Application of a Serological Screening Test for Detecting Double-Stranded RNA Mycoviruses

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ABSTRACT

Antisera to synthetic double-stranded ribonucleotides were specific for double-stranded RNA (ds-RNA) and also detected it specifically in phenol extracts of virus-like particles (VLP's) from *Penicillium chrysogenum* ATCC 9480 or in phenol extracts from mycelium. When the antisera were used to test phenol extracts from 70 selected fungal isolates, 20 isolates reacted positively. Some, though not all, of the positive reactions were correlated with the presence of VLP's in clarified buffer extracts from mycelia, and to ds-RNA detected in phenol extracts by polyacrylamide gel electrophoresis or sucrose density-gradient centrifugation. The utility of ds-RNA antisera as a simple and sensitive aid in screening fungi for ds-RNA mycoviruses, and for assaying their concentration in extracts, is discussed.

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Additional key words: mycoviruses, ds-RNA antisera.

Conventional serological and infectivity tests cannot be used in screening fungi for virus-like particles (VLP's or "mycoviruses"). Serological relatedness of distinct mycoviruses is known in only a few instances (1), and there is no known indicator host which can be readily infected with a wide range of mycoviruses.

Current methods for detecting them involve electron microscope searches for VLP's in concentrated extracts of mycelial homogenates, or in thin-sections of mycelium or spores (2, 6). However, VLP's may be difficult to detect against a background of host components, or they may be difficult to isolate intact.

Double-stranded RNA (ds-RNA) is characteristic of most mycoviruses (1) and in preliminary work (13) antisera specific for ds-RNA were successfully used to detect ds-RNA mycoviruses in four fungal cultures known to contain them. We report here on further application of this technique in a screening program involving 70 fungal isolates selected from various culture collections. Electron microscopy, polyacrylamide gel electrophoresis, and sucrose density-gradient analysis were used to check representative results of the serological tests. An abstract of this work has appeared elsewhere (14).

MATERIALS AND METHODS. — *Serology.* — Antisera to ds-RNA were made against complexes of methylated bovine serum albumen and the synthetic ds-RNA's polyinosinic:polycytidylic acid (polyI:polyC) and polyadenylic:polyduridylic acid (polyA:polyU) as described by Francki and Jackson (5). Antiserum specificity was tested with the following antigens: polyA:polyU; polyI:polyC; polyA; polyU; polyI; polyC; polyG (Miles Laboratories, 1127 Myrtle, Elkhart, IN 46514, all with minimum molecular weights of 100,000); *Escherichia coli* ribosomal RNA; calf thymus DNA, all at 1 mg/ml; RNA prepared from tobacco mosaic virus (TMV) by the method of Diener and Schneider (4); and nucleic acids prepared from mycelium and VLP's of *Penicillium* spp. (13). For the screening program, nucleic acids from mycelium of the fungi tested as unknown were prepared as described below. Serological screening tests and tests of antiserum specificity and titers were done by two-dimensional immunodiffusion at 25 C (13).

Culture of fungi.—*Penicillium chrysogenum* Thom (ATCC 9480) containing VLP's, and VLP-free NRRL 813 were cultured as described earlier (13). Fungal isolates tested as "unknowns" (Table 1) were cultured similarly, in potato-dextrose broth, for 4-7 days.

Virus isolation and purification.—VLP's were isolated and partially purified from cultures of *P. chrysogenum* as described previously (13) by precipitation with polyethylene glycol (PEG, M.W. 6,000) from chloroform-clarified mycelium extracts, and final concentration by cycles of differential ultracentrifugation. With fungi tested as unknowns, the PEG-precipitation step was omitted, and the extracts were concentrated by two cycles of differential ultracentrifugation.

Nucleic acid extraction.—RNA was extracted from partially purified VLP's by the single-phase phenol-ethanol method of Diener and Schneider (4). Nucleic acids were extracted from mycelium by homogenization in phenol as described earlier (13) except that the phenol homogenate was passed through a French press (French Pressure Cell, American Instrument Co., 8030 Georgia Av., Silver Spring, MD 20910), which increased the yields of serologically detectable ds-RNA (see Results). Nucleic acids were concentrated by precipitation with cold ethanol and resuspended in STM buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.01 M $MgCl_2$, pH 7.3), at 1 ml STM/10 g starting with filter-dried mycelium.

