STUDIES ON PHYTOALEXINS

III. THE ISOLATION, ASSAY, AND GENERAL PROPERTIES OF A PHYTOALEXIN FROM PISUM SATIVUM L.

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Summary

An antifungal compound which conforms to Müller's definition of a phytoalexin has been isolated from the endocarp tissues of detached pea pods inoculated with *Monilinia fructicola*. The same compound has been identified in inoculated organs of growing pea plants. This compound, which is an abnormal metabolite of pea tissues, has been named "pisatin".

The details of a quantitative bioassay for the measurement of fungitoxicity are described.

A chemical method for the estimation of pisatin in aqueous solutions of plant extracts is given.

Some of the physicochemical properties of pisatin are:

(1) It has a molecular formula $C_{17}H_{14}O_6$; the crystals are colourless, m.p. 72°C.

(2) Solutions of pisatin have a characteristic ultraviolet absorption spectrum, $\lambda_{max.}$ 286 mμ and 309 mμ in ethanol.

(3) The solubility of pisatin in water at 23°C is 0·03 mg/ml.

Pisatin is stable to heat treatments including autoclaving (15 lb, 20 min). It is also stable to visible light but its antifungal activity is destroyed by high-energy radiation in the 253·7 mμ range of ultraviolet light, and by sunlight.

Pisatin is fungistatic at the concentration (mean value 67·5 μg/ml) it normally occurs in extracts (diffusates) from infected plant tissues. Although at this concentration no toxic effect could be demonstrated towards leaf and pod cells, the growth of wheat roots was significantly inhibited.

The results are discussed in relation to other compounds which appear functionally similar to pisatin in that they are abnormal metabolites with fungitoxic activity and are formed as a response to fungal infection.

I. INTRODUCTION

Hiura (1943), Gaumann, Braun, and Bazzigher (1950), Müller (1956, 1958), Uehara (1958*a*, 1958*b*, 1958*c*), and Condon and Kuć (1960) have each demonstrated the formation of abnormal metabolites, with antifungal activity, as a result of the interaction between fungi and specific tissues of certain higher plants. In each case, with the exception of that of Hiura (1943), the authors have suggested that these compounds are of direct significance to the natural disease resistance of the tissues in which they are formed. Kubota and Matsuura (1953) established the chemical structure of the compound isolated by Hiura (1943). This compound, ipomeamarone, has recently been reinvestigated by Akazawa (1960), who suggests it may also play

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a role in the natural disease resistance of the tissues in which it occurs. Gaumann's work has been followed up by Boller *et al.* (1957), who isolated one of the components of the antifungal complex, namely orchinol. Condon and Kuć (1960) in the course of their work isolated a third antifungal compound from infected roots of carrots.

We now report the isolation and general properties of an abnormal metabolite with antifungal properties, not detected in healthy tissues of *Pisum sativum* L. but produced in hypersensitivity studies similar to those described by Müller (1956, 1958). The name "pisatin" has been proposed for this new compound (Cruickshank and Perrin 1960).

II. Experimental and Results

(*a*) *Sources of Antifungal Activity*

(i) *Detached Pods.*—For the purpose of isolating the compound primarily responsible for the antifungal activity in one of the supplementary host–parasite combinations used by Müller (1958), and in order to obtain sufficient quantity of it for chemical identification, a large-scale inoculation of pea pods was carried out. Three hundred pounds of detached opened pods of field-grown peas (cv. Greenfeast), in 20-lb batches, were set out in large covered "Perspex" trays, and inoculated with a spore suspension (concn. 4×10^5 spores/ml) of *Monilinia fructicola* (Wint.) Honey (synonym *Sclerotinia fructicola* (Wint.) Rehm). The inoculum was applied with constant-flow syphon bottles at approximately 1 ml per pod. The inoculated pods were then incubated for 40 hr at 20°C and, after this period, the liquid ("diffusate") remaining on the inoculated areas of the endocarp tissues was drawn off under vacuum and collected. The inoculum was replaced by sterile water and left a further 24 hr. Sterile water applications were repeated up to three times, depending on the physiological condition of the pods. In each case the liquid was removed and tested for the presence and concentration of antifungal substance before being added to the diffusate bulk.

