Properties of Germination Inhibitors from Stem Rust Uredospores

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Supported by funds from the Graduate School, University of Wisconsin, and by Grants GB-1900 and GB-8098 from the National Science Foundation.

The technical assistance of Barbara Houser and Frances Hutchison is gratefully acknowledged, and we thank Mary Frances Lena and Ron Stroshane for help in growing rust uredospores.

Accepted for publication 28 June 1971.

ABSTRACT

Inhibitor can be extracted from wheat stem rust uredospores with water but not with dry organic solvents. A preliminary extraction of the spores with ether aids in purification. The inhibitor partitions from aqueous solutions into ether at a pH of 8.5 or below, but not at 12.5. It is more stable in ether than in aqueous solution, and inactivates rapidly at higher pH values. Purification was facilitated by partitioning a water extract into ether, evaporating the ether, and reextracting the residue with water. The most active preparations gave 95% inhibition at concentrations of 10-25 ng/ml. Chromatography on cellulose with aqueous solvents resolved the inhibitor into two components, with R_f values slightly lower than those of the two components of bean rust inhibitor. In most chromatographic systems, only one component appeared. Ultraviolet absorption by the inhibitor was indicated, but was not clearly defined because of evidence of impurities even in the most active preparation. Fluorescence at inciting wavelengths of 254 and 366 nm was either weak or lacking. Evidence suggests that the inhibitors are phenolic compounds which are interconvertible, and that they are closely related to, but not identical with, the inhibitors from bean rust uredospores.

Phytopathology 61: 1382-1389.

Additional key words: Puccinia graminis var. *tritici,* self-inhibitors.

Germination of rust uredospores is reversibly inhibited by endogenous inhibitors carried by the spores and released along with many other soluble constituents when the spores are floated on water (3,4,6,15,16,18,23,24,30). Self-inhibition has also been reported in the spores of many other kinds of fungi (11,13,19,25), and the agents responsible are of potentially broad interest as factors in the onset and maintenance of dormancy in fungus spores (26). They may also be related to the germination-inhibiting substances from mycelium studied by Garrett & Robinson (14), Robinson & co-workers (20,21,22) and by Carlile & Sellin (7). Knowledge of the chemical nature and biological action of these agents may also be of great significance in practical problems of disease control, particularly because, as shown in this report, they are active at extremely low concentrations.

Preliminary studies of self-inhibition in the uredospores of rust fungi showed that aqueous extracts of the spores contained inhibitor activity and gave some indications of the properties of the active material (1). Later studies with other species showed that uredospores of most rust fungi are self-inhibited in large populations, and release inhibitor when in contact with aqueous solution (6,15,16,24). The amounts of inhibitor activity and the extent of cross-activity with spores of other species vary considerably (6,16,19,24). Separation of the inhibitory material of *Uromyces phaseoli* from other components by partitioning the aqueous extract into ether was reported by Bell & Daly (6). Paper chromatography showed that at least two different substances were active. Glutamic and aspartic acids, previously reported to be the endogenous inhibitors in this species (29), were definitely excluded from that role by the demonstration that they were inactive in neutral solution. Macko et al. (17) recently presented convincing evidence that the inhibitors of bean rust are the *cis-* and *trans*-isomers of methyl-3,4-dimethoxycinnamate. They inhibit germination 50% at 5 ng/ml. Trimethylethylene, suggested to be the inhibitor in uredospores of *Puccinia graminis* (9), is disqualified because the concentration required to inhibit is more than 10^4 times higher than that of the natural inhibitors (6,17). Acetaldehyde is produced by uredospores and is inhibitory (10), as are other short, straight-chain aldehydes, but the active concentrations are above 10 μg/ml. *N*-nonanoic acid inhibits germination at concentrations of several μg/ml, whereas the homologous aldehyde, *n*-nonanal, stimulates germination (12). The active concentrations of all known endogenous inhibitors except those from bean rust uredospores are therefore relatively high in comparison with many regulatory agents in both plants and animals.

The present paper reports studies on the separation and properties of substances responsible for the self-inhibition of germination in the uredospores of the wheat stem rust fungus. The initial procedures followed were intended to provide maximum assurance that the compounds studied were those responsible for the phenomenon of self-inhibition. Toward this end, the amount and the quality of activity were related to those of the original preparations. Such precautions are necessary because varied substances of biological origin can act as germination inhibitors, and because of the natural occurrence of counter-

acting substances in uredospores (2,27).

