Serological Properties of Oat Necrotic Mottle Virus

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ABSTRACT

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Antiserum prepared with a total of 4.9 mg of oat necrotic mottle virus (ONMV) had a titer in microprecipitin tests of 1/4,096 with 1 mg virus/ml. About 0.12 μg of virus could be detected after absorption of the antiserum with healthy sap and reconcentration of the absorbed antiserum with polyethylene glycol to a titer of 1/4,096. Optimal reacting proportions were 0.5 mg virus/ml and 1:64 dilution of reconcentrated, absorbed antiserum. After clarification of the sap with silver nitrate, oat necrotic mottle virus in sap from oat leaves was readily detectable with the microprecipitin test. In Ouchterlony double-diffusion tests, no immunodiffusion lines were obtained with reconcentrated, absorbed antiserum and ONMV. A broad band near the antiserum well was usually visible with unabsorbed antiserum and purified virus in high-pH ammonia agar gel plates and in 1% agar plates when SDS-ammonium carbonate reagent was added to the virus preparation. The microprecipitin method was the most reliable test for detecting OBMV in partly purified preparations. Antiserum to wheat streak mosaic virus had a titer of 1/8 to ONMV. The ultraviolet absorption spectrum of the virus was maximum at 259 nm and minimum at 247 nm and the average 280:260 and max:min absorption ratios were 0.75 and 1.06, respectively.

Oat necrotic mottle virus (ONMV) is a mechanically transmissible, flexuous filament, 720 nm long and 11 nm in diameter (8, 9). Cylindrical inclusions typical of the potato virus Y group (6) were demonstrated in cells of plants infected by the virus (10). A vector for the virus has not been found, and unlike most viruses of this group ONMV was not transmitted by aphids (8).

An antiserum to ONMV was necessary to aid in the identification of ONMV in plants naturally infected in the field and for use in taxonomic studies of the virus. This work describes the preparation of such an antiserum, the investigation of its properties, and the usefulness of the antiserum for detecting the virus in preparations from infected oats. Also, ONMV was tested against antisera to several possibly related gramineous viruses and the ultraviolet absorption spectrum of the virus was examined.

MATERIALS AND METHODS

Virus purification.—The virus isolate used and its propagation in oats were the same as in an earlier work (9), but the purification method was slightly modified as follows. To increase the yield of virus from infected oat leaves, the fibrous material remaining after the sap had been expressed in the mechanical juice extractor was homogenized in the expressed sap with a Waring Blendor. To increase the purity of the virus preparations, the first centrifugation in the Beckman No. 30 rotor at 70,800 *g* was shortened from 2.5 to 2.0 hours. The virus was then sedimented by two successive centrifugations at 92,600 *g* for 2.25 hours using a method slightly modified from that of Knesek et al. (11). In each of these centrifugations the virus preparation was layered on a column (4.5 ml) of 30% sucrose in citrate buffer (0.1 M sodium citrate, pH 6.3), containing 4% (w/v) of polyethylene glycol, M.W. 6,000, (PEG) in tubes for the Beckman No. 40 rotor. After each sedimentation of the virus the pellet was resuspended in citrate buffer and the insoluble material was removed by centrifugation for 15 minutes at 8,200 *g*. The effect of these modifications on the amount of virus was measured by comparing the areas under the relevant peaks of ultraviolet absorption profiles obtained with the ISCO density-gradient analyzer after centrifugation of the preparations through sucrose density gradients. The ultraviolet absorption spectrum of ONMV was determined with a Beckman Model DK-2A recording spectrophotometer. The amount of virus in purified preparations was estimated assuming an extinction coefficient of 2.4 (A 0.01/261 nm) which was found for tobacco etch virus (14), a virus with morphological similarity to ONMV.

