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THESIS

A HISTOCHEMICAL STUDY OF *ERYSIPHE GRAMINIS HORDEI* EL. MARCHAL ON BARLEY WITH PARTICULAR REFERENCE TO PENETRATION AND PHOSPHATASE ACTIVITY OF THE HAUSTORIA

Erysiphe graminis hordei El. Marchal is an obligate ectoparasite of barley. The germ tubes of this fungus penetrate the epidermis of the host and haustoria, which are specialized absorbing organs, are formed in the epidermal cells.

Histochemical techniques have been utilized only occasionally in the study of host-parasite relationships and this research represents, in large measure, a new approach to the physiological study of parasitism. Specifically, two points were investigated. These were the alteration of the cell wall accompanying direct penetration by *Erysiphe* and the phosphatase activity of the haustoria.

The cell wall alterations were shown, for the first time, to be the result of interaction between the fungus and its host since radioactive carbon (C^{14}) supplied to the host as glucose was localized about the points of penetration. The germ tubes of the fungus as well as the haustoria were found to be rich in acid phosphatase as were the germinated spores of another obligate plant parasite, *Puccinia graminis tritici* Eriks. and Henn. Furthermore, the phosphatase of this latter parasite was shown to possess transferase as well as hydrolase activity.

It has been postulated that phosphatases play a role in the transport of solutes across cell membranes and the possibility was discussed that the phosphatase associated with the haustoria of obligate plant parasites is related to the absorbing function of these organs.

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A HISTOCHEMICAL STUDY OF ERYSIPHE GRAMINIS HORDEI EL. MARCHAL ON BARLEY WITH PARTICULAR REFERENCE TO PENETRATION AND THE PHOSPHATASE ACTIVITY OF THE HAUSTORIA

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by

Thomas Grisedale Atkinson

Saskatoon, Saskatchewan

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LIST OF ABBREVIATIONS

Bios	Bios Laboratories Incorporated
C.	Centigrade
cm.	centimetre(s)
F.C.	foot candle(s)
g.	gram(s)
Harleco.	Hartman-Leddon Company
hr.	hour(s)
M	molar
mg.	milligram(s)
min.	minute(s)
ml.	millilitre(s)
mμ.	millimicron(s)
N	normal
N. B. Co.	Nutritional Biochemicals Corporation
p.p.m.	parts per million
qt.	quart
Will Corp.	Will Corporation
w/v	weight per volume
μc.	microcurie(s)
μg.	microgram(s)
μl.	microlitre(s)
μ moles	micromoles

INTRODUCTION

New and highly refined physiological techniques have largely replaced the descriptive methods of classical cytology originally used in the investigation of host-parasite relations (Shaw and Samborski, 1956; Samborski and Shaw, 1956). Physiological as contrasted to cytological methods, however, usually do not permit distinction between the members of the host-parasite complex or detection of host reactions at the cellular level. With such methods, therefore, it is virtually impossible to investigate the vegetative phase of obligate parasites or to analyze host reactions when these are limited to one or a few cells as, for example, the initial changes following infection of susceptible hosts and the localized reactions of highly resistant hosts. The present research was initiated on the premise that certain aspects of host-parasite relations could best be investigated by histochemical methods since these provide a combined cytological-physiological approach.

The experimental work reported in this thesis falls naturally into three parts. Part I deals with histochemical studies on _Erysiphe graminis hordei_ El. Marchal and is divided into two sections. The first section, dealing with changes in the cell wall accompanying its direct penetration by the fungus, provides an introduction to the second section, which deals with the localization of acid phosphatase in the haustoria. The possibility that this enzyme might be associated with haustoria was investigated because phosphatases are thought to play some role in the active transport of glucose across cell membranes and haustoria are generally considered to be absorbing organs. This section is largely concerned with methods since, when these investigations were initiated, histochemical techniques for the demonstration of acid phosphatase were in the process of evolution. None was completely satisfactory; consequently, the validity of the findings had to be established by the use of several procedures.

Although phosphatase activity in association with haustoria was demonstrated by histochemical methods, similar techniques could not provide answers to the questions immediately raised by this discovery. It was of interest to know, for example, if the enzyme was localized on the surface of the haustorium and if it possessed, as reported for other phosphatases, transferase as well as hydrolase activity. The quantitative investigations of the phosphatase activity of germinated rust spores (*Puccinia graminis tritici* Eriks. and Henn.) described in Part II are an attempt to throw some light on these and related problems.

Finally, Part III deals with an unsuccessful attempt to control powdery mildew of barley by inhibiting the haustorial phosphatase of *E. graminis hordei* with molybdate supplied to the host.

Experimental considerations dictated the use of the obligate parasites *E. graminis hordei* and *P. graminis tritici* for different aspects of this research. *Erysiphe graminis hordei* was chosen for the histochemical work for several reasons. The haustoria of this fungus, unlike those of *P. graminis tritici*, are confined to the epidermal cells of the host. They are, therefore, easily located and their phosphatase activity easily demonstrated, not only because epidermal cells have a low level of the enzyme but also because stripping the epidermis is a simple means of obtaining suitable microscopic material. Furthermore, the use of epidermal strips rather than microtome sections avoided the enzyme inactivation caused by paraffin embedding.

On the other hand, spores of *P. graminis tritici* were used for the quantitative investigations of phosphatase activity because, unlike the conidia of *E. graminis hordei*, they can be collected in large numbers and stored in the refrigerator until needed.

Acid rather than alkaline phosphatase was studied because plant protoplasm is usually buffered in the acid range and Booth (1944) found that wheat phosphomonoesterase had a pH optimum of circa 5.0 with no detectable activity at pH 8 to 10.

LITERATURE REVIEW

Alterations In Host Cell Walls Caused By Directly Penetrating Fungi

Alterations in cell walls accompanying their direct penetration by fungi have often been reported.

Smith (1900), studying the development of *Erysiphe communis* on *Geranium maculatum*, noted that the first intimation of penetration was an increased staining of the inner surface of the wall directly under the point where the hypha contacted it. The wall thickened ahead of the penetration process, forming an outgrowth which, when eventually pierced, formed a collar about the neck of the mature haustorium. De Bary (1870) observed similar structures. The thickened area differed from the unaffected wall in that it stained little with safranin.

Surface views of *Erysiphe communis* on *Poa* and *Erysiphe Cichoracearum* on *Eupatorium* showed areas about the point of penetration which took no stain and which roughly corresponded with the thickened areas revealed in transverse sections. Similar, although slightly larger, colourless areas surrounded the penetration processes of *Erysiphe graminis* on *Poa pratensis* (Smith, 1900).

No conclusions as to the significance of the cell wall thickening were made, but Smith (1900) thought that it was an effort on the part of the host to protect itself since the thickening appeared to be more the result of the addition of new material to its inner surface than a mere swelling of existing cell wall substance.

Mackie (1928), studying _Erysiphe graminis_ on barley, mentioned nonstaining circular areas in the wall about infection threads which he supposed were the result of an enzymatic reaction. He considered that the outgrowth or swelling accompanying the penetration of the infection thread was formed by the host cell.

Corner (1935) studied _E. graminis tritici_, _E. graminis hordei_, _E. graminis agropyri_, _Podosphaera leucotricha_ and _Spaerotheca pannosa_ on susceptible and resistant varieties of their appropriate hosts as well as on a miscellany of inappropriate hosts. Alterations in the staining behaviour of the cell wall about points of penetration were observed in both susceptible and resistant varieties in all cases and on most inappropriate hosts.

He illustrated, in cross-sectional detail, the steps in the penetration of the host cell wall by _E. graminis_. Before there was any sign of the papilla, the name given by Corner (1935) to the local thickening of the wall preceding the penetration process, small circular areas of altered staining behaviour were evident beneath the tips of the germ tubes. These altered areas, which were distinctly swollen and which stained preferentially with certain stains but no longer reacted like cellulose, enlarged as the papilla developed.

Unstained epidermal strips of fern (*Polypodium* sp. and *Adiantum reniforme*) inoculated with *E. graminis* and *Sphaerotheca pannosa* showed bright yellow-brown autostained halos which corresponded with the altered areas of the wall revealed in other hosts by staining.

Corner (1935) did not believe that the infection papilla was formed by deposition from the host cell as a means of resistance against the parasite. Rather, since the methods he used showed the altered area to have a "bleary aspect and blurred outline" (Corner, 1935, p. 189), he considered the changes in the cellulose layer of the cell wall to be caused by a cytase diffusing from the penetration process. The increased thickness of the cell wall about the points of penetration by mildew was considered similar to the swelling reportedly caused by other directly penetrating fungi (*Botrytis cinerea*, Blackman and Welsford, 1916: *Colletotrichum lindemuthianum*, Dey, 1919; *Sclerotinia libertiana*, Boyle, 1921; sporidia of *Puccinia graminis* on barbary, Waterhouse, 1921). The infection papilla was, however, with the single exception of the bryophilous discomycete, Neotiella crozalsiana (Corner, 1929), considered characteristic of penetration by mildews.

Structures formed during the direct penetration of cell walls by other fungi and closely resembling Corner's infection papilla had, however, been previously reported.

Young (1926a) used the term "callosity" to designate the local additions to host cell walls that he described as accompanying the penetration of wheat coleoptiles and other plant tissues by several facultative parasites. He reviewed the literature on this subject, crediting Mangin (1899) with first describing these bodies. According to Young (1926b), Stevens (1922) had previously tentatively named these structures "calluses."

In addition to callosities, which Young believed to be laid down by the host, penetration by these facultative parasites was usually accompanied by a thickening and alteration in the staining behaviour of the cell wall. Some instances of autostained discs surrounding penetration points were also noted.

Although he did carry out some microchemical and staining tests, Young (1926a) considered them too incomplete to justify conclusions concerning the chemical nature of the callosities or the nature of the alterations of the cell walls around infection points.

Similar protuberances in cell walls were described by Fellows (1928) as accompanying the penetration of wheat roots by *Ophiobolus graminis*. He suggested the name "lignitubers" for these structures since microchemical tests indicated that they were composed chiefly of lignin. He stated that lignitubers did not invariably accompany penetrating hyphae.

Robertson (1932), pointing out that *O. graminis* possessed both macro- and microhyphae, found that lignitubers usually accompanied the penetration of cell walls by the former but not by the latter. He considered the protuberances to be ligninlike growth reactions of the cell wall in response to penetration or attempted penetration by macrohyphae.

Russel (1934) noted that, around points where *O. graminis* penetrated wheat coleoptiles, circular areas of the cell wall showed an increased staining with acid fuchsin and Haidenhain's haemotoxylin. He suggested this alteration was caused by some fungal enzyme or toxin. Russel referred to the lignitubers of Fellows (1928) as callosities and stated that they appeared to be laid down by the living host cell in an attempt to shut out invading hyphae.

It is therefore obvious that, contrary to Corner's (1935) statement, protuberances, or papillas, as he called them, accompany the direct penetration of cell walls by fungi other than the mildews.

Smith (1938) studied the penetration of clover leaves by *Erysiphe polygoni*. He described an apparent swelling of the subcuticular wall materials for some distance around each point of penetration and the formation of an ingrowth from the cell wall. This ingrowth appeared to Smith (1938) to be formed by the addition of new materials to the inner surface of the cell wall.

Cherewick (1944), Smith and Blair (1950), and, more recently, Lupton (1956) have observed alterations in the staining reaction of cell walls around points where *Erysiphe graminis* penetrated.

Cherewick (1944) reported that with aceto-carmine the wall about penetration points stained deeper red than the surrounding tissue. Smith and Blair (1950) found that, with cotton blue, stained halos appeared around the penetration points. Like Cherewick (1944), these latter workers inferred that a reaction, probably enzymic, occurred between the host cell and pathogen.

Lupton (1956) related resistance to penetration with the formation of cotton blue halos about points where the germ tubes of *E. graminis tritici* passed through the cuticle of the host. He decided that the changed staining, accompanied by a thickening of the cell wall, indicated unsuccessful penetration of resistant hosts and, under conditions unfavourable for infection, even susceptible ones. Occasionally, however, these modifications of the cell wall were associated with successful penetrations.

Lupton (1956) suggested that the alterations in the wall, which he termed "bruising," were caused by an enzyme produced by either host or parasite or by the physical action of the fungus attempting to penetrate. He apparently looked on the thickening as a swelling which took up the stain. The halos, when occasionally associated with the successful penetration of susceptible hosts, indicated to him slowness in penetration.

It should be pointed out that Lupton (1956) had difficulty in obtaining consistent penetration of his susceptible hosts and that differences in behaviour were frequently observed in different parts of the same leaf. Furthermore, Corner (1935) not only found cotton blue an unreliable stain for detecting cell wall changes but also illustrated cell wall thickening and papilla formation accompanying successful penetration by *Erysiphe graminis*.

Despite the fact that it was claimed to be the exception, the only figure that Lupton (1956) presented illustrating successful penetration in transverse section definitely showed a cell wall thickening like Corner's (1935) infection papilla.

Nutrition Of Obligate Parasites

Absorption of metabolites by haustoria

Visiani, studying the mildew of grapes, was probably the first, according to von Mohl (1853), to see haustoria. He compared them to the roots of higher plants, which suggests that he considered the haustoria to be feeding organs. De Bary (1870), who was the first to study the structure of haustoria, defined them (de Bary, 1887) as special organs of attachment and suction. He noted that the haustoria of *E. graminis* were peculiarly branched. Smith (1900) considered that the elaborate, digitately-branched haustoria which he described for this species were a form of absorbing organ developed as a result of a special effort to obtain abundant food.

Further statements pointing out that the haustorium is usually considered to be a feeding organ have been made by Salmon (1905), Latham (1934), Pady (1935), and Wang (1949), to name but a few.

The view, originally expressed by Lutman (1910), that the haustorium is an organ developed to overcome difficult conditions (haustoria rarely being found in regions of easy feeding) has recently found support in the work of Fraymouth (1956). She concluded from her study of haustoria of the Peronosporales that haustoria were not essential to all these parasites since they were not developed if sufficient food was supplied to the hyphae by diffusion from the host cells. Furthermore, in certain species she found some correlation between haustorial form and the supply of food, simple haustoria being found in regions where it was plentiful, more elaborate ones, presenting a greater surface, where it was in short supply. Rice (1935) regarded elaboration of haustorial form as evidence of a high degree of adaptation by the parasite in securing maximum contact with the host protoplasm. The haustoria of *Erysiphe graminis* were considered among the most highly developed forms.

Invagination without actual invasion of the host protoplast has been a generally-accepted character of the haustorium (Rice, 1935; Arthur, 1929; Corner, 1935). Thatcher (1943), however, was the first to actually demonstrate, by plasmolytic methods, that this was so, at least for the haustoria of *Puccinia graminis tritici*. Recently, Fraymouth (1956) has similarly demonstrated the invagination of the host protoplast by haustoria of the Peronosporales.

Mechanism of nutrient uptake by haustoria

Little is known of how the haustoria of obligate plant parasites absorb nutrients from the host protoplasts which they invaginate. So far as the writer is aware, Thatcher (1939, 1942, 1943) is the only worker who has sought to investigate this problem. He found that obligate plant parasites not only had a higher osmotic pressure than that of their respective hosts but also that they induced an increase in the permeability of susceptible host cells. On the basis of these findings, Thatcher (1939) proposed that the fungus was able to withdraw water from host cells by virtue of its greater osmotic pressure while cell solutes were made available to it as a result of the induced increase in plasma membrane permeability.

Thatcher seemed to believe that these solutes moved into the fungus by passive diffusion across the haustorial membrane. From what is now known of solute transport across other cell membranes however (see later in this review), it is likely that enzymes localized in or on the surface of the membrane play a role.

Specific metabolites required

The nutritional requirements of obligate plant parasites are largely unknown because of the difficulty of distinguishing between their metabolism and that of their hosts and because, with the exception of a few questionable claims (Ray, 1901; Gretchushnikov, 1936; Hotson and Cutter, 1951), it has proved impossible to culture these fungi on artificial media. It is not surprising, therefore, that the literature on this subject is speculative, based largely on studies of the conditions favourable to the development of the parasite and the altered metabolism of infected host tissue. Allen (1954) recently concluded that the nutrient contributions of the host are still largely unknown.

Although, as Allen (1954) stated, the need for a plentiful supply of host carbohydrate for development of obligate parasites is well established, there is, as Trelease and Trelease (1929) pointed out, insufficient information available to show whether it is the carbohydrates themselves or other substances, for example, proteins, whose formation is dependent on carbohydrate, which are utilized by the fungus.

Humphrey and Dufrenoy (1944), studying, with the aid of a rather crude histochemical procedure, the distribution of phosphorus in oat tissues parasitized by Puccinia coronata, found that cells of a susceptible host apparently released phosphorus compounds into the intercellular spaces. They not only suggested that these compounds were remetabolized by the rust hyphae but also that phosphorylated sugars were translocated into the infection court from more remote tissues. Upon degradation of these sugar phosphates the phosphorus supposedly was consumed by the rust while the carbon residues were deposited in the form of glucose, sucrose, or starch. Starch accumulation about rust infections (Allen, 1926; Shaw, Brown, and Rudd-Jones, 1954) and increases in the total starch, reducing sugars and sucrose during the first week following infection with mildew (Allen, 1942), have been reported. Moreover, the accumulation of carbohydrates in the latter instance is accompanied by a slight but definite increase in inorganic phosphate without, however, concomitant increases in labile or ester phosphorus (Allen, 1953). Allen (1953) suggested that mildew toxins act to accelerate the release of inorganic phosphorus and prevent it being tied up in organic form. In the writer's opinion, Humphrey and Dufrenoy's (1944) work was not critical enough to warrant their speculations.

Gottlieb and Garner (1946) fed radioactive phosphorus (P^{32}) as phosphate to intact wheat plants and quantitatively determined the phosphorus distribution in healthy and rust-infected leaves. Although no significant difference in the total content of P^{32} was found between entire rusted and healthy leaves, over twice as much phosphorus was localized in the infected portion of inoculated leaves as in similar parts of healthy leaves. Radioautographs showed the accumulation to be localized about infection courts. That the rust metabolized some form of radioactive phosphorus was evident from the fact that the spores were radioactive. However, since the specific activity of the spores was less than that of noninoculated leaves, the accumulated P^{32} must have been localized either in the fungus mycelium or in the host cells. Results of similar experiments by Shaw, Brown, and Rudd-Jones (1954), in which the mycelium of the ectoparasite Erysiphe graminis was removed prior to radioautography, suggested that the bulk of the accumulated activity was in the host. Gottlieb and Garner (1946) suggested that the augmented phosphorus supply, paralleling as it did the reported accumulation of carbohydrates (Allen, 1942), might permit a greater activity of the phosphorylating enzymes of intermediary sugar metabolism.

Shaw and Samborski (1956) have recently reported the accumulation of radioactivity at uredial and conidial colonies of the obligate parasites *Puccinia* and *Erysiphe*, respectively, following the feeding of various radioactive carbon compounds, phosphorus and calcium. They pointed out, however, that it is not valid to assume that all compounds accumulating at the site of infection are metabolized by the parasite.

Cutter (1951) has recently speculated at length on the nutritional requirements of obligate plant parasites. Referring first to what he considered to be the only serious report of the successful culture of an obligate parasite (*Gymnosporangium juniperi-virginianae*) upon synthetic media (Hotson and Cutter, 1951), he mentioned that, with the exception of biotin, the four isolates obtained seemed to have no special nutrient requirements. These authors considered that the extreme rarity of accomplishing this transformation with identical material suggested the selection of a mutant. The fact that the transformation was preceded by a period during which the rust became pathogenic and destroyed the host tissue culture (*Juniperus-virginiana*) on which it had been growing further indicated such a possibility. The validity of

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this claim is thrown into doubt by the fact that Hotson (1953) in a subsequent paper, reporting the behaviour of this same rust on tissue cultures, did not make any reference to the occurrence of saprophytic growth and did not mention the original claim. Furthermore, Turel (1956) has reported that she has been unable to find any sign of saprophytic growth of mycelium in over 1,000 tissue cultures of *Gymnosporangium* sp.

Neglecting evidence to the contrary, Cutter cited reports which to him suggested the light-conditioned synthesis in green plant cells of an unstable accessory compound required by rusts. His own failure to infect albino corn (*Zea mays*) and the chlorophyll-deficient areas of variegated *Geranium maculatum* with their appropriate rusts was presented as evidence that the infection factor is associated with the presence of chlorophyll. He failed to mention other reports of the successful growth of rusts on albino corn (Mains, 1926) and barley (Wu, 1952) and of mildew (*Podosphaera oxycantha*) on white leaves of hawthorne (Grainger, 1947). Allen (1954) suggested that the supply of carbohydrate may have been the limiting factor in Cutter's experiments although he admitted that something besides carbohydrate may have been lacking.

Referring next to the report by Allen and Goddard

(1938) of the fungus-induced increase in respiration of wheat leaves infected with Erysiphe graminis, Cutter, though admitting that the nature of the responsible compound was still hypothetical, presumed it to be a phosphorylase secreted by the fungus. Cutter also credited this hypothetical phosphorylase with accomplishing the degradation of phosphorylated sugars which Humphrey and Dufrenoy (1944) postulated accumulated about the rust mycelium. It has already been pointed out, however, that Allen (1953) found no detectable increase in labile or ester phosphorus in mildew-infected leaves.

The reports (Hurd-Karrer and Rodenhiser, 1947; Rodenhiser and Hurd-Karrer, 1947) that germ tubes of cereal rusts formed structures resembling appressoria and substomatal vesicles on media containing high concentrations of hexose and inorganic phosphate suggested to Cutter a slight ability of these rusts to synthesize their key requirements (apparently hexose phosphate). This report together with the finding that adenine or adenylic acid stimulated the growth and sporulation of the Gymnosporangium isolates led Cutter to entertain the possibility that rusts require phosphorylated esters. Here, Cutter's terminology is not correct since he should have referred to phosphate esters, not phosphorylated esters.

In Savile's report (1939) that the host nucleus stains like the parasite cytoplasm while the rust nucleus reacts like the host cytoplasm and nucleoli, Cutter saw the possibility that host nucleic acid or its degradation products may be exerting an influence on the rust. Further, Yarwood's (1951) report of increased invasiveness and infectivity of several plant viruses on rust-infected hosts led Cutter to speculate that this was due to the liberation or accumulation of phosphorylated nucleotides.

Accepting the possibility that the key metabolites required by rusts were phosphate esters or sugars, Cutter was confronted with the problem of getting these poorly diffusible compounds into the rust mycelium. For this purpose, he drew both on the report by Rice (1927) that the haustorial membrane in rusts is much less differentiated than the membranes surrounding the remainder of the mycelial cells and Thatcher's (1939) hypothesis already outlined.

Cutter linked the light-conditioned synthesis in green plant cells of an unstable accessory compound required by the rust with phosphate esters by reference to the report by Emerson, Stauffer, and Umbreit (1944) that the absorption of light energy by chlorophyll results in the formation of energy-rich phosphate.

Cutter concluded by suggesting that the major difference between obligate parasites and other facultative parasites might reside in the inability of the rusts to synthesize the necessary energy-rich intermediates with which to carry out the phosphorylation of simple carbohydrates. In the writer's opinion, Cutter's highly speculative article gives no sound basis for this conclusion.

Enzymatic Processes In Cell Membrane Penetration

At one time the cell membrane was considered to be a passive structure allowing solutes to penetrate according to the properties of their molecules, such as size, membrane solubility, and electric charge. More recently, however, it has become evident that enzymes localized in the membrane participate in the transport of many substances.

Rosenberg and Wilbrandt (1952), in a recent review of enzymatic processes in cell membrane penetration, discuss the general principles and possible mechanisms underlying this activity. They confine their attention mainly to the role of surface enzymes in glucose transport.

Reviewing the experimental evidence indicating that phosphorylation processes play a role in the uptake of glucose by muscle cell as well as in other instances, Rosenberg and Wilbrandt (1952) point out that none of the known orthophosphate esters of hexose could be the transport form since, according to general experience, these are unable to penetrate cell membranes. This dilemma was solved (Rosenberg, 1950) by postulating the formation, on the outside surface of the membrane, of a glucose metaphosphate ester which, at least in the case of muscle, would be hydrated to glucose orthophosphate after passage into the cell.

The mediation of surface enzymes in glucose uptake by yeast was indicated by the findings of Barron, Muntz, and Gasvoda (1948) and Rothstein and coworkers (1948, 1949, 1951) that nonpenetrating uranyl salts inhibited the fermentation and oxidation of this sugar. Rothstein and Meier (1951) and Rothstein *et al.* (1951) came to the conclusion that the inhibition resulted from the complexing of uranyl ions with cell surface compounds similar to polymetaphosphates. Rosenberg and Wilbrandt (1952) reinterpreted the data and suggested that although the complexing groups on the yeast cell apparently belonged to the class of phosphates their exact nature could not be decided. Considering other evidence, they thought that the inhibition by uranyl ions might be due to complex formation with adenosine triphosphate which, with hexokinase, could be involved in the process of glucose uptake.

Although the literature reviewed indicated that, beyond doubt, glucose penetration into human red cells was an enzymically controlled process, Rosenberg and Wilbrandt (1952) concluded that too little was known to make definite assumptions concerning the enzymes and reactions involved. However, the fact that several inhibitors affecting the rate of glucose penetration were inhibitors of enzymes in some way related to reactions involving phosphate again pointed to a possible role of phosphorylations.

The complexity of the mechanisms involved was indicated by the fact that the enzymatic pathways for glucose entrance and exit apparently differed. Phosphorylated phloretin, a substance related to phloridzin and incapable of penetrating the membrane, showed virtually no inhibition of glucose penetration from outside to inside but markedly inhibited the outward passage. Phloridzin also showed immediate strong inhibition of glucose exit whereas entrance was inhibited less strongly. This inhibition increased with time, however, apparently reflecting inward penetration of phloridzin. Rosenberg and Wilbrandt (1952) pointed out that the phloridzin group seems to affect the dissociation of a glucose-membrane carrier complex on both sides of the membrane. This point will be referred to later in connection with the reabsorption of glucose by kidney and intestine epithelia.

Rosenberg and Wilbrandt (1952) considered glucose reabsorption by the epithelial cells of both kidney and intestine to be accomplished by the same mechanism. Both of these organs are characterized histologically by brush borders on the lumen side of the glucose-absorbing epithelia, and, furthermore, these brush borders have been shown to be extremely rich in alkaline phosphatase activity. Phloridzin has been found to inhibit glucose reabsorption in both of these organs.

