

Effects of Toxin from *Helminthosporium sacchari* on Nongreen Tissues and a Reexamination of Toxin Binding

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Research was supported by NSF grants PCM-7611916 and PCM-8100711.

Michigan Agricultural Experiment Station Journal Article I0157.

Accepted for publication 24 November 1981.

ABSTRACT

Lesney, M. S., Livingston, R. S., and Scheffer, R. P. 1982. Effects of toxin from *Helminthosporium sacchari* on nongreen tissues and a reexamination of toxin binding. *Phytopathology* 72:844-849.

Green leaves, etiolated shoots, and roots from susceptible clones of sugarcane were sensitive to toxin from *Helminthosporium sacchari*; comparable tissues of resistant clones were not affected. Indications from previous work were that the toxin first affects chloroplasts, and that chloroplasts may be required for other effects. Our results indicate toxic effects on green and nongreen tissues and no requirement for functional chloroplasts. Next, using the established methods, we repeated earlier work on toxin binding. Equilibrium dialysis of ¹⁴C-toxin against membrane preparations gave no indication of toxin binding to preparations from

either resistant or susceptible tissues. Also, there was no indication of toxin binding to high-molecular-weight materials from membrane preparations of susceptible and resistant clones incubated with ¹⁴C-toxin and then fractionated on gel columns. Thus, the mode of toxic action remains an open question. When proper precautions were not taken, quenching of counts and chemiluminescence proved to be problems with preparations of this type. Binding may be involved in toxic action, but a toxin preparation with a more active label than any used to date may be required to prove it.

The eyespot disease caused in sugarcane (*Saccharum officinarum* L.) by *Helminthosporium sacchari* (Van Breda de Haan) Butler, has been used as a model for studies on the molecular basis of disease development and disease resistance in plants (12-14). *H. sacchari* produces in culture a compound that is selectively toxic to sugarcane clones that are susceptible to infection (9,11). With a few possible exceptions, there is a correlation between relative tolerance of clones to the toxin and relative resistance to the fungus (9). The correlation was evident in assays based on visible effects of toxin and on toxin-induced

leakage of electrolytes. Other workers have reported that membrane preparations from susceptible tissues will bind ¹⁴C-labeled toxin, whereas preparations from resistant tissues do not bind toxin (12). The reported binding phenomenon was the basis of a hypothesis concerning action of the toxin (12,14).

There are also claims that toxin acts on chloroplasts in situ (17) and that this action may culminate in leakage across the plasma membrane (7). If so, the toxin of *H. sacchari* (HS toxin) differs in site and mode of action from other host-selective toxins, which affect all tissues of the susceptible genotypes, including roots and etiolated shoots. *H. sacchari* normally infects only the green tissues of its host. Nongreen tissues have not been tested previously for sensitivity to toxin.

The purposes of this study were: to test nongreen tissues for sensitivity to toxin; and to reexamine the reported correlation between ability of cellfree preparations to bind toxin and

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0031-949X/82/07084406/\$03.00/0

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susceptibility of source plants to the toxin and to the fungus. Two susceptible and two resistant clones of sugarcane were used as sources of cellfree preparations from green leaves, etiolated shoots, and young roots. Sensitivity to toxin was determined by measuring toxin-induced leakage of electrolytes, the most quantitative measure of toxin sensitivity available at this time (9).

MATERIALS AND METHODS

Plants. Sugarcane clones 51NG-97 (susceptible to *H. sacchari*), H52-4610 (resistant), and H50-7209 (resistant) were obtained from G. A. Strobel of Montana State University at Bozeman. Clone Co453 (susceptible) was obtained from J. L. Dean of the USDA Sugarcane Research Station, Canal Point, FL. Plants were grown in 18.9-L (5-gal) containers in the greenhouse at temperatures above 18 C. Young, but fully expanded, leaves were used in all experiments that required green tissue. Etiolated shoots were grown from mature buds on stem sections; the stem pieces were planted in vermiculite and the plants were grown in the dark at approximately 22 C. The vermiculite was initially saturated with White's solution (2) and subsequently watered with distilled water. Etiolated shoots were harvested when they were 8–12 cm tall. Root tissues were obtained from young plants grown in the greenhouse in trays containing vermiculite.

Stock cultures of *H. sacchari* were maintained on cane leaf agar (18). The isolate used in this work was obtained from J. L. Dean. The isolate was comparable to many others in pathogenicity and toxin production (9–11).

