Changes in the Ribonuclease Activity of Flax Cotyledons following Inoculation with Flax Rust¹

LEROY A. SCRUBB, ARUN K. CHAKRAVORTY, AND MICHAEL SHAW
Faculty of Agricultural Sciences, University of British Columbia, Vancouver 8, B.C., Canada

ABSTRACT

There was a significant increase in the ribonuclease activity of both resistant (Bombay) and susceptible (Bison) varieties of flax (Linum usitatissimum L.) 3 to 4 days after inoculation with flax rust (Melampsora lini [Pers.] Lev., race No. 3). A second and much greater increase in the activity of this enzyme occurred only in the susceptible host at later stages of disease development. While a similar increase in ribonuclease level was also caused by mechanical injury, evidence is presented showing qualitative differences between the enzyme from parasitized tissue and that from the mechanically injured cotyledons. Comparison of the enzyme from healthy and inoculated cotyledons and from flax rust revealed the presence of a relatively unstable component and some unique catalytic properties in the enzyme from inoculated cotyledons.

Isolation and characterization of RNase from plant tissues have been carried out by several workers (3, 25, 30–34). A general increase in RNase activity has been reported in virus infected plants (23) and in wheat leaves inoculated with wheat rust (25). A rapid increase in the level of this enzyme has also been shown to occur in a number of plant tissues due to mechanical damage (2, 30) and senescence (1, 12), both of which lead to a decrease in the RNA content of the affected tissue (13).

Previous work in this laboratory has demonstrated an increase in the rate of RNA synthesis in inoculated flax cotyledons (10), which contributes to a substantial increase in the RNA content of rust infected host tissue in the later stages of the disease (15). The present study was initiated in an effort to elucidate the role, if any, of RNase in the changes in the transcription pattern of inoculated flax cotyledons. Reported here are experiments on quantitative and qualitative changes in the RNase activity of flax cotyledons following inoculation with flax rust. The results suggest the presence in the inoculated host of an RNase with some properties different from those of the enzymes extracted from both healthy cotyledons and flax rust.

MATERIALS AND METHODS

Flax plants (Linum usitatissimum L. var. Bison) were grown in growth chambers with an illumination of 800 to 1000 ft-c for 16 to 18 hr daily at 25 C. One-week-old plants were sprayed with a suspension (approximately 50 mg of spores per 100 ml of distilled water) of flax rust uredospores (Melampsora lini [Pers.] Lev., race No. 3). The pots containing inoculated plants were transferred to a refrigerator at 4 C in a water-saturated atmosphere, and the electricity was disconnected for 18 hr, thus bringing the interior of the refrigerator up to room temperature (10). The pots were then returned to the growth chamber. The control (healthy) plants were sprayed with water alone but were otherwise treated identically. Under these conditions of inoculation, the number of pustules that developed on each cotyledon varied from 7 to 18, the average (scored at 8 days after inoculation) being 12 pustules/cotyledon.

The rust spores used for inoculation and enzyme extraction were always freshly collected. In all experiments special precautions in handling material and cleaning glassware (16) were rigidly followed.

ENZYME EXTRACTION

Healthy and Inoculated Flax Cotyledons. Approximately 10 g (fresh weight) of flax cotyledons were homogenized in 100 ml of ice cold 50 mm potassium phosphate buffer, pH 6.7, containing 10 g of Polyclay AT powder (polyvinylpyrrolidone, Sigma Chemical Co). The cotyledons were homogenized with an OmniMixer (Sorvall Inc.) set at high speed for four 30-sec periods with cooling between each homogenization (28). The slurry was passed through four layers of cheesecloth. The filtrate was then centrifuged for 30 min at 10,000 rpm in a refrigerated Sorvall RC2-B centrifuge. A fraction of the supernatant was used directly for the estimation of DNase and acid phosphatase. The rest of the supernatant was acidified to pH 5.0 with 1 N HCl and left at 0 to 4 C overnight. The precipitate formed during this interval was removed by centrifugation at 15,000 rpm for 15 min. The supernatant was used for the estimation of RNase activity. In a typical analysis, the total extractable protein and enzyme units (one unit of RNase is defined as the amount of enzyme causing a ΔA260nm of 1.0 under the standard assay conditions described below) from 10 g (fresh weight) flax cotyledons are: healthy: 136 mg and 328 units; and infected: 140 mg and 668 units respectively. After the pH 5 precipitation step, 21% of protein and 96% of the enzyme units are recovered in the supernatant fraction of the extracts from both healthy and infected cotyledons.

