Aldehyde Traps

As Antisporulants for Fungi

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BULLETIN OF THE CONNECTICUT AGRICULTURAL
EXPERIMENT STATION, NEW HAVEN • NO. 694, MAY 1968

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Aldehyde Traps As Antisporulants for Fungi

James G. Horsfall and R. J. Lukens[1]

We would like to discover a practical compound that would aid the control of a fungal plant disease by inhibiting sporulation of the causal fungus. We hope to fill in a gap between the two methods of disease control that presently are used by farmers, (a) to "eradicate" the fungus in diseased tissue, and (b) to protect the healthy plant by killing spores in the infection court. In this research we assume that the fungus has escaped both of these attacks on it. It has established an infection and it has not been killed in the lesion. We assume then, that it is ready to sporulate and set off a new round of disease. Can a living fungus be inhibited from sporulating? Specifically, can a fungus be inhibited from forming conidia?

The modern emphasis in research on plant protectants is to discover specific compounds, i.e., compounds that attack only the target organism, or a specific target in an organism. Since fungi produce conidia, and green plants do not, we have looked for compounds that specifically affect conidiation, that is, we have looked for antisporulants.

This paper is the tenth in a series of reports on antisporulants. It will extend the work reported elsewhere (Horsfall and Lukens, 1966 and 1967, Lukens and Horsfall, 1968) on the hypothesis (a) that the glycolic acid route of respiration is important in producing the energy whereby a mycelial fragment forms a spore almost as large as it is, and (b) that to block the process at any stage is to block sporulation.

We have shown recently (Lukens and Horsfall, 1968) that numerous relatives of glycolic acid will block the oxidation of glycolic acid to glyoxylic acid and will inhibit sporulation. In this paper we test the hypothesis further. Glyoxylic acid is an aldehyde. Presumably, then, sporulation can be inhibited also by blocking the reaction beyond the glyoxylate step with compounds that can react with aldehydes. These compounds may be described as aldehyde traps.

Hydrazines, being classical aldehyde traps, have been used extensively in this study to test the hypothesis.

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[1] Our thanks go to Miss Barbara Wooding for her enthusiastic assistance in following the leads turned up in this research and to Dr. A. E. Dimond and Dr. Saul Rich for numerous suggestions and for reading the manuscript.

The proposed reaction of glyoxylic acid in the cell with a hydrazine-type compound would proceed as follows:

$$\overset{\overset{\displaystyle O}{\|}}{HC}\text{-}COOH + R\text{-}NH\text{-}NH_2 \rightarrow R\text{-}NH\text{-}N = CH\text{-}COOH + H_2O$$

The use of aldehyde traps as antifungal compounds is not new. Several years ago (Horsfall and Zentmyer, 1944) we tested several aldehyde traps as inhibitors of spore germination and reported some action from phenylhydrazine, α-naphthylhydrazine, and benzenesulfohydroxamic acid. We have worked with aldehyde traps from time to time ever since. The possibility that the antifungal action of phenylhydrazine is due to a reaction with the aldehyde group of glucose was discussed by Horsfall (1945). This possibility has a serious practical weakness, however. There is too much glucose to trap.

Reid (1958) was able to inhibit sporulation of *Fusarium* in culture with two aldehyde traps, semicarbazide and sodium bisulfite. He suggested that the trapped aldehyde was glucose. Reid's paper encouraged us to reenter the field and test aldehyde traps as possible antisporulants.

Materials and Methods

The compounds were obtained from commercial sources including many from the Eastman Kodak Company. They were tested without further purification. If this project were to go, we had to find a method that would differentiate between killing the fungus and inhibiting its conidiation. In earlier papers (Horsfall and Rich, 1955) we had used the poisoned food technique, in which these two processes were but poorly separated.

While investigating the sporulation of a hitherto recalcitrant organism, *Alternaria solani*, Lukens (1960) found that the fungus produces conidiophores in the light, conidia in the dark. His method is to grind with a Waring blendor the hyphae produced in shake-culture. The resulting fragments of mycelia are washed in deionized water, centrifuged to free them of nutrients, and then spread over clean filter papers in petri dishes and placed in the light. The fungus, being drastically starved, produces no additional mycelium but conidiophores in great abundance within 24 hours. The organism thus follows Kleb's law that fungi sporulate under conditions of low nutrition. In the field, *Alternaria solani* presumably follows the same law because the major sporulation is in or near the center of the lesion where presumably nutrients are low.

If conidiophores so produced are then placed in the dark, masses of conidia are formed overnight.

The test compound is dissolved in a suitable solvent at 1000 ppm and diluted to give four doses as shown in the data tables. Duplicate pieces of filter paper are dipped into each test concentration and dried. Most compounds were tested on two or more separate days. Thus, each point in the data tables represents a minimum of the four tests of each concentration.

It should be remembered that this test will fail to give a proper assay of volatile compounds that disappear from the test paper during drying.

At the time of treatment the dried papers are placed in separate petri dishes and wetted with a few drops of distilled water. Similar-sized filter papers bearing the conidiophores are placed on top of the test papers and incubated overnight in darkness. The spore production is read next morning.

A treated conidiohore may either (a) collapse and die, (b) produce no spore, (c) produce a spore, or (d) produce an extension which may be of conidiophore cells or hyphal cells. The last criterion distinguishes life from death of cells of the conidiophore. Thus (d) coupled with (b) enables us to examine the physiology of spore differentiation and to discover compounds that will inhibit sporulation without killing the fungus.

The beauty of the method is that the treatment can be withheld until conidiophores are formed. Thus, we can assay the effect of the test compound on the physiology of spore differentiation as distinct from the physiology of mycelial growth, or the physiology of conidiophore formation.

Now that we have worked with the method for some time, we note another unique feature. It is useful for studying fungal translocation. In most bioassays the chemical route is short because the test chemical bathes the spore or hypha. Here the spore is formed on the tip cell of the conidiophore some 125 microns and 5 or 6 cells above the foot of the conidiophore where the compound is. Presumably the test compound must climb through 125 microns of fungal tissue before reaching the terminal cell which produces the spore.