Purification of toxin. Toxin was produced and highly purified, as previously described (6,9). The usual bioassay (9) was used to guide the procedure and to insure a highly active preparation. Toxin samples were weighed and appropriate dilutions were made. ^{14}C -toxin was produced by a method similar to that used by Strobel (12). *H. sacchari* cultures were grown at 21–23 C in still culture in 1-L Roux bottles, each containing 200 ml of modified Fries' medium supplemented with 0.1% yeast extract (8). After 14 days, an aliquot was removed from each bottle and assayed for toxin by the electrolyte leakage method (9). The culture medium in each of the five bottles with the highest toxin assays was then drained and 100 ml of a fresh medium was added aseptically. The replacement medium for each bottle contained 0.5 g ammonium tartrate, 0.1 g monobasic potassium phosphate, 1.0 g nonlabeled sucrose, and 0.1 mCi of [^{14}C]-sucrose (602 mCi/mmol). The cultures were then incubated at 21–23 C in still culture for 12–14 days; bioassays of small samples were used to determine the optimum time for harvest.

The five cultures were harvested by filtration, first through cheesecloth and then through Whatman No. 1 filter paper. The culture filtrate was concentrated at 35 C under reduced pressure to 0.1 the original volume. Charcoal (2 g, Norit A) was added and the preparation was held overnight at 4 C, with constant stirring. Toxin was eluted from the Norit as described previously (6). The preparation was concentrated to 0.75 ml and chromatographed on an LH-20 column (110 × 1.0 cm) with 50% methanol as the solvent. Fractions (30–35, 2 ml each) containing toxin were combined, concentrated, and chromatographed on an SP/QAE column (6). Three to 4 mg of labeled toxin were obtained from 500 ml of replacement culture filtrate, as determined by comparing the dosage-response curve with that of a weighed sample of purified toxin run concurrently. The specific radioactivity of toxin used in one set of experiments was 0.12 nCi/ μg , and in a second set of experiments was 0.48 nCi/ μg . This is approximately fourfold to 16-fold greater than the radioactivity of toxin used in previous studies (12). Toxin was stored in 50% methanol at -15 C.

Radioactivity measurements. Radioactivity was determined with a liquid scintillation counter (Beckman LS 7000 Microprocessor). Samples (0.5 ml) from columns were mixed with 0.3 ml of methanol; scintillation fluid (14 ml of Aquasol from New England Nuclear Co., Boston, MA 02118) was then added. Methanol was not used in the equilibrium dialysis experiments. Scintillation counts were monitored for 2–10 min, to at least the 95% confidence level, with both channel 1 (low-energy range) and

channel 2 (^{14}C peak) of the instrument.

Assays for toxin sensitivity. Sensitivity of leaf, shoot, and root tissues to toxin was determined by a procedure based on toxin-induced leakage of electrolytes from leaf tissue. This is the same procedure used for bioassay of toxin (9). For green leaves, each assay vial contained eight leaf disks, cut with a 1-cm-diameter cork borer. For nongreen tissue, each assay vial contained eight pieces (2 × 5 mm) of tissue from immature, etiolated shoots, or 20 root tip segments, each 1 cm long. Disks or segments were randomly selected for each vial.

Isolation of membranes and membrane proteins. Cellular membranes from several different sugarcane clones were prepared by the methods used by Strobel (12). The preparations were obtained from green leaves, etiolated shoots, and adventitious roots (tip ends, 6 cm long). The same ratios of tissue to buffer (4 g:30 ml) were used in all isolations. Tissues were ground in tris buffer (0.05 M, pH 7.2) and centrifuged at 500 g for 10 min. The pellet was discarded and the supernatant solution was centrifuged again at 48,000 g for 20 min. The pellet was resuspended in tris-HCl buffer (0.01 M, pH 7.2) or in tris buffer with Triton X-100 (1.0%). In some cases, trichloroacetate (1.0 M) was substituted for Triton X-100 in the isolation procedure (15). The preparation was used in equilibrium dialysis experiments and as a source for preparation of membrane proteins.

