STUDIES ON PHYTOALEXINS

I. THE FORMATION AND THE IMMUNOLOGICAL SIGNIFICANCE OF PHYTOALEXIN PRODUCED BY PHASEOLUS VULGARIS IN RESPONSE TO INFECTIONS WITH SCLEROTINIA FRUCTICOLA AND PHYTOPHTHORA INFESTANS

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Summary

In using the inner epidermis of Phaseolus vulgaris pods as host tissue and Sclerotinia fructicola and Phytophthora infestans as pathogens which interact in a hypersensitive fashion, a principle (or principles?) was separated from the former which exerts a strongly inhibitory effect on the growth of the latter. It is shown that this principle (phytoalexin):

(i) Is the result of an interaction between host and pathogen and is absent from non-infected host tissue at concentrations which could exert an inhibitory effect on the pathogen.

(ii) Is formed at a rate and at concentrations which are sufficient to prevent further growth of the pathogen in the diseased tissue.

(iii) Is not specific, and

(iv) Possesses properties from which its nature as an individual chemical factor(s) becomes obvious.

Concerning the action of the phytoalexin(s) under consideration, results as follows were obtained:

(i) The antibiotic activity is not influenced by chemical factors in the host tissue which may serve as nutrients to the pathogen.

(ii) Within a pH range of 4.0–7.5, the hydrogen ion concentration exerts neither an antagonistic nor a synergistic effect on the activity of the inhibitory principle.

(iii) There is strong adsorption of the active principle to non-parasitized cells.

(iv) The output per unit volume of parasitized tissue is dependent on the age of the host tissue.

Six other host–pathogen combinations, which interact in a hypersensitive fashion, were shown to produce, post-infectionally, inhibitory principles at concentrations sufficient to stop the pathogen’s growth.

In the light of these results, the mechanism is discussed which underlies the local lesion reaction. It is stressed that—at least in the cases under discussion—“resistance” is preconditioned by the ability of the host tissue to encounter the metabolic activities of the pathogen with the formation and the accumulation round the infection sites of an antibiotic principle which has been termed “phytoalexin”.

I. INTRODUCTION

The problem of disease resistance in plants has exercised the minds of phytopathologists for more than half a century. More than 50 years ago Ward (1902), investigating the behaviour of Puccinia dispersa in uncoenengial hosts, discovered

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that shortly after penetration the pathogen ceased to spread; simultaneously, the host tissue underwent necrotic changes and a "local lesion" resulted at the point of infection. In considering the fundamental cause of the checking of the pathogen in necrotic tissue Ward mooted the possibility that preformed toxins present in the host might be the actual cause of the arrest of the pathogen. But he failed to demonstrate the presence of such factors in extracts from plants which responded in this way and finally concluded that the antagonism must be due to something much more subtle "than a mere soluble poison oozing from the cells". He suggested that "the onslaught of the pathogen on the resistant host cells is too vigorous", and therefore the infected cells die too rapidly, and since dead cells are unsuitable as a medium for further growth of the mycelium, the parasite dies. Nevertheless, he maintained the view that enzymes or toxins might be involved in the local lesion reaction.

Subsequent authors (e.g. Gibson 1904; Marryat 1907; Stakman 1914; and Allen 1923, 1927) also considered the possibility that toxins and antitoxins were involved in the local lesion reaction, but due to lack of experimental evidence the toxin concept gradually lost ground, and became largely displaced by the nutritional hypothesis, which was fostered by quite a number of workers (e.g. Leach 1919; Wellensiek 1927). In this concept the non-establishment of the pathogen in resistant hosts was explained on the grounds that specific nutrients required by the parasite were absent from these hosts and thus the fungus died from starvation; subsequently necrosis of the host cells took place due to substances diffusing from the dead parasite. A variation of this concept suggested that diffusion substances from the parasite first killed the host cells and thereafter the parasite died of starvation.

Because of lack of experimental evidence in support of the nutritional hypothesis, however, the idea that there might be substances present or formed in the host tissue which could exert an inhibitory effect on the pathogen, continued to linger in many minds. Angell, Walker, and Link (1930) isolated such a toxic principle, protocatechuic acid, from scales of onion varieties resistant to onion smudge (Colletotrichum circinans). This toxin, however, is produced only in the dead outer scales of the bulbs and therefore it cannot be directly connected with the local lesion reaction.

About the middle of the thirties, Vavilov wrote a review in which all aspects of the problem of immunity of plants to infectious diseases were discussed. It includes also the Russian literature on the subject and may therefore serve as a useful guide to the understanding of the situation up to that time.

Müller and his co-workers (1939, 1940, 1949, 1950, 1953) and Meyer (1940) used a new approach. Müller and Börger (1940), working with different strains of Phytophthora infestans and tubers of resistant and susceptible potato hybrids, found that a Phytophthora strain, interacting with the host in a hypersensitive fashion, initiates a change in the parasitized tissue of such a kind that thereafter even virulent strains are not able to establish themselves in the changed tissue. Various strain–host combinations were tested. They always showed a predominance of the less virulent strain when inoculations were made simultaneously. Other
microorganisms, pathogenic and non-pathogenic to the potato tuber, were also checked in tissues which had reacted with an avirulent Phytophthora strain (Müller and Börger 1940). With these results the nutritional concept was ruled out as an explanation of the restriction of the pathogen’s growth on hypersensitive hosts. However, the change in the host tissue was a completely local one. If the speed of the hypersensitive reaction was reduced by pretreating the host tissue with narcotics, then the spread of the parasite increased (Behr 1949; Müller and Behr 1949). Furthermore it was shown in genetically analysed breeding lines that the spread of the pathogen in the host tissue decreased with increasing reaction rate (Müller 1953). From these results it was postulated that a principle, which was post-infectionally formed and non-specific, was actually responsible for checking the parasite in hypersensitive tissue. This principle was named “phytoalexin”, a designation which refers only to the biological (i.e. the defensive) action of the active principle and does not include any suggestion as to its chemical nature (Müller and Börger 1940).

This concept of plants being able to encounter an infection by the formation of “antibiotic bodies” was efficiently supported by subsequent investigations of Gäumann and Jaag (1945) and Gäumann, Braun, and Bazzigher (1950). These workers showed that orchid (Orchis militaris) tuber slices exposed to the metabolites of Rhizoctonia repens produce a fungistatic substance which has to be regarded actually as a new formation by the living tissues of the orchid tuber. The recent work of Kuč et al. (1955, 1956) and Kuč (1957) strongly supports the idea of the plant being able to respond to infections with the production of “antibody”-like chemical compounds. They showed that when slices of potato tubers were inoculated with Helminthosporium carbonum an antibiotic factor developed which not only checked Helminthosporium but also other fungi. These authors (1956, 1957) found an increased amount of chlorogenic and caffeic acid present in the inoculated tissue. The concentrations found, however, were insufficient for complete inhibition of the parasite in vitro. The authors therefore suggested a synergistic effect, in vivo, by other cell constituents which are not themselves fungistic.