Thus, we are able to discuss permeation and migration of a compound in somewhat more meaningful terms than in all our work on spore germination or mycelial growth in treated media.

We realize that a volatile compound could negotiate the climb through the vapor phase outside the cell and not through the liquid phase inside the cell, but we suspect that most compounds with enough vapor pressure to move up in the air will already have vaporized and left the filter paper along with the solvent.

We have found some difficulty in expressing the data. We tried the method of summing the antisporulation percentages to derive an approximate index, but this is inadequate because it includes dead collapsed conidiophores that do not produce spores. We are interested in live conidiophores that do not produce conidia. Then we tried to subtract the non-sporing live conidiophores from the dead collapsed ones, but this too was unsatisfactory. For these reasons the data tables will show for each dose of a test compound the averaged percentage of conidiophores not sporulating (labelled NoS in the tables) and the percentage of dead (collapsed) conidiophores (labeled Col in the tables).

Structure-activity Relationships

The aldehyde traps that we have tested are mainly phenylhydrazines and numerous analogues. From them we have gathered much information on structive-activity relationships with due regard to the role of permeation in the phenomenon.

The data are arranged in tables by structural groupings: hydrazines, hydrazones, semicarbazides, semicarbazones, carbohydrazides, carbohy-

drazones, acid hydrazides, acid hydrazones, and carbazic acid esters. By definition our compounds have a central skeleton of two connected nitrogen atoms. We shall discuss these as N^1 and N^2. If both nitrogen atoms are substituted, no aldehyde can be trapped.

Hydrazines

Because hydrazines are classical aldehyde reagents, we shall deal with them first.

If the hypothesis we have set out to test is correct, then any hydrazine compound that can enter the conidiophore and travel up its length to the tip cell should react with the glyoxylate there, convert it to a hydrazone, and thus inhibit sporulation. Let us see how the data match the hypothesis.

The upper part of Table 1, (through no. 4683) lists the hydrazines that are singly substituted on nitrogen[1] with aryl groups, chiefly phenyl. The lower part (beginning with no. 5679) lists those compounds that are doubly substituted on nitrogen.[1]

In general the hydrazines are effective antisporulants. The most effective compounds in the table are 1, 1-diphenylhydrazine (no. 3410) and its hydrochloride (no. 647). The general conclusion is that the hydrazines do reduce sporulation. The data are thus consonant with the hypothesis that an aldehyde trap should reduce sporulation by interfering at the glyoxylate step in glycolic acid respiration.

It is now time to proceed to a more detailed examination of Table 1 to try to account for the differences between structures.

Considering first the aryl single substitutions, we note that phenyl (no. 766) and biphenylyl (no. 1451) are about equal in potency; naphthyl (no. 610) is weaker and benzothiazolyl (no. 4683) is not active. Presumably these differences are due to reduced ability of the last two compounds with condensed rings to migrate up the long conidiophore and inhibit the formation of a conidium at the end.

There is some evidence for differences in mobility up the conidiophore among some of the compounds with substitutions on the ring of phenylhydrazine. If a sulfonic acid group be inserted in the 4-position (no. 5770), the potency practically vanishes, presumably because the compound is too water soluble to permeate and migrate well.

Chlorines on the ring are known to increase fat solubility and thus to increase permeability (Lukens and Horsfall, 1968). We have data on five halogen-substituted phenylhydrazines. The rising order of potency is; unsubstituted (no. 364)=2, 5-dichloro (no. 5768) < 4-bromo (no. 5767) < 2-chloro (no. 5831) < 4-chloro (no. 5833) < 2,4,6-trichloro (no. 5807).

Usui and Matsumura (1967a) tested the 4-bromo and 2,4,6-trichloro compounds to inhibit the growth of *Corticium sasakii*. They found the reverse order of ours. This is the first of a number of cases in which inhibition of growth differs from sporulation.

We would not hold, of course, that all the differences between our compounds are reproducibly different, but the data do suggest that the ring chlorines do improve potency in part at least by improving mobility up the conidiophore.

On the other hand the ring is an electron sink tending to withdraw

Table 1. Antisporulant activity of hydrazines, R_1-N-N-R_3 (R_2, R_4)

Code No.	Substituents R_1	R_2	R_3	R_4	Salt	Concentration, ppm 1000 NoS	1000 Col	500 NoS	500 Col	250 NoS	250 Col	125 NoS	125 Col
364	Phenyl	H	H	H	—	58	0	14	0	0	0	0	0
766	Phenyl	H	H	H	HCl	98	77[a]	75	6	48	3	22	0
5829	Benzyl	H	H	H	HCl	70	0	45	0	0	0	0	0
5832	Phenylethyl	H	H	H	H_2SO_4	23	0	0	0	0	0	0	0
5826	Phenyl	H	Benzoyl	H	—	22	0	17	0	15	0	17	0
5767	4-Br-phenyl	H	H	H	HCl	97	9	40	0	0	0	0	0
5831	2-Cl-Phenyl	H	H	H	HCl	88	6	39	3	12	0	0	0
5830	2-Cl-Benzyl	H	H	H	HCl	63	12	6	0	0	0	0	0
5833	4-Cl-Phenyl	H	H	H	HCl	100	82	100	25	63	5	13	0
5768	2,5-DiCl-Phenyl	H	H	H	—	31	0	35	0	0	0	0	0
5807	2,4,6-TriCl-Phenyl	H	H	H	—	100	4	100	0	61	0	16	0
648	4-NO_2-Phenyl	H	H	H	—	100	3	100	0	94	0	57	0
630	2,4-DiNO_2-Phenyl	H	H	H	—	100	2[a]	75	0	52	0	25	0
5770	4-SO_3H-Phenyl	H	H	H	—	55	0	—	—	—	—	0	0
1451	4-Biphenylyl	H	H	H	HCl	98	6[a]	33	0	22	0	0	0
610	α-Naphthyl	H	H	H	HCl	62	16	0	0	0	0	0	0
4683	2-Benzothiazolyl	H	H	H	—	0	0	—	—	—	—	0	0
5769	Methyl	Methyl	H	H	—	0	0	—	—	—	—	0	0
649	Phenyl	Methyl	H	H	—	46	0	7	0	0	0	0	0
2802	Phenyl	Methyl	Oxygen	—	—	17	0	0	0	0	0	0	0
607	Phenyl	Isoamyl	H	H	—	59	0	8	0	0	0	0	0
3410	Phenyl	Phenyl	H	H	—	100	90[a]	100	17	100	0	100[b]	2[b]
647	Phenyl	Phenyl	H	H	HCl	100	88	100	21	100	5	80	0
579	Phenyl	Benzyl	H	H	HCl	97	22[a]	29	2	0	0	0	0

