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Physiology of fungal haustoria

(Erysiphales)

by

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Abstract

Haustorial complexes (hc) were isolated from five plant species infected with powdery mildews (including Hordeum vulgare - Erysiphe graminis). Special attention has been given to Pisum sativum infected with Erysiphe pisi where fractions of isolated hc's were obtained in sufficient quantity and purity to permit physiological studies of the haustorium and its host interface both in vivo and in vitro.

To study the nutritional physiology in vivo, hc's were isolated from leaves exposed to $^{14}CO_2$ where the mycelium was either left attached or separated from the leaves prior to the exposure. In each case ^{14}C assimilates were detected in the isolated hc fraction. Light microscope autoradiography and control fractions from uninfected plants indicated that approximately 85% of the ^{14}C in the fraction was in the hc's and $^{14}CO_2$ was released from hc's subsequently incubated in vitro indicating assimilation into the haustorial cytoplasm. Haustorial complex, mycelial and host fractions were also obtained from infected leaves either immediately following a 30 min 'pulse' $^{14}CO_2$ exposure or after a further 20h 'chase' period in CO_2; fractions were then analysed. After the 'pulse', labelled soluble sugars (glucose, fructose, sucrose) and mannitol predominated in the host and mycelial fractions respectively and lipids (mainly triglycerides) and an ethanol insoluble glucan

(not α 1-4 linked) predominated in hc's. The neutral sugar fraction constituted only 3% of the total ^{14}C in the hc's with sucrose and glycerol as the major components. After the 'chase' the total ^{14}C in host and mycelium had declined but the proportions in starch, glycogen, protein, cell wall and nucleic acid fractions had increased; total ^{14}C in hc's increased but a decrease in ^{14}C in sucrose and glycerol and an increase in mannitol were the only changes in biochemical distribution. In all $^{14}CO_2$ exposures the quantity of ^{14}C resident in the hc's was small compared with that in the mycelial and host fractions from the same area of leaf. By sampling ^{14}C in the mycelium and estimating hc frequency and surface area by light microscopy, carbohydrate fluxes of 2.4 ng cm^{-2} sec^{-1} and 0.75 mg cm^{-2} sec^{-1} were calculated for the extrahaustorial membrane and haustorial neck region respectively. These indicate specialised transport processes.

Ethanol soluble carbohydrates were quantitatively analysed and constituted approximately 4, 14 and 1% of the host (mainly, glucose, sucrose, fructose), mycelial (mainly, mannitol, arabitol, erythritol) and hc (mainly, sucrose, glucose) fractions respectively. Infection did not alter host invertase activity. but the mycelium and hc's contained significant activity. The enzyme was soluble in water after extraction involving homogenization.

Isolated hc's *in vitro* showed oxygen uptake which was unaffected by exogenous glucose, sucrose or mannitol. No significant uptake of glucose, sucrose, glycine inulin, UDPG or SCN^- was detected. However, *in vitro*, the systemic fungicide ethirimol (I.C.I. Ltd) was absorbed rapidly reaching equilibrium in less than 30 min. The final concentration attained was directly proportional to, and 20 fold higher than the ambient concentration (5-400 μM). Rates of influx were similar at pH 4.2 and 6.2 but at 0° only half that at 25°. Efflux kinetics comprised two superimposed logarithmic phases, indicating penetration into two compartments in hc's. Ethirimol also entered pea mesophyll protoplasts but was not significantly accumulated against a concentration gradient.

Results are discussed in relation to the role of the haustorium in the transport of nutrient and fungicides from host to parasite.

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Introduction.

Haustoria are characteristic of many biotrophic fungi. Bushnell (1972) has defined the haustorium as 'a specialised organ which is formed inside a living host cell as a branch of an extracellular or intercellular hypha or thallus, which terminates in that host cell and which probably has a role in the interchange of substances between host and fungus'. However despite their discovery by Visiani over one hundred years ago (Smith, 1900) there has been no direct demonstration of the function of haustoria.

The superficial organisation of a powdery mildew colony indicates a dependance upon haustoria which is less obvious in biotrophic fungi with an intercellular mycelium. Powdery mildew hyphae are separated from the host cells by a cuticle which is a considerable barrier to the movement of most nutrients and in Erysiphe graminis, Bushnell (1967) has shown that in a colony with one haustorium, when that haustorium is excised, the rate of hyphal extension immediately declines. However, in 1975 when the present investigation was commenced, the best evidence in any fungus that haustoria absorb nutrients from the host was from studies of primary

infection of Erysiphe graminis by Ellingboe and co-workers (Masri & Ellingboe, 1966; Mount & Ellingboe, 1969; Slesinski & Ellingboe, 1971). They had shown that both development of hyphae and transfer of ^{32}P and ^{35}S from the host to superficial fungal structures depended on the successful establishment of the primary haustorium.

Despite the dearth of information on the function of haustoria there have been numerous investigations of their ultrastructure and that of the host interface. These have been reviewed and discussed in detail by Ehrlich & Ehrlich (1971) and Bracker & Littlefield (1973). These reviews, particularly the latter have concluded that knowledge of the ultrastructure of haustoria has not yielded any information on their function. However, they have revealed that haustoria have highly specialised interfaces with their hosts. The host plasmamembrane is not penetrated but is invaginated by the intruding haustorium and structures not normally formed outside the host plasmalemma are formed between them. Various terms, some with ambiguity, have been applied to the interfacial structures but Bushnell (1972) has simplified nomenclature by defining the invaginated host plasmalemma as the extrahaustorial membrane and the region between it and the

haustorium, the extrahaustorial matrix.

Fractionation of infected tissue has facilitated structural and some physiological studies of the interface. Dekhuijzen (1966) developed a technique to isolate haustorial structures from cucumber infected with powdery mildew and this was improved by Gil & Gay (1977) for pea powdery mildew. The latter termed the isolated structure the haustorial complex which comprises "the haustorium with its cytoplasm, the extrahaustorial membrane around it and the materials and structures between them". This approach has shown haustoria to have significant respiration in vitro (Dekhuijzen, 1966) and permitted a thorough examination of the structure of the interface (Gil & Gay, 1977). Freeze fracture studies and various enzyme and chemical treatments of isolated haustorial complexes have shown that the extrahaustorial membrane is quite distinct from the host plasmalemma (Gil, 1976; Gil & Gay, 1977) which is in agreement with earlier structural studies of flax rust (Bracker & Littlefield, 1973).

The attachment of the extrahaustorial membrane to the haustorial neck previously argued by Bushnell (1972) was clearly visible in isolated haustorial complexes and furthermore the volume of the extrahaustorial matrix could be altered by adjustments of the osmolarity of the ambient medium. These

results agreed with observations by Bushnell (1971) who dissected cells of Hordeum vulgare infected with haustoria of E. graminis.. However, because Gil & Gay's (1977) studies were combined with electron microscopy, the uncertainty of the particular cell membrane or component involved was clarified and they indicated that the extrahaustorial membrane or more unlikely the neck wall, or both, were semipermeable and that the extrahaustorial matrix was liquid and enclosed in an interfacial compartment.

Concurrent with studies on the structure of haustoria numerous papers have been published on the biochemistry of biotrophic associations and with particular regard to their nutrition. Smith et al (1969) have discussed in detail the carbohydrate nutrition of biotrophs. The role of carbohydrates is of prime importance in biotrophic associations of higher plants and fungi, distinct features have been resolved (Smith et al, 1969; Lewis, 1970). First, movement of carbohydrates to affected areas is stimulated and sometimes this is accompanied by an increase in polysaccharide synthesis in nearby uninfected cells. These carbohydrates with those synthesised in infected cells are then released, absorbed by the fungus and converted to fungal storage products. Of these processes, it is those of nutrient release

from host cells and uptake by the fungus which are the most pertinent to studies of haustorial function and it is these stages which are the least understood (Scott, 1972; Lewis, 1976).

Lichens have been used as model experimental systems to study biotrophic transport processes and the nature of the translocate and mechanisms have been resolved using two techniques in particular. The algal partner has been isolated and the rates and nature of ^{14}C - labelled photosynthates released by these cells compared with those from free living forms (Drew & Smith, 1967a). Secondly, transport can be inhibited at the plasmamembranes of autotroph and fungus by either competitive substrates or disruptive agents (Drew & Smith, 1967b; Chambers et al, 1976). The competitive inhibition technique has also been used in studies of intercellular transport in rust infections and mycorrhiza (Lewis, 1976).