MATERIALS AND METHODS.—*Culture and handling of uredospores.*—Uredospores of *Puccinia graminis* Pers. var. *tritici* Eriks. & E. Henn. race 56 were used both as a source of inhibitor and for assay. They were grown in the greenhouse on *Triticum aestivum* L. 'Little Club'. Wheat seed and some supplies of the inoculum were kindly provided by John Rowell and William Bushnell, respectively, Cooperative Rust Research Laboratory, St. Paul, Minn. Wheat plants grown in loam soil were inoculated either by injecting 0.1 ml of an aqueous suspension of spores in 0.1% Triton WR-1339 (Ruger Chemical Company) between the culm and the sheath at the flag-leaf stage in shoot development, or by spraying the leaves in the seedling stage with an oil suspension of spores. For the latter, plants growing in 7-inch pots were watered 10 days after planting with 100 ml of a 0.1% (w/v) solution of the diethanolamine salt of maleic hydrazide and inoculated 2 days later by spraying with a suspension of 20 mg of uredospores/ml of Mobilsol 100 (Standard Oil of New Jersey) or Soltrol 170 (Phillips Petroleum Company). About 2 ml of the suspension was used to inoculate seedlings in 10 pots. In some cases, the seedlings were sprayed immediately afterwards with 10^{-4} *M n*-nonyl alcohol, a germination stimulant which increased the intensity of infection. Immediately following inoculation, the pots were placed in a high-humidity chamber at 20 C and left overnight. The humidity was maintained with a fine spray. Seedlings were returned to the greenhouse bench the following morning.

Spores were harvested by shaking them onto aluminum foil, then stored in vials at 6 C. Only those showing 50% germination or more were used for assays. Germination seldom remained above this level in spores more than 2 months old. Inhibitor was usually extracted within a few months after harvest, but spore age was not an important factor in recovery.

Aqueous solutions were assayed by adjusting to pH 7.0 with phosphate, placing in the center well of a micro-Conway diffusion vessel, and making up to a final volume of 0.5 ml and a final concentration of 0.01 *M* potassium phosphate buffer at pH 7.0 and 0.0001 *M* $Ca(H_2PO_4)_2$ (Ca/K[Calk] buffer). Although assays can also be done on distilled water, the buffered test solutions were used exclusively in this study. Solutions in ether or other organic solvents were evaporated in the center wells, and the residues then dissolved in buffer. Inhibitor concentrations were recorded in terms of mg spore equivalents; i.e., the weight of spores from which the contents of 1 ml of an assay sample were derived, or in terms of the weight of inhibitor-containing oil or volume of solution. Before transfer to the assay vessel, about 0.1 mg spores was spread evenly over the surface of 5 ml buffer solution in a 10-ml beaker and prefloated for 5 min, thus removing some of the endogenous inhibitor. One loopful of spores (ca. 2,000) was then transferred to each Conway vessel with a clean platinum loop. The vessels were sealed using petrolatum and a glass cover, and incubated in the dark at 18 C for 90 min. All germination counts were made directly in the vessels.

Inhibitor activity was estimated by measuring the degree of inhibition at a single concentration of each test sample, or a quantitative assay was carried out with five or more concentrations of each sample. In the second procedure, the per cent inhibition was calculated in relation to internal controls, and the probit of the per cent inhibition (probit of $\frac{\%\text{ germination in control} - \%\text{ in test}}{\%\text{ in control}} \times 100$) plotted as a function of the logarithm of sample concentration (Fig. 1). The level required for 95% inhibition was calculated from the intercept of the line with the probit of 95% inhibition (6.64). A unit of inhibitor is defined as the amount present in 1 ml test solution which gives 95% inhibition under the conditions of assay described. About one-third to one-fifth of this concentration was required for 50% inhibition. The number of inhibitor units present in a μg of dry inhibitor-containing preparation is referred to as the "specific activity" of the preparation, and indicates its degree of purity.

Procedures for the recovery of inhibitor.—Inhibitor was obtained from the spores by extraction with water. Spores were either floated on the surface or were placed at the rate of 500 mg/150 ml Ca/K buffer in each of several 500-ml Erlenmeyer flasks and shaken on a reciprocal shaker for 4-6 hr. Most work, however, was based on direct extraction of the spores with Ca/K buffer at pH 7.0 over a period of a few minutes only.

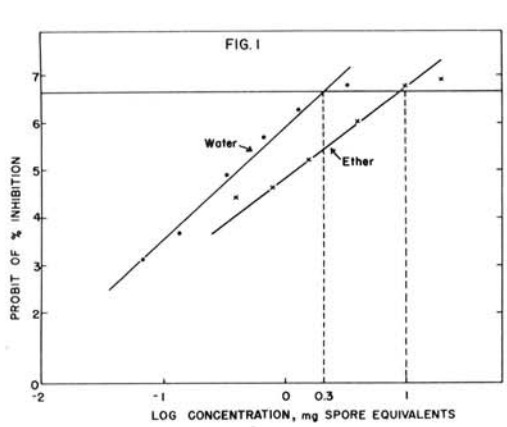
Uredospores were broken wet by grinding with glass beads in a Potter-Elvehjem homogenizer (Erway Glass Blowing, Oregon, Wis.) following the procedure of Williams & Ledingham (28). For dry breakage, a mixture of 0.2 g spores and 0.4 g No. 130 Superbrite glass beads (3M Co., St. Paul, Minn.) was vigorously ground in a mortar for 2-3 min, then extracted with 10 ml of water or ether as required. Breakage in all experiments was high but incomplete (75-90%).