[a] Much more collapse second day.

[b] At 63 ppm it produces 67% without spores and 0 collapse. At 31 ppm the numbers are 14 and 0, respectively.

electrons from the hydrazine grouping and thus to enhance its reactability. Halogen in the 2- and 4-positions will increase the strength of the electron sink and they should enhance the potency, and, of course, they do.

The same would be true also of nitro groups in the 2- and 4-positions. They should increase the potency. That they do is shown by the data on the 4-nitro (no. 648) and the 2,4-dinitro analogue (no. 630).

The theory of the electron sink finds excellent support in another direction. The insertion of a methane bridge between the ring and the hydrazine group should insulate the ring and reduce potency. The validity can be tested by comparing 2-chlorophenylhydrazine (no. 5831) with 2-chlorobenzylhydrazine (no. 5830). The bridge reduces the potency almost to zero.

Other points show up when we turn to the lower part of Table 1 to examine the effect of double substitution on nitrogen[1]. If nitrogen[1] of phenylhydrazine (no. 364) be additionally substituted with alkyl groups (methyl, no. 649 and isoamyl no. 607), the potency does not change greatly, presumably because the alkyl groups do not strengthen the electron sink. If, then, the ring be removed from nitrogen[1] and a second methyl group take its place (no. 5769), the potency falls to zero because the electronegativity falls essentially to zero. If, however, nitrogen[1] be given two benzene rings and still more electronegativity (no. 3410) the potency rises sharply. If now, one of these rings be separated from nitrogen[1] with a methane bridge (no. 579), the potency falls back again to the single ring compound (no. 766).

Two significant compounds for the thesis we are studying are no. 5826 in the upper portion of Table 1 and no. 2802 in the lower portion of the table.

These two compounds concern us because both nitrogens are substituted in both compounds. Therefore, neither can form a hydrazone and neither is a significant antisporulant. It is of further interest to note, however, that no. 5826 is good enough as a growth inhibitor of fungi to have been patented by Smith *et al.* (1956). Here we have another elegant example of selectivity between hyphal growth and sporulation.

All phenylhydrazine data lend support to the hypothesis being tested—that aldehyde traps should inhibit sporulation by reacting with glyoxylic acid. The hydrazines do reduce sporulation. They can react with glyoxylic acid. The evidence, however, does not really distinguish whether the hydrazines react with glyoxylic acid, with some other aldehyde, or even with some other metabolite.

Our phenylhydrazine results offer a biochemical explanation for the action of phenylhydrazine on wheat rust as reported by Livingston (1953) and Jaworski and Hoffman (1963).

It should be noted that Livingston and Jaworski and Hoffman read their data as numbers of rust pustules. Since rust pustules are masses of spores, these authors were actually reading sporulation, and thus they were really showing that phenylhydrazine, inhibits sporulation of rust. Does it do so by blocking the respiration at the glyoxylate stage as it appears to do in *A. solani*? We have no evidence on wheat rust but Staples (1962) says that the germinating bean rust spore metabolizes "most of the acetate . . . via the glyoxylate pathway." If sporulation of

wheat rust also follows the glyoxylate pathway, then phenylhydrazine inhibits sporulation by inhibiting glyoxylate metabolism. The data point in the direction of our hypothesis.

Hydrazones

Given the validity of the conclusion that phenylhydrazines can react with the aldehyde grouping of glyoxylic acid, we move on to the phenylhydrazones which should not be effective because they are incapable of reacting with glyoxylic acid to form hydrazones.

In Table 2, we list 10 hydrazones for use in testing the theory of aldehyde traps as antisporulants. None of them exerted any important antisporulant effect.

A comparison of the effect of several hydrazones with their parent hydrazines is very revealing.

For example, 4-nitrophenylhydrazine is no. 648 in Table 1. It is a very active antisporulant. Its hydrazone is no. 5749 in Table 2. It is inactive. Similarly 2,4-dinitrophenylhydrazine is no. 630 in Table 1. It too is effective. Its hydrazones are nos. 5746, 5750, 2434 and 2435, in Table 2. None shows any activity whatsoever.

We conclude that hydrazones lack antisporulant activity because they cannot combine with the glyoxylic acid in the conidiophore. Admittedly they might not permeate but this seems unlikely.

The only fungitoxic hydrazones that we have seen in the literature are acetylhydrazone acetone as reported by Dvoretskaya *et al.* (1958) and 1-phenyl-2-piperonylidene hydrazone as patented by Ladd (1947). The former was reported effective on tomato leaf mold caused by *Cladosporium fulvum*.

Whether either will inhibit sporulation we do not know. At first glance the rust literature might suggest that hydrazones are effective. Jaworski and Hoffman (1963) published a paper on control of "leaf rust with phenylhydrazones." They concluded, however, after studying 77 hydrazones that "phenylhydrazine is the actual toxicant and that the phenylhydrazones may serve as more stable, less phytotoxic sources of phenylhydrazine." Thus, their data, too, agree with our hypothesis of reactability with glyoxylic acid.