Haustoria are not ubiquitous in lichens and when present they are often found in degenerate host cells (Collins & Farrar, 1978). Thus they are not thought to have a significant role in nutrient transport (Collins & Farrar, 1978). Further, techniques used for lichens cannot be applied directly to study biotrophic transport in haustorial associations of fungi where the major interface is intracellular. There, release of photosynthate

by the host cell cannot be studied directly by isolating infected cells since the haustorial interface is a localised and intimate association between the fungus and host membranes which is difficult to divide (Gil & Gay, 1977). Further, since the extrahaustorial matrix which bathes the haustorium is not in continuum with the leaf apoplast, the competitive inhibition technique is not applicable to the haustorial interface in infected tissue. Thus, the problem for haustorial studies is in obtaining experimental access to the intracellular interface.

In his review of the biology and distribution of haustoria, Bushnell (1972) recognised that because of its intracellular location and intimate relationship with the host cell, new techniques combining structural and biochemical approaches were necessary to study haustorial physiology. He listed isolation of haustoria, micromanipulation, high resolution autoradiography and *in vitro* induction of haustoria as the most promising systems. Some progress has been made using autoradiography and probable differences in the nutritional physiology of intercellular and intracellular haustorial interfaces have been demonstrated in rusts and downy mildews. Andrews (1975) showed that *Bremia lactucae* infecting lettuce cotyledons fed with ^{3}H-glucose or ^{3}H - leucine could utilize glucose prior to penetration and throughout its growth whilst utilization of leucine was delayed

until haustoria were established. Similarly Onoe et al (1973) have shown that whereas infection hyphae of Puccinia coronata incorporated label when infected oat leaves were exposed to $^{14}CO_2$ or fed ^{3}H-glucose, pyrimidine nucleosides were not utilised until haustoria were formed. As yet, auto-radiography has been of limited value because the procedure is both tedious and time consuming and it does not routinely detect soluble compounds. Thus it is unclear whether the lack of utilisation of compounds prior to haustorial development is related to the timing of their assimilation into insoluble compounds or uptake by the haustorium. Also, although the nature of the labelled substrate is known, the composition of the cell components into which it is incorporated cannot be determined. Similarly micromanipulation studies do not readily lend themselves to elucidation of biochemical or membrane transport processes.

Isolation of haustorial complexes from powdery mildew infections is potentially more valuable. It permits direct analysis and in vitro studies of the haustorium and its host interface (Gil & Gay, 1977). This thesis describes experiments which apply this technique to study the function of powdery mildew haustoria and in particular their role in transport of nutrients and fungicides from host to parasite. Parts of the work are

already published, (Gay & Manners, 1977; Manners & Gay, 1977; Manners & Gay, 1978a; Manners & Gay, 1978b; Manners, 1978).

MATERIALS AND METHODS

I) Hosts and Fungi

The plants and pathogens used were as follows (The nomenclature follows Blumer, 1967).

Plant	Common name	Fungus
Pisum sativum L. cv. Onward	Pea	*Erysiphe pisi* DC. ex Saint-Amans
Beta vulgaris L. cv. Bushmono	Sugar beet	*Erysiphe polygoni* DC. ex Saint-Amans
Malus pumila L. cv. Jonathon	Apple	*Podosphaera leucotricha* (Ell & Everh.) Salm.
Vitis rebe L. cv. Mullerthurgau	Grapevine	*Uncinula necator* (Schw.) Burr.
Hordeum vulgare L. cv. Proctor	Barley	*Erysiphe graminis* DC. (Mérat) f. sp. *hordei*

Except where indicated all experiments were carried out on *Pisum sativum* infected with *Erysiphe pisi*.

A) Cultivation

Pea plants were maintained in a greenhouse at

approximately 25°C with a daylength of 17h, supplementary lighting was supplied by mercury vapour lamps (Philips Ltd). High quality plants with larger leaves were obtained in the summer months.

Four week old plants were inoculated by shaking above them a plant which had been infected similarly 15 days before. After seven days, infected plants were removed for experiments. Uninfected controls were maintained identically in a separate compartment.

Experiments on apple, barley, sugar beet and grapevine were carried out at the research station of I. C. I. Ltd, Plant Protection, Jealott's Hill. All plants there were maintained at 21° with a 16h daylength. Apple and barley were grown until the first true leaves were fully formed and were then inoculated by shaking over them plants similarly inoculated 15-20 days before. Mature grapevine and sugar beet were inoculated similarly when five to eight fully expanded leaves were formed and were taken for experiments after 18 and 10 days respectively.

II) Isolation of Haustorial Complexes

A) *Pisum sativum* - *Erysiphe pisi*

The method of Gil (1976) with some amendments, was employed. The method is summarised in the flow diagram (fig. 1). The composition of the isolation medium was 0.2M sucrose, 3mM $MgSO_4$ and 0.1M phosphate buffer pH. 6.8. The degree of comminution of infected leaves was varied to improve purity. Starch grain and cell wall contamination was

Figure 1. Flow diagram for the isolation of haustorial complexes from Pisum sativum infected with Erysiphe pisi.