Serology.—During preparation of purified virus for injection of rabbits the final agarose gel filtration was omitted because of the heavy virus loss incurred in that step. The virus preparation, reconcentrated by ultracentrifugation from the relevant fractions of the density-gradient centrifugation was divided in half. One half was dialyzed overnight at 4 C against 500 ml of citrate buffer containing 0.85% sodium chloride and 0.2% formaldehyde to determine the effect of formaldehyde on the immunogenicity of the virus. The other half was dialyzed against buffer containing only sodium chloride as control. Free formaldehyde was removed from the virus preparation by dialysis against citrate buffer that contained sodium chloride. The formaldehyde-treated virus preparation was emulsified with an equal volume of Freund's incomplete adjuvant and 4 ml of the emulsion

containing 2.2 mg of virus was injected intramuscularly into a rabbit on each of two occasions one week apart. Two weeks after the second injection, 1.5 ml of the formaldehyde-treated virus preparation that contained 0.5 mg of virus was injected intravenously into the rabbit. The same injection schedule was maintained with a second rabbit with virus preparations of the same concentration not treated with formaldehyde. Blood was drawn from the ear on three occasions at weekly intervals commencing 1 week after the intravenous injection to determine the time at which maximum antiserum titer occurred, and subsequently on four occasions over a further period of 7 weeks to obtain a stock of antiserum.

Absorbed antiserum was derived by mixing one part of unabsorbed antiserum by volume with four parts of partly clarified sap. The latter was prepared by expressing sap from frozen and thawed oat leaves, then allowing the sap to stand overnight at 4 C and finally removing the sediment by centrifuging for 15 minutes at 8,200 *g*. After standing 2 hours at room temperature, then overnight at 4 C, the mixture was clarified by centrifuging 15 minutes at 8,200 *g* to yield absorbed antiserum. Reconcentration of the antibodies in the absorbed antiserum was made by precipitation either with ammonium sulphate (18) or with PEG (19). With the ammonium sulphate method three or four precipitations with the salt were effected and the final precipitate was taken up in a volume of citrate buffer equal to half the original serum volume. Excess salt was removed from the final preparation by dialysis against citrate buffer. In the second method PEG was added, with stirring, to the absorbed antiserum to 8% (w/v). When all the PEG had dissolved, the mixture was centrifuged 15 minutes at 8,200 *g* and the pellet was dissolved in a volume of 0.85% sodium chloride solution in water equal to one-fifth that of the original serum.

Microprecipitin tests and Ouchterlony double-diffusion tests with Ionagar No. 2 in plates (normal agar) were performed according to Ball (1), and high-pH ammonia agar immunodiffusion tests according to Langenberg and Ball (12). Optimal reacting proportions of ONMV and its antiserum were determined according to Matthews (13). Healthy oat concentrate and antiserum to sap from healthy oats were the same as used previously (9). Two-fold serial dilutions of antiserum and of virus (1 mg/ml) were made with normal saline and with citrate buffer containing 0.85% sodium chloride, respectively. Degraded virus was prepared from the relevant pooled and reconcentrated density-gradient fractions by resuspending the virus pellet in an aqueous reagent containing 1% SDS, 0.1 M ammonium carbonate, and 0.0001 M EDTA (5). The mixture was allowed to stand overnight at 4 C, then was clarified by low-speed centrifugation and the volume was adjusted with the SDS reagent to the equivalent of 1 mg virus/ml. In some of the agar-gel diffusion tests, the SDS reagent was added to the antigens (1:1, v/v) just before the wells were filled.

To detect the virus serologically in infected oats, microprecipitin tests were done on extracts of systemically infected leaves. Leaves (5g) collected from Clintland oats were frozen overnight at −12 C, thawed, and homogenized in 2 ml of 0.05 M phosphate buffer (potassium dibasic-sodium monobasic phosphate, pH 7) containing 0.85% sodium chloride. The extract was centrifuged twice at 11,000 *g* for 10 minutes and the clear supernatant was divided into three parts. One part was further clarified by shaking with an equal volume of chloroform for 2 minutes. The second part was further clarified by adding an aqueous 0.01 M solution of silver nitrate dropwise with a microliter pipette while agitating the mixture. At the incipient flocculation point an extra volume of silver nitrate was added equal to one-third of that already added. Precipitates were removed by centrifugation for 10 minutes at 8,200 *g*. The third portion of the extract was not clarified (unclarified extract). When determining virus end points, these extracts were diluted with phosphate buffer.

Antisera to wheat streak mosaic virus (WSMV), Hordeum mosaic virus (HMV), and Agropyron mosaic virus (AMV) were kindly donated by J. T. Slykhuis; to ryegrass mosaic virus (RMV) by Y. C. Paliwal and W. G. Langenberg; and to maize dwarf mosaic virus (MDMV), and to sugar cane mosaic virus (SCMV) by R. M. Lister. The homologous antiserum titers were (*personal communications*): WSMV, 1/640; AMV, and HMV, 1/1,280; RMV (Y. C. Paliwal), 1/1,024; MDMV, 1/128; and SCMV, 1/64.