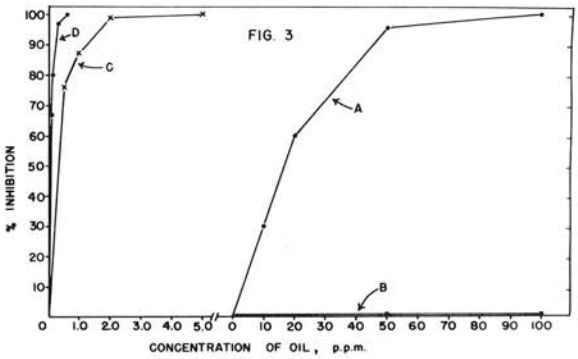
Inhibitor was transferred from aqueous solution to ether as quickly as possible. The ether solutions were stored at −20 C.

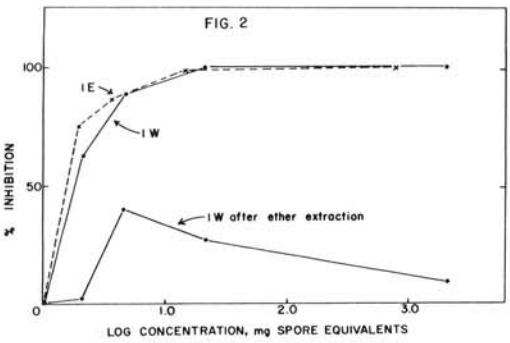
Solvent preparation.—Diethyl ether was redistilled over ferrous sulfate and used for all original extractions of spores or of inhibitor-containing solutions. Other solvents were treated as indicated in the text.

Chromatographic procedures.—Paper to be used for chromatography was first extracted with 50% methanol and/or pure ether to remove germination stimulants (5).Thin-layer plates were spread 0.25 mm thick with an applicator, using MN-cellulose powder 300 G or MN-Silica Gel G (Brinkman), and the silica gel plates were activated at 105 C for 30 min. Both cellulose and silica gel plates were washed in methanol-chloroform (1:1, v/v) and dried in the open. Silica gel plates were then stored in a desiccator until used. Chemically pure-grade solvents were used for developing chromatograms, but were not redistilled.

For column chromatography, Whatman Chro-







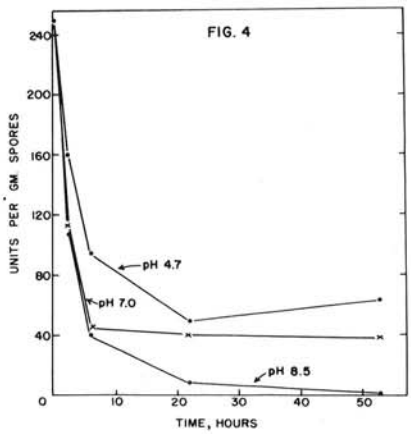


Fig. 1-4. Effect of various treatments on inhibitor activity. **1)** Examples of the assay procedure. Left curve represents the aqueous extract of spores (crude inhibitor) assayed without treatment; right curve represents the inhibitor recovered by partitioning that preparation with ether. **2)** Distribution of inhibitory activity between the aqueous and ether phase at pH 7.0 1W = original aqueous extract of spores; 1E = ether phase after partitioning with one volume of ether and two of aqueous extract. The bottom curve shows the activity of 1W after ether extraction. **3)** Inhibitor activity of the oil recovered from ether extracts. A = ether extract of crude inhibitor; B = ether extract of intact spores; C = ether extract of crude inhibitor recovered from ether-extracted spores; D = oil from C purified by extraction with a small volume of water, and the inhibitor partitioned back into ether. There is a 200-fold increase in specific activity between A and D. **4)** Effect of pH on the decay of inhibitor activity in aqueous solution. A Ca/K buffer extract of spores was made at pH 7.0, and partitioned with ether. The ether solution was dried and the residue dissolved in water and assayed for zero time content of inhibitor. The remaining water solution was divided into three batches diluted with the appropriate mixture to give pH levels of 4.7, 7.0, and 8.5, respectively, all at the same concentration. Samples of each were periodically removed, neutralized if necessary, and assayed immediately without an intermediate extraction with ether.

media cellulose powder, grade CF-11, was employed. Packed columns were washed with 10-column volumes of 50% methanol followed by a similar quantity of water or of 0.01 *M* phosphate buffer (without Ca^{++}) at the pH to be used for chromatography. The eluent emerging at the end of this wash had no significant effect on germination. Samples were applied in aqueous solution, and the fractions of eluate tested as soon as possible. The hold-up volume was measured by eluting a sample of KCl into silver nitrate acidified with H_3PO_4.