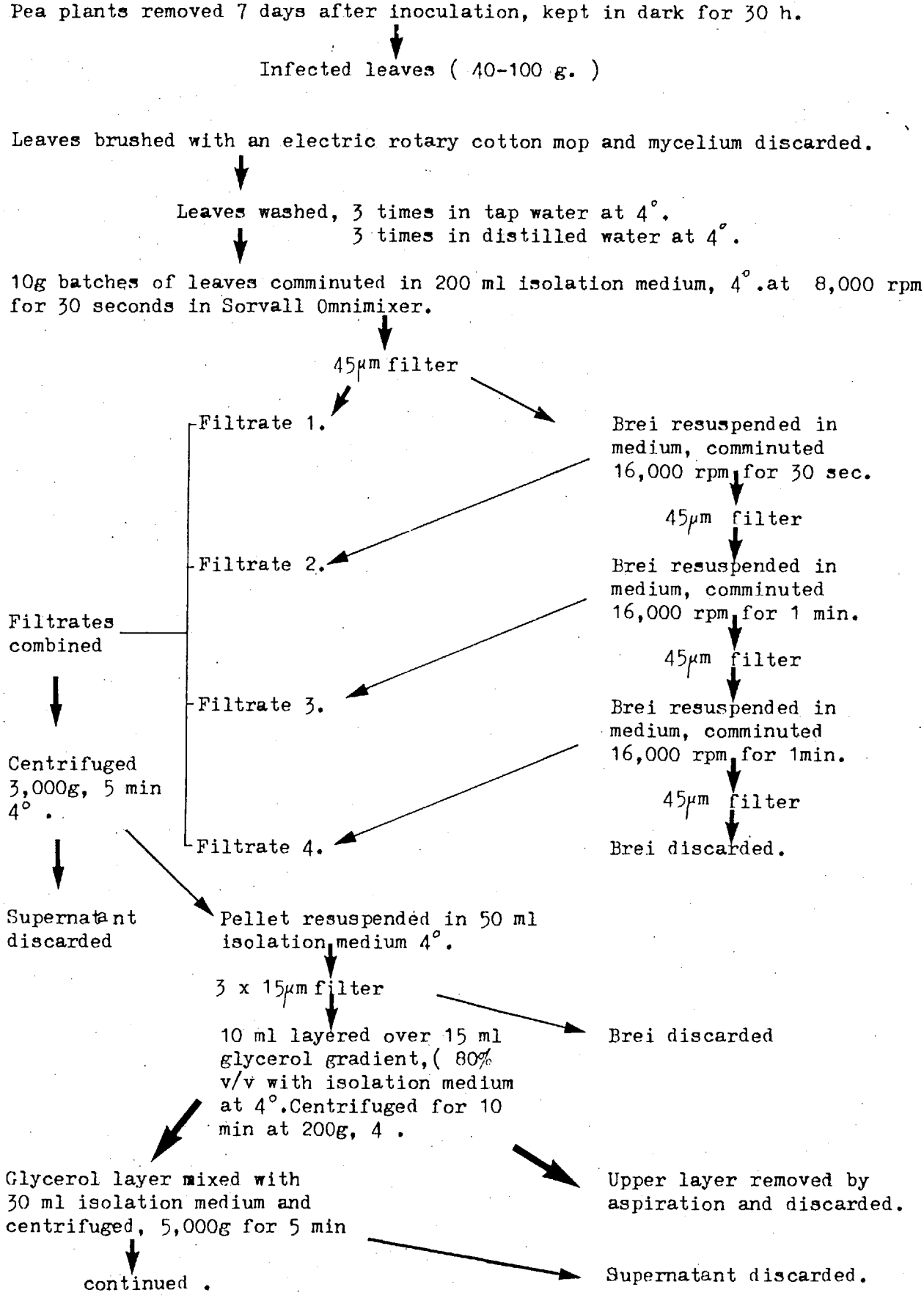
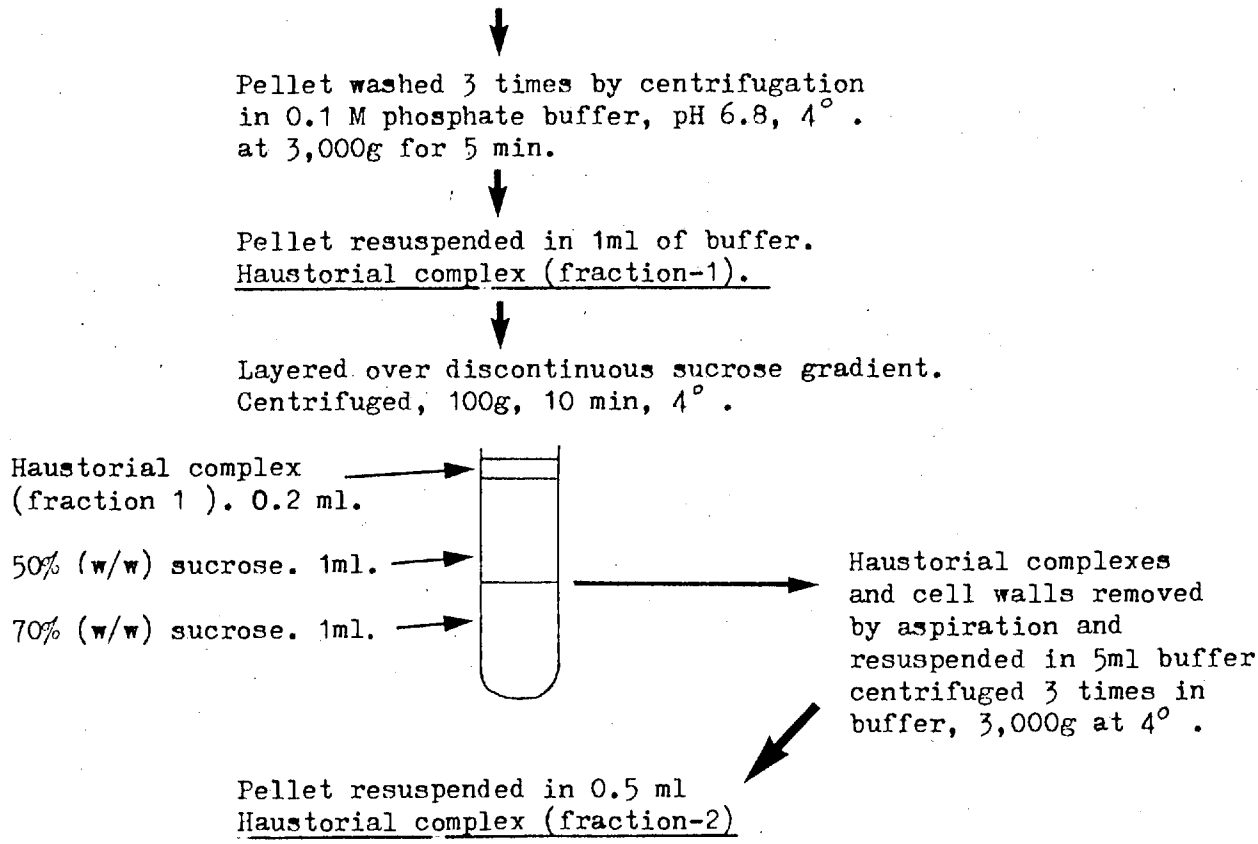


Figure 1. continued



reduced to a minimum by using only filtrates 1 and 2 during the isolation and this procedure was used routinely except where indicated; in some instances, improved yields of haustorial complexes were obtained by using filtrates 3 and 4 also.

When contamination by starch grains was particularly important, it was significantly reduced by a 30 h dark pretreatment of the plants immediately prior to isolation of haustorial complexes or by the additional discontinuous sucrose gradient centrifugation of the haustorial complex fraction 1. A fuller consideration of contamination is given in the results and discussion sections.

B) Other host-parasite combinations

Comminution of whole infected barley leaves resulted in severe damage to the haustoria. Therefore leaves were attached to the bench by double sided sellotape by their upper surfaces, the lower epidermes were then removed with forceps and a razor blade and comminuted at 5,000 rpm. The glycerol gradient was not a necessary prerequisite for the simple morphological observations to be made.

For all other hosts, whole leaves were comminuted. In apple the fraction from the final centrifugation was filtered through 10 µm mesh which retained intact host cells. The final fraction from infected sugar beet leaves was filtered through 5 µm mesh and the haustorial complexes washed from the mesh after filtration, host intracellular

inclusions passing through the mesh. No satisfactory method was found to totally separate the vine haustorial complexes from the intracellular crystalline inclusions released from the host during comminution.

III) Isolation of protoplasts of Pisum sativum

Fully expanded leaves (10-15g) were excised from 4-5 week old plants which had been kept in the dark for 30h. Younger plants give poorer yields of protoplasts, (Von Arnold & Eriksson, 1976). The lower epidermes were removed with forceps and the mesophyll cells plasmolysed by floating the leaves on 0.7M sucrose for 1h. The leaves were then washed in 2.5% (w/v) KCl and 1% (w/v) $MgSO_4 7H_2O$. A mixture of either Macerozyme R-10 (Onazuka) (0.1% w/v) and Cellulase R-10 (Onazuka) (0.5% w/v) or Driselase (2.4% w/v) with either KCl (2.5% w/v) and $MgSO_4.7H_2O$ (1% w/v) or mannitol (0.6M) were used to digest the plant cell walls and release the protoplasts. Carbenicillin was sometimes included (100 µg/ml) to limit bacterial contamination (Watts & King, 1973). The leaves were incubated for 2-3h at 30° in 200-250 ml of the mixture. They were then gently agitated and the liberated protoplasts removed by filtration through nylon mesh (77 µm). The filtrate was centrifuged at 100g for 3min. and washed twice by similar centrifugation in KCl (2.5% w/v) and $MgSO_4.7H_2O$ (1% w/v). The pellet was suspended in 50 ml of 20% sucrose and centrifuged at 1,000g for 3 min. Intact protoplasts formed a band at the surface of the sucrose solution and were removed with a pasteur pipette. Protoplast number and contamination by isolated vacuoles were

estimated by mounting in 20% sucrose in a Fuchs-Rosenthal cell counting chamber, depth 0.2mm.

IV) Microscopy

Simple observations of isolated haustorial complexes or stripped epidermes were made by means of phase contrast or Nomarski interference contrast microscopy. Specimens were usually mounted in water, isolation medium or in 20% lactophenol after staining with acid fuchsin (1% w/v) in lactophenol. Estimates of haustorial numbers were made by means of a haemocytometer slide (0.1 mm depth). Measurements were made with a micrometer eyepiece which had been calibrated against a graduated slide.

A) Estimation of the frequency of haustoria

The frequency of haustoria on the leaves was estimated by peeling upper epidermes and mounting them in water over a haemocytometer grid and counting the number of haustoria per unit area. Total leaf areas were estimated by photocopying detached leaves from 30g of trimmed plants cutting out the paper images and weighing them. Areas were then calculated by proportional comparison.

