Helminthosporoside, a Host-specific Toxin from Helminthosporium sacchari*

(Received for publication, October 19, 1970)

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SUMMARY

Helminthosporoside has been isolated from the sugar cane fungus pathogen Helminthosporium sacchari. This compound is the first host-specific plant toxin to have a structure proposed for it. The toxin produces symptoms only on those clones of sugar cane that are susceptible to the fungus. Based on its spectral and chemical properties, the proposed structure of helminthosporoside is 2-hydroxycyclopropyl-α-D-galactopyranoside. A biological assay for its quantification based on the degree of symptom expression on sugar cane leaves is described. Helminthosporoside-14C can be isolated from cultures of H. sacchari that have been incubated with galactose-1-14C.

Host-specific toxins have been reported for a number of plant-pathogenic species of Helminthosporium and a few other fungi, and there are numerous review articles on these toxins (1-4). These compounds have been termed host specific since they affect only those clones within a plant species that are susceptible to the fungus that produces them. Furthermore, these compounds do not produce symptoms on any plant species not otherwise attacked by the fungus. Several investigators have indicated that characterization of these toxins has been difficult since they are unstable in a homogeneous condition. The published data, however, indicate that they are low molecular weight peptides (2, 3).

Steiner and Byther (5) reported that a host-specific toxin was produced by Helminthosporium sacchari (Van Breda de Haan) Butler. H. sacchari is the causal organism of eye spot disease of sugar cane which occurs in most of the sugar cane-growing areas of the world (6). The fungus causes eye-shaped lesions on the leaves, followed by the development of reddish brown streaks or “runners” extending from the lesions toward the tip of the leaf. The fungus can be isolated from the lesion but not from the runner areas. This observation suggested that a toxic compound from the fungus was causing the runner. Steiner and Byther (5) partially purified a substance that was capable of causing runners only on susceptible clones of sugar cane. Thus, since the toxin had the same host range as the fungus, it could be termed host specific. Their report indicated that the toxin had a low molecular weight and was stable to high temperatures. The partially purified substance is now used to screen clones of sugar cane for resistance to the eye spot disease (5). Because the toxin appeared to be stable, it was a likely candidate for isolation and characterization.

This paper has a 4-fold purpose: (a) to present a method for purification of a host-specific toxin of H. sacchari; (b) to report on the characterization of the toxin based on spectroscopic and chemical analyses; (c) to describe some of the biological properties of the toxin, and (d) to suggest the trivial name helminthosporoside for the toxin, based on its source and structure.

EXPERIMENTAL PROCEDURE

Culturing.—The culture of H. sacchari used in this investigation was originally isolated from naturally infected sugar cane in Hawaii. The organism was maintained on agar made from sugar cane leaf extract (7). For toxin production, the fungus was grown for 18 to 20 days in 1-liter Roux bottles containing 160 ml of Fries medium supplemented with 0.1% yeast extract dialysate (8). The cultures were grown at 22-24°C under stationary conditions.

Biological Assay.—Assays were made on a susceptible clone of sugar cane 51 NG 97, in a manner similar to Steiner and Byther (5). Leaves of the cane were cut into 18-cm sections and 1 μl of the test solution was placed on a needle puncture spot near the base of the leaf. Inoculated leaves were placed into a moist chamber at room temperature and measurements of runner length made after 22 hours unless otherwise indicated. The length of the runner was taken as an indication of the toxic potency of the preparation and a relationship was established between runner lengths and the amount of toxin applied to the leaf. Specific biological activity was arbitrarily taken as the amount of material required to produce a 5-cm runner.

Precursor Experiments.—Galactose 14C (55.7 mCi per μmol), 50 μCi, was added to a culture flask and the incubation time and purification techniques to remove the toxin were identical to the procedures established in this report. Radioactivity was measured with a Nuclear Chicago liquid scintillation counter.
model 6004. The scintillation fluid and method for correction to disintegrations per min were as reported by Strobel (9).

