STUDIES ON THE REACTION OF HOST TISSUE TO INVASION BY SOME OBLIGATE FUNGAL PARASITES

A Thesis

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Abstract

A method was developed for isolating single living mesophyll cells and maintaining them in culture. The middle lamella was dissolved by protopectinase, with prior plasmolysis of the tissue to reduce the toxic factor associated with protopectinase of this enzyme. Experiments with detached cotyledons and portions of cotyledons suggest that the degree of meristematic activity in the tissue of sunflower cotyledons controls the susceptibility of the tissue to rust infection. Intense meristematic activity leads to resistance due to extreme competition for available nutrients.

TABLE OF CONTENTS

List of Text Figures

List of Plates

Introduction

The main difficulty in studying the physiology of obligate parasitism is that it is not possible to investigate the physiological requirements and peculiarities of the parasite for most of its life cycle while it is dependent upon the host. For this reason the precise relationship between the host cells and the infection hyphae is still largely a matter of speculation.

Consideration of the course of rust infection in resistant tissue, particularly in the hypersensitive host, leads to an impression that resistance is a cellular attribute as in the case of potato tissue resistant to Synchytrium endobioticum (Schipb.), Perc., rather than a case of the invaded tissue responding to infection by producing an unfavourable environment for growth of the parasite.

This has led many workers eg. Brown (1948) to suggest that the key to the problem lies in the study of the formation and function of the haustoria in relation to the individual cell. However, more recent work eg. Samborski and Shaw (1956) and Forward (1954) with susceptible and moderately resistant varieties, indicates a control of infection determined by the physiological state of the tissue, the cells of which being susceptible or resistant depending upon the conditions prevailing.

In view of the possible divergence of these hypotheses, the experiments described in this thesis were started along two main lines as follows:-

1. An investigation of the reaction of individual host cells to infection independently of the surrounding tissue.

2. The determination of the extent to which tissue response could modify the reactions of individual host cells.

In the first case a considerable amount of preliminary work was necessary to develop a method for isolating individual mesophyll cells and maintaining them in a living condition, while the most promising approach to the second line appeared to be through a study of the effect upon susceptibility of a progressive reduction of the area of infected tissue.

Literature Review

Fungal parasites pathogenic to plants may be classified into two distinct yet overlapping groups, viz.,

(a) Facultative parasites

(b) Obligate parasites

Faculative parasites normally attack a wide range of host plants. The hyphae vary from being inter- to intracellular and usually kill the infected tissue in advance living as saprophytes upon the dead tissue. Probably the most distinctive feature is the fact that they can be cultured on artificial media for at least part of their life cycle.

In contrast, obligate parasites have narrow host ranges with the ultimate formation of biotypes. The hyphae are normally intercellular with intracellular haustoria and as yet have not been cultured apart from the living host plant (Brown, 1948). The obligate parasites tend to reach a stage of balance within the host plant comparable in appearance to the mutual associations found in symbiosis.

In the later stages of infection necrosis of the host usually develops followed by decrease in vigor and in many cases death of the host tissue or plant. An attempt to explain this phenomenon was made by Thatcher (1939) in his permeability theory of rust infection. He has shown experimentally, that some rust fungi can secrete a substance or substances which promote an increase in the permeability of the host protoplast,

and considers that this secretion could lead eventually to a complete breakdown in the semi-permeable properties of the host protoplast.

The spores of many obligate parasites can be made to germinate on distilled water as well as on a wide range of synthetic media. Thus the process of germination appéars to be dependent only on the presence of adequate moisture, suitable atmospheric conditions and food reserves in the spore (Stock, 1931). There appears to be no way of prolonging this stage, although Yarwood (1948) with Puccinia triticina reported a slight increase in length of the germ-tubes under various conditions. In general, the germ-tubes of obligate parasites, germinated under artificial conditions, undergo a process of lysis about 3-4 days after germination. That the form of the germ-tubes is not independent of environmental conditions has been shown by Dickinson and others who discuss the wide variety of forms produced under various environmental conditions and upon various surfaces (Dickinson, I. 1949; Sharp and Smith, 1952). Also, Dickinson (II. 1949) has shown structures comparable in appearance to appressoria, substomatal vesicles and infection hyphae.

Such obligate parasites as the rusts are considered to draw their food supply from the host by means of the intracellular haustoria, although Rice (1927) has shown that in certain cases considerable growth of rust mycelium occurs with few haustoria present. Allen (1926) drew attention to the possible contact stimulus for the formation of haustoria in

P. triticina. She considers that when the tip of a hypha strikes against a host cell, its growth is forcibly checked for the moment, and changes preparatory to haustorium formation set in. Penetration is usually by way of a plug of narrow bore made at the point of contact similar to that produced by Botrytis cinerea when penetrating strips of cuticle from onion scales (Brown, 1948). Haustoria do not penetrate directly into the host cell but merely cause an invagination of the protoplast wall increasing the area of absorption (Allen, 1926, Fraymouth, 1956, and Rice 1927).

Thatcher (1939) in his study of permeability relationships showed that the osmotic pressure of each of the obligate parasites investigated was considerably greater than that of its host. Hence, with this higher osmotic pressure and resultant suction pressure gradient the fungus is able to remove water from the cell, whereas the increase in permeability brought on by the fungal secretion probably promotes the absorption of foodstuffs by the parasite from the cells of the host. Dickinson (IV, 1949) has reported the formation of haustoria on artificial membranes. Whether or not these were true haustoria or mere artifacts produced under the experimental conditions it is difficult to say.

Early in the study of obligate parasitism it was found that under suitable conditions normal growth of the fungus could be achieved on detached leaf cultures. Beginning about 1880 detached leaves came to be extensively used in studies

of water absorption; transpiration; respiration and photosynthesis. Farlow (1885) was one of the first, if not the first, to use detached leaves successfully for inoculation tests with parasitic fungi. Mains (1917) was the first to demonstrate clearly by means of detached leaves the important relation between the carbohydrate nutrition of the host and the development of a plant pathogen (corn rust). Detached leaf cultures have been used as a substrate for the growing of total cultures of most plant pathogens including the rusts (Arthur, 1906, Berwith, 1936, Clinton and McCormick, 1924).

The principle aspects of plant disease problems studied so far by detached leaf technique are:- carbohydrate nutrition (Mains, 1917); effect of environment on disease development (Butler and Jones, 1949, Fawcett, 1921); host range (Berwith, 1936); heterothallism (Millar, 1932); respiratory and photosynthetic changes induced by infection (Corner, 1935); and volatile fungicides (Yarwood, 1948).

The advantages of the use of detached leaves and not of entire plants for the culture of parasitic fungi are as follows:- (a) economy of space, host material, and inoculum; (b) ease and exactness of observation; (c) reduced danger of contamination; (d) uniformity of experimental units; (e) ease of control and manipulation of environmental conditions; and (f) elimination of necessity of light when sucrose is present in the medium (Yarwood, 1946).

A recent approach in the study of obligate parasitism

have been the attempts to culture such parasites on host tissue cultures. Morel (1948) was the first to report successful culturing of Plasmopora viticola on grape callus tissue and followed this by the cultivation of some other obligate parasites. Little success has been met in this direction with rust fungi. Hotson (1953) using healthy callus tissue of Juniperus virginiana has successfully cultured Gymnosporangium Juniperi-virginianae. The rust produced typical telial horns and a fungal mycelium survived in sub-cultures of the gall callus in serial transfers for over three years. The vegetative mycelium of the fungus growing aerially from the cultured host callus was binucleate, dichotomously branched and without clamp connections.

Heim and Gries (1953) have shown that Erysiphe cichoracearum can be cultured on sunflower tumour tissue. The results showed that, to a very limited extent, the powdery mildew was capable of growth on excised undifferentiated tumour tissue. It was also noted that the cultures which grew most rapidly were on the more rapidly growing tissue sections. It has been shown that obligate parasites thrive best on vigorous hosts (Butler and Jones, 1949). Since rusts do not generally grow on meristematic tissue they are unlikely to grow well on tissue culture and though a few positive results have been obtained, it must be considered, that this will not be a very fruitful field of research.