Mechanically Injured Cotyledons. Each excised cotyledon was cut transversely into three sections which were placed on a filter paper (Whatman No. 1) in a Petri dish. The filter paper was moistened with 10 ml of sterile distilled water containing 6 μg/ml of gamicidin D (Sigma) and in some cases 50 μg/ml cycloheximide (Sigma). After incubation for 6 hr at 25 C, the sections were thoroughly washed with glass distilled water and were used for enzyme extraction on the
same day. The filter papers were also extracted with the same buffer to examine any leakage of enzymatic activity during the incubation period. The extract was centrifuged at 15,000 rpm for 30 min and the precipitant was used for enzyme assay (1). Under these conditions of sectioning and incubation, RNase activity in the mechanically injured cotyledons increased to levels approximately equal to those found in infected cotyledons 6 days after inoculation (Table I).

**Flax Rust Uredospores.** Approximately 150 mg of flax rust uredospores were hydrated by stirring in glass distilled water (10 ml) containing 6 μg/ml gramicidin D (11) for various periods at 25°C. The spore suspension was then centrifuged at 5000 rpm for 15 min and RNase was extracted from the pellet exactly as described for flax cotyledons. The incubation medium was centrifuged again at 15,000 rpm for 30 min and the supernatant was used to estimate any enzyme activity released into the medium during hydration of the uredospores.

**ENZYME ASSAY**

**RNase.** The reaction mixture (final volume, 1 ml) contained: 50 μmoles of sodium acetate buffer, pH 5.5; 0.6 to 1.5 mg of yeast RNA; and enzyme solution containing 0.05 to 0.2 mg of protein. Duplicate samples were also run under identical conditions as enzyme and substrate blanks. All samples were incubated at 37°C for 30 min. The reaction mixtures were chilled, and 2 ml of precipitating reagent (1N HCl in 76% ethanol containing 0.5% Lactate) was added to each sample. The tubes were immediately closed with Parafilm to prevent evaporation, allowed to stand at 0 to 4°C for 15 min, and the samples were then centrifuged in the cold until the supernatant was clear. The supernatant was diluted with distilled water and reads at 260 nm against similarly diluted precipitating reagent. Corrections were made for enzyme and substrate blanks. Under these conditions of assay, RNase activity was linear against time at least for 4 hr.

The RNA used as substrate was a high molecular weight RNA fraction isolated from yeast and purified as described before (7). The 1 M NaCl-precipitated RNA fraction was dialyzed extensively against 5 mM EDTA and then against glacial distilled water to remove any heavy metals (8). Stock RNA solution (8 mg/ml) was stored frozen in small quantities and thawed once prior to use.

The reaction mixtures for the radioassay of RNase activity using [3H]-labeled polyribonucleotides (specific radioactivity 20 μc/μmole P) as substrate were as described above except that yeast RNA was omitted. At the end of the incubation period, 0.1 ml of nonradioactive RNA (8 mg/ml) was added to each tube prior to the addition of the precipitating reagent. After centrifugation, the supernatant was processed for the estimation of radioactivity (7) in a Nuclear Chicago (Mark I) liquid scintillation spectrometer. These assays were performed with three different substrate concentrations (0.5, 1.0, and 2.0 μc) for each polynucleotide, and the results were regarded as valid only when specific radioactive (cpm/mg protein) increased linearly with substrate concentration.

