STUDIES ON PHYTOALEXINS

VIII. THE EFFECT OF SOME FURTHER FACTORS ON THE FORMATION, STABILITY, AND LOCALIZATION OF PISATIN *in vivo*

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Summary

In a study of the effect of some further biological, physical, and chemical factors on the formation of pisatin, the following points were demonstrated:

(1) Pisatin formation following heat treatment (45°C) or anaerobic storage was dependent on the duration of the exposure period. Delayed inoculation, following some treatments, resulted in an increased capacity of the host to form pisatin. Pisatin concentration and host susceptibility were inversely related.

(2) Pisatin formation was stimulated by several saprophytic fungal species.

(3) Detached pea pod tissues retained their capacity to form or release pisatin for 20 days following inoculation.

(4) Pisatin was stable in the tissues of *Pisum sativum* and mycelium of *Ascochyta pisi*.

(5) Pisatin was localized in inoculated tissues and did not diffuse to neighbouring healthy tissues. The formation of pisatin in relation to alterations in host metabolism is briefly discussed.

I. INTRODUCTION

The effect of a range of biological, physical, and chemical factors on pisatin formation in the endocarp tissue of pea pods (*Pisum sativum* L.) has been reported (Cruickshank and Perrin 1963). The present study is an extension of this earlier work and an examination of the stability of pisatin in the tissues of the pea plant and in mycelium of *Ascochyta pisi* Lib., a fungal pathogen of peas, which is tolerant to high concentrations of pisatin ($ED_{50} > 100$ μg/ml). Experiments relating to the localized nature of pisatin formation in inoculated tissues are also reported.

II. MATERIALS AND METHODS

Pea pods (class 2 and 3 host material, see Table 1 of Cruickshank and Perrin 1963) of the varieties Greenfeast or Little Gem were used as the host tissues. *Monilinia fructicola* (Wint.) Honey, a non-pathogen of peas, was used as the primary test fungus in the biological assays of the capacity of the host tissue to synthesize or release pisatin. The standard conditions of fungal culture, preparation of spore suspensions, inoculation and incubation of host tissues, collection of diffusates, and their chemical assay for pisatin concentration have already been described (Cruickshank and Perrin 1963). To allow the chemical assay for pisatin in large numbers of endocarp samples, the assay described earlier (Cruickshank and Perrin 1961) was modified as follows. Weighed samples of endocarp (0·3–0·8 g) were homogenized in 90% ethanol, and quantitatively transferred to volumetric flasks, and adjusted to volume. Two aliquots (5 ml) were withdrawn from each flask and placed in glass-stoppered tubes. Hydrochloric acid [0·2 ml of a solution of 1 vol.

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HCl (sp. gr. 1·18)+1 vol. water] was added to one aliquot. To maintain equal volumes, 0·2 ml of water was added to the second aliquot. Both solutions were mixed by inversion of the tubes and then stood in the dark for 16–22 hr at room temperature (*c.* 23°C). The absorption spectra of the two solutions were recorded over the range 260–370 mμ and the difference in absorbance at 358 mμ between the two spectra was measured and the amount of pisatin calculated. An optical density of 1·00 was equivalent to 8·813 μg pisatin per millilitre of the ethanol homogenate prepared above. For convenience of presentation of results, the pisatin concentrations were converted to micrograms per gram fresh weight of endocarp.

This modified assay makes use of the quantitative conversion of pisatin to anhydropisatin on acidification (Perrin and Bottomley 1962). This change leads to intensification and a bathochromic shift to the 320–370 mμ spectral region which is almost optically transparent for the ethanolic extract of healthy pea endocarp. The concentration of hydrochloric acid and standing time selected yielded most satisfactory results when the anhydropisatin formed gave an absorbance within the range 0·3–1·0. Precautions were taken against exposure of samples to light because anhydropisatin may be changed photochemically.

Triplicate samples were taken from each treatment for the chemical assays, unless otherwise stated. In the experiments involving changes of host reaction type [Sections III(*a*)(i) and III(*a*)(ii)], observations on mycelial growth and sporulation of *M. fructicola* were made 5–7 days after inoculation. Results from typical experiments only are presented.

Experimental details which concern specific experiments are described in Section III.