The above observations show that the culture of obligate

parasites on restricted portions of host tissue is possible, but this does not approach the fundamental problem of culturing the rust on nutrient media other than the living plant. With P. triticina Stock (1931) carried out an extensive series of trials. He attempted to culture rust on 1% agar with several C-N combinations with host autolysate and expressed sap. He concluded from his results and those of plasmolysis and vital staining that the germ-tubes were unable to take up substances in solution. Dickinson (1955) has recently shown that the germ-tubes of P. triticina can take up some vital dyes. Gretchushnikov (1936) reported the saprophytic cultivation of species of Puccinia which were kept growing for 12 days on a culture medium which contained substances that could absorb ammonia and urea produced by the growing mycelium. These compounds had been found to inhibit development of rust spores and susceptibility was considered to depend upon their continuous removal by the host plant.

Since growth completely independent of the host has never been achieved with obligate parasites little is known of their physiology. Therefore, it appears that there is some intimate physiological link between host and parasite which, as yet, has not been obtained in artificial culture. On the other hand, it is possible that the appropriate nutrients are being supplied, but are not being absorbed by the germ-tube.

Hawker (1950) suggests that these obligate parasites may obtain from the host some highly unstable enzymic complex possibly in the nature of an oxidase which they themselves are unable to synthesise.

Materials and Methods

The plant materials used throughout this project were the cotyledons and leaf tissue of Helianthus annuus var. Foundation grown from 1955 seed and using as the inoculum the urediospores of Puccinia helianthus Schw (seed and urediospores were originally supplied by Dr. W.E. Sackston, Laboratory of Plant Pathology, Science Service, University of Manitoba.)

I. The Surface Sterilisation of Sunflower Seeds

Preliminary experiments showed that the surface of normal sunflower seeds carried a heavy potential inoculum of fungal and bacterial spores and on germinating this resulted in the surface contamination of the cotyledons making them unsuitable for culture work.

A standard method of surface sterilising seeds was tried (Machacek, Cherewick, Mead and Broadfoot, 1951). The seeds were immersed and shaken up in a mixture of mercuric chloride and alcohol (1 part of 95% ethyl alcohol in 3 parts of a 0.1% aqueous solution of mercuric chloride). The results were very unsatisfactory. Short periods of immersion of up to 15 minutes did not prevent contamination of the cotyledons, while periods of immersion over 15 minutes resulted in growth abnormalities such as negative geotropism and browning of the roots, and the cotyledons were twisted and curled making them unsuitable for use.

Later, a more convenient method was developed. The seeds

were first shelled and washed during a period of 30-45 minutes in several changes of sterile distilled water. They were then removed aseptically and placed in a petri-dish containing sucrose-peptone agar of the following formula:-

Sucrose	= 10 gms
Bacteriological peptone	= 5 gms
Potassium dihydrogen phosphate	= 2 gms
Magnesium sulphate (crystalline)	= 0.5 gms
Ferric chloride	= 0.01 gms
Agar	= 15 gms
Water	= 1 litre

Within 24 hours contaminated seeds could be easily identified. The aseptic handling of seeds and tissue followed the general techniques set down by White (1943) and by Gautheret (1942).

II. The Growth of Aseptic Plants

Many techniques have been described for growing plants under aseptic conditions (De Ropp, 1946, German and Bowen, 1951, Kathrein, 1951). In almost every case the techniques are elaborate, being designed for prolonged growth of plants, thus, necessitating constant aeration and replenishment of nutrients. Such methods would be suitable, but not convenient for growing large numbers of aseptic cotyledons.

In the early stages of these experiments the surface sterilised seeds were sown on moist vermiculite or sand contained in glass jars plugged with cotton wool and all previously

autoclaved at 15 lbs. pressure for 30 minutes. The cotyledons produced under such conditions were considerably smaller than normal and there was a tendency for incurved growth to occur at the edges of the cotyledon.

Recently, a more convenient method has been developed in which the seeds previously surface sterilised and placed on agar in petri-dishes, as previously described, were removed aseptically after twenty-four hours and placed on sucrose-peptone agar in small glass vessels covered with watch glasses. The size of the vessels, 2" x 2½", was sufficient for germination and for the normal development of the cotyledon stage of the sunflower plant. Normal healthy cotyledons were obtained by this method.

III. The Production of Uncontaminated Urediospores

Sunflower plants grown under normal greenhouse conditions were inoculated with urediospores and the entire plant covered for twenty-four hours with a polythene bag to provide a saturated atmosphere for germination. At the 'flecking' stage of infection, before the sorus breaks through the epidermis, the leaves were washed once with the mercuric chloride/ethyl alcohol solution and then several times with sterile distilled water. The entire leaf was then placed in a sterilised glass tube, plugged with cotton wool and the whole supported by a stand and clamp. Spores produced under these conditions were seldom contaminated.

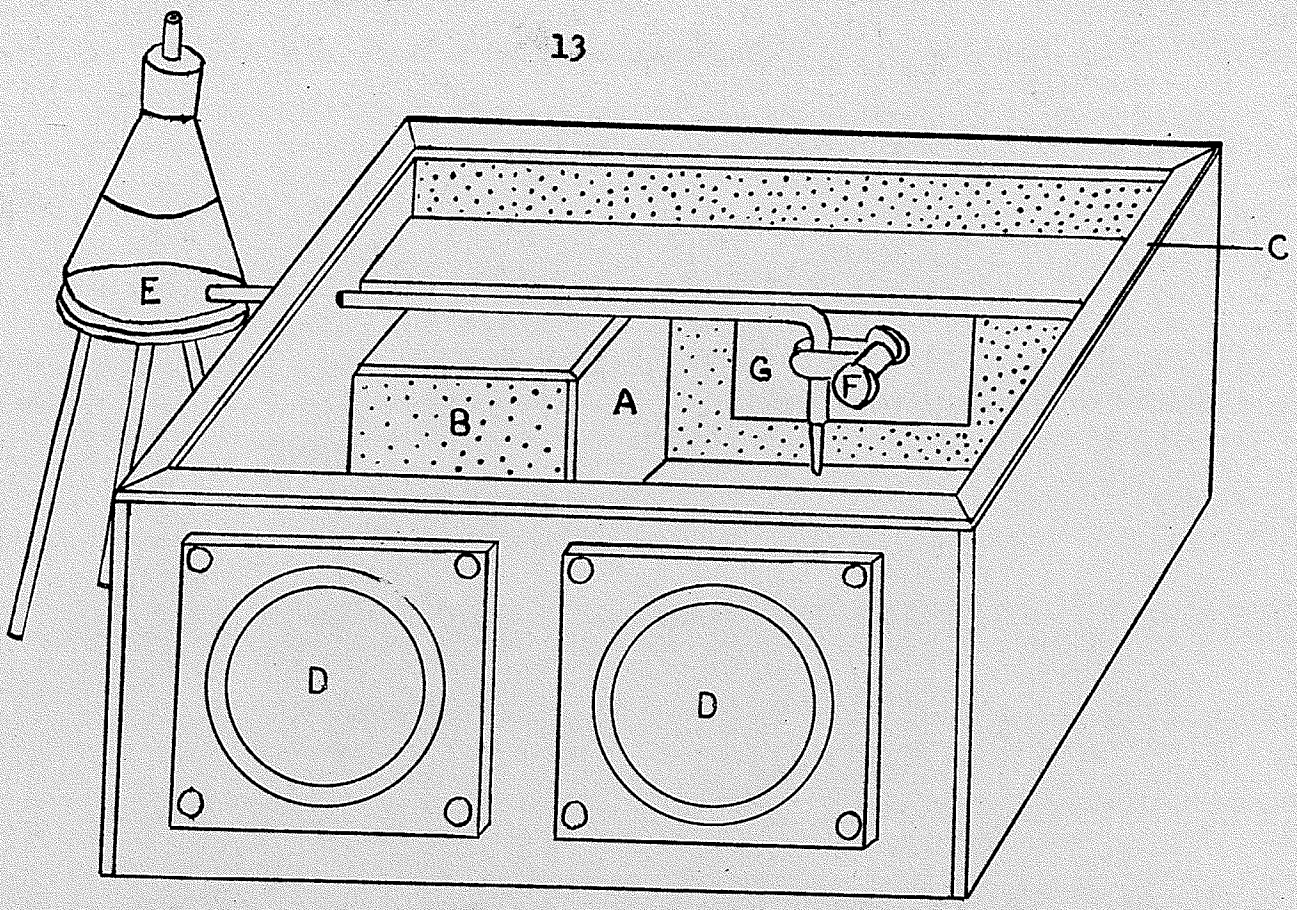
Cultures could be maintained free of contamination by growing them upon aseptic cotyledons, but when grown under these conditions the rate of pustule development was reduced as was the yield of spores.