In considering the nature of the enzymes participating in glucose reabsorption, Rosenberg and Wilbrandt (1952) referred to the experiments with erythrocytes in which phloridzin apparently inhibited an enzyme catalyzing the dissociation of glucose-carrier complex. In addition to the general parallelism between glucose uptake by red cells and epithelia suggesting a priori a similar action of phloridzin on the kidney and intestine, they pointed out that experimental evidence also supports this view. They were led to conclude that the intracellular effect of phloridzin on the dissociation of a substrate-carrier complex, formed presumably by some sort of phosphorylation, must be on a dephosphorylating enzyme. Supporting this view was the finding that phloridzin inhibited phosphatase.

Histochemical investigations, it was also pointed out, indicated in a very impressive way that phosphatases were involved in the transport of glucose. Not only did the distribution of alkaline phosphatase coincide with the regions of sugar absorption, but also the histochemical localizations showed the bulk of the enzyme activity concentrated on the lumen side of the epithelial cells.

Rosenberg and Wilbrandt (1952) suggested the formation of a metaphosphoric ester as a primary step common to all five examples of enzymic transport that they discussed: muscle, yeast cell, erythrocyte, kidney and intestine cell. Subsequent reactions would depend, they said, on the enzymatic conditions in the different cells. It was suggested that dissociation of the substrate-carrier complex was accomplished by transphosphorylation rather than by hydrolysis. The phosphatase-mediated transfer of phosphate from an ester-phosphate substrate to some suitable alcoholic acceptor was originally described by Axelrod (1948) and later confirmed by Morton (1953), who used electrophoretically pure enzyme.

Recently, Chiquoine (1955) has postulated, on the basis of histochemical studies, that a specific glucose-6-phosphatase is responsible for the dephosphorylation which occurs during the passage of glucose through kidney and intestinal epithelia. Although in the introduction to his paper Chiquoine (1955) refers to the role of glucose-6-phosphatase in the transport of glucose across cell membranes, it is evident from his discussion that he is not considering enzymatic processes in cell membrane penetration, but, rather, he is referring to the views of Lundsgaard (1935) and Kalckar (1937) that glucose, after passing unchanged through the membranes of the epithelial cells, is phosphorylated at the free end and dephosphorylated at the basal end of the cell. Rosenberg and Wilbrandt (1952) critically reviewed and rejected this idea of a cytoplasmic rather than a membrane-carrier system.

Rosenberg and Wilbrandt (1952) made no reference to the fact that molybdate inhibition of the surface phosphatases of yeast (Rothstein and Meier, 1948, 1949) had no significant effect on the carbohydrate metabolism of this organism. These surface phosphatases appeared to have no role other than hydrolyzing nonpenetrating organic phosphates in the medium, thereby enabling the yeast to utilize them. Possibly glucose uptake was not affected because the molybdate did not inhibit intracellular phosphatases. However, it is equally possible that phosphatases, despite the suggestive evidence, play no role in sugar transport. Keston (1954), for example, has proposed that glucose reabsorption by the kidney is mediated by the enzyme mutarotase. He considered the phosphorylation theory of glucose reabsorption defective in that too high concentrations of phloridzin are required to inhibit kidney phosphatases. Nevertheless, Rosenberg and Wilbrandt (1952) mentioned that Marsh and Drabkin (1947) found considerable inhibition of alkaline phosphatase in undiluted kidney homogenates of phloridzin poisoned animals.

Enzyme Histochemistry

Enzyme histochemistry, which has, as described in the preceding section, provided such strong presumptive evidence for the participation of phosphatases in glucose reabsorption by the kidney and intestine, is a branch of what is the youngest and at the same time one of the oldest of the biological sciences. Although both Baker (1943) and Pearse (1951, 1953) have pointed out that histochemistry had its origin in the works of Raspail (1825a, 1825b, 1829, 1830, 1833, 1834), whom they consider its founder, it was Lison's (1936) Histochimie Animale which heralded the rebirth and modern development of this science. Indeed, Gomori (1952) considered Lison to be the founder of histochemistry.

Histochemistry could be defined as the chemistry of tissues and cytochemistry as the chemistry of cells (Pearse, 1951; Stowell, 1952). However, the two terms have often been used synonymously. Both Gomori (1952) and Pearse (1951) restrict their use of the term histochemistry to those methods which use microscopic examination to reveal the results of chemical reactions carried out on structurally unaltered tissues. Histochemistry in this restricted sense seeks to identify and localize chemical substances in the tissues, that is, in situ. Gomori (1952) reserved the term cytochemistry for the study of the chemical organization of the cell in general, including those methods in which structure was destroyed or disorganized, as, for example, by differential centrifugation of tissue homogenates. It was the destructive nature of many of the earlier histochemical methods which led Lison (1936) to regard the nondestructive science he introduced as new.

Histochemistry, especially in the last ten years, has undergone a rapid development which is best reflected by the number of recent books and periodicals, exclusive of review and other articles, devoted to this subject. Glick's (1949) *Techniques of Histo- and Cytochemistry* was the first major work to appear following that of Lison's (1936) *Histochimie Animale*. Three years later, Gomori (1952) presented his rather brief treatise *Microscopic Histochemistry*. In 1953, Danielli published his small volume, *Cytochemistry*, in which he maintained that cytochemistry was so recent that anything in the nature of a textbook was premature. In the same year, a completely rewritten second edition of Lison's book appeared under the new title *Histochimie et Cytochimie Animale*. The most comprehensive treatment of the subject to date is Pearse's (1953) *Histochemistry*.

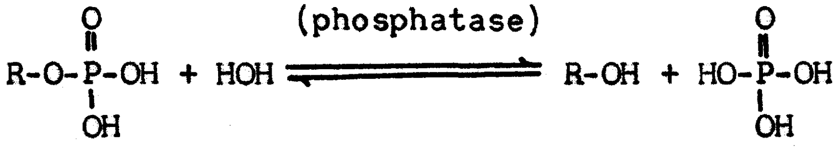
The increasing number of articles devoted to histochemistry was acknowledged by the editors of *Stain Technology* in 1952, when they changed its subtitle from *A Journal of Microtechnique* to *A Journal of*

Microtechnique and Histochemistry. The following year, the newly-formed Histochemical Society issued the first volume of its official publication, The Journal of Histochemistry and Cytochemistry.

Pearse (1953) pointed out that, in its origin, histochemistry was primarily botanical; for some decades, the whole practice of histochemistry in its true sense was in the hands of the botanists. Such is not true of modern histochemistry as should be evident from the fact that both Pearse and Gomori are medical doctors while Glick and Lison are associated with medical colleges. Indeed, Rawlins and Takahashi (1952) pointed out in the preface to their volume, Technics of Plant Histochemistry and Virology, that plant histochemical techniques have been somewhat neglected in America. Their handbook and a review article on the localization of enzymes by Van Fleet (1952) are the only compilations of literature devoted specifically to plant histochemistry of which the writer is aware.

The following review of histochemical literature will be confined to tracing the development of methods currently used for localizing nonspecific acid phosphatase. To do this requires reference to histochemical methods for alkaline phosphatase from which the methods for the acid enzyme have evolved.

Phosphatases, enzymes catalyzing the hydrolysis of phosphate esters, may be divided into three types, mono-, di- and triphosphatases. Histochemistry, however, is usually only concerned with the phosphomonoesterases which exhibit group but usually no absolute specificity. Thus R, the alcoholic moiety of a phosphate ester in the equation below, may be any one of a number of alkyl or aryl radicals.



$$R\text{-}O\text{-}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}\text{-}OH + HOH \xrightleftharpoons{\text{(phosphatase)}} R\text{-}OH + HO\text{-}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}\text{-}OH$$

On the basis of their pH optima *in vitro*, phosphomonoesterases may be divided into alkaline (optimum pH circa 9) and acid (optimum pH circa 5) phosphatases. Histochemical methods for the localization of these enzymes have been developed which depend on the precipitation *in situ* of either their alcoholic or phosphate reaction products.

Modern enzyme histochemistry had its origin when Gomori (1939) and Takamatsu (1939) simultaneously and independently published the same method for localizing alkaline phosphatase. Takamatsu's (1938) earlier publication in an obscure Japanese journal was overlooked by

Western readers.

Their method was based on the principle that an enzyme may be localized by precipitating a product of its activity at the site of formation. Tissue sections were incubated with a suitable phosphate ester at circa pH 9.0 in the presence of calcium ions. As phosphate was enzymically liberated it was precipitated as calcium phosphate, which, being white, could not be detected under the microscope. The site of enzyme activity was rendered visible by transforming the precipitate into silver phosphate, which yielded black metallic silver on photoreduction. Later, Gomori (1941a) developed a cobalt sulphide method which is now widely used for the same purpose. The initial precipitate of calcium phosphate is transformed into black cobalt sulphide via the formation of cobalt phosphate.

Phosphatases may be localized by precipitating the alcoholic, rather than the phosphate, reaction product. This principle was introduced into enzyme histochemistry by Menten, Junge, and Green (1944), who developed the first azo dye method for alkaline phosphatase. Essentially, their procedure consisted in precipitating, and at the same time rendering detectable as a coloured azo dye, the organic moiety of a suitable monoaryl phosphate ester by causing it to couple with diazotized amine immediately upon its enzymic release. Menten et al. (1944) coupled β-naphthol released from calcium β-naphthyl phosphate with diazotized α-naphthylamine. Their method was, however, unsatisfactory for routine use because the diazotized amine was so unstable that it had to be prepared prior to each test and the incubation carried out at low (4 to 6° C.) temperatures.

Manheimer and Seligman (1948) overcame this difficulty by introducing the use of stabilized diazonium salts. Gomori (1951) further improved the method by using not only the more soluble sodium salt but also the alpha, rather than the beta, isomer of the substrate. This enabled the substrate concentration to be increased to its optimum, and, furthermore, decreased the diffusion of the final reaction product since the azo dyes formed with α-naphthol were more insoluble than those formed with β-naphthol.

Further improvements in the azo dye method for alkaline phosphatase by using different stable diazotates and by otherwise modifying the coupling reaction have been reported by Grogg and Pearse (1952a), Pearse (1953), and Burton (1954a).

Gomori (1941b) developed a method for acid phosphatase similar in principle to that which he used for alkaline phosphatase. However, since calcium phosphate was completely soluble at the pH optimum for the enzyme (circa pH 5.0), lead rather than calcium ions were included in the incubation mixture to precipitate the enzymically released phosphate. The white lead phosphate was converted into dark brown lead sulphide with ammonium sulphide.

The many reports (see Pearse, 1953; Gomori, 1950, 1952) complaining of the capriciousness of his technique prompted Gomori (1950) to investigate the reasons for this and to attempt to improve the reliability of the method. He found that the ratio of buffer/substrate was important in obtaining uniform results, yet occasional inexplicable failures still occurred (Gomori, 1952).

Yin (1945), in order to avoid interference from preformed calcium and other insoluble salts, used Menten, Junge, and Green's (1944) azo dye technique at pH 6.0 rather than Gomori's (1939) method to study the distribution of acid phosphatase in plant tissues. Glick (1949) pointed out that this was unnecessary since preformed phosphates could be removed by treatment with citrate buffer.

Seligman and Manheimer (1949) made the first serious attempt to utilize the principle of azo coupling for the histochemical demonstration of acid phosphatase. They used calcium α-naphthyl phosphate as substrate and diazotized 1-amino anthraquinone as coupling agent. Because of the low solubility of the substrate, long incubations (up to 24 hr.) were required and gross diffusion artifacts resulted (Gomori, 1952). Pearse (1953) stated that, at best, results obtained with this method compared unfavourably with the results of the Gomori (1950) method. Grogg and Pearse (1952b) considerably improved the azo dye method by using as substrate the highly soluble sodium salt of α-naphthyl phosphate and as coupling agent, tetrazotized o-dianisidine. Frozen, rather than paraffin-embedded, sections were employed to prevent undue destruction of enzyme.

Burton (1954b) sought to prevent the diffusion artifacts which he claimed, and Pearse (1954) admitted, were evident with the method of Grogg and Pearse (1952b). He pointed out that although azo coupling occurred between pH 5.0 to 9.0, it was slowest in the acid range. He claimed that artifact as a result of diffusion of released naphthol occurred when the enzymic rate of hydrolysis of substrate surpassed the rate of the coupling reaction. To prevent this he suggested accelerating the coupling reaction by incubating at pH 5.5 to 6.0 rather than at the enzyme optimum of pH 5.0, at 37° C. rather than 20° C., by increasing the concentration of tetrazotized o-dianisidine and by using percain as a catalyst. He also advised controlling the enzymic release of naphthol by limiting the substrate concentration.

Rutenburg and Seligman (1955) recently published a postincubation azo coupling technique for demonstrating acid phosphatase. Incubation with 6-benzoyl-2-naphthyl phosphate is carried out at the pH optimum of the enzyme. The released naphthol is insoluble and remains at the site of its formation during the incubation period following which coupling with tetrazotized o-dianisidine is rapidly accomplished at a higher pH.

Pearse (1953) pointed out in the preface to his text that histochemistry can transform the descriptive biological sciences, such as histology and pathology, into dynamic and functional sciences complementary to physiology and biochemistry. Stowell (1952) also stated that the application of histochemistry could aid research in medical pathology. They both stated, however, that besides an increased awareness of the advantages histochemistry had to offer, an appreciation of the limitations of individual methods was required.

MATERIAL AND GENERAL METHODS

Plant Material

Atlas and Atlas 46, the two varieties of barley (*Hordeum vulgare* L.) chosen for this investigation, were genetically similar (Schaller, 1951). Atlas was susceptible while Atlas 46 was resistant to the race of powdery mildew (*Erysiphe graminis hordei*) used. Unless otherwise stated, seed was sown in soil and the plants grown either in the greenhouse or, when isolation was required, in an environment chamber.

Mildew Inoculum

Erysiphe graminis was maintained on Atlas seedlings inoculated simply by brushing heavily sporulating plants over them. The disease reactions it produced on Atlas, Atlas 46 and the differential hosts recommended by Cherewick (1944) are given in Table I. The infection types were scored by two independent observers nine days after the seedlings had been inoculated. The reactions to races 3 and 4 included in the table are those reported by

Table I

A Comparison of the Infection Types* Produced on Various Barley Varieties by the Physiologic Race of *E. graminis hordei* used in this Investigation and by Races 3 and 4

Barley variety	Observer 1	Observer 2	Newton and Cherewick Race 3	Newton and Cherewick Race 4
Chevron	;	1	0-1	0-;
Peruvian	2	2	3-4	1
Heil's Hanna	4	4	4	3-4
Nepal	4	4	4	4
Goldfoil	0-;	;	0	0-;
Black Hull-less	4	4	3	4
Atlas	4	4	4	1-
Atlas 46	;	;	not listed	

*Explanation of symbols (Newton and Cherewick, 1947):

0--immune, no visible signs of infection.
;--highly resistant, chlorotic or necrotic flecks present with no visible development of mycelium.
1--highly resistant, with a slight development of mycelium.
2--moderately resistant, moderate development of mycelium with a slight production of conidia. Chlorotic or necrotic spots present.
3--moderately susceptible, moderate to abundant development of mycelium accompanied by moderate sporulation. Chlorotic or necrotic spots may be present.
4--very susceptible, large pustules abundantly sporulating. No necrotic spots.
(+) and (-) signs indicate quantitative increase or decrease in the infection type.

Newton and Cherewick (1947).

According to Newton and Cherewick's (1947) key, the reactions of the differential hosts eliminated all races except 3 and 4. Although the reaction on Peruvian, critical for this distinction, was not decisive, the mildew was assumed to be race 3 from its behaviour on Atlas.

Rust Spores

Spores of stem rust (*Puccinia graminis tritici*) were collected, unless stated otherwise, from Atlas 46 plants grown in the greenhouse. These plants were inoculated with race 15-B at about the shot-leaf stage by injecting a heavy spore suspension between the stem and leaf sheath. After collection, the spores were passed through a 100-mesh sieve and stored in stoppered vials in the refrigerator.

Procedure For Obtaining And Handling Epidermal Strips

Epidermal strips from *young*, *first* leaves of barley were obtained by the following procedure. A detached leaf was held tautly, abaxial surface uppermost, over the first and second fingers of one hand. Close to the basal end, which was held between the second and third fingers, a tablike piece was raised in the middle of the leaf with forceps and bent over sharply to snap the brittle adaxial epidermis. The abaxial epidermal layer, stripped by pulling slowly on the tab, was floated, cutinized surface uppermost, on a tray of water as it was formed.

Neutral red staining of the strips thus obtained showed that all but the guard and accessory cells were usually ruptured (Fig. 1). On the basis of this criterion, however, haustoria of E. graminis in infected strips were intact (Fig. 2).

To facilitate handling during histochemical processing, the strips were mounted on microscope slides. A slide, brought up carefully under the floating strip, was gradually lifted so that the epidermal layer stretched out flat along its length. After removing excess water and tissue overhanging the ends of the slide, the strip was fastened in place by dipping each end into about one inch of warm embedding wax (Fisher Tissuemat). At the right temperature, the wax hardened into a satisfactory seal almost as soon as the slide was withdrawn. However, if the wax was too hot or the slide held in it too long,

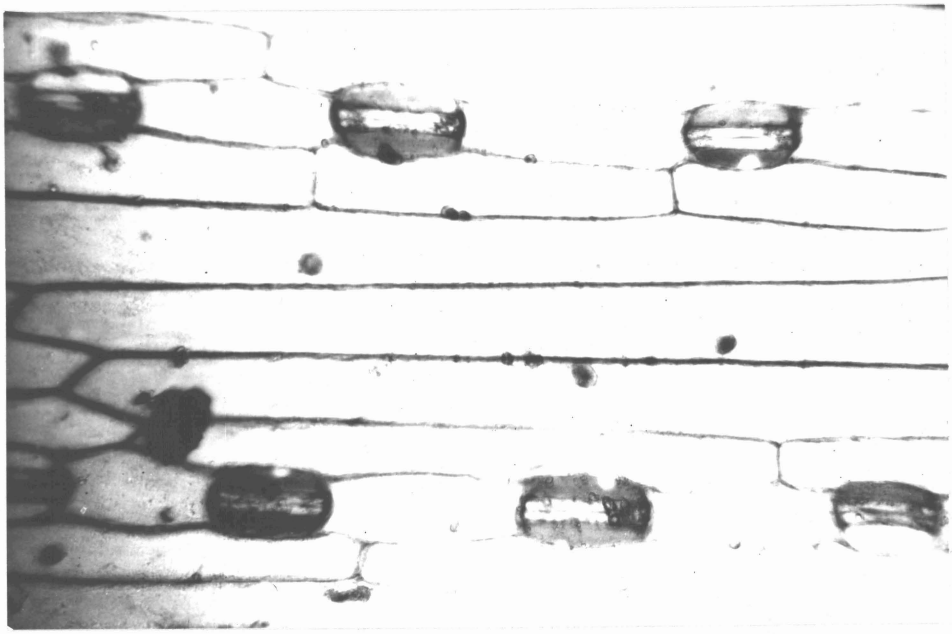


Fig. 1. Epidermal strip of barley showing the normal accumulation of neutral red dye only by accessory and guard cells.

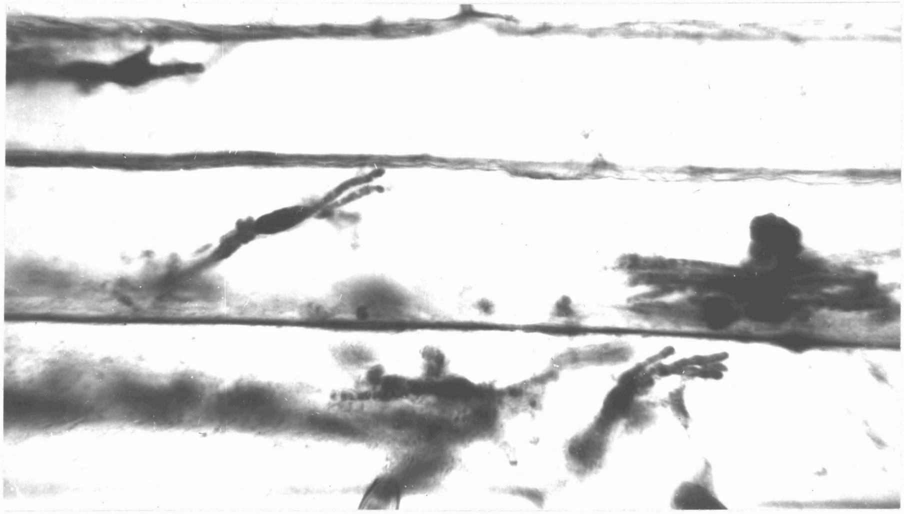


Fig. 2. Haustoria of E. graminis hordei showing accumulation of neutral red dye.

the film was thin and unsatisfactory.

Later, to prevent air pockets between strip and slide from resulting in improper exposure of tissue to reagents, strips were mounted with their inner, rather than cutinized, surface up. The only modification in the foregoing procedure necessary to obtain inverted strips was the addition of a little Aerosol to the tray of water. This lowered the surface tension and allowed strips to be floated cuticle surface down.

EXPERIMENTAL

Part I. Histochemical Studies On *Erysiphe graminis hordei*

A. Penetration

1. *Methods*

The following methods were used to study local changes in the cell wall accompanying penetration by *E. graminis*. All material, except that treated by the periodic acid-Schiff procedure, was first fixed in a mixture of 70% ethanol, formalin and glacial acetic acid (18:1:1 volumes, respectively). Carnoy's fluid (6:3:1 volumes of absolute alcohol, chloroform and glacial acetic acid, respectively) was usually used as fixative for periodic acid-Schiff-treated material although the other fixative was occasionally used with identical results. Strips were fixed for 2 hr., then either used or stored in 70% ethanol.

(a) Staining with acid and basic dye

One per cent (w/v) solutions of acid (Will Corp.) and basic (Harleco.) fuchsin in 70% ethanol were used. Strips immersed in the acid dye for 2 hr. were rinsed in 70%

ethanol, quickly dehydrated in 95% ethanol and mounted in Diaphane. Strips similarly treated with the basic dye were quickly rinsed with water and mounted in glycerine. Johansen (1940) listed the acid dye as a histological stain for cutinized and cellulose cell walls.

(b) Microchemical tests for cellulose

Two microchemical tests for cellulose were tried. The first method was used by Corner (1935) studying the same problem. Strips were soaked in an iodine-potassium iodide solution (1 g. of iodine and 3 g. of potassium iodide in 300 ml. of deionized water) for 4 to 5 min. and then transferred to 66% sulphuric acid for 5 to 10 min. After thoroughly washing in water, the strips were mounted in dilute glycerine. Corner (1935) reported that cellulose cell walls normally stained dark blue but that circular patches about penetration points were left unstained.

The second method was developed by Post and Laudermilk (1942), who reported that cellulose was stained various colours, depending on its source. Strips were treated for 30 sec. with an iodine-potassium iodide-glycerine solution (20 ml. of 2% iodine in 5% potassium iodide plus 180 ml. of distilled water and 0.5 ml. of glycerol), blotted dry with filter paper and mounted in a saturated aqueous solution of lithium chloride.

(c) Periodic acid-Schiff procedure for polysaccharides

Pearse's (1953) modification of a method originally proposed by Hotchkiss (1948) was followed except that a sulphite bath (Hotchkiss, 1948) was used after treatment with Schiff's reagent.

Strips were immersed for 5 min. in a periodic acid solution (0.4 g. of $HIO_4 \cdot 2H_2O$ in 35 ml. of ethanol, 5 ml. of 0.2 M sodium acetate and 10 ml. of distilled water), rinsed in 70% ethanol and then treated for 1 min. in a reducing bath (1 g. of potassium iodide and 1 g. of sodium thiosulphate in 30 ml. of ethanol and 20 ml. of distilled water to which 0.5 ml. of 2 N hydrochloric acid was added). Following a rinse in 70% ethanol, the strips were immersed for 20 min. in Schiff's solution (de Tomasi, 1936) and then, after a short rinse in a sulphite bath (0.5 ml. of concentrated hydrochloric acid and 2 ml. of 10% potassium metabisulphite in 50 ml. of deionized water), washed for 10 min. in running tap water. After dehydrating the strips in 95% ethanol, they were mounted in Diaphane. Glick (1949) reported that starches, cellulose, hemicelluloses and pectins were the chief substances in plant tissues that were stained by this method. More exactly, any compound having C-C bonds present as 1:2-glycol groups (which periodic acid oxidatively breaks) will give a reaction as long as it and its oxidation product do not diffuse away (Hotchkiss, 1948).

(d) Microradioautography

Epidermal strips from leaves fed radioactive (C^{14}) glucose were processed for microradioautography according to Maclachlan's (1954) procedure. Strips picked up on slides in the usual manner were placed between several layers of absorbent paper and pressed between weighted steel plates. Gentle heat from a small electric heater was applied to one of the plates for about 30 min. and the strips left undisturbed for at least 24 hr. In the darkroom, the prepared epidermal strips were securely fastened in apposition with the emulsion of nuclear track plates (Ilford). The plates, wrapped in lightproof paper, were then exposed in the refrigerator.

The exposed plates were developed (Ilford D-19 developer, diluted 1:3 with water) for 20 min., rinsed in 2% acetic acid, fixed (30% sodium thiosulphate) for twice the time necessary for clearing and, finally, washed in cold running water for 30 min. The steps preceding fixation were carried out under a Wratten Safelight No. 1 and the temperature of successive solutions was kept between 10 and 15° C. Local concentrations of C^{14} in the tissue were revealed as dark areas on developed plates.

2. Results

(a) Changes in the cell wall accompanying penetration

The cell wall about penetration points showed an altered reaction to all procedures used.

Circular patches about penetration points were stained more intensely than unaffected regions with acid fuchsin (Fig. 3) while with the basic dye this area remained unstained (Fig. 4).

As Corner (1935) reported, colourless or yellowish, circular patches appeared on a background of deep blue when the iodine-potassium iodide-sulphuric acid method was used. However, despite strict adherence to Corner's procedure, this method proved unsatisfactory for a careful study of the changes accompanying penetration because the epidermal strips soon disintegrated. The Post and Laudermilk (1942) microchemical test for cellulose was much more satisfactory for this purpose.