III. Experimental and Results

(*a*) *Pre-inoculation Treatment of the Host*

(i) *Effect of Temperature*

Detached, unopened whole pods were exposed to a range of air temperatures from −20°C for 3 hr to 100°C for 10 min. Direct readings of air temperature among the pods were determined by suitable positioning of mercury-in-glass thermometers. Above 20°C, but excluding 100°C, an equilibration time of 15 min prior to the treatment period was allowed. At −20°C a much longer period was required for equilibration. At 100°C the total period of treatment including the equilibration period was 10 min.

Following the temperature treatments, the whole pods were divided into two equal samples. The pods of one sample from each treatment were split into half pods and the seeds discarded. The endocarps were immediately inoculated and incubated under standard conditions. The pods of the second sample were stored whole for 3 days at 20°C in cotton-stoppered containers prior to dissection, inoculation, and incubation.

The results presented in Table 1 show that temperatures from 2 to 40°C for exposures up to 3 hr did not affect the capacity of the endocarp of pea pods to produce pisatin. This condition was not affected by subsequent storage of the treated

host tissue for 3 days prior to inoculation. The normal resistance of the host tissue to *M. fructicola* was not affected by these treatments. Pods treated at −20°C and 50°C for 3 hr, and 100°C for 10 min permanently lost their capacity to form pisatin. The endocarp tissues following these treatments were susceptible to *M. fructicola* and various saprophytic species of bacteria. The results of the 45°C treatments were time-dependent. Exposure periods of 3 hr to 45°C destroyed the host tissue's capacity to form pisatin and resulted in host susceptibility to *M. fructicola*. The

TABLE 1

EFFECT OF PRE-INOCULATION TEMPERATURE TREATMENT OF PEA PODS ON PISATIN FORMATION IN DIFFUSATE SOLUTIONS FOLLOWING INOCULATION WITH MONILINIA FRUCTICOLA

Temperature (°C)	Time (hr)	Mean Pisatin Concentration (μg/ml)	
		Immediate Inoculation	Delayed Inoculation
−20	3	Nil*	Nil*
2	3	39·9	32·6
20	3	35·7	36·2
36	3	54·4	34·1
40	3	32·3	29·9
40	2	42·5	34·3
40	1	42·5	36·8
45	3	Nil*	Nil*
45	2	Nil*	27·2†
45	1	41·7	30·6
45	0·5	47·3	41·3
50	3	Nil*	Nil*
100	10 min	Nil*	Nil*
Control (water only)		2·9	4·0

* Mycelial growth and sporulation observed 5–7 days after inoculation.
† Limited mycelial development, no sporulation.

situation was not altered by storage prior to inoculation. Immediate inoculation of the endocarp of pods treated at 45°C for 2 hr was followed by a susceptible host reaction and no pisatin was formed. Host tissue storage for 3 days following the treatment for 2 hr at 45°C prior to inoculation resulted in a highly significant level of pisatin production. It also resulted in a reversion of the host reaction to almost the normal resistance of untreated pea pod endocarp towards *M. fructicola* (cf. Plate 1 of Jerome and Müller 1958). Treatment periods of 0·5 and 1 hr at 45°C did not affect the capacity of pods to form pisatin or the normal resistance of pea pod endocarp to the test fungus.

(ii) *Effect of Anaerobic Storage*

Whole pea pods were stored at 4°C in four air-tight chambers connected in series to a source of oxygen-free nitrogen gas. The system initially was flushed extensively with nitrogen and nitrogen was passed continuously (1 lb/sq in) through

the system over the entire anaerobic exposure period. Whole pod samples were taken for bioassay after storage for 0, 3, 6, 9, and 12 days. As in Section III(*a*)(i), one portion of pods from each sample was immediately inoculated and incubated under standard conditions while a second portion of the pods was stored for a further period of 3 days at 4°C in air prior to inoculation. Concurrently, with these biological assays of pisatin production, further aliquots of pods were taken from each sample and the endocarps treated with an aqueous solution of mercuric chloride (1×10^{-4}M) to determine if similar responses to those from fungal inoculation would occur following application of a chemical solution.

