High pH-Ammonia Agar Immunodiffusion for Plant Viruses

Willem G. Langenberg and Ellen M. Ball

Research Plant Pathologist and Research Associate, respectively, Plant Science Research Division, ARS, USDA, and Department of Plant Pathology, University of Nebraska, Lincoln 68503.

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ABSTRACT

A simple method for immunodiffusion of long flexuous viruses was developed. No chemical degradation or other treatment of the virus or plant juice was necessary, and existing antisera to undegraded viruses were used. Two long flexuous, two shorter rod-shaped, and three icosahedral plant viruses, or their components, formed precipitin lines in pH 9.1 agar plates containing free ammonia and detergent. Precipitin lines were visible as early as 6 hr after charging of the wells with virus and antiserum when the dishes were incubated at 37 C. Double-diffusion plates were prepared by pouring into tight-lid petri dishes 4 ml of a 1:1 mixture of 1% autoclaved agar and an unautoclaved solution of 0.2 M dibasic ammonium phosphate, 1% 1,3-propane diamine, and 0.2% Igepon T-73 (active ingredient 28-30% N-methyl-N-oleoyl taurate).

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Many methods for the analysis of virus-antibody reactions are in existence. Immunodiffusion in agar is routinely used to study interactions of plant viruses, their components, and their antisera, and is a reliable diagnostic tool. However, difficulties often arise in the diffusion of rod-shaped viruses through the agar medium, and special treatments are necessary to obtain visible precipitin lines. A few examples are: tobacco etch virus (TEV) (13), maize dwarf mosaic virus (MDMV) (15), barley stripe mosaic virus (BSMV) (9), wheat streak mosaic virus (WSMV) (4), and tobacco mosaic virus (TMV) (16). Rod-shaped viruses often form precipitin lines close to the antigen well only after prolonged incubation whereas icosahedral viruses usually diffuse readily into the agar medium.

Efforts to circumvent the slow, or no, diffusion of long flexuous viruses or virus inclusions have included chemical degradation of the virus (12, 13, 14, 15) and the addition of detergents to agars used for immunodiffusion (4, 8, 9, 11). Some of the procedures may be cumbersome or time-consuming, or can give rise to false reactions (8).

Precipitin lines produced in agar gels at pH 7, 8, and 9 by MDMV corresponded with lines of MDMV protein (Langenberg & Ball, *unpublished data*). The higher the pH of the agar, the heavier the precipitation lines, suggesting progressively enhanced virus degradation at higher pH levels. When other rod-shaped viruses were tested in agar double-diffusion at pH levels higher than customarily used, some broke down partially (BSMV), and others not at all (WSMV, TMV).

Detergent incorporation in pH 7.2 agar plates broke down BSMV, but not WSMV, sufficiently fast to show precipitin lines in 24 hr of incubation.

Antigens of MDMV, WSMV, and BSMV diffused poorly or not at all in immunodiffusion plates at pH 7.0. The diffusion of TMV in neutral agar and the relation of electrolytes to the formation of precipitin lines has been extensively investigated (16).

The method reported below differs from other procedures employing alkaline degradation in that pH levels higher than 9.1 were avoided, alkaline and detergent exposures of antigens were combined in one step, and degradation took place in the immunodiffusion plates. No addition of chemicals to the antigen preparation was required. Reaction products were immediately available for antigen-antibody reactions, and it was not necessary to dialyze chemical- or alkaline-degraded virus preparations before they were used for the serological test.

MATERIALS AND METHODS.—*Viruses.*—Tobacco mosaic virus, American Type Culture Collection (ATCC) (2) No. 2 (common strain) and potato virus X (PVX), Nebraska isolate, were maintained in *Nicotiana tabacum* L. Xanthi and *Xanthi-nc*, respectively. Maize dwarf mosaic virus, B strain, Nebraska isolate, was maintained in sweetcorn, *Zea mays* L. 'Goldencross Bantam'. Wheat streak mosaic virus, ATCC No. 29, was maintained in Cheyenne wheat, *Triticum aestivum* L., whereas barley stripe mosaic virus, type strain, ATCC No. 69 and brome mosaic virus, ATCC No. 66, were maintained in Moore barley, *Hordeum vulgare* L.