The altered reaction of the cell wall about penetration points revealed when this procedure was used is shown in Fig. 5. The contrast between the light blue reaction of native cellulose and the unstained penetration areas was increased by using yellow light. Small, unstained circles were the first evidence of change in the cell wall. As these circles enlarged, a stained halo formed

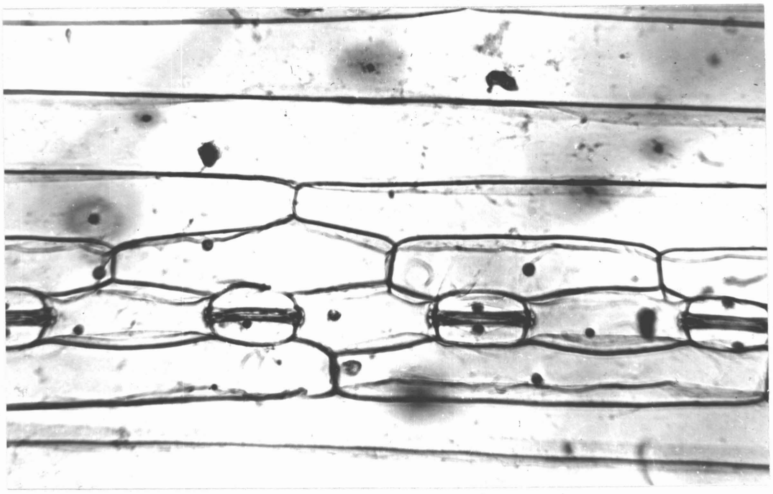


Fig. 3. Epidermal strip from a barley plant inoculated with E. graminis 48 hr. previously and stained with acid fuchsin. Note that the cell wall about the points of penetration stains more intensely than the unaltered wall.

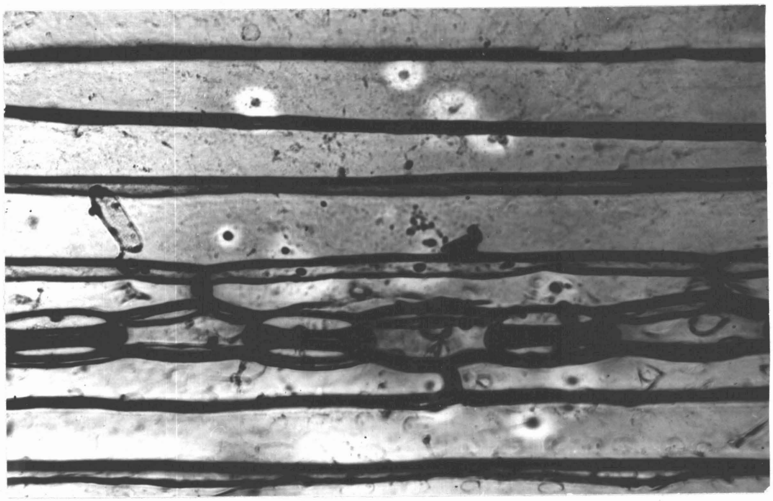


Fig. 4. Epidermal strip from a barley plant inoculated with E. graminis 48 hr. previously and stained with basic fuchsin. Note that the cell wall about the points of penetration is not stained.

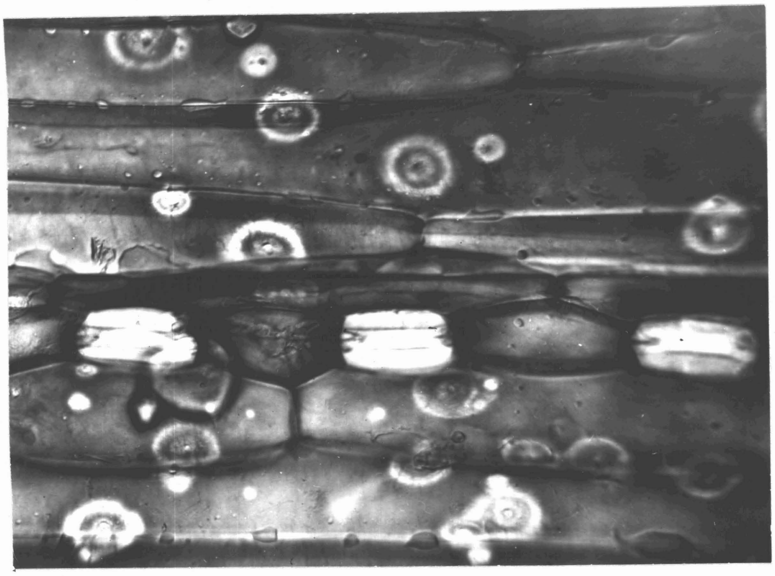


Fig. 5. Epidermal strip from a mildew-infected barley plant stained by Post and Laudermilk's procedure for cellulose. Note that as the nonreactive circular areas about points of penetration enlarge, reactive halos form within them.

within them and the cell wall at the periphery stained slightly darker than the adjacent unaltered cellulose.

Changes in the cell wall about points of penetration were also revealed by the periodic acid-Schiff method for polysaccharides. The altered staining behaviour is shown in Fig. 6. The wall surrounding the intensely stained, red penetration process initially reacted more strongly than the unaffected cellulose. Then, as the altered area enlarged, the staining intensity of the central portion decreased, forming a red halo about the penetration process. Eventually, within some of these penetration halos another halo appeared.

Since both susceptible (Atlas) and resistant (Atlas 46) varieties exhibited these characteristic alterations in the cell wall about penetration points, it is unlikely that the responsible reactions are concerned with resistance to the fungus. However, the succession of staining patterns revealed by both the Post and Laudermilk (1942) and periodic acid-Schiff methods suggests that interactions between host and parasite, rather than merely alterations of original cell wall material, are involved. Moreover, direct evidence for this view is presented later.

(b) Penetration and host cell integrity

In the Literature Review it was pointed out that

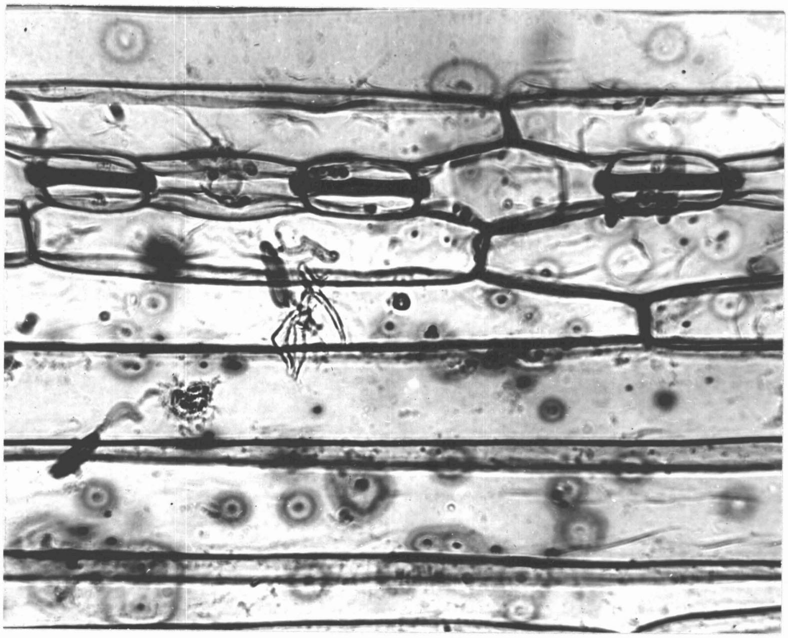


Fig. 6. Epidermal strip from a barley plant inoculated 36 hr. previously and treated by the periodic acid-Schiff procedure. Note that the cell wall about points of penetration initially shows an increased reactivity which soon disappears except at the periphery of the altered area. A halo within a halo is shown at top left.

Corner (1935) considered that the changes in the cell wall accompanying penetration by E. graminis were caused by the diffusion of an enzyme from the penetration process. He suggested, but apparently did not try, inoculating strips of dead epidermis to decide whether or not the host cells played an active role in these cell wall alterations.

This point was investigated by inoculating epidermal strips floated on deionized water. No changes in the cell walls of these strips were detected although strips from detached leaves, inoculated at the same time and held under the same conditions, showed the characteristic alterations in staining behaviour.

In another experiment, the entire abaxial surface of detached leaves was inoculated after partially stripping off the epidermal layer and replacing it in contact with the exposed mesophyll. Following incubation in a humid atmosphere, the epidermis was completely stripped off and stained by the periodic acid-Schiff procedure. Areas of altered reaction were detected only on that portion of the epidermis which had not been stripped prior to inoculation.

Although no changes in the staining reactions were detected when epidermal strips were inoculated, it was uncertain whether or not the fungus had actually

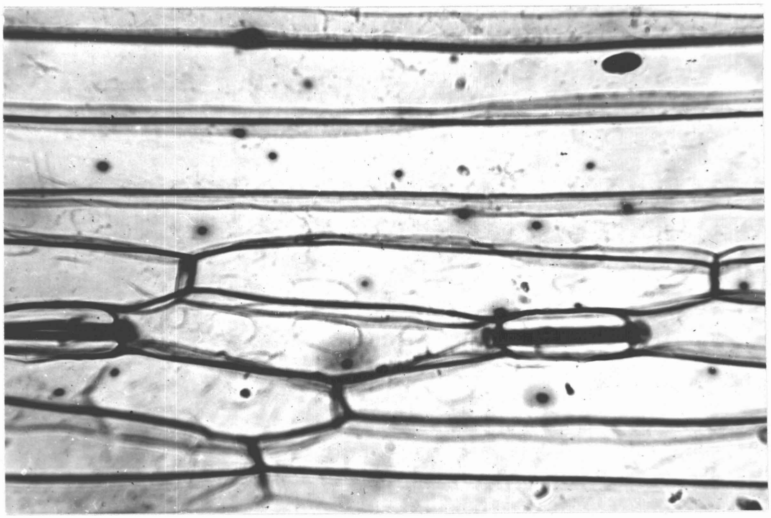


Fig. 7. Epidermal strip from a detached leaf inoculated 15 hr. previously and treated by the periodic acid-Schiff procedure. Note the reactive penetration hyphae and the altered reaction of the cell wall about some of them.

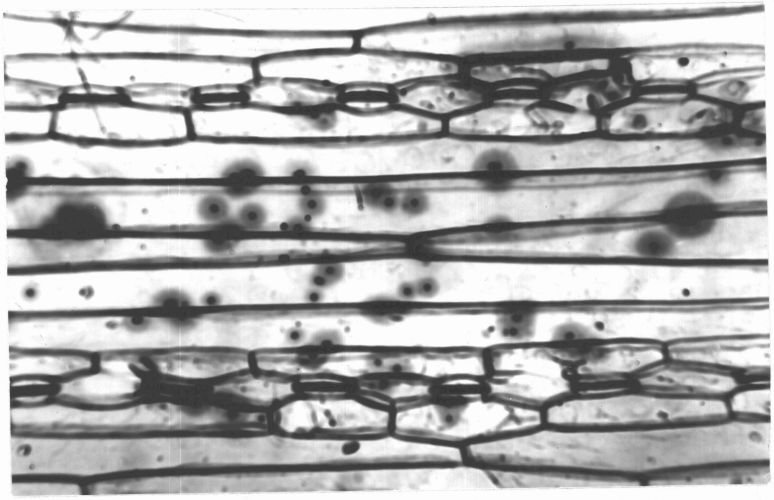


Fig. 8. Epidermal strip from a detached leaf inoculated 48 hr. previously and treated by the periodic acid-Schiff procedure. Note the solidly-stained, circular areas about points of penetration (cf. Fig. 6).

penetrated the cell walls. This point was investigated by the following procedure, which was devised to circumvent the technical difficulties involved in embedding and sectioning epidermal strips.

The epidermis of detached leaves was partially stripped under water and laid back in contact with the mesophyll. The surface of the leaves was then dried and inoculated. After incubation in a humid atmosphere for 15, 36 and 48 hr., the epidermis was completely stripped off and mounted, inner surface up, on microscope slides. The strips were then fixed, processed by the periodic acid-Schiff method and mounted in Diaphane.

Fifteen hours after inoculation, small, intensely stained red spots were found only on that part of the epidermis which had not been stripped prior to inoculation (Fig. 7). These were taken to be the chitinous penetration processes of the fungus. Even after 48 hr., neither penetration processes nor areas of changed cell wall reaction were noted on that portion of the epidermal strip which had been detached prior to inoculation. By that time, large circular areas of increased staining intensity were evident on the intact portion (Fig. 8). These solidly stained circles differed from the halo pattern previously described (Fig. 6). This difference may have been due to the fact that, here, detached leaves were used whereas in the previous experiment the epidermal layers were stripped from leaves which had been inoculated while they were on the plant.

Since the fungus did not penetrate ruptured (stripped) cells, it was impossible to determine, using epidermal strips as Corner (1935) suggested, whether or not the metabolism of the host participated in the cell wall changes accompanying penetration. However, the fact that the cell wall changes showed different patterns in detached and intact leaves indirectly suggested that the host played a role in these changes since it is well known that the metabolism of leaves is profoundly altered by detachment. More positive evidence for this view was obtained by microradioautography.

(c) Cell wall changes and host metabolism

Young, freshly inoculated, excised first leaves of Atlas and Atlas 46 were stood individually in 5-ml. beakers to which uniformly labelled (C^{14}) glucose (2 µc. in 0.2 ml.) had been added. Deionized water was periodically added during the 48 hr. that the leaves remained in a fume cupboard where they received continuous fluorescent light (circa 250 F.C.). The inoculated epidermal layers were then stripped off, placed cuticle surface down on microscope slides and processed as described in Methods. They were then placed in apposition with nuclear track plates which were developed nine days later. The strips were treated by the periodic acid-Schiff procedure.

A stained strip of Atlas 46 is shown in Fig. 9a and a photograph of the corresponding radioautograph is shown in Fig. 9b. It is evident that there is a general correspondence between areas of altered staining and localization of radioactivity. The same result was obtained with inoculated leaves of Atlas. However, no similar localizations of radioactivity were detected on a plate exposed to an uninoculated but otherwise identically treated leaf.

This result demonstrates that carbon compounds are laid down in the walls of host cells in response to penetration by _E. graminis_. To this extent, at least, the metabolism of the cell is concerned with the changes accompanying penetration.

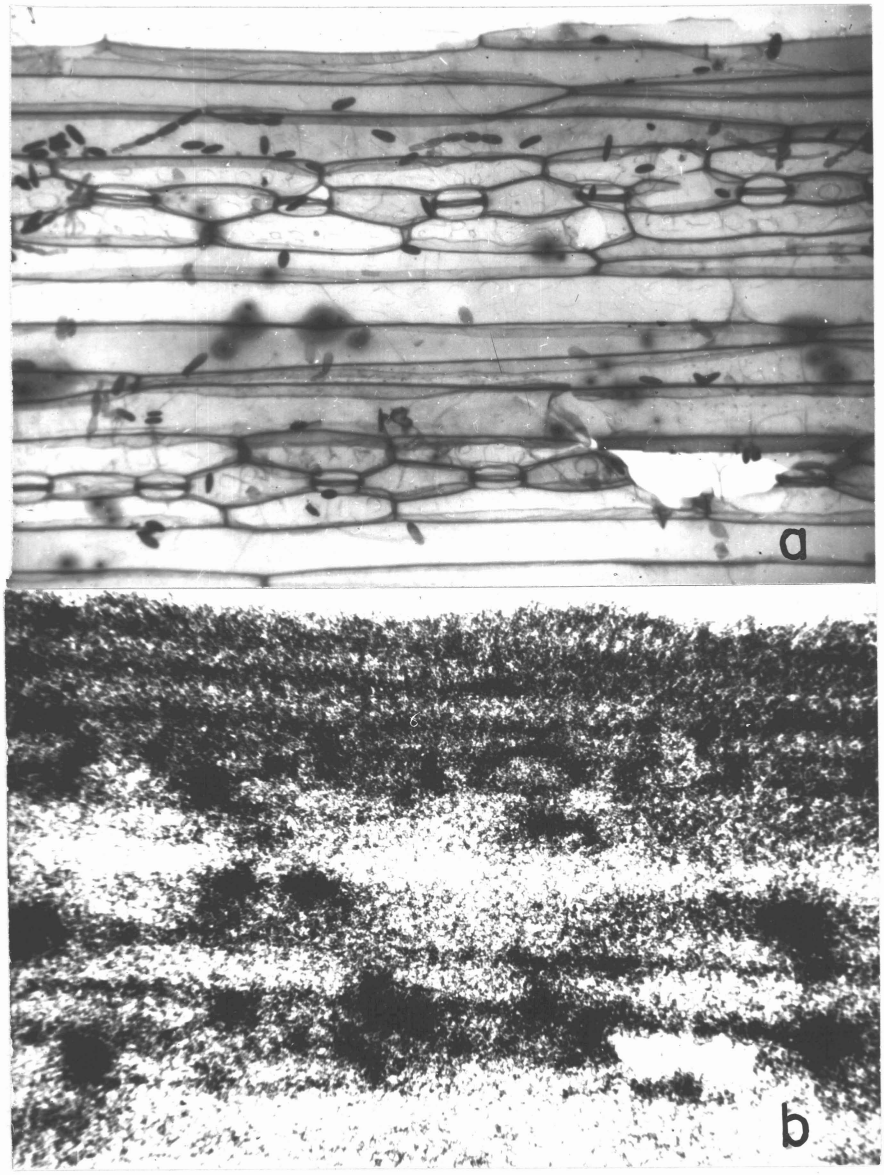


Fig. 9. (a) Photograph and (b) microradioautograph of epidermal cells stripped from a barley leaf 48 hr. after it had been detached, inoculated with E. graminis and placed in radioactive glucose. Note the general correspondence between areas of altered staining (periodic acid-Schiff procedure) and localized radioactivity.

B. Demonstration Of Phosphatase In Haustoria

1. Methods

(a) Localization of enzyme activity

Four histochemical procedures were used to test for acid phosphatase associated with the haustoria of *E. graminis hordei*. Three were azo dye methods and the fourth was Gomori's (1950) improved lead-phosphate technique.

The first azo dye method tried was Menten, Junge, and Green's (1944) original coupling technique for alkaline phosphatase modified, as suggested by Yin (1945), for demonstrating the acid enzyme. Except for buffering the incubation mixture at circa pH 5.0 (0.01 M acetate buffer), the technique was carried out as described in the original paper. The substrate, calcium β-naphthyl phosphate, was purified of free β-naphthol by repeated extractions with ether. α-naphthylamine was purified by quasisublimation before it was diazotized.

Later, Burton's (1954b) modification of Grogg and Pearse's (1952b) azo dye technique for acid phosphatase was used. The incubation mixture he suggested had the following standard composition:

Sodium acid α-naphthyl phosphate	5 mg.
Tetrazotized o-dianisidine	20 mg.
0.1 M acetate buffer	10 ml.

The monosodium salt of α-naphthyl phosphate was synthesized according to Friedman and Seligman (1950). The stable coupling salt, tetrazotized o-dianisidine, was supplied by Canadian Industries Limited.

Several modifications suggested by Burton in this standard formulation were also tried. They are described in the appropriate section of the results.

Following incubation, the slides were exposed to 1% hydrochloric acid in 80% ethanol until all soluble dye was removed. They were then dehydrated to 95% ethanol and mounted in Diaphane.

The final azo dye method used was Rutenburg and Seligman's (1955) newly published, post-coupling method. The incubation mixture used in this technique had the following composition:

Sodium acid 6-benzoyl-2-naphthyl phosphate	25 mg.
0.5 M acetate buffer pH 5.0	20 ml.
Distilled water	80 ml.

The sodium acid 6-benzoyl-2-naphthyl phosphate was obtained from Dajac Laboratories. The coupling mixture consisted of cold (4° C.), freshly prepared aqueous tetrazotized o-dianisidine (1 mg./ml.) made alkaline with sodium bicarbonate.

Gomori's (1950) lead-phosphate technique was extensively used. Unless mentioned otherwise, his procedure was followed closely. In experiments concerned only with the localization of enzyme activity, the substrate was a mixture of alpha (25%) and beta (40%) disodium glycerophosphates (Eastman-Kodak). The incubation mixture was prepared as follows: To 100 ml. of 0.05 M acetate buffer, pH 5.0, containing 1.2 g. of lead nitrate per litre was added 10 ml. of 0.1 M disodium glycerophosphate. After keeping the mixture at 37° C. for 24 hr. and filtering off the precipitate formed, it was ready for use.

Variations necessary when other substrates were used are described in the next section. However, in all experiments using the Gomori method, the steps following incubation were the same. The slides were rinsed in distilled water and then immersed in dilute acetic acid (1 to 2%) for about 1 min. After rinsing in distilled water, the slides were immersed in weak ammonium sulphide (a few drops in 50 ml. of distilled water) and again thoroughly rinsed in distilled water. They were then dehydrated in alcohol and mounted. The precipitate of lead sulphide localizing enzyme activity faded badly when Diaphane was used for mounting. The slides were therefore dehydrated up to absolute alcohol, treated briefly with xylol and mounted in Canada Balsam.

(b) Testing enzyme activity towards various substrates using Gomori's method

Using the Gomori procedure outlined in the preceding section, various phosphate esters were tested as substrates for the haustorial phosphatase. Barley seedlings for these experiments were grown at 26° C. in an environment chamber where they received 12 hr. of light (800 F.C.) in every 24. Eight to 10 days after planting, the abaxial surface of first leaves was inoculated with powdery mildew conidia.

Following inoculation the plants were removed to a constant temperature room (20 to 23° C.), where they continued to receive the same amount of light. Three or four days after inoculation, the parasitized epidermal layers were stripped off. The strips, mounted with their cuticle surface down, were fixed in 80% ethanol for 2 hr. and washed for 30 min. in running tap water. The slides were then thoroughly rinsed in deionized water before being incubated in the appropriate mixture at 37° C.

The procedures followed in preparing and using the various substrates are outlined below.

α- and β-glycerophosphates. The alpha-isomer was obtained as its calcium salt (Bios) while the beta-isomer was in the form of its disodium salt (N. B. Co.). Incubation mixtures of these two substrates were prepared following the previously outlined procedure, that is, by adding one part of 0.1 M substrate solution to 10 parts of the buffer-lead mixture. The mixtures were kept at 37° C. for 10 hr. prior to use. The precipitate formed during this time was filtered off.

So that results with the two isomers would be strictly comparable, the calcium salt of α-glycerophosphate was converted to the corresponding disodium salt in the following manner: With the addition of a little 1.0 N hydrochloric acid, 0.4202 g. of the calcium salt was dissolved in 15 ml. of deionized water. The calcium ions were precipitated by the addition of approximately 0.2120 g. of sodium carbonate. The calcium carbonate was centrifuged down and the supernatant tested with a pinch of sodium carbonate. When no further precipitation occurred, the supernatant was made up to 20 ml. (0.1 M) and the required amount added to the buffer-lead solution.

Glucose-1-phosphate. This substrate was obtained as the dipotassium salt (N. B. Co.), in which form it was used. Gomori (1950) made no recommendations for the preparation of an incubation mixture containing this substrate; consequently, his procedure for glucose-6-phosphate was followed. For this substrate, he advised a buffer-substrate ratio of six.

The incubation mixture was prepared by adding 10 ml. of 0.0833 M glucose-1-phosphate to 100 ml. of standard buffer-lead mixture. After allowing the mixture to stand for 1 hr. at 37° C., the slight amount of precipitate that formed was filtered off.

Phosphoglyceric acid. This substrate was obtained as the barium salt (N. B. Co.). Since there was no information in the literature pertaining to the preparation of a Gomori-type incubation mixture using this substrate, the same procedure as that outlined above for glucose-1-phosphate was first tried. Later, other concentrations of the substrate were tested as described in the appropriate section of the results.

The barium salt was converted into the corresponding disodium salt as follows: The substrate (0.2677 g.) was dissolved in 5 ml. of deionized water by adding a few drops of 1.0 N hydrochloric acid and the barium ions precipitated by the addition of 0.1183 g. of sodium sulphate. After allowing the mixture to stand for 1 hr. at room temperature, the precipitate was centrifuged down and the supernatant tested with a pinch of sodium sulphate. When no further precipitation occurred, the supernatant was adjusted to pH 5.0 with sodium hydroxide and made up to 10 ml. (0.0833 M). This solution was added to 100 ml.

of buffer-lead mixture and the resulting precipitate removed by filtration.

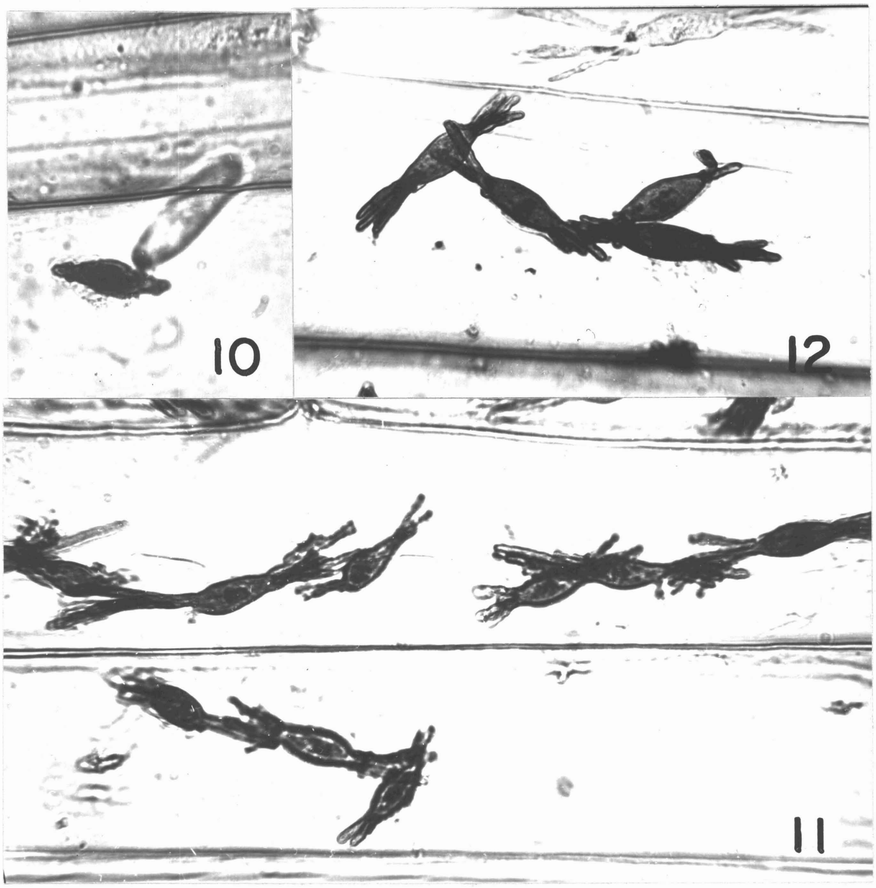
2. Results

(a) Localization of enzyme activity

The first method tried in testing for the presence of an acid phosphatase in association with the haustoria of *E. graminis* was the modified Menten, Junge, and Green (1944) azo dye technique. It was found, as the originators suggested, that this procedure was too intricate for routine use. However, before the method was abandoned, the presence of phosphatase associated with haustoria was indicated when, after incubating unfixed, infected epidermal strips in the refrigerator for 40 min., a red azo dye was found localized on these structures.