We must not overlook hydrazones that have been reported to inhibit growth of fungi in culture. Usui and Matsumura (1967a) report reasonable effectiveness of three hydrazones against *Corticium sasakii*. Here is another case of a compound in our series where the effect on growth differs from that on sporulation.

Semicarbazides

We move next to the semicarbazides as shown in Table 3. These compounds can form hydrazones as long as nitrogen[1] is unsubstituted. Therefore, they should inhibit sporulation.

The data in Table 3 show clearly that those that can form hydrazones (nos. 555, 553, 5780, 5779, and 631) are more or less active antisporulants depending on their permeation properties. Those that have substitutions on both hydrazine nitrogens (nos. 5786, 485, and 5778) cannot form hydrazones and they are inactive antisporulants.

Table 2. Antisporulant activity of hydrazones, $R_1\text{-}N(R_2)\text{-}N{=}R_3$

Code No.	Substituents R_1	R_2	R_3-Parent aldehyde or ketone	Concentration, ppm 1000 NoS	1000 Col	500 NoS	500 Col	250 NoS	250 Col	125 NoS	125 Col
5772	Phenyl	H	Acetone	18	0	0	0	0	0	0	0
5771	Phenyl	H	Benzaldehyde	55	0	23	0	0	0	0	0
5773	Phenyl	Methyl	Benzaldehyde	0	0	0	0	0	0	0	0
5749	4-Nitrophenyl	H	Benzaldehyde	0	0	0	0	0	0	0	0
5746	2,4-Dinitrophenyl	H	Acetone	0	0	0	0	0	0	0	0
5750	2,4-Dinitrophenyl	H	2,3-Butanedione oxime	0	0	0	0	0	0	0	0
2434	2,4-Dinitrophenyl	H	2,4-Pentadienal	0	0	0	0	0	0	0	0
2435	2,4-Dinitrophenyl	H	α-Hydroxyvaleraldehyde	0	0	0	0	0	0	0	0
2302	2-Benzothiazolyl	H	4-Acetylaminobenzaldehyde	0	0	0	0	0	0	0	0
2317	2-Benzothiazolyl	H	Anisaldehyde	0	0	0	0	0	0	0	0

Table 3. Antisporulant activity of semicarbazides, $R_1\text{-}N(R_2)\text{-}C(=O)\text{-}NH\text{-}N(R_4)\text{-}R_3$

Code No.	Substituents R_1	R_2	R_3	R_4	Oxygen or Sulfur	Salt	Concentration, ppm 1000 NoS	1000 Col	500 NoS	500 Col	250 NoS	250 Col	125 NoS	125 Col
555	H	H	H	H	Oxygen	HCl	18	0	0	0	0	0	0	0
553	H	H	H	H	Sulfur	—	79	0[a]	32	0	0	0	0	0
5780	Phenyl	H	H	H	Oxygen	HCl	98	0	35	0	17	0	7	0
5779	Phenyl	H	H	H	Sulfur	—	98	0	84	0	15	0	0	0
631	Phenyl	Phenyl	H	H	Oxygen	—	100	30[a]	100	0	100	0	22	0
5786	H	H	Phenyl	H	Oxygen	—	0	0	0	0	0	0	0	0
485	H	H	Phenyl	H	Sulfur	—	5	0	0	0	0	0	0	0
5778	Phenyl	H	Phenyl	H	Oxygen	—	0	0	0	0	0	0	0	0

[a] Much more collapse second day.

Here again as in Table 1, the 4,4-diphenyl derivative (no. 631) is by far the most active as an antisporulant. The 1,4-diphenyl derivative (no. 5778) is just as dramatically inactive. A second ring in the fourth position adds great potency, but a second ring in the first position quenches activity, presumably because it inhibits hydrazone formation.

In general the sulfur-containing compounds are more active than their oxygen analogues. Presumably the sulfur ones can form more stable hydrazones than the oxygen ones. They also can form chelates, however, and thus part of their activity could be due to chelation. We have shown on several occasions that chelation is an active mechanism of antisporulation (Horsfall and Rich, 1955).

There are some rust data. As we have said, rust is read as sporulation. Hence we find it not surprising that Fuchs and Bauermeister (1958) should have followed closely after Livingston (1953) in showing that thiosemicarbazide will reduce wheat rust. It can form hydrazones and is effective. Similarly Heitefuss and Fuchs (1960) showed that it can inhibit *Peronospora brassicae* on *Brassica*.

Rudolph (1963) suggested that rust inhibition could be due to the general action of semicarbazides in blocking keto compounds in the cell but also to interference with glycine utilization because he found large quantities of glycine accumulating in the diseased tissue.

Rudolph's explanation comes close to ours but he did not conceive of his results as interference with sporulation.

1-Phenylthiosemicarbazide (no. 485) and its oxygen analogue (no. 5786) are worth special consideration in a discussion of antisporulation. Since nitrogen[1] is substituted in these compounds, they cannot form hydrazones and they are not active.

We collected a sample of 1-phenylthiosemicarbazide many years ago in connection with our earlier study of the aldehyde reagents. It was only very weakly active in inhibiting germination of spores (Horsfall, 1945). Unfortunately, it was erroneously figured as 4-phenylthiosemicarbazide which can form hydrazones.

Pluijgers and Sijpesteijn (1966), however, report that 1-phenylthiosemicarbazide inhibits growth of several fungi in culture. We find, too, that it inhibits *Monilinia fructicola* in culture. Here, then, is another example of a compound that inhibits hyphal growth without inhibiting sporulation or germination.

In a second paper Pluijgers and Berg (1966) show that the thione group is oxidized by hydrogen peroxide in an unusual fashion to give a $>C=S=O$ group. The resonance thus generated gives the compound a red color. Their evidence is that the red compound is fungitoxic to their growing fungi. We have not tested the red form here except to learn that our sample does undergo reddening in the presence of hydrogen peroxide. The evidence suggests that their fungi growing on agar conduct a lethal synthesis by oxidizing the compound to a toxic substance and that our germinating spores of *Monilinia* and *Stemphylium* do not.

