Studies on an Antagonist of the Phytotoxin Fusaric Acid

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By

R. Kalyanasundaram

With 9 figures


I. INTRODUCTION

Fusaric acid is one of the wilt toxins produced by the wilt pathogens Fusarium lycopersici Sacc. and Fusarium vasinfectum Atk. and a few other members of the family Hypocreaceae (Gäumann, Naef-Roth and Kobel 1952, Gäumann 1957). This toxin has now been demonstrated in the infected tomato and cotton plants, the respective host plants of the above mentioned pathogens (Lakshminarayanan and Subramanian 1955, Kalyanasundaram and Venkata-Ram 1956, Kern and Kluepfel 1956). In fact, the evidence along these lines is increasing and recently it has been reported that fusaric acid is also a toxin of the Panama disease of banana plants infected by Fusarium cubense F. M. Smith (Page 1959). Thus, toxins do play a role in plant diseases, even in tissues which themselves do not contain the parasite. The action of toxins is therefore no more in the realm of hypothesis, but it rests on experimental evidence: we thus find a similar situation to that observed in certain human diseases.

Studies on the mode of action of fusaric acid on the susceptible organs or organisms indicate that this toxin acts by interfering with different
functions at different substructures of the host (Gäumann, Naef-Roth and Kobel 1952, Gäumann 1958). Hence, Gäumann calls its action pleiotropic (Gäumann 1958). The spectrum of action of fusaric acid is wide. It has been demonstrated to be toxic to algae, bacteria, yeasts, fungi and higher plants (Gäumann, Naef-Roth and Kobel 1952, Gäumann 1958). In the classical sense of the term, fusaric acid is a weak antibiotic.

Studies with fusaric acid up to now show that the action of fusaric acid on susceptible organs apparently takes two different courses:

1. Injurious effect on the water permeability of the protoplasts, and
2. Injury to the ferment systems of the host (Gäumann 1958).

We have not as much experimental evidence on the latter effect of fusaric acid as on the former (Bachmann 1956, 1957, Gäumann and Loeffler 1957). The results in this direction were the effect of this toxin on the tissue respiration of tomato and yeast (Naef-Roth and Reusser 1954, Reusser and Naef-Roth 1956). Recently it has also been shown that fusaric acid competitively inhibits the polyphenoloxidase (Bossi 1960). On the basis of the chemical configuration of the molecule, it seems possible that fusaric acid may form chelates, thereby causing heavy metal deficiency in host tissues (Deuel 1954).

Although the action of fusaric acid is manifold, we may reasonably expect that some of the injuries caused are basic and more fundamental in nature, and therefore common to all organs and organisms susceptible to fusaric acid. If this is so, the other host specific and tissue specific injuries play an additive role in the clinical picture, or, they superimpose on the ground effect.

Ever since the discovery by Woods (1940) that sulfanilamide acts in bacteria by competitively replacing one of the essential metabolites of the bacterium, p-aminobenzoic acid, evidence is growing that many toxins and antibiotics affect the susceptible organisms by antimetabolite action. There are many examples of competitive antagonism to an essential metabolite in the field of antibiotics and antivitamins (Woolley 1952). However, very few cases have been demonstrated in the field of phytopathologically interesting toxins. That such possibilities do exist is shown in the wild fire disease of tobacco. Its pathogen Pseudomonas tabaci Wolf and Foster produces a toxin, which has been shown to be an antimetabolite of the amino acid methionine (Braun 1955).

With this in view, we asked ourselves the following questions:

Is the toxicity of fusaric acid fully or in part attributable to its antagonism of some essential metabolite of the susceptible organisms or tissues? If so, is it possible to remove its toxicity to susceptible organisms and organs by administering the hypothetical metabolite?

Since it is easier to demonstrate a metabolite-antimetabolite relationship with microorganisms, the studies were initiated with this method. However, the ultimate aim was to see if a similar mode of action of fusaric acid also exists in tomato plants.
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II. MATERIAL AND GENERAL METHODS

The following cultures of microorganisms have been used in the course of these investigations:

Organisms:

- *Fusarium lycopersici* Sacc.
- *Ustilago zeae* (Beckm.) Ung.
- *Ustilago sphaerogena* Burr. ex Ellis et Everh.
- *Candida vulgaris* auct.
- *Saccharomyces cerevisiae* Hans.
- *Bacillus subtilis* Cohn
- *Escherichia coli* (Mig.) Cast.
- *Staphylococcus aureus* Rosenb.

Media used for cultivation and maintenance:

**Bacteria**

<table>
<thead>
<tr>
<th>Broth medium</th>
<th>Synthetic medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>1.0 g.</td>
</tr>
<tr>
<td>Peptone</td>
<td>2.5 g.</td>
</tr>
<tr>
<td>Water (distilled)</td>
<td>1000 ml.</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₂SO₄</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td></td>
<td>MgSO₄</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Water (distilled)</td>
</tr>
</tbody>
</table>

**Fungi and Yeasts**

<table>
<thead>
<tr>
<th>Malt water</th>
<th>Richard's medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt extract</td>
<td>10.0 g.</td>
</tr>
<tr>
<td>Water (distilled)</td>
<td>5.0 g.</td>
</tr>
<tr>
<td></td>
<td>2.5 g.</td>
</tr>
<tr>
<td></td>
<td>0.02 g.</td>
</tr>
<tr>
<td></td>
<td>50.0 g.</td>
</tr>
<tr>
<td></td>
<td>1000 ml.</td>
</tr>
<tr>
<td></td>
<td>NH₄NO₃</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>MgSO₄</td>
</tr>
<tr>
<td></td>
<td>FeCl₃</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Water</td>
</tr>
</tbody>
</table>

For agar media these were supplemented with 1.8% agar-agar.

III. SCREENING OF CERTAIN ESSENTIAL GROUPS OF METABOLITES FOR THEIR ACTIVITY AGAINST FUSARIC ACID ON BACTERIAL SPECIES

Our aim is to see whether, among the different groups of vital metabolites, there exists a substance which could reverse partially or fully the toxicity of fusaric acid to certain bacterial species. It was therefore planned to study the effect of the following groups of substances: vitamins, phenols, purine and pyrimidine bases, certain substances known to be involved in detoxication mechanisms in biological systems, yeast extract and beef extract. The latter two substances are composed of known and unknown biologically essential metabolites.
1. Minimum level of fusaric acid inhibitory to *Escherichia coli* and *Bacillus subtilis*

Before proceeding with the actual investigation, it was thought worthwhile to cultivate the two bacterial species in a simple nutrient solution rather than in a broth medium which contains unknown growth factors. Since fusaric acid is a weak antibiotic, fairly high concentrations are needed to inhibit the growth of bacteria, in the range of $10^{-3}$ to $10^{-4}$ molar (Gäumann, Naef-Roth and Kobel 1952). It was also our intention to see whether the sensitivity of the two organisms to fusaric acid could be increased in a simple nutrient solution. Experiments were set up to study the minimum dose of fusaric acid inhibitory to the bacterial species in the broth as well as in the synthetic medium.

a) Preparation of the medium and dilution series

Serial dilution was used as the assay method. The range of fusaric acid concentration was maintained from 0.25 μg/ml to 100 μg/ml medium. The dilution technique was slightly modified, and the following concentrations of fusaric acid were used: 0.25, 0.75, 2.5, 5.0, 7.5, 25.0, 40.0, 50.0, 60.0, 80.0, and 100.0 μg/ml of the medium.