RESULTS

Virus purification.—Homogenization of the macerated leaves in the sap obtained from the juice extractor resulted in an average two-fold increase in the amount of virus recovered compared to that obtained when homogenization was not used. The addition of 0.1% "Igepon T73" to the citrate buffer used to resuspend the pelleted virus resulted in destruction of the virus. This detergent has been used as a dispersing agent for barley stripe mosaic virus (2). The infectivity of ONMV was better preserved by resuspending it in 0.1 M borate buffer, pH 8.4, than in citrate buffer (9). But when this buffer was tried as a solvent for the sucrose density gradients, the amount of virus recovered decreased to one-half to one-fifth of that obtained when citrate buffer was used as the solvent. Profiles in the ISCO density-gradient analyzer showed that the amounts of impurities in virus preparations centrifuged in the No. 40 rotor through citrate buffer containing 30% sucrose and 4% PEG were usually much smaller than in virus centrifuged through the same volume of citrate buffer containing 5% sucrose. The amount of virus recovered was either unaffected or only slightly reduced.

Yields of virus obtained by the complete purification procedure from source material inoculated in December to February (a favorable period for virus propagation) ranged from 1.7 to 3.2 mg per kg of leaves. These preparations at a concentration of 1 mg virus/ml did not react to healthy-oat antiserum in microprecipitin tests. When the final step of agarose-gel filtration was omitted from the purification, dilution end points of the virus preparations averaged about 1/8 for healthy-oat antiserum diluted 1/2. Tests in two trials indicated that virus losses of 46% and 33% resulted when the preparation was passed through the agarose gel column and then reconcentrated by centrifugation.

The ultraviolet absorption spectrum of the virus was typical of that for nucleoproteins, with a maximum at 259 nm and a minimum at 247 nm. The average 280:260 and maximum:minimum ratios (uncorrected for light

scattering) were 0.75 (range 0.73 to 0.77), and 1.06 (range 1.01 to 1.09), respectively. A prominent shoulder occurred at about 290 nm.

Antiserum properties determined by microprecipitin tests.—The antiserum titer for each rabbit determined with 0.25 mg of virus/ml was 1/512 4 weeks after the first injection of virus, and it reached a maximum of 1/4,096 at 7 weeks. Results were the same whether the antigen in the tests was virus or virus treated with 0.2% formaldehyde solution. Immunoprecipitates were amorphous and a pale bluish-gray in color. In grid titrations with antiserum of 1/4,096 titer the virus end point in several experiments varied from 1/2,048 (0.49 μg virus/ml) to 1/8,192 (0.12 μg virus/ml). The antiserum reacted to healthy oat concentrate with a titer of 1/16 and a healthy host antigen end point of 1/4.

Partly clarified sap was used to absorb the virus antiserum because the volume of healthy oat concentrate required to eliminate host antibodies was twice that of clarified sap. Absorbed antiserum did not react to healthy oat concentrate, but absorption resulted in a decrease of antiserum titer from 1/4,096 to 1/64 in one trial and to 1/128 in another. When the absorbed antiserum was reconcentrated two-fold with ammonium sulphate, titers in two trials were 1/256 and 1/512. When reconcentrated five-fold with PEG, titers were 1/4,096 and 1/2,048 in two other trials and from 0.12 to 0.24 μg of virus could be detected. The PEG method of antiserum reconcentration was better than the ammonium sulphate method for eliminating colored impurities of the added sap from the serum. Alpha optimal reacting proportions for virus and reconcentrated, absorbed antiserum were, 0.5 mg virus/ml with 1/64 antiserum dilution, and 0.25 mg virus/ml with 1/128 antiserum dilution. Similar proportions were obtained by visual estimations of the amounts of precipitate in microprecipitin grid titrations.

No reactions were observed when serial dilutions of ONMV were tested against the undiluted antisera to RMV, AMV, HMV, MDMV, or SCMV. With WSMV antiserum, however, a virus dilution end point and an antiserum titer each of 1/8 was obtained. The immunoprecipitates were similar in appearance to those between ONMV and its antiserum. The end point with ONMV and the homologous, reconcentrated, absorbed antiserum was 1/2,048. None of the antisera reacted to healthy oat concentrate.