**Chromatography** Descending paper chromatography was conducted on Whatman No. 1 filter paper using the following solvent systems: a, 1-butanol-acetic acid-H₂O, 4:1:5 v/v/v; b, 1-propanol-NH₄OH-H₂O, 6:3:1 v/v/v; c, 1-butyric acid-1-butanol-H₂O, 2:2:1 v/v/v; d, 1-butanol-ethanol-H₂O, 5:1:4 v/v/v; e, ethyl acetate-formic acid-acetic acid-H₂O, 18:3:1:4. Thin layer chromatography was performed on plates of Adsorbosil-5 with the following solvents: f, hexane-ethyl acetate, 85:15 v/v; g, benzene-methanol, 95:5 v/v; h, 1-butanol-acetic acid-H₂O, 3:1:1 v/v/v; i, 1-butanol-acetone-H₂O, 4:5:1 v/v/v; j, methyl ethyl ketone-acetic acid-methanol, 6:2:2 v/v/v; k, benzene-acetic acid-methanol, 2:2:16 v/v/v. The toxin was detected by spraying the chromatograms with a chloroform solution saturated with either antimony trichloride or antimony pentachloride and heating at 90° for 3 or 4 min. The toxin appeared as a reddish spot with antimony trichloride and as a yellow spot with antimony pentachloride. Both reaction products with the antimony compounds fluoresced under ultraviolet light. The toxin was eluted from chromatograms and checked for biological activity.

**Harvest, Isolation, and Purification**—The fungus was removed from the medium (250 ml) by straining through four layers of cheesecloth and filtering through Whatman No. 1 and No. 50 filter papers, respectively. The filtrate was concentrated to 0.10 of the original volume under vacuum at 55°C. Then, 4 volumes of acetone at −15°C were added with stirring. The precipitate was removed by centrifugation at 20,000 × g for 5 min and discarded. Acetone was removed by flash evaporation, and the remaining solution was partitioned against 3 volumes of chloroform. The aqueous phase was concentrated to about 0.02 of the original volume. At this state of purification, the toxin could be stored for extended periods of time without loss of activity. Further purification was accomplished by extracting the aqueous phase (5.0 ml) with three 10-ml volumes of water-saturated 1-butanol. After removing the butanol by flash evaporation, the material was suspended in 1.0 ml of H₂O and applied to a column (1.5 × 48 cm) of Sephadex G-15 and eluted with distilled H₂O. Fractions (1 ml) were collected. The tubes (35 through 48) after the void volume containing biological activity, were pooled, flash evaporated to dryness, and chromatographed on water-washed Whatman No. 541 filter paper in Solvent a. The area containing biological activity was eluted with water and rechromatographed in Solvent b. The toxin was eluted with water and stored in a desiccator over P₂O₅. The yield of pure toxin from 1 liter of culture medium was approximately 7 to 9 mg. Small weight measurements were made on a Cahn electrobalance.

**Gas-Liquid Chromatography**—Sugars were determined quantitatively and qualitatively by gas-liquid chromatography after acid hydrolysis and neutralization of the samples. After 0.05 ml of a mixture containing hexamethydisilane, trimethylchlorosilane, and pyridine, 3:1:9 v/v/v, was added to the sample, the mixture was incubated at room temperature for 30 min in a capped vial (10). The silyl ether derivatives of the sugars were subjected to gas chromatography on an F and M gas chromatograph equipped with a column of 3% SE-30 on Gas Chrom Q (0.4 × 180 cm). The column oven temperature was run isothermally at 175°C with a detector temperature of 250°C and a carrier gas flow rate of 30 to 50 cc per min. The silyl ether derivatives of the standard sugar were chromatographed for quantitative and reference purposes.

Other products of acid hydrolysis of the toxin were detected by injecting 5-μl aliquots of the aqueous-acid hydrolysate directly on to a column (0.4 × 180 cm) of Porapak Q. The column oven temperature was run isothermally at 190°C and all other conditions of gas chromatography were as described above.