Following the limited, yet suggestive, results obtained with the azo dye method, Gomori's (1950) improved lead-phosphate technique was used. Parasitized epidermal strips mounted cuticle-surface up and fixed for 2 hr. in 80% ethanol were processed by this technique.

After incubation periods of from 2 to 4 hr., dark brown deposits of lead sulphide, indicative of phosphatase activity, were found localized on haustoria, in guard cells and on nuclei of accessory and other epidermal cells (Figs. 10 and 11). A lesser staining of the cytoplasm



Figs. 10, 11, 12. Demonstration of acid phosphatase activity in haustoria of E. graminis hordei by the Gomori method. Fig. 10, a young haustorium with attached conidium; Fig. 11, mature, digitately-branched haustoria; Fig. 12, an unstained haustorium adjacent to stained ones, indicating the occasional capriciousness of the Gomori method.

seemed somewhat darker in the long cells found between the stomatal rows than in the shorter cells adjacent to these rows. Since no precipitate was evident when incubation was carried out in the absence of substrate, in the presence of 0.005 M sodium fluoride (a powerful inhibitor of acid phosphatase), or after holding the tissue in a jet of steam for a few minutes, the positive results must have been due to acid phosphatase activity.

As Gomori (1950, 1952) and others (Grogg and Pearse, 1952b) reported, the results even with this improved technique were occasionally erratic. Fig. 12 shows a haustorium in one cell devoid of activity while those in adjacent cells are strongly stained.

The histochemical literature contained numerous references to the fact that acid phosphatase was readily inactivated by fixation, especially in alcohol (Rabinovitch _et al._, 1949; Stafford and Atkinson, 1948).

Fixation in cold absolute acetone (Gomori, 1952; Rabinovitch _et al._, 1949) or 10 to 15% cold neutral formalin (Grogg and Pearse, 1952b; Seligman, Chauncey, and Nachlas, 1951) was generally considered to give the best preservation of this enzyme. Danielli (1953) found a mixture of pyridine, alcohol and formaldehyde to be a good fixative for alkaline phosphatase. Nevertheless, it was a fact that, of the few investigations concerned with the histochemical demonstration of acid phosphatase in plant tissues, most involved alcoholic (70 to 95%) fixation (Hilbe and Marron, 1940; Yin, 1945; Glick and Fischer, 1945a,b, 1946; Kugler and Bennet, 1947; McGregor and Street, 1953). It seemed desirable, therefore, before proceeding further, to determine which fixative was the most suitable for the material used.

In a preliminary experiment, healthy epidermal strips were fixed in absolute acetone (12 hr. at 4° C.), 15% formaldehyde (12 hr. at 4° C.), 80% ethanol (2 hr. at circa 22° C.) and in a mixture containing 20% pyridine, 70% ethanol and 4% formaldehyde (2 hr. at circa 22° C.). After the strips were incubated in Gomori's mixture for ½, 1, 2, 4 and 8 hr. and subsequently processed to reveal phosphatase activity, fixation treatments were compared on the basis of staining intensity. Checks included unfixed strips and fixed strips incubated for 8 hr. in the absence of substrate.

Two hours' fixation in 80% ethanol caused slightly less inactivation of the enzyme than did 12 hr.' fixation either in cold absolute acetone or in 15% formaldehyde. Moreover, the staining of tissue fixed in formaldehyde was erratic, not only within but also between strips. The pyridine-ethanol-formaldehyde mixture gave the greatest inactivation of enzyme. These results, along with the fact that slides incubated in the absence of substrate showed no staining, are illustrated in Fig. 13.

On the slide beneath one of the ethanol-fixed strips incubated for only ½ hr., a deposit of lead sulphide was found. Diffusion of enzyme or of phosphate or lead phosphate from the strip could have caused this result.

Because different fixation times and temperatures were used, the results of the foregoing experiment were not conclusive. Furthermore, the formaldehyde was not neutralized as was generally recommended. Another more comprehensive experiment was therefore carried out.

Healthy epidermal strips were fixed for 2, 4 and 12 hr. at 4° C. in absolute acetone, absolute ethanol, a mixture of 50% acetone and 50% ethanol, 80% ethanol, and in 15% formaldehyde adjusted to pH 7.0 with sodium bicarbonate. Fixation in 80% ethanol was followed by a 2-hr. immersion in absolute ethanol to reduce enzyme diffusion (Danielli, 1946). Strips fixed in 80% ethanol only were used to reveal enzyme inactivation caused by the absolute ethanol treatment.

Before incubation in Gomori's medium for 1, 2, 4 and

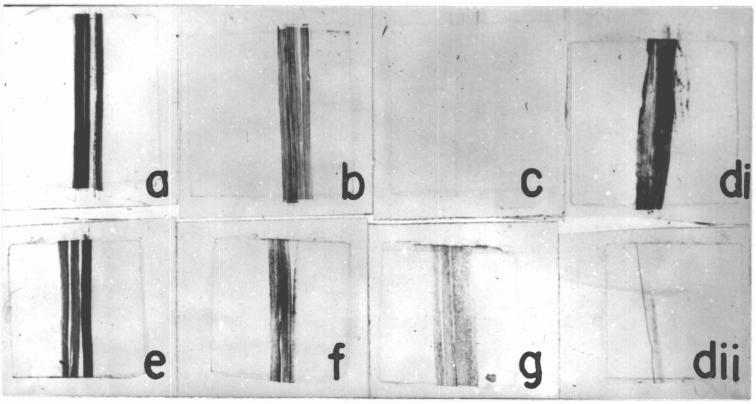


Fig. 13. Demonstration, by Gomori's method, of the phosphatase activity remaining in epidermal strips after various fixation procedures: (a) no fixation; (b) 12 hr. in absolute acetone, 4° C.; (c) representative result when incubation was carried out in the absence of substrate; (di), (dii) and (f) 12 hr. in 15% formaldehyde, 4° C.; (e) 2 hr. in 80% ethanol, room temperature; (g) 2 hr. in pyridine-ethanol-formaldehyde, room temperature. All strips incubated for 2 hr. except those in (c), (di) and (dii), which were incubated for 8 hr.

8 hr., strips fixed in absolute acetone, absolute ethanol, 50% acetone:ethanol mixture and 80% ethanol were stepped down to water through 95, 80, 70, 50, 30 and 10% ethanol (10 min. in each). Strips fixed in formaldehyde were rinsed well with deionized water before incubation. Treatments were compared, as before, on the basis of staining intensity.

Visual comparison of the mounted strips showed that inactivation of enzyme was greatest in the neutral formalin, 50:50 acetone-ethanol and absolute ethanol fixed strips. Although the 2-hr. treatment with absolute ethanol following fixation in 80% ethanol did cause some inactivation, the slides of this series showed as good, if not better, staining than slides fixed in absolute acetone.

Slides in the absolute acetone and 80% ethanol series showed some tendency towards increased intensity of staining with increasing duration of fixation suggesting, perhaps, an activating effect of these fixatives on acid phosphatase. A similar increase, following an initial decrease, in acid phosphatase activity after prolonged fixation (72 hr.) in acetone and 80% ethanol is evident in the results of Stafford and Atkinson's (1948) quantitative study. However, these authors made no reference to this curious result.

Microscopic examination of the slides generally revealed enzyme activity localized in guard cells and in nuclei, especially those of accessory cells. There were, however, many instances of obvious artifact in which diffuse staining was evident about accessory cells (Fig. 14). Occasionally, too, the cytoplasm of cells close to the edges of a strip was completely devoid of activity in contrast to the usual faint but definite staining. It was thought that some of the poor cytological detail was due to improper exposure of the strips, which, in these experiments, were mounted cuticle-surface up, to the reagents.

For this reason, epidermal strips used in all subsequent experiments were mounted on microscope slides with their noncutinized, inner surface up. Furthermore, fixation was always carried out in 80% ethanol for 2 hr. since this treatment appeared to give the best preservation of enzyme as well as satisfactory cytological detail. The absolute ethanol treatment following fixation was not used since no difference other than reduced enzyme activity was noted when this step was included.

Intracellular localization of acid phosphatase by Gomori's (1941b, 1950) method has often been considered unreliable, with the position of "active" sites depending

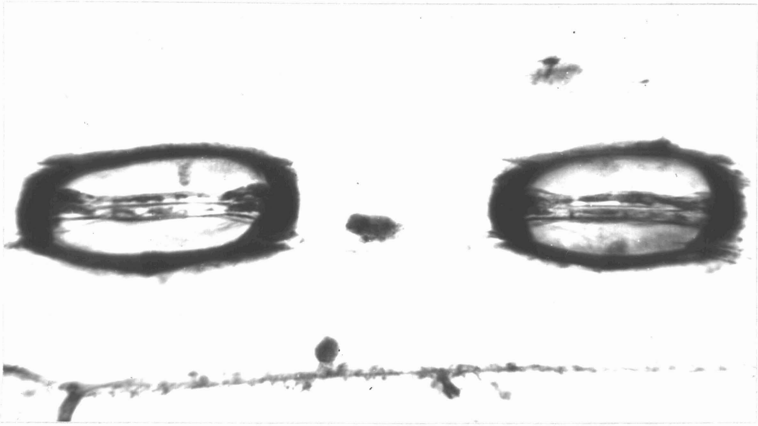


Fig. 14. Diffusion artifact about accessory cells revealed when an epidermal strip fixed for 2 hr. in absolute ethanol was incubated in Gomori's mixture for 2 hr.

upon physico-chemical conditions, such as diffusion and adsorption of lead phosphate or its constituent ions, rather than upon the actual site of the enzyme (Newman, Kabat, and Wolf, 1950; Grogg and Pearse, 1952b). It was therefore desirable to substantiate the results obtained by some other completely unrelated method. Azo dye procedures, depending upon the localization of the alcoholic rather than the phosphate moiety of the hydrolyzed substrate, seemed to have the requirements and, indeed, were the only alternative procedures available. At the time, the most satisfactory azo dye method for demonstrating acid phosphatase activity appeared to be Burton's (1954b) modification of Grogg and Pearse's (1952b) method.

Burton claimed that his modifications eliminated diffusion artifact by ensuring rapid azo coupling following enzymic release of α-naphthol. These modifications included using high concentrations of the coupling salt (tetrazotized o-dianisidine), low concentrations of substrate (sodium acid α-naphthyl phosphate), percain to catalyze the coupling reaction and buffering at pH 5.6 to 5.8 rather than at pH 5.0.

Formation of a black azo dye, insoluble in acidic ethanol, rather than a red, soluble one, was considered indicative of rapid coupling. The experimental conditions necessary to obtain maximum formation of black dye varied, depending on the enzymic rate at which α-naphthol was being released in the tissue.

Preliminary experiments with healthy epidermal strips were therefore conducted to determine the optimal conditions for this material. Using the standard formulation of Burton's incubation mixture, enzymic activity, as revealed by staining intensity, was greatly reduced at pH 5.8 compared to pH 5.0. This effect was completely offset by doubling the substrate concentration. However, the azo dye formed was not uniformly black.

Percain (0.5 ml. of 5% aqueous solution per 10 ml. of incubation mixture) greatly increased the amount of black dye formed at pH 5.0 but not at pH 5.8 when the standard substrate concentration was used. However, when the substrate concentration was doubled, percain increased the amount of black dye formed at pH 5.8 but not at pH 5.0. These results, confirming Burton's observations, suggested that the limiting factor in the formation of black dye at pH 5.0 was the rate of coupling, at pH 5.8, the rate of enzymic hydrolysis.

An experiment to determine the optimal pH for balancing these two counteracting effects was therefore set up. Strips were incubated for 2 hr. at pH 5.0, 5.2, 5.4, 5.6 and 5.7 in mixtures containing percain and having one and two times the standard substrate concentration.

Optimal formation of black dye occurred when the standard substrate concentration was used at pH 5.6. Doubling the substrate concentration at this pH and at pH 5.7 increased the amount of black dye but microscopic examination revealed diffusion artifacts. Fig. 15 shows phosphatase activity localized in all nuclei except those of the guard cells and in the cytoplasm of all but the accessory cells. The dye was gray or black except in the cytoplasm of the guard cells, where it was reddish.

The optimal conditions determined, localization of phosphatase activity was studied in strips from plants inoculated with *E. graminis* three days previously. Strips were incubated at pH 5.6 and 5.7 for 2, 4, 6 and 8 hr. The medium was renewed at the end of every 2 hr.

Figs. 16 and 17 show young and fully differentiated haustoria stained with black dye. These results, in close agreement with those previously obtained by the unrelated Gomori method, suggested both that the localizations were accurate and that, at least with this material, the Gomori procedure was valid.

The opportunity to use, in these investigations, a new azo dye procedure for acid phosphatase (Rutenburg and

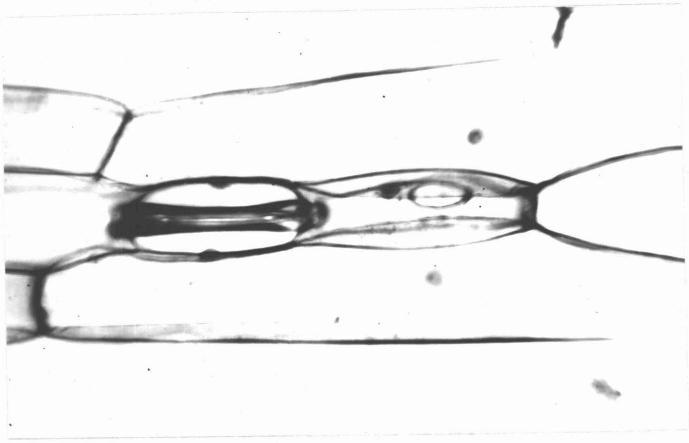
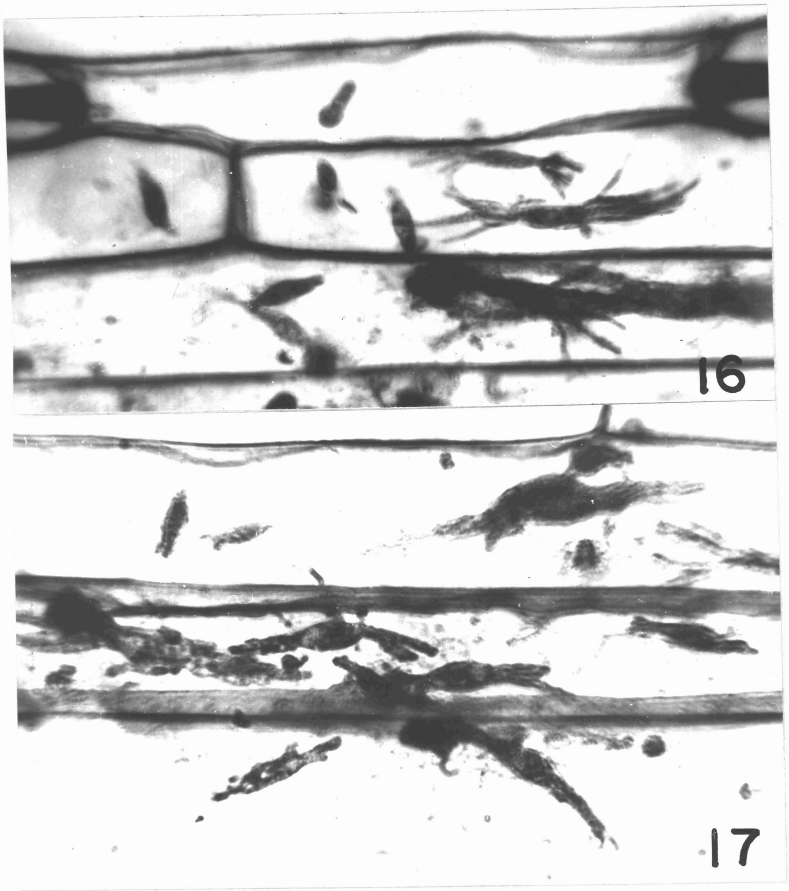


Fig. 15. Acid phosphatase activity demonstrated by Burton's azo dye method in epidermal and accessory cell nuclei but not in the dumb-bell shaped nuclei of the guard cells. Cytoplasm of the epidermal cells lightly stained, of the guard cells darkly stained and of the accessory cells, unstained.



Figs. 16, 17. Demonstration of acid phosphatase activity in haustoria of E. graminis hordei by Burton's azo dye method. Incubation for 8 hr. at pH 5.6.

Seligman, 1955) was made possible through the kindness of Dr. Rutenburg, who supplied details of the method prior to publication.

Epidermal strips from young Atlas and Atlas 46 plants inoculated three days previously with E. graminis were incubated for ½, 1 and 2 hr. in the recommended mixture at 26° C. Following this they were washed three times (10 min. each) in cold tap water and transferred to the coupling solution. After 3 to 5 min.' agitation in this mixture, the slides were washed three times in cold water and mounted in glycerine.

The localization of activity was as previously found. Haustoria and guard cells were intensely stained, even after incubation for only ½ hr., and the nuclei, especially of accessory cells, appeared only slightly less active (Fig. 18). The staining of the cytoplasm in all cells was considerably lighter. Not only the haustoria but also, perhaps to a lesser degree, the mycelium showed activity. Equally active haustoria were observed in both the susceptible (Atlas) and resistant (Atlas 46) varieties. Localization was so precise with this method that the phosphatase activity in haustoria was seen to be confined by a thin membrane or wall (Fig. 19) and in hyphae by a somewhat thicker wall. Phosphatase activity

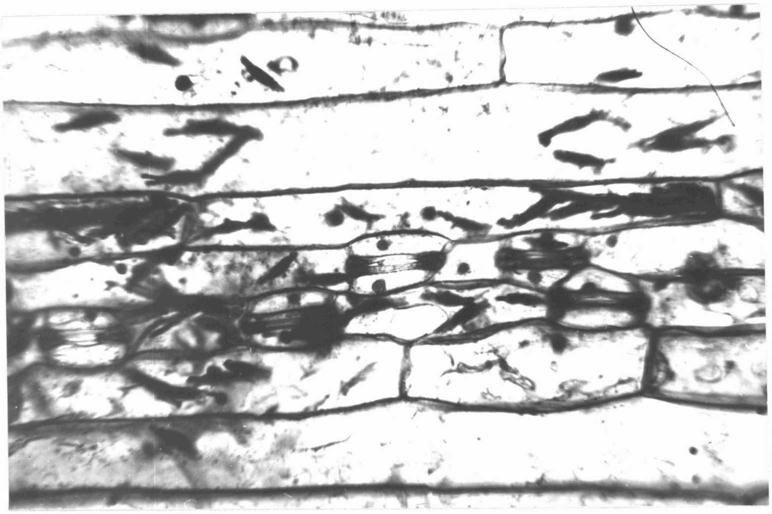


Fig. 18. Phosphatase-active haustoria, nuclei, guard cells and, to a lesser extent, cytoplasm, as demonstrated by Rutenburg and Seligman's azo dye method. Incubation for 1 hr. at pH 5.0.

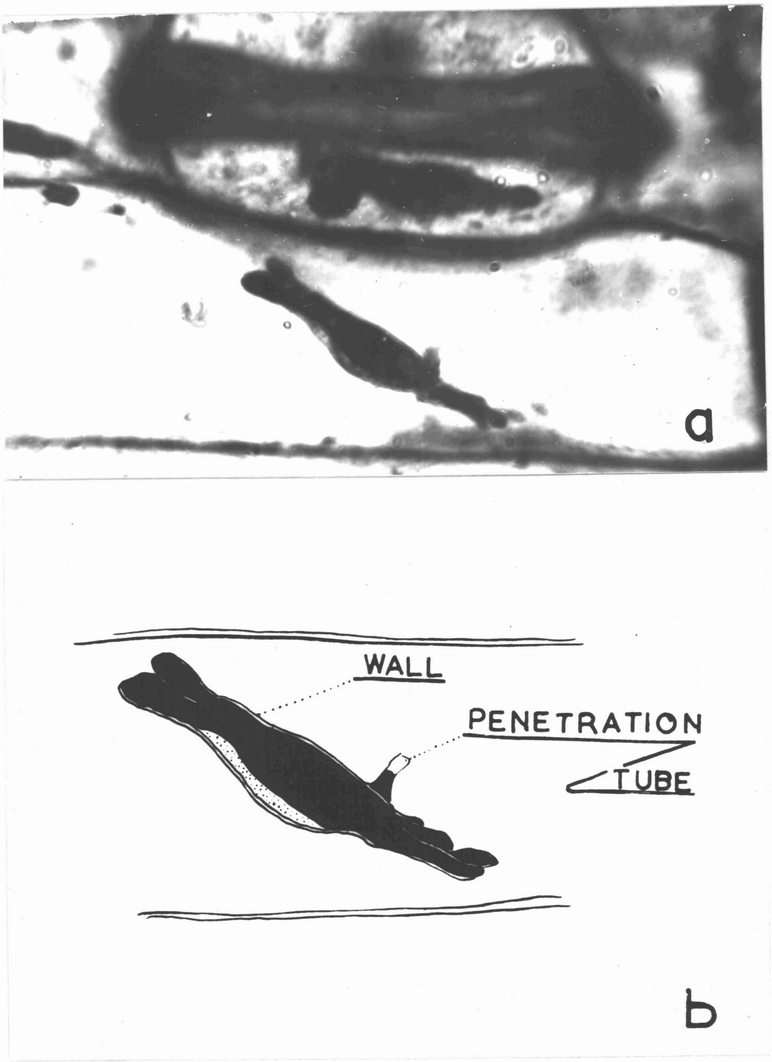


Fig. 19. Demonstration, by Rutenburg and Seligman's method, that the haustorial phosphatase activity of E. graminis hordei is localized within the haustorial wall. (a) oil-immersion photograph (note also the haustorium in the accessory cell); (b) camera-lucida drawing showing clearly the detail observable.

in a germinated conidium was localized mainly at the tips of the germ tubes (Fig. 20). These results definitely showed that the phosphatase associated with the haustoria was an enzyme of the fungus.

After the last slide had been removed, the incubation mixture was made alkaline with sodium bicarbonate and a little tetrazotized o-dianisidine was added. The mixture soon turned pink, indicating, according to Rutenburg and Seligman (1955), that some enzyme had diffused out of the tissue into the medium.

Demonstration of acid phosphatase activity in association with the haustoria of E. graminis hordei and the nuclei and guard cells of the host by two azo dye methods as well as by the unrelated lead-phosphate technique would appear to be unequivocal evidence for the validity of these localizations. Although the association of phosphatase activity with haustoria of E. graminis is obviously accurate, the intracellular localization of the enzyme in the host may be subject to question. Palade (1951), using biochemical methods, as well as Grogg and Pearse (1952b), Burton (1954b), and Rutenburg and Seligman (1955), using azo dye histochemical methods, have reported that in animal tissues the bulk of the acid phosphatase activity is localized in the cytoplasm with little or none

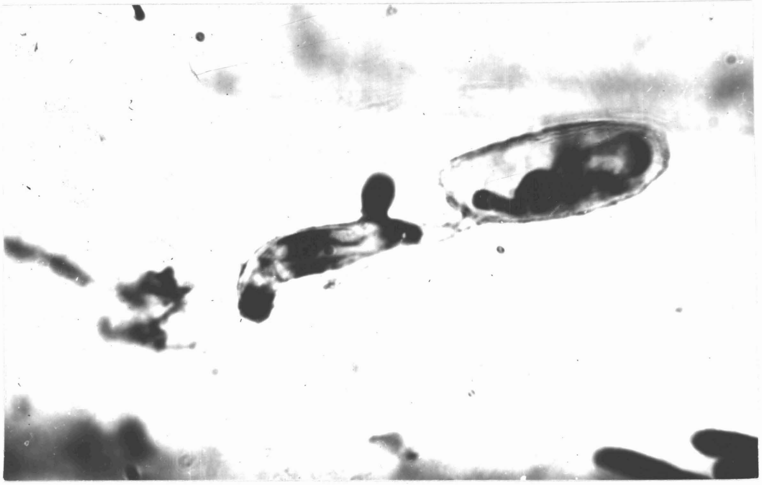


Fig. 20. Acid phosphatase activity in a germinated conidium of E. graminis hordei. Note the concentration of enzyme at the tips of the germ tube. Rutenburg and Seligman's method; incubation, 1 hr.

associated with the nuclei.

(b) Testing enzyme activity towards various substrates using Gomori's method

Although the enzyme had been demonstrated by three different histochemical methods, little was known about the activity of the haustorial phosphatase except that a mixture of α- and β-glycerophosphates, α-naphthyl phosphate and 6-benzoyl-2-naphthyl phosphate were suitable substrates. It was considered desirable to determine, if possible, what additional phosphate esters were hydrolyzed by this enzyme or group of enzymes. Although the choice of substrate was severely limited with azo dye techniques because the alcoholic moiety had to couple with diazotized amines, all substrates possessed the phosphate moiety which the Gomori method localized. This method was therefore utilized to test additional substrates since its accuracy for the localization of haustorial phosphatase had been apparently confirmed by the azo dye methods.

In practice, the choice of substrate was limited even with Gomori's technique because the lead ions present in the incubation mixture formed insoluble lead salts with many phosphate esters. A few substrates whose lead salts were sufficiently soluble were, however, tested.

α- and β-glycerophosphates. In the previous experiments concerned only with localization of phosphatase activity by Gomori's technique, a mixture of α- and β-glycerophosphates was used. It was thought worthwhile to test pure preparations of these isomers. Strips were incubated for ½, 1, 2 and 6 hr. Heat-inactivated strips and incubation in the presence of 0.005 M sodium fluoride were used to check the enzymic nature of the reaction.