Polyacrylamide gel electrophoresis.—Electrophoresis

TABLE 1. Results of screening fungal isolates for ds-RNA mycoviruses, using serology and polyacrylamide gel electrophoresis (PAGE) to detect ds-RNA, and electron microscopy to detect virus-like particles (VLP's)

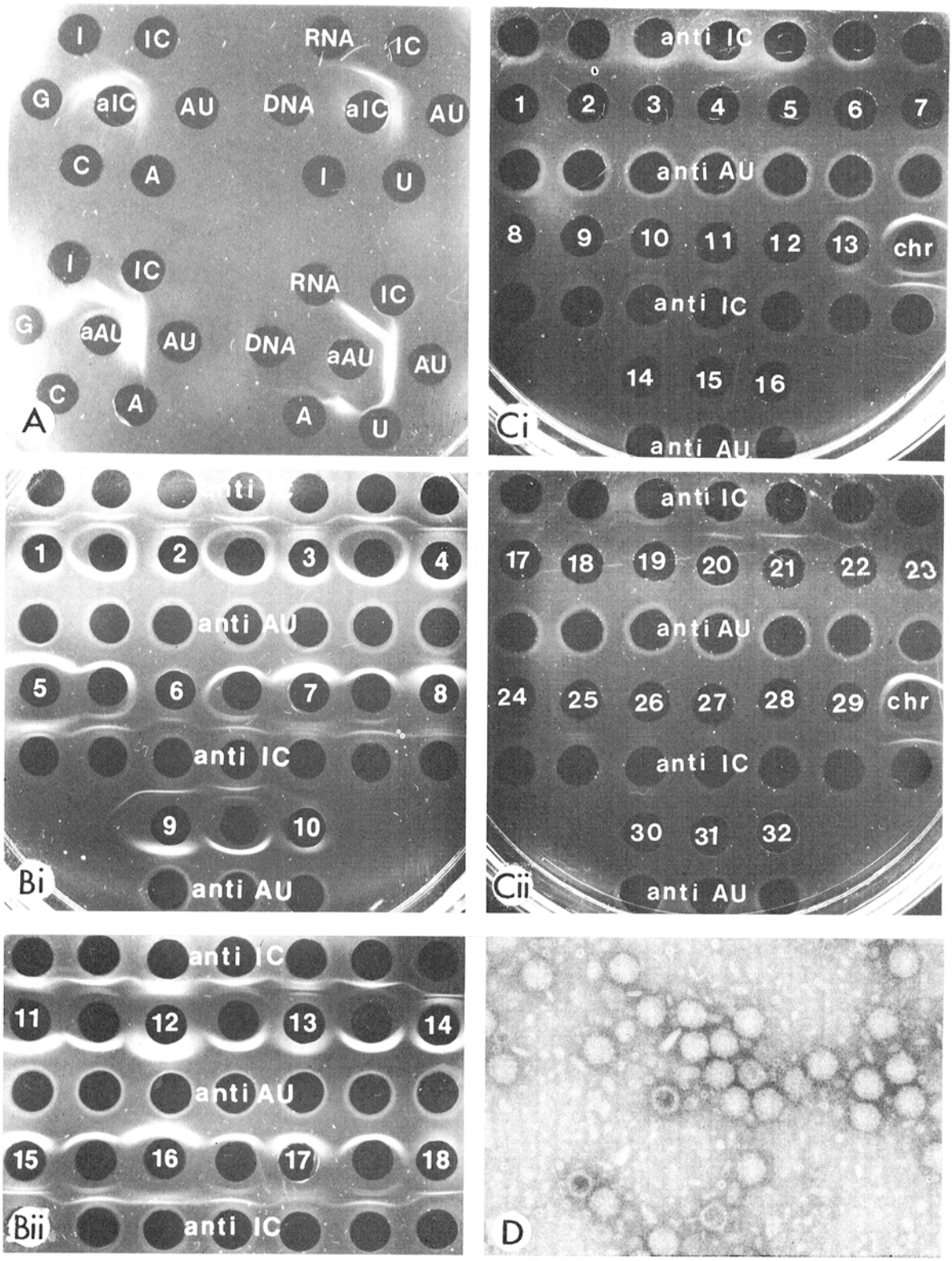
Fungal isolate	Culture number or source	Reason for screening	ds-RNA detected by		
			Serology[h]	PAGE[h]	VLP's detected[h]
Penicillium funiculosum	RC 2[a]	cultural characteristics	+	–	–
P. funiculosum	RC 20[a]	cultural characteristics	–		–
P. funiculosum	RC 29[a]	cultural characteristics	–	–	–
P. oxalicum	RC 4[a]	cultural characteristics	–	–	–
P. oxalicum	RC 17[a]	cultural characteristics	–	–	
P. oxalicum	RC 13[a]	cultural characteristics	–		
P. cyclopium	RC 19[a]	cultural characteristics	–		–
P. cyclopium	RC 2[a]	cultural characteristics	–		
P. cyclopium	RC 15[a]	cultural characteristics	+[g]	–	–
P. viridicatum	RC 4[a]	typical isolate	–		
P. viridicatum	RC 32[a] (ATCC 18411)	ochratoxin production	–		
P. viridicatum	RC 33[a]	no ochratoxin	–		
P. viridicatum	RC 41[a]	no ochratoxin	–		
P. viridicatum	RC 38[a]	citrinum production	–		
P. variable	RC 7[a]	typical isolate	–		
P. brevi-compactum	RC 3[a]	typical isolate	–		
P. brevi-compactum	RC 18[a]	cultural appearance	–		
P. brevi-compactum	RC 19[a]	cultural appearance	–		
P. citrinum	RC 4[a]	citrinum production	–		
Aspergillus ochraceus	RC 1[a]	ochratoxin production	–	–	–
A. ustus	RC 1[a]	typical isolate	–		
A. parasiticus	2221[b]	aflatoxin production	–		
A. parasiticus	15517[b]	aflatoxin production	–		
A. parasiticus	11906[b]	no aflatoxin	–		