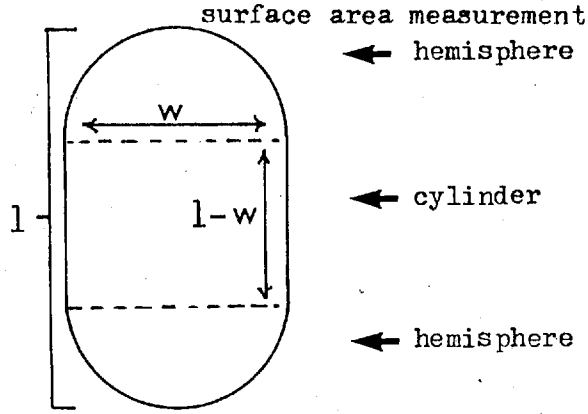
B) Estimation of surface areas and volumes

i) Haustorial complexes

The volume of the haustorial complexes was calculated from measurements of the average lengths and widths of complexes in the

fraction. The idealised shape of an haustorial complex was taken to be as shown in fig. 2.

Figure 2 The idealised shape of an haustorial complex for volume and surface area measurements.



The average volume and surface area were thus calculated from length and width measurements with the following formulae.

$$\text{Volume} = \frac{4}{3}\pi \left(\frac{w}{2}\right)^3 + \pi\left(\frac{w}{2}\right)^2 (l - w)$$

$$\text{Surface area} = 4\pi \left(\frac{w}{2}\right)^2 + 2\pi\left(\frac{w}{2}\right) (l - w)$$

ii) Plant Protoplasts

Isolated pea mesophyll protoplasts were spherical and their surface area and volumes were estimated from measurements of average diameters.

V) Leaf disc experiments

A) Photosynthesis, dark $^{14}CO_2$ fixation and uptake by the mycelium.

Batches of discs (12mm diameter) were placed on a moist filter paper in small perspex chambers (volume 27 ml) and each chamber sealed with a supaseal. Injection of 20 μCi of $NaH^{14}CO_3$ (60 mCi/mmol) was followed by 0.5 ml of 50% lactic acid. Chambers were then incubated at 25° and illuminated by an Atlas tungsten reflector lamp providing 10,000 lux at the chamber surface. After 5 min, 1 ml of 2N KOH was injected and the discs removed. Dark fixation of $^{14}CO_2$ was estimated by light-proofing the chambers with aluminium foil. For photosynthesis experiments discs were removed from the chambers and dropped directly into boiling 80% ethanol. Uptake by the mycelium on infected discs was estimated by gently brushing the discs with a camel hair brush, the mycelial brushings and brushed discs were then also dropped into boiling 80% ethanol. Ethanol extraction was for 2h and the insoluble material then subjected to boiling 1.5 N H_2SO_4 for 2h. The acid was neutralised by adding $BaCO_3$ (Lewis & Harley, 1965). The ethanol and acid extracts were then reduced to a known volume at 50°C and the radioactivity estimated by liquid scintillation counting.

B) Leakage of ^{14}C photosynthates

Discs (3 or 6 mm in diameter) were cut from uninfected leaves and from infected leaves where the mycelium had been removed with a camel hair brush. They were washed for 3 min in distilled water, blotted and placed on moist filter paper in a perspex chamber (volume 420 ml) which was sealed.

A quantity of $NaH^{14}CO_3$ (60 mCi/mmol) containing 100μCi activity was injected and followed by 1ml of 50% lactic acid. The chamber was illuminated as above (V,A). After the exposure the discs were removed; 10 were taken and their dry weight estimated after heating in an oven at 80° for 48h. The remainder were placed into 3 x 25 ml flasks (10 per flask) and incubated in 10 or 15 ml of distilled water at 25° on a shaking waterbath. At hourly intervals discs from successive flasks were removed by filtration with sintered glass. The discs were then extracted in 80% ethanol and 1.5 N H_2SO_4 as above. Radioactivity in the extracts and incubation medium was estimated by scintillation counting. The ion content of the incubation medium was also estimated with a conductivity bridge.

VI) In Vivo uptake of ^{14}C photosynthates by haustorial complexes

To study uptake kinetics $^{14}CO_2$ was fed to infected leaves, haustorial complexes were subsequently isolated and radioactivity estimated. Two experimental procedures were used and will be described separately both here and in outline in the results section.

A) Short exposure procedure

Detached infected and healthy leaves were used for these experiments. For infected leaves the mycelium was either left attached or removed by gentle brushing with an electrically rotated cotton mop prior to exposure. Seven gram quantities of leaves were arranged on a rectangular perspex rack which was placed in a modified McIntosh and

Fildes glass container (31) normally used for incubation of bacterial cultures. A high humidity was maintained by moist tissue paper. After closing, CO_2 - free air was passed through the container for 5 min. Both taps were then closed and 1mCi of $NaH^{14}CO_3$ (60 mCi/mmol) injected through a rubber supaseal so that it collected in a shallow dish. This was followed by 1ml of 50% lactic acid to liberate the $^{14}CO_2$. The vessel was then illuminated by 2 Atlas tungsten reflector lamps (each 150W) which provided 13,000 lux at its surface. A perspex tray of cold water was placed immediately beneath the lamps to prevent overheating. For some experiments (dark controls) the vessel was light-proofed by enveloping it in black plastic.

Exposures to $^{14}CO_2$ were ended by injecting 1ml of 4M KOH and blowing air through the vessel for 1 min. The leaves were removed, briefly washed in cold water and haustorial complexes isolated. If the mycelia were attached during the exposure, they were removed by brushing immediately before haustorial complex isolation. Haustorial complex fraction - 2 (IIA) was isolated to minimize contamination by the starch grains.

B) Long exposure procedure

Whole plants were used. The stems were trimmed with a razor blade to remove all leaves without confluent mildew colonies on their upper surfaces, and those showing etiolation or browning. For the experiments where the mycelia were to be separated from the leaves before $^{14}CO_2$

feeding, extreme care was taken not to damage or detach the leaves during brushing. The stems of these plants were then cut at the base using a wet razor blade. The plants were arranged on a perspex plate over a preweighed tray of water. The stems projected through perforations so that their cut ends were immersed in water. Batches of 130g of prebrushed plants or uninfected plants were arranged in this manner. (Preliminary experiments had shown that 138g of infected plants would yield approximately 130g after removal of the mycelium). The tray assembly was then placed in a rectangular perspex exposure chamber (55l) and the apparatus sealed. A solution of $NaH^{14}CO_3$ (1ml, 2mCi, 60 mCi/mmol) was injected through a rubber supaseal into a shallow container and $^{14}CO_2$ released by subsequent injection of 2 ml of 50% lactic acid. Air was circulated in the chamber by a small electric fan. Illumination was by two Atlas tungsten reflector lamps (each 150W) providing 13,000 lux at the surface of the chamber. Temperature within the chamber was $25^{\circ} \pm 1^{\circ}$. At the end of exposure, 4 ml of 2N KOH was injected and air passed through the chamber for 1 min. The plants were removed and the leaves detached and placed in a mixture of ice and water. The unbrushed leaves were placed in a cold room at 4° and small quantities removed for brushing. Afterwards these leaves also were immersed in an ice-water mixture until the brushing was completed.

Further treatment of all leaves was then identical.

i) Twenty 7 mm discs were punched out of leaves chosen at random and placed in 95% ethanol.

ii) The mycelium was recovered by means of a suction trap consisting of a funnel attached to a vacuum cleaner. Mycelia were collected from a 45 µm nylon mesh fixed in the narrow orifice of the funnel. Samples of these were placed in 95% ethanol.

iii) The leaves were washed 5 times in an ice-water mixture and twice in distilled water at 4°C and haustorial complexes (fraction - 1) then isolated as described previously. No additional gradient was used to reduce starch contamination.

VII) In vitro uptake of solutes.