**DNase.** The incubation mixture (final volume, 2.0 ml) contained: 50 μmoles sodium acetate buffer, pH 5.8; 10 μmoles MgCl2; 0.5 mg of native or heat denatured DNA (highly polymerized calf thymus DNA, Calbiochem); enzyme solution containing 0.05 to 0.2 mg of protein. The incubation and estimation of DNase activity were carried out exactly as described for RNase.

**Phosphodiesterase.** The reaction mixture (final volume, 2.5 ml) contained: 50 μmoles tris-acetate buffer, pH 8.5; 10 μmoles of magnesium acetate; 30 μmoles KH2PO4; 1.75 mg calcium bis[p-nitrophenyl] phosphate; and enzyme solution containing 0.05 to 0.2 mg of protein. After incubation for 1 hr at 37°C, the tubes were read at 410 nm against a reagent blank.

**Acid and Alkaline Phosphatase.** The reaction mixture for acid phosphatase (final volume, 3.0 ml) contained: 0.4 mg of carboxyphenyl phosphate; 300 μmoles of sodium acetate buffer, pH 5.0; and 0.1 to 0.2 mg of enzyme protein. After incubation for 30 min at 37°C, the absorbancy of the reaction mixture was read at 300 nm against a substrate blank. The same assay procedure was followed for alkaline phosphatase except that the reaction mixture (final volume, 3.5 ml) contained: 0.8 mg of carboxyphenyl phosphate; 400 μmoles of glycine buffer, pH 8.8; 25 μmoles of MgCl2; and 0.1−0.2 mg of enzyme protein. The above procedures for phosphodiesterase and phosphomonoesterase assay are modified from those described by Udvardy et al. (30).

Unless otherwise stated the results of all enzymatic activities are expressed as specific activity which is ΔA at the appropriate wavelength/mg of protein under the experimental conditions specified above. Protein concentration in the enzyme extracts was determined by the method of Lowry et al. (21) using bovine serum albumin (fraction V, Calbiochem) as standard.

**RESULTS**

**Changes in RNase Levels in Flax Cotyledons following Inoculation with Flax Rust.** Soluble RNase activities from healthy and inoculated flax plants were determined at various stages after inoculation with flax rust. Resistant and susceptible varieties of flax plants were compared in these experiments. The results are presented in Figure 1. The ratios of specific RNase activities (inoculated/healthy) in the two varieties of flax plotted against time after inoculation demonstrate that in both resistant (Bombay) and susceptible (Bison) plants there is a conspicuous rise in RNase specific activity 3 days after inoculation resulting in an increase in the ratio to about 1.5. In the resistant combination the ratio decreases to 1 by the
5th day and there is no further change up to 10 days after inoculation. In the susceptible combination, on the other hand, the ratio increases again starting on the 5th day and it continues to rise up to the 10th day after inoculation. In all cases, the amount of protein in the RNase extract (pH 5 supernatant) remains essentially constant (28.5–30 mg protein/10 g cotyledons), indicating that the observed increases in specific activity are due to actual increase in soluble RNase activity rather than to a decrease in extractable protein.

The pattern of increase in the ratio of RNase specific activity in the susceptible host-pathogen complex is bimodal. A similar bimodal curve for the increase in RNase activity has been reported for rust-infected wheat leaves (25). However, in wheat leaves both susceptible and resistant combinations exhibit an increase in RNase content in the later stages of disease development, whereas in flax cotyledons the second increment is observed only in the susceptible combination.

**Effect of Mechanical Injury and Inoculation on RNase and other Hydrolytic Enzymes of Flax Cotyledons.** Mechanical injury of plant tissues has been shown to result in demonstrable increases in the activities of hydrolytic enzymes (2, 34). The following experiments were designed to ascertain whether or not the observed increases in RNase activity of flax cotyledons (Fig. 1) were in fact due to the damage caused by the pathogen growing intercellularly in the host tissue. In these experiments the specific activities of several hydrolases from healthy and infected flax (Bison) cotyledons were compared with those from mechanically injured cotyledons. The results presented in Table I reveal that sectioning and incubation of healthy cotyledons result in an increase in RNase activity to the same level as observed in infected cotyledons 6 days after inoculation. There is no increase in the specific activities of DNase, nonspecific phosphodiesterase or acid and alkaline phosphatases in the infected cotyledons while the specific activities of all these enzymes have increased by varying degrees in the injured cotyledons. The effects of inoculation and those of mechanical injury are therefore distinct inasmuch as inoculation does not cause a general increase in the levels of a number of hydrolytic enzymes.