Detection of compounds separated by chromatography.—To locate the inhibitor after chromatography, the filter paper or thin layer of cellulose was divided into segments, each segment was transferred to a separate tube and extracted for 2-24 hr with 2-10 ml ether, and the resulting solutions were used for bioassays. Papers were also examined with long (366 nm) and short (254 nm) wavelength ultraviolet (UV), sometimes after exposure to NH_3 vapor to enhance fluorescence, and were sprayed with various reagents to detect reactive compounds.

The activity of column fractions was determined by bioassay of their ether extracts. Alkaline eluates were acidified to pH 6.7 immediately upon elution and before ether extraction.

Absorption properties of the fractions were determined with a Beckman DU spectrophotometer, or with a Gilford recording spectrophotometer.

For calculating concentrations, or determining specific activity, samples were dried to constant weight as determined with an analytical balance.

RESULTS.—*Preliminary experiments on production and recovery of inhibitor.*—Crude aqueous inhibitor (1W) prepared by extracting spores at pH 7.0 with Ca/K buffer was partitioned with ether in a separatory funnel, using two volumes of aqueous extract to one of ether. Most of the inhibitor moved

TABLE 1. Comparison of germination inhibitor yields from *Puccinia graminis* spores with different extraction procedures and spore concentrations

Method of extraction[a]	Spore conc, g/100 ml	Inhibitor yield, units/mg spores in	
		Aqueous extract (1W)	Ether extract (1E)
Floating	2.0	0.25	0.13
Suspended			
without breaking	10.0	0.70	0.50
without breaking	30.0	1.25	0.75
with 80% breakage	10.0	None detected	0.50

[a] Tests were made with different batches of spores, and the initial extraction was with 0.01 *M* Ca/K buffer, pH 7.0.

into the ether phase (1E) (Fig. 2). Recombining the materials present in the aqueous and ether phases in their original proportions gave nearly the same inhibition as the original extracts.

The transfer of inhibitor to ether from neutral or acid solutions gave yields varying from 50 to over 100%. Sufficient inhibitor was recovered to account for the self-inhibition. Most of the inhibitor was released from spores immediately upon contact with water. It was found that detergent was not necessary, and that mechanical suspension in a Potter-Elvehjem homogenizer without breakage of spores permitted good and rapid recovery of inhibitor. If the spores were broken, more substances were released which interfered with detection of inhibitor in the aqueous extract, but the inhibitor was found in a subsequent ether extract in yields comparable with those from unbroken spores. An example of comparative yields from different extraction methods is presented in Table 1. Good recovery was obtained by mechanical suspension with spore concentrations as high as 30 g/100 ml.

When 1E solutions were evaporated at 34 C, a residue of light yellow oil containing the inhibitor activity was obtained (Fig. 3-A). This crude product gave 95% inhibition at concentrations ranging from about 10-50 μg/ml.

Attempts to extract the inhibitor with ether directly from dry spores were not successful. Although considerable amounts of oil and wax were extracted, they were inactive (Fig. 3-B) even at concentrations as high as 500 μg/ml (containing the extract from 50 mg spores in 1 ml test solution). The inhibitor was recovered in good yields after an ether extraction of the spores if they were dried and re-extracted with buffer. Thus preliminary extraction with ether removed some of the inert lipids which constitute about 20% of the spore carbon (8); and the residual oil recovered from a subsequent water extract had a higher specific activity (Fig. 3-C) than that recovered from unextracted spores (Fig. 3-A). Other organic solvents, including acetone and hexane, also failed to extract the inhibitor from dry spores, but a partial recovery was obtained with 75% ethanol/25% water (v/v).

Enrichment was also achieved by extracting, with a small volume of water, the oil obtained after evaporating the ether from 1E solutions. The inhibitor was dissolved, leaving behind most of the less water-soluble inactive components of the oil. If inactive materials were first removed from the spores with ether, this procedure yielded a product with a specific activity as high as 10 (i.e., 95% inhibition by 0.10 μg/ml) (Fig 3-D). The highest activity ever obtained was shown by a preparation made by a somewhat different procedure. 1E was evaporated to dryness, followed by ether extraction of the residue. Only part of the inhibitor was dissolved in the ether, and the remainder was subsequently extracted from the flask with water after removal of the ether solution. The water extract was then transferred to ether, dried, weighed, and assayed. This procedure gave low yields but high specific activity, up to 100 (95% inhibition at 0.01 μg/ml).