The pisatin concentration of diffusates collected after 40 hr was not a constant value. If the solutions of diffusate recovered from the 15 batches of peas used in the above extraction series are considered, the concentration range was 52–97 μg/ml (mean value 67·5 μg/ml). With the exception of one or two of the lowest values, these concentrations were sufficient to completely inhibit germination of *M. fructicola* under the conditions of the bioassay described below. The lowest values, although inadequate to prevent germination, were, however, in excess of the concentration required to prevent colony growth of this organism *in vitro* (Cruickshank, unpublished data).

(ii) *Organs Attached to Growing Plants.*—The waxy bloom was removed from leaves and stems of pea plants growing in pots by gently rubbing them between the fingers. They were then inoculated by placement of drops of spore suspension of *M. fructicola* on these areas, and incubated under conditions of high humidity (temp. 20°C, R.H. 100%, time 40 hr). Pods attached to plants, growing both in the glass-house and field, were inoculated by injection of a spore suspension of *M. fructicola* directly into the cavity of the pods with a hypodermic syringe, and left for 40 hr. Care was taken to prevent contact of the injection wound with the spore suspension.

Diffusates were collected from the leaf and stem inoculations as described for detached pods and analysed in the same way (see below). Endocarp analysis (see below) was necessary in the case of injected pods to identify and determine the concentration of the antifungal compound formed in the course of these tests. In each instance pisatin was detected at concentrations comparable to those in the diffusate solutions in the detached pod experiments.

(*b*) *Bioassay of Pisatin*

(i) *Antifungal Activity of Extracts.*—Supernatants of centrifuged diffusates were assayed for antifungal activity, using a technique similar to that described by Müller (1958). For the purpose of guiding the chemical extraction and purification of the antifungal component from the diffusate, and to determine whether a close correlation existed between the antifungal compound and the light-absorbing component in the extract, a more quantitative bioassay was developed. The following procedure was followed, using *M. fructicola* as the test organism.

Three spore suspensions (concn. 4×10^5 spores/ml) were prepared by blending the spores of three lots of four 5–7-day-old slant cultures grown at 20°C on potato dextrose agar in the dark. The spore suspensions were prepared in the cold in order to prevent premature germination. Six tubes of double-autoclaved Difco prune agar were dissolved and cooled to 40°C in an accurately controlled water-bath with stirrer. 1·5 ml of each spore suspension blend were used to seed each of two agar tubes. Each tube was held in the bath for 30 sec and the contents were then poured into a level flat-bottomed sterile petri dish (9 cm dia.). The latter were stored at 2°C prior to use.

Solutions to be tested were prepared either in water or 5% ethanol, using a dilution series with an 0·75 dilution interval. The dilution series, consisting of six solutions and a water control, were dispensed into open watch-glasses (0·45 ml/watch-glass) set out on trays lined with moist filter-paper, and covered with a "Perspex" sheet. The seeded agar in the petri dishes was cut into 5 by 5 by 1 mm blocks with a sterile stainless steel disk-cutter. One agar block from each of the six petri dishes was placed into each test solution (for statistical purposes each agar block was treated as a single replicate). The assays were carried out at 20°C in the dark. After 6 hr the spores were killed by addition of a drop of 1% formalin and cotton-blue lactophenol. Percentage germination was determined by classifying the first 100 spores in each agar block into germinated and non-germinated. The convention, spore tube length > spore diameter = germination, was used.

(ii) *Statistical Transformation of Bioassay Results.*—The mean percentage of germinated spores, corrected for control germination was plotted against log concentration of extract, and a free-hand curve drawn. The curve was not of the symmetrical form given by the cumulative normal distribution. It had a long arc towards maximum germination relative to the short opposing arc approaching zero germination. In consequence, transformation to probits did not give a linear relation. An empirical transformation to achieve this end was determined graphically by drawing a straight line through the point of median germination and cutting the base line at a concentration slightly in excess of that which gave no germination. Scores to correspond to the percentage germinations were read off the percentage scale as

the ordinates of the line and curve at the same concentration. The score at the median was 50.