The synthetic as well as the broth medium was divided into twelve 90 ml. aliquots and a stock solution of 1 mg/ml fusaric acid was maintained in a 0.2 molar phosphate buffer at pH 6.2. From this a second set of working stock solutions was prepared in such a way that each solution had a concentration ten times that which was actually needed for the medium. For each concentration, 10 ml of this fusaric acid solution was added to 90 ml of medium to give the necessary dilution. Thus, when a dilution of 5 μg/ml of fusaric acid is needed in the medium, 0.5 ml is taken from the first stock solution containing 1000 μg/ml, and diluted to 10 ml with phosphate buffer. These 10 ml (fusaric acid concentration 50 μg/ml) were added to 90 ml of the medium to give the working concentration of 5 μg/ml. The control received 10 ml of the buffer solution. The pH of the medium after autoclaving was 6.2. From each 100 ml aliquot, 5 ml were pipetted into test tubes which subsequently were inoculated with the organism. Each dilution had four replicates. Pipetting was done under sterile conditions, as was the adding of fusaric acid. In order to maintain uniform volumes, sterilisation was not performed in the test tubes.

b) Preparation of the inoculum

*Escherichia coli* grown for 18 hours in broth medium was diluted 100 times with 0.9% NaCl, and used as the inoculum. For *Bacillus subtilis* however, sterile spore suspensions were prepared with saline straight from beef-extract agar slants in which the bacteria were grown at 37 °C for 18 hours. The constancy of the inoculum was checked by the turbidity of the solution in the spectrophotometer at 420 μ. The spore suspensions could be stored in the cold for one to two months. One drop of the spore suspension was added to each tube. The tubes with synthetic medium were incubated for 24 hours, and the others for 18 hours at 37 °C.
c) Results

The growth of the organism was measured turbidimetrically. The results presented in Table 1 show that the growth of both *Bacillus subtilis* and *Escherichia coli* in the synthetic medium was inhibited at a fusaric acid concentration of 5 μg/ml. The minimum dose of fusaric acid just inhibiting the growth of the two species in the synthetic medium, lies between 2.5 and 5.0 μg/ml. However, in the broth medium, the minimum amount of fusaric acid for inhibiting growth lies between 50 and 60 μg/ml. Thus the sensitivity of both test organisms to fusaric acid is increased about 15 times in the synthetic medium. For further investigations the synthetic medium was used, as fusaric acid here acted at a physiologically lower level.

**Table 1**
The minimum dose of fusaric acid needed to inhibit the growth of *Escherichia coli* and *Bacillus subtilis* in synthetic and broth media

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Media</th>
<th>Concentration of fusaric acid in μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Synthetic</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Broth</td>
<td>—</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Synthetic</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Broth</td>
<td>—</td>
</tr>
</tbody>
</table>

— no growth; + growth.

2. Vitamin supplement to the medium and the toxicity of fusaric acid to *Bacillus subtilis* and *Escherichia coli*

The following vitamins were used for the investigation: thiamine, riboflavin, pyridoxin, nicotinic acid, nicotine amide, panthothenic acid, α-amino benzoic acid, biotin, inositol and ascorbic acid. All the vitamins except ascorbic acid were supplied to the medium at three different levels: 0.1, 5 and 10 μg/ml of the medium. Ascorbic acid, however, was supplied at the level of 5, 10 and 50 μg/ml of the medium. The dilutions were made under sterile conditions in the same way as before: 1 ml vitamin solution was added to 99 ml of the medium which already contained the appropriate concentration of fusaric acid. None of the vitamins tried, except ascorbic acid, could reverse the toxicity of fusaric acid to *Bacillus subtilis* and *Escherichia coli*. Even with ascorbic acid amendment to the medium, the growth of *Bacillus subtilis* was observed, up to 7.5 μg, and *Escherichia coli* up to 25 μg of fusaric acid per millilitre. The corresponding level in the control, i.e. with no ascorbic acid, was 2.5 μg fusaric acid per ml.

Experiments were then set up to try the effect of increasing concentrations of ascorbic acid in the medium on the toxicity of fusaric acid. Higher levels of ascorbic acid, viz. 200 and 500 μg/ml were themselves toxic to the growth of the bacteria and sometimes growth was belated. So
it is concluded that the partial reversal of toxicity of fusaric acid to the bacteria by ascorbic acid may not be a direct effect of antagonism, but only a change in the redox potential of the medium. It is worthwhile to note that pyridoxin and nicotinic acid, which are structural analogues of fusaric acid, show no antagonism towards fusaric acid.

3. Role of phenolic compounds in reversing the toxicity of fusaric acid to Bacillus subtilis and Escherichia coli

Fusaric acid has been shown to inhibit the activity of polyphenoloxidase competitively (Bossi 1960). Assuming that this inhibition follows the normal pattern, it might be expected that fusaric acid competes with the normal substrates of this enzyme. Conversely, if an external source of this substrate were to be supplied, it should be possible to eliminate the inhibition of polyphenoloxidase by fusaric acid. An experiment was set up to try the effect of fusaric acid in the synthetic medium on the two species of bacteria in the presence of certain phenolic compounds. Protocatechic acid, paraphenyl and phloroglucinol were tried at one concentration. Tyrosine, cresole and pyrocatechol were tried at three different concentrations. Preliminary experiments showed that higher concentrations were toxic.

Results: tyrosine was observed to reverse the toxicity of fusaric acid in both organisms. But this was limited. Increasing concentrations of tyrosine failed to increase the minimum dose of fusaric acid. Other compounds gave no positive response (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Supplements in μg/ml</th>
<th>50</th>
<th>25</th>
<th>7.5</th>
<th>2.5</th>
<th>1.0</th>
<th>0.75</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine 100</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine 200</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine 300</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cresol 100</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cresol 200</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cresol 300</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyrocatechol 100</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyrocatechol 200</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyrocatechol 300</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Protocatechic acid 100</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Paraphenyl 100</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phloroglucinol 100</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

— no growth; + growth.
4. Addition of certain purine and pyrimidine bases to the medium and the inhibition of growth of the two species of bacteria by fusaric acid

Adenine, hypoxanthine, guanine, xanthine among purine bases, cytosine, uracil, thymine, cytidine, uridine, thymidine among pyrimidine bases and adenosine, guanosine and xanthosine among purine ribosides were used in this experiment. Two different concentrations of the individual amendments were used, viz. 100 and 500 µg/ml. None of these compounds had any effect in reversing the toxicity of fusaric acid to Bacillus subtilis and Escherichia coli.

5. Addition of certain detoxicating agents to the medium and the effect of fusaric acid on the growth of Bacillus subtilis and Escherichia coli

Certain metabolites in human and animal tissues when they reach toxic levels are rendered harmless through the action of certain enzymes, or through conjugation reactions. This process is termed detoxication. Glycine, cysteine and glucuronic acid are known to play a specific role in conjugation reactions. The pyridine ring, in particular, when administered to the animal body is detoxicated by methylation (Williams 1947). Choline, methionine, sarcosine and the numerous betaines have been shown to be the methyl donors. In fact, when the minimum dose of fusaric acid was administered to tomato plants, i.e. 150 mg/kg fresh weight, a portion of it was metabolised into N-methyl fusaric acid derivative (Kluepfel 1957). Therefore it was planned to study the effect of certain of these substances responsible for detoxication in biological systems, on the toxicity of fusaric acid to the two bacteria.