A) Haustorial complexes

Although the concentrations of the solutes varied with experiments (see results section) the method used for detecting uptake was similar. Haustorial complexes (fraction - 1) were isolated by the method described above (IIA) and suspended in 0.1M phosphate buffer pH 6.8. Uptake was then measured by the following two methods.

i) Fractions of 0.2 ml of the haustorial complex suspension were placed in 5 ml centrifuge tubes. 0.1 ml of the ^{14}C labelled solution was added to give the final required concentration. Tubes were then incubated at 25° on a shaking water bath. To end the uptake process 4 ml of cold buffer was then added and the haustorial complexes separated from the medium by 3 centrifugations (4,000g) in cold buffer at 4°C.

ii) To study uptake kinetics of single compounds, the haustorial complex fraction (1.5 ml) was added to a 50 ml tube and 0.5 ml of the ^{14}C - labelled solution added. The tube was incubated on a shaking water bath as above and at various time intervals 0.2 - 0.4 ml were removed and haustorial complexes separated from the medium by centrifugation as above (VII, A, i).

B) Protoplasts

For protoplasts, a suspension was added to a known volume of radioactive solution to give the required final concentration. This was incubated at 25° in a shaking water bath and 0.5 ml aliquots removed at intervals. These were added to 20 ml of solution of KCl (2.5% w/v) and $MgSO_4\ 7H_2O$ (1% w/v) in 50 mM phosphate buffer pH 6.8 and washed twice by centrifugation at 500g for 2 min. The pellet was then resuspended in 20% (w/v) sucrose and centrifuged at 1,000 g for 3 min. The intact protoplasts were recovered from the surface of the sucrose solution by a pasteur pipette. Their cell density was estimated and the radioactivity of the protoplast suspension and the supernatant from the final centrifugation was estimated by scintillation counting.

XIII) Oxygen uptake by isolated haustorial complexes

Oxygen uptake by haustorial complexes (fraction 1) isolated from two batches of 80g of brushed leaves was measured by suspending them in 0.5 ml of 0.1M phosphate buffer pH. 6.8, and 0.1 - 0.4M mannitol, sucrose and glucose in buffer in a Clark oxygen electrode chamber at 25°.

After 20 min measurements were begun and continued over the subsequent 15 min incubation. Cell densities were estimated after each incubation. The electrode was calibrated against distilled water (oxygen concentration 240 nmoles O_2 / ml) and water containing crystals of sodium dithionite.

IX) Autoradiography

The stripping film technique of Berriman et al (1950) and Domiack and Pelc (1950) as described by Rogers (1973) was employed.

A) Preparation of slides

Glass slides were prewashed by the following procedure.

12h - in 100 g of potassium chromate,
100 ml of H_2SO_4 (conc) and
850 ml of H_2O.

4h - in running tap water

3 x 1h - in distilled water

2 x 1h - in double glass distilled water

They were then allowed to dry in a dust free atmosphere. One drop of a suspension of labelled or unlabelled haustorial complexes (10^5/ml) in double distilled water or 70% (v/v) ethanol was then placed at one end of the slide and allowed to spread over a 400 mm length of the slide. A label was then attached at the other end and the slide dried.

B) Application of emulsion

AR-10 (Kodak Ltd) stripping film was used. Darkroom conditions were critical; humidity was 60%, temperature 24° - 25° and a Kodak No 1 (red) safelight filter with a 25W bulb was placed a distance of 1½m from the workbench and dimmed to a minimum.

Glass plates with the film attached were held at their edges and, with a scalpel blade, the emulsion slides were scored 10 mm from the edge. The central rectangle of film was then scored into 6 smaller rectangles, each 80 x 40 mm. The films became detached at their edges and were then taken by forceps and gently peeled from the glass. Flashes of static electricity were avoided by breathing on the film during the stripping process.

The plates were stripped just above a trough filled with double distilled water at 25°. As each film left the plate it was laid on the water surface with the emulsion side down and left to imbibe water and expand for 2 - 3 min. The film was then picked up by holding the slide at the end furthest from the smear with the specimen uppermost and dipping it into the water under the film and was lifted so that the film's ends wrapped around the slide and smoothly covered the specimen. The slide was dried in a vertical position for 1 h at low humidity (30%) and then overnight in a desiccator. Slides were stored in light proof boxes at 4° for 5 days after applying the emulsion.

C) Development and Fixation

The slides were immersed for 5 min in Kodak D-19 developer at 16°, rinsed for 30 - 60 sec in distilled water (14°) and fixed in 30% (w/v) sodium thiosulphate at 12° - 14° for 5 min. They were finally washed in 4 changes of double distilled water at 12° and dried in a desiccator for 12 h.

D) Experimental Controls

The following controls were used,

i) An unlabelled suspension of haustorial complexes to detect positive chemography (Rogers, 1973) was treated similarly.

ii) A slide of labelled complexes was exposed to the light prior to development and fixation, this permits detection of negative chemography (Rogers, 1973).

X) Analytical Techniques

A) Homogenisation of samples

Prior to all analysis, samples of haustorial complexes, leaves and mycelia were homogenised. The following two techniques were employed.

i) Sonication

Haustorial complex fractions only were subjected to 5 periods each of 10 sec with a Sonicator (MSE - Mullard 7685/2).

The temperature was maintained at 4° by immersing the sample tube in an ice bath during sonication. Microscopic examination showed this completely disrupted all the complexes in the fraction.

ii) Potter homogenisation

Suspension of leaf discs, mycelia and haustorial complexes were disintegrated by 30 strokes in a light fitting glass Potter homogeniser at 4° .

B) Assay of Radioactivity

i) Scintillant mixtures

Two scintillant mixtures (S1 and S2) were used in this study.

S1) This was adopted from Spencer and Banerji (1971). The dimethyl sulphoxide necessary to solubilize blood plasma was omitted . The composiion of the scintillant mixture was as follows. All reagents were of scintillation grade.

10 g	Fumed silica (Cab - O - Sil)
5 g	Naphthalene
0.35 g	2.5 - Diphenyloxazole.
0.015 g	1,4 - Di - 2 - (5-phenyloxazolyl) - benzene.

These were then made up to 50ml in 1,4 Dioxan.

This scintillant mixture forms a gel and was particularly valuable for counting particulate suspensions, e.g. sonicated cells and ethanol insoluble residues. Since it has a maximum water capacity of 5 - 6%, aqueous and ethanolic solutions were also counted with it. Aliquots of

0.2 - 0.5 ml of sample were incorporated into 10 ml of scintillant. The efficiency of counting was usually between 65 and 75%.

S2) This scintillant mixture was adapted from Patterson and Greene(1965) and includes the non-ionic detergent Triton X-100 which permits the counting of larger proportions of aqueous samples by emulsifying them in the scintillant mixture. The composition of this mixture was:

34 ml	Toluene
16 ml	Triton X-100
0.2 g	2,5 - Diphenyloxazole
0.005 g	1,4 - Di - 2 - (5-phenyloxazolyl) - benzene

This had a water capacity of up to 15% and at that level there was little loss of counting efficiency. Aqueous and ethanolic samples of 0.2 - 1 ml were added to 10 ml of scintillant. Efficiency of counting was usually between 70 - 80%.

All counting was carried out in polypropylene counting vials (Packard Ltd) except where dry weight estimates were made prior to counting, when glass vials were used. The polypropylene vials have the advantage of very low background counts, (20 - 24 cpm). Counting was done in a Tracerlab Corumatic - Spectromatic Scintillation counter.

ii) Internal Standardisation

To estimate the efficiency of counting, the experimental vial containing the scintillant mixture and sample were weighed after counting and 0.1 ml of ($U - {}^{14}C$) hexadecane (1.13×10^{6} dpm/g) was added and the vial

reweighed. The hexadecane is completely miscible in the solvent and permits estimation of the efficiency of both the counter and scintillant and the degree of quenching by chemicals in the sample. This system was preferable to the channels ratio method (Bush, 1963) which gave inconsistent results with the S1 scintillant mixture.

C) Assay of Protein

Protein was determined by the method of Lowry et al (1951). Samples containing 60 - 150 µg protein were measured into test tubes with a microlitre syringe and made up to 100 µl with water. If the sample contained insoluble protein (e.g. membrane components), 0.4 ml of 10% (w/v) sodium deoxycholate was added to solubilize it; otherwise 0.4 ml of water was added. Then 5 ml of Na_2CO_3 (2% w/v) in 0.1N NaOH, 50 µl $CuSO_4 . 5H_2O$ (1% w/v) and 50 µl sodium-potassium tartrate (2% w/v) was added, mixed thoroughly and left to stand for 10 min at room temperature. A 0.5 ml quantity of a mixture of 1 part Folin - Ciolcalteau phenol reagent with 2 parts water was added whilst mixing vigourously with a vortex mixer (Whirlimix, Fisons). When the tubes had stood for 30 min at room temperature, optical densities were estimated at 600 nm. Bovine serum albumin was used as a standard.