Table 1 (continued)

Fungal isolate	Culture number or source	Reason for screening	ds-RNA detected by Serology[h]	PAGE[h]	VLP's detected[h]
A. parasiticus	20245[b]	no aflatoxin	−		
A. flavus	18166[b]	aflatoxin production	−		
A. flavus	15546[b]	aflatoxin production	−		
A. flavus	10124[b]	no aflatoxin	−		
Helminthosporium sp.	Dodd 537[c]	cultural appearance	+		−
H. maydis race O	8780 9A#1[c]	typical isolate	−		
H. maydis race O	(Florida)[c]	slow growing	+	+	+
H. maydis race T	(Union Co.)[c]	typical isolate	−		
H. ravenellii	136[c]	typical isolate	−		
H. pedicellatum	RT 50[c]	typical isolate	−		
H. pedicellatum	135[c]	typical isolate	−		
H. carbonum race I	1972[c]	typical isolate	+		+
H. carbonum race I	+ tester[c]	typical isolate	+		+
H. carbonum race I	1966[c]	typical isolate	+		+
H. carbonum race I	− tester[c]	typical isolate	−	−	−
H. carbonum race I	1965 (143)[c]	typical isolate	+	−	−
H. carbonum race I	1964 (141)[c]	typical isolate	+		−
H. carbonum race II	1959 (51)[c]	typical isolate	−		
H. carbonum race II	1963 (75)[c]	typical isolate	+[g]		−
H. carbonum race II	1958 (20)[c]	typical isolate	−		−
H. turcicum	(corn) 72[c]	typical isolate	+		−
H. turcicum	(Johnsongrass)[c]	typical isolate	+		−
H. turcicum	1973	typical isolate	+		−
Colletotrichum graminicola	Miss.[c]	pathogenic on sorghum	+		−
C. graminicola	Princeton, Indiana[c]	pathogenic on corn	−		−
C. graminicola	N.C. #2-1[c]	pathogenic on corn	−		
C. graminicola	2 Texas[c]	pathogenic on sorghum	−		
C. coccoides	MC[c]	weak pathogen on tomato	−		
C. falcatum	Miss.[c]	pathogenic on sugar cane	+[g]		
Gibberella zeae[c]		pathogenic on corn	−		
Curvularia lunata[c]		pathogenic on corn	−		
Glomerella gossypii	41[c]	pathogenic on cotton	−		
Phyllosticta maydis	160[c]	pathogenic on corn	−		
Kabatiella zeae	168[c]	pathogenic on corn	−		
Pythium butleri	171[c]	pathogenic on corn	+		−
Fusarium roseum[d]		cultural appearance	+[g]		
Ophiobolus graminis	3[d]	low pathogenicity	+	+	+
O. graminis	2[d]	high pathogenicity	−		−
Endothia parasitica	1502 Michigan[e]	high pathogenicity	−	−	−
E. parasitica	1503 France[e]	low pathogenicity	+	−	−
E. parasitica	1504 France[e]	high pathogenicity	−		−
E. parasitica	1505 France[e]	low pathogenicity	+	−	−
Neurospora crassa	ATCC24560	slow-growing mutant, VLP's	−		
N. crassa	FGSE1578[f]	"poky" mutant, VLP's	−		

[a]From R. Caldwell, Purdue Univ., West Lafayette, Indiana.
[b]From G. A. Bean, Univ. of Maryland, College Park.
[c]From A. J. Ullstrup, Purdue Univ., West Lafayette, Indiana.
[d]From D. M. Huber, Purdue Univ., West Lafayette, Indiana.
[e]From K. J. Kessler, USDA Forest Service, St. Paul, Minnesota.
[f]From R. W. Tuveson, Univ. of Illinois, Urbana.
[g]Positive serological test with anti polyI:polyC only.
[h]No entry means no test was done.

of nucleic acids was according to Loening (12), on 2.4% gels at 25 C for 6 hours at 6 mA per tube, with 0.2% sodium dodecyl sulfate in the running buffer, which was 36 mM Tris-Cl, 30 mM Na-acetate, 1 mM Na_2 EDTA, pH 7.7. Samples were in STM buffer + 10% sucrose, and they contained 0.2-0.5 A_{260} pure VLP RNA, or 0.5-10 A_{260}



mycelial RNA. Gels were scanned at 254 nm with a Joyce-Loebl densitometer and recorder.