Detection of ONMV in infected plants and partly purified virus preparations.—In microprecipitin tests with reconcentrated, absorbed antiserum (titer 1/2,048), virus was detectable in sap that had been extracted from infected leaves with a pestle and mortar and then clarified with silver nitrate. Virus dilution end points and antiserum titers were each 1/16. Unclarified sap from infected leaves and sap that had been clarified with chloroform were unstable and spurious precipitates rapidly occluded the immunoprecipitates. With the usual virus purification procedure, clarified sap and the concentrate prepared with the No. 30 rotor (concentrated virus) had virus dilution end points of 1/16 and 1/512, respectively, and antiserum titers of 1/32 and >1/32. No reactions occurred with similarly treated preparations from healthy oats.

Ouchterlony double-diffusion tests with absorbed ONMV antiserum, reconcentrated either with ammonium sulphate or PEG, gave no immunodiffusion lines with pure or partly purified whole virus preparations. A weak reaction resulted with degraded ONMV near the antiserum well in normal agar and high pH-ammonia agar plates. With unabsorbed antiserum and either purified virus or concentrated virus in 0.5% normal agar plates, a sharply defined line occurred within 2 days close to the antigen well. With unabsorbed antiserum and high pH-ammonia agar plates or normal agar plates with SDS reagent added to the antigens, results were erratic and, at best, reaction lines were of low intensity and often diffuse. A broad virus antigen line near the antiserum well was usually observed with purified ONMV and degraded ONMV. This line together with a host antigen line, sometimes poorly separated from each other, were observed with some of the impure virus preparations. Plates with normal agar in citrate buffer, pH 6.3, produced results superior to agar in citrate buffer, pH 8.0, or in phosphate buffer, pH 7.0, 7.5, or 8.0. No lines resulted with agar in water or normal saline. Lines were more distinct with agar at a concentration of 1% than with agar of lower concentrations.

DISCUSSION

Yields of purified virus from oats were low. The advantage of a two-fold increase in virus yield, obtained with the homogenization step, was to some degree nullified by the extraction of larger amounts of host components, presumably from organelles such as the chloroplasts. Thus, with homogenization, a larger volume of silver nitrate solution was required for clarification and a green coloration was sometimes difficult to remove from the virus preparation. The agarose gel filtration was a significant factor in removing impurities in the final stages of purification, but virus losses with this step were high. The value of 0.75 obtained as the 280:260 ultraviolet absorption ratio for the virus was low compared to many rod-shaped viruses, but was close to the value of 0.73 for WSMV (4).

The reaction between WSMV antiserum and ONMV suggested a distant relationship between the two viruses, although the reciprocal test has yet to be applied. Distant serological relationships also have been found between WSMV and AMV, and between AMV and HMV (15). Wheat streak mosaic virus and AMV, and another virus of grasses, RMV, are transmitted by eriophyid mites (6). All, including ONMV, are mechanically transmissible, and none has been transmitted by aphids or other vectors. Particle lengths are in the approximate range of 683 to 720 nm (15, 16, 19) and the viruses induce the formation of cylindrical inclusions in infected cells (16). Therefore, although a vector of ONMV has not been found, it seems that ONMV, together with WSMV, AMV, HMV, and RMV, constitute a taxonomic group. These viruses have been included in the PVY group (6), but a case might also be made for their segregation into a separate group or subgroup on the basis of vector, particle morphology and length, and possibly also, serological relations.

The virus is a good antigen since only about 4.9 mg produced an antiserum with a titer of 1/4,096. Formaldehyde, which is known to stabilize viruses (7),

did not increase the antiserum titer when virus treated with this chemical was injected into the rabbit. The optimum reacting proportions of the virus and its antiserum were close to those for WSMV and its antiserum (3). Oat necrotic mottle virus could be satisfactorily detected in clarified sap from greenhouse-grown, infected oats with reconcentrated, absorbed antiserum and the microprecipitin test. Naturally-infected oats in the field, however, may have a lower level of virus, particularly if symptoms are at the necrotic stage. In this case, oats grown in a greenhouse and inoculated with sap from the field plants might be required as virus-source material. Grid titrations with the virus indicated that the antiserum diluted 1/8 could be used satisfactorily for this test.