IV. A Chamber for Aseptic Handling of Tissue

A special chamber was constructed for the purpose of prolonged handling of tissue etc. under aseptic conditions (Fig. 1). All materials and instruments previously sterilised must pass through a double-baffled entrance box (A) inserted through the rear of the chamber. This box is constructed with a hinged door (B) at either end and inside each door hangs a cloth baffle impregnated with Lysol or a similar disinfectant. With the inside door closed materials may be inserted from the rear, the inside of the box sprayed with Lysol, and the rear door closed again. The materials can then be brought into the chamber by lowering the inside door. Thus, at no point in the operation is there any direct contact between the atmosphere inside and outside of the chamber. The glass covered top (C) is removable and allows for frequent sterilisation of the chamber while the floor is covered with a cloth impregnated with Lysol.

For handling material within the chamber the operator's hands are slid into rubber gloves fitted to circular openings (D) at the front of the chamber. The gloves are of fine texture and allow almost complete freedom of movement of the hands.

The reservoir (E) containing sterile distilled water is



Text - Fig. 1. Diagram of chamber used for aseptic handling of tissue etc.

A. - Double baffled entrance-box.

B. - Hinged door.

C. - Glass covered top.

D. - Point of attachment of rubber gloves.

E. - Reservoir of sterile distilled water.

F. - Outlet fitted with pinch-cock.

G. - Rear window.

connected by glass tubing to an outlet (F) so a supply of sterile water is available within the chamber.

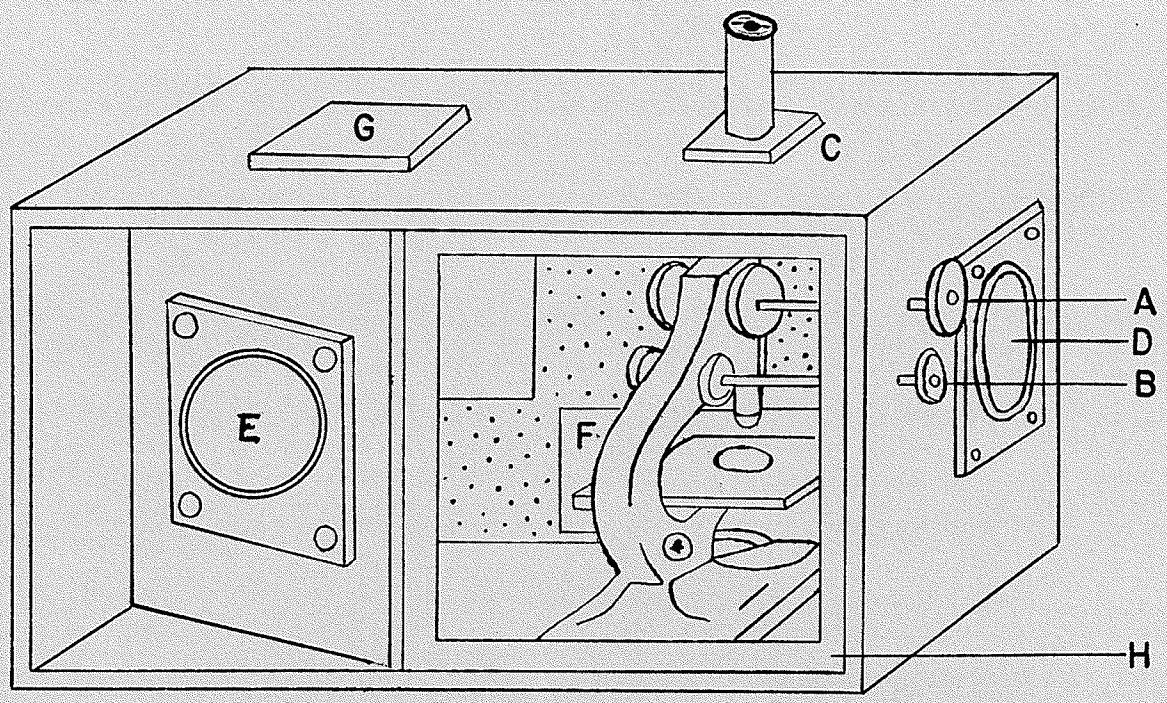
V. A Chamber for Microscopic Examination of Tissue under Aseptic Conditions.

In order to maintain aseptic conditions during microscopic examination of tissue a special chamber was constructed (Fig. 2). The coarse and fine adjustments (A and B) of the microscope are controlled from the outside by connecting-rods. A rubber connection (C) seals the projecting eyepiece to the surface of the chamber while still allowing full upward movement of the tube. The mirror and condenser can be adjusted through a rubber glove connection attached at (D). Materials to be examined are passed through a self-sealing opening made of overlapping sheets of dentine (E). Light enters the chamber at (F, G, H).

VI. Extraction and Preparation of Macerating Enzymes

A protopectinase or macerating enzyme was required in this study to dissolve the protopectin component of plant cell walls. Two commercial protopectinases were used in preliminary experiments, but neither were completely satisfactory. A fresh source of protopectinase extracted from potatoes infected with Pythium de Baryanum Hesse had very strong macerating properties.

When P. de Baryanum attacks a susceptible variety of potato tuber a characteristic soft rot develops. The diseased



Text - Fig. 2. Diagram of chamber used for aseptic microscopic examination of tissue.

A. - Coarse adjustment of microscope.

B. - Fine adjustment of microscope.

C. - Flexible rubber seal.

D. - Points of attachment of rubber gloves.

E. - Self-sealing opening.

F. - Rear window for microscopic lamp.

G. - Windows for general illumination.

H.

tissue becomes soft and watery due to the presence of the protopectinases which have dissolved away the protopectin of the cell wall. The effect is to separate the cells from each other with a certain amount of destruction of the cell wall, and subsequently the death of the protoplast. The particular strain of Pythium used in these experiments was isolated from 'damped-off' cress seedlings and has since been maintained on a sucrose-peptone medium pH 6. The stock cultures were kept on agar slopes at 5°C. and subcultured at least once a month.

The potatoes were first washed and dried and the part to be inoculated washed with 95% ethyl alcohol which was then allowed to evaporate. A plug of tissue, approximately 0.5 cm. in depth, was removed with a sterile No. 2 cork borer and a similar plug of agar, taken from the advancing edge of a 2 day old Pythium culture, was inverted into the cavity and sealed with low-melting point wax (Isaac, 1951) of the following composition.

Petroleum Jelly	75% by weight
Paraffin Wax (M.p. 52°C)	25% by weight

The inoculated tubers were then incubated for 3-4 days at 25°C., after which time a characteristic soft-rot had set in and most of the potato had become a brown watery mass due to the action of the macerating enzymes. This disorganised tissue was scraped out and squeezed through a double layer of muslin and the solution collected was centrifuged at c. 20,000

x g for 20 minutes to remove all cell debris. The supernatant fluid was further purified by acetone precipitation of the proteins at -10°C in the ratio of 2 volumes acetone to 1 of enzyme solution. A copious white flocculent precipitate was formed and collected by centrifuging at 20,000 x g for 10 minutes. The residual acetone was removed under vacuum and the final dry precipitate ground to a fine powder and stored at -10°C. For experimental use, weighed samples of the powder were dissolved in sodium-citrate-citric acid buffer pH 6.5. The amount of protopectinase present in a solution is measured by the 'maceration time' ie., the time required by a section of tissue of standard thickness, immersed in a solution of the protopectinase, to lose coherence (Brown, 1915).

VII. Method used for Studies on the Respiration of Cotyledon Tissue

Standard Warburg techniques were employed in the respiration studies. A standard weight of tissue (0.15 gms) previously injected with water to allow uniform penetration of the bathing solution was used in each experiment and the volume within the flask made up to 3.0 ml with the solution to be tested. All experiments were performed at 29.5°C.