Since inhibitor could not be extracted from intact spores with ether, attempts were made to extract it from broken spores. Dry spores were ruptured by grinding in a mortar with glass beads, then extracted. No significant activity was found in the ether extract. Partial purification and assay of the ether extracts showed less than one-tenth as much activity in the ether extract as in the water extract of dry broken spores.

Inhibitor loss into the volatile fraction was not evident when ether solutions of inhibitor were distilled. Something stimulating germination was, however, recovered in the final 10% of the ether condensate. Contamination with stimulants would lead to underestimation of the amount of inhibitor partitioned into ether from the first water extract. In most experiments, however, the stimulants were probably removed upon evaporation of the solvent from the micro-Conways, if not already lost at an earlier stage. In terms of total activity, far less stimulant than inhibitor was recovered.

Germination of the spores in the presence of the stimulant *n*-nonyl alcohol at 10^{-4} *M* did not prevent the subsequent recovery of inhibitor from the incubation water.

Stability.—Inhibitor preparations stored at 6 C often lost part of their activity, particularly in aqueous solutions, but frequently also in ether solution. Storage at −20 C did not prevent losses. The most stable preparations were ether solutions free of water and with low content of other contaminating compounds from the spores. Losses in activity almost

always accompanied manipulations of the inhibitor, with occasional exceptions in the original transfer from water to ether (Fig. 2). Quantitative recovery in subsequent treatments was seldom obtained.

The loss from aqueous solutions was most rapid in the first few hours after preparation (Fig. 4), and tended to be greater when high activity was found in the original extract. At first, activity dropped off at all pH levels, but after the first few hours further losses occurred only at an alkaline reaction. At pH 8.5, most activity was lost within 1 day, whereas at pH 12.5, inactivation was essentially complete in a few minutes. When transferred to ether and stored, either at 23 C or at −20 C, no detectable loss in activity occurred over a period of 7 days. Initial activity in the ether solution had, however, been reduced during the additional processing to about the same level as that reached after storage in neutral aqueous solution.

Good, but not maximum, yields of inhibitor (about 100 units/g spores) were recovered from the filtrate of boiled suspensions of spores, and also from water extracts boiled after removal of spores. The inhibitor was not destroyed by mild oxidizing or reducing agents. Incubation with H_2O_2 (2%), sodium thiosulfate, sodium hydrosulfite, potassium ferricyanide, or sodium thioglycollate (all at 10^{-2} *M*) did not destroy or increase activity. Paper chromatography of the product obtained after incubation with sodium hydrosulfite gave the same distribution of activity as the untreated inhibitor.

Effect of pH on recovery and partitioning.—Mildly acid or alkaline conditions made little difference in the release of inhibitor from the spores, in the partitioning of inhibitor from water into ether, or in its re-extraction by water from dried oil preparations. No definitive differences in behavior were observed over a range from pH 4.7 to 8.7. With aqueous solutions at very high pH, however, partitioning of the inhibitor into the ether phase did not occur. Because of rapid inactivation at pH 12.5, separation and neutralization of the aqueous phase was carried out as quickly as possible (less than 1 min). In a typical partitioning experiment, all of the activity recovered (60% of the original) was found in the ether phase at pH 8.3, whereas it was all in the aqueous phase at pH 12.5 (35% of the original).

Separation of inhibitors by chromatography.—Separation and recovery of activity on paper or thin layers of cellulose with 0.01 *M* phosphate buffer at pH 7.0 revealed two components (Fig. 5-A), one with R_f about 0.40 to 0.45 (component I), the other with R_f 0.2 to 0.25 (component II). Development with alkaline phosphate (pH 8.3) or acid phosphate (pH 4.7) also showed two components, but their chromatographic behavior and relative intensities varied slightly (Fig. 5-B, C). Development with distilled water gave similar results, but the two components were better resolved and the R_f values of both were somewhat higher (0.3 and 0.55, respectively (Fig. 6,7). Several other solvent systems were tested, but organic solvents generally did not clearly resolve the inhibitor into two components. Associated with the