Ribonuclease sensitivity.—VLP and *E. coli* RNA were tested for ribonuclease (RNase) sensitivity in SSC buffer (0.15 M NaCl, 0.015 M Na-citrate, pH 7.2) and also in 0.1 × SSC, by incubating mixtures of 1 A_{260} unit of nucleic acid and 10 μg of pancreatic ribonuclease I per ml, at 37 C for 4 minutes.

Similarly mycelial nucleic acid preparations (0.2 ml of the final products in STM) were treated by incubating at 27 C for 90 minutes with RNase (0.05 ml of a solution containing 20 mg/ml in water) and bovine pancreatic deoxyribonuclease I (DNase) (0.05 ml of a solution containing 80 Kunitz units per ml of 1 M $MgSO_4$).

Sucrose density-gradient analysis.—Rate zonal density-gradient analysis of partially purified VLP's was in 5-20% sucrose gradients made in 0.1 M potassium phosphate buffer, pH 7, in Spinco SW 41 tubes. Centrifugation was at 39,000 rpm for 45-90 minutes at 10 C. The tube contents were analyzed at 254 nm in an ISCO density-gradient fractionator and ultraviolet (UV) analyzer (3).

Electron microscopy.—Preparations were negatively stained with 1% phosphotungstate at pH 7 on carbon-coated formvar membranes, and examined in a Philips EM200 electron microscope. Particle images were measured on enlarged photographs with a magnifying comparator (11).

RESULTS.—*Characteristics of antisera to ds-RNA.*—Antisera to polyI:polyC reacted in gel diffusion tests with both polyI:polyC and polyA:polyU. Similarly, antisera to polyA:polyU reacted with both synthetic double-stranded ribonucleotides. Typically, multiple precipitin lines were observed, and in arrangements involving homologous and heterologous reactions against a common antiserum well, spur formation occurred with antisera to polyA:polyU (Fig. 1-A). Neither type of antiserum reacted with *E. coli* ribosomal RNA, TMV-RNA, RNA from a VLP-free *P. chrysogenum* culture, calf thymus DNA, or with the synthetic single-stranded polynucleotides polyI, polyA, polyC, polyG, or polyU. These results confirmed specificity for RNA double-strandedness. Interestingly, however, in arrangements where the appropriate single-stranded polyribonucleotides were placed in adjacent wells, reactions sometimes occurred in positions where annealing could have been expected (Fig. 1-A).

Minimum detectable amounts of synthetic ds-polynucleotides, as measured by titration in gel diffusion plates, were:

polyA:polyU antiserum:	10 μg polyA:polyU/ml
	4 μg polyI:polyC/ml
polyI:polyC antiserum:	10 μg polyA:polyU/ml
	4 μg polyI:polyC/ml

Precipitin lines were most intense after 24 hours, after which spreading and fading occurred. The antisera to polyI:polyC consistently gave the clearest and sharpest lines.

As little as 0.5 μg ds-RNA per ml was detectable in ring precipitin tests.

Properties of ds-RNA from Penicillium chrysogenum VLP's.—RNA from partially purified *P. chrysogenum* VLP's reacted in gel diffusion tests with antisera to polyI:polyC and polyA:polyU. Ribonuclease treatment did not affect serological activity, nor did repeated freezing and thawing. The RNA migrated slower than *E. coli* rRNA when electrophoresed on 2.4% polyacrylamide gels, forming three barely resolvable, but characteristic, peaks (cf. Fig. 2-A). These three components withstood pancreatic ribonuclease at 5 μg/ml under conditions which completely digested single-stranded *E. coli* rRNA. Preparations made by the same procedures using the VLP-free *P. chrysogenum* (NRRL 813) as starting material yielded no UV-absorbing peaks.