Experimental Results Part I

A major part of this project consisted of developing a technique for isolating single living cells from sunflower cotyledons and to observe the effect of germinating uredospores of P. helianthus among such cells.

The isolation and maintenance of single living cells has been achieved by Chayen, 1952, and De Ropp, 1955, the tissue used was meristematic, either, root tips or callus tissue. These methods were unsuitable for the differentiated mesophyll tissue of sunflower cotyledons. A number of chemical reagents which react with calcium have been tried by Tribe (1955) as a means of dissolving the calcium pectate middle lamella viz. ammonium oxalate and sodium pyrophosphate, fluoride, carbonate and hexametaphosphate, but no positive results were obtained.

The nature and composition of this lamella is still a matter of conjecture. Evidence is accumulating for the existence of linkages other than 1:4 glycosidic bonds between individual galacturonic acid residues and may take the form of multivalent metallic ions, particularly calcium, between carboxyl groups of adjacent chains associated through esterified phosphoryl groups or proteins (Kertesz, 1951, Wood, 1955). With view to developing a method of isolating single living cells a detailed study of the properties of protopectinase (syn. cytase; pectinase) was carried out. The principle aspects

of study were the macerating and toxic properties of the protopectinase, and ways of reducing or eliminating this toxic factor were sought.

I. A Study of the Toxic and Macerating Properties of the Protopectinase Isolated from Pythium infected Potatoes

Protopectinases, the enzyme or group of enzymes which dissolve the protopectin, the water insoluble substance which occurs in the middle lamella of plant cells, are present in most tissues infected with soft-rot pathogens, and examination of the infection spot has shown that the cells are isolated from each other and are dead.

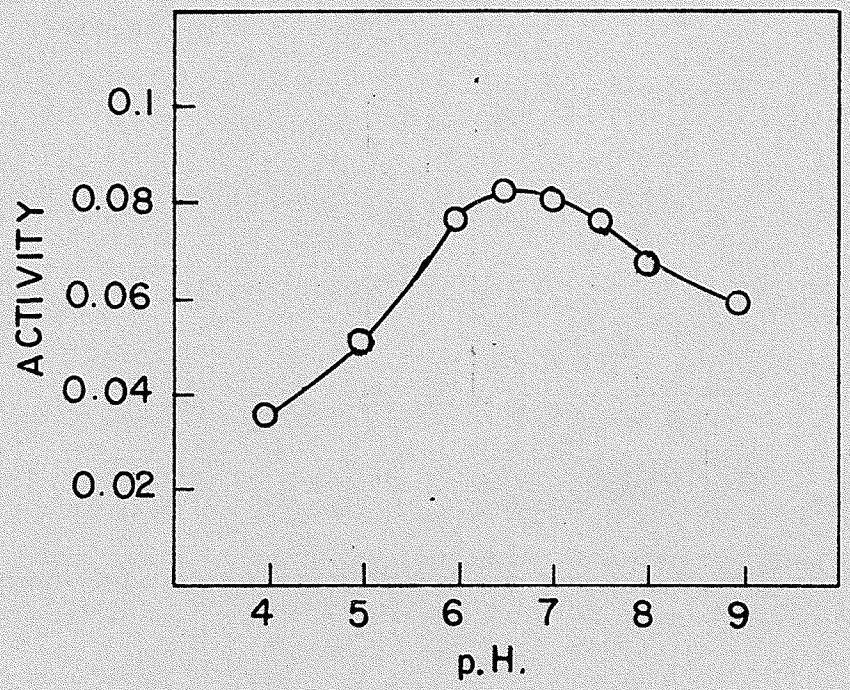
Brown (1915) began a study of the physiology of such pathogens and using extracts prepared from germ-tubes of Botrytis cinerea he was unable to separate the macerating and toxic effect of this thermolabile preparation. Two theories for this phenomenon were postulated.

(1) That both factors, maceration and toxicity, originate from the same substance.

(2) That they are separate factors and that maceration renders the protoplast permeable to a colloidal toxin.

(a) The Effect of pH on the activity of the Protopectinase

The macerating and toxic properties of the protopectinase were tested over a pH range of from pH 4.0-8.5 using two buffers; from pH 4.0-7.0 sodium citrate-citric acid buffer was used and from pH 7.0-8.5 sodium borate-boric acid buffer was used. Both



Text - Fig. 3. The effect of pH on the macerating activity of the protopectinase. Activity was measured as the reciprocal of 'maceration time' in minutes.

buffer solutions were brought to the correct pH with NaOH or HCl respectively. Maceration was followed by Brown's technique using uniform sections of cotyledon tissue previously injected with distilled water to assist the penetration of the enzyme solution. The relationship between activity and pH is shown in Fig. 3. The activity was taken as the reciprocal of the 'maceration time' in minutes.

The effect of the pH on toxicity was followed by the neutral red staining technique of Tribe (1955). Sections of cotyledon tissue were immersed in enzyme solutions over a pH range of 4.0-8.5 units. At five minute intervals sections were removed from each solution washed quickly in distilled water and placed in a drop of the following solution (M.KNO_3 8.5ml; 0.1% neutral red chloride 2.0 ml; citrate buffer pH 7.0 a few drops). Within 2-5 minutes protoplasts which were still alive would round off and take up the vital stain eventually becoming spherical and darkly stained. Plasmolysis and vital staining were taken as criteria of life within the cell and when neither of these phenomena would occur the cell was considered dead. A close parallel was found between degree of maceration using Brown's technique and toxicity as measured by vital staining and plasmolysis. After maceration few cells would plasmolyse, and it was shown that the pH of the solution did not have a differential effect on the macerating and toxic action of the protopectinase.

(b) The effect of dialysis on the activity of the protopectinase

Dialysis of the protopectinase preparation against distilled water for 18 hours produced almost complete inactivation. Partial recovery was obtained by adding an equal volume of boiled protopectinase solution to the dialysate.

(c) The effect of plasmolysing concentrations of salts and non-electrolytes on the macerating and toxic properties of the protopectinase

Tribe (1955), using protopectinase extracts from Botrytis cinerea and Bacterium aroideae, has recently shown that various plasmolysing concentrations of salts and non-electrolytes reduce the rates of both the maceration and toxicity of the protopectinase and that the latter is affected to a much greater degree. He has also shown that the concentration of plasmolysing solution just below that which produced plasmolysis had little effect in prolonging the life of the protoplasts, and that a slight increase in the concentration of the plasmolysing agent, sufficient to bring about plasomysis, caused a 100-fold increase in the number of protoplasts surviving at the end of a twenty hour period of immersion.

In view of these results experiments were carried out in this project, to study the effect of plasmolysing concentrations of salts and non-electrolytes on the macerating and toxic properties of the protopectinase isolated from Pythium infected potatoes. It was found that the molar concentrations

of KNO_3 and $MgSO_4$ (0.5-0.75) that were required to plasmolyse the cells of the cotyledons had a toxic action on the cells after prolonged immersion. Solutions of non-electrolytes such as sucrose and glucose of equivalent molarity did not have this toxic action and tissue could be left in 1M sucrose for twenty four hours without obvious damage. When sections of cotyledon tissue were placed in a solution of equal volumes of 2M sucrose and protopectinase solution to give a final molarity of unity, the sections would still respond to vital staining after three hours immersion. In a protopectinase control without sucrose the tissue would not respond to vital staining and plasmolysis after twenty minutes immersion. In both experiments 'maceration time' was approximately twenty minutes.

Results such as these were considered by Tribe (1955) to be due to an increase in the resistance of the protoplast by plasmolysis, rather than an inhibition of the toxic agent by the plasmolyticum.

(d) <u>The effect of protopectinase on the respiration of sunflower cotyledons</u>

Respiration is a vital phenomenon of living tissue and this series of experiments were carried out to see what effect protopectinase had on the system and this in conjunction with plasmolysis and vital staining would be a further check on the viability of the tissue.