**Effect of Cycloheximide on Increases in RNase Activity.**

Increases in the enzymatic activity of excised *Avena* coleoptiles and *Rheo* leaf sections have been shown to be due to enzyme synthesis which was prevented by inhibitors of RNA and protein synthesis (12, 30). The results of similar experiments with sectioned flax cotyledons are presented in Table II. It appears that cycloheximide, an inhibitor of protein synthesis, completely prevents the increase in RNase activity which normally occurs during the 6-hr incubation of the sectioned cotyledons. It has been noted that some of the hydrolytic enzymes of the sectioned cotyledons leak out into the incubation medium (Table I). In the presence of cycloheximide there is no leakage of RNase into the incubation medium.

The effects of cycloheximide in vivo on the RNase activity of healthy and infected cotyledons were examined in the following series of experiments. Inoculated plants and healthy control plants of corresponding age were sprayed lightly with 50 μg/ml cycloheximide at different times after inoculation. The plants were allowed to grow for several days and soluble RNase was subsequently extracted from the cotyledons. The results of two groups of experiments are presented in Table III. The plants in group A were sprayed with cycloheximide once (2nd day after inoculation) or twice (2nd and 4th day) and RNase was extracted on the 6th day after inoculation. The results show that in these plants only one application of cycloheximide (on the 2nd day) strikingly inhibits the increase in RNase activity that is expected to occur 6 days after inoculation. A second application of cycloheximide does not cause any further decrease in RNase specific activity. It is also noteworthy that cycloheximide has no apparent effect on the RNase activity of healthy control plants.

Group B plants were treated with cycloheximide once (4th day) or twice (4th and 6th day) and RNase was extracted on the 10th day after inoculation. The results show that regardless of the number of cycloheximide treatments, the specific activities of RNase in cycloheximide-treated, infected plants are reduced by 30 to 40% as compared to those of the untreated inoculated plants but are still nearly twice as high as those of the healthy plants.

In group A plants, although there is no visible effect of cycloheximide on the growth of healthy and inoculated plants,

---

### Table I. Changes in the Activities of Hydrolytic Enzymes of Flax Cotyledons in Response to Inoculation with Flax Rust and to Mechanical Injury

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity Ratios</th>
<th>ΔA/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Infected (I)</td>
<td>Sectioned (S)</td>
</tr>
<tr>
<td>RNase</td>
<td>11.3</td>
<td>20.9</td>
</tr>
<tr>
<td>DNase1</td>
<td>8.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Phosphodiesterase1</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>21.6</td>
<td>26.2</td>
</tr>
<tr>
<td>Alkaline phosphatase2</td>
<td>10.6</td>
<td>17.5</td>
</tr>
</tbody>
</table>

1 Similar results were obtained with native and denatured DNA as substrate.

2 Approximately 5 g of cotyledons were homogenized in 20 ml of 0.10 M tris-acetate buffer, pH 8.5, and 5 g of Polyclar AT, filtered through cheesecloth and centrifuged at 15,000 rpm for 30 min. The pH of the supernatant was adjusted to 8.5 with 1 N NaOH. This extract was used for the estimation of phosphodiesterase and alkaline phosphatase activities.

### Table II. Effect of Cycloheximide on the RNase Activity of Mechanically Injured Flax Cotyledons

Healthy flax cotyledons were sectioned and incubated in sterile distilled water containing 6 μg/ml gramicidin D (see “Materials and Methods”) prior to extraction of RNase. Intact cotyledons were collected from plants grown in the same pot just prior to the extraction of RNase. The results for sectioned cotyledons were corrected for the activity that leaked out into the incubation medium and the values are presented in parentheses.