The reason for the failure of the reconcentrated, absorbed antiserum to react in agar-gel double-diffusion tests with ONMV treated with SDS reagent or in high-pH ammonia plates is not known. A similar absence of visible reactions in agar-gel tests has been reported (17) with absorbed pea seed-borne mosaic virus (PSbMV) antiserum and PSbMV treated with virus-degrading agents. The erratic results or the poor resolution of the lines obtained with the unabsorbed ONMV antiserum, and high pH-ammonia agar plates, or normal agar plates with SDS reagent added to the antigens, indicated that these methods could not be used with confidence to detect the presence of ONMV in infected preparations, except possibly when clarified with silver nitrate. These tests have shown, however, that degradation of ONMV by certain agents produces small molecular weight entities, probably viral protein, that still retain antigenicity, and are able to diffuse rapidly in agar.

LITERATURE CITED

1. BALL, E. M. 1974. Serological tests for the identification of plant viruses. The American Phytopathological Society, St. Paul, Minnesota. 31 p.
2. BRAKKE, M. K. 1959. Dispersion of aggregated barley stripe mosaic virus by detergents. Virology 9:506-521.
3. BRAKKE, M. K., and E. M. BALL. 1968. Purification and antigenicity of wheat streak mosaic virus. Phytopathology 58:963-971.
4. BRAKKE, M. K., and N. VAN PELT. 1968. Photometric scanning of a centrifuged gradient column at several wavelengths. Anal. Biochem. 26:242-250.
5. BRAKKE, M. K., and N. VAN PELT. 1970. Properties of infectious ribonucleic acid from wheat streak mosaic virus. Virology 42:699-706.
6. EDWARDSON, J. R. 1974. Some properties of the potato virus Y-group. Fla. Agric. Exp. Stn. Monogr. 4. 398 p.
7. FRANCKI, R. I. B., and N. HABILI. 1972. Stabilization of capsid structure and enhancement of immunogenicity of cucumber mosaic virus (Q Strain) by formaldehyde. Virology 48:309-315.
8. GILL, C. C. 1967. Oat necrotic mottle, a new virus disease in Manitoba. Phytopathology 57:302-307.
9. GILL, C. C. 1971. Purification of oat necrotic mottle virus with silver nitrate as clarifying agent. J. Gen. Virol. 12:259-270.
10. GILL, C. C. 1974. Inclusions and wall deposits in cells of plants infected with oat necrotic mottle virus. Can. J. Bot. 52:621-626.
11. KNESEK, J. E., G. I. MINK, and R. O. HAMPTON. 1974. Purification and properties of pea seed-borne mosaic virus. Phytopathology 64:1076-1081.
12. LANGENBERG, W. G., and E. M. BALL. 1972. High pH-ammonia agar immunodiffusion for plant viruses. Phytopathology 62:1214-1217.
13. MATTHEWS, R. E. F. 1957. Page 43 *in* Plant virus serology. Cambridge University Press. Cambridge, England. 128 p.
14. PURICIFULL, D. E. 1966. Some properties of tobacco etch virus and its alkaline degradation products. Virology 29:8-14.
15. SLYKHUIS, J. T., and W. BELL. 1966. Differentiation of Agropyron mosaic, wheat streak mosaic, and a hitherto unrecognized Hordeum mosaic virus in Canada. Can. J. Bot. 44:1191-1208.
16. SLYKHUIS, J. T., and Y. C. PALIWAL. 1972. Ryegrass mosaic virus. No. 86 *in* Descriptions of plant viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol.; Kew, Surrey, England.
17. STEVENSON, W. R., and D. J. HAGEDORN. 1973. Antiserum preparation and serodiagnosis of pea seed-borne mosaic virus. Phytopathology 63:1467-1469.
18. WILLIAMS, C. A., and M. W. CHASE. 1967. Methods in immunology and immunochemistry. Vol. I. Academic Press, New York. 319 p.
19. WOLF, P., and K. SCHMELZER. 1973. Polyethylene glycol as a means for improving titers of antisera against plant viruses. Acta Phytopathol. Acad. Sci. Hung. 8:351-352.