Two aspects of respiration were studied

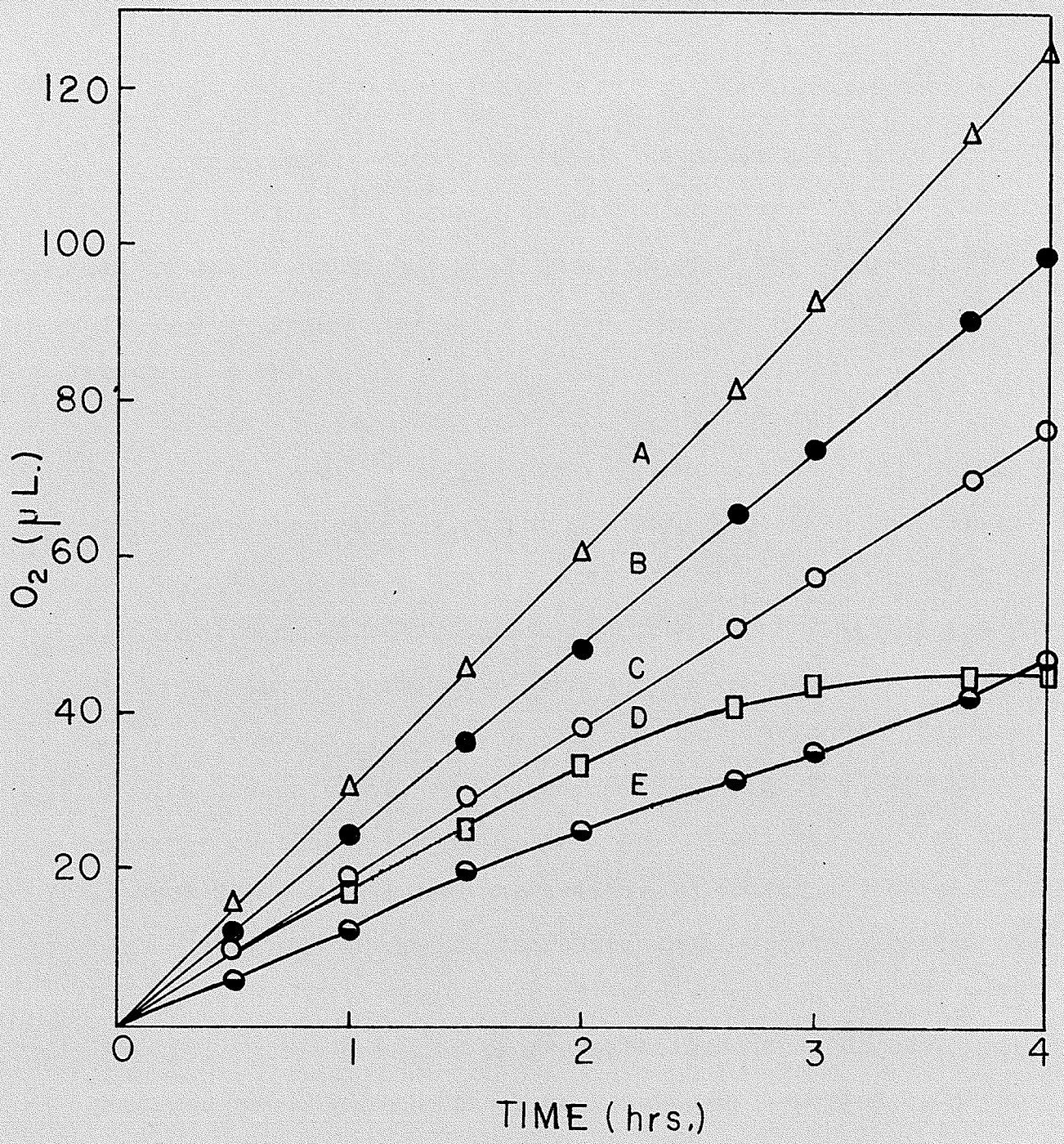
(1) Oxygen uptake

(2) Total respiration

(1) Oxygen uptake

Experiments were carried out using both the enzyme solution extracted from potato and centrifuged to remove cell debris, and the acetone powder extract of such a solution taken up in buffer. Fig. 4 shows clearly that in the direct enzyme solutions (A and B) there was an increased and uniform uptake of oxygen by the tissue, compared with the control in water (C), over a period of three hours. The increase occurred in both the direct enzyme extract in water (A) and the direct enzyme extract in sucrose (B) but was greater in the former case. This oxygen uptake continued beyond the point of death of the cells as shown by plasmolysis and vital staining. When the acetone extract was used, as a source of protopectinase, both in water (D) and sucrose (E) the increase in oxygen uptake beyond the control did not occur. Bennet-Clark and Bexon (1946) showed that there is a decrease in gas exchange in plasmolysed tissue compared to normal tissue and this accounts for the difference between A and B, and between C and D.

It is considered, that the increase in oxygen uptake over the control or true value is due to the oxidation of phenolic compounds present in the enzyme solution and would account for the browning of the solution on prolonged exposure to the atmosphere. A polyphenol oxidase is certainly present as the protopectinase solution was taken from potato. Acetone purification precipitates the proteins and substances such as phenols are left in the supernatant fluid. In the acetone extract solution there is a gradual decrease in oxygen uptake by the tissue and this finally stops after approximately 3 hours.



Text - Fig. 4. The effect of protopectinase on oxygen uptake of sunflower cotyledons.

A. - Direct Enzyme Extract in Water

B. - " " " " Sucrose

C. - Control in Water

D. - Acetone Powder Extract in Water

E. - " " " " Sucrose

(2) Total Respiration

Oxygen uptake cannot in itself be taken as a true criterion of life as oxidative processes involving oxygen uptake can occur in dead tissue, but when measured together with the carbon dioxide output will point to a truer indication of life within the cell.

In Fig. 5 the normal gas exchange of tissue respiring in water is shown. The gas exchange was constant throughout with the carbon dioxide output slightly less than the oxygen uptake. Fig. 6 shows the gas exchange of tissue respiring in a plasmolysing concentration of sucrose. Here, again, the gas exchange was constant. The decrease in gas exchange between unplasmolysed and plasmolysed tissue has previously been explained (Bennet-Clark and Bexon, 1946). Fig. 7 shows the gas exchange of tissue respiring in a protopectinase extract in water. The gas exchange was relatively constant over the first three hours but gradually decreased until all respiration had ceased by the five hour stage of immersion. In Fig. 8, with the tissue in a solution of protopectinase and sucrose, respiration remained relatively constant over a period of seven hours.

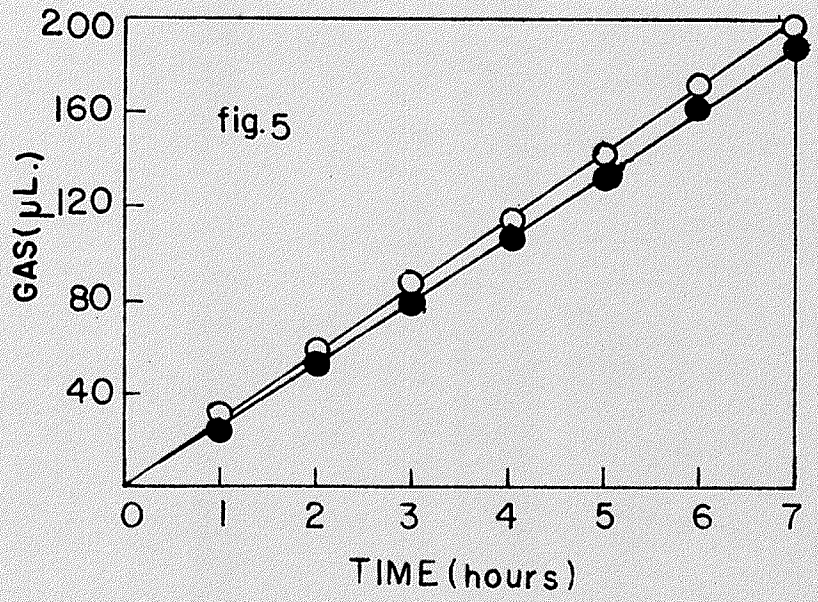
These experiments show clearly that protopectinase had little effect on the respiration of plasmolysed tissue over a period of seven hours immersion. In contrast, unplasmolysed tissue had ceased to respire after four hours immersion in the protopectinase.