Recent work reported from Japan and Australia may be mentioned in this connection. The Japanese workers (Tomiyama 1955, 1956; Tomiyama et al. 1956a, 1956b; Takakuwa and Tomiyama 1957) investigated cytomorphological and chemical changes in the tissue of potato tubers of various degrees of resistance to Phytophthora infestans, after infection with that fungus. Their results are in good accordance with those obtained by Müller and his co-workers and support the idea that post-infectional changes of the host tissue are the actual cause of the pathogen being checked in the resistant tubers. In Australia, Scott, Millerd, and White (1957) reported that they isolated from barley leaves a phenolic compound which inhibits the growth of Erysiphe graminis. This factor, however, was found to be present in healthy leaves of susceptible varieties as well. The authors therefore suggested that the difference in the reaction of susceptible and resistant varieties was due to the particular sensitivity of the protoplasm of the latter to some metabolites of the pathogen. As “cell collapse is associated with the release of this compound”, the metabolic activities of the pathogen’s haustoria are prematurely stopped.
Müller (1956) elaborated his phytoalexin theory on purely experimental lines by demonstrating the occurrence of an antibiotic factor in infected tissues under conditions which exclude any doubt that the inhibitory principle is the result of an interaction between host cell and pathogen and is responsible for arresting the growth of the latter in local lesions. Six preconditions had to be fulfilled for this purpose:

1. No preformed substances inhibitory to the pathogens used must be present.
2. The pathogen must be able to be grown on ordinary nutrient media.
3. The interaction between host tissue and pathogen (and so the formation of the antibiotic principle) must take place under conditions which exclude interference by contaminants.
4. Chemical extraction methods which may affect the active principle must be avoided.
5. Mechanical injury which may bring about the formation of other inhibitory factors must not be involved.
6. It must be possible to demonstrate that the antibiotic substance is present in vivo at concentrations sufficient to stop the pathogen’s growth.

Material which complied with these conditions was found in the epidermal tissue of seed cavities of bean pods (Phaseolus vulgaris). This tissue reacts to an infection with Sclerotinia fructicola, Phytophthora infestans, and other fungi, with local lesions. The present paper describes the experiments concerned in more detail, and results obtained will be discussed in relation to work of previous authors in the field of “reactive resistance” in plants.

II. MATERIAL AND METHODS

The basic test material was the epidermal tissue lining the seed cavities of Phaseolus vulgaris pods. Fruits of Pisum sativum, Vicia faba, and Capsicum annuum were also used.

Sclerotinia fructicola and Phytophthora infestans (strain B) were used as pathogens. Conidia and zoospore suspensions were applied as inocula. The Sclerotinia conidia were taken from pure cultures, from 4 to 10 days old, grown on potato dextrose agar; the Phytophthora zoospores were obtained from cultures maintained upon living potato tubers.

In addition to the fungi mentioned above, Colletotrichum lindemuthianum, Botrytis cinerea, Uromyces trifolii, Rhizoctonia solani, and Pythium ultimum were investigated with regard to their sensitivity to phytoalexin obtained from Phaseolus pods which had been inoculated with Sclerotinia or Phytophthora.

The method used to obtain the toxic interaction products has already been described (Müller 1956). It consists of inoculating the seed cavities with spore suspensions and collecting the latter after a certain period of time. The re-collected liquids which have been purified from ungerminated spores and mycelial fragments by centrifuging are referred to as “diffusates”.
The diffusates were examined by various methods for contamination with other microorganisms before being centrifuged. The number of contaminants, chiefly bacteria, varied between 0 and 800 per ml in nine tests.

The antibiotic activities of the crude diffusates were tested by preparing the following dilution series: $n$, $n/2$, $n/4$, $n/8$, $n/16$, or $n$, $2n/3$, $n/2$, $n/3$, $n/4$—where $n$ is the original concentration of the diffusate. Flat blocks of water agar (1 by 5 by 5 mm), seeded with conidia of *Sclerotinia* ($10^3$–$10^4$ per block) were placed in watch-glasses and covered with c. 0·1 ml of test liquid. No agar block was used in testing the sensitivity of *Phytophthora*. The test liquid was added to a thick zoospore suspension at an approximate ratio of 3:1. Water controls were used in each test series.

![Fig. 1.—Dosage–effect curves demonstrating the mode of calculation of phytoalexin concentration in two diffusates. *Sclerotinia fructicola* was used for inoculation and for bioassay.](image)

In using *Sclerotinia*, antibiotic activity was measured (1) by estimating the germination rate and the growth of the fungus after 18 hr (temp. 23°C) on a—to $+++++$ scale where — means no inhibition (=water control) and $+++++$ no germination (limit error at 15 per cent.); or (2) by measuring the length of 30 germ tubes lying completely within the focus of the microscope 9–13 hr after starting the test. It was found that alteration of constant temperatures within the range 8–25°C did not affect the sensitivity of the test organism. A critical examination of the reliability of this method was made by Messrs. Dudzinski and McIntyre and is given in Appendix I.

Dosage–effect–curves were used to determine, by graphic interpolation, the degree of dilution at which the mean germ tube length was reduced to 50 per cent. of the water control. The reciprocal of this degree of dilution was used as a numerical index of the antibiotic activity of the undiluted diffusate. Figure 1 illustrates the mode of calculation. In addition, this value was used to designate the amount of active units present in a diffusate; the phytoalexin (PA) unit was defined as that amount of PA which, when dissolved in 0·001 ml water, reduces the length of the germ tubes of *Sclerotinia* to 50 per cent. of the control.
Where Phytophthora was used as test organism, the standard for the antibiotic activity of the diffusate was taken to be that degree of dilution at which about 50 per cent. of the zoospores (water control = 100) still reached the stage of germination. Since, as we shall see in a later section, the sensitivity of the zoospores decreases with increasing age, only newly hatched zoospores were used.

Cytological changes which occurred in the infected host cells were studied using hand-sections and staining them with rhodamine B (10 p.p.m.).

III. Histological Observations

The proportion of Phytophthora germ tubes which penetrate into the epidermal cells is relatively high (up to 80 per cent.), and the actual penetration is usually preceded by swelling of the hyphal tips. The actual perforation of the outer wall of the epidermal cell is carried out by a part of the infection peg which is of very small diameter; immediately after perforation, however, when it enters the cell lumen, it expands to a globular organ filled with dense cytoplasm. It is at this stage that growth usually ceases in the type of infections under discussion. In the case of Sclerotinia, however, the proportion of germ tubes which enter the cell lumen is considerably smaller than in that of Phytophthora. Most of the developing germ tubes grow in close contact with the outer wall of the epidermis, without actually penetrating into the cell. There is here no swelling at the tips of the successful germ tubes such as is seen in Phytophthora. At this stage again, soon after penetration, growth of the infection hyphae ceases.

Six to 8 hr after penetration, the cytoplasm of the host cells becomes granular, and simultaneously the nuclei start to swell. After a further 2–4 hr a slight affinity to rhodamine B becomes apparent in the infected cell. This affinity gradually increases and becomes very obvious during the next 10 hr. At this stage in tissues inoculated with Phytophthora the staining is restricted to the infected cells (Plate 1, Fig. 1); where Sclerotinia is the parasite, however, many cells in the infection area, though showing no evidence of penetration, but only contact with the infection hypha, exhibit stain accumulation (Plate 1, Fig. 2).