Properties of ds-RNA extracted directly from Penicillium chrysogenum mycelium.—Serology, polyacrylamide gel electrophoresis, and enzyme sensitivity showed that some of the nucleic acid extracted by phenol treatment from *P. chrysogenum* (ATCC 9480) [but not from *P. chrysogenum* (NRRL 813)] mycelium was ds-RNA similar to that characteristic of partially purified VLP's. In some experiments, resolution of the three electrophoretic components was impaired by the presence of other nucleic acids. These were readily

Fig. 1[A, B (i and ii), C (i and ii), D]. A) Gel diffusion tests showing specificity of antisera made against ds-RNA. Antisera made against either polyI:polyC (aIC) or polyA:polyU (aAU) are in center wells. Antigens tested: polyI (=I), polyI:polyC (−IC), polyA:polyU (=AU), polyA (=A), polyC (=C), polyG (=G), polyU (=U), calf thymus DNA, and *Escherichia coli* ribosomal RNA, all at 1 mg/ml. **Bi and Bii)** Selected serological tests indicating presence of ds-RNA in fungal isolates screened as "unknowns". Antisera are in rows marked "anti IC" and "anti AU". Nucleic acids from fungal mycelia are in numbered wells; *Penicillium chrysogenum* VLP ds-RNA is in alternating unmarked wells. Unknowns: 1) *Helminthosporium carbonum* race I, 141; 2) *H. carbonum* race I, + tester; 3) *H. carbonum* race I, 1974; 4) *H. carbonum* race I, 143; 5) *H. carbonum* race I, 4; 6) *H. carbonum* race II, 75; 7) *H. turcicum,* Johnson grass; 8) *H. turcicum,* 1972; 9) *H. turcicum,* 1973; 10) *Helminthosporium* sp.; 11) *H. maydis* race O, slow-growing; 12) *Pythium butleri;* 13) *Colletotrichum graminicolum,* Miss.; 14) *Endothia parasitica* 1503; 15) *E. parasitica* 1505; 16) *Ophiobolus graminis* 3; 17) *Pencillium funiculosum* RC 2; 18) *P. cyclopium* RC 15. All indexed positive with one or both antisera. **Ci and Cii)** Selected serological tests mostly indicating absence of ds-RNA in fungal isolates screened as "unknowns". Antisera are in rows marked "anti IC" and "anti AU". Nucleic acids from fungal mycelia are in numbered wells; "chr" = *P. chrysogenum* VLP ds-RNA. 1) *Penicillium viridicatum* RC 4; 2) *P. brevi-compactum* RC 18; 3) *P. citrinum* RC 4; 4) *P. variable* RC 7; 5) *Aspergillus ustus* RC 1; 6) *Helminthosporium maydis* race O, 8780 9A#1; 7) *H. ravenellii* 136; 8) *H. pedicellatum* RT50; 9) *H. maydis* race T, Union County; 10) *Colletotrichum graminicolum;* 11) *Gibberella zeae;* 12) *Curvularia lunata;* 13) *Glomerella gossypii;* 14) *Neurospora crassa ATCC24560; 15) N. crassa* FGSC1578; 16) *Colletotrichum graminicolum;* 17) *Phyllosticta;* 18) *Kabatiella zeae;* 19) *Colletotrichum coccoides;* 20) *Fusarium roseum;* 21) *Colletotrichum falcatum;* 22) *H. carbonum* race II, 51; 23) *H. carbonum* race II, 20; 24) *Ophiobolus graminis* 2; 25) *Endothia parasitica* 1502; 26) *E. parasitica* 1504; 27) *Aspergillus flavus* 10124; 28) *A. flavus* 18166; 29) *A. flavus* 15546; 30) *A. parasiticus* 11906; 31) *A. parasiticus* 2221; 32) *A. parasiticus* 15517. Isolates 20 and 21 reacted weakly with anti IC only. **D)** VLP's from hypovirulent isolate of *Ophiobolus graminis.*

removed by treatment with RNase and DNase. Similar treatment of control material from the VLP-free *P. chrysogenum* isolate confirmed that the contaminating nucleic acids included normal single-stranded RNA (ss-RNA) and DNA (Fig. 2-A).

Direct phenol extraction of mycelium, rather than of VLP's purified from mycelium, appeared to be a more efficient route for preparing antigenic ds-RNA. Thus, in a typical experiment (Table 2) in which equal weights of mycelium were processed using the two routes of extraction, serological endpoints indicated that direct extraction from mycelium was up to eight times more efficient. Possibly, however, these calculations are misleading, because visualization of the serological endpoints of mycelial ds-RNA may be enhanced by impurities that co-precipitated with the ds-RNA:antibody complex.

In earlier tests (13), phenol homogenates had not been passed through a French press. In the present study, use of a French press improved antigen yields, but drastically