The curvilinear relation of scores to percentages was graduated and graphed. The variance of a score corresponding to a percentage P, to a first approximation, is given by

$$P(100-P)/n.(\mathrm{d}s/\mathrm{d}P)^2,$$

where $(\mathrm{d}s/\mathrm{d}P)$ is the slope of the tangent to the curve at entry P and n is the number of spores on which P is based. The inverse of the variance is the weight to be given the observation in fitting a linear regression line to scores on log concentration. From the regression line obtained the ED_{50} value of the test solution was read off. The fungitoxicity of a solution in phytoalexin germination units (PAG units) is given by $(100/ED_{50})$.

(c) *Analysis of Bioassay*

(i) *Test of Homogeneity of Dosage Response Slope.*—Weighted regression lines were fitted for seven samples and departures of residues from expected values tested, using χ^2. Two of the lines showed some departure from expectation, which could be due to factors other than the binomial variation in the experimental set up, or to non-linearity of the score/log dilution line. From the plot of germination score values (Fig. 1) for the several dilution series, however, no systematic departure from linearity was evident. An analysis of variance (see Table 1) made of the homogeneity of slopes in the lines and the deviation of slopes from the mean slope was not significant. Germination percentages corresponding to the score values are also given in Figure 1.

(ii) *Test of Variation.*—From the analysis of variance (Table 1), the mean value for the within-test variation was shown to be 1·78 times the variation one would expect from binomial variation. This corresponds to a mean value of 4·4 reported by McCallan, Wellman, and Willcoxon (1941), using the standard slide germination technique.

A series of reference sample values were compared in eight experiments carried out over a period of 6 weeks. The variation between PAG values obtained for the series relative to the expected variation deriving from the binomial variation within dilutions was 5·41. Using the standard slide germination technique McCallan, Wellman, and Willcoxon (loc. cit.) reported an equivalent value of 23·7 where *M. fructicola* was used as the test fungus and inorganic compounds were bioassayed.

(d) *Chemical Assay of Pisatin*

The characteristic ultraviolet absorption spectrum of pisatin, and the partition of pisatin between light petroleum and water have been used to develop a chemical assay for pisatin in inoculum diffusates. 5-ml aliquots of diffusates were centrifuged at 1900 *g* for 10 min and the supernatant liquid transferred to 6 by $\frac{3}{4}$ in. glass-stoppered tubes. The sediment in the centrifuge tube was washed with 1 ml of distilled water, centrifuged at 1900 *g* for a further 5 min, and the wash fluid transferred to the diffusate supernatant. The combined liquids were then extracted four times with an equal volume of light petroleum (b.p. 55–60°C) and the combined extracts

taken to dryness at a temperature less that 40°C *in vacuo*. The residue of pisatin was dissolved in 5 ml of ethanol (redistilled, suitable for spectrophotometry to 260 mμ),