Visual comparison of the two sets of slides showed that those incubated in α-glycerophosphate were more darkly stained than those incubated in β-glycerophosphate. Microscopic examination revealed, however, that the precipitation pattern, although, in general, similar, was much more regular and precise in the strips incubated in β-glycerophosphate than those incubated in α-glycerophosphate. Occasionally, too, parts of α-glycerophosphate-incubated strips showed black rather than the normal dark brown staining, and, in such areas, the localization was atypical. For example, guard and accessory cells were devoid of staining and adsorption artifact was indicated when penetration halos appeared as clear, unstained areas on a darkly stained background (Fig. 21). Strips incubated in β-glycerophosphate also occasionally showed variable results, structures normally staining showing

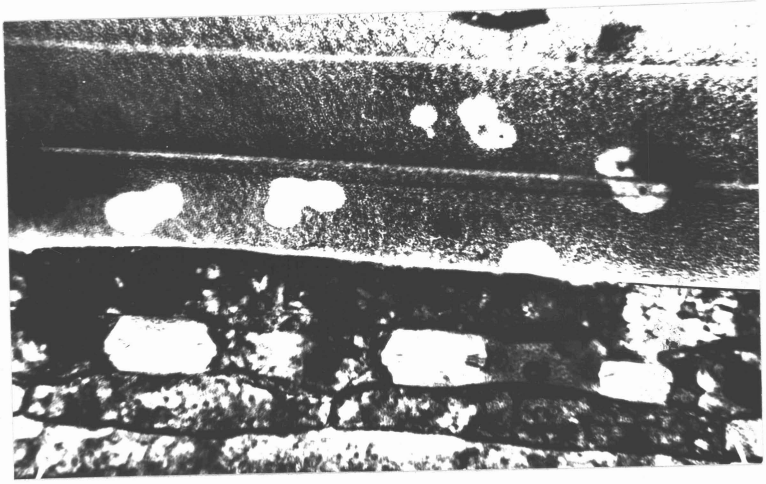


Fig. 21. Adsorption artifact found when a mildew-infected epidermal strip was incubated in Gomori's medium. Note that the cell wall about points of penetration is unstained.

little or no activity in some instances.

Incubation for ½ to 1 hr. gave slides most suitable for microscopic examination. Fig. 22 shows enzyme activity localized in the nuclei of guard, accessory and other epidermal cells and also in the cytoplasm, especially of the large epidermal cells. Haustoria were usually found to be strongly stained after incubation in both substrates. Although less frequent, the haustoria in Atlas 46 appeared no different either in development or enzymic activity. Haustoria, some much more strongly stained than others, are shown in Fig. 23, not only in epidermal but also in accessory cells. As was originally pointed out, accessory, in contrast to epidermal, cells are usually not ruptured by the stripping process. The demonstration of phosphatase-active haustoria and nuclei (by azo dye as well as by lead-phosphate methods) in both cell types indicates, therefore, that these enzyme localizations were not artifacts resulting from the stripping procedure.

Virtually no staining was observed in both groups of check slides incubated for 6 hr., indicating that the reactions were enzymic.

Glucose-1-phosphate. The slides were incubated for 1, 2, 4 and 8 hr. Sodium fluoride checks were incubated for 8 hr.

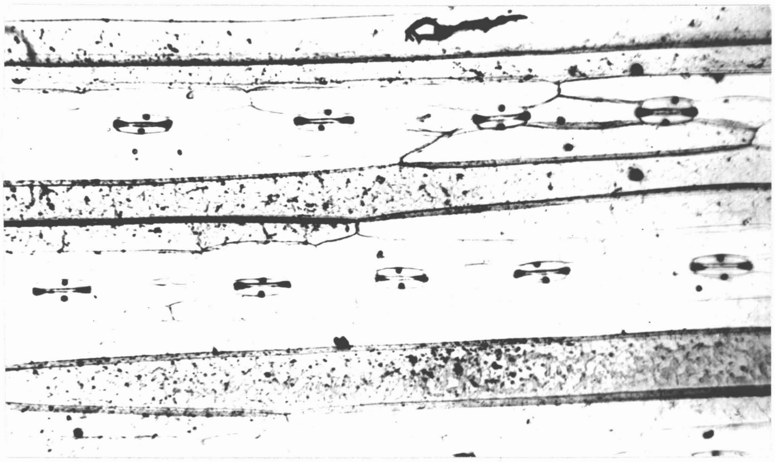


Fig. 22. Localization of phosphatase activity in a barley epidermal strip as revealed by Gomori's method using β-glycerophosphate.

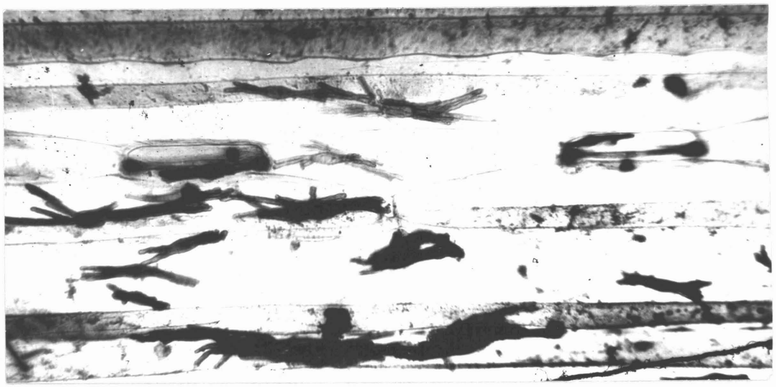


Fig. 23. Phosphatase-active haustoria in accessory as well as in epidermal cells as demonstrated by Gomori's method using β-glycerophosphate.

Localization of activity was not uniform, especially after incubation for 1 and 2 hr. At the end of 1 hr. only a few nuclei of accessory and other epidermal cells and the occasional haustorium were stained. At the end of 2 hr. the only change was the appearance of slight activity in some guard cells and in the cytoplasm of a few accessory cells. Haustorial staining ranged from none to dense. After 4 hr., general staining of nuclei, guard cells and cytoplasm of accessory and long epidermal cells was evident. Most haustoria showed strong staining after this period of incubation. The only change noted in material incubated for 8 hr., apart from an increase in intensity of staining, was slight activity in the short epidermal cells adjacent to the stomatal rows.

The slides incubated with sodium fluoride were devoid of staining. Therefore, although variable, these results were due to the activity of phosphatase. Phosphorylase also releases phosphate from glucose-1-phosphate but the activity of this enzyme is not inhibited by fluoride.

Phosphoglyceric acid. A large amount of precipitate formed when the substrate solution was added to the buffer-lead mixture. After filtering off the precipitate, incubation was carried out for 1 and 2 hr. Sodium fluoride checks were also incubated for 2 hr.

Little staining was evident after 1 hr. but at the end of 2 hr. it was strong. The checks were devoid of activity. Microscopic examination of slides incubated for 1 hr. revealed strong staining of some haustoria and irregular diffuse staining of epidermal cell cytoplasm. After 2 hr., most cells showed a general staining of cytoplasm and haustoria, both in Atlas and Atlas 46, were densely stained. Nuclear staining was variable. Nuclei were conspicuously stained in some strips while in others they were no more strongly stained than the cytoplasm. Guard cells showed little or no activity.

It was realized that the large amount of precipitate formed in the preparation of this incubation mixture may have reduced the lead concentration to a point where enzyme localization was affected. Therefore, after experimentally determining that 0.0125 M phosphoglyceric acid gave only a slight precipitate when combined with the buffer-lead mixture, the histochemical test was again carried out using this level of substrate. Incubation periods of 1, 1½, 2 and 2½ hr. were used and sodium fluoride checks were included.

Whereas no staining occurred after 1 hr., considerable staining was evident in slides incubated for 1½ hr. After 2½ hr., staining intensity was comparable to that in slides of the previous experiment incubated for 2 hr. The sodium fluoride checks, incubated for 2½ hr., showed no activity, demonstrating that the results were due to the activity of phosphatase. Microscopic examination revealed that after 1½ hr. activity was regularly localized only in haustoria with nuclear and cytoplasmic staining variable, suggesting that the fungal as compared to the host enzyme was either in higher concentration or had a greater activity towards this substrate. After 2 and 2½ hr., however, activity was more or less regularly localized in nuclei, cytoplasm and guard cells.

The results of these experiments suggest that phosphatases associated with haustoria, nuclei and, to a lesser extent, the host cytoplasm are capable of hydrolyzing α- and β-glycerophosphates, glucose-1-phosphate and phosphoglyceric acid. However, the accuracy of the nuclear localization of acid phosphatase is, as before, questionable. Although, on the basis of the incubation times required to produce a distinct precipitate, the various substrates appear to be hydrolyzed at different rates, it must be remembered that different substrate concentrations were used in some instances and possibly existed in others. It seems valid, therefore, to conclude only that the haustorial phosphatase(s) of _E. graminis_ hordei is (are) capable of hydrolyzing all phosphate esters tested.

Part II. Quantitative Studies On Rust Spore Phosphatase

A. Methods

1. General procedures

The steps involved in studying the phosphatase activity of germinated rust spores were, briefly, as follows: Known weights of spores were dispersed in a small volume of deionized water and germinated under controlled conditions. A mixture of buffer and substrate was then added and the spore dispersion incubated for the desired time under carefully controlled conditions. After incubation, the cell-free reaction mixtures were analyzed by quantitative and qualitative methods. The detailed procedure used in each of these steps is given below.

(a) Dispersion of known weights of spores

Two methods for dispersing equal quantities of spores in experimental flasks were tried.

The first may be called a volumetric method since it involved the use of equal volumes of a standard spore suspension. An accurately-weighed sample of uredospores was added to a known volume of deionized water in a glass-stoppered Erlenmeyer flask and dispersed by vigorous shaking. Equal volumes of the suspension were then delivered to experimental flasks with a graduated pipette.

The second method used in dispersing spores into the experimental flasks was a gravimetric one and, theoretically at least, more accurate. Spores for each flask were weighed out on small glass trays using the Sartorius semi-micro balance. The weighed spores, stored until needed in grease-sealed dishes kept in the refrigerator, were then dispensed to the flasks as follows: The glass trays were carefully placed in dry Erlenmeyer flasks (usually 50 ml. capacity) so that they were suspended between the side and bottom of the vessel and the spores dumped off by flicking over the trays with the end of a glass rod or forceps. The trays were then removed. After the addition of an accurate volume (usually 5 ml.) of deionized water, the spores were dispersed.

In early experiments, spores were dispersed simply by gently shaking the mixture. A more efficient procedure was later developed using a vibro-tool (Burgess Battery

Co.) clamped upright to a heavy ring stand and adjusted to give short strokes. When the bases of the flasks were held firmly against a dull tool, spores spread quickly and evenly over the surface of the water.

(b) Germination procedure

Germination and germ tube growth took place while the dispersed spores were held for 24 hr. or longer at 20 to 23° C. in a constant temperature room.

(c) Buffer-substrate preparation

Enzyme substrates, dissolved in acetate buffer, were prepared so that the addition of a specified volume to the spore dispersions gave the desired final concentrations.

Sodium acid α-naphthyl phosphate, the substrate used in most experiments, was chosen mainly because the naphthol as well as the phosphate moiety could be colorimetrically estimated. It was synthesized as previously mentioned and recrystallized twice from aqueous methanol. Since it was strongly acidic, this substrate was adjusted to the desired pH with sodium hydroxide before being combined with buffer.

(d) Incubation procedure

Incubation, timed from the moment of substrate addition, was usually carried out in a water bath at 38° C.

The flasks were kept in place by attaching weights to them.

(e) Stopping the enzymic reaction

Several procedures for stopping the enzymic reaction were tried. The most satisfactory method simply involved filtering off the spores.

2. Analytical procedures

Quantitative colorimetric and qualitative chromatographic procedures were used to analyze the reaction mixture filtrates.

(a) Phosphorus determinations

Both inorganic and total phosphorus were determined by Waygood's (1948) modification of Lowry and Lopez's (1946) method, which permits the estimation of inorganic phosphorus in the presence of labile phosphate esters.

After allowing 15 min. for colour development, the optical density at 650 mμ. was read in a Beckman D.U. spectrophotometer. A blank prepared at the same time was used to zero the instrument. Optical densities were converted to micrograms of phosphorus by reference to a standard curve (Fig. 24) which was prepared using dilutions of a standard potassium dihydrogen phosphate solution.

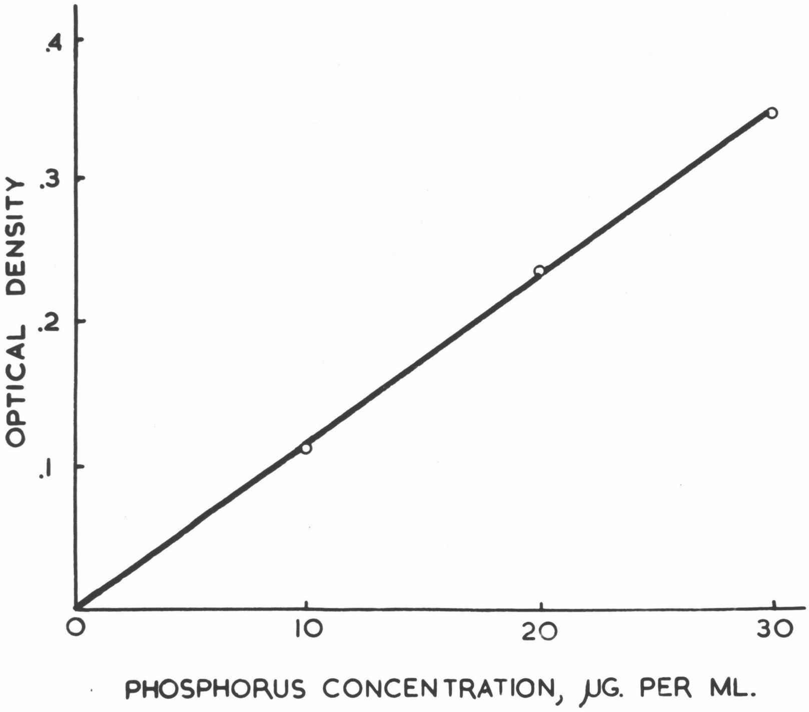


Fig. 24. The calibration curve for phosphorus.

(b) Naphthol determinations

A quantitative colorimetric method for naphthol was devised using Folin and Ciocalteu's (1927) reagent for phenols.

A standard α-naphthol solution was prepared in the following way: Five millilitres of a sodium acid α-naphthyl phosphate solution containing circa 10 mg. per ml. were refluxed for 36 hr. in a boiling water bath with 5 ml. of 2.0 N hydrochloric acid. The hydrolysate was then quantitatively transferred to a 500-ml. volumetric flask using 20 ml. of 1.0 N sodium acetate and sufficient deionized water to make to volume. A blank consisting of 5 ml. of deionized water and 5 ml. of 2.0 N hydrochloric acid was prepared in the same manner and at the same time. The level of inorganic phosphorus in the hydrolysate was then determined and the concentration of α-naphthol which must have been simultaneously released from the naphthyl phosphate calculated.

The method devised to estimate α-naphthol colorimetrically was based largely on the findings of Gottschalk (1948), who carefully studied the conditions optimal for colour development using phenol and the Folin-Ciocalteu reagent. This reagent was prepared exactly as described by Folin and Ciocalteu (1927).

An aliquot of a naphthol-containing solution (usually 2 ml., but, if less, sufficient deionized water was used to make up the difference) was added to a clean test tube. To this, in turn, was added 5 ml. of deionized water, 3 ml. of Folin-Ciocalteu reagent diluted 1:3 with deionized water and 10 ml. of 5% (w/v) aqueous sodium carbonate (anhydrous). After stirring the mixture vigorously with a glass rod, the tubes were stoppered and incubated in a water bath at 38° C. for 1 hr. to fully develop the colour.

A standard curve for the estimation of α-naphthol (Fig. 25) was prepared by using dilutions of the standard naphthol solution and following the procedure for colour development outlined above. Optical densities were determined using a Beckman D.U. spectrophotometer set at 755 mμ, the wave length found to give maximum absorption (Fig. 26).

(c) Chromatographic procedures

Transferase reaction mixtures were analyzed by ion-exchange and filter paper chromatographic methods. Ion-exchange resins were used to separate any phosphate esters formed by the transferase activity of phosphatase from the high levels of glucose required in these experiments. Subsequently, paper chromatography was used to identify any phosphate esters thus isolated.

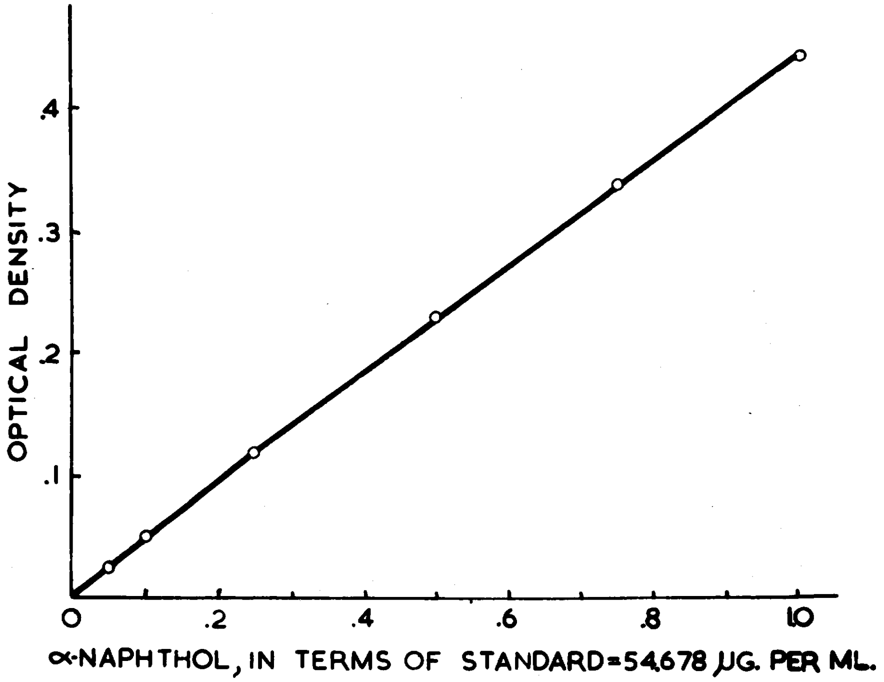


Fig. 25. The calibration curve for α-naphthol.

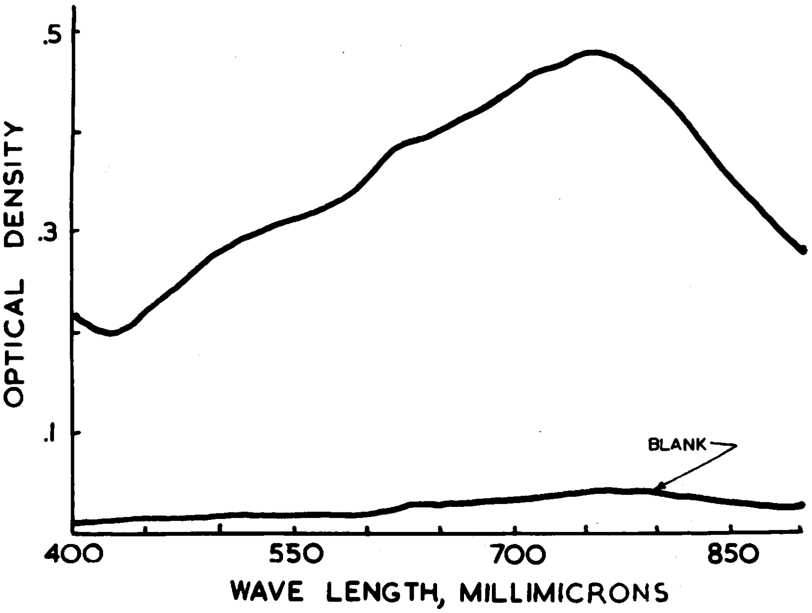


Fig. 26. Absorption spectra of α-naphthol and reagent blank using the Folin-Ciocalteu reagent. Maximum absorption at 755 mμ.

Cationic (Amberlite IR-120) and anionic (Amberlite IR-4B) synthetic resins were used in the form of columns (1.0 x 20 cm.). Prior to use, the cationic and anionic resins were regenerated with 10% hydrochloric acid and 4% sodium carbonate, respectively, and then thoroughly washed with deionized water.

The transferase reaction mixtures, followed by deionized water, were first passed (15 drops per min.) through the cationic column and the eluate collected until it no longer showed the presence of glucose using Dreywood's anthrone reagent (Morris, 1948). The eluate, followed by deionized water, was then passed through the anionic column. Washing with water was again continued until a positive reaction with anthrone reagent was no longer obtained. The eluate was discarded.

Four per cent ammonium hydroxide was used to elute the anionic resin. This eluate, collected until it showed a pH of about 10 to 11, was then concentrated in the draft of a fume cupboard.

The concentrated eluate was then qualitatively analyzed by one-dimensional paper chromatography. Whatman #1 filter paper was washed, twice in each direction, first with 0.1 N hydrochloric acid and then with frequently-changed deionized water (Hanes and Isherwood, 1949). The papers were allowed to dry between washings. A Gilmont ultramicro burette was used to spot the unknown and known samples.

Two solvent mixtures recommended by Hanes and Isherwood (1949) for the separation of phosphate esters were tried. The first, a basic, water-miscible one, consisted of n-propanol-concentrated ammonia-water (6:3:1 volumes, respectively). The second, an acid, water-immiscible solvent, was composed of tert.-amyl-alcohol-water 90% formic acid (3:3:1 volumes, respectively). The papers, placed in all glass cabinets, were allowed to equilibrate overnight before adding the solvent system. After removal, the papers were dried in the fume cupboard.

Colour development was essentially as described by Hanes and Isherwood (1949). After spraying with an acid molybdate reagent, the papers were heated for 10 min. at 80° C. (Isherwood, 1954) and then removed to a hydrogen sulphide atmosphere where the full colour developed.

B. Results

1. Preliminary experiments

(a) Demonstration of phosphatase activity

Procedures for measuring phosphatase activity of germinated rust spores were worked out in preliminary experiments. In the first of these, all procedures were asceptically performed since it was not known if bacterial contaminants would be a complicating factor in the assays. Flasks, pipettes and deionized water were autoclaved and the buffer-substrate mixture, consisting of sodium acid α-naphthyl phosphate in acetate buffer (pH 5.0), was Seitz filtered.

Three of four 125-ml. Erlenmeyer flasks containing 8 ml. of deionized water were "seeded" with approximately 5.68 mg. of rust spores by pipetting in 2 ml. of a spore suspension. The possibility that bacterial contaminants on the rust spores might possess phosphatase activity was taken into account by adding 2 ml. of spore suspension filtrate to the fourth flask. The flasks, stoppered with cotton plugs, were then placed in the constant temperature room for 24 hr., at the end of which time one of those containing spores was autoclaved. To this and all but one spore-containing flask, 5 ml. of deionized water were added. Five millilitres of 0.004 M sodium molybdate, a strong inhibitor of acid phosphatase, were added to the remaining flask. The addition of buffer-substrate to all flasks gave final buffer, substrate and molybdate concentrations of 0.01, 0.005 and 0.001 M, respectively.

Following an 18-hr. incubation at 37° C., the reaction mixtures were filtered. Four-millilitre aliquots of these filtrates were dispensed to 25-ml. volumetric flasks and inactivated by the addition of 10 ml. of cold 15% trichloracetic acid. The pH was adjusted to 4.0 by the addition of 6 ml. of 2 N sodium acetate and the solution made to volume with deionized water. Inorganic phosphorus determinations on 4-ml. aliquots gave the results shown in Fig. 27. At the end of the experiment the pH of the reaction mixture filtrates was about 5.1 to 5.2.

These results indicated that germinated rust spores possessed acid phosphatase activity which could be quantitatively estimated by a procedure similar to that used. They also suggested that bacterial contaminants of rust spores would not prove to be a complicating factor in these experiments since the spore filtrate showed negligible activity.

(b) Determination of pH optimum

Initial experiments to determine the optimal pH for

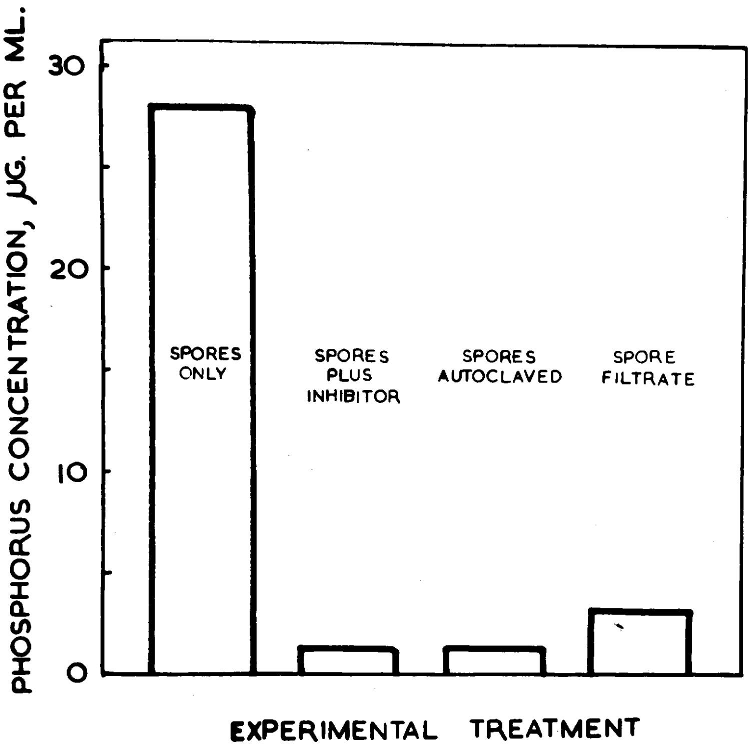


Fig. 27. Phosphatase activity of germinated rust spores and its inhibition by 0.001 M sodium molybdate. Note the low phosphatase activity of the spore filtrate as shown by the fact that the phosphorus level is only slightly in excess of that due to spontaneous hydrolysis of substrate (cf. autoclaved spores). Substrate, 0.005 M α-naphthyl phosphate (pH 5.0).

the enzyme, although unsatisfactory from that point of view, were of value in the development of techniques.

For example, in the first experiment the experimental flasks were immersed for ½ hr. in boiling water at the end of the incubation period in order to stop the enzymic reaction. However, the high and variable phosphorus levels of check flasks containing no spores indicated that the substrate was too labile for this to be an acceptable procedure.

In the second pH experiment, the feasibility of stopping the enzymic reaction simply by filtering off the spores was shown by the fact that the inorganic phosphorus level of reaction mixtures, simply filtered after incubation with rust spores, did not vary greatly over the 24-hr. period they remained at room temperature. Incidentally, this result also demonstrated that it was unnecessary to employ sterile technique since neither had the apparatus used in this experiment been autoclaved nor the buffer-substrate mixture Seitz filtered. Highly variable results between triplicate flasks were considered to be caused by spores sticking to the sides of flasks following their dispersion by gentle shaking.