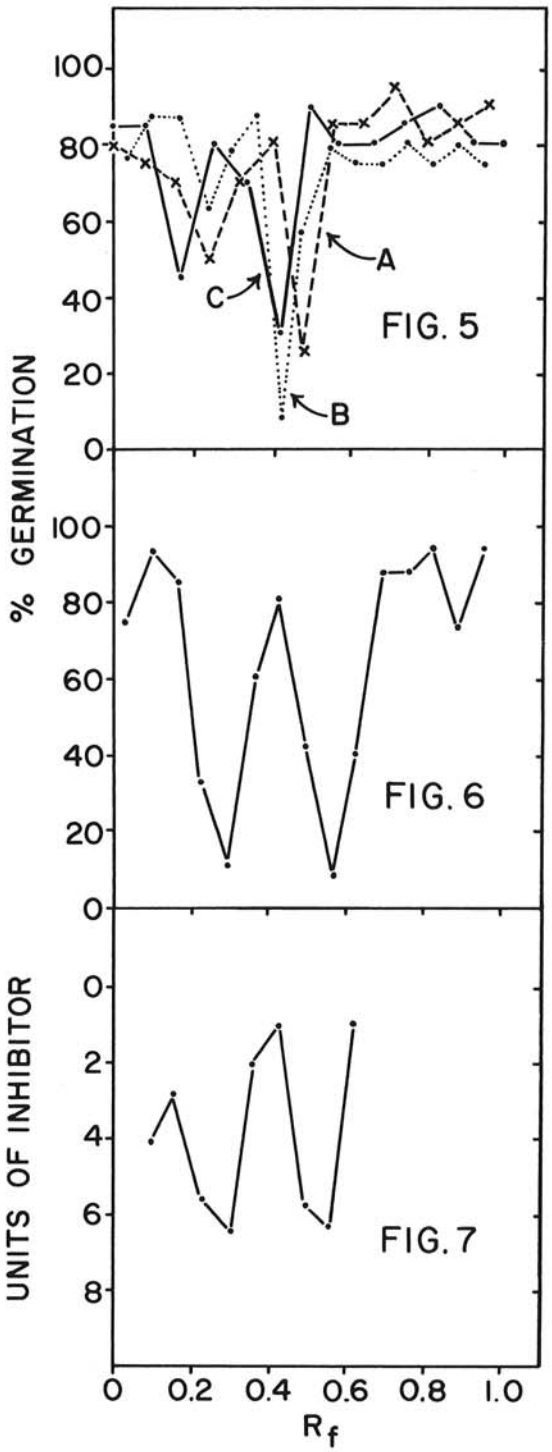


Fig. 5-7. Separation of components I and II on thin layer cellulose or paper chromatograms developed with aqueous solvents. **5)** Whole extracts developed with phosphate buffer: A at pH 7.0 (4.5 units); B at pH 8.3 (6 units); C at 4.7 (4.5 units). **6)** Cellulose column zone from 1.4 hold-up volumes developed with distilled water. **7)** Cellulose column zone from 1.1 hold-up volumes, developed with distilled water. Data represent distribution of inhibitor determined by assay of successive segments of paper.

inhibitor zone in some runs was a faint blue fluorescence (at 366 nm) which turned yellow green on exposure to NH_3 vapors. This spot was not, however, always detectable at the locus of inhibitor, possibly because fluorescence was too weak to manifest itself with the quantities of inhibitor usually present. An inactive contaminant can not be completely discounted as the source of this fluorescence.

Column chromatography with aqueous solvents sometimes (but not always) resulted in separation of the inhibitor into two zones. With 0.01 *M* phosphate buffer at pH 8.0, the two zones peaked after elution of 1.1 to 1.2 times and 1.4 times the holdup volume, respectively. A typical pattern of elution is shown for phosphate buffer at pH 8.0 in Fig. 8. The late-eluting peak did not always appear, but the early eluting peak did appear and had most of the activity. Losses were always sustained during column chromatography, with yields usually not exceeding 50%. The recovery was as high at pH 8.0 as at 6.0, but resolution was better at the higher pH.