After 14–16 hr the epidermal tissue under the inoculation droplets appears to be slowly losing its turgidity. At the same time, the pH decreases significantly within the infected cells, as demonstrated by using di-ethyl red as an indicator. The infected cells turn bright red, the non-infected ones stain yellow. By the use of a variety of indicator stains it was found that the pH drops from 6.3 to around 4.2. In the Sclerotinia infections, many cells which have not been penetrated but have only been in superficial contact with the fungus show the red stain when treated with di-ethyl red.

About this same stage in the infection it can be seen that the wall, cytoplasm, and nuclei of the infected cells begin to show an increasing brown pigmentation. The nucleus gradually shrinks, and the whole cell is apparently dying or dead. Since the parasite does not continue to spread after the first incubation period of 24 hr, the inoculated areas now appear to the naked eye as sharply bordered circular surfaces (Plate 2, Figs. 1 and 2).
During the course of the above reactions, the parasite also undergoes significant changes. After an incubation period of 20 hr, the germ tubes also begin to show an affinity for rhodamine B, regardless of whether they have been able to penetrate into the host tissue or not, and about this time also the growth of the parasite in the inoculation drops ceases. No further development of the parasite was observed 24 hr after inoculation. However, if necrotic tissue, which includes the restricted parasite (Sclerotinia), is inoculated into plums or apples, growth of the parasite is resumed. This shows that the parasite is still alive.

Summing up the morphological changes which have been found in the epidermis of the seed cavities of bean pods as a result of infection with Phytophthora or Sclerotinia, we may state that the histological changes observed comply with those which have been found in many other plants after infection with pathogens which interact with the host in a hypersensitive fashion.

IV. Experimental

(a) Basic Experiments Demonstrating the Appearance of Antibiotic Factors in Tissue Inoculated with Sclerotinia or Phytophthora

Before describing the inhibitory effects of the diffusates collected from inoculated tissue, it is necessary to mention some experiments designed to show that the tissues do not contain any preformed substance which could be responsible for checking the growth of the pathogens. Three series of experiments carried out for this purpose were:

1. Slices of tissue consisting of epidermis and subepidermal parenchyma were dissected from pod cavities and treated with (i) a temperature of 110°C for 10 min; (ii) temperatures ranging from 45 to 65°C for 1 hr; or (iii) a temperature of about —10°C for several hours. Immediately after treatment the tissue pieces were inoculated with S. fructicola. The fungus developed copiously with luxuriant sporulation. In untreated controls no fungal development took place and local lesions only were seen.

2. A drop of dense zoospore suspension was added to exudates from tissue which had been pretreated with temperatures of 110 and —10°C. Five hours later, high germination rates and normal germ tube development were observed.

3. In order to eliminate the possibility that preformed substances may have been destroyed by the treatments used in (1) and (2) above, a third series of experiments was carried out as follows: Parenchyma tissue from the cavities, to which a minimal amount of water had been added, was homogenized, and the resulting pulp was then centrifuged. The supernatant was used immediately in dilution series in a bioassay using S. fructicola and P. infestans as test organisms for the presence of any inhibitory substances. The germination rates proved normal with both fungi. Only slight inhibition of germ tube growth was observed at concentration n.

(i) Demonstration of the Antibiotic Activity of Diffusates Obtained from Inoculated Bean Pod Cavities.—After incubation at 20°C for 24 hr, the diffusates collected from cavities inoculated with S. fructicola or P. infestans appeared brown in colour. After centrifuging, the resulting liquids were perfectly clear and transparent. Their
pH varied from 6-0 to 7-0; their buffering capacity was low. Osmotic concentration varied from 0-5 to 0-6 atm. Surface tension was reduced by 10–14 per cent., in comparison with pure water.

The antibiotic activities of two representative diffusates are shown in Table 1. It can be seen that the undiluted diffusates completely prevent germination, this effect decreasing with dilution to zero at n/32. The diffusates obtained from Phytophthora inoculations showed a higher activity towards the pathogen, than did the Sclerotinia diffusate. The diffusate collected from cavities which had been inoculated with sterile water only, showed no inhibition either of germination rate or of germ tube length.

**Table 1**

**ANTIBIOTIC ACTIVITY OF DIFFUSATES TOWARDS THE HOMOLOGOUS FUNGI**

Diffusates were collected 20 hr after inoculation. The results were read after an incubation period of 18 hr.

<table>
<thead>
<tr>
<th>Diffusate Obtained after Inoculation with:</th>
<th>Concentration of Diffusates</th>
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<tr>
<td></td>
<td>n</td>
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<tr>
<td><strong>S. fructicola</strong>*</td>
<td>+ + + +</td>
</tr>
<tr>
<td><strong>P. infestans</strong>†</td>
<td>+ +</td>
</tr>
<tr>
<td>Control‡</td>
<td>–</td>
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</table>

*+++, no germination; +++, some germination, very short germ tubes; +, normal germination rate, length of germ tubes <50 per cent. of control; –, germ tube length about 50 per cent. of control; –, no inhibition.
†+++, no germination; +, germination rate about 50 per cent. of control; –, germination as in control.
‡Cavities "inoculated" with distilled water; testing carried out with both Sclerotinia and Phytophthora.

These results, together with those which have been obtained from experiments with non-inoculated tissue, give strong indications that the inhibitory activity of the diffusates collected from inoculated cavities is due to the post-infectious production of an antibiotic principle.

According to the PA hypothesis it must be expected that tissue from hosts which are susceptible to either of the two pathogens will be unable to produce an inhibitory factor in response to the attack by the congenial parasite. Apricot leaves and slices from fruits of plum, apricot, and apple were tested for their ability to respond to an infection by Sclerotinia with PA production. The diffusates obtained showed no antibiotic activity towards the parasite.

Similar experiments were carried out with slices from the tuber parenchyma of a potato variety which is susceptible to the Phytophthora strain used in these investigations. Petroleum ether extracts from exudates (see p. 284) collected 24 hr
after inoculation showed no toxicity; after 40 hr incubation, however, a clear inhibitory effect could be observed in the exudate bioassay.

(ii) The Mode of Action of the Toxic Principle on the Pathogens.—Spores of *Sclerotinia*, exposed to concentrations of PA which inhibit germination, turn brown and show affinity to rhodamine B. At dilutions which sustain germ tube growth at 5–10 per cent. of the water control, the hyphae are seen to be markedly thicker than those grown in sterile water only; also branching occurs much earlier. After extraction of the toxic principle from the diffusate germ tube growth was markedly better than that of water control. This is obviously a nutritional effect, due no doubt to some nutritional factors in the inoculation drop which have diffused out from the dying host cell. Many hyphae also show curling and twisting in their growth, under the influence of this PA.