Because of this difficulty, the use of equal volumes of a standard spore suspension rather than the theoretically more accurate gravimetric method seemed the most reliable way, at that time, of obtaining reasonably close agreement between duplicate flasks.

In the next experiment, using a graduated pipette with a wide orifice, 10 ml. of a suspension equivalent to 5 mg. of spores were delivered into 50-ml. Erlenmeyer flasks. After 24 hr. in the constant temperature room, 10 ml. of the appropriate buffer-substrate mixture were added to each flask. The final concentrations of the sodium acid α-naphthyl phosphate and acetate buffer used were 0.005 and 0.01 M, respectively. Duplicate experimental flasks, as well as check flasks having no spores, were set up at pH 4.0, 4.5, 5.1, 5.2 and 5.5. Incubation at 38° C. was ended after 12 hr. by filtering off the spores. Inorganic phosphorus determinations on 1-ml. aliquots gave the results shown in Fig. 28.

Because the filtrates showed pH increases of 0.05 to 0.1 units and because considerable variation between duplicate flasks was found in the region of the pH optimum, the experiment was repeated. The only change in procedure was the use of a stronger buffer (final concentration 0.03 M) at pH 4.0, 5.0, 5.1, 5.2, 5.3, 5.4 and 5.5. Incubation was ended after 10 hr. by filtering off the spores. Fig. 29 represents the results of inorganic phosphorus

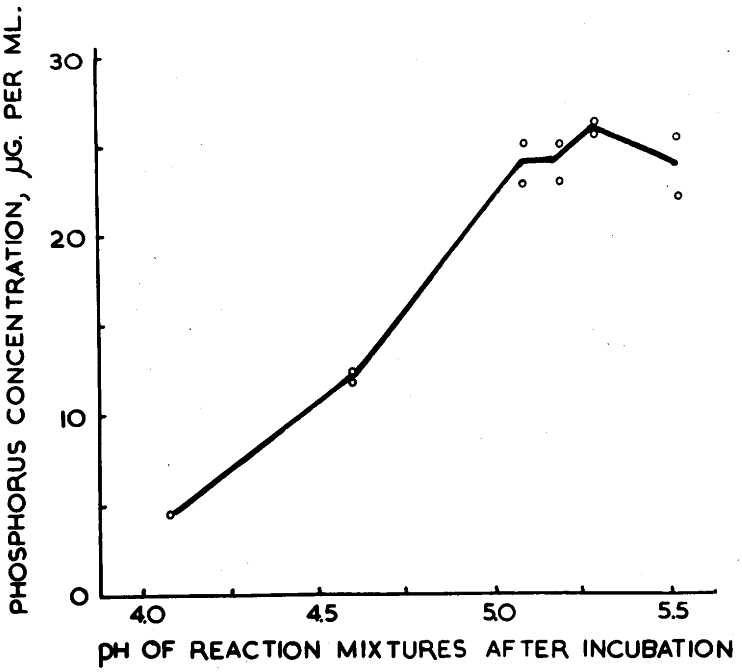


Fig. 28. Effect of pH on the hydrolysis of 0.005 M α-naphthyl phosphate by germinated rust spores in duplicate flasks. Sodium acetate buffer, 0.01 M.

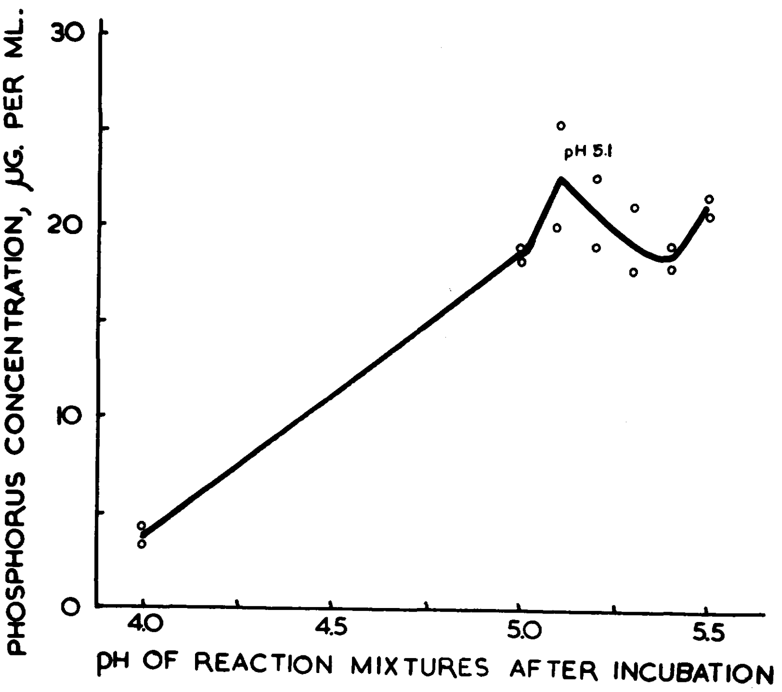


Fig. 29. Effect of pH on the hydrolysis of 0.005 M α-naphthyl phosphate by germinated rust spores in duplicate flasks. Sodium acetate buffer, 0.03 M.

determinations made on 1-ml. aliquots.

Although no change in the pH of these reaction mixtures was detected, variable results between duplicate flasks were again evident in the region of the pH optimum. The increased activity at pH 5.5 is not in agreement with the previous experiment but the accuracy of this result was not checked. The fact that the variation was greatest at pH 5.1 and progressively decreased with increasing pH suggested that pH 5.1 was near optimal for the enzyme since differences due to unequal numbers of spores in duplicate flasks would be most obvious under conditions of greatest activity. This pH was therefore used in all subsequent experiments.

The validity of stopping the enzymic reaction simply by filtering off the spores was further quantitatively substantiated in this experiment. Filtrates at pH 5.1 from both a blank and a seeded flask were stored at 21° C. and inorganic phosphorus determinations periodically made on 1-ml. aliquots. The data in Table II show that, although a certain amount of substrate was hydrolyzed after filtration, the difference in inorganic phosphorus level between control and seeded flasks varied little even after four days. Hydrolysis of substrate by filterable rust spore contaminants was therefore considered

Table II

Mean Optical Densities found when Inorganic Phosphorus Determinations were made on Filtrates from Seeded and Unseeded Flasks over a Four-Day Period

Source of filtrate	Determination		
	Initial	After one day	After four days
Seeded flask	0.348	0.353	0.366
Unseeded flask	0.060	0.062	0.070
Difference	0.288	0.291	0.296

negligible.

2. Hydrolysis of glucose-1-phosphate by germinated and ungerminated spores

An experiment in which glucose-1-phosphate was used as substrate demonstrated not only the greater phosphatase activity of germinated as compared to ungerminated spores but also suggested that the enzyme was localized on the surface of the spores and germ tubes.

Four of 11 50-ml. Erlenmeyer flasks containing 5 ml. of deionized water were seeded with 5 mg. of field-collected rust spores (probably a mixture of stem and leaf types) which were then dispersed using the vibro-tool. All the flasks were left for 24 hr. in the constant temperature room. Then, after first dispersing 5 mg. of spores in four of the unseeded flasks, dipotassium glucose-1-phosphate in acetate buffer (pH 5.05) was added to all flasks. The three unseeded flasks served to measure nonenzymic hydrolysis of the substrate. The final concentrations of the buffer and substrate were 0.03 and 0.005 M, respectively.

After incubation at 22 to 23° C. for 10 hr., the spores were filtered from all but one flask of each of the two inoculated groups and phosphorus determinations made on 1-ml. aliquots. Germination checks were made on the spores in the two unfiltered flasks.

Spore germination in the flask inoculated 24 hr. prior to the addition of the reaction mixture was strong, with a mycelial-like mat of germ tubes formed. Virtually no germinated spores were observed in the other seeded flask. The results of the phosphorus determinations are shown in Fig. 30. Obviously, the germinated spores hydrolyzed over twice as much substrate as ungerminated ones. But even the ungerminated spores hydrolyzed considerable substrate, as comparison with noninoculated flasks shows.

Since glucose-1-phosphate penetrates living cell membranes only with difficulty, its ready hydrolysis by rust spore phosphatase suggests that the enzyme is surface localized. This possibility is strengthened by the fact that the germinated spores hydrolyzed nearly twice as much substrate as ungerminated ones. Although this result could be due to the activation of phosphatase during germination and/or to easier penetration of the substrate, it is also explainable on the basis of the increased surface area provided by the germ tubes of germinated spores.

3. Transferase activity

(a) Demonstration of transferase activity

Since phosphatases have been reported capable not only of hydrolyzing phosphate esters but also of

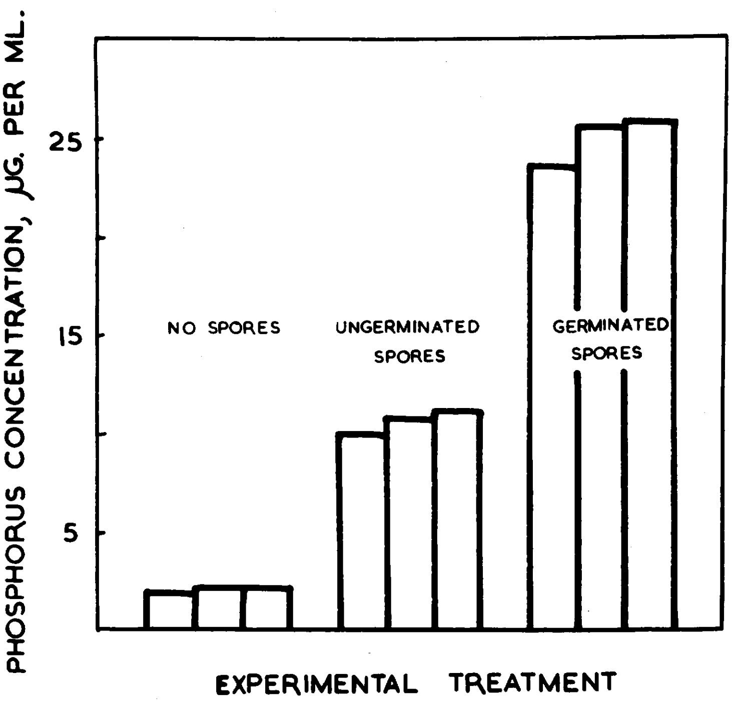


Fig. 30. Phosphorus released from 0.005 M glucose-1-phosphate by spontaneous hydrolysis (no spores) and by the phosphatase activity of germinated and ungerminated rust spores in triplicate flasks.

transferring a portion of the ester phosphate to suitable acceptor compounds, it was of interest to determine whether or not the acid phosphatase(s) of rust spores possessed such activity. An experiment was therefore set up to test this possibility, using α-naphthyl phosphate as donor substrate and glucose as potential phosphate acceptor.

Because it was not known what concentration of acceptor would provide maximum transferase activity, various levels of glucose dissolved in a mixture of sodium acid α-naphthyl phosphate and acetate buffer (pH 5.1) were used.

Due to unforeseen difficulties, the flasks seeded with 5 ml. of a suspension equivalent to 5 mg. of spores remained in the constant temperature room for 52 hr. before addition of the reaction mixtures. The final glucose concentrations were 0.00, 0.313, 0.625, 1.25 and 2.50 M while final buffer and substrate concentrations were 0.03 and 0.005 M, respectively. Each level of glucose was added to one unseeded and three seeded flasks, the former providing a check on nonenzymic hydrolysis. Incubation at 38° C. was ended after 6 hr. by filtering off the spores.

Naphthol and phosphorus determinations were made on 1-ml. aliquots of the filtrates and the ratio, μ moles naphthol/μ moles phosphorus, calculated. Transferase activity was indicated when this ratio exceeded unity, the value theoretically expected if the substrate was simply hydrolyzed.

The results obtained are shown in Fig. 31. Obviously, maximum transferase activity was obtained with glucose concentrations of 1.0 M or greater. The hydrolase flasks (no glucose supplied) gave ratios somewhat higher than the expected value of unity; however, this discrepancy was thought to be due to slight inaccuracies in the estimation of naphthol and/or phosphorus. Corrections can be made by dividing the ratios obtained in the presence of the acceptor by the average ratio obtained in the absence of the acceptor (cf. Davison-Reynolds *et al.*, 1954). Although this experiment was conducted before the vibro-tool method for dispersing spores had been perfected, the variations between similar flasks could not have been due to unequal numbers of spores caused by some sticking to the sides of the vessel since the results are expressed as a ratio. A technical feature of this experiment worthy of note was the finding that naphthol values varied linearly with glucose concentration in check flasks containing no spores (Fig. 32), indicating some reaction between glucose and the Folin-Ciocalteu reagent.

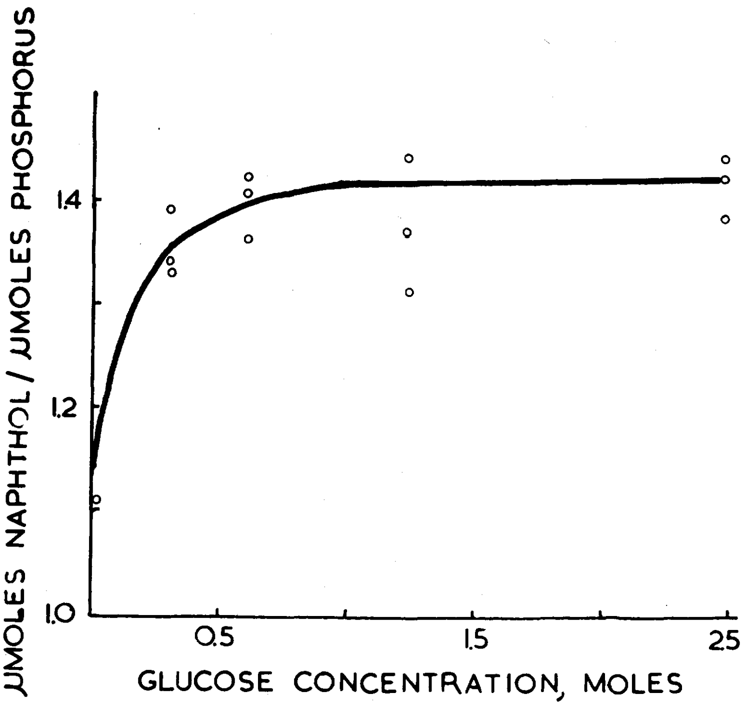


Fig. 31. Transferase activity shown by germinated rust spores in triplicate flasks containing varying levels of glucose and 0.005 M α-naphthyl phosphate. (µ moles naphthol/µ moles phosphorus = transferase ratio)

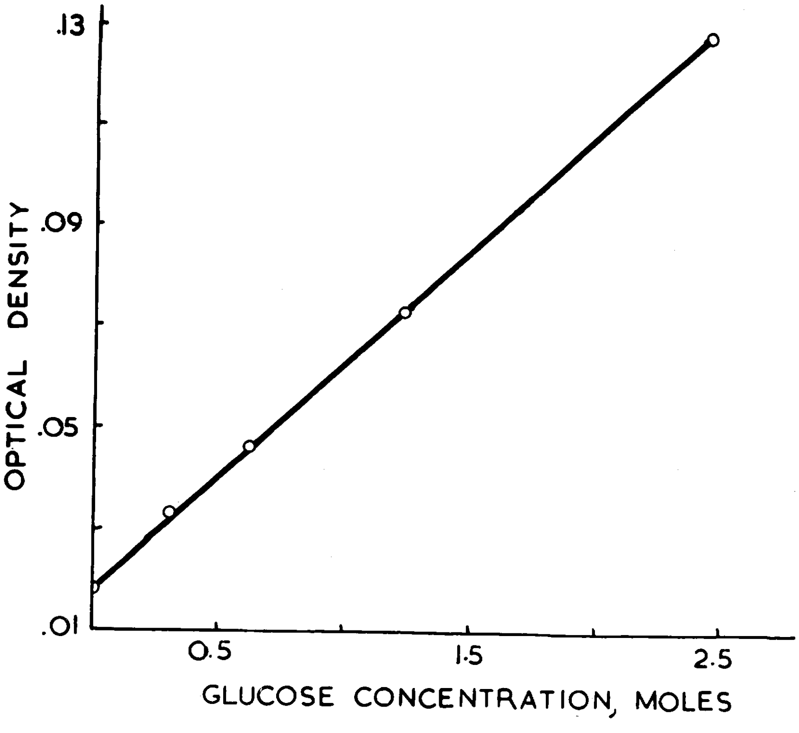


Fig. 32. Linear variation of optical density with glucose concentration found when naphthol determinations were made on unseeded flasks and indicating some reaction between glucose and the Folin-Ciocalteu reagent.

(b) Inhibition by molybdate

Initial measurements on the activity of rust spore phosphatase (Fig. 27) showed that, on the basis of phosphorus determinations, 0.001 M sodium molybdate was strongly inhibitory. In order to determine if molybdate inhibited transferase as well as hydrolytic activity, an experiment was designed to demonstrate its effect on both.

All hydrolase and transferase reaction mixtures contained sodium acid α-naphthyl phosphate dissolved in acetate buffer (pH 5.05). In addition, transferase mixtures contained glucose and inhibited preparations contained sodium molybdate.

Sixteen flasks, 12 containing 5 mg. of vibro-tool-dispersed spores in 5 ml. of water and four containing 5 ml. of water only, were left in the constant temperature room for 25 hr. prior to the addition of the reaction mixtures. Each mixture (hydrolase and transferase control and inhibited) was supplied to one unseeded and three seeded flasks, the former serving as a check on nonenzymic hydrolysis. The final concentrations of naphthyl phosphate, buffer, glucose and sodium molybdate were 0.005, 0.03, 1.0 and 0.001 M, respectively.

Incubation at 38° C. was stopped at the end of 5 hr., the spores filtered off and phosphorus determinations made on 1-ml. aliquots. Naphthol levels in uninhibited flasks were determined on 1-ml. aliquots but 2-ml. aliquots were required for the determinations on inhibited reaction mixtures.

When it was found that flasks containing molybdate gave naphthol values lower than the corresponding phosphorus determinations indicated that they should have been, an experiment to check this anomaly was carried out. Determinations were made on a standard α-naphthol solution both in the presence and absence of 0.001 M sodium molybdate. The values were 2.187 µg./ml. lower in the presence than in the absence of the inhibitor, indicating some interaction between sodium molybdate and the Folin-Ciocalteu reagent. The naphthol values previously obtained for the inhibited flasks were therefore corrected and the ratios, µ moles naphthol/µ moles phosphorus, calculated.

Fig. 33 graphically presents the results of this experiment. It should be noted that the ordinate scale for the inhibited series is 1/20 of that for the control series. Although molybdate inhibited 95% of the hydrolytic activity, the ratios obtained in both control (average 1.08) and inhibited (average 1.04) flasks were of the same order of magnitude. The ratio averaged 1.32 for the

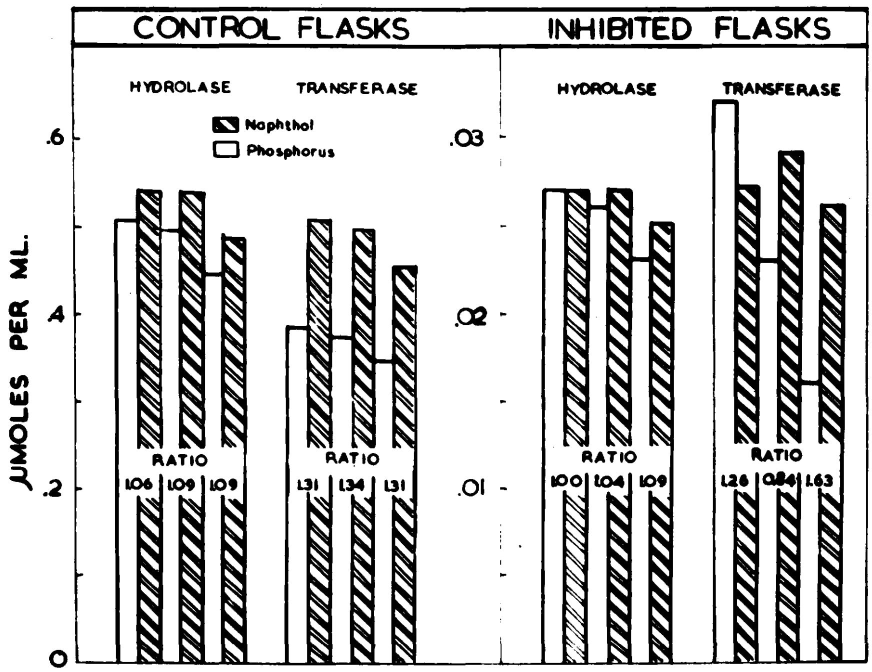


Fig. 33. The effect of inhibitor (0.001 M sodium molybdate) on the naphthol and phosphorus levels and transferase ratios (µ moles naphthol/µ moles phosphorus) found when germinated rust spores in triplicate flasks were incubated with α-naphthyl phosphate in the presence (transferase) and absence (hydrolase) of glucose. Note that the ordinate scale for the inhibited series is 1/20 of that for the control series.

control transferase flasks but reliable ratios were not available for the inhibited transferase flasks because of obvious errors (large variations between triplicate flasks) in phosphorus determinations. However, the naphthol determinations for this group appeared reliable (close agreement between flasks) and calculations based on these values yielded the desired information.

The ratio, μ moles naphthol/μ moles phosphorus, theoretically unity in the presence of hydrolytic activity, averaged 1.08 for the hydrolytic control flasks of this experiment. This slight discrepancy, considered due to errors inherent in the method used to estimate naphthol and/or phosphorus, was corrected for in calculating the transferase ratio. The latter (average 1.32) was divided by 1.08 to give a corrected transferase ratio of 1.22. This meant that, of the total amount of naphthol released in the transferase flasks, 82% was due to hydrolytic breakdown and 18% to transferase breakdown of the substrate. The calculation was as follows:

$$\frac{\text{Naphthol}}{\text{Phosphorus}} = \frac{1}{x} = 1.22$$

$$x = 0.82$$

That is, only 82% of the phosphorus expected, on the basis of the amount of naphthol found, was actually detected. The other 18% was apparently transferred to glucose via the transferase activity of phosphatase. Consequently, the average naphthol level of 0.484 μ moles/ml. found in control transferase flasks was made up of 0.396 μ moles resulting from hydrolytic and 0.088 μ moles resulting from transferase activity. Therefore, if molybdate completely inhibited hydrolase activity but had no effect on transferase activity, at least circa 0.088 μ moles/ml. of naphthol should have been found in the inhibited transferase flasks. Fig. 33 shows that this was not so, only an average of 0.027 μ moles/ml. being found.

That this was the correct order of magnitude to be expected if hydrolase and transferase activities were similarly affected by molybdate was shown by calculations based on the 95% inhibition of hydrolytic activity actually obtained. Thus, 5% of 0.396 and 0.088 μ moles gave values of 0.020 and 0.004 μ moles, respectively. The sum of these, 0.024 μ moles, was closely approached by the average amount actually determined (0.027 μ moles/ml.) in the inhibited transferase flasks.

(c) Attempts to substantiate transferase ratios

On the basis of transferase ratios, the foregoing experiments indicated that rust spore phosphatase possessed transferase as well as hydrolase activity. It was thought worthwhile, however, to substantiate these results by demonstrating the presence of a new phosphate ester (presumably glucose phosphate) in the reaction mixtures. This was attempted by qualitative paper chromatography.

Filtrates remaining from the transferase control flasks of the preceding experiment were combined to give a total volume of approximately 45 ml. and passed through cationic and then anionic ion-exchange columns as described under Methods.

Elution of the anionic column with 4% ammonium hydroxide recovered the phosphate esters (unused α-naphthyl phosphate as well as the suspected newly-formed glucose phosphate) and anions (phosphate and acetate) in the form of their ammonium salts. The eluate was reduced to 2 ml. in the draft of a fume cupboard, which also removed excess ammonia.

Co-chromatography of 200 µl. of the "transferase eluate," 75 µl of potassium dihydrogen phosphate (containing 30 µg. phosphorus per ml.) and 100 µl. of 0.001 M glucose-1-phosphate was carried out using the basic solvent system (n-propanol-ammonia-water). Running time was 48 hours.

The developed chromatogram showed no glucose phosphate (glucose-1-phosphate and glucose-6-phosphate having the same R_F value in this solvent) in the reaction mixture. However, in addition to a large orthophosphate spot, a small positively reacting spot was noticed at the origin.

In order to determine whether or not this spot was due to some of the obviously large amount of orthophosphate present in the mixture lagging at the origin, co-chromatography was again carried out. Two hundred microlitres of the eluate and 200 µl. of potassium dihydrogen phosphate, both calculated to contain the same level of phosphorus, were spotted and run in the basic solvent for 48 hr.

Despite the fact that the spot at the origin of the test solution did behave like orthophosphate, turning yellow immediately after spraying but becoming blue on further treatment, no similar spot was detected at the origin of the known. Fig. 34 is a photograph of the resulting chromatogram.

When the eluate was chromatographed in an acid solvent system (tert.-amyl alcohol-formic acid-water), no spot was detected at the origin (Fig. 35). Besides the large orthophosphate spot, however, a faint spot, shown by

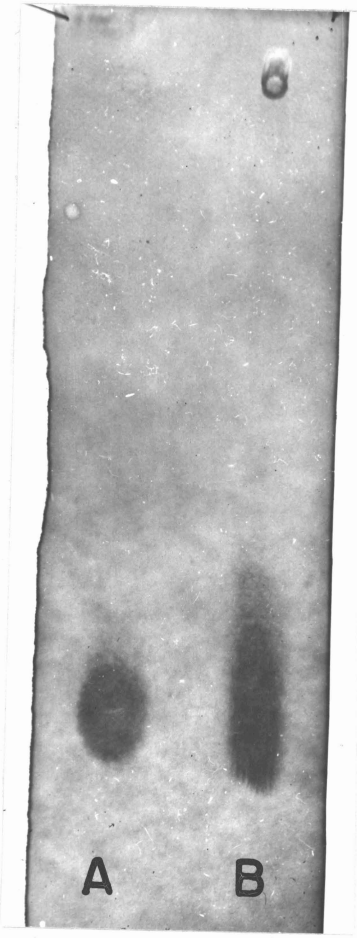
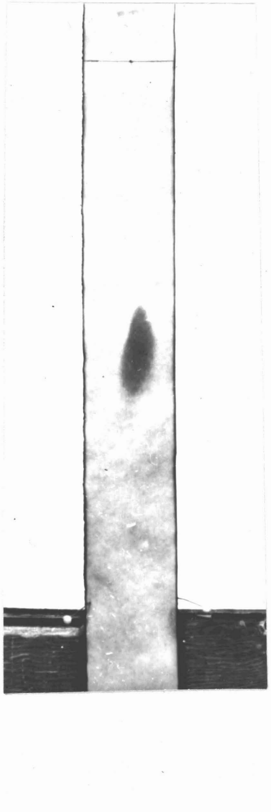


Fig. 34. Paper chromatogram showing the behaviour of (A) potassium dihydrogen phosphate and (B) the eluate separated from transferase reaction mixtures in a basic solvent (n-propanol-ammonia-water). Note the reactive spot at the origin of (B).