Attempts to identify the two column peaks with

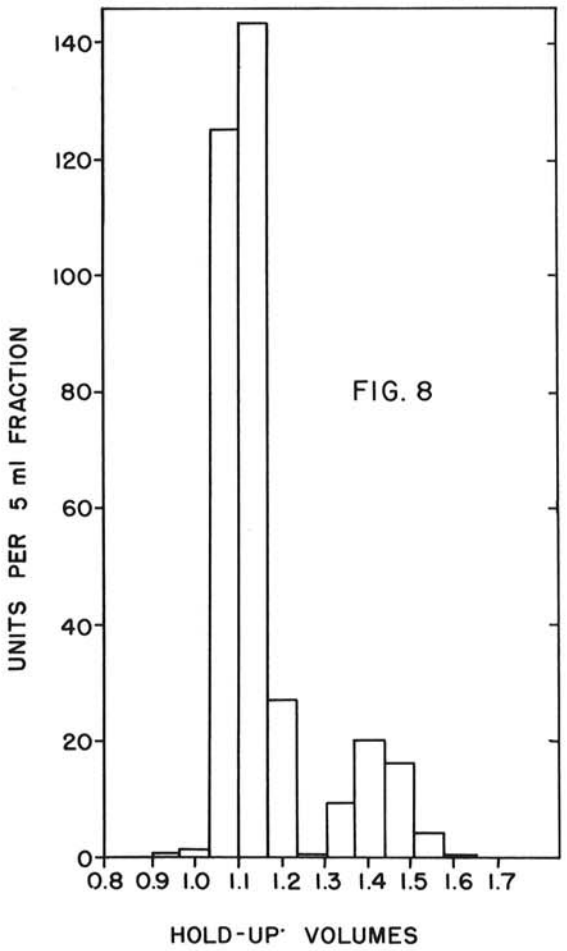


Fig. 8. Separation of two inhibitor zones on cellulose column. Elution with 0.01 *M* potassium phosphate buffer, pH 8.0. Samples acidified to pH 6.7 with monobasic phosphate and extracted with ether for assay.

inhibitor spots on paper chromatograms gave equivocal results. In most tests, fractions taken from either peak gave both component I and II when rechromatographed on cellulose. The front peak, however, tended to be low in component II, and in some cases the leading edge of the first peak was nearly free of this component. The inhibitor from the late emerging peak always showed both components when rechromatographed on paper with phosphate buffer. Both components also appeared when chromatograms were developed with distilled water (Fig. 6, 7). Characteristically, the R_f values were slightly higher than with phosphate buffer.

Paper chromatography with phosphate buffer of the products recovered in ether at pH 8.7 and in the aqueous phase at 12.5 indicated that both consisted of the same two components as originally present.

On thin layer plates of silica gel, separation of the inhibitor from some accompanying contaminants was achieved with organic solvents in some instances. With benzene:methanol:acetic acid (45:8:4 v/v) as developing solvent, inhibitor was recovered, usually at R_f 0.6-0.8. The same single zone was obtained with the original ether extract and with material from either of the two peaks eluted from cellulose columns with phosphate buffer.

Ultraviolet absorption and fluorescence.—The ether-soluble fraction of aqueous extracts showed strong absorption in the UV, including a peak at about 270-280 nm. The material responsible for this absorption remained in the oil after water extraction (Fig. 9-A), although most of the inhibitor was extracted by the water. In the water extract, absorption in this region was nevertheless reduced more sharply than at other wavelengths (Fig. 9-B). Neither of the two fractions recovered after elution from cellulose columns had much absorption above 250 nm. Even the purest preparations, however, showed

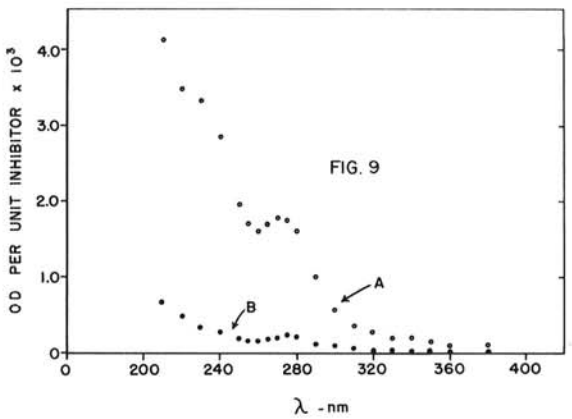


Fig. 9. Absorption curves for impure and purified inhibitor dissolved in ethanol. Optical densities (OD) per unit of inhibitor are given for 25 μg of oil containing 45 units of inhibitor and dissolved in 3.0 ml ethanol (A) and 18 μg of oil containing 260 units in the same solvent volume (B). A = the first ether extract of aqueous inhibitor after evaporation of ether and extraction with water. B = the water solubles after two additional cycles of extracting with ether and dissolving the residue in water.

absorption below 250 nm. Absorption in this region has not been dissociated from the inhibitor, but it is clear that contaminants with strong absorption at 210 to 280 nm are present and account for most of the absorption in crude inhibitor preparations (Fig. 9). Absorbing spots were not consistently detectable at the locus of inhibitor activity on paper chromatograms with UV radiation at 254 or 366 nm.

Fluorescence at 254 and 366 nm, sometimes but not always associated with inhibitor activity on chromatograms, was not visually detectable in purified inhibitor solutions.

Other tests for inhibitor.—Chemical tests revealed many spots on paper and silica gel chromatograms, but in no case was there a consistent correlation between the presence of inhibitor and a color reaction. The tests employed included alkaline $FeCl_3$, diazotized benzidine, diazotized *p*-nitroaniline, and bromination.