D) Assay of Carbohydrate

i) Anthrone assay for total carbohydrate, (Dische, 1962). Up to 500 µg of carbohydrate in 1 ml of water was added to a tube and followed by 4 ml of anthrone reagent (0.2%) in concentrated sulphuric acid.

The tube was heated to 100° for 10 min. When cool, the optical density was estimated at 620 nm and compared to that of glucose standards.

ii) Nelson - Somogyi method for reducing sugars (Nelson, 1944). This is a sensitive assay and requires careful preparation of the following reagents.

Copper reagent A.

25 g	anhydrous sodium carbonate
25 g	sodium-potassium tartrate
20 g	sodium bicarbonate
200 g	sodium sulphate.

These were dissolved in 800 ml of water and then diluted to 1 l filtered and stored at room temperature.

Copper reagent B.

15 g of $CuSO_4 . 5 H_2O$ and one or two drops of concentrated H_2SO_4 were dissolved in 100 ml of water.

Arsenomolybdate reagent

Ammonium molybdate (25 g) was dissolved in 450 ml water and 21 ml of concentrated H_2SO_4, 3 g of $Na_2HAsO_4 . 7H_2O$ in 25 ml water were added sequentially and the solution incubated at 37° for 48 h. The reagent was then stored at room temperature in a brown glass bottle in the dark.

Assay procedure:

Copper reagents A & B were mixed on the day of use in the proportions of 25 parts A to 1 part B. The test sample (0.2 ml, containing up to 50 µg/ml reducing sugar) was added to 0.8 ml water and 1 ml of copper reagent, this was heated at 100° for 30 min. After cooling, 1 ml of arsenomolybdate reagent was added and thoroughly mixed with a 'Whirlimix' until CO_2 evolution had ceased. The blue colour produced was estimated by spectrophotometry at 660 nm against a water blank. Glucose solutions (between 5 and 50 µg/ml) were used as standards.

iii) Dinitrosalycilic acid assay for reducing sugars, (Broderson and Ricketts, 1949).

To make up the reagent, 1 g of dinitrosalycilic acid was dissolved in 5 ml of water with constant stirring and heating. Dropwise additions of 20 ml of 2N NaOH were followed by 30 g of sodium-potassium tartrate and the volume made up to 100 ml.

One ml of this reagent was added to 1.5 ml of sample and boiled for 5 min. When cool the optical density of the solution was measured at 540 nm. Glucose (50-500 µg/ml) solutions were used as standards.

iv) Thiobarbituric acid assay for uronic acids, (Preiss and Ashwell, 1963).

A 1 ml sample was added to 5 ml of thiobarbituric acid (0.01M) and 2.5 ml HCl (0.5M) in a test tube and mixed well. The tube was capped and heated in a boiling water bath for 30 min. After cooling the optical

density was measured at 515 nm and compared to that of galacturonic acid standards (200μg/ml).

Ion exchange chromatography was carried out on some samples prior to assays. To separate basic and acidic compounds, aqueous solutions were passed through the following ion-exchange resins.

i) Amberlite IR - 120(H^+)

This cation exchange resin was regenerated by washing in 4 bed volumes of 2N HCl and washing with double distilled water until a constant pH was attained.

ii) Amberlite IR - 45 (OH^-).

This anion exchange resin was regenerated by washing in 4 volumes of 4% NaOH followed by double distilled water until constant pH was attained.

iii) Dowex - 50 (x 8 mesh H^+)

This was regenerated with 4 volumes of 2N HCl and 10 volumes of deionised water.

iv) Dowex - 1 (x 10 mesh CH_3COO^-form)

This was regenerated from the chloride form by flushing with 4 volumes of 0.5 M NaOH, 8 volumes deionised water, and washing overnight in 4 volumes of 0.5N acetic acid and then with deionised water to a constant pH.

Columns of the above resins (2-3g) were made up in pasteur pipettes and aqueous extracts passed through.

E) Qualitative analysis of neutral sugars and polyols

Sugars and polyols were separated from extracts and identified by paper chromatography.

i) Extraction of ethanol soluble compounds.

Mycelial, leaf and haustorial complex fractions were homogenised in 80% ethanol and the homogenate refluxed for 2 - 3 h. Insoluble material was separated and washed twice in 80% (v/v) ethanol by centrifugation at 12,000 g. The supernatants were combined and reduced at 40° on a rotary evaporator to near dryness in a preweighed flask. If the dry weight was to be estimated the flask was then dried under vacuum for 30h at 50° and then reweighed. The dry weight of the insoluble residue was estimated similarly. The samples were then dissolved in water.

ii) Clearing

To remove from the extracts, coloured materials which may interfere with chromatography, a technique modified from that of Yemm (1935) was used. The extracts after solution in water, were mechanically shaken with equal volumes of 20% aluminium hydroxide suspension in a conical flask for 15 min and then filtered.

iii) Deionization

When cleared extracts were to be deionised, a 10 g resin mixture consisting of Amberlite IR - 120 (H^+) and Amberlite IR - 45 (OH^-)in equal proportions to 10 ml of the extract (Lewis and Smith,1967b). The extracts and resin were shaken for 30 min and then filtered through glass wool leaving solutions of neutral compounds only.

iv) Chromatography

a) Chromatography papers

Large sheets (46 x 57 cm) of Whatman No 1 and No 4 unwashed chromatography papers were used.

b) Spotting chromatograms

Extracts were reduced to small volumes (0.2 - 1 ml). Aliquots containing 30 - 100 µg of standard or of carbohydrate previously estimated by Anthrone assay, were then spotted on to the origin with a microsyringe. Individual additions were limited to 2 - 3 µl so that the spot was no larger than 5 mm in diameter.

c) Development of chromatograms

Single dimensional descending chromatography was employed essentially as described by Smith (1960). The end of the paper was serrated to allow the solvent to drip evently off the paper. All chromatograms were run in deep glass chromatography tanks at room temperature. The solvents used are listed in table 1 which includes their abbreviations used throughout this text.

Table 1 Solvent systems used in paper chromatography for sugars & polyols

Solvent system (composition by volume)	Abbreviation	Compounds separated & comments.	Paper used	Duration of run	Reference
n - propanol - 7 ethyl acetate - 1 water - 2	PEW	Best for disaccharides, myo-inositol. Some value for hexoses, pentoses.	No 1	24 - 30h	Smith (1960)
ethyl-methyl ketone - 9 acetic acid - 1 sat. boric acid - 1	MAB	Will separate isomeric polyols; good separation of hexoses & polyols.	No 1	24 - 30h	Rees and Reynolds (1958)
ethyl acetate - 14 acetic acid - 3 water - 3	EAW	Best general solvent, separates hexoses/polyols and of some value for disacchorides.	No 1 No 4	24 - 30h 18h	Smith (1960)
ethyl acetate - 50 acetic acid - 12 pyridine - 18 water - 10	EAPW	General solvent, separates most sugars but especially useful for separating glucose and galactose, and uronic acids.	No 1	30 - 36h up to 72h for uronic acids.	Jarvis and Duncan (1974)

d) Detection reagents

Several reagents were used to detect sugars and their specific reactions are listed on table 2. They were prepared and used as follows.

Silver Nitrate:

Saturated aqueous $AgNO_3$ solution was added to acetone in the proportion of 0.5 ml to 100 ml. Water was then added in drops until all the silver nitrate dissolved. Chromatograms were cut into strips, dipped into this solution and dried for 5 min before spraying with ethanolic NaOH (2% w/v). When dry, to permanently fix the spots, chromatograms were dipped in sodium thiosulphate solution (10% w/v) and washed for 30 min in cold water.

When the MAB solvent was used chromatograms were dipped into 5% (v/v) hydrofluoric acid in acetone to remove residual borate prior to applying the silver nitrate reagent (Britton, 1959).

p - Anisidine hydrochloride:

A 100 ml quantity of a 3% (w/v) solution of p - anisidine - HCl was prepared in moist butanol, 2 ml of acetic acid was added. Chromatograms were sprayed, dried for 8 min and then heated initially at 60°, inspected and then heated at 100° for 3 - 5 min. Spots were not permanent and were marked immediately.