TABLE 2

EFFECT OF STORAGE OF PEA PODS AT 4°C UNDER ANAEROBIC CONDITIONS ON THEIR PISATIN-PRODUCING CAPACITY FOLLOWING INOCULATION WITH MONILINIA FRUCTICOLA AND MERCURIC CHLORIDE APPLICATION

Duration of Storage (days)	Mean Pisatin Concentration (μg/ml) in Diffusate Solutions			
	Fungal Inoculation		Mercuric Chloride Application (1×10^{-4}M)	
	Immediate	Delayed	Immediate	Delayed
0	47·7	—	35·9	—
3	41·3	48·5	39·0	54·4
6	22·1*	52·3	29·1	40·4
9	13·6*	41·5	13·6	40·4
12	6·5*	13·6*	6·0	13·2

* Mycelial growth and sporulation observed 5–7 days after inoculation. Intensity of sporulation increased from the 6- to the 12-day treatment.

The results shown in Table 2 indicate that an exposure period of 3 days to anaerobic conditions did not affect pisatin production or the normal resistant reaction of the host tissues. This result was not affected by subsequent aerobic storage. Exposure for periods of 6, 9, and 12 days followed by immediate inoculation resulted in the formation of progressively lower concentrations of pisatin by the endocarp. In each instance the host reaction was one of susceptibility to *M. fructicola* (sporulation intensity increasing with decreasing pisatin concentration). Aerobic storage subsequent to exposure to anaerobic conditions, and prior to inoculation, resulted in increased pisatin concentrations. Following these subsequent aerobic treatments the host reaction of the pods stored under anaerobic conditions for 6 and 9 days, was one of resistance. The host reaction after the 12-day exposure treatment was not altered by subsequent aerobic storage. These results, in so far as pisatin formation was concerned, were fully confirmed by the results following application of mercuric chloride solution.

(*b*) *Pisatin Induction by Saprophytic Fungi*

To determine if the formation of pisatin may be induced by fungal species not pathogenic to plants, various saprophytic species were tested as inocula (Table 3). The species used were grown on potato dextrose agar. The preparation of spore suspensions and the inoculation and incubation of the pod tissues were carried out under standard conditions.

TABLE 3

CONCENTRATIONS OF PISATIN FORMED FOLLOWING INOCULATION OF THE ENDOCARP OF PEA PODS WITH SOME MISCELLANEOUS FUNGAL SAPROPHYTES

Fungal Species	Mean Pisatin Concentration after 72 Hr (μg/ml)
Aspergillus nidulans (Eidam) Wint.	72·3
Aspergillus repens (Corda) de Bary	11·1
Cladosporium herbarum (Pers.) Linle	30·6
Rhizopus nigricans Ehrenberg	61·6
Pithomyces chartarum (Berk. & Curt) Ellis	89·3
Trichoderma viride Pers. ex Fr.	8·0
Neurospora crassa Shear & Dodge	55·0
Mucor mucedo (Linne) Brefeld	11·5
Controls:	
Monilinia fructicola (Wint.) Honey	70·7
Water	6·4
Least significant difference at 5% level	0·86
Least significant difference at 1% level	1·19

Some germination and restricted mycelial growth occurred with all fungal species used as inocula, but in no instance was the host tissue susceptible. The results (Table 3) show that all the fungi tested induced the formation of pisatin at concentrations significantly above the levels of the water control.

(*c*) *Formation of Pisatin Resulting from a Single Inoculation*

Freshly picked field-grown pea pods (cv. Greenfeast, class 2 host material) were inoculated under standard conditions with *M. fructicola* and incubated for 48 hr. The diffusate solution was then withdrawn from the inoculated tissues and replaced by a similar volume of sterile distilled water. The incubation period was continued for a further 24 hr after which time the new diffusate was withdrawn and again replaced by a similar volume of sterile distilled water. This sequence of addition and withdrawal of sterile distilled water and diffusate was repeated daily for 20 days. The samples collected were chemically assayed for pisatin concentration.

The results shown in Table 4 indicate that detached pea pod tissues may form or release pisatin for at least 20 days subsequent to inoculation.

(*d*) *Stability of Pisatin*

(i) *Plant Tissues*

Endocarp tissues of half pea pods in one treatment were inoculated with *M. fructicola* and in a second treatment were treated with an aqueous solution of mercuric chloride (1×10^{-4}M). Both lots of treated half pods were incubated under standard conditions for 40 hr. The diffusate solutions were then withdrawn and the host tissue placed in a cold room (4°C). Sequential samplings of both lots of pods were made at 2-day intervals over a 25-day storage period. The treated endocarp was removed from the pods, extracted, and assayed for pisatin concentration.