Protein to be used in toxin-binding experiments was prepared as described by Strobel (12). The membrane preparation in tris-Triton solutions was incubated in an ice bath for up to 4 hr, then centrifuged at 40,000 g for 10 min. ^{14}C -toxin was added to the supernatant solution, incubated on ice for 8 hr, and chromatographed (descending) on a BioGel P-100 column. In some experiments, the preparations were incubated at 30 C rather than on ice, with the same results. A 95 × 1.5-cm column was used in the earlier experiments; a shorter column (24 × 1.5 cm) gave comparable results and was used in all the later experiments. Each eluate fraction from the column was 2 ml, and radioactivity in each fraction was determined from a 0.5-ml aliquot. Bound toxin should be eluted in the early, high-molecular-weight fractions. Free toxin was eluted in later fractions, as determined by scintillation counting of pure toxin preparations passed through the column. In some experiments, the fractions were oxidized according to a standard procedure using a 1:2 ratio of 60% perchloric acid and 30% H_2O_2 .

The initial differential centrifugations of green leaf homogenates gave preparations that were dark green. The green color was retained in the early (high-molecular-weight) fractions from BioGel

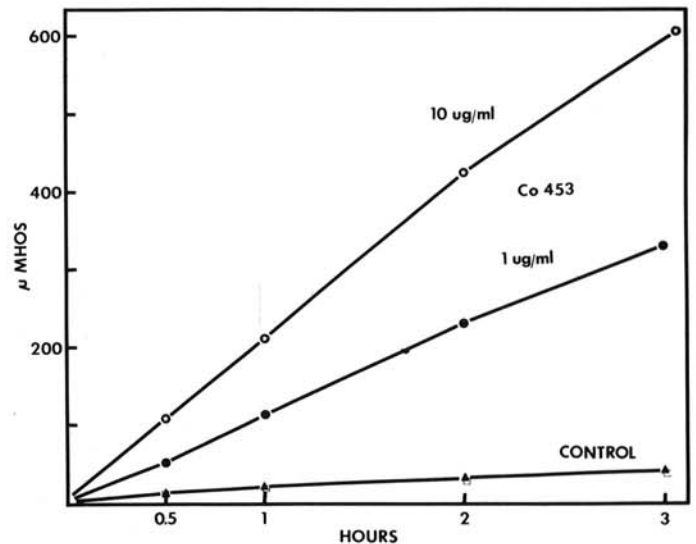


Fig. 1. Toxin-induced leakage of electrolytes from etiolated shoot tissues of sugarcane clone Co453, which is susceptible to *Helminthosporium sacchari*. Tissues were exposed to toxin at 0, 1.0, and 10.0 $\mu\text{g}/\text{ml}$ for 1 hr, rinsed, and placed in water at time 0. There was no toxin-induced leakage from a resistant clone (data not shown). Total disruption of these tissues by freezing and boiling give a solution with 2,050 μMHOS conductance.

P-100 columns, as reported previously by Kenfield (3). These fractions were described elsewhere as "purified binding protein" (12). Comparable preparations from etiolated shoots and roots were brownish in color.

Equilibrium dialysis methods. A standard dialysis chamber (2 ml, total volume) was used with membrane preparations from green leaves, etiolated shoots, and roots. Cell membrane suspensions (0.5 ml) were placed on one side of the dialysis membrane (exclusion limit, 6,000 mol wt; from Technilab Instruments, Pequannock, NJ 07440) and solutions containing various concentrations of ^{14}C -toxin were placed on the other side. Dialysis was at 22–23 C with gentle rotary shaking for 12–14 hr, to obtain equilibrium. Bound toxin was estimated from differences in counts in the half-cells before and after equilibrium dialysis (12). When preparations from green leaves were used, radioactivity in the green side of the dialysis membrane could not be determined because of the viscous nature and the dark green color, which

TABLE 1. Equilibrium dialysis assays to determine possible binding of ^{14}C -toxin to membrane preparations from susceptible (S) and resistant (R) sugarcane^a

Source clone		Total added ^b	Counts per minute		Percent in toxin half-cell ^e
			Toxin half-cell ^c	Membrane half-cell ^d	
51NG-97	(S)	4,600	2,183	—	48
51NG-97	(S)	823	459	—	56
51NG-97	(S)	823	425	—	52
H52-4610	(R)	823	438	—	53
H50-7209	(R)	1,320	578	—	44
Co453	(S)	1,320	572	—	43
Co453 (roots)	(S)	1,320	578	538	52
Co453 (etiolated)	(S)	1,320	592	632	48

^aTissue (4 g) was homogenized in 50 mM tris-HCl buffer (pH 7.2), filtered, centrifuged at 500 g to remove debris, then centrifuged at 48,000 g. Pellets were resuspended in 0.75 ml of tris-HCl (10 mM, pH 7.2). Green leaves were used except as indicated.