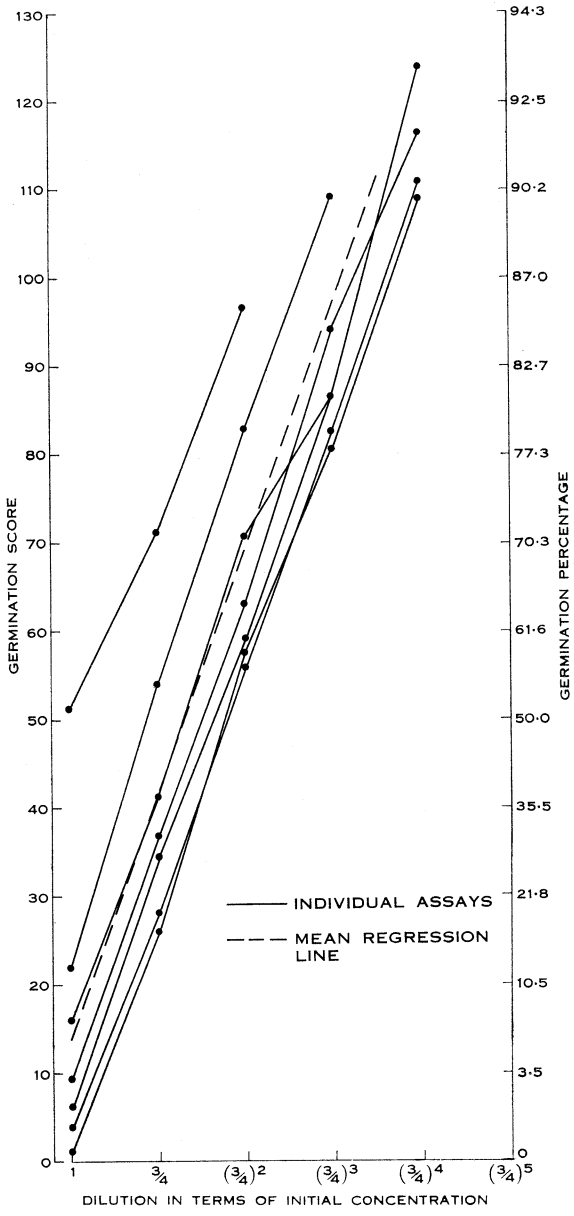

Fig. 1.—Dosage response curves of pisatin solutions against *M. fructicola* (for details of bioassay and statistical transformation see Section II(*b*) of text).

and the optical density (O.D.) of the resulting solution measured at 286 mμ and 309 mμ using a 1-cm cell in an ultraviolet spectrophotometer. For spectrophotometry,

samples were diluted as required to give O.D. readings within the accurate range of the instrument used. The concentration of pisatin in diffusates was calculated from the O.D. at 309 mμ, taking an O.D. value of 1·00 for a 5-ml solution as equivalent to 43·8 μg/ml of the original solution. When pisatin was the only light-absorbing species present the ratio $O.D._{309\ m\mu}$ to $O.D._{286\ m\mu}$ was 1·47.

(*e*) *Biophysical Correlation*

In the early stages of this work biological activity was the only criterion available to guide the extraction of the antifungal compound in the diffusates. Once purified extracts were available, however, it became apparent that the ultraviolet absorption spectrum of the biologically active fraction could be used for its identifi-

TABLE 1

ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*
Mean slope	1	6963·66	6963·66	
Variation between slopes	6	20·85	3·48	1·95*
Residues	20	35·57	1·78	

* Not significant.

cation and estimation, provided a high level of correlation existed between the biological and physical tests.

Results from four separate experiments were considered. The PAG values of the samples in each experiment (three sets in water, one set in 5% ethanol) were plotted against the $O.D._{309\ m\mu}$ values in ethanol. The results are presented in Figure 2. This corresponds to a correlation coefficient between 0·8 and 0·9 and strongly suggests that the light-absorbing species present corresponds to the fungitoxic component of the solutions. This was later confirmed by the isolation and testing of pure samples of pisatin.

(*f*) *Isolation and Purification of Pisatin*

The diffusate collected from the endocarp of pea pods inoculated as described above was clarified by centrifuging. It was then extracted four times with an equal volume of light petroleum (b.p. 55–60°C) (Müller 1958). The aqueous residue after these extractions was assayed as described above and shown to be non-toxic. Endocarp tissue stripped from the inoculated pods was extracted by maceration of this tissue in 80% ethanol at room temperature. The ethanol extract was centrifuged and the supernatant collected. The latter was then evaporated to small volume in a rotary film evaporator and the alcohol replaced by an appropriate volume of water.

The resulting aqueous solution was extracted with light petroleum as described for diffusates.

The various constituents from light petroleum extracts from diffusates and the corresponding endocarp tissues were separated on paper chromatograms (Whatman No. 3 paper; solvent *n*-propanol–water, 20 : 80 v/v; ascending). The paper used was purified by chromatographic washing with acetic acid and ammonia (Isherwood and Hanes 1953). The chromatograms after development were eluted with 50% ethanol (v/v). The ethanol was then removed and the eluates tested for antifungal activity in aqueous solution. In all extracts with initial antifungal activity a fungus-inhibiting