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td>Sensitivity of freshly hatched Phytophthora infestans zoospores and of zoospores just beginning to germinate to a homologous diffusate</td>
</tr>
<tr>
<td>++, no germination; +, germination rate about 50 per cent. of control; −, germination as in control</td>
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<tr>
<td>Zoospores</td>
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<td>-----------</td>
</tr>
<tr>
<td>Freshly hatched</td>
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<tr>
<td>Just starting germination</td>
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*Phytophthora* zoospores respond to higher concentrations of PA with swelling and bursting (plasmoptysis). This process (see Müller 1956, Fig. 4) is preceded by premature contraction of the flagella from the tip, and rapid rounding off of the cell contents. The rapidity with which these changes take place depends on the concentration of the active principle and the temperature. At high PA concentrations and a temperature of 20°C, practically all of the spores have been ruptured after 60 sec. At lower temperatures and at lower PA concentrations it takes more than 30 min to complete this process. Freshly hatched zoospores are more sensitive than those which have already settled down and developed a cell membrane (Table 2). There is no doubt, from microscopical evidence, that PA affects first the structure of the plasmalemma, causing lack of cohesion, and thus rupture of the spores. Occasionally the zoospores do not show plasmoptysis at lethal concentrations and in these cases shrinkage only takes place.

At concentrations around the threshold value of 6 PA units, the inhibition of the spores of *Sclerotinia* has been shown to be only static in effect. If higher concentrations are used, e.g. 10 PA units, then a cidal effect is seen. This was determined by exposing such spores to various concentrations higher than 6 PA units for 24 hr.
and thereafter transferring them to sterile water for observation of the germination rate.

(b) Some Physicochemical Properties of the Active Principle

(i) Resistance to High and Low Temperatures.—Samples (1 ml) in sealed, air-tight test tubes were treated at 65°C or at 98°C for 2 hr or at 110°C for 1 hr. There was no reduction in antibiotic effect. Diffusates stored at −10°C for several months retained their full activity.

(ii) Dialysability.—The active principle passes through “Cellophane” and collodion membranes. However, the PA yield is reduced by 15–60 per cent. by this treatment.

(iii) Adsorbability.—The diffusates lost their activity within 60 min after being mixed with animal charcoal and shaken vigorously. Ordinary filter paper adsorbed 86 per cent. of the antibiotic principle in less than 1 hr. “Cellophane” behaved similarly. Starch, on the other hand, showed no effect.

(iv) Lipophily.—The active principle may be extracted from aqueous solutions by means of petroleum ether or cyclohexane (yield from the conventional method, 35–60 per cent.). The residual aqueous fraction possesses no antibiotic effect.

(v) Sensitivity to Ultraviolet Light.—The antibiotic activity is lost rapidly if the diffusates are treated with short-wave radiation as transmitted by a Wood’s filter.

These results dispel any doubts that a chemical principle or principles is involved which cause the inhibition of the pathogen in the necrotic tissue.

(c) Experiments on the Dynamics of the PA System

(i) Relationship between the Concentration of the Diffusates and their Antibiotic Effect.—A number of dosage–effect curves were subjected to formal analysis. As is generally known, no direct proportionality exists between the concentration and the biological effect of an antibiotic; graphic representation gives curves which deviate to a greater or less extent from the type of a straight line. This is illustrated in Figure 1, which is based on data obtained with two diffusates collected after infection of the seed cavities with Sclerotinia.

The relationship is almost certainly disturbed at the lowest concentrations by nutrients in the diffusates and there is some uncertainty about its precise form in the absence of nutrient contamination. In the data examined in Appendix I the curvature of the regression of log length on concentration was convex to the base line and significant.

For this data extrapolation to increasing concentration gave an estimated mean length of 1 per cent. of the control at a PA concentration of 3·65 units and 0·1 per cent. of the control at a PA concentration of 4·66 units. A conservative upper limit based on these and other series for virtually no development would be about 6 PA units. There is, naturally, a dilution effect involved in our tests. This effect depends on the relative volumes of the agar blocks in which the conidia are suspended to the liquids to be tested. The PA values mentioned have therefore to be considered as relative ones.
(ii) *Time Relationship of PA Formation.*—The following problems were investigated:

(1) How long does it take before PA is detectable in the inoculation droplets?
(2) How rapidly and to what height does the PA concentration rise in the infection droplets?
(3) Is the "PA production potential" of the infected tissue exhausted when the maximum PA concentration is reached?

The procedure in test (1) was as follows:

Very concentrated suspensions of *Sclerotinia* and *Phytophthora* were applied to the seed cavities as rapidly as possible at a temperature of 5°C; about 300 seed cavities were used in each case. Fifty inoculation droplets were collected at each of several stated intervals (6, 10, 14, 25, and 29 hr after application of the suspensions) and their activity tested with the homologous parasites. The presence of PA was first detectable after an incubation period of 14 hr in both series of tests. Thereafter, the PA content rose steeply in both series of tests. The increase after an incubation period of 20 hr was insignificant.

In concentrating the samples to one-eighth by the dry-freezing method and re-testing them, PA was found to be present already after an incubation period of 10 hr. The resultant preparation showed an activity of $<5$ and $>3$ PA units. Since about 4 hr elapse before the germination of the conidia, we must conclude from this result that the supply to inoculation droplets is already in progress 6 hr after the parasite has begun to germinate. Since, as it will be shown later on, the host cells retain the accumulated PA to a very considerable extent by "inner" adsorption and release only the excess to the inoculation droplets, the true reaction time may be even shorter.

In our numerous experiments, the PA concentration of the diffusates never exceeded the value of 13 units, even with incubation times of more than 24 hr. The most reasonable explanation for this fact is that PA production is connected with the metabolic activity of the parasite. Since the latter ceases to grow 20 hr after coming into physiological contact with the host tissue, PA production probably also ceases. If after 24 hr a new diffusion gradient was established by removing the inoculation droplets and replacing them by sterile water, and the second series collected and bioassayed, a high activity was obtained.

(iii) *Age of the Host Tissue and PA Production.*—At the very beginning of these investigations it was found that, although the test conditions remained constant, the PA yield might vary quite considerably with the different pod samples. As already stated (Müller 1956), the PA yield depends upon a number of factors, including the external conditions under which the seed pods are kept before being inoculated. The influence of temperature is discussed in detail in Part II of this series (Jerome and Müller 1958). The "physiological" age of the host tissue also is involved here. This was shown by experiments such as the following:

Pods from *Phaseolus* plants raised in a glass-house were divided into three groups according to their stage of development; the seed cavities were inoculated with the same *Sclerotinia* conidia suspension. The resulting diffusates were collected after
24 hr and tested for their PA activity. The results are given in Table 3. The PA production of the older pods (group 3) was found to be more than double that of the younger ones.

The high PA content of the diffusates in group 3 led us to carry out another experiment, using the above method, in order to determine whether the marked inhibition of the parasite in the diffusates of the older seed pods was due to the additional effect of preformed inhibitors. This test gave a negative result.

(d) The Antibiotic Character of the Diffusates

The results obtained from tests with Phytophthora-resistant strains of potatoes (Müller and Börger 1940) indicate that the antibiotic principle occurring in diffusates has a wide range of operation. The findings of Kuč et al. (1955) and Kuč (1957) point towards the same conclusion; their tests showed that the antibiotic factor obtainable from slices of potato after infection with Helminthosporium carbonum is not only effective against this organism, but also against Fusarium oxysporum.

In the tests discussed below, the effectiveness of the diffusates was tested in relation to the following fungal organisms: Colletotrichum lindemuthianum, Uromyces trifolii (uredospores), Botrytis cinerea, Pythium ultimum, and Rhizoctonia solani.