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>RNase Specific Activity</th>
<th>Specific Activity Ratios (Sectioned/Intact)</th>
<th>ΔA/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cotyledons</td>
<td>10</td>
<td>19 (20.2)</td>
<td>1.8 (2.0)</td>
</tr>
<tr>
<td>Sectioned cotyledons (no cycloheximide)</td>
<td>10.8 (10.8)</td>
<td>1.1 (1.1)</td>
<td></td>
</tr>
<tr>
<td>Sectioned cotyledons (cycloheximide, 100 μg/ml)</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table III. Effect of Cycloheximide on the RNase Activity of Healthy and Inoculated Flax Cotyledons

Healthy and inoculated flax plants were sprayed with cycloheximide solution (50 μg/ml of sterile distilled water) as indicated. Control plants were sprayed with water alone. RNase was extracted from the cotyledons 6 days after inoculation in Group A and 10 days after inoculation in Group B.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group A</th>
<th></th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.3</td>
<td>28.2</td>
<td>9.2</td>
</tr>
<tr>
<td>Cycloheximide (Sprayed once, 2nd day after inoculation)</td>
<td>11.4</td>
<td>14.8</td>
<td>9.25</td>
</tr>
<tr>
<td>Cycloheximide (Sprayed twice, 2nd and 4th day after inoculation)</td>
<td>11</td>
<td>14.3</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Table IV. Effect of Hydration on the RNase Activity of Flax Rust Uredospores

The rust spores were hydrated for various periods of time in sterile distilled water containing 6 μg/ml of gramicidin D in the presence or absence of cycloheximide (50 μg/ml).

<table>
<thead>
<tr>
<th>Hydration Treatment</th>
<th>RNase Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spores</td>
</tr>
<tr>
<td>None</td>
<td>8.0</td>
</tr>
<tr>
<td>Hydration 2 hr</td>
<td>7.4</td>
</tr>
<tr>
<td>Hydration 2 hr + cycloheximide</td>
<td>6.8</td>
</tr>
<tr>
<td>Hydration 4 hr</td>
<td>4.1</td>
</tr>
<tr>
<td>Hydration 4 hr + cycloheximide</td>
<td>6.9</td>
</tr>
</tbody>
</table>

the growth of the fungus in the inoculated plants is retarded and sporulation which normally occurs at 7 or 8 days after inoculation is delayed by 3 to 5 days, depending on the number of cycloheximide treatments. In group B plants, however, the fungus is not noticeably affected by cycloheximide and there is no evidence of delay in sporulation. The conclusion that can be drawn from these experiments is that the growth of fungus in the host tissue is inhibited and the increase in RNase activity in the inoculated cotyledons is substantially prevented by cycloheximide only if the drug is administered within the first 2 days after inoculation.

The foregoing results (Table II) and those reported by other workers (2, 30) have suggested that the increase in RNase activity of mechanically injured flax cotyledons is due to the synthesis of new RNase molecules. The observed effect of cycloheximide in preventing an increase in RNase activity of infected cotyledons (Table III) may be interpreted alternatively as indicating (a) an inhibition of parasitically induced synthesis of new enzyme in host cells, (b) an inhibition of new RNase synthesis in the growing pathogen, which may normally contribute substantially to the RNase content in the infected tissue or (c) an inhibition of the release of particulate bound enzyme in the host cells. The last mentioned possibility was investigated by isolating subcellular particulate fractions by differential centrifugation (8, 25) and assaying for active and latent RNase activity under appropriate conditions (8, 9). It has been observed that the activity of particulate-bound enzyme does not change significantly for up to 6 days after inoculation with or without cycloheximide treatment (unpublished observation) suggesting that the increase in soluble RNase activity (Fig. 1) is not due to a release of particulate bound enzyme. Similar results were reported earlier by Rohringer et al. (25).