The results of these investigations can be seen in Table 3. It will be noticed that of all the substances tried, glucuronic acid removed the toxicity of fusaric acid to some extent. Thus with 1000 µg/ml glucuronic acid, the minimum dose of fusaric acid needed for inhibiting the growth of the

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
</table>

**The effect of certain detoxicating agents on the possible inactivation of the antibiotic potency of fusaric acid. Test organisms, Bacillus subtilis and Escherichia coli**

| Supplements in 1000 µg/ml | Fusaric acid concentration µg/ml |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | 80  | 40  | 25  | 7.5 | 2.5 | 0.75 | 0.25 |
| Choline | - | - | - | - | + | + | + |
| Betaine | - | - | - | - | + | + | + |
| Methionine | - | - | - | - | + | + | + |
| Cysteine | - | - | - | - | - | - | - |
| Sarcosine | - | - | - | + | + | + | + |
| Glucuronic acid | - | - | + | + | + | + | + |
| Betaine, choline and Sarcosine | - | - | + | + | + | + | + |
| Glycine | - | - | - | + | + | + | + |
| None | - | - | - | - | - | - | - |

- no growth; + growth.
two organisms was above 25 μg/ml compared with 2.5 μg/ml in the control. Further increase in glucuronic acid concentration had no effect on the toxicity of fusaric acid. A somewhat similar situation was observed with sarcosine.

It is difficult to assess the role of glucuronic acid and sarcosine in decreasing the toxicity of fusaric acid to *Escherichia coli* and *Bacillus subtilis*. The possibility of a portion of the fusaric acid molecule undergoing detoxication reactions is not ruled out. These substances may also act by enhancing the nutrition which is restricted in the synthetic medium.

These experiments were repeated with the normal broth medium to rule out the second possibility. Glucuronic acid, however, still lowered the toxicity of fusaric acid, but the concentration of glucuronic acid used to reverse the toxicity was very high (Table 4) in both cases. The question remains whether such a high concentration of glucuronic acid as 10 mg/ml can be expected in normal biological systems. Sarcosine had no effect in this highly nutritive medium.

### Table 4

**The effect of sarcosine and glucuronic acid in reversing the toxicity of fusaric acid to *Bacillus subtilis* and *Escherichia coli* in the broth medium**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Amendment at 10 mg/ml</th>
<th>Fusaric acid μg/ml</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>75</td>
<td>60</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Glucuronic acid</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Sarcosine</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Glucuronic acid</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Sarcosine</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

— no growth; + growth.

6. On the possibility of a factor being present in yeast extract, beef extract, casamino acid or peptone which may antagonise the toxicity of fusaric acid

Woods (1940) observed that the potency of sulfanilamide was considerably lowered when the bacteria were grown in a medium supplemented by yeast extract. Subsequent work resulted in the discovery of p-aminobenzoic acid which is present in yeast extract and which accounted for the lowering of the potency of this drug. It has been observed in the first experiment that the potency of fusaric acid is lowered by about fifteen times in the broth medium compared to that in the synthetic medium. So it was thought profitable to investigate certain common biological substances of indefinite composition like yeast extract (Difco), beef extract, peptone and casamino acids (Difco) for a possible fusaric acid antagonist.

The substances were supplied at a concentration of 1000 and 3000 μg/ml to approximate the concentrations normally used in the media for growing bacteria. The results are presented in Table 5.
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Table 5
The effect of yeast extract, beef extract, casamino acids and peptone
in the synthetic medium on the toxicity of fusaric acid to the bacterial species,
Bacillus subtilis and Escherichia coli

<table>
<thead>
<tr>
<th>Supplements in µg/ml</th>
<th>Fusaric acid in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80</td>
</tr>
<tr>
<td>Yeast extract 1000</td>
<td>—</td>
</tr>
<tr>
<td>Yeast extract 3000</td>
<td>—</td>
</tr>
<tr>
<td>Casamino acids 1000</td>
<td>—</td>
</tr>
<tr>
<td>Casamino acids 3000</td>
<td>—</td>
</tr>
<tr>
<td>Peptone 1000</td>
<td>—</td>
</tr>
<tr>
<td>Peptone 3000</td>
<td>—</td>
</tr>
<tr>
<td>Beef extract 1000</td>
<td>—</td>
</tr>
<tr>
<td>Beef extract 3000</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
</tr>
</tbody>
</table>

— no growth; + growth.

It will be seen that yeast extract and beef extract do reduce the toxicity of fusaric acid considerably, but peptone and casamino acid do not have a similar effect. Although peptone and casamino acid increase the minimum dose of fusaric acid, once a particular level is reached they have no influence. On the other hand, with yeast and beef extract the situation is quite different. With increasing concentrations of these two substances the minimum dose of fusaric acid for growth inhibition also increases. Especially with yeast extract one notices that the reversal of the toxicity of fusaric acid is competitive within the concentrations tried.

Hence further investigation was undertaken to look for the “probable antagonist” of fusaric acid in yeast.

IV. THE SEPARATION OF THE “PROBABLE ANTAGONIST” OF FUSARIC ACID FROM YEAST EXTRACT

1. Modification of the technique to demonstrate competitive antagonism
   a) Paper disc or agar cup method

Haenel (1956) described a simple test on agar plates to demonstrate non-competitive and competitive antagonism between a metabolite and its anti-metabolites. On agar containing the test organism, two porous cups are placed some distance apart, one containing the metabolite and the other the anti-metabolite. Material diffuses from the cups to give concentration gradients of the metabolites and the anti-metabolites. The organism grows well when it receives the metabolite (M). The non-competitive inhibitor (N) prevents growth as far as it diffuses in the agar and gives a circle of complete inhibition. The competitive inhibitor (C) also has a circle of no growth.
around it, but this circle is interrupted as the metabolite concentration changes. This produces a straight demarcation line of growth between the metabolite and the anti-metabolite (Figs. 1 and 2).

**Fig. 1.** Diagram of competitive and non-competitive antagonism demonstrated with paper disc method.

**Fig. 2.** Competitive antagonism between yeast extract and fusaric acid demonstrated with the paper-disc method. Test organism *Candida vulgaris*. Concentration of fusaric acid $5 \times 10^{-2}$ molar, that of yeast extract 500 mg/ml

#### b) Cross strip technique

Two filter paper strips, 5 mm broad and 6 cm long, are dipped in the solution of metabolite and anti-metabolite respectively, and are dried between blotting paper. The two strips then are laid at right angles on the agar containing the test organism (Fig. 3). If the two substances diffuse identically, they produce an area of diffusion taking the shape of a cross as shown in Fig. 3. This may be possible when the two substances are structurally similar and of a similar molecular size. Where the two substances meet and overlap there are points where the concentrations of both substances are the same. These points lie on two lines running at an angle of $45^\circ$.

**Fig. 3.** Diagram of competitive antagonism with the cross strip technique. The horizontal strip contains the antimetabolite and the vertical one the metabolite.
to the axes. At a particular concentration of the metabolite and the anti-metabolite, these two lines pass through the coaxial point. At this concentration the ratio of the amount of metabolite to anti-metabolite gives the inhibition index. (Juillard 1957).

2. Competitive antagonism of yeast extract to fusaric acid
as shown by cross strip technique

*Escherichia coli* and *Bacillus subtilis* were grown in the synthetic agar medium in Petri dishes. In each Petri dish 17.5 ml of agar were covered with 5 ml of agar containing the test organism. The second layer was poured after the first layer had cooled and solidified. The agar received a constant amount of spore suspensions from cultures of the same age.

Preliminary experiments showed that a fusaric acid concentration of $5 \times 10^{-8}$ molar absorbed on the filter paper, produced inhibition zones on both test organisms.