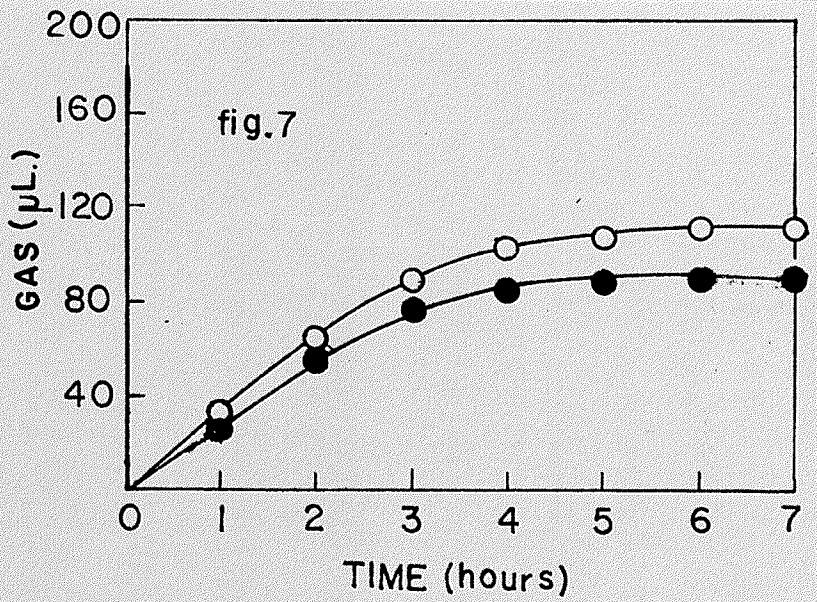
Text - Fig. 5. Control experiment. 0.15 gms tissue and 2.85 ml. distilled water.

Text - Fig. 6. Control experiment for plasmolysed tissue. 0.15 gms tissue and 2.85 ml. molar sucrose.

Text - Fig. 7. Acetone enzyme extract in water. 0.15 gms tissue, 1.45 ml. enzyme extract and 1.4 ml. distilled water.

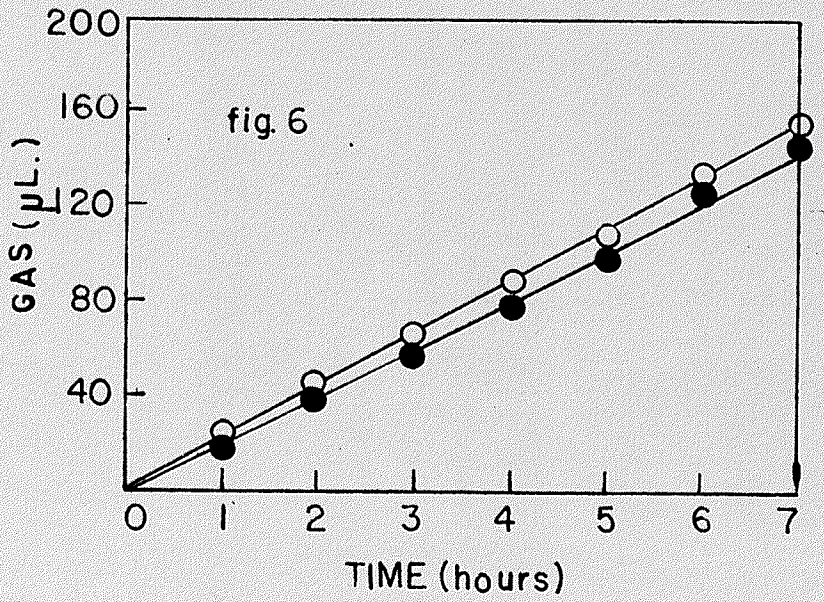
Text - Fig. 8. Acetone enzyme extract in sucrose. 0.15 gms tissue; 1.45 ml. enzyme extract and 1.4 ml. 2 molar sucrose.

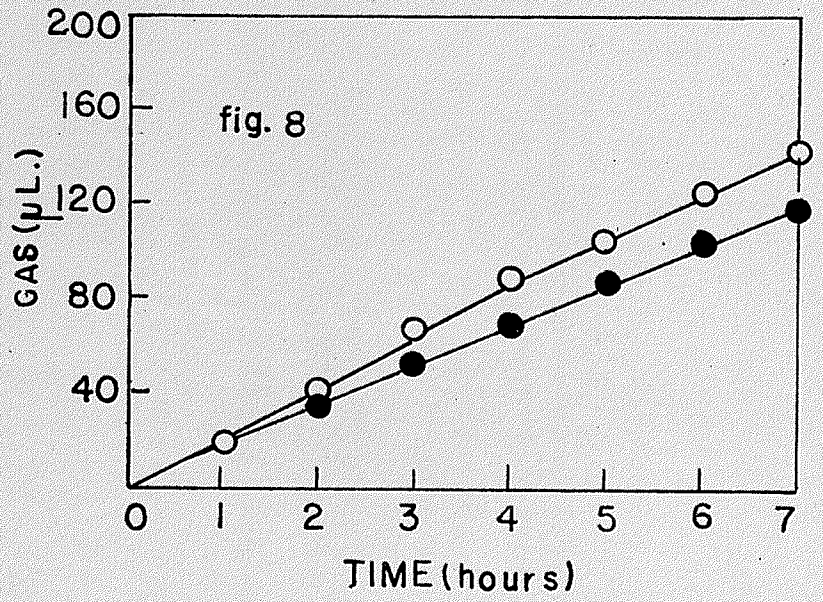




O—O O_2 UPTAKE

●—● CO_2 OUTPUT





2. The Isolation of Single Living Cells

Single cells and groups of cells, which respond to plasmolysis and vital staining, have been obtained experimentally by bringing the calcium pectate middle lamella into partial solution and separating the cells from each other by microdissection or mechanical shaking. The acetone powder extract of the macerating enzymes previously described at a concentration of 0.7 gms/20 ml. citric buffer pH 6.5 was used as the source of protopectinase. The cotyledons of the sunflower were found to be excellent material for this experiment because of the large size of the individual mesophyll cells (C.200μ diam.) and the relatively few points of contact between neighboring cells. This is due to the high percentage of the total volume of the cotyledon occupied by the intercellular space.

Early experiments showed that a higher percentage of single cells were obtained by using cotyledons taken 3-4 days after germination. The cotyledons were injected with water and transverse sections 0.5 mm. thick were cut and immersed in a plasmolysing concentration of sucrose (1M) for approximately 60 minutes until they had reached the final stage of plasmolysis and the protoplasts had become spherical.

The sections were then removed and 0.15 gms of tissue placed in a solution of 1 ml. of 2M sucrose and 1 ml. of the enzyme solution. The period of immersion in this solution varied from 90-120 minutes and the correct period of immersion

had to be found for each experiment by micro-dissection at regular time intervals. The viability of the tissue was followed by vital staining with neutral red chloride. When the correct period of immersion was ascertained, the tissue sections were washed in a 1M sucrose solution and removed to a glass slide where the tissue was gently teased apart with micro-dissecting needles. Large numbers of isolated plasmolysed cells could be obtained and these were removed by micro-pipetting to other solutions and deplasmolysed in decreasing concentrations of sucrose. A 0.5M sucrose solution was isotonic to the protoplasts. The cells were able to plasmolyse again and take up the vital stain neutral red chloride. Cells isolated in this way have been kept alive for 48 hours in a 0.5M sucrose solution.

Prolonged immersion in the enzyme solution resulted in a disintegration of the protoplast wall, the chloroplasts became a disorganised mass and collected at one end of the intact spherical tonoplast. Since there is no mention of pectin as a structural component of plant protoplasts in modern treatments of protoplast structure (Frey-Wyssling, 1948), it is possible that this disintegration of the protoplast wall was not due to the protopectinase enzymes but to some other enzyme system present in the solution.

In control experiments with the enzyme solution alone a large number of isolated cells were obtained which, under microscopic examination, appeared perfectly normal and turgid

but could not be plasmolysed or stained by vital dyes. Total collapse of these cells did not occur until immersed for approximately 24 hours.