Southern bean mosaic virus, SBMV, ATCC No. 17, was maintained in *Phaseolus vulgaris* L. 'Bountiful'. Cowpea mosaic virus, CPMV, was maintained in cowpea, *Vigna unguilata* (L.) Walp. and has been identified as the Sb strain of CPMV [originated from J. B. Bancroft, Purdue University, identified by Agrawal (1)].

Purification.—Four of the viruses were received as purified preparations or in diseased tissue and were used as expressed sap: BMV and SBMV from M. K. Brakke; CPMV from G. D. McLean; and BSMV from A. O. Jackson.

TMV was purified by repeated (5 times) precipi-

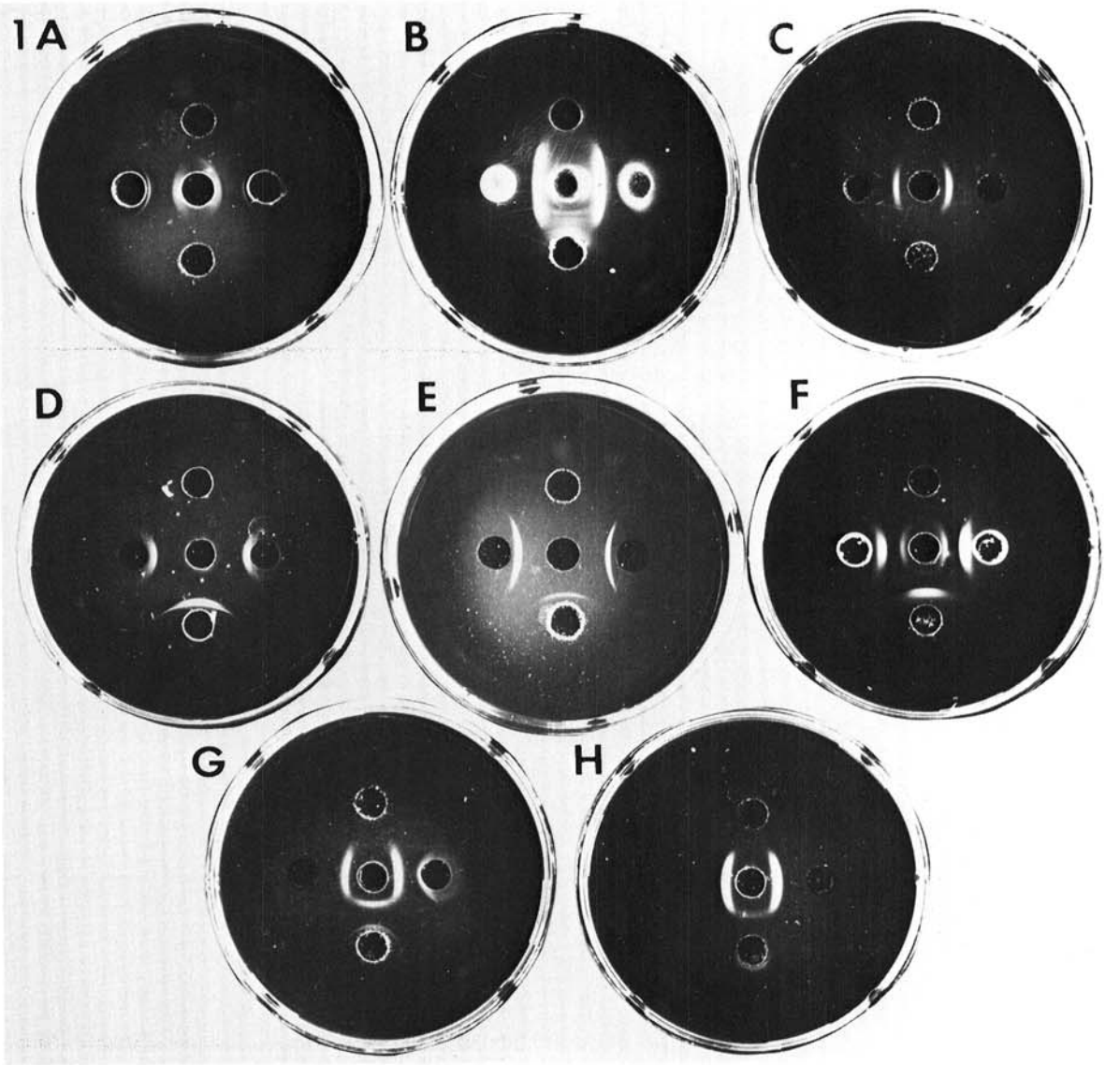

Fig. 1. A). Potato virus X (PVX) immunodiffusion in 0.5% agar at pH 9.1 and containing 0.05 M K_2HPO_4 only. Center well: anti-PVX antiserum; top and bottom well: healthy tobacco juice, left and right wells: juice from PVX-infected tobacco. Rapidly diffusing antigen formed precipitin lines close to antiserum well, and slowly diffusing antigen lines close to antigen wells. Developed in 18 hr at 37 C. B-H) plates prepared with 0.5% agar containing 0.1 M dibasic ammonium phosphate, 0.1% Igepon T-73, and 0.5% 1,3-propane diamine, final pH 9.1. All plates developed at 37 C for 18 hr. B) Barley strip mosaic virus (BSMV): peripheral wells clockwise from top: healthy barley juice; 1 mg/ml BSMV; BSMV-infected barley juice; BSMV protein, 0.3 mg/ml. Center: BSMV antiserum. C) Wheat streak mosaic virus (WSMV): clockwise from top: healthy wheat juice; WSMV, 1 mg/ml; WSMV-diseased wheat juice; and WSMV, 1 mg/ml. Center: WSMV antiserum. Concentration of WSMV in diseased wheat is too low to form a visible precipitin line. D) Brome mosaic virus (BMV): clockwise from top: healthy barley juice; juice from BMV-diseased barley; BMV, 1 mg/ml; juice from barley diseased with BMV. Center: BMV antiserum. E) Southern bean mosaic virus (SBMV): clockwise from top: healthy bean leaf