^bInitial CPM for ^{14}C -toxin (specific activity, 0.12 nCi/ μg) placed in the toxin side of the half-cell.

^cFinal CPM for ^{14}C -toxin in the half-cell.

^dFinal CPM for the half-cell containing membrane preparation. Preparations from green leaves could not be counted because of quenching; these are designated by minuses (—).

^ePercent of total counts that remained in the toxin half-cell. The counts varied $\pm 3\%$.

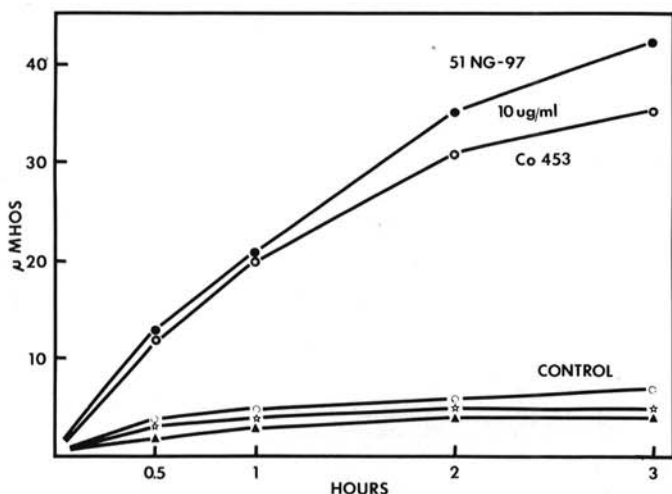


Fig. 2. Toxin-induced leakage of electrolytes from young roots of sugarcane clones that are susceptible and resistant to *Helminthosporium sacchari*. Clones were 51NG-97, toxin treated (●), and control (○); Co453 toxin-treated (▲) and control (☆); and H50-7209 toxin-treated and control (▲). Toxin was used at 10 $\mu\text{g}/\text{ml}$.

caused severe quenching. When preparations from etiolated shoots and roots were used, radioactivities on both sides of the dialysis membrane were determined.

Lipid extractions. Sugarcane tissues (3 g) were ground in a chloroform-methanol (4:1) mixture with a Sorval Omni-Mixer, in an ice bath. The aqueous phase was discarded and the chloroform-methanol phase was concentrated to 3–4 ml. Lipids from crude membrane preparations and pooled green fractions from a BioGel P-100 column (24 \times 1.5 cm) also were extracted with chloroform.

RESULTS

Toxin-induced leakage of electrolytes from roots and etiolated shoots. Etiolated shoots from a susceptible clone (Co453) and two resistant clones (H52-4610 and H50-7209) were tested for sensitivity to nonradioactive, highly purified toxin (6). Tissue sections were held in toxin solutions (10 $\mu\text{g}/\text{ml}$) for 1 hr, then were washed and monitored for 3 hr for electrolyte leakage. Toxin caused significant leakage from tissues of the susceptible clone, but not from tissues of the resistant clone (Fig. 1). Root tip segments were also incubated with toxin, washed, and monitored for leakage. Toxin caused significant efflux of electrolytes from roots of both susceptible clones (Co453 and 51NG-97) used in the experiment (Fig. 2). There was no toxin-induced leakage from roots of the resistant clone (H50-7209). Variability in these data was comparable to that given in an earlier report (Fig. 4 in ref. 9). Thus, roots and etiolated shoots from susceptible sugarcane had the same relative sensitivity to HS toxin as did the green leaf tissue used in all earlier work.