Tests were also carried out to determine whether the diffusate obtained after infection with Sclerotinia also possesses antibiotic properties with respect to Phytophthora, and vice versa.

Spores which were seeded directly into the test liquid were used in the experiments with the first three organisms. Small blocks of agar (1 by 5 by 5 mm), which were cut from young potato dextrose agar cultures and placed in the undiluted diffusates, were used in the tests on Pythium ultimum and Rhizoctonia solani. Here, the rate at which the hyphae grew out of the blocks of agar was taken as an index of the antibiotic action of the test liquid.

Results obtained with undiluted diffusates after an incubation period of 18 hr are given in Table 4. They are much as expected: the diffusate derived

<table>
<thead>
<tr>
<th>Group of Age:</th>
<th>Longitudinal Diameter (cm) of:</th>
<th>Colour of Epidermis of Cavities</th>
<th>Phytoalexin Content of Diffusates (units)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Cavities</td>
<td>Embryos</td>
<td></td>
</tr>
<tr>
<td>1 (youngest)</td>
<td>&lt;1</td>
<td>&lt;0·3</td>
<td>Deep green</td>
</tr>
<tr>
<td>2</td>
<td>1–1·5</td>
<td>0·3–1·2</td>
<td>Light green</td>
</tr>
<tr>
<td>3 (oldest)</td>
<td>&gt;1·5</td>
<td>&gt;1·2</td>
<td>Silvery green</td>
</tr>
</tbody>
</table>
### Table 4
DEVLOPMENT OF SEVEN FUNGI IN DIFFUSATES OBTAINED FROM CAVITIES WHICH HAD BEEN INOCULATED WITH SCLEROTINIA FRUCTICOLA OR PHYTOPHTHORA INFESTANS

Data compiled from four experiments

<table>
<thead>
<tr>
<th>Diffusate from Cavities Inoculated with:</th>
<th>Phytoalexin* (units)</th>
<th>Fungus</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. fructicola†</td>
<td>P. infestans†</td>
<td>Colletorichum lindemuthianum†</td>
</tr>
<tr>
<td>S. fructicola</td>
<td>6·8</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>4·0</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2·6</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2·0</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>1·3</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>1·0</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>P. infestans</td>
<td>9·0</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>4·5</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2·25</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>1·12</td>
<td>++</td>
<td>±</td>
</tr>
</tbody>
</table>

*Refers to reaction of S. fructicola.
†−, no germination; ±, germination of only a few spores; +, germination rather strong, but significantly less than in water control; ++, germination as in control.
‡−, no outgrowth of hyphae; ±, length of outgrown hyphae less than 25 per cent. of control; +, length of hyphae 25–50 per cent. of control; ++, length of hyphae 50–100 per cent. of control.
from tissue infected with *Phytophthora* completely inhibited the germination of the *Sclerotinia* conidia; the diffusate obtained from tissue infected with *Sclerotinia* had the same effect with *Phytophthora* zoospores. Germination was likewise inhibited in the case of *Colletotrichum lindemuthianum*, the fungus responsible for bean anthracnose. This organism, as shown by another series of tests, appears to be slightly more susceptible than *Sclerotinia*.

The results obtained with *Pythium* and *Rhizoctonia* merit closer consideration. No signs of growth were visible with both fungi after an exposure period of 18 hr. The situation altered during the next 24 hr, however; numerous hyphae had meanwhile grown out of the blocks of agar. The rate of growth was considerably less than in the case of the water control, but finally the two fungi succeeded in growing completely through the test liquid. Further investigations must be carried out before a satisfactory explanation for this phenomenon can be given, i.e. whether there is an “adaptation” of these two fungi to the antibiotic factor or whether the toxic principle is destroyed by the metabolic activity of the fungi.

- (e) Influence of Nutrient Concentration and of pH of the Substrate on PA Activity towards *Sclerotinia*

The *Sclerotinia* fungus is readily cultivable, a feature which enabled us to investigate the extent to which the PA sensitivity of the parasite and thus the clinical result of an infection is affected by the content in the host tissue of substances which the parasite is able to use as nutrients. It could be possible, for example, that an abundant supply of nutrients may reduce the PA sensitivity of the parasite and thus increase its virulence. Similar ideas have recently been voiced by certain authors who have treated “host–parasite relationships” primarily as a nutritive–physiological problem. Lewis, for example, states in a recent paper (1953): “We find substances that enhance and substances that inhibit the activities of parasites. We find also that enhancement or inhibition depends on the nutritional environment in which the substances operate. Is it not readily conceivable that these or similar factors determine the success or the failure of a parasite in its host?”

From a methodological point of view, too, it seemed desirable to ascertain whether an antagonistic or a synergistic relationship exists between the PA content and the nutrient content of the substrate. It is true that we investigate the PA sensitivity of the organisms, which give rise to the PA formation, in a substrate to which no nutrients are added. However, the “natural medium” in which the interaction between parasite and host cell occurs is very different from the conditions under which the PA sensitivity is tested in *vitro*. If, therefore, substances which may be used as nutrients by the parasite do actually influence its PA sensitivity, it becomes extremely difficult to apply the results of our *in vitro* tests to *in vivo* conditions.

The following experiments were carried out: *Sclerotinia* conidia were suspended in agar blocks at increasing nutrient concentrations. As “sources of nutrients”, boiled filtered juice from potato tubers, plum fruits, or from the parenchyma of bean pods were used. Small cubes of agar, about 25 mm³ in size, were taken from each concentration and placed in test solutions of varying PA content.
The result of an experiment, in which juice from the parenchyma of bean pods was used as nutrient, may be taken as a typical example. As we see from the results in Table 5, the added nutrients had no significant influence upon the PA sensitivity of the parasite.

Filtered juice from potato tubers or plums was used in certain other tests. Here, too, the results proved to be negative.

In the above experiments filtered juices, which contained practically no solid cell constituents or proteins, were used. If non-filtered juices were used, i.e. those containing abundant proteins and cell particles, a slight increase of the germ tube length was noted with a PA concentration corresponding to 2·65 units (see Table 5).

**Table 5**

DEVELOPMENT OF SCLEROTINIA AT DIFFERENT PHYTOALEXIN CONCENTRATIONS AND NUTRIENT CONTENTS

Homologous diffusate was used. Results refer to the relative germ tube length.

<table>
<thead>
<tr>
<th>Nutrient and Treatment</th>
<th>Nutrient Conc. (%)</th>
<th>Concentration of Phytoalexin (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10·6</td>
</tr>
<tr>
<td>Bean sap, boiled and filtered</td>
<td>60</td>
<td>0·0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0·0</td>
</tr>
<tr>
<td></td>
<td>3·75</td>
<td>0·0</td>
</tr>
<tr>
<td>Bean sap, boiled only</td>
<td>60</td>
<td>0·0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0·0</td>
</tr>
<tr>
<td></td>
<td>3·75</td>
<td>0·0</td>
</tr>
<tr>
<td>No bean sap (control)</td>
<td>0·0</td>
<td>0·0</td>
</tr>
</tbody>
</table>

Since, as mentioned on page 280, the acidity of the host cells clearly increases soon after the PA formation commences, the pH of the substrate was next considered as an additional factor having a possible influence on PA activity. It is known, too, that the effectiveness of many antibiotics, such as streptomycin, is partly determined by the pH of the substrate. A series of experiments was therefore carried out, as described in the following paragraph.