extract; SBMV, 1 mg/ml; juice from SBMV-diseased bean leaves; SBMV, 1 mg/ml. Center: SBMV antiserum. F) Cowpea mosaic virus (CPMV): clockwise from top: healthy cowpea juice; CPMV, 1 mg/ml; juice from CPMV-infected cowpea leaves; CPMV, 1 mg/ml. Center: CPMV antiserum. Lines around antiserum well indicate some CPMV breakdown products. G) Tobacco mosaic virus (TMV): clockwise from top: healthy tobacco juice; TMV, 1 mg/ml; TMV-diseased tobacco juice; TMV-X protein, 0.3 mg/ml. Center: TMV antiserum. Extensive, but not complete, breakdown of TMV into X-protein. H) Maize dwarf mosaic virus (MDMV): clockwise from top: healthy maize juice; MDMV, 1 mg/ml; juice of MDMV-infected corn; MDMV protein, 0.3 mg/ml. Center: MDMV antiserum. Complete breakdown of MDMV into protein.

tation with polyethylene glycol (10) and low-speed centrifugation, and was dissolved in 0.01 M phosphate buffer, pH 7.2. TMV protein was prepared by the acetic acid method of Fraenkel-Conrat (7).

We purified MDMV-B by grinding systemically infected sweetcorn leaves in 3 times their weight of cold 0.1 M TAC extraction buffer (0.1 M Tris [tris(hydroxymethyl) amino methane] 0.05 M citric acid; pH adjusted to 8.2 with 1,3-propane diamine (practical grade); 0.8% polyvinylpyrrolidone, and 0.2% 2-mercaptoethanol). Green material was removed by calcium phosphate precipitation, and the virus precipitated from the cleared supernatant with 6% polyethylene glycol, MW6000. The precipitated virus was dissolved in 0.01 M TAC buffer (the pH lowered to 7.2 by a reduction of the amount of 1, 3-propane diamine) and layered on sucrose density-gradients for the final purification and concentration. A more detailed procedure will be published at a later date. MDMV-SDS protein was prepared by degradation of MDMV with an ammonium carbonate-sodium dodecyl sulfate (SDS) system (5), and excess SDS was removed by dialysis.