Mautner *et al.* (1956), stimulated by the tuberculosis work, tested 2-phenylsemicarbazide, 2-phenylthiosemicarbazide, and 2-phenylselenosemicarbazide on *Botrytis cinerea* and other fungi growing in culture. All three compounds were potent, but none could form a hydrazone.

They ascribe the effect to chelation. Potency was in the order indicated and so was the strength of chelation.

Hiruki (1964) has reported that both the 1-phenyl and the 4-phenylthiosemicarbazides can kill zoospores of *Olpidium brassicae*. One can form a hydrazone and one cannot. Clearly, hydrazone formation is not the explanation. The effect is more probably due to chelation as first suggested by Carl and Marquardt (1947) to account for the action of semicarbazide on tubercle bacteria.

Semicarbazones

We turn next to the semicarbazones. If the hypothesis we are testing has validity, then a preformed semicarbazone should not act as an antisporulant. The data in Table 4 show that in general they do not inhibit sporulation. The most dramatic example of this principle is, of course, the semicarbazone of glyoxylic acid itself (no. 5824) as shown in Table 4. It had no antisporulant effect whatsoever. In fact, no oxygen analogue had any significant antisporulant effect (nos. 1240, 1318, 5777, 2261, 2439, 5824, 5781, and 2849).

The exceptions to the rule of no hydrazone-no effect are the thiosemicarbazones. Mautner *et al.* (1956) showed clearly that the growth-inhibiting property of the thiosemicarbazones is due to chelation—that the sulfur analogues chelate much more strongly than the oxygen ones and, hence, are more potent. Gingras *et al.* (1965) agreed, and so did Addy and Mitra (1966).

We tested the thiosemicarbazones in Table 4 with copper sulfate. All showed color changes. Presumably all chelated the copper.

Why, then, are not all the sulfur analogues active antisporulants through the chelation route? Obviously, we must look further for an explanation. We suggest that permeation provides the simplest explanation. Consider benzaldehyde thiosemicarbazone (no. 4118). It is among the top three antisporulants. If, however, a hydrophilic hydroxyl group be added to the ring as in nos. 4111 and 4121, the potency vanishes, presumably because the molecule is not hydrophobic enough to pass up the tall conidiophore and inhibit the metals necessary for the formation of a conidium on the upper end.

Let us consider next the alkyl aldehydethiosemicarbazones towards the top of Table 4. The potency rises with the number of carbons 3 carbons (no. 4108) $<$ 4 carbons (no. 4115) $<$ 6 carbons (no. 4105) $<$ 7 carbons (no. 4116). This series indicates pretty clearly that increased permeation means increased antisporulant activity. Benns *et al.* (1960) show that potency rises up to 11 carbons for inhibiting growth of fungi.

We conclude, then, that thiosemicarbazones can and will inhibit sporulation but that they act by a metal robbing route and not via the glyoxylate route.

We must conclude further that the activity is in proportion to the stability constant and to translocation through the long conidiophore.

Carbohydrazides

A carbohydrazide is symmetrical around the carbonyl group as shown in Table 5. The first three compounds can form hydrazones, the latter

Table 4. Antisporulant activity of semicarbazones, $NH_2\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}NH\text{-}N{=}R$

Code No.	R=Parent aldehyde or ketone	Oxygen or Sulfur	Concentration, ppm 1000 NoS	1000 Col	500 NoS	500 Col	250 NoS	250 Col	125 NoS	125 Col
1240	5-Chloro-2-thienylmethyl ketone	Oxygen	0	0	0	0	0	0	0	0
1318	5-Nitro-2-furaldehyde	Oxygen	0	0	0	0	0	0	0	0
4108	Acetone	Sulfur	28	0	0	0	0	0	0	0
5777	Acetone	Oxygen	35	0	18	0	0	0	0	0
4115	2-Butanone	Sulfur	44	0	14	0	0	0	0	0
2261	α-Acetylvaleronitrile	Oxygen	0	0	0	0	0	0	0	0
2439	2,4-Pentadienal	Oxygen	0	0	0	0	0	0	0	0
5824	Glyoxylic acid	Oxygen	0	0	0	0	0	0	0	0
4105	Cyclohexanone	Sulfur	94	0	64	0	21	0	0	0
4116	*n*-Heptanol	Sulfur	100	51[a]	100	53	100	8	60	0
4118	Benzaldehyde	Sulfur	100	9[a]	100	5	91	0	53	0
4111	Vanillin	Sulfur	0	46	0	0	0	0	0	0
4117	Piperonal	Sulfur	60	0	53	0	22	0	18	0
4121	Salicylaldehyde	Sulfur	0	0	0	0	0	0	0	0
5781	Salicylaldehyde	Oxygen	19	0	0	0	0	0	0	0
4102	Acetophenone	Sulfur	100	6[a]	100	3	98	0	39	0
2849	Acetophenone	Oxygen	15	2	13	0	6	0	5	0

[a] Much more collapse second day.

Table 5. Antisporulant activity of carbohydrazides, $R_1\text{-}NH\text{-}NH\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}NH\text{-}NH\text{-}R_2$

Code No.	Substituents R_1	Substituents R_2	Oxygen or Sulfur	Concentration, ppm 1000 NoS	1000 Col	500 NoS	500 Col	250 NoS	250 Col	125 NoS	125 Col
5783	H	H	Oxygen	67	0	0	0	0	0	0	0
5782	H	H	Sulfur	100	58	100	26	95	19	89*	5*
3413	NH_2-C(O)-	H	Oxygen	0	0	—	—	—	—	0	0
633	Phenyl	Phenyl	Oxygen	26	0	—	—	—	—	0	0
470	Phenyl	Phenyl	Sulfur	0	0	—	—	—	—	0	0
5784	4-Nitrophenyl	4-Nitrophenyl	Oxygen	0	0	—	—	—	—	0	0

* At 63 ppm 69 and 0. At 31 ppm 61 and 0.