Solutions at six different degrees of dilution were prepared from a diffusate obtained from tissue infected with *Sclerotinia*. These were mixed with phosphate buffer solutions (0·2M) at pH values of 3·5, 4·5, 5·0, 6·0, 7·0, and 8·0 in the ratio of 1 : 1. Pure water and buffer solutions served as control. *Sclerotinia* was used as test organism. The result of a typical test series shows that the pH of the substrate exercises no effect upon PA activity in the range between pH 4·0 and 7·5.
(f) Sorption Phenomena

At the commencement of our investigation there were many indications that the amounts of PA occurring in the diffusates represented only a part of the total PA formation. This hypothesis was supported by the following experiment: Seed cavities were treated with a diffusate containing 3.7 PA units. The latter was recovered after an incubation period of 20 hr and tested for its PA activity; the result showed a reduction in the antibiotic effectiveness to <0.5 units.

If we apply this result to the conditions obtaining in the host tissue, we must postulate that large PA amounts given off by the infected cells to the adjacent cells are immediately fixed and accumulated by the latter. Such a mechanism would therefore exclude free diffusion of the "excess" amounts of PA into the surrounding host tissue.

Further experimental data were needed to confirm this hypothesis. Several problems had to be answered:

1. What is the rate at which the "free" PA present in the diffusates is fixed by living tissue?
2. Does total binding of the free PA occur?
3. Is dead host tissue also capable of binding PA?

The following procedure was adopted in the experiments carried out in connection with the above problems:

Tissue taken from the parenchyma of bean pods was placed in test tubes, which were then filled with sufficient amounts of a standardized PA solution to ensure that the samples of tissue were completely covered by the solution. The test tubes were then transferred to a shaking apparatus. The liquid was tested for its PA activity at specified intervals. Since the volumes of the particles of tissue and of the diffusates used were known, it was possible to determine the extent and the rate of PA fixation by the particles of tissue from the decrease in the antibiotic effectiveness of the supernatant liquid.

From the diagram in Figure 2 (curves A and B), we see that more than 80 per cent. of the PA contained in the diffusate had already been fixed by the living tissue after the first 10 min. Since no further decline in the PA activity in the supernatant liquid after this time was apparent, a stable equilibrium between the amount of PA bound by the tissue and the "free" PA must have been established within 10 min.

The same experiment was carried out with tissue which had been killed by heating to 100°C. The result is given in Figure 2 (curves C and D). It shows that the dead tissue was also capable of binding considerable amounts of PA. However, the rate at which the liquid lost its antibiotic effectiveness in the test with dead tissue did not appear as great as with the living tissue. The final values for PA fixation were also lower.

The results of these tests may also provide a satisfactory explanation for the apparent decrease in the PA sensitivity of the parasite in unfiltered cell sap, as described on page 289. Part of the PA present in the test liquid is evidently fixed by adsorption by the abundant proteins and cell fragments, and the PA concentration...
thereby reduced. However, the possibility must be considered that rather than being only adsorbed the PA may be inactivated.

*(g) Phytoalexin Formation in other Hosts than Phaseolus vulgaris after Infection with Sclerotinia or Phytophthora*

We have hitherto been concerned solely with the antibiotic activity of diffusates obtained from infected *Phaseolus* tissue. The question that now arises is whether the reaction mechanism described above has a wider significance. Three other host plants were investigated to determine whether the local lesion reaction is associated with PA formation in these particular cases also.

![Graph showing phytoalexin adsorption](image)

**Fig. 2.**—The amount of phytoalexin adsorbed to the host tissue after different intervals.

The test plants selected were *Pisum sativum*, *Vicia faba*, and *Capsicum annuum*; *Sclerotinia* and *Phytophthora* were again used as parasites, and the inner epidermis of the fruits was again used as host tissue. It was found that these three plants also respond to infection by local lesions. After an incubation period of 24 hr the PA content in the infection droplets was found to be relatively high. The PA activity of the crude diffusates corresponded approximately to that found in the tests with *Phaseolus*. It was possible, in all cases, to separate the antibiotic principle from the crude diffusates by means of petroleum ether extraction.

These plants were likewise tested in the manner described above (see p. 281), in order to ascertain whether the antibiotic activity of the diffusates is due to the exosmosis of preformed inhibitors from the dying host cells. The behaviour of the germinal hyphae of *Sclerotinia* upon tissue previously treated at low or high temperatures (−10 or +50°C) lends no support to such a hypothesis. In these cases, too,
there is therefore not the slightest doubt that the inhibition of the parasite in the affected tissue is due to the action of antibiotic factors which first develop in the course of the interaction between parasite and host.

V. DISCUSSION

The main purpose of the investigations described in Section IV was to determine whether the inhibition of the parasite in the local lesion tissue is due to the post-infectional formation of antibiotic agents. The results of the basic experiments may be summarized as follows:

1. The epidermis of the seed cavities of Phaseolus vulgaris which produces local lesions in response to infection with Sclerotinia fructicola or Phytophthora infestans, releases—after a relatively brief incubation period—a principle (PA), which exerts an antibiotic effect upon the parasites.

2. Under the particular conditions of these tests, the effective principle occurred in amounts which were quite sufficient to totally inhibit growth of the parasites.

3. This principle was not detectable in healthy tissue.

4. There was no formation of an antibiotic factor (or only after a relatively long incubation period) in hosts, which are susceptible to the above-mentioned parasites.

5. The principle separated from the host tissue possessed physical and chemical properties which exclude all doubts regarding the existence of biologically active chemical compounds in the diffusates.

6. A basic condition for its development is that the host tissue should be physiologically "normal".

These results may be taken as confirmation of the hypothesis that the inhibition of the parasite in the local lesion tissue is due to the activity of an antibiotic principle, which does not occur "preformed" in the host cell, but which owes its origin to an interaction between the host and the parasite. In view of this finding, there can be no further doubt that—in the present case—the changes occurring in the reactive tissue must be regarded as defensive reactions.

Supplementary tests were made with Pisum sativum, Vicia faba, and Capsicum annum. Here, too, infection with Sclerotinia or Phytophthora resulted in the development of local lesion and, at the same time, in abundant PA formation. Kuč et al. (1955, 1956) and Kuč (1957) have also demonstrated the post-infectional formation of an antibiotic factor following the infection of potato tubers with Helminthosporium; it follows, therefore, that a more general significance must be attached to the defensive mechanism which is the subject of the present discussion.

Given the present state of our knowledge of this matter, however, it would be premature to attempt to interpret every local lesion reaction in the light of the PA concept. It is conceivable, for example, that preformed inhibitors only begin to take effect after the structure of the plasmalemma of the host cell has been destroyed by metabolic products of the parasite, and the inhibitors thus come into direct
contact with the hyphae of the parasite for the first time. In this case, too, interaction would result in the death of both partners. This idea has been proposed by previous workers who found inhibitory factors in susceptible hosts as well; for instance, Newton and Anderson (1929) investigated the phenolic compounds in eight wheat varieties resistant in varying degrees to rust and found no correlation between content of phenolic compounds and rust resistance. Scott, Millerd, and White (1957) employ a similar concept in an article on the local lesion reaction occurring in barley after infection with non-virulent Erysiphe strains. They also postulate such a reaction mechanism because they had found that susceptible plants also contain a preformed phenolic factor, which has an inhibiting effect upon the parasites. Unfortunately, no information is given as to whether this factor is present in concentrations sufficient to check the parasite in vivo.