**Instrumental Analyses**—The infrared spectrum was obtained on a Beckman microspectra using a micropellet of KBr. Mass spectral data were obtained from a Varian CH-5 with 100 Μamp on the filament and the probe heated to 195°C. Ultraviolet analyses were performed on a Beckman DU spectrophotometer. Specific optical rotation of the compound was determined in a Zeiss circle polarimeter with 0.76 mg of the sample dissolved in 0.1 ml of Η₂Ο and placed in a tube with a light path of 50 mm at 20°C. Amino acid analyses were performed on a Technicon automatic analyzer. The total carbon in each tube from samples obtained from Sephadex column chromatography was measured directly in a Beckman CO₂ analyzer.

**Materials and Services**—All ¹⁴C-labeled compounds were purchased from Amersham/Searle Corporation. D₂O was obtained from Bio-Rad Laboratories, Richmond, California. Adsorbosil-5 and all materials for gas-liquid chromatography were purchased from Applied Science Laboratories, State College, Pennsylvania. β-Galactosidase was obtained from Worthington. α-Galactosidase was a generous gift of Dr. P. Albersheim and Dr. Ken Kegstra, University of Colorado, Boulder, Colorado. Sugar cane dyes 51 NG 97 and H50-7209 were kindly supplied by Drs. R. Coleman and R. Hebert of the United States Department of Agriculture. Guinea pigs and mice were supplied and handled by Dr. N. Reed of this department. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tennessee. Galbraith Laboratories also did a molecular weight determination by membrane osmometry. Mr. L. F. Johnson of the Varian Applications Laboratory kindly furnished the NMR spectrum of the unknown. All compounds used were either reagent or spectroscopic grade.

**RESULTS**

Fig. 1 is a flow chart illustrating the procedure used to isolate the toxin. The effectiveness of each step in removing contaminating materials is shown in Table I. The specific biological activity increased with purification. The toxin could not be demonstrated with the biological assay in either of the precipitates following the steps of the addition of acetone or chloroform to the culture filtrate. Trace amounts of the toxin were detectable in the water phase after partitioning against butanol. Altogether from 60 milligrams of Sephadex G-15 removed a large amount of contaminating materials, tubes 35 through 48 containing most of the toxin still had additional contaminants (Fig. 2).

Purification of the toxin was completed by chromatography in Solvent a, followed by elution and chromatography in Solvent b. However, after chromatography in Solvent a, two bands possessing toxic activity were observed on the chromatogram, confirming previous results that two toxins were present after Sephadex column chromatography (Fig. 2) (5). The toxin (belminiosporoside) in Peak 1 (Fig. 2) was the substance chosen for investigation in this study since it was the more biologically potent of the two.

†The abbreviation used is: NMR, nuclear magnetic resonance.
Helminthosporoside

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150 ml Culture Filtrate of H. Saccberi

Concentrate, 15 ml

4 Volumes Acetone

Discard ppt.

Concentrate, 15 ml

3 Volumes Chloroform

(3 washes)

Discard chloroform fraction

Concentrate, 3 ml

Equal Volumes Butanol

(4 washes)

Discard butanol fraction

Concentrate, 1 ml

Sephadex G-15

Chromatography, Butanol, HAc, H2O

Paper Chromatography, Butanol, HAc, H2O

Paper Chromatography, NH4OH-Propanol

FIG. 1. A flow sheet summarizing the procedure used to purify helminthosporoside.

Table I

Effectiveness of each step in purification scheme in isolation of helminthosporoside

| Step in purification* | Dry weight | Specific biological activity |
|-----------------------|------------|----------------------------|
| Crude culture filtrate | 9880       | 1.64                       |
| Acetone               | 4808       | 0.55                       |
| Chloroform            | 3960       | 0.35                       |
| Butanol               | 1120       | 0.31                       |
| Sephadex G-15         | 425        | 0.17                       |
| Chromatography, Solvent a | 30 | 0.037                      |
| Chromatography, Solvent b | 7.8      | 0.038                      |

For each step in the purification procedure, the total dry weight and specific biological activity were determined. The data are based on 1.0 ml of crude culture filtrate as the starting material.