TABLE 4

PISATIN FORMATION FOLLOWING A SINGLE INOCULATION OF THE ENDOCARP OF PEA PODS WITH MONILINIA FRUCTICOLA FOLLOWED BY SUBSEQUENT DAILY APPLICATIONS OF STERILE DISTILLED WATER

Total pisatin formation 978·4 μg per 68 mg of endocarp*

Time after Original Inoculation (days)	Pisatin Concentration in Diffusate Solutions (μg/ml)	Time after Original Inoculation (days)	Pisatin Concentration in Diffusate Solutions (μg/ml)
2	69·9	12	59·9
3	68·6	13	52·9
4	62·5	14	47·6
5	66·9	15	45·5
6	69·9	16	35·8
7	69·9	17	28·8
8	40·2	18	28·0
9	48·5	19	31·0
10	54·2	20	38·0
11	60·3		

*Mean fresh weight of endocarp covered by 1 ml of spore suspension is 68 mg.

The results presented in Table 5 show that, under the storage conditions used in this experiment, pisatin concentration in the endocarp tissues reached a maximum 4 days after inoculation and remained at approximately this concentration for the duration of the storage period. The data in the case of the mercuric chloride treatment varied in detail from the inoculation treatment but confirmed in principle the stability of pisatin in plant tissues.

(ii) *Fungal Mycelium*

Erlenmeyer flasks (capacity 25 ml) were sterilized and dried to constant weight. Pisatin in ethyl ether (Cruickshank 1962) was aseptically dispensed into the flasks to give a final concentration of 500 μg of pisatin per flask. The ethyl ether was removed by partial immersion of the flasks in a water bath at 50°C for 10 min and ethanol (analytical reagent grade) was added to give a final concentration of 1%. 10 ml of a basal semisynthetic nutrient broth (Lilly and Barnett 1951) was

aseptically dispensed into each flask. The treatment flasks were inoculated with a 5 by 5 by 1 mm disk of *Ascochyta pisi* grown on potato dextrose agar and incubated at 20°C, without shaking, for 21 days in the dark.

TABLE 5

STABILITY OF PISATIN *in vivo* AT 4°C

Time (days)	Mean Concentration of Pisatin in Endocarp Tissues of Pea Pod (μg/g fresh wt.)	
	Inoculation with *M. fructicola*	Mercuric Chloride Application (concn. 1×10^{-4}M)
0	Nil	Nil
2	35	63
4	201	122
6	177	171
8	185	102
10	167	145
12	173	152
14	118	215
16	193	259
18	191	167
20	168	155
22	184	178
25	197	112

Samples of the broth for pisatin analysis were withdrawn from uninoculated control flasks at the beginning and termination of the incubation period. At the termination of the incubation period the inoculated flasks were divided into two sets.

TABLE 6

STABILITY OF PISATIN TOWARDS ASCOCHYTA PISI CULTURED IN SEMI-SYNTHETIC BROTH FOR 21 DAYS AT 20°C IN THE DARK

	Mean Pisatin Concentration (μg/ml)		
	Broth	Mycelium*	Total
Before inoculation and incubation	51·4	—	51·4
After inoculation and incubation	Nil	53·3	53·3
After incubation (uninoculated control)	53·2	—	53·2

*Mean dry weight of mycelium after incubation was 56 mg.

One set of cultures were centrifuged and the supernatant and mycelial residue separately extracted and analysed for pisatin. A second set of cultures was washed and the amount of mycelial growth determined gravimetrically after drying at 70°C to constant weight. Six replicates were used in all phases of this experiment.

The results presented in Table 6 show that pisatin was removed from the nutrient broth by *A. pisi* during the incubation period. It was recovered, however, without significant loss from the mycelium.

(*e*) *Distribution of Pisatin in Pea Pod Tissues*

Half pea pods were set out under standard incubation conditions. A rectangular area of endocarp (0·5 mm wide) at the centre of each pod and extending the width of the pod was inoculated with a standard spore suspension (0·08 ml per pod). Sterile distilled water was used in the control series. Fluid on the inoculated areas was removed after 72 hr.

