its previous peak level.

The quick screen for CaMV was relatively fast and efficient and gave sharp quantitative rockets (Fig. 4-I). In reconstruction experiments in which purified virus CM4-184, added to uninfected leaves, was worked up through the screen procedure and assayed with the rocket system, approximately 80% of the virus was recovered. The amount recovered was reproducible at each mixture level tested. It is likely that in assays for wild-type CaMV-B, in which higher virus concentrations are present initially, much less plant extract (eg, 100 μl) will be needed to obtain a good quantitative set of rockets.

Virus concentrations in infected plant leaf and infected plant root tissue were compared by using the quick screen purification and rocket assays. Root tissue from infected plants contained no detectable virus. The rocket assay suggests that the normal level of CM4-184 virus is about 6 μg/g of infected leaf tissue. As described above, as little as 25 ng of virus per gram can be detected by this assay. Reconstruction experiments with uninfected root suggest that as little as 50 ng of virus could have been detected in root tissue. Therefore, we conclude that the root tissue from infected plants contains less than 2% of the virus found in the leaves.

Callus growth from plugs of infected cultivar Just Right turnip leaves could be established with the 2,4-D medium in about 2 wk. Although agar medium was slower than liquid in establishing callus growth and development, the success rate was higher than for liquid. Uninfected turnip leaf tissue died on this medium in a few days. No sign of callus growth was detected in any of hundreds of uninfected leaf plugs. More recently, using a similar medium in which benzyladenine was substituted for 2,4-D, growth was

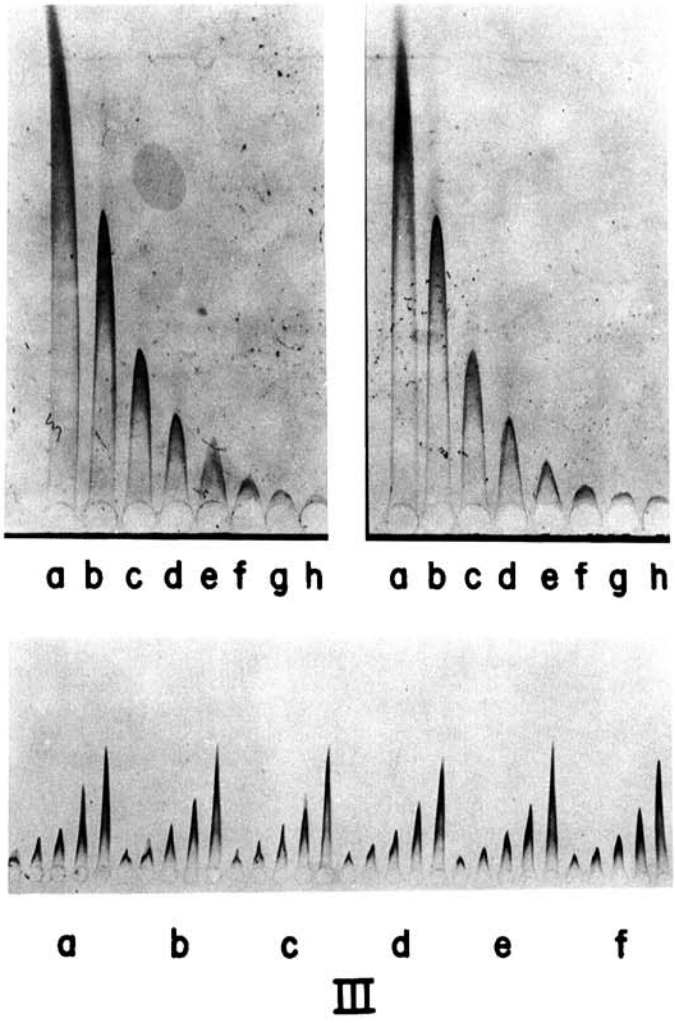

Fig. 2. I and **II,** Reproducibility of pouring two separate rocket gels. Identical amounts of buffered agarose and anti-CM4-184 IgG were poured in two separate rocket gels. Samples "a" through "h" contained 3, 1.5, 0.75, 0.37, 0.19, 0.1, 0.045, and 0.025 μg of purified CM4-184. **III,** Reproducibility across a single rocket gel. The rocket gel contained 40 ml buffered agarose and 60 μl purified IgG fraction on a 10 × 24-cm rocket gel. Sample groups "a" through "f" contained a serial dilution of 0.2, 0.4, 0.8, 1.6, and 3.2 μg of purified CM4-184 in each well.