*This is defined as the amount of material required to produce a runner on a susceptible sugar cane leaf 5 cm long 22 hours after inoculation. A 5-cm lesion was arbitrarily set as a standard lesion length. The length of a lesion obtained from a given test was extrapolated from Fig. 5 to yield the amount of toxin present in the preparation.

Table II

| Clones of sugar cane | Response |
|----------------------|----------|
| Clone 1              | 0.3%     |
| Clone 2              | 0.5%     |
| Clone 3              | 0.7%     |
| Clone 4              | 0.9%     |
| Clone 5              | 1.1%     |
| Clone 6              | 1.3%     |
| Clone 7              | 1.5%     |
| Clone 8              | 1.7%     |
| Clone 9              | 1.9%     |
| Clone 10             | 2.1%     |
| Clone 11             | 2.3%     |

The toxin caused no visible symptoms in mice receiving alcoholic ninhydrin and with the reducing sugar test of Trevally, Procter, and Harrison (12), respectively. In all of the chromatographic systems tested (a through k), the biological activity eluted at the place corresponding to the RF on the chromatogram, giving a positive test with the antimony trichloride reagent. The compound was considered homogenous.

Biological Activity Studies—Sugar cane leaves inoculated with the pure toxin caused symptoms that were identical with those produced by the fungus (Fig. 3). The first visible indication of toxicity to the treated leaf was the development of a light green area surrounded by the dark green area of the nonaffected portion of the leaf. Eventually the light green area developed into a reddish brown runner.

The length of visible runners on susceptible sugar cane leaves as a function of time and concentration of toxin was determined (Fig. 4). Based on these results, subsequent assays relied on a 22-hour incubation period in order to allow for full symptom development.

After 22 hours of incubation, runner length, as a function of toxin concentration, was virtually a linear relationship when plotted in a semilog scale (Fig. 5). Using this inoculation technique, symptoms on leaves were observable in amounts of helminthosporoside as low as $5 \times 10^{-12}$ moles.

Helminthosporoside was tested on 11 clones of sugar cane differing in their susceptibility to H. sacchari. In all cases, the plants responded in the same manner to the fungus as they did when helminthosporoside was placed on the leaf (Table II). In addition, the toxin did not produce symptoms on several varieties of wheat, corn, sorghum, and native grass species that were tested.
FIG. 3. A comparison of symptoms produced by the toxin and the fungus pathogen *Helminthosporoside sacchari* on the leaves of susceptible sugar cane. The leaf on the left was inoculated with a spore suspension of *H. sacchari*. A runner had developed from the primary lesion area (arrow) after 14 days. The leaf on the right was injected on both sides of the midrib with 20 μg per μl of *helminthosporoside* (arrows). Good symptom development as shown in this figure took 22 hours.

FIG. 4. The relationship between runner length and time as a function of toxin concentration. Susceptible sugar cane leaves were inoculated as indicated in the text and runner length was measured.

FIG. 5. Runner length as a function of toxin concentration. Runners were measured 22 hours after inoculation of the toxin onto the leaves as described in the text.

TABLE II

*Comparison of reaction of 11 clones of sugar cane to inoculation with helminthosporoside and H. sacchari.*

Approximately 15 μg of the toxin were applied at the site of inoculation. The reactions resulting from fungus and toxin inoculation were evaluated as + (runner development) or − (no runner development).

| Clones        | Reaction With Toxin | Reaction With Fungus |
|---------------|---------------------|----------------------|
| 51 NG 97      | +                   | +                    |
| H-109         | +                   | +                    |
| Lahaina      | +                   | +                    |
| H61-7207      | +                   | +                    |
| H38-5029      | +                   | +                    |
| H49-823       | +                   | +                    |
| H49-5         | −                   | −                    |
| H50-7209      | −                   | −                    |
| H57-5174      | −                   | −                    |
| H52-4610      | −                   | −                    |
| H61-5433      | −                   | −                    |

intravenous injections of 1.0 mg of toxin in a 0.85% NaCl solution. Likewise, guinea pigs treated epidermally with 1.0 mg of the toxin did not develop an inflammatory reaction.