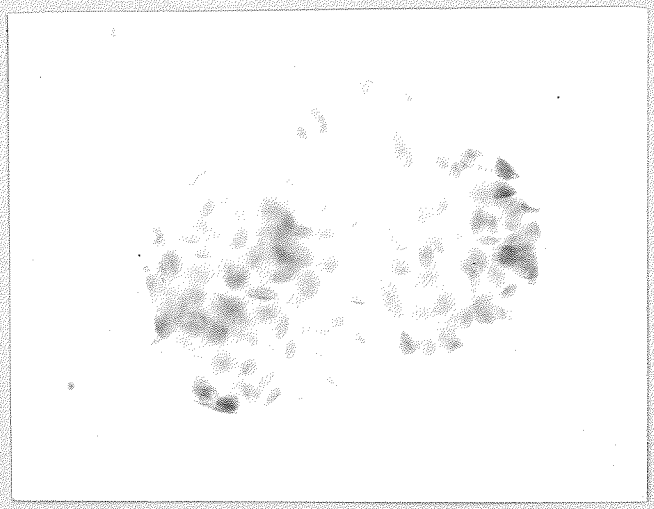


Plate No. 1. Photomicrograph (magn. 450x) of a mesophyll cell isolated from sunflower cotyledon by treatment with normal enzyme solution.

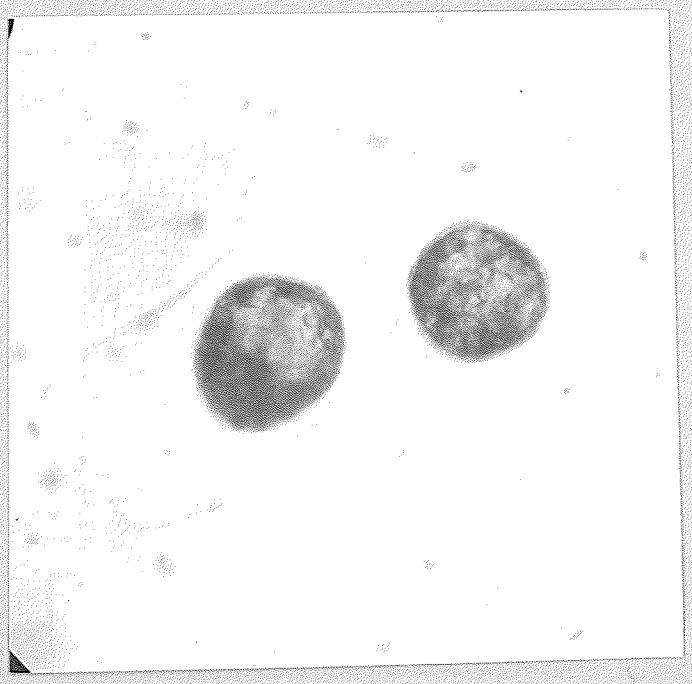


Plate No. 2. Photomicrograph (magn. 450x) of mesophyll cells isolated from a solution of enzyme and sucrose of 1M concentration. The cells were capable of being plasmolysed and stained by vital dyes.

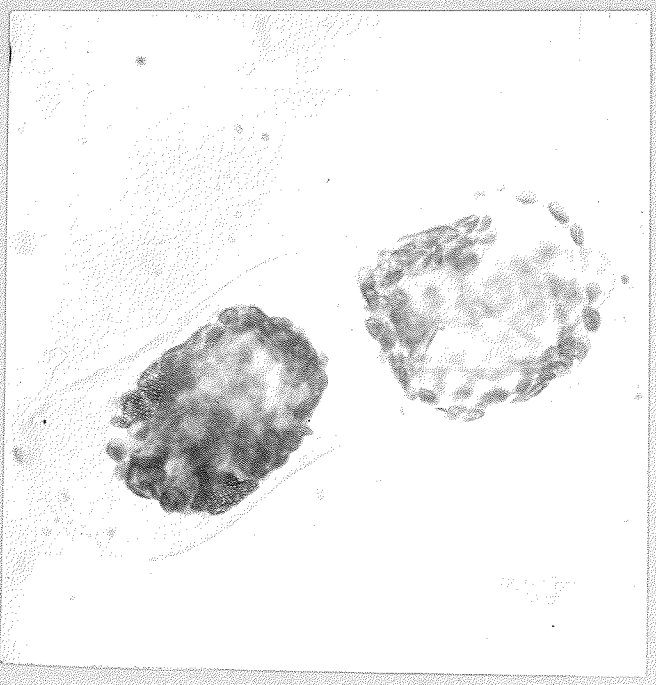


Plate No. 3. As above but showing early stages of deplasmolysis in water.

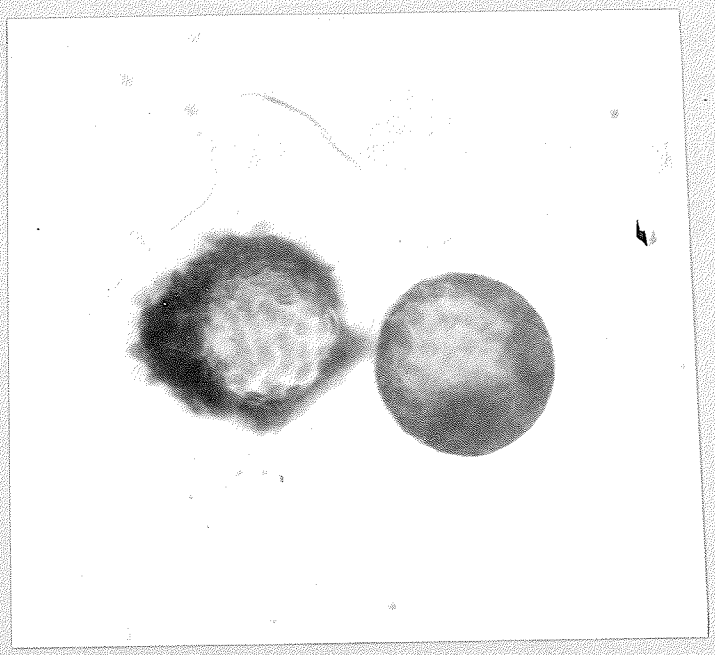


Plate No. 4. Photomicrograph (magn. 450x) of isolated mesophyll cells showing effect of prolonged immersion in the solution of enzyme and sucrose. The protoplast on the right had begun to disintegrate.

Experimental Results Part II

Experiments with detached leaves (Yarwood 1946) have shown that successful growth of obligate parasites is possible on isolated portions of plant tissue, however the work of Samborski and Shaw (1956) suggests that available nutrient supply is a limiting factor in the development of infection. Thus, it appeared possible that by the addition of substances to an area of tissue, just smaller than that required to support an infection, valuable information might be obtained on the nutritional requirements of the parasite. With this object in mind the following series of experiments were conducted.

1. To Find the Smallest Area of Tissue that will support a Rust Infection.

(a) Inoculation of Detached Whole Cotyledons:-

Detached cotyledons were placed aseptically on sucrose-peptone agar in small petri-dishes and inoculated with urediospores. The petri-dishes were set in closed vessels over water for germination and placed in a position fully exposed to normal greenhouse conditions for 36 hours. Following this, the dishes were removed and set on the laboratory bench and approximately 14 days later distinct 'flecking' was apparent. The entire process, from inoculation to pustule formation, takes between 3-4 weeks as compared to the normal 10 days on attached cotyledons.

One very important point of difference is that the number

of pustules is greatly reduced. On a normal attached cotyledon up to 200 pustules may be formed depending on the degree of inoculation; whereas on detached material the maximum number has been 4.

The size of the pustule depends on the degree of infection; on heavily infected tissue the pustules are very small and increase in size in relation to the area of tissue available. On detached cotyledons the pustule size was approximately the same as that of pustules occupying a similar area of tissue on attached cotyledons.

On many of the detached cotyledons meristematic growth commenced at the base of the petiole from which green shoots developed.

(b) Inoculation of Portions of Cotyledon Tissue.

Portions of cotyledons varying in size from half to one quarter of the whole cotyledon were placed on nutrient agar and inoculated with urediospores. Though the cultures were kept free of contamination for up to 6 weeks and periodically subcultured to prevent dessication, there was no external manifestation of infection. Subsequent sectioning and staining with aniline blue showed that the urediospores had germinated and that the germ-tubes could be seen on the surface but no penetration had occurred.

When cotyledons were cut in such a manner as to leave only portions varying from half to one quarter of the usual cotyledon still attached to the plant and inoculated, normal infection occurred on these portions. Within two days of sporulation these attached portions of cotyledon turned yellow

and shrivelled up.