Further tests were carried out in conjunction with the basic experiments, primarily for the purpose of studying the mechanism of the local lesion reaction.

The antibiotic action of the PA derived from Phaseolus is non-specific. This agrees with the findings of Kuc and co-workers, who state that the antibiotic substance isolated from potato slices infected with Helminthosporium is also effective against Fusarium oxysporum. The non-specific nature of PA, as postulated by Müller and Börger (1940), is thereby confirmed.

It may seem difficult, at first glance, to reconcile the non-specific character of PA with the fact that strongly marked specialization exists with many host–parasite combinations. With rust infections, for example, the mere presence or absence of a single gene, on the side of either the host or the parasite, is sufficient to determine whether the interaction between the two partners will result in local lesions. Catcheside (1951) does not hesitate, therefore, to draw a parallel between the mutual relationship between the genes of the host and the parasite and the well-known antigen–antibody reaction from the field of animal pathology. However, there is no fundamental difference here. If, following Müller (1950), we extend our observations to all theoretically conceivable host–parasite combinations, then the situation is reversed. For we find that the characteristic of specificity does not pertain to the local lesion reaction, but rather to its opposite, i.e. the mutual tolerance between host and parasite. However, this weakens the argument advanced previously against the non-specific nature of the factor which causes the inhibition of the parasite in the local lesion tissue. There is another argument which supports the idea that the end-result of the interaction between host cell and parasite need not necessarily be a specific one: there is no doubt that a "local lesion reaction" on the one hand, and a "mutual tolerance" of the two partners on the other are the extremes of the same category of interaction, reflecting differences only in degree. This quantitative approach is justified by a number of facts: In the first place, the reaction of intermediate host types may change with changes in environmental conditions and age of the host, towards the one or the other extreme. Furthermore, it has been shown that the rate of reaction of the host has a strong impact on the clinical outcome of the interaction between the two partners; the greater the interaction rate, the earlier the pathogen's growth is checked (Müller and Börger 1940; Müller 1953). In view of the fact that the principle inhibitory to the pathogen is
active against other organisms also, we are compelled to the conclusion that the specificity of an interaction (actually that of mutual tolerance between pathogen and host) rests on particular factors, both of host and pathogen, which meet in the infected host cell and determine the rate with which the non-specific principle inhibitory to the pathogen appears at the infection site.

Thus, in the light of our considerations, there is no disease resistance as such, but only the host being able to render the parasitized tissue inhospitable to the pathogen with due speed. This would mean in terms of our PA concept: there should be a threshold concentration of PA which must be reached within a certain period of time; otherwise the pathogen will continue to spread in the host tissue.

This concept of the nature of "active" or "protoplasmic" resistance is supported by experimental results obtained in this work: (1) the interaction starts within a few hours of the pathogen contacting the host cells, and (2) a few hours later, the PA produced per unit volume of the reacting tissue exceeds by far that amount at which no further growth of pathogen occurs. Further evidence is given in Part II (Jerome and Müller 1958).

Further results, which may be regarded as decisive for the causal understanding of the mechanism upon which the local lesion reaction in our test objects is based, are as follows:

(1) A high affinity exists between the antibiotic principle and the host tissue.
(2) The amount of PA eventually obtained is partly determined by the age of the host tissue.
(3) The PA's found to date possess lipophilic properties.

In the interpretation of our test results, it should be remembered that, when our method is used, the conditions under which the interaction between parasite and host tissue takes place are very different from those under which it occurs in the natural environment. The ratio between the volumes of infection drops used and of the host tissue is "unnaturally" high; the diffusion equilibrium between the PA concentration in the infection droplet and in the reactive tissue must therefore necessarily occur at a relatively late stage. This implies, in any case, that where parasite and host tissue are in direct contact, the PA concentration must be considerably higher than in the inoculation drops. Furthermore, the diffusion equilibrium is delayed by adsorption of PA by the reacting host cell. That is, we must conclude from our experiments that "free" PA only becomes available for release to the infection drops after the host cell has become "saturated". In addition, the competitive action on the part of the non-infected neighbouring cells must also be taken into account; these cells, in their turn, fix large amounts of PA.

It has already been mentioned that the release of PA to the infection drops was found to be in operation about 6 hr after germination of the parasites. From the above observations it appears that the first amounts of PA accumulate considerably earlier within the reactive cells. This also corresponds to the fact that the development of the parasite does not, as a rule, pass beyond the formation of short infection hyphae.
As already stated in an earlier publication (Müller 1956), the interaction product exercises a toxic effect, not merely upon the parasite, but upon the host cell as well. A preparation, semi-purified by dialysis, with a PA activity of about 16 units produced increased rhodamine B affinity in the treated epidermal cells. At the present time, unfortunately, it is impossible to decide whether the principle acting upon the parasites is identical with the one which causes the necrotic changes in the host cells. Only the chemical identification of the active principles can supply an answer to this problem.

As shown in a recent paper (Müller 1958), the PA released per unit volume of infected host tissue is extraordinarily high. Under the conditions of our experiments, it is more than a thousand times the amount required to check the parasite at the site of infection. If we consider that in the diffusates only those amounts of PA can be detected which have escaped fixation by the host tissue, we see that the total PA output must be estimated at an even higher figure than that quoted in the above-mentioned work.

The high adsorption characteristics of the active principle may also be important in another respect for a causal understanding of the local lesion reaction. Many observers have stressed the fact that it is not only the cells in direct contact with the parasites, but also the neighbouring cells as well—often merely the adjacent membranes with the corresponding protoplasm—which reveal histological changes corresponding to those observed in the case of the infected cell. This indicates that a factor is given off, from the cells which interact directly with the parasites, to the neighbouring cells where it is fixed, and that the changes are caused by this factor. This probably also applies to the amounts of free PA which are released by the reacting cell to its surroundings after saturation is complete. Consequently, as our sorption tests indicate, free diffusion through the adjoining tissue must be impossible, and a zone of high PA content is formed, which surrounds the seat of infection like a protective wall. This presumably prevents the parasite from escaping from the seat of infection into the adjacent tissue. The fact that the formation of PA begins anew once a fresh diffusion gradient has been created (see p. 285), may also be regarded as an additional safety device.

The fact that the isolated antibiotic factors possess lipophilic properties indicates that free PA is primarily adsorbed to the lipid particles of the host cells. Since the lipoids, owing to their surface activity, occur preferentially in the plasmalemma, the neighbouring cells probably adsorb the greater part of the free PA at their surfaces. However, this is the place where the hyphal tips of the pathogen come into contact with the neighbouring cell first.