The stability of helminthosporoside was determined by placing 150 μg into each of many vials. Two sets of vials were sealed under vacuum and one set stored at −15°C, the other set at room temperature, respectively. Two other sets of vials were covered with paraffin and also stored in the same fashion. Samples from the four sets of vials were tested periodically. No loss of biological activity was noted in any of the treatments up to 57 days, at which time the experiment was terminated.

Properties of *Helminthosporoside*—At room temperature and humidity the purified compound was a yellowish syrup. How-
Table III

$R_F$ values of helminthosporoside and another toxin produced by
H. sacchari

| Solvent | Helminthosporoside | Other toxin |
|---------|--------------------|-------------|
| a. 1-Butanol-acetic acid-H$_2$O, 4:1:5 | 0.30 | 0.15 |
| b. 1-Propanol-NH$_2$OH-H$_2$O, 6:3:1 | 0.80 | 0.70-0.80 |
| c. 1-Butyric acid-1-butanol-H$_2$O, 2:2:1 | 0.14 | 0.03 |
| d. 1-Butanol-ethanol-H$_2$O, 5:1:4 | 0.34 | 0.21 |
| e. Ethyl acetate-formic acid-acetic acid-H$_2$O, 18:3:1:4 | 0.54 | N.D. |
| f. Hexane-ethyl acetate, 85:15 | 0.0 | N.D. |
| g. Benzene-methanol, 95:5 | 0.0 | N.D. |
| h. 1-Butanol-acetic acid-H$_2$O, 3:1:1 | 0.70 | N.D. |
| i. 1-Butanol-acetone-H$_2$O, 4:5:1 | 0.54 | N.D. |
| j. Methyl ethyl ketone, acetic acid-methanol, 6:2:2 | 0.53 | N.D. |
| k. Benzene-acetic acid-methanol, 2:2:6 | 0.56 | N.D. |

* Substance from Peak 2, Fig. 2. This toxin was located on chromatograms by elution with water and bioassaying on sugar cane leaves.

* Not determined.

Fig. 6. Infrared spectrum of helminthosporoside. The toxin was dried over P$_2$O$_5$ and redried after the preparation of a mixture with KBr. A micropellet was prepared and the sample was run in a Beckman Microspectrophotometer. The X axis is wave length in microns.

Table III shows the $R_F$ values of both helminthosporoside and the other toxin (Peak 2 in Fig. 2) in several solvents by both thin layer and paper chromatography. Helminthosporoside moved well in polar solvents, suggesting that it was a polar compound.

The infrared spectrum of the toxin revealed a strong absorption band at 3.0 $\mu$m indicating the presence of $-\mathrm{OH}$ groups on the molecule (Fig. 6). The lack of a strong band around 6.0 $\mu$m assured the absence of a carbonyl group either as an acid or an aldehyde. The remainder of the spectrum initially suggested that the toxin could be a glycoside, which would be in keeping with the chromatographic data. The ultraviolet spectrum revealed no absorption bands above 210 $\mu$m, eliminating the presence of aromatic and conjugated systems in the molecule. The specific optical rotation of the compound was $[\alpha]_D^{25} = -49.0$.

Mass spectroscopy of the toxin gave a discernible molecular ion peak of 236 $m/e$ (Fig. 7). That the peak of 236 represented the molecular ion was confirmed by a molecular weight determination by membrane osmometry near 250. The peaks at 218 and 200 each represent the loss of 1 water molecule, respectively (Fig. 7).

Elemental analysis of helminthosporoside yielded the following percentages:

| Compound    | C | H | O |
|-------------|---|---|---|
| C$_4$H$_{14}$O$_7$ | 45.8 | 6.8 | 47.4 |
| Found       | 45.7 | 7.3 | 46.1 |

Based on a molecular weight of 236, an empirical formula C$_4$H$_{14}$O$_7$ could be written for this compound. Only a trace of nitrogen was found in the samples.