Difco yeast extract was tried in three different concentrations for its antagonism to fusaric acid with the cross strip technique. The concentrations tried were 100, 500 and 1000 mg/ml, while that of the fusaric acid was $5 \times 10^{-3}$ molar. Similar experiments were set up with the two species of bacteria grown on broth agar. The fusaric acid concentrations used were $10^{-2}$ molar and $2.5 \times 10^{-2}$ molar. After placing the strips of fusaric acid and yeast extract in their proper positions, the plates were incubated at 37 °C for eighteen hours in the case of broth medium and thirty-six hours for the synthetic medium. In the synthetic medium the inhibition of both test organisms by fusaric acid was reversed by yeast extract. Growth and inhibition zone formed an angle of $45^\circ$ to the axis. This effect was most pronounced with 1000 mg/ml yeast extract, but could not be distinctly observed with test organisms grown in broth agar.

Since the concentration of yeast extract used was fairly high, it is reasonable to conclude that the antagonist to fusaric acid is probably present in small concentrations. So it was decided to tackle this problem from two sides. Firstly to look for an organism that needs smaller amounts of the fusaric acid antagonist, and secondly, to remove most of the interfering ingredients selectively from the extract.

3. Selection of a more sensitive test organism

In the relationship between a metabolite and an anti-metabolite the concentration of the one needed to nullify the effect of the other varies from organism to organism. Thus with 0.01 µg/ml of thiamine in the medium, the amount of the anti-metabolite pyrithiamine needed to reverse the growth is 0.02 µg/ml with *Cerastomella fimbriata*, 0.33 µg/ml with *Saccharomyces cerevisiae*, and more than 20,000 µg/ml with *Escherichia coli* (Woolley 1952).

As the probable antagonist of fusaric acid has been found in yeast extract, it occurred to us that yeast and yeast-like organisms might be more sensitive than bacteria. With this in view we undertook our further studies
with Candida vulgaris and Saccharomyces cerevisiae, which have already been shown to be sensitive to fusaric acid (Gaumann et al. 1952).

The two test organisms were grown on malt agar. The concentration of fusaric acid used on the paper strips was $5 \times 10^{-2}$ molar. Compared with the bacterial species, the concentration of fusaric acid used here was high. In spite of fusaric acid being a weak antibiotic, use of this potentiality is necessary to demonstrate the presence of an antagonist. Moreover, the filter strip technique though accurate, is not very sensitive. It is not the concentration but the absolute amount of the antibiotic on the filter paper which is responsible for the inhibition. Although the dilution technique is more sensitive, the strip technique is preferred since it avoids a large number of replications. In addition, the visual demonstration of inhibition and its reversal is more convincing and easily to observe in Petri dishes.

It was found that 500 mg/ml of yeast extract could competitively reverse the toxicity of fusaric acid to Candida vulgaris. Here the line of 45° passes through the coaxial point of the strips. At this concentration, however, the same result was not to be observed with Saccharomyces cerevisiae. Candida vulgaris therefore was used as the test organism for further studies.

4. Extraction procedures to isolate the "probable antagonist" of fusaric acid

For the extraction of the anti-fusaric acid factor from yeast extract, we followed the method schematically represented below.

50 g of Difco yeast extract were extracted with 1000 ml of cold acetone overnight in a sintered glass percolator. The acetone extract was evaporated to dryness and dissolved in 50 ml of water. The residue of yeast extract was tested for its activity and found to be active against fusaric acid. Further work was undertaken with the acetone insoluble portion of the yeast.

25 g of this acetone insoluble portion were extracted with cold methanol and ethanol respectively. Neither the ethanol nor the methanol soluble portions contained the factor antagonistic to fusaric acid, but all the activity remained in the residue. This activity was undiminished when compared with that of the original yeast extract.

5. Selective adsorption technique

Since the factor antagonistic to fusaric acid could not be extracted with acetone, methanol or ethanol, experiments were carried out to see whether it could be adsorbed on the two natural adsorbents, floridine and frankonite.

20 g of Difco yeast extract were dissolved in 200 ml of distilled water and divided into two equal portions. Each portion was supplemented by 5 g of frankonite and floridine respectively. After thorough shaking, the suspensions were centrifuged and the supernatant tested. The sediments were dried at room temperature and extracted separately with 500 ml distilled water and the same amount of ethanol — 1 N HCl (80 : 20). The extracts were evaporated to dryness at 50 °C in vacuo. The residues were redissolved in 10 ml of water, the pH of which was adjusted to 5 with 0.1 N Na₂CO₃. These solutions were tested for their biological activity against fusaric acid.
The results showed that most of the factor is adsorbed by frankonite and not by floridine. From frankonite the factor can be quantitatively recovered with ethanol HCl. This method made it possible to get rid of most of the other substances in the yeast extract and to proceed with the separation of the anti-fusaric acid factor.

V. SEPARATION OF THE FACTOR BY PAPER CHROMATOGRAPHY

1. Unidimensional chromatography

The ethanol water extract containing the active factor was spotted on Whatman No. 1 filter paper. The chromatograms were run in a solvent of 70% ethanol for 12 hours. Since the nature of the fusaric acid antagonist is unknown, it was not possible to locate the substance by spraying with a specific reagent; so the chromatograms were bioassayed by the two following techniques.

The substance was applied in six spots, and after running, the whole chromatogram was transversely cut into strips of $1.5 \times 25$ cm. It was possible to cut 25 such strips. Each strip was individually eluated with water by placing the narrow end of the strip between glass and immersing in a trough of water. The other tapering end was directed towards a beaker into which the liquid dropped. The whole experiment was carried out inside a moist chamber. At the end of 24 hours most of the substances were completely eluated from the paper strips. The eluates were evaporated to dryness at 50°C. Each sample was dissolved in a small amount of water and tested against fusaric acid on test plates of Candida vulgaris, using the cross strip technique. Antagonism to fusaric acid was observed on strip 11, and to a greater extent on strip 12. From this it could be found that the $R_t$-value of the antagonist was 0.61.

In the second technique the samples were applied to the paper in the form of a thin line. After running and drying, the chromatogram was cut lengthways into strips of $38 \text{ cm} \times 6 \text{ mm}$. Every strip was then divided again into pieces $2 \text{ cm} \text{ long}$. Pairs of these numbered strips, corresponding to the same $R_t$-values, were placed exactly over each other on test plates and bioassayed by the usual cross strip technique. More details of this experiment are diagrammatically shown in Fig. 4. It will be seen that the antagonist to fusaric acid activity is in strip 12.
is at one end of strip 11 and at both ends of strip 12. A reconstruction of these strips shows that the anti-fusaric acid factor was a spot extending from 22—25 cm, the centre of the spot lying at 22.5 cm. This gives an $R_f$ of 0.61.

Thus it was possible to show that the "probable antagonist" of fusaric acid could be separated from yeast extract and located on paper chromatograms as a substance having a definite $R_f$-value.

a) Character of the antagonist on paper chromatograms

After establishing the $R_f$ of the antagonist, it was intended to study some of its special characteristics both physical and chemical on the chromatograms. It is needless to mention that the factor, though it has been established on paper chromatograms, is not yet pure. Before embarking on further separation with another solvent, the chromatograms were observed under an ultra-violet lamp (Osram HQV 500 B, with 100% emission at 3650—3663 Å). At the spot where the biologically active substance was found, a dark blue fluorescent spot with a bright blue-white fluorescing border was noted. The spot was oval with the lower end broader than the upper and the border fluorescence more conspicuous at the top. When the substance was streaked as a thin line the blue-white fluorescing tops joined to form a wavy line.

2. $R_f$-value of the factor in different solvent systems

The samples were chromatographed with different solvent systems in order to establish that the biologically active substance was identical with the one showing the characteristic fluorescence in ultra-violet light. If this proved to be so, the active spot could be located by merely inspecting the chromatograms under ultra-violet light.