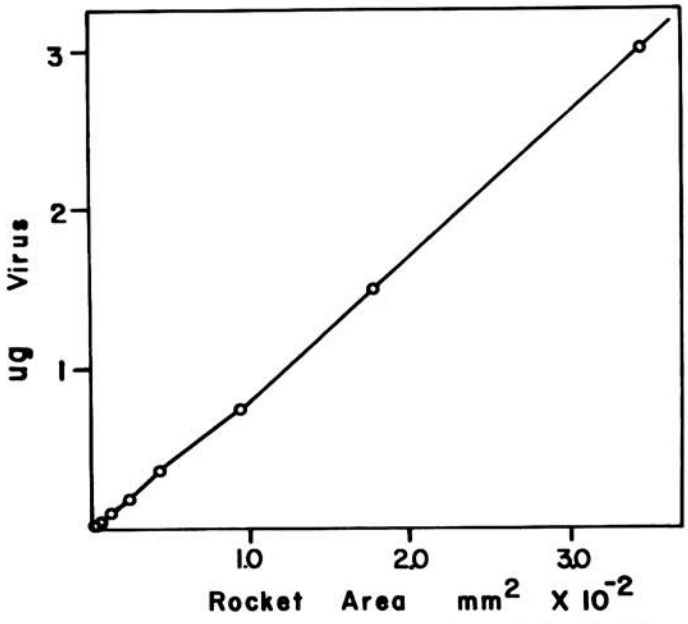

Fig. 3. Linearity of rocket assay. Plot shows the linear relationship of virus concentration with rocket area. Area was calculated as the product of peak height and peak width measured at half peak height. Peak height was measured from the top of the sample well. These data points were taken from the rocket assays presented in Fig. 2-II.

obtained for uninfected tissue plugs and no growth occurred with infected tissue (R. B. Meagher, *unpublished*).

Six independent 2,4-D callus suspension cultures established from CM4-184 infected leaf tissues were assayed for virus using the rocket assay. One gram of callus from each culture was screened. No detectable virus was found in cultures within 6 wk of placing leaf tissue into callus culture. Reconstruction experiments demonstrated that purified virus was recovered at expected levels when homogenized with callus tissue.