Table 6. Antisporulant Activity of Carbohydrazones, $R_1NH\text{-}NH\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}N{=}N\text{-}R_2$

Code No.	Substituents R_1	Substituents R_2	Oxygen or Sulfur	Concentration, ppm 1000 NoS	1000 Col	500 NoS	500 Col	250 NoS	250 Col	125 NoS	125 Col
537	Phenyl	Phenyl	Sulfur	0	0	—	—	—	—	0	0
2721	2-Naphthyl	2-Naphthyl	Sulfur	0	0	—	—	—	—	0	0

three cannot. One of the first three is a sulfur compound (no. 5782). It is far more active than its two oxygen analogues (nos. 5783 and 3413). We suspect that the two inactive ones are not hydrophobic enough to climb the long conidiophore.

Carbohydrazones

We had available only two carbohydrazones (Table 6). Neither was an active antisporulant even though both are sulfur analogues. The results support the theory being tested. The compounds are not able to form hydrazones with glyoxylic acid and they are not active.

Acid hydrazides

An acid hydrazide can react with aldehydes to form hydrazones. In fact we acquired a sample of 3-nitrobenzhydrazide (no. 641) many years ago when we first became interested in the possible fungicidal action of aldehyde traps. It was not toxic to germinating spores nor to fungal growth in culture, and Table 7 shows that it is also not an antisporulant. Neither is 4-nitrobenzhydrazide (no. 5751).

These are just examples of a generalization that the hydrazides are no more than nominally effective as antisporulants. Thus, here is a whole family of exceptions to the hypothesis that we are testing. Here is a whole series of hydrazone-forming compounds that are essentially inactive as antisporulants.

This is dramatically shown by comparing the highly effective 4-nitrophenylhydrazine (no. 648 in Table 1) with its acid hydrazide analogue (no. 5751) in Table 7. The latter is only 1/6 as potent as the former.

The carbonyl moiety in the phenyl analogues of the acid hydrazides destroys the activity completely. Apparently, the carbonyl moiety inhibits permeation of the molecule to sites of action within the fungus. There is further evidence for this in Table 6. The carbonyl moiety can affect permeation in two ways. First, it withdraws electrons from the terminal nitrogen atom, makes that atom more positive, and thus increases reactivity at this center. The increase in reactivity of the acid hydrazide over that of the hydrazine permits it to be more easily captured by cellular components that it encounters along the route up the conidiophore to the site of action. And second, through an increase in polarity of the molecule the carbonyl moiety decreases hydrophobic bonding of the molecule which reduces the chance that the compound can penetrate the lipoid barrier of cellular membranes. When the effects of the carbonyl moiety are nullified, in part, by the insertion of the amide nitrogen in a semicarbazide (Table 3), activity is restored to four-fifths that of the original hydrazine (compare data of phenyl and diphenyl analogues of hydrazine and semicarbazide in Tables 1 and 3). We suspect that if thione were substituted for the carbonyl moiety permeation and potency would be improved.

The nicotinic acid hydrazides at the bottom of Table 7 are interesting. These compounds have become powerful tools in the fight against tuberculosis in humans, but they are totally inactive as fungal antisporulants.

Similarly, maleic hydrazide is a potent compound on green plants, but like other acid hydrazides it has no antisporulant properties.

Table 7. Antisporulant activity of acid hydrazides, $R_1\text{-}\underset{\underset{O}{\|}}{C}\text{-}NH\text{-}NH\text{-}R_2$

Code No.	Substituent R_1	Substituent R_2	Concentration, ppm 1000 NoS	1000 Col	500 NoS	500 Col	250 NoS	250 Col	125 NoS	125 Col
5775	Phenyl	H	0	0	—	—	—	—	0	0
641	3-Nitrophenyl	H	25	0	0	0	0	0	0	0
5751	4-Nitrophenyl	H	42	0	14	0	0	0	0	0
5776	2-Aminophenyl	H	46	0	12	0	0	0	0	0
5835	4-Aminophenyl	H	79	5	47	4	19	0	0	0
5774	2-Hydroxyphenyl	H	68	0	7	0	0	0	0	0
2071	2,4-Dihydroxyphenyl	H	0	0	0	0	0	0	0	0
2918	2,5-Dihydroxyphenyl	H	0	0	0	0	0	0	0	0
5836	3,4-Dichlorophenyl	H	100	15[a]	100	18	85	10	48	0
4003	Benzyl	H	30	0	0	0	0	0	0	0
5834	$(C_6H_5)_2C(OH)$ -	H	54	0	22	0	0	0	0	0
3785	$CN\text{-}CH_2$-	H	25	0	0	0	0	0	0	0
5839	$NH_2C(O)$ -	H	0	0	0	0	0	0	0	0
5838	$NH_2\text{-}NH\text{-}C(O)$ -	H	20	0	0	0	0	0	0	0
1399	Methyl	Phenyl	0	0	0	0	0	0	0	0
5843	Propyl	H	86	12	80	5	55	4	40	0
2848	Propyl	Phenyl	100	0	—	—	—	—	37	0
5826	Phenyl	Phenyl	20	0	17	0	16	0	16	0
2811	Phenyl	Benzoyl	0	0	0	0	0	0	0	0
3036	3-Pyridyl	H	0	0	0	0	0	0	0	0
2919	3- (2-Aminopyridyl)	H	30	0	—	—	—	—	0	0
2004	4-Pyridyl	H	28	0	0	0	0	0	0	0
5837	3-Methylindole	H	54	0	22	0	0	0	0	0

[a] Much more collapse second day.

Acid hydrazones

Acid hydrazones (Table 8) exert no more antisporulant action than the regular hydrazones as shown in Table 4.