For chromatography with different solvent systems, samples were first collected on a large scale. The frankonite eluate from yeast extract was chromatographed with 70% ethanol on Whatman Nr. 3 MM. The samples were streaked at the starting line. The active zone, 2 cm broad, ($R_f$ 0.62) which showed ultra-violet fluorescence, was eluated from ten chromatograms by soaking small strips in warm 80% ethanol. Subsequent experience showed that the addition of a small amount of 0.2 N HCl gave better and quicker extraction. The dried extract weighed about 20 mg. This was dissolved in 5 ml of water and used for further chromatography.

0.1, 0.2 and 0.5 ml of the solution were spotted on Whatman No. 1 paper. The different solvent systems used, and the $R_f$-value of the biologically active spots are given in Table 6.

The $R_f$-value of the active spot was established by the characteristic fluorescence. The spots were cut out and eluated as mentioned above. After
Table 6
Rf-value of the anti-fusaric acid factor in different solvent systems

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethanol : water (70 : 30)</td>
<td>0.62</td>
</tr>
<tr>
<td>isopropanol : water (70 : 30)</td>
<td>0.58</td>
</tr>
<tr>
<td>n-butanol saturated with 3% ammonia</td>
<td>0.18</td>
</tr>
<tr>
<td>sec. butanol : formic acid : water (75 : 15 : 10)</td>
<td>0.41</td>
</tr>
<tr>
<td>n-butanol : acetic acid : water (4 : 1 : 5)</td>
<td>0.44</td>
</tr>
</tbody>
</table>

adjusting the pH to 4.5, the eluates were separately tested for their activity on plates of Candida vulgaris. All spots eluted were biologically active, thus showing that the factor could be located on paper chromatograms by its characteristic fluorescence in ultra-violet light.

3. Attempt to detect the active spot with chemical reagents

The chromatograms were sprayed with different specific reagents. Positive reactions were shown only by substances which were not identical with the active factor. These substances showed Rf-values of 0.15, 0.31 and 0.62 in n-butanol, acetic acid, water (4 : 1 : 5) where the active substance had an Rf-value of 0.44, Table 6.

a) Tests for amino groups

An 0.25 % solution of ninhydrin in 96 % ethanol was sprayed. The chromatograms were then heated to 80 °C for fifteen minutes. The substances with Rf-values of 0.15 and 0.62 gave a positive reading in contrast to the antagonist of fusaric acid and the substance with an Rf-value of 0.31.

b) Tests for phenolic groups and aromatic amines

Diazonium salts react readily with phenols and aromatic amines to form bright coloured azo compounds. Diazotized sulfanilic acid or benzene-diazonium chloride are used as reagents. Colours are generally produced by these reagents with phenols and tertiary amines having a free position in ortho or para to the hydroxyl or amino group. After development in the second solvent system no reaction of the biologically active spot was observed, although there was sometimes a slight positive reaction after development in the first solvent system.

c) Tests for pyridine and allied substances

Two reagents have been widely used for pyridine derivatives such as picolinic acid, fusaric acid, nicotinic acid etc.

Dried chromatograms are exposed for an hour to cyanogen bromide vapour. Then they are sprayed with the following solution of p-amino-benzoic acid: this is prepared by dissolving 2 g of substance in 75 ml of
0.75 N HCl and adding 25 ml ethanol. Colours produced by this reagent vary between red, orange and yellow.

The Dragendorff reagent was prepared according to the method of Linskens (1955). Fusaric acid and certain other carboxylic acids give a rosy-orange spot with this reagent.

The antagonist of fusaric acid did not react with either reagent.

d) Tests for acidic or basic groups

The reagent was prepared by dissolving 40 mg of bromocresol green in 100 ml of distilled water and adjusting the colour to a dark blue with NaOH. Then it was sprayed on the chromatograms. A yellow spot on a green-blue background was distinguishable which coincided with the factor. This reaction suggests that the factor is acidic in character.

VI. BEHAVIOUR OF THE FACTOR ON ION EXCHANGE RESINS

The behaviour of the factor on anion and cation resins might give additional information on its chemical nature, and it might also be helpful in large scale extraction. In this investigation Dowex 2, a strongly basic anion resin (X 8, 100—200 mesh, OH\textsuperscript{-}-form), and Dowex 50, a strongly acidic cation resin (X 8, 100—200 mesh, H\textsuperscript{+}-form) were used. The resins were used in columns 2 cm broad and 6 cm high.

The Dowex 2 column was prepared by washing the resin with 60 ml 1 N NaOH followed by water until the eluate was neutral. After applying 2 ml of the biologically active solution from unidimensional chromatograms, the column was first eluted with 60 ml of water and then with 80 ml of 1 N acetic acid. Both eluates were evaporated in vacuo to dryness and tested for activity. It was found that only the second eluate was active; thus further indicating that the factor is acidic in character.

A similar amount of active sample was applied to the Dowex 50 column, which was prepared by washing with 1 N HCl and water until the eluate was neutral. The column containing the factor was treated with 60 ml of water and the same amount of 0.5 N HCl. The eluates were again evaporated to dryness, redissolved and tested for activity, having adjusted the pH to 4.5. Only the water eluate showed activity. This also shows that the factor is acidic.

VII. BIOLOGICAL ACTIVITY OF THE FACTOR

1. The nature of the antagonism between fusaric acid and the factor

a) Inhibition index

In a competitive antagonism the potency of the inhibitory analogue may be expressed as a number called the inhibition index. This is the quotient of the concentration ratio of the two substances where their effects are just counterbalanced (Woolley 1952). In the present in-
vestigations it was possible to obtain a picture similar to that theoretically expected (Fig. 3) by varying the concentration of one of the analogues. In order to determine this figure, the following procedure was adopted. Twenty-four spots of the active substance were eluated from two dimensionally run chromatograms. The eluate, evaporated to dryness, weighed 2.48 mg. This was dissolved in 2 ml of water and diluted to $\frac{1}{2}$, $\frac{1}{4}$ and $\frac{1}{8}$. The four concentrations were tested against $5 \times 10^{-2}$ molar fusaric acid by the cross strip technique. The test organism was again *Candida vulgaris*. Fig. 5 shows that the action of fusaric acid and antagonist were just counterbalanced at the original concentration of the factor. The results suggest that the inhibition index of fusaric acid is in the order of about 10. However, it is possible that this figure is too small since the extract contained an unknown amount of impurities.

![Fig. 5. Competitive antagonism between fusaric acid and the factor from yeast extract after separation on paper chromatograms. The action of the metabolite in this figure just counterbalances that of the antibiotic. From the concentration of the two the inhibition index is calculated. Test organism *Candida vulgaris*](image)

**b) Competitive nature of the antagonism**

When the inhibition index is constant over a wide range of concentrations the antagonism is said to be competitive (Woolley 1952). In the cross strip technique by varying the concentration of one of the analogues (factor), the border line between inhibition and growth zone undergoes a parallel shift. Only at one concentration, however, does the line of 45° pass through the coaxial point (Fig. 5). Thus we have positive evidence that **fusaric acid inhibits the growth of Candida vulgaris by competitively inhibiting the action of a metabolite (factor from yeast extract).**

2. The spectrum of action of the factor

The antagonism between the factor and fusaric acid could not only be demonstrated with the fungus *Candida vulgaris*, but also with certain bacteria. This proved to be difficult since the bacteria had to be grown in a medium containing as small an amount of factor as possible though still permitting growth of the bacteria.