Fig. 35. Paper chromatogram showing that the spot at the origin (cf. (B), Fig. 34) behaves like orthophosphate when run in an acid solvent (tert.-amyl alcohol-formic acid-water).



co-chromatography to be α-naphthyl phosphate, was detected immediately behind the solvent front.

Finally, an unsprayed chromatogram, originally spotted with eluate and run in the basic solvent system, was sprayed after rechromatography in the acid solvent system. The spot, left behind at the origin in the first solvent, travelled like orthophosphate in the second solvent system.

Chromatographic analysis of transferase reaction mixtures did not, therefore, reveal the presence of any newly-formed phosphate esters. This failure to substantiate the apparent transferase activity of rust spore phosphatase (ratios greater than unity) was probably due to hydrolytic loss of the small amount of new ester phosphate formed. However, it was also possible that the spores possessed no transferase activity but that, in the presence of glucose, they selectively accumulated phosphorus following enzymic hydrolysis of the substrate. Such a mechanism would result in ratios similar to those found. An experiment was therefore designed to test this possibility. It was reasoned that if spores did take up phosphorus in the presence but not in the absence of glucose, the total phosphorus levels (organic as well as inorganic) in transferase reaction filtrates should be lower than the total phosphorus levels in hydrolase reaction filtrates.

Transferase and hydrolase reaction mixtures like those described previously, but buffered at pH 5.1 rather than at pH 5.05, were prepared. Each mixture was added to six flasks, three containing 5 mg. of spores dispersed in 5 ml. of water and three containing 5 ml. of water only. The flasks had remained in the constant temperature room for 24 hr. prior to this step. Following 5 hr. incubation at 38° C., phosphorus and naphthol determinations were made on 1-ml. aliquots and transferase ratios calculated.

Table III presents the results of these calculations. The ratios for both the hydrolase (average 0.858) and transferase (average 1.19) flasks were much lower than the values previously obtained in similar experiments. No explanation of these discrepancies, other than possible errors in the estimation of naphthol and/or phosphorus, can be given. Davison-Reynolds *et al.* (1954) likewise could not understand why ratios of 0.96 to 0.94 were obtained for some phosphatase preparations acting in the absence of acceptor whereas other preparations gave ratios of 0.84 to 0.88. However, the *corrected* transferase ratio (1.39) was actually higher than those previously obtained.

Samples for total phosphorus determinations on the

Table III

Naphthol and Phosphorus Levels (μ moles/ml.) and Transferase Ratios (μ moles naphthol/μ moles phosphorus) of Hydrolase and Transferase Reaction Mixtures after Incubation with Germinated Rust Spores

Reaction mixture	Flask	Naphthol	Phosphorus	Ratio
Hydrolase	A	0.443	0.516	0.859
	B	0.402	0.473	0.850
	C	0.455	0.526	0.865
Transferase	A	0.474	0.403	1.18
	B	0.474	0.391	1.21
	C	0.466	0.395	1.18

Table IV

Total Phosphorus Levels (μg./ml.) of Hydrolase and Transferase Reaction Mixtures after Incubation in the Presence and Absence of Spores

Reaction mixture	Flasks without spores		Flasks with spores	
	A	B	A	B
Hydrolase	15.1	15.0	14.7	14.9
Transferase	15.0	14.7	15.0	15.3

four groups (transferase seeded and unseeded, hydrolase seeded and unseeded) were obtained by combining 10-ml. aliquots from each of the three flasks in each group. Duplicate 5-ml. aliquots of these combined mixtures were wet ashed with 10 ml. of concentrated nitric acid, 4 ml. of 60% perchloric acid and 2 ml. of concentrated sulphuric acid. The cleared and cooled samples were then quantitatively transferred to 50-ml. volumetric flasks with 40 ml. of 1.0 N sodium acetate and made to volume with water. Phosphorus determinations were made on 1-ml. aliquots. Despite the fact that 40 ml. of 1.0 N sodium acetate had previously been added to the digested samples, it was necessary to use 5 ml. of 1.0 M acetate buffer (pH 4.0) rather than the usual 2 ml. of 0.1 M buffer to achieve buffering satisfactory for the colorimetric determinations.

The results presented in Table IV show no significant difference in the total phosphorus level in the presence and absence of glucose and therefore do not uphold the view that the transferase ratios are the result of phosphorus accumulation by the spores.

4. Comparison of glucose-1-phosphate and ribose-5-phosphate as substrates for rust spore phosphatase

An experiment designed to determine the relative hydrolysis of glucose-1-phosphate and ribose-5-phosphate by the phosphatase of germinated rust spores revealed also that some compound, detectable as inorganic phosphorus, was released by the spores.

This experiment was set up in the usual manner. Flasks containing 5 mg. of spores dispersed in 5 ml. of water and flasks containing 5 ml. of water only were left in the constant temperature room for 25 hr. prior to the addition of the reaction mixtures. These consisted of the two phosphate esters in acetate buffer (pH 5.1) and acetate buffer without any substrate. Each mixture was added to three seeded and three unseeded flasks. The final concentrations of buffer and substrate were intended to be 0.03 and 0.005 M, respectively. Later, however, it was found that the concentration of ribose-5-phosphate was considerably lower than that of glucose-1-phosphate. This point will be referred to again.

The barium salt of ribose-5-phosphate was dissolved in a little water by the dropwise addition of 1.0 N hydrochloric acid and converted to its disodium form by the use of sodium sulphate. After centrifuging down the barium sulphate, more sodium sulphate solution was carefully added until no further precipitation occurred. The pH of the ribose phosphate solution was adjusted to pH 5.1 with sodium hydroxide before combining it with buffer.

The glucose phosphate was in the form of its dipotassium salt.

After incubation at 38° C. for 5 hr., phosphorus determinations were made on 1-ml. aliquots of the reaction mixture filtrates. Table V reveals that an average optical density of 0.010 was found in seeded flasks containing buffer without substrate. Buffer in the absence of spores showed no absorption at the wave length used.

This finding indicated that optical densities of seeded flasks containing substrates should be corrected by subtracting not only the average value of their respective control (unseeded) flasks but also the average value of 0.010 obtained for the seeded flasks containing only buffer.

These corrected optical densities and the corresponding phosphorus levels are presented in Table VI. This data suggested that glucose-1-phosphate was more readily hydrolyzed than ribose-5-phosphate. However, total phosphorus determinations showed that the concentration of ribose-5-phosphate was considerably less than that of glucose-1-phosphate, making any comparison difficult.

After the analyses for free phosphorus had been completed, the filtrates remaining from the unseeded control flasks in each series were combined and duplicate

Table V

Optical Densities found when Inorganic Phosphorus Determinations were made on Various Reaction Mixtures after Incubation in the Presence and Absence of Spores

Reaction mixture	Unseeded flasks			Seeded flasks		
	A	B	C	A	B	C
Glucose-1-phosphate	0.026	0.026	0.025	0.337	0.336	0.331
Ribose-5-phosphate	0.011	0.011	0.009	0.182	0.178	0.188
Buffer only	0.001	0.000	-	0.010	0.010	0.009

Table VI

Corrected Optical Densities of Reaction Mixtures (see Table V) and the Corresponding Phosphorus Levels in Micrograms per Millilitre

Reaction mixture	Flask	Optical density	Phosphorus level
Glucose-1-phosphate	A	0.301	25.8
	B	0.300	25.7
	C	0.295	25.4
Ribose-5-phosphate	A	0.168	14.4
	B	0.162	14.0
	C	0.158	13.7

5-ml. aliquots wet ashed with 5 ml. of 60% perchloric acid. The digests were transferred to 50-ml. volumetric flasks with 40 ml. of 1.0 N sodium acetate and made up to volume with deionized water. Phosphorus analyses were made on 1-ml. aliquots.

The optical densities obtained are presented in Table VII. Not only is it obvious that the concentration of ribose-5-phosphate was less than that of glucose-1-phosphate (probably due to losses suffered in converting the barium salt of this phosphate ester to its sodium form) but also that the buffer contained no organic phosphate which the spores could have hydrolyzed. This last finding proves that the compound, detected as inorganic phosphorus when spores were incubated with buffer (Table V), must have been released into the medium by the spores themselves.

The fact that the two substrates were present in different concentrations makes an accurate comparison of their ease of hydrolysis by rust spore phosphatase impossible. However, the per cent organic phosphorus hydrolyzed was calculated in each case. The average optical density for the total phosphorus determinations (Table VII) made on glucose-1-phosphate (0.181) and ribose-5-phosphate mixtures (0.127) were equivalent to 15.5 µg. and

Table VII

Optical Densities found when Total Phosphorus Determinations were made on Various Reaction Mixtures

Reaction mixture	Sample		Average
	A	B	
Buffer only	0.000	0.000	0.000
Glucose-1-phosphate	0.179	0.184	0.182
Ribose-5-phosphate	0.126	0.128	0.127

Table VIII

A Comparison, in Terms of Per Cent Organic Phosphorus Hydrolyzed, of Two Phosphate Esters as Substrates for Rust Spore Phosphatase

Substrate	Flask		
	A	B	C
Glucose-1-phosphate	16.6	16.6	16.4
Ribose-5-phosphate	13.1	12.7	12.5

11.0 µg. of phosphorus/ml., respectively. Since the 5-ml. samples used for these determinations had been diluted to 50 ml., the actual phosphorus values were 155 and 110 µg./ml. These data were combined with that of Table VI to calculate the per cent organic phosphorus hydrolyzed (Table VIII). The data in Table VIII suggest that the glucose-1-phosphate was more readily hydrolyzed than ribose-5-phosphate by the rust spore phosphatase.

Part III. Effect Of Molybdate Level On Susceptibility To Powdery Mildew

A. Methods

Atlas barley grown in sand culture was supplied with varying levels of molybdate prior to inoculation with powdery mildew.

Stocks of the major and minor elements required for Hoagland's #2 nutrient solution (see Bonner and Galston, 1952) were made up at a concentration 20 times that desired in the final solution with the following modifications. Sequestrene iron (Alrose Chem. Co.) replaced ferric tartarate and no molybdenum was included in the microelement stock solution. The basic nutrient solution was prepared by combining 50 ml. of each of the major stock solutions, making up to one litre with deionized water and adding 1 ml. of the microelement stock solution.

Molybdate levels of 0, 2, 4, 6, 8 and 10 p.p.m. were supplied to the plants. Stock solutions of sodium molybdate containing 200 times these concentrations and with sufficient sodium sulphate to equalize the level of sodium in each were prepared. Thus, apart from molybdenum, sulphate was the only variable in these treatments. Irrigating solutions containing the desired level of molybdenum as well as the other elements were obtained by adding 1 ml. of a molybdate stock solution to 199 ml. of the basic nutrient solution referred to earlier.

B. Results

Atlas barley planted in glazed crocks (1-qt. capacity) containing acid-washed quartz sand was thinned to five plants per crock. Prior to inoculation the barley was grown in the environment chamber, where it received continuous light (800 F.C.). Each level of molybdate-nutrient solution (pH 5.1) was supplied as required to duplicate crocks which were periodically flushed with water.

Nineteen days after planting, the plants were removed to the greenhouse, where they were dusted heavily with mildew conidia. No differences either in growth or susceptibility to mildew were noted between treatments.

DISCUSSION

The literature dealing with alterations in plant cell walls which accompany direct penetration by a number of fungi is largely descriptive, being based on the results obtained with nonspecific staining methods. Apart from Corner's (1935) observation that circular areas about the points where *E. graminis* penetrate no longer react positively for cellulose and the finding of Fellow's (1928) that the cell wall protuberances accompanying penetration by *Ophiobolus graminis* are composed largely of lignin, there is no information concerning the nature of the induced changes. Whether or not host metabolism participates in these cell wall alterations has been the subject of discordant speculation.

The exact nature of the cell wall alterations accompanying penetration by *E. graminis hordei* cannot be deduced from the results of the limited variety of tests conducted in the present investigation. However, the fact that circular areas about penetration points stained with acid but not with basic fuchsin (Figs. 3 and 4, respectively) immediately suggests that, whatever their exact nature, the changes are associated with a net increase in electrostatic charge. But caution is required in interpreting this result. Singer (1952) pointed out that cellulose is dyed by colour ions (anions) having the same charge (negative) as the fibre, and consequently, forces other than electrostatic ones must be invoked to explain the uptake and fixing of the dye. This behaviour was evident in the present experiments, for whereas the cell walls of epidermal strips treated with acid fuchsin retained the dye during dehydration with alcohol (in which the fuchsins are soluble), those treated with the basic dye did not. Strips treated with basic fuchsin were, therefore, rinsed with water (in which this dye is virtually insoluble) and then mounted in glycerine. The very fact that, when handled in this way, the unaltered but not the altered areas of the cell wall were stained seems to indicate not only a certain amount of electrostatic binding of dye by unaltered cellulose but also an increase in the net charge of the altered region. Further suggestive evidence of a difference in electrostatic charge between the unaltered and altered cell wall is provided by the pattern of the adsorption artifact shown in Fig. 21. Apparently the lead cations present in Gomori's incubation mixture were initially adsorbed by the unaltered but not by the altered cell wall and were then transformed into lead sulphide during the normal processing routine.

The literature, however, provides no consistent supporting evidence for the binding of acid but not basic dyes by the altered penetration areas. Smith (1900), working with E. graminis, found that safranin, a basic dye, stained the normal but not the altered cell wall. On the other hand, Corner (1935), studying the same organism, reported that this dye stained the altered part. Corner also found that gentian violet, another basic dye, stained the altered region. Russel (1934) reported that circular areas about the points where O. graminis penetrated stained more intensely than the unaltered wall with acid fuchsin. More refined experiments are obviously required to fully investigate this point.

The results obtained with Post and Laudermilk's (1942) microchemical test (Fig. 5) confirm Corner's (1935) finding since they show that, initially at least, circular areas of the cell wall about penetration points no longer react positively for cellulose. An initial apparent increase in the polysaccharide content of this region is suggested by the results obtained with the periodic acid-Schiff procedure (Fig. 6). However, since this increase in reactivity is shortly followed by a decrease, except at the periphery of the altered part, an alternative explanation must be considered. Before speculating, it is interesting to note that Corner (1935) reported similar staining patterns with cotton blue. He believed that this acid dye was bound only by the part of the wall that was being altered because small blue circles in the initial stages of penetration developed into blue halos. Young (1926b), using the acid dyes Congo red and acid fuchsin, also found that either solidly stained circles or halos appeared around the points where *Alternaria*, *Helminthosporium*, *Cephalosporium* and *Colletotrichum* penetrated wheat coleoptiles. The halos he illustrated were apparently much larger than those reported by Corner (1935) or found in the present investigation since they had a diameter equal to five cell widths. He thought that perhaps some chemical diffused from the penetration hypha and caused differential chemical alteration of the cell wall in different places. Contrary to Lupton (1956), who thought it possible that attempted penetration by *E. graminis* caused the wall to swell and thus take up the stain, Young (1926b) did not consider it likely that physical alteration in the cell wall, such as increased porosity, affected the retention of the stain.

As Corner (1935) and others have proposed, an enzyme diffusing from the penetration process of *E. graminis* is probably responsible for at least the initial alterations in the cell wall accompanying penetration. A β-glucosidase, for example, would release potential aldehyde groups possibly capable of oxidizing Schiff's reagent and could thereby account for the initial apparent increase in polysaccharide (Fig. 6). The subsequent decrease in staining intensity except at the periphery of the altered part could be explained by postulating that the continued activity of the enzyme produces low molecular weight residues capable of diffusing away. The fact that the altered penetration areas demonstrated in the present investigation are, in contrast to those reported by Corner, mainly clear and sharply outlined makes the enzyme hypothesis no less attractive.

It is acknowledged that this hypothesis does not explain why the altered cell wall increases in thickness, as Corner reported, why the enzyme apparently stops acting on the cellulose except at the periphery of the diffusion zone and why the staining pattern is different when detached leaves are used. However, its validity, as far as it goes in explaining the initial increase in reactivity of the altered region to the periodic acid-Schiff procedure, could easily be checked by treating infected epidermal strips directly with Schiff's reagent. Free aldehyde groups, if present as postulated, would oxidize the

Schiff reagent and produce the characteristic red dye.

Although the initial changes in the cell wall accompanying penetration may be due entirely to an enzyme, or enzymes, diffusing from the fungus, the results of these investigations strongly suggest, contrary to Corner's (1935) views, that the metabolism of the host participates in at least some of the alterations. The reappearance of cellulose and polysaccharide reactive material as halos within initially nonreactive areas (Figs. 5 and 6, respectively) suggests that the host lays down new cell wall material. Unequivocal proof of such activity is provided by the microradioautograph (Fig. 9b), which demonstrates the deposition of radioactive carbon compounds in the region of penetration. This evidence suggests that the penetration papillas, which Corner (1935) thought resulted both from the swelling of existing cell wall materials by a fungal enzyme and the thrust of the penetration process, are, in fact, due to localized syntheses of cell wall material. Although this view has been expressed by other workers, no evidence has previously been presented for its validity. Possibly, synthetic activity on the part of the host is also responsible for the increased thickness of the cell wall which Corner (1935) observed throughout the altered area. Indeed, the microradioautograph suggests that this is true, but obviously the pattern of changes shown by these detached leaves (Fig. 9a) differs from that shown by intact leaves (Fig. 6). To determine the normal pattern of host reactions, microradioautographs of both microtome sections and epidermal strips should be made using leaves inoculated and fed radioactive glucose while attached to the plant.

In the illustrations of cell wall changes presented thus far, no haustoria are shown. There is no reason to believe, however, as Lupton (1956) suggested, that these alterations usually occur only in instances of unsuccessful or slow penetration. As was pointed out in the Literature Review, Lupton's (1956) observations are subject to question because he used cotton blue, a stain which Corner (1935) found unreliable for the detection of cell wall changes. Furthermore, Corner illustrated and described cell wall changes accompanying successful penetration. Probably no haustoria were noticed associated with the cell wall changes illustrated in previous figures because the infections were young (none of the leaves were stripped more than 48 hours after inoculation) and haustoria were not specifically looked for. Fig. 36, however, shows haustoria obviously associated with cell wall alterations. Curiously, the staining shown by this

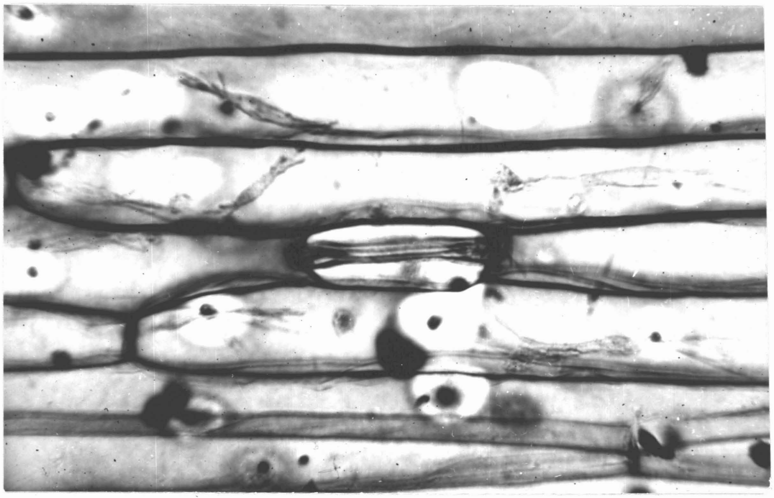


Fig. 36. Haustoria of E. graminis hordei accompanied by alterations in the cell wall about points of penetration.

strip was never repeated. It resulted when the epidermal strip, which had previously been processed by the Feulgen procedure and which had stood for some days in water, was stained with toluidine blue. There is, however, no reason to consider this an exceptional instance of cell wall alterations accompanying successful penetration, as Lupton (1956) admitted occasionally occurred. Cell wall alterations were always evident about penetration points on both the susceptible and resistant hosts, and, in contrast to Lupton's (1956) experience, there was never any difficulty in obtaining successful infection of susceptible hosts.

The numerous reports of cell wall alterations accompanying the direct penetration of a miscellany of susceptible and resistant hosts by various obligate and facultative parasites suggest that it is a common host-parasite interaction and not peculiar to the complex studied in these investigations. It would be interesting to examine the penetration of mesophyll cell walls by rust fungi for such changes. Dickinson (1949) reported that, although germ tubes of P. triticina were not able to penetrate the walls of mesophyll cells exposed by stripping off the epidermis, both the cell wall and the germ tube stained more intensely than normally with cotton blue when the two came in contact. No such changes were, however, observed when the normal infection hyphae penetrated the mesophyll cell walls. Here again, some question of the reliability of this observation arises since Dickinson used cotton blue stain.

Histochemical methods for localizing enzymes in tissues have been severely and often justly criticized. It is quite untrue, however, as Palade (1951) intimated, that nothing can be demonstrated by these methods which is not better demonstrated biochemically. For example, in the present investigation, the haustorial phosphatase could hardly have been more satisfactorily demonstrated by biochemical procedures simply because haustoria are normally associated with host tissues. Similarly, it is suggested, histochemical methods are better suited than physiological or biochemical methods for the detection of host reactions limited to one or a few cells. If, however, histochemistry is not to fall into disrepute, it must be applied critically and with an awareness of the defects and deficiencies of particular procedures.

The results obtained both with the lead-phosphate and azo dye techniques suggest that not only the haustoria of _E. graminis hordei_ but also the nuclei, guard cells and, to a lesser extent, the cytoplasm of the host possess acid phosphatase activity. Before these localizations can be accepted as accurate, two major sources of error must be considered. False localization may have resulted first from adsorption, at other than active sites, of either the enzymic reaction products or substances used in the incubation mixture, or second, from the diffusion of the enzyme itself from its original site and its adsorption at another.

Intracellular localizations of acid phosphatase by lead-phosphate methods (Gomori, 1941b, 1950) have often been considered artifacts caused by nonspecific adsorption of lead or lead phosphate (Lassek, 1947; Newman _et al._, 1950; Palade, 1951). However, artifact due to the adsorption of lead ions is eliminated in the present investigation because no staining occurred when incubation was carried out either in the absence of substrate or in the presence of sodium fluoride (Newman _et al._, 1950). This result also indicates that preformed phosphates were not responsible for the localizations obtained. Diffusion of lead phosphate from enzyme sites and its subsequent adsorption, especially by nuclei, were considered by Palade (1951) to be responsible for the lack of correlation between the results of his comparative biochemical and histochemical study. However, this does not appear to be a source of error in the present investigations since identical localizations were obtained with the azo dye procedures. Although not impossible, it seems unlikely that nuclei, guard cells and haustoria would have an affinity for such unrelated compounds as phosphate or lead phosphate produced in Gomori's (1950) procedure and α-naphthol or 6-benzoyl-2-naphthol, or their azo dyes, formed in the coupling methods of Burton (1954b) and Rutenburg and Seligman (1955), respectively. Furthermore, animal tissues processed by these azo dye techniques show no nuclear staining (Burton, 1954b; Rutenburg and Seligman, 1955). The demonstration, by the method of Rutenburg and Seligman (1955), that azo dye is localized within and not on the outside of the haustorial wall (Fig. 19) proves that this result, at least, is not a diffusion artifact. This finding and the demonstration that the germ tubes of the fungus also give a positive reaction (Fig. 20) are considered conclusive evidence that the haustoria of *E. graminis hordei* possess acid phosphatase activity.

Despite the agreement between the localizations obtained by the lead-phosphate and azo dye methods, the acid phosphatase activity shown by host nuclei, however, must still be questioned from the point of view of enzyme diffusion. Not only, as was previously mentioned, has acid phosphatase been localized in the cytoplasm and not the nuclei of animal tissues by both biochemical and azo dye histochemical methods, but also the same distribution of this enzyme has recently been reported for plant tissues by Jensen (1956). He found that only cytoplasmic particles, which he considered to be mitochondria, were stained when paraffin-impregnated sections of frozen-dried root tips (*Allium cepa*, *Vicia faba* and *Pisum sativum*) were processed by Gomori's (1950) acid phosphatase method. The sections were incubated before removing the embedding wax in order to avoid the use of organic solvents and thereby maintain the almost complete preservation of cell organelles that results from the use of freeze-drying. This procedure, originally proposed by Goetsch and Reynolds (1951), was criticized by Grogg and Pearse (1952b), who pointed out that the presence of paraffin in sections necessarily interferes with the establishment of conditions suitable for precipitation to occur.

Jensen (1956) reasoned that, since fixation in 80 per cent alcohol destroys the mitochondria, it is impossible to conclude that acid phosphatase is present in the nuclei of the living cell on the basis of activity in the nuclei of alcohol-fixed cells. He suggested that the localizations of acid phosphatase demonstrated by other investigators (Glick and Fischer, 1946; McGregor and Street, 1953) using alcohol-fixed plant tissues were artifacts, caused by the diffusion and subsequent adsorption of enzyme by nuclei and other cell structures following its release from the mitochondria. Adsorption of enzyme normally localized in the mitochondria could be responsible for the nuclear staining obtained in the present investigation since alcohol-fixed tissue was used and diffusion of some enzyme out of the strips was shown with the Rutenburg and Seligman (1955) method. Adsorption of enzyme would, of course, explain the identical localizations obtained with the lead-phosphate and azo dye methods.

This point, however, must still remain undecided for several reasons. Although Jensen (1956) is certainly justified in maintaining that freeze-drying rather than chemical fixation should be used in histochemical studies since only in that way are cell structures preserved, he is not justified in concluding that the phosphatase activity shown by nuclei in alcohol-fixed tissues is caused by the adsorption of enzyme. Jensen, however, did not admit this even when he found that frozen-dried root sections treated for two hours in 80 per cent alcohol showed no nuclear staining after incubation for two hours in Gomori's

(1950) medium. Rather, since the enzyme activity was greatly reduced by the alcohol treatment, he reasoned that the enzyme had been released by the dissolution of the mitochondrial membrane and, because sectioned not bulk tissue was used, had diffused right out of the cells. If this is correct, it is surprising that nuclear staining is observed when alcohol-fixed epidermal strips are used. Obviously, most of the decreased activity can just as readily be explained by the alcohol-inactivation of the enzyme.