DISCUSSION.—The quantity of spores required to give an inhibitory aqueous extract was of the same order of magnitude as the quantity of spores which produced self-inhibition on the same volume of liquid (1-5 mg/ml for 95% inhibition). The inhibitor recovery upon partitioning into ether was frequently 100% or more, the higher yields probably reflecting removal of interfering substances. As purification progressed, there was generally a loss of total activity, but the specific activity, an indicator of enrichment in inhibitor, increased greatly. Preparations with the highest activity showed 95% inhibition at concentrations as low as 10-25 ng/ml. By our definition of the unit, this material would have 40-100 units inhibitor/μg oil. Since preparations with this level of activity were evidently not completely pure, 10 ng/ml represents an upper level for the concentration of pure inhibitor giving 95% inhibition. The material is therefore similar in specific activity to the bean rust inhibitor, which inhibits by 50% at 5 ng/ml (17). Inhibition at these levels eliminates the compounds previously suggested as representing the self-inhibitor (9), as well as all the inhibitory compounds such as ferulic acid, *p*-hydroxybenzoic acid, and *o*-coumaric acid isolated from uredospores by Van Sumere et al., but not inhibiting by 95% until concentrations above 100 μg/ml were used (27). Obviously the agents involved in self-inhibition are active at levels comparable with those of hormones in other biological systems.

Since the yields of purified preparations did not account for the total inhibitor present in the original ether extract, the purified preparations may not represent the full complement of inhibitors extracted from the spores. There is some evidence that inhibitory compounds may undergo changes during processing, and there may be preferential losses of some components.

Fluorescing, UV-absorbing, and color-producing (with diazotized benzidine or $FeCl_3$) substances with the same R_f as the inhibitor were found after chromatography, but most of them were clearly not the inhibitor, as further chromatography separated visually detectable spots from the locus of biological activity. Although inhibitor extracts sometimes contain colored compounds, these are probably inactive constituents, as enriched preparations are colorless.

In contrast with these preparations of wheat stem rust inhibitor, the bean rust inhibitor has several distinct absorption peaks in the UV (17). Since the absorption data for *Puccinia graminis* still refer to impure preparations, no definite conclusions are warranted concerning the absorption by the inhibitor. Conclusive evidence concerning the absorption properties must depend upon greater assurance of purity and stronger evidence from bioassay that most of the original activity has been recovered in the purified product.

The levelling off in the decay of inhibitor activity during storage at intermediate pH levels suggests a conversion of the initial inhibitor leading to an equilibrium before inactivation is complete. In view of the clear resolution of two inhibitory compounds, by chromatography with water on cellulose, interconversion of these two components could account for the observed changes in activity if one component is less active than the other. The two components of the bean rust inhibitor are, however, reported to have similar molar activities (17). Alternatively, a third component may be lost or an equilibrium may be established with an inactive derivative. In any event, interconversion of the two components undoubtedly occurs, as indicated by the recovery of both components on paper from either of the two peaks eluting from cellulose columns irrigated with aqueous solvents. It is also indicated by the variable proportions of components I and II in the column eluates. At alkaline reactions, both components must be labile, as all activity is lost in 1 day or so at pH 8.5, and within a few minutes at pH 12.5.

There remain other perplexing aspects of the stability of inhibitor, such as the apparent stability to heating in aqueous extracts on the one hand and the loss on storage in crude ether solutions on the other. The losses during purification are also greater than would be expected in view of the heat stability. Although probably not explainable as oxidations or reductions, losses appear to be accelerated by reaction with some impurities.

Failure to extract inhibitor from spores with dry organic solvents, also noted by Bell & Daly (6), indicates that it is in some way bound to spore constituents, possibly chemically combined. Upon hydration, it is quickly released. This might represent an actual hydrolysis of a precursor such as a glycoside, or possibly a release from structures whose hydration permits diffusion out from otherwise inaccessible sites such as the interior of the wall.

The faster-running component on cellulose (I) in neutral phosphate buffer has a distinctly lower R_f (0.45) than the front-running component from the bean rust (0.65). The R_f in distilled water is also lower, though not as much so. This evidence indicates that the inhibitors from the two genera of rust fungi are not identical, but the cross activity of crude inhibitors (2), together with the general resemblance

in their physical properties, supports the idea that they are chemically related.

Partitioning between ether and water at different pH levels shows that the inhibitors do not have a free carboxyl group. Decrease in ether solubility and complete transfer to the aqueous phase at pH 12.5 suggests that the molecules become dissociable at this pH, and the rapid irreversible inactivation indicates that the dissociated compounds are quite labile. Since quick separation and neutralization of the alkaline aqueous phase permits recovery of the inhibitor, the dissociation must be reversible. These properties suggest that the active compounds of wheat stem-rust inhibitor contain a free-dissociating, phenolic hydroxyl group, a property which would also distinguish them from the bean rust inhibitors (17).

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