Glycone Portion of Helminthosporoside—Helminthosporoside (300 $\mu$g) was refluxed in 0.3 ml of 0.5 $\times$ H$_2$SO$_4$ for 2 hours at which time an excess of BaCO$_3$ was added to the solution to neutralize the acid. H$_2$O (2 ml) was added to the suspension and it was centrifuged to remove the precipitate. The supernatant liquid was dried in a stream of warm air and the residue was treated with the silylation reagent and subjected to gas chromatography.

Two peaks with the same retention times as $\alpha$- and $\beta$-galactopyranoside were observed. Theoretically, 228 $\mu$g of galactose would be the amount recoverable from this experiment, whereas the actual value was 160 $\mu$g. The identity of galactose was confirmed by paper chromatography in Solvent a and in a system consisting of ethylacetate-pyridine-H$_2$O, 8:2:1 v/v. In both systems the unknown had $R_F$ values identical with authentic galactose. After neutralisation, acid-refluxed solutions of helminthosporoside were not phytotoxic.

The proton magnetic resonance spectrum of helminthosporoside run in D$_2$O is shown in Fig. 8. Table IV summarizes the integrals for each of the resonance peaks. Peak A, equivalent to 1 proton had the correct chemical shift for the proton at the anomeric carbon at a glycosidic linkage (13). Peak B (4 protons) was assigned to protons on carbon atoms having a secondary alcohol function (13). Three of these protons would be contributed by the galactose moiety of the toxin (13).

Two protons on the carbon atom with a primary alcohol function on the C-6 of galactose, and the proton on C-5 of the hexopyranose ring were collectively attributed to Peak Area C which integrated for 3 protons and had the correct chemical shift for this assignment (13). The protons on the $-\mathrm{OH}$ of sugars are readily exchanged for deuterium and thus not seen in a sample that is dissolved in D$_2$O.

Helminthosporoside-14C with a specific activity of 1.43 $\mu$Ci per mmole was isolated from a culture of H. sacchari that had been administered galactose-14C. After acid hydrolysis of 40 $\mu$g of the toxin and chromatography of the neutralized hydrolysate in Solvent a, at least 73% of the radioactivity was recovered as galactose. This lends further support for galactose as the glycone moiety and is an effective way of preparing labeled helminthosporoside for physiological studies.

Aglycone Moiety—The molecular weight of the aglycone moiety minus the glycosidic oxygen atom must be 57 if galactose was contributing 179 to the total molecular weight. The base peak at 73 in the mass spectrum was attributed to the aglycone fragment carrying the glycosidic oxygen atom, and the large peak at 57 was assigned to the aglycone fragment alone (Fig. 7).
Cleavage on either side of an oxygen atom involved in a linkage of this type is a well established phenomenon (14). Based on the empirical formula, a mass of 57 leaves only C₆H₅O for the aglycone fragment. The chemical shift position and the complex nature of absorption bands F and G in the NMR spectrum suggested that these protons were associated with a cyclopropane ring (Fig. 8). This assignment was in close agreement with those assignments reported for other cyclopropane compounds such as phenylcyclopropane and 1-methyl-1-phenylcyclopropane (15). Furthermore, each peak integrated for only 1 proton, indicating the presence of a functional group on the remaining carbon atom. This functional group could only be an —OH for reasons relating to empirical formula and spectral considerations. It was suggested that the proton on the carbon carrying the secondary alcohol functional group on the cyclopropane ring was located under Peak B, accounting for the 4th proton in that peak (Table IV). The proton on the carbon atom of the cyclopropane ring linked to the glycosidic oxygen was assigned to