Equilibrium dialysis experiments. Differential centrifugation was used to prepare cell membranes from green leaves, etiolated shoots, and young roots. Preparations from the two susceptible and the two resistant clones were tested by equilibrium dialysis for evidence of toxin binding. ^{14}C -toxin was added to one side of the membrane in the half-cell, and plant preparations were added to the other. If binding occurred, there should have been a disproportionate number of counts (<50%) in the side of the half-cell without membranes. The experiment, repeated many times, gave no evidence that toxin was bound to the plant membrane preparations (Table 1). Comparable negative results were obtained

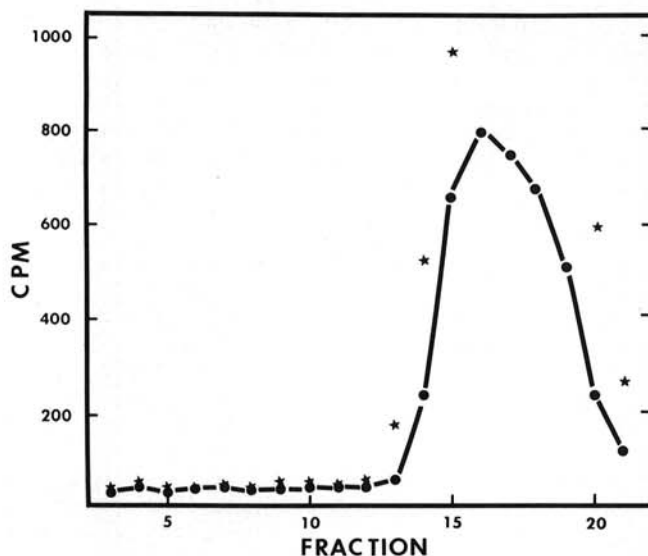


Fig. 3. Column chromatography of toxin after incubation with dissolved membrane preparations from etiolated shoots of sugarcane clones that are resistant (H52-4610, ●) and susceptible (Co453, ☆) to *Helminthosporium sacchari*. Membrane preparations from 13 g (susceptible) or 6.5 g (resistant) of tissue were incubated with ^{14}C -toxin (6 nCi for susceptible and 3 nCi for resistant; specific activity, 0.12 nCi/ μg) prior to chromatography on a BioGel P-100 column (24 \times 1.5 cm). Each fraction was 2 ml; void volume was determined with Blue Dextran 2000, which gave a peak in fraction 7. The large peaks represent unbound toxin. Counts were taken to the $P = 0.05$ level.

regardless of the source clone (resistant or susceptible) or type of tissue. When preparations from green leaves were used, counts were taken on only one side of the dialysis membrane, because the preparation caused severe quenching; this was not a problem with preparations from roots and etiolated shoots. The equilibrium dialysis data showed no measurable binding, either firm or reversible.

Tests for toxin binding to membrane components from nongreen tissue. Crude membrane preparations were obtained, by differential centrifugation, from etiolated shoots of each of the two susceptible and the two resistant clones. These preparations were incubated with ^{14}C -toxin, then chromatographed on BioGel P-100 columns. Eluate fractions showed no evidence that toxin was bound or associated with membrane components, regardless of the source clone (Fig. 3). Membrane proteins also were prepared from young roots of clones 51NG-97 and H52-4610, incubated with ^{14}C -toxin, and chromatographed. The results (Fig. 4) were essentially the same as for preparations from etiolated shoots. The large peaks in fractions 18 to 20 from all P-100 columns (Figs. 3 and 4) result from the free ^{14}C -toxin. These experiments were repeated several times, with similar results. Trichloroacetate rather than Triton X-100 was used to prepare membranes in one experiment with etiolated tissue; again, assay of fractions from the column gave no indication of toxin binding.

Tests for toxin binding to membrane components from green tissue. Membrane preparations from green leaves of each of the four sugarcane clones were incubated with ^{14}C -toxin and chromatographed on columns as described above. In some experiments, fractions from the column were oxidized as recommended for scintillation counting; in other experiments the fractions were not oxidized. In all cases, both channels of the scintillation counter were used. Counts on channel 2 (^{14}C -peak) gave no evidence that toxin was bound or associated with membrane components, regardless of the source clone (resistant or susceptible) or treatment of the fraction. The experiment was repeated many times, following the procedure described elsewhere (12,13) and with other variations. The results were always comparable to those given in Figs. 3 and 4. The experiment was also repeated with an excess of ^{14}C -toxin, again with comparable results