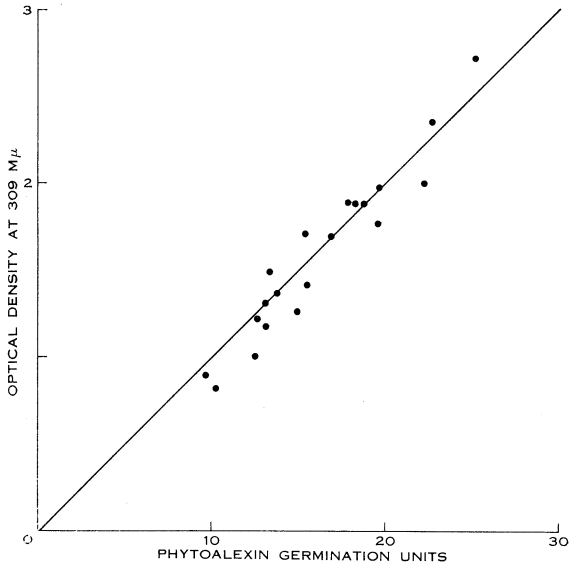

Fig. 2.—Biophysical correlation diagram.

fraction was found at R_F 0·60–0·70. A characteristic ultraviolet-light-absorbing species was also observed in this fraction. No other antifungal components could be detected. Representative tests for other groups of compounds on the chromatograms described and on chromatograms developed in other solvent systems indicated that the extract of the diffusate was essentially chromatographically homogeneous.

An antifungal compound corresponding to the above fraction was subsequently separated from a light petroleum extract by repeated recrystallization from light petroleum and from aqueous ethanol. This extraction procedure, used in the large-scale preparation of pisatin from the diffusate solution described in Section II(*a*)(i), yielded a colourless needlelike crystalline compound with a melting point of 72°C. The yield from 300 lb of pea pods was approximately 5 g.

(*g*) *Physical and Chemical Properties of Pisatin*

A preliminary account of some of the physicochemical properties has already appeared (Cruickshank and Perrin 1960). Further details are now reported, including studies on solutions of pure pisatin and plant extracts (diffusates) containing pisatin.

(i) *Recrystallized Pisatin.*—Pisatin contains only carbon, hydrogen, and oxygen and has the molecular formula $C_{17}H_{14}O_6$. A preliminary report on the structure of pisatin has been given elsewhere (Perrin and Bottomley 1961). The characteristic ultraviolet absorption spectrum, $\lambda_{max.}$ 286 mμ (log ϵ 3·68) and 309 mμ (log ϵ 3·86) in ethanol, has formed the basis of the quantitative chemical assay described above and has been used in investigations of chemical and physical characteristics of pisatin.

Distribution coefficients for pisatin have been determined between light petroleum (b.p. 55–60°C) and water (2·3 : 1), cyclohexane and water (3·6 : 1), and between oleyl alcohol and water (100 : 1). Oleyl alcohol was selected as representative of fatty acids found in cytoplasmic membranes (Albert and Hampton 1954).

Pisatin is stable in neutral and alkaline solutions. There is no change in the ultraviolet absorption spectrum even in 0·05M sodium hydroxide. This fact, together with its ready extractability from alkaline solutions, indicates the absence of phenolic or other acidic groups. Pisatin is, however, extremely acid labile, forming a compound which has been recrystallized and which analyses as $C_{17}H_{12}O_5$. This substance contains one molecule of water less than pisatin, and has been designated anhydropisatin (Perrin and Bottomley 1961). The dehydration of pisatin is accompanied by a considerable bathochromic shift and intensification of the ultraviolet absorption spectrum with maxima at 339 mμ (log ϵ 4·58) and 358 mμ (log ϵ 4·60). The quantitative formation of anhydropisatin from pisatin as measured at these maxima, has been used to confirm the identity of pisatin and the presence of low levels of this substance in the chemical assay described above.

The solubility of pisatin at 23°C is 0·03 mg/ml in water, 0·5 mg/ml in light petroleum, 6 mg/ml in oleyl alcohol, and >42 mg/ml in ethanol.