With *Bacillus subtilis* it was possible to show a competitive antagonism between fusaric acid and the factor, using a synthetic agar medium (Fig. 6). A similar situation was observed with *Staphylococcus aureus* grown in the synthetic medium supplemented by 0.25 % yeast extract. This addition was necessary for good growth since this organism is heterotrophic to a number of essential metabolites. The concentrations of fusaric acid used were $5 \times 10^{-3}$ molar for *Bacillus subtilis* and $10^{-2}$ molar for *Staphylococcus aureus.*
With the fungus *Ustilago sphaerogena* a competitive antagonism between the factor und fusaric acid could be demonstrated only in a medium of 25 % soil extract and 0.3 % yeast extract. In a synthetic medium, however, growth was very poor. The concentration of fusaric acid used was $10^{-2}$ molar and $2 \times 10^{-2}$ molar.

With *Escherichia coli* the factor eluated from frankonite showed non-competitive antagonism, as did the spot eluated from the chromatogram. The concentration of fusaric acid used was $5 \times 10^{-3}$ molar.

A similar result was observed with yeast (*Saccharomyces cerevisiae*) grown on malt agar. The concentration of fusaric acid was $5 \times 10^{-2}$ molar.

3. Influence of pH on the biological activity of the factor

A solution of the factor was divided into six equal portions. The pH of the solutions, which originally was 4.5, was adjusted to 3, 4, 5, 6, 7 and 8 with HCl and Na₂CO₃, respectively. With *Candida vulgaris* the antagonism could be observed over the whole pH range; while at the pH of 3 and 4 a slight toxicity was observed at the coaxial point of the strips, an optimum antagonistic effect appeared at pH 5. With bacteria however, no antagonistic activity was found at pH 4 or 5; it started at pH 6 and was optimum at pH 8.

Control experiments were done to ensure that at the higher pH the antagonism to fusaric acid is only due to the factor, and not to the dissociation of fusaric acid, which depends on the pH. Buffer solutions, with a pH varying from 3 to 11, were made from 1 molar Na₂CO₃ and 1 molar KCl and H₃BO₃. Even at pH 10 and 11 the buffers had practically no effect on the growth inhibition caused by fusaric acid. The effect of these buffers on the toxicity of fusaric acid to all the test organisms used was studied with the cross strip technique.

VIII. ORGANISMS PRODUCING THE ANTAGONISTIC FACTOR

On the basis of the theory of competitive antagonism, fusaric acid acts by inhibiting the action of a metabolite. Its toxicity to the organism could be removed by externally supplying the metabolite i.e. the factor from yeast extract. Therefore, the antagonistic factor is produced by all the organisms mentioned above, though probably in differing quantities.
The anti-fusaric acid factor was not only detected in yeast extract but also in Tryptone (Difco), N—Z amine and wheat corn residue. The latter source suggests that the factor is produced by higher plants as well as by microorganisms.

1. Production of the antagonist to fusaric acid by *Fusarium lycopersici*

During investigations with the culture filtrate of *Colletotrichum fuscum* it was accidently observed that this fungus produced a substance with similar chromatographic properties as the anti-fusaric acid factor. Closer studies showed that *Colletotrichum fuscum* actually produces the substance in question\(^1\).

Since *Fusarium lycopersici* produces fusaric acid it may also produce closely related substances, such as the anti-fusaric acid factor. In these investigations *Fusarium lycopersici* was grown for three weeks. One litre of culture filtrate was shaken with increasing concentrations of acetone. After the addition of 4 litres of acetone, precipitation was completed. The precipitate was lyophilised and weighed 1.48 g. 50 mg of this amount were dissolved in water and tested against fusaric acid with the cross strip technique. Here the action of fusaric acid was competitively antagonised. However, after hydrolysing the material in 4 N HCl for four hours at 80 °C and neutralising in an atmosphere of NaOH, the activity of the factor increased about five times.

2. Paper chromatography of the factors produced by *Fusarium lycopersici* and *Colletotrichum fuscum*

The hydrolysed extracts of *Fusarium lycopersici* and *Colletotrichum fuscum* were chromatographed with different solvent systems on Whatman No. 1 paper. The biologically active antagonistic factor showed characteristic fluorescence at the same *R*\(_f\)-values in two different solvent systems (Table 7). On this basis it is concluded that the factors produced by *Fusarium lycopersici*, *Colletotrichum fuscum* and contained in yeast extract are identical.

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Source</th>
<th><em>R</em>(_f)</th>
<th><em>R</em>(_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-butanol : acetic acid : water 4 : 1 : 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difco yeast extract</td>
<td>0.44</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td><em>Fusarium lycopersici</em></td>
<td>0.42</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td><em>Colletotrichum fuscum</em></td>
<td>0.44</td>
<td>0.61</td>
</tr>
</tbody>
</table>

\(^1\) The author wishes to express his gratitude to Dr. R. N. Goodman for kindly supplying material and suggesting methods for this investigation.
IX. ANTAGONISM BETWEEN THE FACTOR AND STRUCTURAL ANALOGUES
OF FUSARIC ACID

1. Toxicity of certain pyridine derivates to *Candida vulgaris*

Extensive studies have been made on the effect of fusaric acid and pyridine derivatives on the water permeability of plant protoplasts (Bachmann 1956, 1957). These studies showed that the specific injury caused by fusaric acid at concentrations above $5 \times 10^{-4}$ molar is, to a large extent, due to the length of the aliphatic side chain of the pyridine ring.

It was therefore of interest to investigate the activity of the factor against structural analogues of fusaric acid with differing lengths of the aliphatic side chain.

In these experiments the following substances with a decreasing length of aliphatic side chain were used: fusaric acid (5-n butylpyridine-2-carboxylic acid), dehydrofusaric acid (5-n butylenepyridine-2-carboxylic acid), 5-ethyl-picolinic acid (5-ethylpyridine-2-carboxylic acid), 5-methyl-picolinic acid (5-methylpyridine-2-carboxylic acid) and picolinic acid (pyridine-2-carboxylic acid). In addition, one pyridine derivative was used which did not contain a carboxyl group, namely, 3-n butyl-pyridine.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusaric acid</td>
<td>21.5 mm</td>
</tr>
<tr>
<td>Dehydro fusaric acid</td>
<td>20.5 mm</td>
</tr>
<tr>
<td>Ethyl picolinic acid</td>
<td>10.0 mm</td>
</tr>
<tr>
<td>Methyl picolinic acid</td>
<td>7.5 mm</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>0.0 mm</td>
</tr>
<tr>
<td>3-n-butyl pyridine</td>
<td>0.0 mm</td>
</tr>
</tbody>
</table>

Experiments were conducted to study the antibiotic potency of all these substances using the paper disc method on plates of *Candida vulgaris*. The results are presented in Table 8. α-picolinic acid and 3-n butyl-pyridine showed no toxicity but with all the other compounds tested, greater toxicity was observed with an increasing length of the aliphatic side chain.

2. The effect of the antagonist on structural analogues of fusaric acid

Fusaric acid, dehydrofusaric acid, ethyl picolinic acid and methyl picolinic acid were tested against the same concentration of the factor antagonistic to fusaric acid. All the pyridine derivatives were used at a concentration of $5 \times 10^{-2}$ molar.
All the pyridine compounds were competitively inhibited by the anti-
fusaric acid factor (Figs. 7, 8 and 9). However, with the decreasing toxicity of ethyl- and methyl-picolinic acid, the activity of the antagonist is increased. Using the same amount of antagonist the parallel shift in the 45° line between growth and inhibition zones obtained with these compounds is the same as may be observed with low concentrations of fusaric acid. Though fusaric acid and dehydrofusaric acid are equally toxic, the antagonist was more active with the former than with the latter (Figs. 7 and 8).

Figs. 7, 8 and 9. Competitive antagonism between the factor isolated from yeast extract and fusaric acid (Fig. 7), dehydrofusaric acid (Fig. 8) and ethyl-picolinic acid (Fig. 9). Test organism Candida vulgaris. The concentration of the antagonist is the same against all the toxins.