Palade (1951), who found that mitochondria were destroyed by acetone, concluded that the nuclear staining shown by acetone-fixed animal tissues incubated in Gomori's (1950) medium was due to the adsorption not of enzyme but of lead phosphates formed elsewhere in the cell. He reached this conclusion after demonstrating that the artifact occurred in inactivated sections coated with a thick film of celloidin when they were incubated with acid phosphatase added to the medium. Under these conditions, he suggested, the enzyme could not reach the section but the phosphates could. The fact that Burton (1954b) reported no nuclear staining when he incubated acetone-fixed animal tissues according to his azo dye procedure for acid phosphatase suggests that Palade's

(1951) conclusion is valid. Like Jensen (1956), however, Burton used paraffin-impregnated sections. Although Grogg and Pearse (1952b) and Rutenburg and Seligman (1955) also found only cytoplasmic staining of animal tissues with azo dye methods for acid phosphatase, these reports cannot be taken as additional evidence that nuclear adsorption of enzyme does not occur. These workers used tissues fixed in formalin, which, unlike acetone and alcohol, does not dissolve away the lipid membranes of the mitochondria and therefore does not release the enzyme (Pearse, 1954). The results of the fixation experiments carried out in the present investigation, however, show that formalin is unsatisfactory for use with plant material.

Diffusion and adsorption of enzyme have also been considered as possible sources of error in histochemical localizations of alkaline phosphatase. Novikoff (1951), although admitting that diffusion of enzyme probably occurred, considered adsorption of enzyme a less important practical problem than the adsorption of calcium phosphate in the Gomori (1946) method. He found no nuclear staining when acetone-fixed animal tissues were incubated in an azo dye medium (Manheimer and Seligman, 1948). Grogg and Pearse (1952a), however, reported that with their azo dye method nuclear staining was often observed with acetone-fixed, paraffin-embedded sections but never with formalin-fixed frozen sections. Pearse (1953) concluded that this indicated either the occurrence of enzyme diffusion during acetone fixation or the activation of phosphatase in occasional nuclei by precipitation of their proteins with acetone. As Pearse (1953) concluded with reference to the nuclear staining reported by Loveless and Danielli (1949), likewise, with the results of the present investigation it seems unlikely that diffusion of phosphatase and its adsorption by nuclei could have taken place (in 80 per cent alcohol) in sufficient amount to have caused the observed result.

More studies using frozen-dried material are obviously required before the intracellular localization of acid phosphatase in plant tissues is established beyond doubt. In this connection, epidermal strips might prove useful since their use eliminates the need for paraffin embedding and would therefore avoid the equally questionable practices of incubating either before or after deparaffinization. Rutenburg and Seligman's (1955) new azo dye procedure, rather than the Gomori (1950) method that Jensen used, would obviously be the method of choice for the histochemical demonstration. Because this post-coupling procedure employs a pH optimal for the enzyme rather than one, as in Burton's (1954b) method, dictated by a compromise between enzymic and coupling reactions, the incubation period is brief and therefore the time available for diffusion artifact to occur is reduced to a minimum. Furthermore, Rutenburg and Seligman (1955) pointed out that Burton (1954b), by using acidic ethanol to remove soluble red azo dye after incubation, overintensified the difference between areas of high and low enzyme activity and thereby introduced an artifact. This may explain why, in the present investigation, guard cell nuclei showed no activity when this method was used (Fig. 15).

Possibly polyvinyl alcohol will prove to be a suitable chemical fixative for enzyme histochemistry, since Chayen and Miles (1953) reported that it preserves the mitochondria of plant cells. Whether or not, however, enzymes are inactivated by this reagent was not stated.

The slight increase in phosphatase activity accompanying increased exposure to 80 per cent alcohol or acetone suggested by the results of the present histochemical investigation and quantitatively demonstrated in the results of Stafford and Atkinson (1948) might be due to the release of bound, inactive enzyme. Release of bound enzyme by the freezing and thawing incurred in the preparation of formalin-fixed frozen sections was suggested by Grogg and Pearse (1952b) to explain why such sections had an apparently higher phosphatase activity than frozen-dried tissues. Loss of enzyme inhibitors normally present in the tissue is also a possible explanation of these increased activities.

The localization of acid phosphatase in association with the haustoria of _E. graminis hordei_ immediately suggests that this enzyme plays a role in the uptake of nutrients by the fungus. Unfortunately, there is no direct evidence for this view, but a role analogous to that postulated by Rosenberg and Wilbrandt (1952) for alkaline phosphatase in the uptake of glucose by kidney and intestinal epithelia is very compelling. Possibly phosphatases on the inner surface of the haustorial membrane function as transphosphorylases, like Rosenberg and Wilbrandt (1952) suggested, and transfer phosphate from a phosphorylated substrate-membrane carrier complex to various intracellular acceptor compounds. It is impossible, however, to demonstrate histochemically the transferase activity of phosphatases. The further discussion of this aspect of phosphatase activity in relation to nutrient uptake will therefore be postponed until the results of the quantitative studies on rust spores are considered.

If, indeed, special phosphorylated membrane transport forms are involved in the uptake of substrates by obligate parasites, the fungus itself must be capable of synthesizing them since Shu (1956) and Ali (1956) have shown that radioactive glucose and other compounds are taken up and metabolized by germinated spores of *P. graminis tritici* and conidia of *E. graminis hordei*, respectively.

Of course, it is possible that the phosphatase is, as in yeast (Rothstein and Meier, 1948, 1949), localized on the outer surface and serves only to dephosphorylate phosphate esters. If so, although Humphrey and Dufrenoy (1944) believed that only the phosphate moieties of phosphorylated compounds were taken up by *Puccinia coronata*, it seems more likely, as Rothstein and Meier (1949) demonstrated for yeast, that the organic portion is also utilized. It is impossible, on the basis of these histochemical results, to decide whether the enzyme is localized on the outer or inner surface of the membrane or if it is distributed throughout the cytoplasm. Microtome sections of haustoria incubated in Rutenburg and Seligman's (1955) mixture should show if the enzyme is distributed throughout the cytoplasm or localized at the surface. In the latter instance it might be possible, with the aid of the electron microscope, to show whether the enzyme is on the inner or the outer surface of the haustorial membrane. Brandes and Elston (1956) have recently used the electron microscope in an effort to increase the accuracy of alkaline phosphatase localizations by Gomori's method.

One possible way of determining if phosphatases play a role in the uptake of nutrients by haustoria is suggested. Epidermal strips containing living haustoria could be floated on solutions of radioactive compounds in the presence and absence of phosphatase inhibitors. If microradioautographs of these strips showed that haustoria accumulate the radioactive compound, for example, glucose, in the absence but not in the presence of phosphatase inhibitors, the evidence would be suggestive that phosphatases play a role in the uptake of that substance. However, further experiments demonstrating that the inhibitor affected only phosphatase activity would be required before the result could be considered conclusive. Sodium molybdate would probably be a satisfactory inhibitor for such experiments, since Bossard (1947) has shown that the effect of molybdate on phosphatases is specific and that molybdate does not affect the activity of β-glucosidases, proteases, lipase, oxidases, peroxidase, catalase, dehydrogenases and carboxylases found in extracts of plant tissues.

Since haustoria merely invaginate host protoplasts, nutrients required by the fungus must be released, as Thatcher (1939) and Humphrey and Duffrenoy (1944) supposed, across the plasma membrane of the host cells after an alteration in their permeability. This and other changes induced in the host, for example, the augmented respiration normally accompanying disease, probably arise from an interchange of chemical substances between the invading parasite and its host (Allen, 1953). It is interesting to consider the possibility that phosphatases are concerned in the secretion of such substances. Bradfield (1950), for example, has suggested that phosphatases may be concerned in the secretion of substances from excretory tubules.

The fact that haustorial phosphatase activity was observed in both susceptible (Atlas) and resistant (Atlas 46) hosts indicates that, at least in this instance, resistance is not a result of the absence or inhibition of this enzyme.

Although haustorial phosphatase hydrolyzed all substrates tested, it is impossible to decide from the results of these limited experiments whether one or several enzymes are involved. The stronger staining obtained when α- rather than β-glycerophosphate was used may indicate a more rapid hydrolysis of this isomer. Booth (1944), for example, observed that the total free phosphomonoesterase activity of wheat flour was greater in respect to α- than β-glycerophosphate. Sulkin and Kuntz (1947), on the other hand, found the opposite to be true of the acid phosphatase in animal tissues that they tested histochemically. The lesser staining shown by the material incubated in β-glycerophosphate may, however, be due to a lower substrate concentration, since Gomori (1952) pointed out that the lead salt of β-glycerophosphate has a lower solubility than that of α-glycerophosphate. It is impossible to make comparisons with the other substrates since experimental necessity dictated the use of different concentrations. However, the relatively short incubation (1½ to 2½ hours) required to obtain distinguishable precipitates with phosphoglyceric acid indicates a rapid hydrolysis of this substrate since a very low concentration (0.0125 M) was used. Possibly the phosphatase activity shown to be present in the haustoria is due to the activity of a mixture of isodynamic enzymes with quite narrow substrate specificities such as Roberts (1956) has reported for wheat.

Apparently the haustoria of E. graminis do not possess a glucose phosphorylase since no phosphate was released from glucose-1-phosphate in the presence of sodium fluoride, which does not affect the activity of this enzyme. There appears to be, therefore, little basis for Cutter's (1951) speculation that obligate parasites secrete a phosphorylase.

Although the quantitative studies using rust spores were carried out to complement the histochemical findings, it is recognized that the results of the two investigations bear only an indirect, analogous relationship to each other. The phosphatases present in the haustoria of _E. graminis hordei_ and the spores of _P. graminis tritici_ are probably not identical enzymes or groups of enzymes and such is not implied in the present investigation. However, a functional similarity seems a priori likely. Just as there is no reason to believe that there is any difference between the acid phosphatase demonstrated histochemically in germinated conidia and haustoria of _E. graminis_, so, too, the haustoria of _P. graminis_ probably possess the same phosphatase activity as that shown to be present in the spores. For the convenience of discussion, "enzyme" and "phosphatase" will henceforth be used, unless the text obviously indicates otherwise, with the understanding that the phosphatase activity shown by rust spores is probably due to more than one enzyme.

Pearse (1953) stated that the pH optima reported for acid phosphatases from various sources varies from pH 5.0 to 6.0, usually being about pH 5.2. Varma and Srinivasan (1954), however, reported two acid phosphatases in Aspergillus flavus with pH optima at 2.3 and 3.6 while Rothstein and Meier (1948, 1949) found the surface phosphatases of yeast to have pH optima of 3.4 (adenosinetriphosphatase and glucose-6-phosphatase) and 3.4 to 5.5 (glucose-1-phosphatase). Fig. 29 shows that the phosphatase of germinated rust spores hydrolyzing α-naphthyl phosphate in the presence of sodium acetate buffer has a pH optimum circa 5.1. Considering the range possible, this is remarkably close to the optimal pH of 5.15 reported by Booth (1944) for the phosphomonoesterase of wheat flour and suggests that the fungal enzyme enjoys an optimal pH environment in its host. The increased activity shown at pH 5.5 in Fig. 29 may be an error (cf. Fig. 28) but it might also indicate the activity of another, isodynamic, phosphatase. Obviously, a full pH activity curve using various phosphate esters should be obtained in future investigations.

The fact that the levels of inorganic phosphorus remained essentially constant after filtering the rust spores from incubated reaction mixtures (Table II) indicates not only that the phosphatase activity of bacterial and other contaminants was small but also that, under these experimental conditions at least, the enzyme was not free in the medium. Therefore, if intracellular, the enzyme is unable to diffuse through the cell membrane, or, if localized on the external surface, it is firmly bound. Rothstein and Meier (1948) similarly found that the surface phosphatases of yeast did not diffuse into the medium. Thus, it does not seem possible that diffusion or secretion of the haustorial phosphatase into host cells is responsible for the fact that the slight increase in total phosphorus accompanying infection by *E. graminis* is accountable for as inorganic, not ester, phosphate (Allen, 1953).

Although the hydrolysis of glucose-1-phosphate by germinated and ungerminated rust spores (Fig. 30) initially suggested that the phosphatase was surface localized (hexose phosphate esters penetrate cell membranes slowly), it has since been found that, after incubation under these conditions, most germinated rust spores do not appear normal when examined with the phase contrast microscope. Thus the ready hydrolysis of glucose-1-phosphate may indicate only the loss of semipermeability by injured cell membranes and therefore no conclusion regarding enzyme localization can be made. However, the results of this experiment are not without interest. Since germinated spores hydrolyzed over twice as much substrate as ungerminated ones, either the phosphatase activity of spores actually increases upon germination or, more probably, as was shown histochemically with E. graminis (Fig. 20), the enzyme is distributed throughout the germ tubes and thus is more readily accessible to the substrate. Probably the best way in which to determine whether or not the phosphatase of rust spores is surface localized is by the use of nonpenetrating inhibitors such as phosphorylated phloretin. Nonpenetrating inhibitors would also be valuable in studying the role of phosphatase in glucose uptake by haustoria of E. graminis, as previously outlined.

Why germinated rust spores were injured by incubation in buffered glucose-1-phosphate is not known. Either the sodium acetate buffer (0.03 M, pH 5.05) or the dipotassium glucose-1-phosphate (0.005 M) was responsible since similar germinated spores incubated at the same time but in the absence of buffer and substrate appeared normal. Although Shaw (1954) found that 0.2 per cent (circa 0.005 M) dipotassium glucose-1-phosphate in 0.025 M acetate buffer (pH 5.8 to 6.0) was toxic to wheat-leaf cells, 0.003 M glucose-1-phosphate (barium salt) did not injure yeast cells (Rothstein and Meier, 1949). However, Rothstein and Meier did not use buffer but merely adjusted the medium to the required pH with sodium hydroxide or hydrochloric acid. It is not possible, therefore, to decide whether the spores were affected by the high concentration of acetate buffer used or by the glucose-1-phosphate. This point, of course, should be checked in future work.

The demonstration that the phosphatase of rust spores possesses transferase as well as hydrolase activity (Fig. 31) suggests that the haustorial phosphatase of _E. graminis_ is also able to carry out transphosphorylations (see page 124). This finding illustrates the value of the quantitative studies since transferase activity of phosphatase is not histochemically demonstrable.

Morton (1953), studying the transferase activity of purified alkaline phosphatase with creatine phosphate as substrate and glucose as acceptor, found that the percentage transfer (μ moles glucose phosphate formed x 100/μ moles creatine liberated) reached a maximum value at about 2.0 M glucose and was unchanged by further increase in glucose concentration. Similarly, Fig. 31 shows that the transferase ratio (µ moles naphthol liberated/µ moles phosphorus hydrolytically liberated) of rust spore phosphatase increased with increasing glucose concentration up to circa 1.2 M glucose and thereafter remained constant. Morton (1953) showed the percentage transfer to be independent not only of donor concentration but also, contrary to Meyerhof and Green (1950), of donor bond energy. In fact, as Morton (1953) pointed out, the transfer from β-glycerophosphate to glucose forms an ester of higher bond energy than the donor. Partially-purified acid phosphatase gave essentially similar results. Morton (1953) concluded that certain compounds containing hydroxyl groups can successfully compete with water for a site at the enzyme surfaces of the nonspecific alkaline and acid phosphatases but not at those of the substrate-specific phosphatases. He stated that the group-specific acid and alkaline phosphatases clearly have a measurable affinity for acceptors such as glucose and glycerol, as well as water, and suggested that those enzymes which show a strict specificity toward the donor also show strict specificity toward water as the phosphate acceptor.

Needham (1952) pointed out that the significance of this method of phosphate transfer is still uncertain, especially as the concentration of acceptor needed, at any rate to show measurable effects *in vitro*, is very high, viz., 1.0 to 2.0 M. She thought it possible that conditions might arise in the cell under which phosphorylation by this mechanism might spare energy, for example, phosphorylation of glucose by hexose diphosphate.

Danielli (1951, 1953), referring to Goldacre and Lorch's (1950) theory that secretion is accomplished by contractile proteins, advanced the general hypothesis that alkaline phosphatases have their main function in providing energy for changes in shape of contractile protein systems. He cited the cytological distribution of the enzyme on chromosomes, newly-formed collagen and at the secretory borders of a wide variety of secretory cells in support of this view and further pointed out that the contractibility of the protein myosin is closely associated with adenosinetriphosphatase activity. According to Danielli's (1953) scheme, molecules in the external environment are transported across the membranes of secretory cells, for example, kidney and intestinal epithelia, by the folding of contractile proteins to which they become attached. This folding is caused by reaction of the intracellular portion of the protein with a substance such as adenosine triphosphate. Phosphatase is considered the enzymic centre through which the energy of adenosine triphosphate is transferred to the contractile protein. Danielli (1951) thought it probable that, for the most part, the enzyme acts not as a phosphatase, that is, hydrolytically, but as a phosphokinase, transferring a phosphate group from a high-energy bonding, as in adenosine triphosphate, to a lower-energy bonding. In such a process, the difference in energy between the two bondings would be available for protein contraction. He suggested that the transferred phosphate group might appear on the transported molecule, giving the transfer the over-all character of a transphosphorylation. Needham (1952), however, pointed out that Morton's results suggest a fundamental difference between the phosphatases and the phosphokinases in that, with the former, water is activated and acts as an acceptor competing with the organic acceptors. To this, Danielli (1951) might possibly reply that the protein of purified isolated phosphatase may well be much modified from that originally present in the cell, and that the remaining enzyme activity may be a very poor guide to the function of the enzyme in the cell. Danielli even speculated that the coexistence inside cells of phosphatases and phosphate esters is made possible by the so-called phosphatases having little or no phosphatase activity when in their correct cellular environment.

Pearse (1953) has pointed out that the finer localizations of phosphatase in much of the older histochemical work were certainly inaccurate. He suggested, therefore, that observations like those of Danielli (1951) on the association of alkaline phosphatase with contractile proteins such as chromosomes and newly-formed collagen may need revision.

Obviously, although there is little agreement as to how phosphatase participates in the transport of glucose across cell membranes and actually little direct evidence that the enzyme is involved in this process (Bradfield, 1950), many workers consider the accumulated indirect evidence more than coincidental. The demonstration that phosphatase is associated with the haustoria of obligate parasites provides additional suggestive evidence that this enzyme is involved in transport of solutes across cell membranes since haustoria are considered to be absorbing organs.

The finding (Fig. 33) that molybdate inhibits the transferase, as well as hydrolase, activity of phosphatase indicates that the continued transferase activity of yeast surface phosphatases cannot explain the uptake of glucose by this organism in the presence of molybdate (Rothstein and Meier, 1949). As was pointed out in the Literature

Review, however, it is possible that intracellular phosphatases participating in glucose transport were not affected by the inhibitor. This is not so unlikely as might at first be thought. The general metabolism of the cell is so concerned with phosphorylations that the intracellular presence of a phosphatase inhibitor such as molybdate might be expected to disrupt normal cellular activities. The carbohydrate metabolism of yeast cells was not, however, altered by the presence of molybdate (Rothstein and Meier, 1949), strongly suggesting that only surface phosphatases were inhibited. Spencer (1954) (see later), on the other hand, has presented data which suggest that the molybdate inhibition of phosphatases in tomato roots (and probably leaves) does not visibly affect the functioning of this plant.

The failure to detect glucose phosphate chromatographically in transferase reaction mixtures does not, it is considered, throw the transferase ratios into serious question. It is more than likely that the small amounts of phosphate ester involved were lost by hydrolysis during the preliminary ion-exchange separations. In investigations of this sort, Slater's (1951) enzymic method for estimating glucose phosphate should be used.

It is not surprising that rust spores, in the absence of glucose, did not accumulate phosphorus released from α-naphthyl phosphate (Table IV) since phosphate uptake is an energy-consuming process requiring the presence of a respirable substrate. However, the fact that the spores did not accumulate any phosphorus even in the presence of 1.0 M glucose, which, according to Shu (1956), is metabolized by germinated rust spores, suggests either that the permeability of the cell membranes was affected by incubation in buffer or substrate or that the amount accumulated could not be detected by the method used.

The demonstration (Table V) that some substance detectable as inorganic phosphorus is released when rust spores are incubated with buffer might again suggest that spores are damaged by 0.03 M acetate buffer. However, it is equally possible that the fungus secretes some compound into the incubation mixture. Whatever the cause, it is now clear that flasks containing spores but no substrate should have been included as checks in all the experiments. Unless some compound reacting with the Folin-Ciocalteu reagent is also released by the spores, the transferase ratios (μ moles naphthol/μ moles phosphorus) are, therefore, actually higher than calculated.

The ability of rust spore phosphatase to hydrolyze ribose-5-phosphate (Table VIII) is worth noting. Since this compound is an intermediate in the Horecker pentose cycle (hexose monophosphate shunt) by which most of the augmented respiration of rust- and mildew-infected plants is mediated (Samborski and Shaw, 1956), it perhaps is available to the fungus as a substrate.

It is not surprising that the barley plants supplied with 10 p.p.m. of molybdenum showed no toxicity effects since Arnon and Stout (1939) found that tomato plants exhibited distinct injury from excess molybdenum only if the concentration in nutrient solutions exceeded 10 p.p.m. This suggests that inhibition of phosphatases does not unduly upset the metabolism of these plants since Spencer (1954) demonstrated that the phosphatases of intact tomato roots were inhibited by circa 75 per cent when the concentration of sodium molybdate in the external solution was 10^{-4} M ($\equiv$ 10 p.p.m. Mo). Although there is no proof, there seems little doubt that leaf phosphatases were similarly affected.

While the molybdate concentration in the leaves of the treated barley plants may have been lower than in the roots, it is possible that a local concentration of the inhibitor was present about the mildew pustules since Shaw and Samborski (1956) have shown that many substances accumulate at infection sites. If the haustorial phosphatase is involved in the uptake of nutrients by the parasite, it is surprising that plants receiving 10 p.p.m. molybdate did not show an altered disease reaction. However, no conclusion about the role of the haustorial phosphatase can be made from the negative results of this experiment because it is not known whether or not the enzyme was inhibited.

This research, although limited to an investigation of the cell wall changes accompanying penetration by *E. graminis hordei* and the phosphatase associated with the haustoria of this fungus, has demonstrated the value of a modern histochemical approach in the study of host-parasite relations. Histochemistry, by combining the attributes of physiology and biochemistry with those of cytology, enables the analysis of the host-parasite complex to be carried down to the cellular, if not the subcellular, level and thereby supplies information not otherwise attainable.

Histochemical methods, however, have their defects and deficiencies, as is evident in these investigations, and, as Pearse (1953) pointed out, those who apply them should be aware of the danger of drawing absolute conclusions from single tests. This is the criticism that must be directed at Humphrey and Dufrenoy's (1944) histochemical study of host-parasite relations. Neither their methods nor their approach seem sufficiently critical to warrant much confidence in the results they obtained. Possibly for this reason, the histochemical approach has not, until the present investigation, been utilized in subsequent studies of host-parasite relations.

The author anticipates that, in conjunction with biochemical and tracer techniques, modern histochemical methods will be increasingly applied to problems in physiological plant pathology during the next five years.

SUMMARY

1. Histochemical and histological staining methods, as well as microradioautography, were used to study changes in the cell walls of both susceptible (Atlas) and resistant (Atlas 46) barley varieties accompanying their direct penetration by *Erysiphe graminis hordei*. The exact nature of the altered cell wall could not be decided from the limited variety of tests used, but the results suggested that the changes were accompanied by an increase in electrostatic charge.

An enzyme, possibly a β-glucosidase, diffusing from the fungus was considered responsible for the initial cell wall changes. However, the staining patterns shown by the penetration area when histochemical methods for cellulose and polysaccharide were used indicated that the metabolism of the host participated in later alterations. Microradioautographs showing that carbon compounds were laid down about the point of penetration further demonstrated that the host plays a role in the cell wall changes.

2. Three histochemical methods for acid phosphatase showed enzyme activity associated with the haustoria of *E. graminis hordei*. Although the nuclei, guard cells and, to

a lesser extent, the cytoplasm of the host also showed phosphatase activity, only the haustorial localization was considered beyond question.

All substrates tested, viz., α- and β-glycerophosphate, glucose-1-phosphate, phosphoglyceric acid, α-naphthyl phosphate and 6-benzoyl-2-naphthyl phosphate, were hydrolyzed by both the fungal and host enzymes and it was suggested that probably more than one phosphatase was present.

3. Quantitative studies on the phosphatase activity of germinated rust spores (_Puccinia graminis tritici_) supplemented the histochemical demonstration that acid phosphatase was associated with the haustoria of _E. graminis_. Rust spore phosphatase showed optimal activity at circa pH 5.1, did not diffuse from the spores and possessed transferase as well as hydrolase activity, both of which could be inhibited with molybdate. The enzyme or enzymes concerned hydrolyzed glucose-1-phosphate, α-naphthyl phosphate and ribose-5-phosphate. Although the spores readily hydrolyzed glucose-1-phosphate, a compound which penetrates living cell membranes only with difficulty, it could not be concluded that the enzyme was surface localized. Spores incubated with buffered glucose-1-phosphate did not appear normal and it was considered possible that the semipermeability of the cell membranes had been

destroyed.

4. The significance of the phosphatase activity present in the haustoria of E. graminis and the spores of P. graminis was discussed in relation to its possible role in the uptake of nutrients by these parasites. It was pointed out that localization of phosphatase activity in association with haustoria, generally considered to be absorbing organs, adds to the accumulated suggestive evidence that this enzyme participates in the transport of solutes across cell membranes. On the other hand, it was suggested that the phosphatase may be surface localized and serve only to hydrolyze nonpenetrating phosphate esters or it may play a role in the secretion of substances into host cells

5. Barley plants supplied with 10 p.p.m. molybdenum exhibited no toxicity effects and no altered reaction to E. graminis. However, no conclusion could be made from the results of this experiment as to whether or not the haustorial phosphatase plays a role in the uptake of nutrients by the fungus because inhibition of the enzyme was not demonstrated.

6. It was concluded that, in conjunction with biochemical and tracer techniques, critically-applied, modern histochemical methods offer a valuable approach to

the study of host-parasite relations.

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