(ii) *Plant Extracts containing Pisatin.*—In extracts or diffusates from infected plant tissues, pisatin occurs in a biologically active state at concentrations of the order of two to three times its maximum solubility in distilled water. Experiments were undertaken to study the stability of pisatin in such a system with the following results. The level of pisatin in a diffusate solution (pisatin concentration 61·3 μg/ml) was not lowered as a result of autoclaving (15 lb for 20 min), heating to 96–98°C for 30 min, or heating to 65–70°C for 15 hr. There was no sedimentation of pisatin from the diffusate by centrifuging at 3200 *g* for 30 min, but there was a slight loss (*c.* 3%) following centrifuging at 18,400 *g* for 30 min. Filtration through a "Millipore"* filter of mean pore size 0·45μ removed 14% of the pisatin from the diffusate, while the pisatin content of a filtrate from a 0·01μ pore size filter was close to 30 μg/ml, i.e. the value for the solubility of pisatin in distilled water. Biological activity paralleled the physical measurements of pisatin.

Pisatin is adsorbed on to or dissolves in certain plastic surfaces such as polyvinyl choride or cellulose nitrate plastics, when the plastic surfaces are brought in contact with diffusate solutions. Pisatin is also strongly adsorbed on to many chromatographic adsorbents such as celite, lactose, sucrose, calcium carbonate, alumina, and charcoal, but it can be removed fairly readily from celite, the sugars, and calcium

* Manufactured by Millipore Filter Corporation, Watertown, Massachusetts.

carbonate with aqueous ethanol. The adsorption or loss of pisatin on to plant surfaces from saturated aqueous solutions and from diffusates with a similar concentration of pisatin (30·3 μg/ml) were studied by placement of drops of the solutions on pea and tobacco leaf surfaces and on the endocarp tissue of pea pods. The plant tissues were incubated at 20°C in the dark for 24 hr, the solutions withdrawn from the surfaces, and the pisatin concentration measured. No loss of pisatin occurred on to the leaf surfaces. The mean loss (four replicates) from the solutions placed on endocarp tissues was 41% for the diffusate solution and 62% for the solution of pure pisatin respectively.

TABLE 2

EFFECT OF LIGHT ON PISATIN SOLUTIONS

Light Treatment	Wavelength (mμ)	Period of Treatment (min)	Pisatin Concn. (μg/ml)
Untreated			61·3
Ultraviolet light	365	120	54·3
Sunlight (approx. 5000 f.c.)		180	26·3*
Ultraviolet light	253·7	15	57·4
Ultraviolet light	253·7	30	53·4
Ultraviolet light	253·7	45	49·5*
Ultraviolet light	253·7	60	48·2*
Ultraviolet light	253·7	90	44·2*

* No antifungal activity detected.

To study the effect of light, including ultraviolet, samples of diffusate (pisatin concentration 61·3 μg/ml) were subjected to various types of irradiation and the residual pisatin concentration measured. The results, presented in Table 2, indicate that the previously established correlation between biological activity and pisatin concentration was not valid after the sunlight and 253·7 mμ irradiations. It was found that the pisatin-like compound remaining in the irradiated solutions did not dehydrate readily under mild acid conditions with the formation of anhydropisatin. Careful examination of the ultraviolet spectrum of samples following irradiation treatment showed a gradual change to a substance which is spectroscopically similar to pisatin ($\lambda_{max.}$ 286 mμ and 311 mμ but without the inflexion at 281 mμ). It is suggested that this substance is chemically close to pisatin but that its biological activity has been changed by the high-energy ultraviolet irradiation.

(*h*) *Biological Properties of Pisatin*

The nature of the antifungal activity of pisatin was studied in two types of experiment. In the first, drops of freshly collected diffusate containing spores of *M. fructicola* were taken from a standard inoculation experiment (pisatin concn. 60 μg/ml). The germinated spores, whose growth had been stopped by the pisatin in the diffusate, were removed from the diffusate by centrifugation and resuspended in sterile distilled water. Single drops of the latter were placed on slices (2·5 cm dia.,

1 cm thick) of banana mesocarp, which had previously been shown to be highly susceptible to *M. fructicola*, under aseptic conditions in petri dishes and incubated for 5 days at 20°C. Nine replicates were used, with sterile water serving as control. Abundant sporulation occurred on all banana slices to which the drops of spore suspension had been added. No sporulation occurred on the controls. In the second test, six seeded agar disks (prepared as for bioassay) were incubated in a pisatin solution in 5% ethanol (pisatin concn. 118 μg/ml) for 48 hr. No germination occurred. The seeded agar blocks were then removed from the pisatin solution and washed in three changes of distilled sterile water. Finally the blocks were reincubated in sterile water for a further 24 hr at 20°C. The resultant mean percentage germination was 94·8%. However, germ tubes were short, distorted, and branched. The results of these tests show that pisatin possesses fungistatic rather than fungicidal activity.