On many of the portions of detached cotyledons meristematic activity occurred at the cut edges and small buds of callus tissue gradually developed.

(c) Inoculation of Epidermal Strips:

Strips of adaxial epidermal tissue were removed from cotyledons, washed several times in sterile distilled water and placed on sucrose-peptone agar. The strips were inoculated with urediospores and within 24 hours 50% germination had occurred. The form of the germ-tubes varied considerably. In most cases they were long and unbranched while others were short with frequent branching. A small percentage on germinating grew to about 50 μ in length and produced a globular swelling at the apex of the germ-tube and on one or two a small peg-like structure developed. This globular structure appeared comparable to a substomatal vesicle and the peg-like structures to infection hyphae.

In some cases there was anastomosing of the germ-tubes over the stomata, and hyphae could be seen entering the open stomata and a few almost transparant hyphae were present under the epidermal strip. There was no further development from this stage.

Penetration had only occurred where there was air trapped between the surface of the agar and the epidermal strip.

(d) Inoculation of a Suspension of Single Cells and Groups of Cells.

Cotyledon tissue was macerated as previously described and a suspension of cells and groups of cells poured onto the surface of sucrose-peptone agar and dusted with urediospores. Normal germination of the spores occurred. The types of germination varied considerably.

When spores, which were not in contact with cells or groups of cells, germinated, the germ-tubes grew at random and were normally long and unbranched. It was clear that the tissue present in the suspension had no controlling effect on the direction of growth of the germ-tubes.

When spores germinated on groups of cells they behaved to a certain extent in a similar manner but, in a few cases, there was anastomosing of the germ-tubes. They formed an irregular pattern on the surface of groups of cells and many peg-like structures developed from the network. During a winter experiment hyphae were observed to have grown into a small group of cells and were more intra- than inter-cellular. A few of the hyphae appeared to grow through cells in a manner similar to facultative parasites. No haustoria could be seen.

These results could not be repeated in a summer experiment.

2. <u>To Study the Effect of Detaching Infected Tissue.</u>

Whole cotyledons and portions of cotyledons detached from the plant at the flecking stage of infection were surface sterilised and placed on nutrient agar. In almost all cases the normal process of infection continued and pustules were

formed.

On a small number of the portions of tissue, pustules did not develop but the coloration of the developing pustule gradually changed from pale yellow to deep red or normal color of a mature spore. Sectioning of the tissue showed that the spores, already present before detachment, had continued to mature but that the hyphal growth required to produce the pressure necessary to break through the epidermis had not occurred.

In some cases pustules continued to develop and uredio-spores form even when the tissue around the pustule was apparently dead and would not respond to vital staining and plasmolysis.

Table I

Type of Cotyledon	Time from 'flecking' stage to pustule termination in days
Attached whole cotyledons	2
Detached whole cotyledons	3-4
Attached portions of cotyledons	2
Detached portions of cotyledons	6-10

3. The Inoculation of Dead Tissue.

In the case of the previous experiments a few isolated examples of rust infections continuing to sporulate on apparently dead tissue were observed. Examples of this occurred on whole cotyledons and also portions of such.

In all modern treatments of obligate parasitism it has been considered that obligate parasites will not attack dead tissue and that established infections die when the surround-

ing tissue dies. Since it was apparent, from the above observations, that sporulation was occurring on dead tissue, a series of experiments were carried out in an attempt to throw some light on this problem.

A technique involving killing the tissue by immersion in liquid air was employed to reduce the physical and chemical damage to the cells. Preliminary experiments showed that the extremely low temperature (-189°C.) had little effect on the bacterial and fungal contaminants, so, for the following experiments, cotyledons were grown under sterile conditions.

Whole cotyledons were suspended by a glass rod in liquid air for 30 seconds, removed and placed aseptically on sucrose-peptone agar and inoculated with urediospores. Within 2 hours the tissue had lost the green coloration but microscopic examination showed little mechanical damage due to freezing. The cultures were kept free of contamination for 6 weeks when microscopic examination showed that the urediospores had germinated and the germ-tubes could be seen growing along the surface but no penetration was apparent.

Whole cotyledons and portions of leaf tissue, at the 'flecking' stage of infection, were dipped in the liquid air and placed on sucrose-peptone agar. The pustules did not break through but the colour of the pustules gradually changed from pale yellow to dark red, signifying, that the spores had continued to mature after treatment.

Discussion

As was stated in the introduction the key to the problem of rust infection appears to be in the study of the formation and function of the haustoria in relation to the individual cell.

Dickinson (IV, 1949) has shown that structures comparable in appearance to haustoria can be formed by the infection hyphae of P. graminis tritici Erikss. and Henn. in contact with special membrane surfaces. No attempt was made to show that these structures were true haustoria in that they acted as organs of absorption.

An investigation of the reaction of individual host cells to infection has been studied. In the first case a considerable amount of work was necessary to develop a method for isolating, individual mesophyll cells and maintaining them in a living condition. Owing to the sensitivity of the tissue to mechanical damage, an enzymic method was employed to dissolve the middle lamella. In common with Tribe (1955), it was found that prior plasmolysis would reduce the effect of the toxic factor normally associated with these macerating enzymes and single living cells could be isolated after treating the tissue with a plasmolysing agent. The viability of the cells was tested by means of plasmolysis and vital staining which, together, are characteristic of living cells (Guilliermond,1941). A study of the respiration of the tissue during immersion in the enzyme solution showed that normal respiration continued long after

the tissue was dead according to plasmolysis and vital staining tests. This suggests that the systems controlling plasmolysis and vital staining are affected directly by the toxin in the enzyme solution, and that the eventual decrease in respiration is a secondary reaction.

When urediospores were germinated among the single cells the growth of the germ-tubes appeared little different from that occurring on agar. There was no apparent attraction between the cells and the hyphae and no penetration of single cells was observed. However, in a few instances, a definite association did occur with very small portions of tissue mixed with the single cells, and hyphae could be seen to be growing in both an intracellular and intercellular fashion. The size of these portions (c. 50-100 cells) was such as to make it unlikely that they were capable of a tissue response in the normal sense and makes it appear that the inability of the single cells to support infection was due to alteration during separation rather than to any inherent resistance.

The experiments with sunflower cotyledons showed that detachment and further division of the cotyledon, resulted in a resurgence of meristematic activity in the differenciated mesophyll cells with the concommitant reduction of susceptibility. Shaw and Samborski, (1956) and Samborski and Shaw (1956) working with resistant Kapli, showed that inactivation of meristematic activity with maleic hydrazide lead to a rapid

increase of soluble carbohydrates and amino acids in the leaf and also lead to the leaf becoming susceptible. Similarly, they found, that by detaching a leaf of Kapli and standing it in a nutrient solution there was a build up of soluble carbohydrates and amino acids and the leaf became susceptible. From these results, they have suggested that in the normal resistant Kapli plant there is competition from all parts of the plant for the available soluble substances, and that when meristematic activity is stopped either by maleic hydrazide or detachment of the leaf there is a surplus of soluble carbohydrates and amino acids available in the leaf to support rust infection.

Thus, with the portions of cotyledon tissue it appears that the actively dividing cells, in close proximity to the site of infection, act as efficient competitors for the essential nutrients. With detached whole cotyledons the area of meristematic activity is small in relation to the total cotyledon, and though competition for the available nutrients is present, a reduced number of infections can occur. In the case of attached portions of cotyledons, it appears that infection can occur, but the resultant competition between the actively dividing cells at the margin of the tissue and the rust infection is too extreme and the tissue dies.

It must also be noted that the hormonal level within the tissue may have a direct inhibitory effect on the rust urediospores preventing penetration and establishment of infection.

Summary

1. A method developed for growing plants under aseptic conditions and apparatus constructed for prolonged handling and examination of tissue under aseptic conditions is described.

2. The macerating and toxic properties of a protopectinase solution were studied.

3. A technique was developed for isolating and maintaining single living mesophyll cells of Helianthus annuus.

4. Experiments, with detached cotyledons and portions of cotyledons, suggest that areas of meristematic activity act as competing sources for available nutrients in the tissue and that it is the degree of meristematic activity which controls the susceptibility of the tissue to rust infection.

Acknowledgments

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