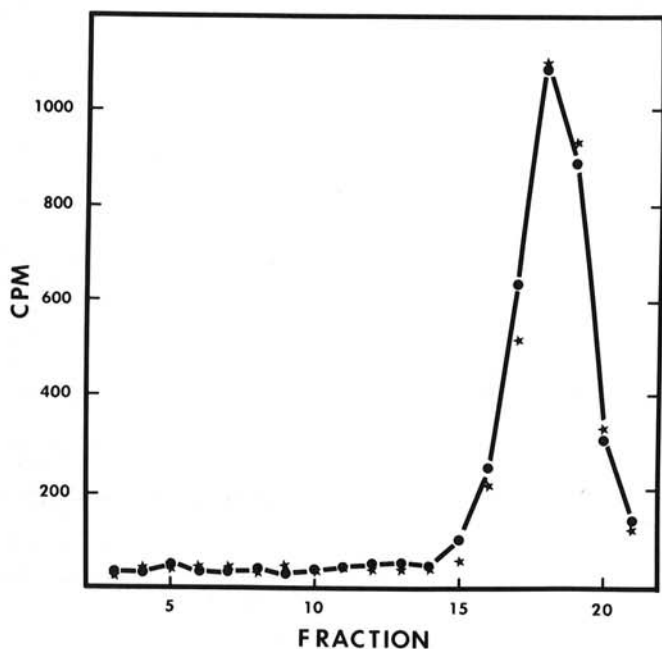


Fig. 4. Column chromatography of toxin after incubation with dissolved membrane preparations from young roots of sugarcane clones that are resistant (H520-7209, ★) and susceptible (Co453, ●) to *Helminthosporium sacchari*. Membrane preparations from 8 g of tissue were incubated with ^{14}C -toxin (3 nCi; specific activity, 0.12 nCi/ μg). Other conditions of the experiment are given in Fig. 3.

(Fig. 5). With unoxidized fractions, channel 1 always gave very high chemiluminescence counts (>1000 cpm) for the fractions containing chlorophyll (especially fractions 7 and 8). When fractions were oxidized as recommended, the counts with channel 1 sometimes were at the background level. However, the samples often did not appear to be completely oxidized and after brief exposure to diffuse light, gave counts that were above background. Preparations from susceptible and resistant clones gave comparable counts in all cases. When ^{14}C -toxin solutions with known counts were added directly to the scintillation fluid, the expected counts were obtained with both channels of the instrument.

The high counts associated with the green fractions were examined further. Counts were taken before and after column chromatography and on membrane preparations that had not been exposed to ^{14}C -toxin. The channel 1 counts for fractions 7 and 8, which contained chlorophyll, were equally high for preparations that were incubated with and without ^{14}C -toxin. Also, preparations from corn leaves gave comparable results, regardless of toxin exposure. The phenomenon was light-dependent; high counts were evident in the active fractions that were prepared and stored in the light. Incubation of the fractions in the dark for 6 hr and dark transfer to the scintillation counter reduced counts to near the background level. Counts for the fractions containing free ^{14}C -toxin were not affected by light (Fig. 5). Reexposure of the green fractions to light restored the counts; low light intensity resulted in low counts and high light intensity resulted in high counts. Lipid extracts from the green membrane fractions also gave comparable counts. Obviously, in experiments of this kind, precautions must be taken against chemiluminescence or photoluminescence.