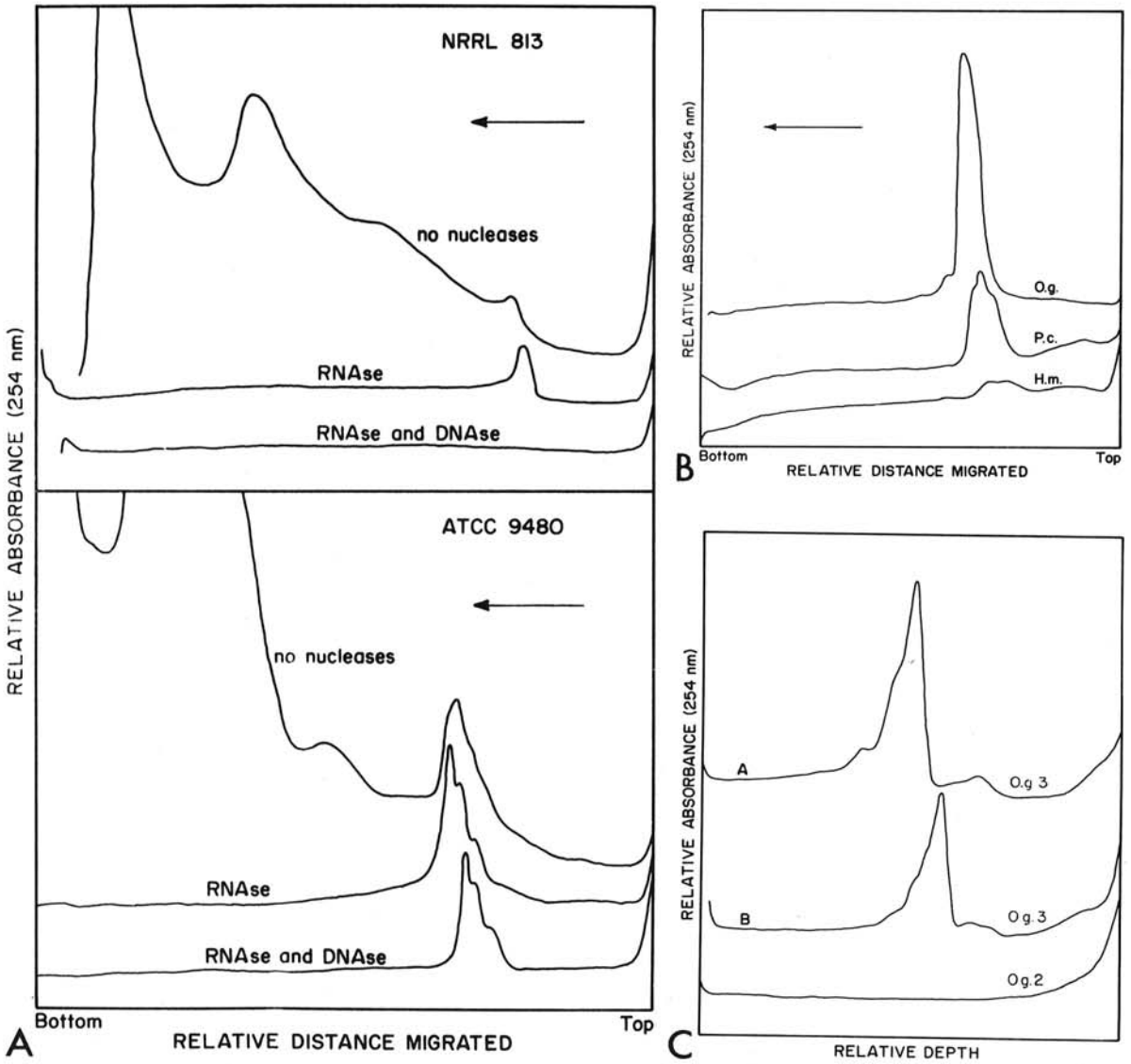


Fig. 2-(A to C). **A)** Ultraviolet absorbance profiles of polyacrylamide gel electrophoresis of nucleic acid from mycelium of VLP-containing (ATCC 9480) or VLP-free (NRRL 813) *Penicillium chrysogenum* with or without ribonuclease (RNase) and deoxyribonuclease (DNase). Electrophoresis was in 2.4% gels for 6 hours at 6 mA per gel. **B)** Ultraviolet absorbance profiles of polyacrylamide gel electrophoresis of nucleic acids from "unknowns" and VLP-containing *P. chrysogenum* (P.c.); O.g., nucleic acid from VLP's of *Ophiobolus graminis;* and H.m., nuclease-treated nucleic acid from mycelium of a slow-growing isolate of *Helminthosporium maydis* race O. Electrophoresis was in 2.4% gels for 6 hours at 6 mA per gel. **C)** Ultraviolet absorbance profiles of sucrose density-gradient centrifugations of VLP's from hypovirulent *Ophiobolus graminis* (O.g. 3), and a concentrated, chloroform–clarified extract of virulent *O. graminis* (O.g. 2). Centrifugation was for 50 minutes (A) and 40 minutes (B) at 39,000 rpm in a Spinco SW41 rotor.

reduced the proportion of nucleic acid resolvable as the typical three-component electrophoretic peak of VLP ds-RNA (Table 2). Presumably, shearing in the press caused fragmentation of ds-RNA into small pieces which still retained antigenicity. Small sequences of about five nucleotide pairs may be adequate to form a binding site for antibodies to ds-RNA (15).