In order to decide between possibilities (a) and (b), a comparative study of the properties of RNase from healthy and inoculated cotyledons as well as from flax rust was undertaken.

RNase from Flax Rust. In order to ascertain the presence of RNase in the soluble fraction of flax rust uredospores, extracts from dry as well as hydrated spores were assayed for RNase activity. The results presented in Table IV reveal the presence of demonstrable amounts of RNase activity in both dry and hydrated rust spores. It is also evident that there is no change in the specific activities of the enzyme in the spores hydrated for up to 4 hr. There is, however, a considerable release of the enzyme into the medium during the 4-hr hydration period. This is completely prevented by cycloheximide in the incubation medium.

Soluble RNase was also extracted from flax rust mycelia grown in axenic culture (29) by the same procedure as described for flax cotyledons (see 'Materials and Methods'). The yield of the enzyme is greater if lyophilized mycelia are used as the starting material.

Properties of RNase from Healthy and Inoculated Cotyledons and from Flax Rust, pH Optima. Figure 2 shows the pH activity curves of RNase from various sources. Maximal activity for RNA hydrolysis by RNase from both healthy and infected cotyledons occurs between pH 5.5 and 5.7. The enzyme from flax rust has a higher pH optimum, around pH 5.7.
6.5. In all cases, there is only one major peak of activity, suggesting that either there is only one RNase component in the extract with a pH optimum in the acidic range, or that all the major isozymes present (31, 33) have similar pH optima. There is a slight increase in activity at pH values higher than 7.0 in extracts from healthy and inoculated cotyledons. The enzyme from flax rust spores does not exhibit a similar increase at the corresponding pH values. The pH-activity curves for the enzymes from mechanically injured cotyledons and from rust mycelium grown in axenic culture (data not included in Fig. 2) are identical to those of the enzymes from healthy cotyledons and rust spores respectively.

Temperature Stability. The stability of RNase at higher temperatures was studied by adjusting the pH of the enzyme solution to 8.5, holding it at a given temperature for 10 min, followed by cooling and readjusting the pH to 5.0. The enzymatic activity was then examined. The results presented in Figure 3 show that the enzymes from healthy cotyledons and rust spores are remarkably stable and lose very little activity even at 100°C. Under identical conditions, 50 to 60% of the enzymatic activity in extracts from infected cotyledons is destroyed. The enzyme from healthy cotyledons exhibits a 50% decrease in activity when held at 60°C for 10 min. This highly reproducible phenomenon has also been reported to occur in sugarcane ribonuclease (28). Much less activation (10–15%) occurs in the enzyme from rust spores at a higher temperature (80°C). The enzyme from inoculated cotyledons shows no reactivation at 60°C. At 80°C, there is a very small increase in activity, but unlike the enzymes from healthy cotyledons and rust spores, that from inoculated cotyledons has much lower activities at all temperatures above 50°C. Thus it appears that the enzyme extracted from infected cotyledons contains a temperature sensitive RNase component which is not found in healthy cotyledons or in the rust spores.

The existence of an unstable component in the inoculated enzyme is also suggested by the observation that while the RNase from healthy plants and rust spores (or mycelia) can be stored at 0°C for at least 10 days without any apparent loss of activity, the enzyme from inoculated cotyledons loses over 35% of its activity after storage for 6 days.

The temperature stability curves shown in Figure 3 are highly characteristic of the source of enzyme. This is indicated by the fact that identical temperature-stability curves are obtained with the enzymes from healthy and mechanically injured cotyledons on the one hand and those from rust spores and mycelia grown in axenic culture on the other (unpublished observation).