### Table IV

| Peak | Shift position | Area |
|------|----------------|------|
|      | ppm | cm² | cm² |
| A    | 5.32 | 7.0 | 7.0 |
| B    | 4.10 | 28.0 | 28.0 |
| C    | 4.10–3.75 | 20.0 | 21.0 |
| D    | 3.0–2.3 | 7.5 | 7.0 |
| E    | 1.8 | 6.0 | 7.0 |
| F    | 1.6–1.35 | 8.1 | 7.0 |
| G    | 1.35–1.2 | 5.0 | 7.0 |
| H (impurity) | 0.9 | 3.5 | 0 |

*a* Refer to Fig. 8 for a reference for peak location in the NMR.
Peak Area D (Fig. 8). Peak Area D integrated for 1 proton (Table IV) and was heavily split by the protons on the adjacent carbon atoms of the cyclopropane ring as would be expected (15). The splitting of the spectrum of this proton was in agreement with that occurring in the spectrum of methyl-cyclopropyl-carboxylate (15); however, Peak D was shifted downfield because of an adjacent oxygen atom. The spike Peak E, integrating for 1 proton, was assigned to the proton on the —OH on the cyclopropane ring. After prolonged periods in D2O, the intensity of this peak diminished, which was consistent with the idea that this proton is exchangeable, but not readily so.

Paper chromatography of the residue, after hydrolysis of helminthosporoside (0.8 mg) in 1.0 ml HCl for 2 hours in Solvent a only yielded a spot corresponding to galactose when the chromatogram was treated with alkaline silver nitrate. In addition, no spots became evident on a similar chromatogram treated with the antimony trichloride reagent, indicating that hydrolysis was complete and the aglycone and products thereof were volatilized.

In addition to the NMR evidence for the presence of a cyclopropane ring as the carbon skeleton of the aglycone, the infrared spectrum showed a weak but detectable band in the region of 9.8 μ (16), further supporting this contention. The toxin reacted with the antimony trichloride reagent producing a reddish spot that fluoresced under ultraviolet light. However, this same reagent did not react with galactose and other free sugars, and several methyl glycosides tested produced a slightly yellowish reaction product. Furthermore, isopropanol and propanol did not react with this reagent and acrolein gave a brown reaction product. However, cyclopropyl derivatives, including cyclopropyl carboxylic acid, cyclopropyl nitrile, and cyclopropyl ethanol, all produced pink to reddish spots with this reagent and fluoresced under ultraviolet in a manner similar to that of the intact toxin.

That the cyclopropyl ring possessed a hydroxyl function was supported by the detection of a substance in acid hydrolysates with the same retention time as acrolein. Samples (5 μl) of the sulfuric acid hydrolysate of the toxin were directly subjected to gas chromatography on Poropak Q. Compounds with retention times of 2.9, 5.2, and 7.4 min were detected. The acrolein peak (2.9 min) accounted for approximately 25% of the volatile products detected. The presence of acrolein was expected as a product of acid hydrolysis of the toxin. Its presence was explained by an elimination of the hydroxyl proton on the cyclopropyl ring resulting in bond reorganization and eventual cleavage of the glycosidic bond. The other volatile products of acid hydrolysis were not identified.

Galactosidic Linkage Incubation of 200 μg of helminthosporoside with 0.05 μg of purified β-galactosidase from E. coli in 0.06 ml of 0.05 M phosphate buffer, pH 7.5, for 18 hours at room temperature yielded no free galactose. However, incubation of 590 μg of helminthosporoside with 40 μg of purified α-galactosidase in 0.4 ml of 0.23 M acetate buffer, pH 4.9, at room temperature yielded free galactose. At the end of the incubation period, the reaction mixture was passed through a small Dowex 50 (H⁺ form) column and rinsed with H2O. The effluent was taken to dryness and checked for the presence of galactose. The identity of galactose was confirmed by its Rf values, identical with authentic galactose in Solvent a and in ethylacetate-pyridine-H2O, 8:2:1 v/v, as well as the retention time of its silylated ether derivative by gas chromatography. The yield of galactose was less than 1% of the theoretical amount. No galactose was detected in a control flask incubated without the enzyme and treated in the same manner. From these results it is concluded that the galactosidic linkage in the toxin is α.