Application of serological test in screening program.—The serological reaction lines in Fig. 1, Bi and ii, and Ci and ii are typical results of the screening test. Reactions with anti polyI:polyC were more sharply defined than the reactions with anti polyA:polyU, and they sometimes consisted of several parallel precipitin lines. In a few cases, reactions with anti polyA:polyU were negative or very doubtful when reactions with anti polyI:polyC appeared to be positive (e.g., Nos. 20, 21; Fig. 1, Cii). Nucleic acids from VLP-infected *P. chrysogenum* mycelium were included in tests as checks, and as a basis for scoring unknowns as positive or negative. Results of the screening tests are listed in Table 1. Significant results were obtained for:

—1) *Penicillia.*—Of 19 isolates chosen for their unusual cultural appearance or toxin production, two reacted weakly in serological tests.

—2) *Aspergillus.*—All seven *A. flavus* and *A. parasiticus* isolates, whether toxin producers or not, were negative for ds-RNA. No VLP's have been observed in spores or mycelia of these isolates (G. A. Bean, *personal communication*).

—3) *Helminthosporium.*—Cultures tested were representative virulent cultures isolated in the past 15 years in this department. Some isolates of *H. carbonum* race I, *H. turcicum,* and a slow-growing *H. maydis* race O isolate reacted positively. One isolate of *H. carbonum* race II only reacted weakly.

—4) *Ophiobolus graminis.*—Of two isolates tested, one (avirulent) isolate reacted positively; the virulent isolate was negative.

—5) *Endothia parasitica.*—Two isolates which were not pathogenic were strongly positive for ds-RNA (Nos. 14 and 15 in Fig. 1, Bii); two wild-type, virulent isolates were negative, except that one gave an extremely weak reaction, judged to be nonspecific, against anti polyI:polyC (No. 25 in Fig. 1, Cii).

—6) *Neurospora crassa.*—Ds-RNA was not detected in *N. crassa* isolates in which VLP's had been reported (17). Recently a different isolate of *N. crassa* was found to contain ss-RNA VLP's (9).

Comparison of screening tests with electron microscopy and analysis by sucrose density-gradient ultracentrifugation and polyacrylamide gel electrophoresis.—Representative cultures that reacted negatively or positively in the serological screening tests were examined for VLP's by electron microscopy of chloroform-clarified ultracentrifugal concentrates of buffer extracts. Also, some mycelial nucleic acid preparations were analyzed by polyacrylamide gel electrophoresis after nuclease treatments, and by rate-zonal sucrose density-gradient centrifugation. The results (Table 1) sometimes confirmed the results of the screening test, but sometimes they did not, viz.:

—1) *Helminthosporium.*—The slow-growing isolate of *H. maydis* race O contained a nucleic acid component, in both the mycelial nucleic acid extracts and in phenol-treated chloroform extracts, which migrated electrophoretically as fast as *P. chrysogenum* VLP ds-RNA (Fig. 2-B). Phenol-treated chloroform extracts reacted with the ds-RNA antisera, but no UV-absorbing bands in positions appropriate for VLP's were evident in sucrose density-gradient analysis of the concentrated chloroform-clarified extracts. However, a few VLP's were observed in electron micrographs.

Similarly, four of five *H. carbonum* race I isolates which reacted serologically contained VLP's, but only a few were observed, and for the representative one tested (141, Table 1), neither nucleoprotein bands nor nucleic acid components were resolved in chloroform or phenol extracts. We infer that VLP's occurring in these isolates were not readily concentrated by the procedures used.

—2) *Ophiobolus.*—UV profiles of samples ultracentrifuged in sucrose density gradients suggested three or possibly four VLP components in the avirulent

TABLE 2. Yield of double-stranded RNA extracted from virus-like particle (VLP)-infected *Penicillium chrysogenum* mycelium or from purified *P. chrysogenum* VLP's

Method of ds-RNA preparation[a]	Source	A_{260}[b]	Reciprocal serological dilution endpoint[c]	Minimal reacting absorbance[d]	ds-RNA[e] (%)	ds-RNA[f] (%)	Specific antigen yield (per gram mycelium)[g]
Waring Blendor	mycelium	122	128	0.95	17	34	13
Waring Blendor and French press	mycelium	232	256	0.91	18	5	25
Phenol extraction	purified virus	5.4	32	0.17	100	100	3

[a]Twenty grams of mycelium were homogenized in phenol and the homogenate was divided for passage or no passage through a French press. Another 10 g of mycelium was homogenized in chloroform for virus purification; purified virus was then extracted in phenol.

[b]Final nucleic acid product dissolved in 1 ml STM (0.1 M NaCl, 0.01 M Tris-HCl, 0.01 M $MgCl_2$, pH 7.3) and used for dilution series.