X. THE EFFECT OF THE ANTAGONIST ON THE SYMPTOMS OF FUSARIC ACID INJURY IN TOMATO SHOOTS

It has been shown that the removal of toxicity of the antimetabolite by the metabolite varies both qualitatively and quantitatively with different test organisms. The antagonistic effect between a specific metabolite and antimetabolite could be shown with bacterial species but not in animals or human beings or vice versa (Woolley 1952). A demonstration of an antagonistic effect also proved to be difficult in our experiments with tomato plants. Moreover, it was difficult to obtain sufficient quantities of the factor with very few interfering substances, to test with a large number of tomato plants.

Preliminary experiments with the extract from yeast extract, which was previously found active on test plates of Candida vulgaris, showed no toxic injury to tomato plants even though it contained certain impurities.

Three series of twenty cut shoots of tomato plants (four-leaf stage) were allowed to take up fusaric acid, anti-fusaric acid factor and fusaric acid plus anti-fusaric acid factor, respectively. 225 mg of toxin per kg fresh weight was applied in a concentration of $2.5 \times 10^{-3}$ molar. The concentration of the antagonist was so chosen that it just counterbalanced the effect of a
standard concentration of fusaric acid in tests with *Candida vulgaris*. The solutions applied to the tomato plants had a pH of 4.3.

Two separate experiments were conducted with samples of antagonist from two different extractions. The results are presented in Table 9. The Wilt index was calculated as detailed in earlier works (Gäumann, Naei-Roth and Kobel 1952). In both experiments antagonism to the effect of fusaric acid is seen only in the leaves. In the first experiment the toxicity of fusaric acid to the leaves is reduced by about 70 % and in the second by about 75 %. The injury to the stem, however, remains undiminished.

**Table 9**

*Symptom production by fusaric acid on tomato in the presence of the antagonist*

Concentration of the toxin in all experiments was $2.5 \times 10^{-3}$ molar

<table>
<thead>
<tr>
<th>Exp.</th>
<th>pH of test solution</th>
<th>dose fusaric acid in mg/kg</th>
<th>Wilt index 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>fusaric acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>stem</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>stem</td>
</tr>
<tr>
<td>1</td>
<td>4.3</td>
<td>225</td>
<td>2.20</td>
</tr>
<tr>
<td>2</td>
<td>4.3</td>
<td>225</td>
<td>1.85</td>
</tr>
<tr>
<td>3</td>
<td>4.3</td>
<td>150</td>
<td>1.10</td>
</tr>
<tr>
<td>4</td>
<td>4.3</td>
<td>150</td>
<td>1.10</td>
</tr>
<tr>
<td>5</td>
<td>6.5</td>
<td>150</td>
<td>0.10</td>
</tr>
</tbody>
</table>

1) The Wilt Index is a measure of the damage to tomato plants where 0 indicates no damage and 4 complete collapse of the plant.

The dose of fusaric acid applied in the above experiments is considerably higher than the dosis minima (150 mg/kg). It seemed reasonable to assume that if fusaric acid were to be antagonised in the stem this was more likely to occur at a lower dose, with no excess of toxin. Therefore in the following experiments (No. 3 and 4, Table 9), cut shoots of tomato were dosed with 150 mg/kg fusaric acid at a concentration of $2.5 \times 10^{-3}$ molar. The concentration of the antagonist obtained from two different extractions was again so chosen that it just counterbalanced the effect of the standard concentration of fusaric acid in tests with *Candida vulgaris*. The pH of the test solutions was 4.3. The results are presented in Table 9.

In both these experiments with the lower dosage the stem injury caused by fusaric acid is reduced by 40—45 % respectively, and the leaf injury again by about 70 %.

In one experiment solutions of the toxin and the antagonist were applied to tomato shoots at a pH of 6.5. The toxin dose was 150 mg/kg fresh weight and the concentration of the antagonist was chosen in the same way as in the previous experiments.

At this pH, fusaric acid only slightly affected the stem; the injury was seen on the leaves. With the toxin and antagonist the stem was not damaged at all and the leaf injury was reduced by about 35 %.
XI. DISCUSSION

The antimetabolite nature of fusaric acid

In the case of competitive antagonism between two substances in a biological system, it is generally assumed that one of them is the substrate for an enzyme reaction and the other its structural analogue (Woods and Tucker 1958). The latter combines with the same active centres of the enzyme as the substrate, but the combination does not result in enzyme activation and so the analogue is not metabolised.

\[
\text{Enzyme} + \text{Substrate} \xrightarrow{\text{Analogue}} \text{Product} + \text{Enzyme}
\]

Thus, in a competitive antagonism the substrate can overcome the inhibition caused by the analogue, and the concentration required will rise in direct proportion to the concentration of the analogue.

However, certain other substances in addition to the substrate can prevent or overcome the inhibitory action of the analogue. If the product of the above enzyme reaction, or substances biosynthetically derived from it, were to be available preformed in the medium, they could also affect the inhibitory action of the analogue. The amount of these products needed is fixed by the requirements of the organism. Thus, the effect of an inhibitor in any biological system is overcome competitively normally by its analogous metabolite.

Our experiments have proved conclusively that the toxicity of fusaric acid to certain microorganisms could be competitively eliminated by the analogous metabolite (factor from yeast extract). Conversely, fusaric acid possesses the character of an antimetabolite.

The part of the fusaric acid molecule responsible for its toxicity

Fusaric acid has an n-butyl group at position 5 of the pyridine ring (Gaumann, Naef-Roth and Kobel 1952). When this is substituted by an ethyl or methyl group, the toxicity is progressively reduced. Dehydrofusaric acid, which has a butylene group instead of the n-butyl group of fusaric acid, shows almost the same toxicity as fusaric acid.

Given the basic structure of a-picolinic acid, the length of the alkyl side chain of the analogues determines the magnitude of their toxicity. A similar observation was made by Bachmann (1956, 1957) in her studies on the injury caused by various pyridine derivatives to plant protoplasts.

A somewhat similar situation occurs with the vitamin thiamine and its structural analogues. The vitamin has a methyl group at position 2 of the pyrimidine ring. The corresponding ethyl analogue has only 10% of the original vitamin activity. The butyl analogue, however, is an antimetabolite (Woolley 1952).
The possible nature of the factor antagonistic to fusaric acid

In contrast to the situation with thiamine, even complete removal of the aliphatic side chain of fusaric acid (leading to α-picolinic acid) did not give rise to the analogous metabolite.

It appears that the metabolite in question (factor from yeast extract) may be a derivative of fusaric acid with a change in the pyridine ring. We have the following evidence for this view:

a) The factor from yeast extract gave no reaction for pyridine derivatives on paper chromatograms.

b) Nicotinic acid does not antagonise the toxicity of fusaric acid; thus a simple shift of the carboxyl group, which in fusaric acid is essential for its toxicity, does not lead to the metabolite in question.

c) The most potent antimetabolites, some of which are antibiotics, are structurally only slightly different from their analogous metabolites (Woolley 1952). Since fusaric acid is a weak antibiotic, it is to be expected that the corresponding metabolite may differ considerably in its structure from the toxin.

d) The factor is capable of antagonising all toxic pyridine derivatives investigated so far.

It has been recently shown that certain piperidine derivatives play an important role in biological systems (Grobbelaar, Pollard and Steward 1955). Certain piperidine-carboxylic acids have nicotinic acid activity in certain organisms. The piperidine ring is derived from the pyridine ring by complete hydrogenation.