Table 2 Chromatographic detection reagents employed for sugars & polyols

Detection Reagent	Aldo-pentoses	Aldo-hexoses	Keto-hexoses	Polyols	References
Silver nitrate	brown	brown	brown	brown	Trevelyan et al (1950)
p-Anisidine (HCl) + acetic acid	red	brown	yellow	no reaction	Hough et al (1950)
Bromocresol purple	no reaction	no reaction	no reaction	yellow on blue background.	Bradfield & Flood (1950)

Bromocresol purple:

Bromocresol purple (40 mg) and boric acid (100 mg) were dissolved in a mixture of 100 ml of 95% methanol and 7.5 ml of 1% (w/v) aqueous sodium tetraborate. This reagent could only be used with the neutral PEW solvent as it is very sensitive to pH. Spots were ephemeral and were marked immediately after detection.

e) Radioactive carbohydrates:

To detect radioactive compounds on the chromatograms, X-ray film (Kodak Ltd) was placed against the chromatogram and stored at - 20° for periods of up to eight weeks. After development areas corresponding to silver grains were cut from the chromatograms and eluted by cutting the paper into fine slivers and heating at 100° in each of two changes of water for 1 h the paper was removed by filtration and the eluates reduced to known volumes and counted in scintillant mixture S2. However, in some instances, particularly for the extracts from haustorial complexes, insufficient counts were present in each sample for detection by X-ray film and in these cases the chromatogram was cut into strips 10 or 5 mm wide and their radioactivities counted by dropping them directly into scintillant mixture (Boffey & Northcote, 1975). Peaks from these scans and spots from X-ray film were identified by comparison with standard sugars developed simultaneously on the chromatograms.

F) Quantitative analysis of neutral sugars and polyols

Sugars and polyols were analysed as their trimethylsilyl derivatives by gas - liquid chromatography (G.L.C.).

i) Extraction and preparation of fractions.

Sugars were extracted and leaf and mycelial fractions were cleared and deionised as described above. Haustorial complex fractions were deionised by passing through columns (each of 2 g) of Dowex 50 (H^+) and Dowex - 1 ($CH_3\ COO^-$). This was a necessary alternative to Amberlite IR - 120 and IR - 45 resins used in preparation of samples for paper chromatography, because they contaminated the sample with substances which interfered with the estimation of myo-inositol. This was especially important for the haustorial complex fraction since only small quantities of sugars were extracted.

ii) Preparation of trimethylsilyl derivatives.

A method adapted from Sweeley et al (1963) was employed.

Samples containing up to 1 mg of carbohydrate were dried in Eppendorf tubes in a vacuum desiccator. Anhydrous pyridine (1 ml) was then added to tubes containing standards, mycelial and leaf extracts and 0.2 ml for extracts of haustorial complexes. The tubes were then capped and heated at 80° for 4 h with occasional stirring. Extreme care was taken to ensure water did not contaminate the mixture (Holligan & Drew, 1971). Then, depending on the sample volumes, 0.2 or 0.05 ml of hexamethyldisilazane and 0.1 or 0.025 ml of trimethylchlorosilane were added.

The tubes were then capped and left overnight at room temperature. The following morning, the tubes were centrifuged at 3,000 g to remove the precipitates and the supernatants kept for analysis.

iii) Gas - liquid chromatography.

Glass columns (1.5 m long and 6 mm internal diameter) containing a stationary phase of 3% methyl phenyl silicone gum, SE52, were used. The supporting material was Chromasorb W. (80 - 100 mesh). A Pye 'Series 104' dual column gas chromatograph with flame ionisation detectors was used with argon as a carrier gas at a flow rate of 50 ml min^{-1}. A temperature programme of 4 min at 132° increasing to 260° at a rate of 4° or 6° per min, was employed. Samples (up to 10 µl) were injected into the columns by a Terumo micro syringe with a bent needle.

iv) Identification of compounds.

Compounds were identified by comparison of retention times and elution temperatures with those for known polyols and sugars. This was verified by simoultaneous injection of standards and samples.

G) Thin layer chromatography of lipids

Lipid extracts were analysed by ascending thin layer chromatography by the method of Skipski et al (1965). Preprepared silica gel plates were used, (Schleicher and Schull, T. L. C. plastic sheets, F1500. L5254, Silica with luminescer) and samples were streaked or spotted 20 mm from one edge of each plate. Plates were developed initially in isopropyl ether - acetic acid (96: 4 v/v) until the front had moved up the plate. The plate was dried for 45 min and then redeveloped in the same direction in

petroleum ether (40° - 60° boiling point) - ethyl ether - acetic acid (90: 10: 1 v/v) which was allowed to run to 195 mm from the bottom of the plate.

Plates were removed and after drying lipids were detected by lightly spraying with chlorosulphonic acid (Martin & Juniper, 1970) and heating at 150° for 10 min. To detect radioactive lipids the chromatogram which was usually 2 mm wide was cut across into 20 x 10 mm segments and each was dropped directly into 10 ml of scintillant mixture S2 and the radioactivity counted. The peaks of radioactivity along the chromatogram were identified by comparison of their position with the standards.

XI) Enzyme assays

A) Invertase

Enzyme extracts (soluble and insoluble) of the mycelium and haustorial complexes were assayed for invertase activity as follows.

A mixture containing 0.2 ml enzyme solution or suspension
0.3 ml H_2O.
0.5 ml 0.5 M sucrose
1.5 ml buffer (citrate - phosphate or phosphate buffers 0.1 M)

was incubated for 30 min at 32°. The reaction was stopped by adding 1.5 ml of dinitrosalycilic acid and boiling for 10 min.

Host (plant) invertase was estimated by the method of Bacon et al (1965) as adapted by Long et al (1975). Discs were made from infected leaves and healthy leaves and were washed in cold water and then shaken gently with cold ethyl acetate for 30 min. The discs were then washed again in cold distilled water. Thev were then resuspended in 10 ml of 0.1 M sucrose in 0.08 M citrate-phosphate buffer in a 25 ml conical flask. The flasks were then sealed and shaken for 3h at 32°. Afterwards, 2.5 ml aliquots were removed and assayed for reducing sugars. The discs were retained, blotted and their dry weight estimated after drying at 80° for 40 h.

B) Pectic enzymes

Enzyme extracts were assayed for the following three classes of pectic enzymes.

i) Pectin methyl esterase.

A cup-plate assay method was used (Mann, 1962); 2.5 g of citrus pectin was added to 245 ml of water and the pH adjusted to 6.0, methyl red solution (5.0 ml), salicylanilide (0.025 g) and agar (5.0 g) were added and the mixture sterilized by autoclaving for 15 min at 120°. Aliquots (25 ml) were pipetted into 90 mm diameter petri-dishes and when set, 9 mm diameter wells were cut in the agar with a No. 5 cork borer. Then 0.1 - 0.2 ml of enzyme sample was pipetted into each well and the plates incubated for up to 70 h. Acid liberated from pectin by the action of pectin methyl esterase is detected as a red zone around the well.

ii) Polygalacturonase and pectin transeliminase.

The reaction mixtures for this assay were as follows.

	Polygalacturonase	Pectin transeliminase
Subtrate	4 ml 1% citrus pectin or sodium polypectate in buffer	4 ml 1% citrus pectin or sodium polypectate in buffer.
Buffer	0.1M sodium citrate pH 5.0.	0.1M tris- HCl pH 9
$CaCl_2$	none (0.5 ml H_2O)	0.5 ml 10 mM $CaCl_2$
Enzyme sample	0.5 ml	0.5ml

Reaction tubes were incubated for up to 5 h at 30°. At intervals, 0.5 ml samples were removed from both sets and assayed for reducing sugars by the Nelson - Somogyi method. Galacturonic acid was assayed by the thiobarbituric acid assay.

C) Glucan synthetase

This enzyme was assayed by a method modified from Ray et al (1969). A 0.1 ml sample was added to 0.1 ml of uridine diphosphate ^{14}C-glucose (312 mci/mmol) with an activity of 10^6 dpm/ml. It was incubated for 30 min at 25° and then 5 ml of 70% alcohol added and the pellet washed three times by centrifugation at 10,000 g. Glucan synthetase was assayed as dpm ethanol insoluble 2×10^{-5} haustorial complexes $30\ min^{-1}$.