Effect of Diethylpyrocarbonate. Table V shows the inhibitory effect of a maximally effective concentration of diethylpyrocarbonate (DEP) on the enzymes extracted from healthy and inoculated cotyledons, from rust spores hydrated for 4 hr and from flax rust mycelia grown in axenic culture. DEP, which has been shown to be a potent RNase inhibitor (18), inhibits the RNase from healthy flax cotyledons by 24%. The inhibition of the enzyme from rust is about 50% but the enzyme from inoculated cotyledons shows the highest degree of susceptibility to DEP. These results again suggest the presence of an RNase component in the infected cotyledons which is inactivated by DEP to a much greater extent than the enzymes from either healthy flax cotyledons or rust spores. When this component is inactivated by preincubating the enzyme solution at 80°C (Fig. 3), DEP inhibits the remaining activity by 43%, a value intermediate between those obtained for the enzymes from healthy cotyledons and flax rust.

Substrate Specificity. The substrate specificity of RNase

---

Table V. Effect of Diethylpyrocarbonate on the RNase Activity of Healthy and Inoculated Cotyledons and of Rust Spores

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Inhibition of RNase Activity by DEP %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy cotyledons</td>
<td>24.3</td>
</tr>
<tr>
<td>Infected cotyledons (6 days after inoculation)</td>
<td>77.2</td>
</tr>
<tr>
<td>Infected cotyledons (enzyme preincubated at 80°C for 10 min at pH 8.5)</td>
<td>42.8</td>
</tr>
<tr>
<td>Rust spores</td>
<td>51.2</td>
</tr>
<tr>
<td>Rust mycelium</td>
<td>55.0</td>
</tr>
</tbody>
</table>

---

from various sources was studied by examining the relative rate of degradation of synthetic homopolymers of cytidylic, adenylic, uridylic, and guanylic acids with identical concentrations of enzyme and substrates. The results presented in Table VI reveal that the RNase preparations from healthy and infected cotyledons hydrolyze poly C at remarkably different rates, the enzyme from infected cotyledons being 6 to 7 times more active. The RNase from both these sources hydrolyzes poly U and poly A at similar rates and poly G is not hydrolyzed at all. The substrate preference of the enzyme from healthy cotyledons is (in decreasing order) poly U > poly A > poly C, and that for the enzyme from infected cotyledons is poly C > poly U > poly A. With 3 M urea in the incubation mixture, the enzymes from both healthy and inoculated cotyledons can hydrolyze poly G, suggesting that the lack of activity in the absence of urea was due to the highly ordered structure of poly G resulting from extensive hydrogen bonding between
guanulate residues. The hydrolysis of poly C is slightly stimulated by urea. Thus, the enzymes from both these sources are capable of hydrolyzing phosphodiester bonds next to all four bases, although at significantly different rates.

The RNase from rust spores (or mycelia) preferentially hydrolyzes the four polynucleotides in the order: poly C > poly A > poly U = poly G. The rust RNase differs from the enzymes from healthy and infected flax in its ability to hydrolyze poly G in the absence of urea. Also, with the possible exception of poly C and poly G, it hydrolyzes the other polynucleotides at a higher rate than the enzymes from healthy and infected cotyledons. These results demonstrate that the RNase from infected cotyledons differs from that of the healthy cotyledons in its preference for the phosphodiester bonds between different nucleotides and from the rust enzyme in the rate at which it hydrolyzes poly A and poly U. The rust enzyme is unique in its ability to hydrolyze poly G in the absence of urea. Since the RNase preparation from infected cotyledons undoubtedly includes RNase derived from the rust mycelia it is surprising that it cannot hydrolyze poly G in the absence of urea.

The experiments on the properties of RNase described above were conducted with a relatively crude enzyme preparation. In the course of this study, the enzymes from healthy and infected cotyledons were purified over 350-fold following a procedure described for sugar cane RNase (28). However, it was observed that starting with the 50 to 80% ammonium sulfate fraction, there was a progressive loss of the labile component (Fig. 3) in the enzyme preparation from infected cotyledons. Since the purpose of this investigation was to study quantitative and qualitative changes in RNase activity during disease development, extensive purification of the enzymes before a study of their properties was therefore avoided.