Structure of Helminthosporoside—Based on the available evidence, the proposed structure of helminthosporoside is 2-hydroxycyclopropyl-α-D-galactopyranoside as shown in Fig. 9. A study of the location of the hydroxyl group on the cyclopropane ring indicated that positions 2 and 3 were identical. Whether the hydroxyl group is cis or trans to the cyclopropane ring has not been determined.

**DISCUSSION**

Our results confirm the fact that H. sacchari produces two plant toxins (Table III) (5). One of these two toxins was isolated and shown to be pure based on chromatographic tests. The final product of purification produced symptoms on leaves in amounts as small as 5 x 10⁻¹⁴ moles. Unlike many of the previous reports on the instability toxins from the genus Helminthosporium (1), the toxin of H. sacchari was stable, which allowed for structural studies to be made on it.

The establishment of a straight line relationship between runner length and toxin concentration was important in determining the effectiveness of the purification procedures. The development of symptoms (runner length) in this test was directly proportional to the log of the concentration of the toxin applied to the leaf (Fig. 5). This observation implied that the toxin may affect some portion of the plant cell, such as the vacuole, causing the release of degradative enzymes (17), ultimately producing greater cell damage and accounting for the logarithmic effect. Some confirmation of this hypothesis has recently been provided by electron microscopic studies of toxin-treated sugar cane leaves in which Hess' has shown that helminthosporoside damages cellular membranes.

The absence of nitrogen showed that the toxin was not a peptide or a heterocyclic nitrogen-containing compound as is implied to be involved in the structures of other host-specific toxins. Nevertheless, it is possible that the other toxin (Fig. 2, Peak 2) produced by H. sacchari is an amino acid or peptide derivative of the toxin that was characterized (Fig. 2, Peak 1).

Some concern was expressed that the various steps used in toxin purification may have resulted in the production of an artifact possessing toxic activity. Artifact production may have occurred at the steps of paper chromatography, since highly acidic and basic conditions were present. This concern was
dispelled when it was learned that the purified toxin had the same elution volume from Sephadex G-15 as did the biological activity in an earlier step of purification (Fig. 2). Furthermore, the purified toxin had the same biological specificity to sugar cane clones as did the pathogen H. sacchari (Table II).

Based on the available spectroscopic data and chemical evidence, the structure of the host-specific toxin of H. sacchari studied is proposed to be 2-hydroxycyclopropyl-α-D-galactopyranoside (Fig. 9). Evidence for galactose being the glycone portion of the toxin is based on its identification by several chromatographic means after acid hydrolysis of the toxin. Furthermore, galactose-1-14C was incorporated into the glycone portion of the toxin. That the aglycone portion of the toxin is 2-hydroxycyclopropane resides principally with NMR spectral data. According to Bhacca, Johnson, and Shoolery (15) and Coffey (16), single highly split proton resonance peaks with a chemical shift in the NMR spectrum around 1 ppm are distinctive for protons on a substituted cyclopropane ring (Fig. 8, Peaks F and G). No other arrangement of 3 carbons, 5 hydrogens, and 1 oxygen could account for these other than that for this assignment.

Furthermore, the reddish reaction product of the toxin with the antimony trichloride was identical to that obtained with the toxin alone. Chemical evidence for the presence of a hydroxyl group on the aglycone since galactose itself did not react with this reagent. The reaction of several cyclopropyl compounds with antimony trichloride was identical to that obtained with the toxin alone. The reaction of several cyclopropyl compounds with antimony trichloride was identical to that obtained with the toxin alone.

Acknowledgments—The authors wish to thank Drs. Bradford Mundy and P. Jennings of the Chemistry Department, Montana State University, who assisted in the interpretation of spectroscopic data. In addition, the help of Dr. G. Baker, Department of Chemistry, Florida Technological University of Orlando, Florida, is also acknowledged.

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*J. Biol. Chem.* 1971, 246:4350-4357.

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