Therapy of fusaric injury to tomato plants

The toxic injury of fusaric acid to cut shoots of tomato plants could be partly removed by the factor isolated from yeast extract. With 225 mg of toxin per kg fresh weight of tomato shoots and a particular amount of the antagonist there is a 70% reduction in the injury to the leaf. With 150 mg of the toxin per kg fresh weight and a correspondingly smaller amount of the antagonist there is nearly the same reduction in the leaf injury. Thus, the antagonism appears to be competitive in the leaves. However, the situation appears to be different with the stems of these plants. Only with the lower dose of toxin there is some reduction to the injury of the stem.

Previous work has shown that in contrast to the stem, the leaves do not react so strongly to changes in the pH of the toxin solution (Gäumann 1958). We therefore draw the following conclusions:

a) Within limits of experimental error it has been shown that the antagonism between fusaric acid and the factor is competitive in tomato plants.
b) This is most conspicuous in the leaves, where damage caused by fusaric acid is little affected by pH changes. Moreover the leaves, being the active centre of metabolism, offer more reactive groups than the stems.

c) Therefore a part of the injury to tomato shoots caused by fusaric acid is attributable to its antimetabolite character. The situation is analogous in some human and animal diseases.

d) The visible injury to the stem is not due to the antimetabolite nature of fusaric acid alone.

e) This discrepancy between stem and leaf injury is also due to the problem of non-uniform translocation of the toxin and the antitoxin in the plant.

**Role of the metabolite in defence reactions**

It has been shown that fusaric acid and its antagonist are produced by the same pathogen, *Fusarium lycopersici*. If the biosynthesis were largely to follow the same pathway for both substances, excess production of one or the other will occur with only a slight shift in the growth conditions at some stage of the biosynthesis. This kind of change may play an important role in the pathogenic wilting.

Fusaric acid has now been established to be one of the toxins responsible for pathogenesis in the wilt diseases of cotton and tomato plants (Kalyanasundaram and Venkata-Ram 1956, Kern and Kluepfel 1956). Infection, generalisation and toxin production are essential prerequisites for a successful pathogenesis (Gäumann 1951). In some of the resistant varieties of host plants the pathogens do generalize but do not cause any toxic symptoms (Gäumann 1951, Kalyanasundaram and Braun 1958). This was explained until now to be due to a lack of toxin synthesis. However, on the basis of our experimental evidence of the production of fusaric acid and its antagonist by the same pathogen, this may partly be attributable to an overproduction of the metabolite by the pathogen. The substrate offered for the growth of the pathogen may play a decisive role in the end stage of pathogenesis; thus after infection and generalization, an unbalanced production of toxin and antitoxin by the pathogen may determine ultimately the susceptibility of the host.

The amount of fusaric acid antagonist present in the tissues of host plants must be also taken into consideration with defence reactions. The minimum dose of fusaric acid needed to cause injury to any organism or organs depends on the proportional distribution of the antagonist. This could partly explain the differing sensitivity of various host plants to fusaric acid. Thus maize and pea are ten times less sensitive than tomato, which again is equally less sensitive than cotton (Gäumann 1957).
As yet we have not directly demonstrated the antagonist of fusaric acid in any of these host plants, but our assumption that they may produce it is based on the following facts:

a) The antagonist of fusaric acid is a necessary metabolite for a wide variety of organisms viz. bacteria, yeasts and fungi.

b) A part of the toxicity of fusaric acid to tomato shoots is due to its replacing the function of an essential metabolite (antagonist).

Summary

Fusaric acid (5n-butyl picolinic acid), one of the wilt toxins produced by *Fusarium lycopersici*, is also an antibiotic. The sensitivity of *Bacillus subtilis* and *Escherichia coli* to this antibiotic is increased about fifteen times in a synthetic medium than in a medium containing beef or yeast extract.

This lowered potency of fusaric acid is due to an antagonist present in these two extracts.

A number of known metabolites like phenols, vitamins, purine and pyrimidine bases etc. were not antagonistic to fusaric acid.

The antagonist of fusaric acid present in yeast extract could be separated by adsorption on frankonite and subsequent elutions. In two dimensional chromatograms the substance could be located as a single spot. The Rf values of the factor in different solvent systems are also given.

Results obtained by paper chromatography and adsorption on ion exchange resins point to the fact that the antagonistic factor has acidic character.

The antagonism between the factor from yeast extract and fusaric acid is competitive with *Candida vulgaris*, *Ustilago sphaerogena*, *Bacillus subtilis* and *Staphylococcus aureus*; it is non-competitive with *Saccharomyces cerevisiae* and *Escherichia coli*.

The antagonist was also found in certain other substances commonly used in biological media. In addition it is produced by *Fusarium lycopersici* and *Colletotrichum fuscum*.

The antagonist is active not only against fusaric acid but also against certain other toxic pyridine-derivatives which are structurally related to fusaric acid.

The antagonist is capable of removing the toxicity of fusaric acid to tomato shoots. The nature of antagonism was competitive in the leaves. The possible role of this antagonistic factor in the anti-toxic defence reactions of the host plants is discussed.

The toxicity of fusaric acid to a wide range of organisms is due to its competitive inhibition of the action of a metabolite. It seems likely that this metabolite, which has not yet been isolated in the pure form, is most probably structurally analogous to fusaric acid. Its molecule has possibly undergone a change in the pyridine ring.
Zusammenfassung

Fusarinsäure (5n-Butyl-Picolinsäure), eines der Welketoxine aus Fusarium lycopersici, ist auch ein Antibioticum. Bacillus subtilis und Escherichia coli werden durch dieses Antibioticum auf synthetischen Nährböden 15mal stärker gehemmt als auf solchen mit Hefee- oder Fleischextrakt.

Die geringere Toxizität der Fusarinsäure wird auf einen antagonistischen Faktor zurückgeführt, der in den beiden Extrakten enthalten ist.

Eine Anzahl bekannter Metaboliten wie Phenole, Vitamine, Purin- oder Pyrimidinbasen wurden auf ihre emthemmende Wirkung geprüft. Keiner der untersuchten Stoffe erwies sich als aktiv.

Der im Hefeextrakt nachgewiesene Antagonist wurde durch Adsorption an Frankonit angereichert. In zweidimensionalen Chromatogrammen konnte er als eine einheitliche Substanz nachgewiesen werden. Die R₁-Werte dieses Stoffes werden für verschiedene Fließmittel angegeben.

Das Verhalten dieses Stoffes bei der Papierechromatographie und an Ionenaustauschern läßt vermuten, daß es sich um eine Säure handelt.

Der Antagonismus zwischen Faktor und Fusarinsäure ist bei Candida vulgaris, Ustilago sphaerogena, Bacillus subtilis und Staphylococcus aureus kompetitiv, während er bei Saccharomyces cerevisiae und Escherichia coli nicht-kompetitiv ist.

Der antagonistische Faktor läßt sich auch in verschiedenen anderen für Nährböden verwendeten Substanzen nachweisen; überdies wird er von Fusarium lycopersici und Colletotrichum fuscum gebildet.

Der Antagonist hebt nicht nur die Wirkung der Fusarinsäure, sondern auch diejenige von anderen toxischen Pyridinderivaten auf.


Die Giftwirkung der Fusarinsäure gegen verschieden Organismen beruht darauf, daß sie einen essentiellen Metaboliten zu ersetzen und so dessen Wirkung kompetitiv aufzuheben vermag. Wahrscheinlich ist dieser Metabolit, der sich bisher noch nicht rein darstellen läßt, der Fusarinsäure strukturell analog. Es ist zu vermuten, daß der Metabolit im Vergleich zur Fusarinsäure einen kleinen Unterschied im Pyridinring aufweist.

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