[c]Reciprocal of greatest dilution of RNA preparation which still reacts against antiserum to polyI:polyC.

[d]A_{260} of undiluted sample × serological dilution endpoint.

[e]Calculated from minimal reacting absorbance, assuming RNA from purified virus is 100% ds-RNA.

[f]Determined from scans of polyacrylamide gel electrophoresis.

[g]A_{260}/ minimal reacting absorbance per gram of mycelium.

isolate of *O. graminis* (Fig. 2-C). Electron microscopy of unfractionated preparations (Fig. 1-D) revealed VLP's with diameters ranging from 25 to 33 nm. Many of the smaller particles were penetrated by stain. Nucleic acids prepared by phenol extraction of these VLP's reacted with ds-RNA antiserum, migrated slowly during polyacrylamide gel electrophoresis, and were resistant to RNase and DNase (Fig. 2-B). There was no resolution of separate ds-RNA species in these tests. Preparations of the VLP's did not react with antisera to VLP's of *P. chrysogenum* or *P. stoloniferum*.

—3) *Endothia*.—For unknown reasons, precipitation occurred when nucleic acid samples treated with nucleases were layered on polyacrylamide gels and electrophoresed; hence, even though these tests gave no evidence of ds-RNA, they were inconclusive. However, electron microscopy and sucrose density-gradient analysis gave no evidence of VLP's in any of the four isolates.

DISCUSSION.—Even though mycoviruses with nucleic acids other than ds-RNA have recently been reported (7, 8, 9), serological tests as described here should be widely applicable in screening fungi for ds-RNA mycoviruses. The validity of such tests is predicated on the specificity and sensitivity of the antisera with respect to ds-RNA. In this regard, our antisera did not react with synthetic or naturally occurring ss-RNA or DNA, unlike some others reported (10, 16). Further, we obtained no reactions in tests with several fungal isolates known to be virus-free, indicating that the antisera did not react spuriously with normal host components or with chemicals used in the extraction process.

We cannot account for the differences in specificity between our ds-RNA and those of others, unless the source of the polynucleotide duplex used is critical. But with our antisera, ds-RNA in the mixture of nucleic acids extractable from mycelium should be reliably detectable if concentrated sufficiently, although the results suggest that it may be desirable to confirm results obtained with anti polyI:polyC by testing also with anti polyA:polyU.

Direct extraction of ds-RNA from mycelium rather than purified VLP's has the advantage of simplicity, and it is also likely to be more efficient, avoiding losses during VLP purification, especially where procedures for optimum VLP yield have not been worked out. Use of ds-RNA antisera would also provide a convenient assay for ds-RNA in the preparatory fractions obtained when developing purification procedures for ds-RNA VLP's.

Serological detection should be more sensitive than electron microscopy of tissue extracts or thin-sections, because it bypasses VLP extraction and viewing difficulties. Loss of VLP's during extraction and clarification could account for our failure to confirm all positive serological tests by electron microscopy and density-gradient analysis. However, since unencapsulated pathogenic RNA's are well known in plants, it is also reasonable to suspect that pathogenic "free" ds-RNA's i.e., nonencapsidated ds-RNA's, can occur in fungi. These would probably be detectable using ds-RNA antisera, but not by electron microscopy. Possibly, *E. parasitica* may be an example of this, for we detected ds-RNA in hypo-virulent isolates in which neither we nor others (K. J. Kessler, *personal communication*) have found VLP's after extensive electron microscopy.

The results suggest that ds-RNA viruses, or perhaps "free" ds-RNA, are widespread among fungi, for ds-RNA was detected in over 20% of the fungal isolates screened. This value compares with Bozarth's (1) estimate of VLP's in 10-15% of randomly sampled fungi observed by electron microscopy.

Because it is difficult to infect VLP-free fungi with VLP's, understanding the effects of mycoviruses currently depends mainly on showing an association between then and modified fungal pathogenicity or biochemistry—a task involving extensive screening of cultures. Serological testing with ds-RNA antisera could simplify the work involved.

Although our tests were not extensive enough for definite conclusions, they do not conflict with the notion that fungal virulence may in some cases be modified by mycovirus infection. Avirulent, but not virulent, isolates of *E. parasitica* and *O. graminis* were positive for ds-RNA, whereas some virulent isolates of *Helminthosporium* were positive for ds-RNA. Reduced pathogenicity would be expected where mycovirus infection reduces fungal vigor: increased pathogenicity could reflect increased toxin production where this is a crucial factor in pathogenicity. However, the tests also suggest that toxin production in the Aspergilli and Penicillia (Table 1) is not related to the presence of VLP's, a conclusion in agreement with work of G. A. Bean and G. A. Sansing *(personal communication)* who found no VLP's in these and similar isolates.

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