TABLE 7

OCCURRENCE OF PISATIN IN PEA POD TISSUES IN RELATION TO THE SITE OF INOCULATION WITH MONILINIA FRUCTICOLA

	Mean Concentration of Pisatin (μg/g fresh wt.)	
	M. fructicola	Water
Lateral distribution in endocarp:		
Section No. 4 (apex of pod)	1·6	3·3
3	3·3	3·3
2	8·3	5·0
1 (inoculated section)	456·6	3·3
2′	5·0	1·6
3′	3·3	1·6
4′ (base of pod)	6·6	3·3
Vertical distribution		
Endocarp	476·0	3·0
Mesocarp	Nil	Nil

In one experiment, designed to study the lateral diffusion of pisatin into uninoculated cells of the endocarp, the pods were dissected into the central inoculated section and three 0·5-mm wide sections in linear series on either side of the central section. The endocarp from the pod sections was removed and corresponding endocarp pieces from 10 pods were bulked, extracted, and assayed for pisatin. In a second experiment, the endocarp and mesophyll tissues of the central inoculated region of the pod were separated, extracted, and chemically assayed to determine the vertical distribution of pisatin in the tissues immediately under the inoculated area. In addition the capacity of the mesocarp tissue of pea pods to form pisatin, following fungal inoculation, was tested under standard conditions of inoculation and incubation after first removing the endocarp layer.

The results of the experiments on the distribution of pisatin presented in Table 7 show that it occurred only in inoculated tissue. Inoculated mesocarp tissues formed 200–300 μg of pisatin per gram fresh weight of plant tissue.

IV. Discussion

Jerome and Müller (1958) reported on the basis of results from biological assays that pre-inoculation treatments of detached pods of French beans (*Phaseolus vulgaris* L.) at various temperatures affected the production of phytoalexin and the disease reaction of this host to *M. fructicola*. Their results indicated a close correlation between host reaction type and level of toxicity of the phytoalexin in the diffusate solutions assayed. The data reported in Sections III(*a*) and III(*b*) fully confirm the report of Jerome and Müller (1958) for a second host–parasite combination, and for a known phytoalexin, pisatin. They also show that similar time-dependent reversibility of host reaction can be obtained by anaerobic storage. These data, together with those of Jerome and Müller (loc. cit.), support the concept of the qualitative similarity in the physiological reaction of the host tissues following inoculation irrespective of the final phenotypic expression of disease. Within a given host–parasite model, such as the combination used in the present study, the expression of disease was closely associated with the concentration of pisatin as measured in the diffusate solutions. In culture the ED_{100} for pisatin towards *M. fructicola* is *c.* 30 μg/ml of agar (Cruickshank 1962). While it is necessary to use *in vitro* data cautiously in the interpretation of *in vivo* results, it appears more than coincidental that *c.* 30 μg/ml appears to be the critical pisatin concentration necessary for inhibition of growth and sporulation *in vivo* of *M. fructicola*. The parallel relationship demonstrated between pisatin concentration and host reaction is suggestive of a causal relationship between these two phenomena. It supports the concept that susceptibility or resistance to a given fungus, other things being equal, is directly dependent on the quantitative aspects of phytoalexin formation.

The formation of pisatin by pea pod endocarp following its inoculation with saprophytic species of fungi provides evidence that this phenomenon is not confined to phytopathogenic fungi. This result is interesting as it means that biochemically well-known fungal species such as *Neurospora crassa* and its biochemical mutants may now be used to study the role of the fungi in the induction of the biosynthesis of pisatin and probably other phytoalexins. The reason for the quantitative differences in the concentration of pisatin formed (Table 3) is not understood. A consideration of the scheme describing the major biological steps in the biosynthesis of pisatin (Cruickshank 1965) would, however, suggest that it may, in part, be due to differences in germination rates of the several fungal species on the endocarp surface. In addition fungal species may inherently differ in their capacity to induce pisatin formation.

The mode of action of pisatin towards fungi is not known. The basis of its selective toxicity towards pathogens and non-pathogens is especially interesting in relation to its postulated function in pathogenicity. Uehara (1964) has recently claimed that the basic reason for the pathogenicity of *Ascochyta pisi* and *Fusarium oxysporum* towards the garden pea lies in their ability to decompose pisatin in advance of their growth presumably by the production of extracellular detoxifying compounds. Uehara's conclusions were based on observations of the disappearance of opaqueness around colonies of these two species grown in agar in the presence of extracts of pisatin.