Hypersensitivity in plants is normally associated with necrosis of infected host cells. The following experiments were carried out to determine if pisatin at concentrations normally present in diffusates is phytotoxic. In the first test, drops of diffusate (pisatin concn. 75·2 μg/ml) were placed on proliferated endocarp cells of the pea pod and left for 48 hr. The viability of the cells under the drops was determined using 0·1% neutral red (Tribe 1955). In the second test, tobacco seedlings and young pea plants were sprayed to incipient runoff with diffusate (pisatin concn. 65·8 μg/ml). The plants were incubated at high humidity for 24 hr in the dark at 18°C and then returned to the glass-house. They were examined for phytotoxicity symptoms 24 hr, 48 hr, and 1 week after treatment. In neither of these experiments was any evidence obtained to suggest that pisatin was toxic to pod cells or leaf tissues.

A third experiment was carried out to determine the effect of pisatin on growth. Ten pregerminated wheat seeds (coleoptiles approx. 1 mm in length) were placed in a petri dish containing 3 ml of one of the following solutions: diffusate (pisatin concn. 65·8 μg/ml), diffusate residue (pisatin extracted), and distilled water. Ten dish replicates were used. The seeds were incubated at 20°C and the mean lengths of the primary roots (radicles) after 48 hr were 21·51, 37·58, and 31·77 mm, respectively. Difference for significance at the 0·1% level was 5·48 mm. The results show that pisatin inhibited the growth of the primary root by one-third if the length in distilled water is taken as the reference length.

III. Discussion

Allen's (1959) primary conclusion regarding the nature of the plant's defence against pathogenic attack was that "it is not a condition of the plant which constitutes resistance but a process of response". This concept of a response or interaction between the host and parasite with the formation of new antifungal compounds is the basic concept in the phytoalexin theory postulated by Müller and Börger (1940) on the basis of important indicative evidence. As a part of this response or interaction respiration increases in diseased tissues (Millerd and Scott 1956; Shaw and Samborski 1957), and biochemical changes occur. Changes in normal metabolites have been reported (Kuć, Ullstrup, and Quackenbush 1955) but the compounds concerned were not adequate in themselves to explain in full the inhibition of growth of the invading fungus. Three previous reports have presented data on the isolation of abnormal

metabolites, namely ipomeamarone (Hiura 1943) formed in sweet potato tissues infected with *Ceratostomella fimbriata* (Ell. & Hals.) Elliot, orchinol (Boller *et al.* 1957) which results from the interaction between *Rhizoctonia repens* Bern. and orchid tubers, and an antifungal compound (Condon and Kuć 1960) isolated from carrot root tissue infected with *Ceratostomella fimbriata*, which may be considered to be formed as a "process of response" to fungal infection. In these reports, however, the preparation of the host tissue involved extensive cell damage, and the conditions and duration of the incubation of the tissues after inoculation in the second and third examples might cast reasonable doubt on the primary nature of the compounds isolated. Ipomeamarone was originally thought to be primarily an uncoupling agent; however, phytoalexin action has recently been envisaged for it at the higher concentration levels (Akazawa 1960).

Pisatin is formed as a response to inoculation of the endocarp tissues with *M. fructicola*. The suggestion that pisatin is the primary antifungal compound formed as a response of pea pod endocarp tissues to inoculation with *M. fructicola* results from the following observations. There is a close association between the hypersensitivity symptoms of the inoculated endocarp tissues, the limited growth of the fungal germ tubes in the inoculum at the end of the incubation period, and the concentration of pisatin in the resultant diffusate. The technique involves a minimum of damage to host cell tissues. The incubation period required for the formation of inhibitory levels of pisatin is relatively short. The drop-diffusate technique takes advantage of the plant cell membrane as the primary "filter" in the extraction procedure. The simplicity of the subsequent extraction and purification, which utilizes only light petroleum and ethanol, is strong evidence that pisatin occurs naturally in infected tissues, and is not an artefact of our extraction technique, as has been shown to be the case in some recent papers reporting the isolation of some other antifungal compounds (Virtanen and Hietala 1960). Thus it would appear that pisatin would qualify as an example of the biologically active class of compounds defined by Müller (1953) as "phytoalexins".