Tests with leaf lipids. Lipid extracts from green leaves of resistant

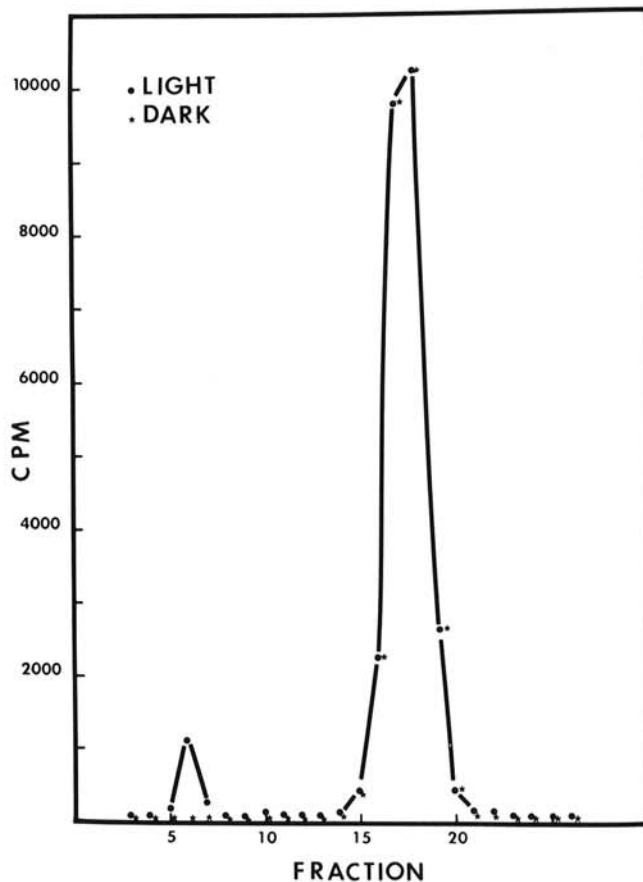


Fig. 5. Column chromatography of toxin after incubation with dissolved membrane preparations from green leaves of susceptible sugarcane (clone Co453). Preparations from 4.0 g of leaves were incubated with ^{14}C -toxin (76 nCi; specific activity, 0.48 nCi/ μg); other conditions of the experiment are given in Fig. 3. Unoxidized fractions from the column were incubated in light (●) or in darkness (★) prior to counting.

and susceptible clones were incubated briefly in the light with ^{14}C -toxin. Control samples were treated the same, except that no ^{14}C -toxin was in the solution. The leaf lipids gave high counts with scintillation channel 1, even when no ^{14}C -toxin was in the preparation. Incubation for 6 hr in the dark did not entirely eliminate chemiluminescence counts. When channel 2 (^{14}C peak) was used, only the preparations containing ^{14}C -toxin gave counts above background; there were no differences between vials held in light or in darkness, or for lipids from resistant and susceptible clones (Table 2). Thus, all the light-induced counts were in the lower energy level covered by channel 1, as was the case with the membrane preparations. This is typical of chemiluminescence, according to the manufacturers of the scintillation fluid. The experiment shows that the light-sensitive counts are associated with the lipophilic fraction; there would be little or no water-soluble protein in this fraction. The findings are consistent with the fact that most of the plant pigments (chlorophyll and carotenoids) likely to be sources of chemiluminescence are lipid-soluble.

DISCUSSION

The green leaf is the usual site of infection by *H. sacchari*, and the leaf is sensitive to the host-selective toxin. However, etiolated shoots and roots of susceptible clones are as sensitive to HS toxin as are green tissues, when sensitivity is determined by toxin-induced leakage of electrolytes. Green and nongreen tissues from resistant clones are equally tolerant of the toxin. It is likely that the fungus will infect nongreen tissues of susceptible clones, although this apparently has not been reported. *H. victoriae*, *H. carbonum* race 1, and other *Helminthosporium* species are known to infect roots, coleoptiles, and leaves of susceptible genotypes; the toxins from these fungi affect the same tissues. There is no evidence that *H. sacchari* and its toxin differ from the others in this respect.

Previous work on HS toxin has shown an early effect on chloroplasts (17). Other workers suggested that functional chloroplasts may be needed for toxin-induced leakage of electrolytes (7). However, our data show a selective toxic effect on nongreen tissues of susceptible clones of sugarcane. Obviously, a functional chloroplast is not required for toxic action, even though the chloroplast may be affected in green tissue.

The basic conclusion from previous work (12) was that susceptibility of sugarcane clones is correlated with the unique ability of cellfree preparations from susceptible leaves to bind ^{14}C -toxin. The conclusion was based primarily on equilibrium dialysis data. Unfortunately, the only data given were scintillation counts for the free-toxin side of the dialysis membrane. The usual procedure, with less chance for error, is to measure radioactivity on both sides of the membrane. We attempted to repeat the work, using the same procedures except that counting was attempted on both sides of the dialysis membrane. However, we obtained a dense "cell-membrane" slurry which gave severe quenching of channel 2

counts, and extra counts from chemiluminescence with channel 1 of the scintillation counter. Thus, accurate estimates of toxin binding by equilibrium dialysis were impossible to obtain. Preparations from nongreen tissue gave less quenching and no chemiluminescence. When samples were oxidized, chemiluminescence counts were reduced, but not always eliminated. We failed to confirm the previous claims (12) in any of the experiments, which were repeated many times. More sensitive techniques or much higher levels of radioactivity in the toxin preparations are needed for conclusive results.