The concentrations of the purified viruses were determined by ultraviolet light absorption at 260 nm.

Antiserum to viruses.—Existing antisera to whole virus only were used. All sera had been stored frozen without preservatives, some for almost 20 years (TMV and SBMV).

Preparation of agar gels.—Solutions of 1% agar (No. 02-106 or No. 02-694, Baltimore Biological Laboratory, Md.) were autoclaved for 10 min and mixed immediately at 1:1 with the following pH 9.7 solution at room temperature: 0.2 M dibasic ammonium phosphate, 1% 1,3-propane diamine (practical grade), and 0.2% Igepon T-73* [28-30% sodium N-methyl-N-oleoyl taurate, 65% sodium chloride, 1.5% water, and not more than 3% sodium oleate; Brakke (3)]. Agar was poured into tight-lid petri dishes (50 X 12 mm, Falcon Plastics, Oxnard, Calif.) in a volume of 4 ml/dish. After the agar hardened, wells were cut with a No. 2 cork borer. The pH of the hardened agar was 9.1 before and at the conclusion of the experiments.

Agar wells were charged with freshly expressed juice of healthy and diseased tissue, purified virus at 1 mg/ml, and, where available, protein of degraded virus, as indicated under RESULTS. The petri dishes were incubated at 37 C for 15-18 hr.

RESULTS.—The high pH, ammoniacal agar was selected after comparison of agars at pH 7.0 and 9.0 with and without NH_4^+ or detergent. Only when agar at pH 9.0 and containing both NH_4^+ and detergent was used did all of the viruses, except PVX, diffuse rapidly to form clear precipitin lines.

Results of the immunodiffusion tests are illustrated in Fig. 1. In the complete agar, WSMV, MDMV, BSMV, and PVX degraded extensively; and TMV, partially (Fig. 1-G).

PVX protein may not form a precipitate with antiserum to the complete virus (14). When high pH alone (9.1) was tested in 0.5% agar that contained 0.05 M K_2HPO_4, PVX formed two lines (Fig. 1-A). One was close to the antiserum well and one close to the antigen well. In plates that contained ammoniacal, high pH, detergent agar, PVX showed no reaction with antiserum to undegraded PVX. Antiserum to degraded PVX was not used.

TMV formed a precipitin line close to the antigen well and a line confluent with the X-protein precipitin band (Fig. 1-G). Similarly, MDMV-SDS protein formed a common precipitin line with the bands from the crude juice well and the whole virus well (Fig. 1-H). The BSMV protein from phenol-degraded virus formed precipitin lines that coalesced with the lines from the crude juice and whole virus wells (Fig. 1-B). WSMV also degraded extensively to rapidly diffusing units (Fig. 1-C).

In contrast, two of the three icosahedral viruses, BMV and SBMV (Fig. 1-D, E), did not form precipitin lines characteristic of the virus breakdown products, since the only lines formed were close to the antigen wells. The CPMV (Fig. 1-F), on the other hand, showed some breakdown of the whole virus and also had lines close to the antiserum well. It is possible that BMV and SBMV did degrade in high pH agar but that, analogous with PVX, the protein subunits did not react with antiserum to the whole virus.

DISCUSSION.—The use of high pH-ammonia agar should be widely applicable to rod-shaped viruses which diffuse with difficulty in agar. It is possible that more viruses will be found which show the same conformational changes of the protein subunit as PVX so that antiserum to the whole virus cannot be used. However, we know of only one other instance where such a possibility may occur. Tobacco streak virus (TSV) in tobacco juice rarely reacted with homologous antiserum, but did form a precipitin line in agar diffusion plates when in bean juice (6).

The high pH ammoniacal gel diffusion has several advantages that may be summarized: (i) ease of preparation of buffered agar; (ii) speed of precipitation zone formation; (iii) no need for antimicrobial agents to be incorporated in the agar plates that might react with the antigens; (iv) no requirement for separate chemical degradation of virus; (v) no need for special antisera against degraded virus to be prepared so that existing antisera will suffice in most instances; (vi) no need for plant juice to be centrifuged before the charging of antigen wells.

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