### DISCUSSION

This study has demonstrated both quantitative and qualitative changes in the soluble RNase extracted from flax cotyledons that had been inoculated with a virulent race of flax rust. Mechanical injury of the cotyledons gives rise to a similar increase in RNase activity (Table I). However, the mechanisms underlying the changes in the RNase activity of parasitized tissue may be quite different from those caused by mechanical injury. This is indicated by the following observations: (a) mechanical injury results in an increase in the activities of several hydrolytic enzymes in addition to RNase and (b) only a quantitative change is induced by mechanical injury; the properties of RNase from injured tissues are essentially identical to those of the enzyme from healthy (intact) tissue.

Treatment with cycloheximide on the 2nd day after inoculation prevents the increase in RNase activity of the cotyledons (Table III) but it also causes a retardation of fungal growth in the host tissue. Hence, in the case of group A plants in Table III, the lack of increase in RNase activity measured 6 days after inoculation may have resulted from the inhibition of fungal growth by cycloheximide rather than from a direct effect of the drug on protein synthesis. In Group B plants, however, cycloheximide does not noticeably inhibit fungal growth but it does significantly reduce the increase in RNase activity. The combined results of these two sets of experiments suggest, but do not conclusively prove, that the increase in RNase levels in parasitized tissue requires continued synthesis of the enzyme at all stages of disease development.

The relatively small but highly reproducible increase in the RNase activity in both resistant and susceptible varieties of flax 3 to 4 days after inoculation (Fig. 1) probably represents a part of the metabolic defense mechanism of the host (5, 14, 17, 24, 27). The remarkable increase in soluble RNase observed in the later stages of the disease occurs only in the susceptible host. This increase in RNase activity of inoculated flax cotyledons and wheat leaves (25) coincides with a dramatic increase in the rate of synthesis (4, 10) and accumulation (4, 15) of RNA in the host cells. Biochemical analysis of the newly synthesized flax RNA indicated that much of the RNA that accumulates is cytoplasmic ribosomal RNA (10). Surprisingly, despite a general increase in the levels of soluble RNase, there is no evidence for a large scale degradation of cytoplasmic RNA in the infected tissue. Two possibilities may be considered to account for this phenomenon: (a) the rates of synthesis and degradation of RNA in the cells are in a dynamic equilibrium that favors a net increase in RNA content and (b) the soluble RNase in the inoculated cotyledons may be reversibly inactivated in vivo by inhibitor molecules. A similar parallelism between an increased total RNase content and enhanced accumulation of RNA has been reported in other organisms (6, 9, 19). In neoplastic cells, the increased RNase is inactivated by the accelerated production of a glyco- protein inhibitor (26) which prevents indiscriminate degradation of cytoplasmic RNA (9). A similar polypeptide RNase inhibitor has not been found in plant cells (22). Nevertheless, in inoculated flax cotyledons there is a conspicuous increase in phenolic compounds and their derivatives which are known to be potent inhibitors of many enzymes (20).

The RNase from infected cotyledons (6 days after inoculation or later) shares very few properties with the enzymes from healthy cotyledons and from flax rust (Fig. 3: Table V and VI). The temperature stability of the enzymes of these three sources clearly suggests the presence of a relatively unstable and highly DEP-sensitive component in the enzyme from infected cotyledons (Fig. 3: Table V). When this component was selectively inactivated, the remaining activity appears to have DEP sensitivity intermediate between that of the enzymes from flax cotyledons and flax rust. It is not known whether this component is synthesized after inoculation or is a pre-existing protein whose properties change in the course of disease development. Changes in the catalytic properties of the rust enzyme in the infected tissue are indicated by the observations that the RNase from infected cotyledons is incapable of hydrolyzing poly G in the absence of urea (Table VI) and that its pH-activity curve is quite different from that of the rust enzyme (Fig. 2).

Further research will be necessary to ascertain whether the
increased RNase in parasitized tissue is produced by the host cells or by rust mycelia and what part, if any, it plays in host-parasite interactions. Since the substrate preference of the RNase from infected cotyledons is noticeably different from that of the enzymes from flax and flax rust (Table VI), it is conceivable that it may have a specific role in selectively hydrolyzing certain RNA molecules.

LITERATURE CITED