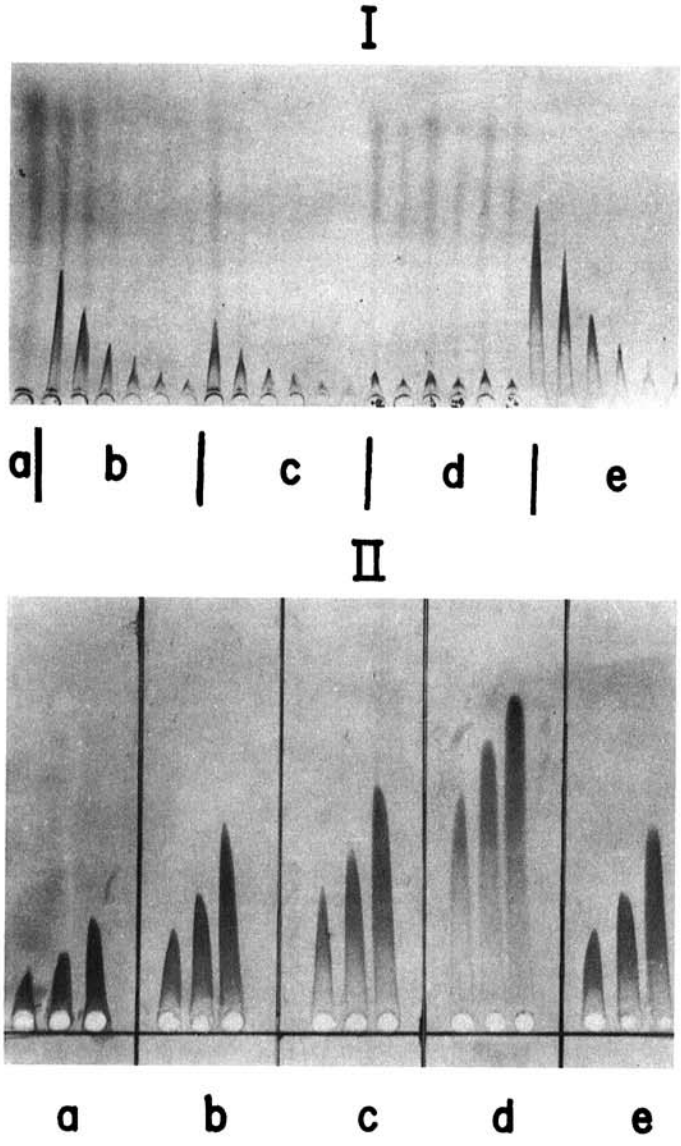

Fig. 4 I, Rocket immunoelectrophoresis of miniscreens of CM4-184. This rocket gel was prepared from 46 ml of buffered agarose containing 30 μl of purified anti-CM4-184 IgG and poured on a 10 × 20-cm plastic sheet. The wells contained 20-μl samples as follows: (a) miniscreen of control plant, leaf tissue; (b) reconstruction experiment in which a known quantity of virus was mixed with control tissue during homogenization; (c) miniscreen of infected leaf tissue (the largest rocket contained one-half of the screened material from 0.26 g of leaf and the next five rockets are twofold dilutions); (d) three pairs of rockets contained one-half and one-quarter of the high-speed pellet before sucrose gradient centrifugation of an extract from 25 mg of the infected leaf tissue from three separate plants; and (e) standards: rockets representing 3, 1.5, 0.75, 0.37, 0.19, and 0.01 μg of purified CM4-184 virus, respectively. **II,** Rocket immunoelectrophoresis comparison of crude anti-CM4-184 serum. Each set of three rockets was produced from 1, 2, and 4 μg of purified CM4-184. Each of the five rocket strips contained 5 ml of buffered agarose and 4 μl of serum. Samples of antiserum from a single rabbit were prepared (a) 10 days after the secondary injection (see Methods), (b) 21 days, (c) 45 days, (d) 75 days, and (e) after a 10-μg booster injection of CM4-184, given at 75 days, serum collected at 85 days.

DISCUSSION

The rocket immunoelectrophoresis assay used on CaMV was simple and sensitive, allowing as little as 25 ng of virus to be detected. Assays on 30 samples required only 30 μl of crude serum. In contrast, assays using Ouchterlony's (3) double-diffusion plates required large amounts of serum (100 μl) to handle several samples. Also, due to the very low diffusion rate of virus, up to 3 days were required to form precipitation bands, which were usually curved and indistinct. CaMV radioimmune precipitation assays were more sensitive and required a time period comparable to that of the rocket system (8). However, our attempts to use the radioimmune assay often yielded artifacts and it could not be internally controlled as easily as the rocket system.

The rocket system should be ideal for studying the physiology of virus-infected plants. More work is needed on DNA-containing plant virus systems to determine kinetics of virus synthesis, conditions for optimum virus production, and immunological relatedness of virus strains.

The observations that infected turnip leaves will yield callus on 2,4-D enriched medium when uninfected tissue will not, and that (within the the limits of this assay) no virus was observed in callus tissue derived from infected tissue, are both puzzling. The data suggest a number of possibilities: that there is in fact a low level of infection not detectable by this assay; that the cells may no longer be infected but are in a permanently altered physiological state; or that the cells may be in a transformed state. For example, plant tissue containing DNA fragments of the Ti plasmid from *Agrobacterium tumefaciens* grows as callus in a defined medium without added hormones. It would be worthwhile to examine callus derived from CaMV-infected tissue for integrated or free viral DNA.

LITERATURE CITED

1. Al Ani, R., Pfeiffer, P., Lebeurier, G., and Hirth, L. 1979. The structure of cauliflower mosaic virus. 1. pH-Induced structural changes. Virology 93:175-187.
2. Gamborg, O. L., Kartha, K. K., Ohyama, K., and Fourke, L. 1978. Protoplasts and tissue culture methods in crop plant improvement. Pages 265–278 in: Proceedings of Symposium on Plant Tissue Culture. Science Press, Peking.
3. Garvey, J. S., Cremer, N. E., and Sussdorf, D. H. 1977. Pages 218 and 313 in: Methods in Immunology. W. A. Benjamin, Inc., London.
4. Ghabrial, S. A., and Shepherd, R. J. 1980. A sensitive radioimmunosorbent assay for the detection of plant viruses. J. Gen. Virol. 48:311-317.
5. Hull, R. 1978. The possible use of plant viral DNA in genetic manipulation in plants. Trends Biochem. Sci. 3:254-256.
6. Hull, R., Shepherd, R. J., and Harvey, J. D. 1976. Cauliflower mosaic virus: an improved purification procedure and some properties of virus particles. J. Gen. Virol. 31:93-100.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
8. Meagher, R. B. 1977. The development of a molecular cloning system in higher plants. Pages 129–158 in: A. Hollaender et al, eds. Genetic Engineering for Nitrogen Fixation. Plenum Press, New York.
9. Meagher, R. B., and McKnight, T. D. 1980. A model for a molecular cloning system in higher plants: Isolation of plant viral promoters. Pages 63–75 in: C. J. Leaver, ed. Genome Organization and Expression in Plants. Plenum Press, New York.
10. Meagher, R. B., Tait, R. C., Bethlach, M., and Boyer, H. W. 1977. Protein expression in *E. coli* minicells by recombinant plasmids. Cell 10:521-536.
11. Oxford, J. S., and Schild, G. C. 1977. Quantification of influenza virus structural proteins using rocket immunoelectrophoresis. J. Gen. Virol. 38:187-193.
12. Shepherd, R. J. 1976. DNA viruses of higher plants. Pages 305–339 in: M. A. Lauffer et al, eds. Advances in Virus Research. Vol. 20. Academic Press, New York.
13. Shepherd, R. J. 1979. DNA plant viruses. Annu. Rev. Plant Physiol. 30:405-423.
14. Verbruggen, R. 1975. Quantitative immunoelectrophoretic methods: a literature survey. Clin. Chem. 21:5-43.
15. Williamson, D. H., and Fennell, D. J. 1975. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. Methods Cell Biol. 12:335-351.