In an earlier paper in this series (Cruickshank and Perrin 1961), it was shown that spore germination of *M. fructicola* could be inhibited by solutions of pisatin for 48 hr, and that after this period the toxin could be removed by mild washing with water. The inhibition of germination of the spores in the presence of pisatin, followed by high percentage germination following washing, suggested that the pisatin was adsorbed by the spore but that it was not metabolized.

Our present studies on the stability of pisatin have involved both host and parasite studies. In the former, where a non-pathogen of peas was used and the inoculated tissues were stored for over 3 weeks at 4°C under aerobic conditions (Table 5), the results did not indicate that pisatin was unstable. The stability of pisatin was independent of fungal stimulation as similar results were obtained after application of mercuric chloride (1×10^{-4}M). In the latter case where *A. pisi* was used as the test fungus the data indicated that pisatin was readily taken up from the culture solution by this pathogenic species but did not establish that pisatin was metabolized or otherwise decomposed. On the basis of the present evidence it may be concluded that pisatin is a stable molecule when present in either plant or fungal cells and that the selective toxicity of this compound is not dependent on the differences in the capacities of pathogens and non-pathogens of peas to decompose it.

The pea pod–*M. fructicola* combination may be looked upon as a host–parasite model suitable for the study of hypersensitivity. The histologically and cytologically restricted nature of the host and parasite cells involved in hypersensitive reactions is well documented (White and Baker 1954; Tomiyama 1956; Müller 1958; Anderson and Walker 1962). The technical difficulties involved prevented the chemical assay of pisatin on a single cell level. However, the absence of the detection of significant concentrations of pisatin in any but inoculated tissue (Table 7) strongly supports the histological data of earlier workers and also indicates that this compound does not diffuse from inoculated to healthy cells.

Studies of some physiological factors relating to pisatin formation following fungal inoculations have already been discussed (Cruickshank and Perrin 1963). Studies on the stimulation of pisatin formation by non-microbiological agents have also received some attention (Perrin and Cruickshank 1965). A consideration of some of the results presented in this paper appear relevant in relation to pisatin formation and will be briefly discussed from this viewpoint.

Half pea pods inoculated and incubated under standard conditions for 40 hr yielded about 70 μg of pisatin per millilitre of diffusate (Table 4). With continued serial application and removal of fluid from the pods at 24-hr intervals, a total of 978 μg of pisatin per 68 mg of endocarp was collected over 20 days. Under initially similar conditions the yield of pisatin [Section III(*d*)] per gram fresh weight of endocarp tissue was 200 μg (Table 5). Storage at 4°C of the inoculated pods prevented further formation of pisatin. In the former situation, metabolism was not interfered with, while in the latter, it was inhibited by low temperature. Further data are available from the pod pre-inoculation experiments (Tables 1 and 2). The effect of the −20°C treatment *inter alia* would be the physical destruction of the unity of the membranes of the cell protoplast and the ordered respiratory mechanism

of the tissue. It would not be expected to lead to the destruction of preformed pisatin substrates. The milder treatments which resulted in reversible situations on subsequent storage prior to inoculation could be envisaged as resulting in partial inactivation of an essential enzyme, the utilization of an essential substrate, or the accumulation of metabolic inhibitors, in short, in the formation of a temporary chemical lesion which interferes with the process involved in the formation of pisatin.

The biochemical nature of pisatin formation in the tissues of the garden pea must remain unknown until direct experimental evidence is available. Attempts to isolate pisatin from fresh, physiologically normal, healthy pea plant tissues (Cruickshank and Perrin 1963) have been negative. Attempts to synthesize pisatin from cell-free extracts of pea plant tissue by chemical or enzymic hydrolysis (Perrin and Cruickshank 1965) have also proved unsuccessful. On the other hand, pisatin is readily formed in pea plant tissue as a result of fungal inoculation and the rate of its formation may be readily altered by many physiological conditions. The evidence at present available suggests that an enzyme-controlled biochemical synthesis of pisatin is involved and that it is unlikely that naturally occurring compounds present in uninfected plant tissues are merely chemically degraded by hydrolysis or oxidation during infection.

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