Frohberger's (1956) compound, quinoneoximebenzoylhydrazone (our no. 3788), is interesting here. He reported good antifungal properties to inhibit growth, but it is essentially bland for us on sporulation. Here is another compound that can inhibit mycelial growth, but does not inhibit sporulation. We do not know why.

Carbazic acid esters

We have tested six carbazic acid esters and a related phosphate ester as shown in Table 9. The phosphate ester was negative, but the four others were more or less active despite their inability to form hydrazones. Presumably, such a structure can chelate if nitrogen[1] is free to be substituted. We tested the four with copper sulfate. Nos. 3257 and 3572 gave bright yellow precipitates, whereas nos. 3577 and 3268 gave none. The antisporulation activity of the two that chelate is very high while that on the two that do not is much lower. The case of no. 3268 is reasonably clear. The nitrogen is methylated. It should not and does not chelate and is not very active. The case of no. 3577 is hazy. The *o*-chlorine presumably inhibits the chelation through steric hindrance, but it does not inhibit the antisporulant activity. One striking aspect of its activity is the peak in the curve at 500 ppm. In other words, potency rises as concentration falls from 1000 to 500 ppm. This was a repeatable result. Altogether this compound is anomalous and we offer no explanation.

We can say that Usui and Matsumura (1967b) tested several carbazic acid esters on growth of several fungi in culture including *Alternaria solani,* our fungus. All were about as toxic as the phenylhydrazine parents.

In this case, our data on antisporulation agree reasonably well with their data on growth.

They also made and tested our no. 3604, the phosphate ester analogue. It was negative for them as well as for us.

Sumina (1965) has reported a protective action of zinc dithiocarbazate against *Colletotrichum* on cucumber.

Aberrances in the Fungus

During these studies several aberrant types of conidia and conidiophores were observed. Two seem to warrant description, although we do not really know what they mean.

In one type the conidia or conidiophores or both are dotted with crystals that are insoluble in water but are soluble in acetone. A typical case is illustrated in Figure 1. We assume until we have further data that the crystals are those of the test compound that has been translocated to the site and excreted. The following compounds have been found to do this: no. 649, 1-methyl-1-phenylhydrazine, no. 5751, 4-nitrobenzhydrazide, no. 4102, acetophenonethiosemicarbazone, no. 4108, acetonethiosemicarbazone, and no. 4115, 2-butanonethiosemicarbazone.

The other and perhaps more striking phenomenon can be described

(*Text continues on page* 25)

Table 8. Antisporulant activity of acid hydrazones, $R_1\overset{\overset{\displaystyle O}{\|}}{C}\text{-}NH\text{-}N{=}R_2$

Code	Substituents		Concentration, ppm							
			1000		500		250		125	
No.	R_1	R_2, Parent ketone or aldehyde	NoS	Col	NoS	Col	NoS	Col	NoS	Col
3788	Phenyl	4-Quinone oxime	29	0	0	0	0	0	0	0
3092	Phenyl	Piperonal	0	0	0	0	0	0	0	0
3100	4-Nitrophenyl	Piperonal	0	0	0	0	0	0	0	0
3088	4-Nitrophenyl	Butyraldehyde	0	0	0	0	0	0	0	0
3087	4-Nitrophenyl	2-Chlorobenzaldehyde	0	0	0	0	0	0	0	0
3085	4-Nitrophenyl	Methylphenylketone	0	0	0	0	0	0	0	0
3219	2-Phenylthiol	2,5-Dichlorobenzaldehyde	0	0	0	0	0	0	0	0

Table 9. Antisporulant activity of analogues of carbazic acid, R_1-N-NH-COO-R_3*
R_2

Code No.	Substituents R_1	R_2	R_3	Concentration, ppm 500 NoS	500 Col	1000 NoS	1000 Col	250 NoS	250 Col	125 NoS	125 Col
3257	Phenyl	H	$-CH_2-CH_2-Cl$	100	0[a]	75	0	45	0	31	0
3572	Phenyl	H	$-CH-(CH_3)_2$	100	5[a]	100	0	40	0	22	0
3577	2,5-Dichlorophenyl	H	$-CH(CH_3)_2$	49	6	91	12	52	2	23	0
3268	Phenyl	Methyl	$-CH(CH_3)_2$	98	0	21	0	0	0	0	0
5842	H	H	$-CH_2CH_3$	65	6	41	0	17	0	0	0
5841	H	H	$-C(CH_3)_3$	35	0	0	0	0	0	0	0

* The dimethyl phosphate ester was wholly inactive.
[a] Much more collapse second day.

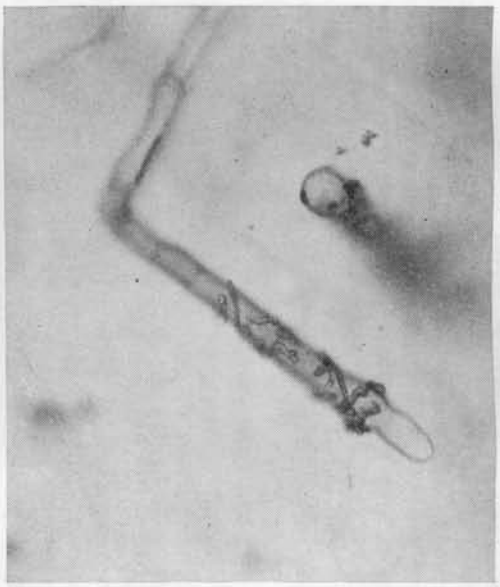


FIGURE 1. Crystals on conidiophore Compound no. 649, 1-methyl-1-phenylhydrazine.