Our knowledge of the antibiotic activity of PA fixed by adsorption is still very far from complete. However, it seems reasonable to assume that destruction of the plasmalemma by the metabolic products of the parasite results in the release of previously adsorbed amounts of PA, which consequently recover their antibiotic effectiveness. This process of alternate fixation and liberation of PA may perhaps explain why the parasite—provided it is not inhibited by the primarily infected cells—encounters increasingly high concentrations of PA, until it is finally obliged to cease growing altogether.
As already mentioned in an earlier report (Müller 1956), the mechanism underlying the formation of PA is inhibited by brief preliminary treatment of the host tissue at high, but not lethal, temperatures. The relationship of the PA formation to the physiological state of the pod tissue was further confirmed in these experiments by the fact that the PA production potential increases in the course of the individual development of the pods. This phenomenon appears to be similar to the well-known increase in the sensitivity of many plants and thus in increasing "reactive resistance" to fungal parasites during the individual development of the host plant.

Although it is now possible to offer a reasonably satisfactory solution to the cardinal problem studied in these investigations, i.e. the demonstration of the principle inhibiting the parasite in the infected tissue, the work as a whole gives the impression of incompleteness; in the first place, because the query as to the chemical structure of the active principle is left unanswered. The principal aim, however, was to separate from the interacting host tissue the factor which is the actual cause of the inhibition of the parasite and study some biological aspects of the problem; the question of its chemical structure was thus essentially a secondary problem. Most earlier writers on this subject endeavoured, by the use of conventional methods of analytical chemistry, to demonstrate a presumed factor which makes a resistant plant resistant. In the majority of cases, the plant was used in the uninfected state. It will be evident from the results discussed above why the goal could not be reached in this way: it is completely impossible to trace this particular factor in healthy plants, simply because it only appears at concentrations sufficient to stop the pathogen after physiological contact between host and parasite has been established. The reverse procedure was adopted in our investigations: whilst "sparing" the physiological structure of the interacting partner as far as possible, the principle inhibiting the parasite was separated from the host tissue and tested quantitatively for its antibiotic effectiveness.

The most important tasks facing us now are to determine the chemical structure of the active principle, which the present author called "phytoalexin" almost 20 years ago and later (Müller 1953) defined as "an antibiotic which is the result of an interaction of two different metabolic systems", and to identify the factor which, released by the parasite into the host cell, gives rise to the formation of phytoalexin. Thanks to modern biochemical methods, in conjunction with the special methods of production and testing employed in our investigations, it should not be difficult, once the chemical structure of these two factors is known to set up a theoretical model of the mechanism by which the plant is enabled to defend itself actively against potential parasites.

VI. ACKNOWLEDGMENTS

The author wishes to acknowledge the help received from Mrs. S. M. R. Jerome and Miss R. Bochert in assisting in the experimental work. He is also indebted to Mr. I. A. M. Cruickshank in assisting in the drafting of the present paper and reading the typescript.
STUDIES ON PHYTOALEXINS. I

VII. References


**APPENDIX I**

**BIOLGICAL ASSAY USING LENGTH OF GERM TUBES AS THE MEASURE OF RESPONSE**

By M. L. Dudzinski* and G. A. McIntyre*

Data was available on individual lengths of 30 germ tubes of *Sclerotinia fructicola* for successive levels of twofold dilutions of two samples (A, B) of diffusate on each of two days. Between these assays the samples were stored at —10°C and there was little likelihood of any deterioration taking place in the interval. During development of the hyphae the temperature control was imperfect but all levels of dilution for each sample together with the corresponding control on each test were subjected to the same conditions. This implies that for comparative purposes the development at the various levels of dilution must be expressed in terms of the corresponding control. Analytically this implied the use of logarithms of germ tube length in fitting regression curves. This transformation is also favourable in the sense of making the variances within treatments more uniform.

To reduce the influence of outlying observations the means of six successive germ tube lengths were taken as the primary entries into the analysis, giving five means per level. The variances of the logarithms of these means were examined for homo-

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geneity between treatments. The variance was greater for treatments with low than with high means. The trend was linear and variances for particular means were estimated from a fitted line. The reciprocals of the variances were used as weighting factors in the subsequent estimate of constants by least squares.

![Graph showing relation between log germ tube lengths and concentration of diffusates for two samples on each of two days.](image)

Fig. 3.—Relation between log germ tube lengths and concentration of diffusates for two samples on each of two days.

The form of regression equations used was

First day \((A_1)\) \(Y_1 = a_1 + b_1x + b_2x^2\),
\((B_1)\) \(Y_2 = a_2 + b_1(kx) + b_2(kx)^2\),
Second day \((A_2)\) \(Y_3 = a_3 + b_1x + b_2x^2\),
\((B_2)\) \(Y_4 = a_4 + b_1(kx) + b_2(kx)^2\),

where \(Y = \log\) (germ tube length) and \(x\) is the concentration expressed as a fraction of the initial concentration.

The first and second equations and likewise the third and fourth are related on the assumption that the second preparation can be regarded as a dilution of the first so that its effect can be represented by the same expression but with a change
in concentration \( x \) by factor \( k \). It was assumed \( k \) would remain constant from the first to the second day. The equations imply that a vertical displacement will bring \( A_2 \) into coincidence with \( A_1 \), and \( B_2 \) with \( B_1 \). With a common ordinate at zero concentration the ratio of the abscissa, \( B \) to \( A \), for any hyphae length is the relative concentration \( k \).

This set of equations was fitted to the data by least squares using an iterative procedure. The final iteration gave the equations

\[
Y_1 = 1.3475 - 1.7728x - 6.8096x^2, \\
Y_2 = 1.3247 - 1.7728(kx) - 6.8096(kx)^2, \\
Y_3 = 0.8722 - 1.7728x - 6.8096x^2, \\
Y_4 = 0.9462 - 1.7728(kx) - 6.8096(kx)^2.
\]

The data and fitted curves are presented in Figure 3.

The factor of relative concentration, \( k \), is 0.739 with a standard error of 0.034, which is a satisfactory level of precision. The value of \( \chi^2 \) for goodness of fit was 6.94 for seven degrees of freedom so that there is no statistical evidence that the model is at fault.

The value of \( x \) at which the length is half the control is the value corresponding to \( Y \) less than the control by 0.301(log 2). Defining this as the unit concentration of phytoalexin the values of \( x \) for A and B are 0.1171 and 0.1585. The strength of the undiluted samples is then 8.53 and 6.30 phytoalexin units.

The ratio of \( b_2 \) to \( b_1 \) is almost 4. The curves in this instance could be made linear by a transformation \( z = (x + 4x^2) \). The data of this analysis are almost certainly disturbed by the effects of nutrients in the diffusates and it is possible that with nutrients eliminated that either a transformation would not be necessary or one could find a standard transformation of this form which would give a linear relation between log length and the transformed concentration. This would simplify the statistical procedures and calculation of errors of estimates.
Host cells infected by *Phytophthora infestans* (Fig. 1) and *Sclerotinia fructicola* (Fig. 2). Incubation period: 20 hr at 20°C. Stain used: rhodamine B (10 p.p.m.). × 470.
Areas of seed cavities inoculated with *Phytophthora infestans* (Fig. 1) and with *Sclerotinia fructicola* (Fig. 2). Incubation period: 48 hr at 20°C. × 15.