We tried without success to confirm previous reports (12, 14) that ^{14}C -labeled HS toxin will bind in vitro to protein preparations from membranes of susceptible, but not resistant, sugarcane. The exact methods used in previous work, plus variations, were followed. We found that all the green leaf preparations, which were said to bind toxin, also exhibited photoilluminescence or chemiluminescence, whether or not toxin had been added. If precautions are not taken, this is a possible source of error in ^{14}C -toxin binding experiments, because it can give spurious scintillation counts. In previous work (12) the fractions and dialysis samples were always green (3), and they were not oxidized prior to counting. However, we have no evidence that chemiluminescence will account for the difference between our results and those of Strobel. His data showing very low counts for resistant preparations are not expected if chemiluminescence is the basis.

The impurities in protein preparations from membranes pose problems for accurate determination of toxin binding, whether channel 1 or channel 2 of the scintillation apparatus is used. Channel 1 can give spurious counts from chemiluminescence, even when samples are oxidized. Even 6 hr in the dark did not completely eliminate counts from chemiluminescence (Table 2). Channel 2 eliminates the chemiluminescence problem, but quenching is a problem. Plant materials, especially pigments, are known to cause such quenching. Thus, the use of impure membrane proteins, such as those described so far (12), would preclude an accurate determination of toxin binding.

Kenfield (3-5) recently described a new procedure that gave a cleaner protein preparation than did Strobel's procedure. Strobel reported that 4 g of tissue gave 250 μg of binding protein (12); we found (*unpublished*) that chloroform will give a heavy precipitate from such a preparation. Kenfield's procedure gave 70-80 μg of protein from 100 g of leaf tissue. Kenfield's binding protein had negative cooperativity, with no further binding of toxin at concentrations $>1.0 \mu\text{g}/\text{ml}$. In contrast, Strobel reported strong binding effects at concentrations up to at least 340 $\mu\text{g}/\text{ml}$ (12). If Kenfield had a binding protein that is involved in toxic action, then it is unlikely that meaningful toxin binding to the cruder preparations would be detectable. The conclusion that a selective protein is the receptor or binding agent for HS toxin is based entirely on data obtained with Strobel's preparations (12-14).

The several discrepancies described above, plus the failure to confirm toxin binding to plant preparations, raises doubts about prior claims concerning action of HS toxin. All conclusions must now depend on the recent work by Kenfield (3-5), who reported that toxin-binding proteins can be isolated from mint, tobacco, wheat, barley, beet, and from one susceptible clone of sugarcane. Proteins isolated by the same procedures from corn, potato, and one clone of resistant sugarcane did not bind toxin (3,5). The putative binding protein, which still contains impurities, was said to bind α -galactosides. Tests for biological activity, such as those reported in Strobel's earlier work (12), have not been done with the more purified protein.

There is an additional factor to consider in the toxin-binding experiment. Toxin was first reported to be an α -galactopyranoside (11), which fits with the claim that toxin acts by binding to an α -galactoside receptor protein (12, 14, 16). Toxin is now known to be a β -galactofuranoside (1,6), which may not bind well to a receptor for α -galactosides.

The mechanism of action of HS toxin is still an open question. There must be a receptor or at least a sensitive site for the toxin, but it is unlikely that the site can be detected by the methods used to date. The site is not necessarily in the plasma membrane, nor is the

TABLE 2. Chemiluminescence or photoluminescence of chloroform-methanol extracts of green sugarcane leaf tissues from susceptible (51NG-97; Co453) and resistant (H50-7209; H52-4610) clones. No ^{14}C -labeled toxin was added

Source clone ^a	Channel 1 cpm for		
	Diffuse light ^b	Bright light ^c	Darkness ^d
51NG-97	164	836	36
Co453	260	853	119
H50-7209	398	3419	75
H52-4610	168	2757	67
Aquasol blank	37	41	30

^aTissue (3 g) was extracted, concentrated to 0.4 ml, and added to 10 ml Aquasol for counting with channel 1 of the scintillation counter. No counts above background were recorded with channel 2 (^{14}C peak).

^bMembranes were prepared and vials were held under normal room lighting.

^cVials were exposed to bright light for 2 min before counts were taken.

^dVials were held in darkness for 6 hr before counts were taken.

toxin necessarily tightly bound. Our data illustrate some of the complexities and potential pitfalls involved in such attempts to determine sites of action. A toxin preparation with much higher radioactivity than any used to date will be required. It is doubtful that sufficient radioactivity can be introduced by growing the fungus on ¹⁴C-labeled sugar.

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