Some of the general properties of pisatin have been studied in this paper. When these are compared with those reported by Müller (1958) for the phytoalexin from French beans (*Phaseolus vulgaris* L.) it will be seen that pisatin is similar in some of its properties. Both are lipophilic in so far as they can be extracted into light petroleum. Solutions of both are stable to high temperatures (*c.* 100°C). Solutions of pisatin are stable to autoclaving (15 lb, 20 min). The compounds differ, however, in their stability to light. The French bean phytoalexin (Müller 1958) rapidly loses biological activity on exposure to ultraviolet light ($\lambda = 365\ \mathrm{m}\mu$). This radiation has only a slight effect on the biological activity of pisatin even after extended exposure (2 hr). Irradiation by sunlight or ultraviolet light ($\lambda = 253{\cdot}7\ \mathrm{m}\mu$), however, rapidly destroys the biological activity of pisatin (cf. Uehara 1958*c*).

The low solubility of pisatin in aqueous solutions has been a major problem in the study of its biological properties. A similar problem has been discussed by Gaumann and Kern (1959) where it is stated that the concentration of orchinol in water at pH levels characteristic of those of the host tissues is roughly 10 times too low to produce inhibition areas of similar size to those produced by infected tuber

fragments. In our results, it is shown that the saturation level of pisatin in water is approximately 30 μg/ml. However, the concentration of pisatin in the diffusates is normally two to three times this concentration. Gaumann and Kern (loc. cit.) explained the discrepancy in their solubility results by suggesting the existence of a solubilizing agent which keeps orchinol in excess of saturation. The results presented above provide strong evidence that pisatin exists in diffusates both in true solution and in a fairly stable dispersed form in which it is biologically active. High adsorption was reported by Müller (1958) as an important character of French bean phytoalexin. Pisatin, as discussed above, occurs in diffusates in excess of its maximum solubility in water. Under such conditions it is lost readily from solution on to various types of surfaces. The adsorption characteristics of pisatin in true solution are, however, quite normal. The drop in pisatin concentration in samples placed on endocarp tissues could be adsorption; on the other hand it could be due to destruction by enzymatic activity. No evidence is at present available on this point.

Müller (1958) stated that French bean phytoalexin was fungistatic at lower concentrations and fungicidal at the higher levels. Pisatin at concentrations equivalent to the maximum concentrations obtained in diffusates has been shown to be only fungistatic, and it is only by the maintenance of the pisatin concentration at inhibitory levels that growth of the fungus is prevented. Resistance, and in particular the hypersensitive reaction in plants, is characterized by the necrosis of host cells surrounding the infection site. This type of reaction is clearly demonstrated in the work of Müller (1958) on French beans. A similar, but less intense necrotic response occurs in peas. The response varies in peas but is not generally apparent over the inoculated areas until 72 hr after inoculation. Moreover it occurs more rapidly if the inoculum liquid is removed after 40 hr than if it is left on the tissue surfaces. The results of phytotoxicity tests suggest that mature cells are not sensitive to pisatin, but that pisatin has a significant effect on rapidly dividing cells, and thus affects rate of growth (cf. acti-dione (Hawthorne and Wilson 1952)). Thus, pisatin is phytotoxic, although there would appear to be a fairly large concentration differential between the equivalent effect of pisatin on fungi and on green plant cells. It may account for the necrosis of host cells *in vivo*, but at this stage of our work the evidence in favour of this is not very strong.

Detached pods were used in the bulk extraction of pisatin; however, pisatin formation is not limited to pod tissues. Its isolation from inoculated pea leaf, stem tissue, and pods on the growing plant indicate the wide distribution of the capacity to form pisatin in pea plant tissues, and suggest that the results obtained in terms of the pod tissues are of pathological significance in relation to the whole pea plant.

IV. Acknowledgments

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