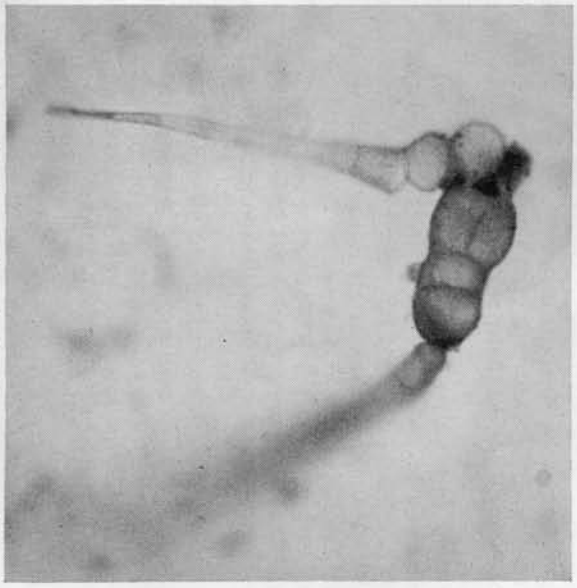
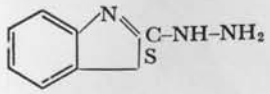
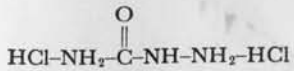
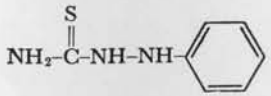
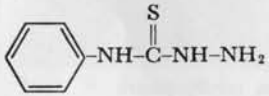
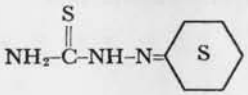
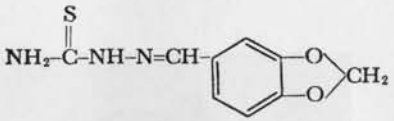
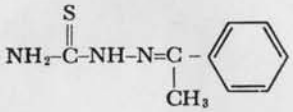
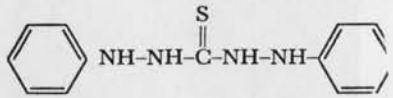
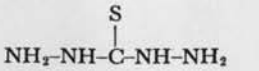
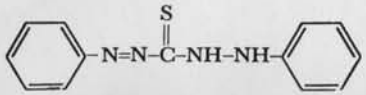


FIGURE 2. A conidium broken by compound no. 4102, acetophenonethiosemicarbazone.

Table 10. Compounds producing "broken" conidia

Code No.	*Structure*	*Antisporulant Data in Table*
4683		1
555		3
485		3
5779		3
4105		4
4117		4
4102		4
470		5
3409		5
537		6

With one single exception these compounds are all thione derivatives. One must hasten to say, however, that several other thione compounds were inactive, or at least no broken conidia were noticed as they were being tested.

as a broken conidium (Figure 2). The long conidium looks as if it had been broken across one's knee. It appears as if internal pressure in the cells had forced two of them to split apart, presumably along the line where they are cemented together. The pressure would presumably apply the greatest force at the edge. If one side let go before the other, then the sundered cells would break apart as if they were hinged on one side. Whereupon the sundered cells seem to round off.

We have observed this phenomenon with 10 compounds as shown in Table 10.

Discussion

We started the investigation with some evidence that the glycolic acid pathway of respiration is involved in sporulation of *Alternaria solani*. If so, then aldehyde traps such as phenylhydrazine and thiosemicarbazide should inhibit sporulation.

The evidence presented is strong. With rare exceptions, the hydrazine derivatives that we have tested act as antisporulants if they can form a hydrazone; but not if they cannot form hydrazones. The results then lend additional support to the probability that the glycolic → glyoxylate pathway is involved in sporulation.

There is some confirmatory evidence from the literature. Phenylhydrazine has been shown by Livingston (1953) and others to reduce the number of wheat rust pustules. Restated in our terms, phenylhydrazine reduces sporulation of wheat rust. Staples (1962) has shown that germinating bean rust spores obtain about 80 percent of their energy from the glyoxylic acid pathway. Perhaps, wheat rust does, too, and if so the effect of phenylhydrazine is to block the path at the glyoxylate step.

A few compounds shown herein are active antisporulants even though they do not form hydrazones. For the most part they are metal chelators. We suggest that these sequester the metals required by the fungus for sporulation.

The exceptions above are compounds that are active and *cannot* form hydrazones. The acid hydrazides are on the obverse of the coin. They are very weak antisporulants and they *can* form hydrazones. We offer the possibility that the acid hydrazides are unable to climb the 125-micron ladder to the site of action out on the end of the conidiophore, (a) because they are not hydrophobic enough, and (b) because they are more reactive than say a hydrazine and, therefore, are more easily captured than a hydrazine by cellular components that they encounter along the route up the conidiophore.

As mentioned in the introduction, we are embarked on a trip to discover compounds that are truly selective for sporulation with the assumption that sporulation is a characteristic of fungi that is not matched in the green host plant. We do not know yet how our compounds will effect green plants, but most of the compounds do distinguish sporulation from growth of the fungus.

We consider it sporulation only when the conidiophores do grow further in the presence of the compound.

It is not surprising then that the literature records many compounds that inhibit fungal growth but did not inhibit sporulation for us. Such are some of the hydrazones of Usui and Matsumura (1967a), of 1-phenyl-

thiosemicarbazide of Pluijgers and Sijpesteijn (1966) and Hiruki (1964), of the semicarbazones of Mautner *et al.* (1956) and Addy and Mitra (1966), the acid hydrazide of Smith *et al.* (1956), and the quinoneoxime-benzoylhydrazone of Frohberger (1956).

It appears that the biochemistry of spore production clearly has some features that distinguish it from the biochemistry of growth. It is to this that we must turn our attention.

Summary

Earlier evidence of ours suggested that the energy for sporulation of *Alternaria solani* is derived by way of the glycolate pathway, and that sporulation can be inhibited by blocking the step between glycolate and glyoxylate with relatives of glycolic acid. The data in this paper suggest that the step beyond glyoxylate can be inhibited by using aldehyde traps to combine with the aldehyde moiety of glyoxylate.

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