Results

I) The isolation and some light microscopic observations of haustorial complexes from apple, barley, beet, pea and vine.

The development of a satisfactory experimental system for mechanically isolating haustorial structures from the host plant has proved invaluable for investigating both structural (Gil, 1976; Gil & Gay, 1977; Dekhuijzen & Van der Scheer, 1969) and physiological aspects, (Gil & Gay, 1977 ; Dekhuijzen, 1966) of the host haustorium interface. It is now clear that for the powdery mildews investigated so far (*Erysiphe pisi* by Gil & Gay, 1977; and *Sphaerotheca fuliginea* by Dekhuijzen, 1966) that other interfacial components are released from the host and retained in association with the haustorium. The composite isolated structure has been termed the haustorial complex (Gil & Gay, 1977).

This section describes attempts to obtain purified fractions of haustorial complexes from other powdery mildews and in particular from *Erysiphe graminis* infections. It also compares structural and physiological features of haustoria of powdery mildews of gramineous and non-gramineous hosts.

A) Experimental procedures.

For host-parasite combinations other than pea mildew, 15 - 20 g fresh weight of infected leaves were used in each experiment. For pea up to 150g of leaves were used. Haustorial complexes were isolated and observed as outlined in the methods (II, A & B)

The osmotic potential of the extrahaustorial matrix was estimated for haustorial complexes isolated from pea. Isolated haustorial complexes were suspended in distilled water for 40h at 4° to permit the matrix to swell to its maximum size. The widths of haustorial complexes were then measured midway along their length with a micrometer eyepiece. The fraction was then divided into four batches which were each washed three times by centrifugation (2,000 g) in either, 0.5M sucrose, 0.1M sucrose, 0.05M sucrose or water. They were then each resuspended in one of these media and incubated at 4° for 24h and their widths remeasured.

The retention of semi-permeability after treatment with detergent was also studied. Complexes were transferred to 2% Triton X-100 at 4° for 1 min and then suspended in water and their widths measured after 2h. They were then transferred to 0.5M sucrose and the average width re-estimated. The effect of EDTA on swelling was estimated by suspending complexes for 5h in a 5mM solution and subsequently transferring them to water for

24h and measuring their widths. For each average width estimation 100 haustorial complexes were measured.

The presence of oxido-reductive reactions within haustorial complexes was monitored by suspending them in 1.5% (w/v) nitroblue tetrazolium; the presence of crystals within the complexes indicated reduction of the salt.

B) Results

i) Barley infected with Erysiphe graminis f. sp. hordei

Collection of intact haustorial complexes from barley leaves was virtually impossible unless the epidermis was first detached and then comminuted for a short period at low speeds. Even then, the long appendages of the mature haustoria were usually broken (plate 1) and in attempts when the whole leaf was comminuted, only fragments of the haustoria were obtained. Young haustoria and complexes whose lobes had not developed fully were released intact from epidermes (plates 2 to 7) but only small numbers of these were recovered in the final fraction.

In isolation medium the extrahaustorial membrane was usually not visible but when the fraction was suspended in water, the membrane quickly distended and separated from the central body in a small proportion of the haustorial complexes (plate 5). Treatment with acid fuchsin in lactophenol also caused distension of the extrahaustorial membrane (plates 1 to 4) and occasionally the collapse of the haustorial cytoplasm (plate 4). However,

Plates 1 to 9, E. graminis f. sp. hordei:
Plates 10 to 12, E. polygoni.

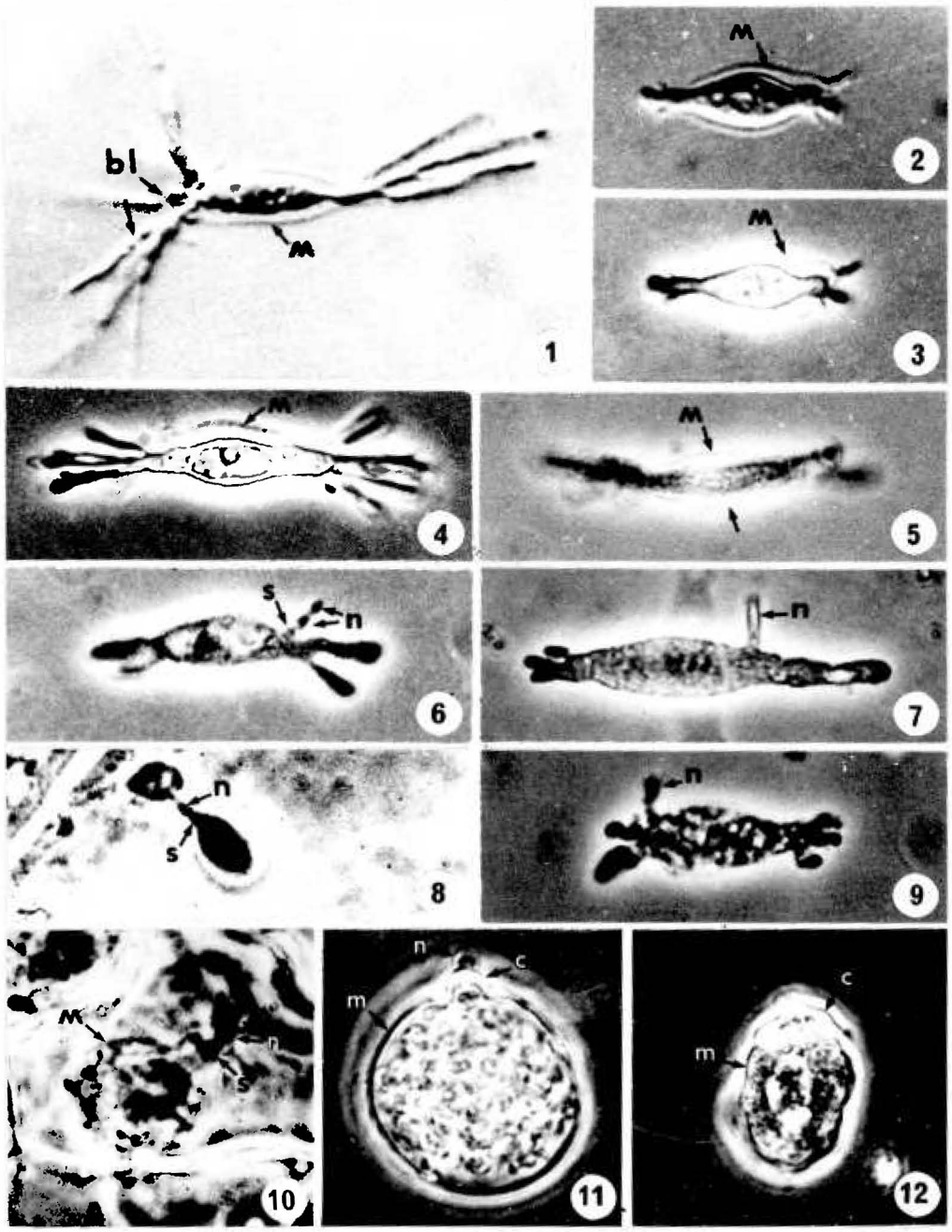
Plate 1. Mature isolated haustorial complex, 4 h after isolation from the epidermis. The lobe appendages at one pole of the haustorium are broken (bl) while those of the opposite appear intact. The extrahaustorial membrane (m) surrounds the central body of the haustorium but seems to be attached to the appendages. Mounted in 1% acid fuchsin in 20% aqueous lactophenol. Interference contrast optics. x 1100.

Plates 2 and 3. Young isolated haustorial complex, 4 h after isolation. The lobes are not yet developed and the membrane (m) can be seen around the haustorium. Mounted in 1% acid fuchsin in 20% aqueous lactophenol. Plate 2. Interference contrast optics. Plate 3. Phase contrast optics. x 950.

Plate 4. Isolated haustorial complex showing the extrahaustorial membrane (m) surrounding the haustorial body. The haustorial wall is shown clearly due to contraction of the haustorial cytoplasm. Mounted in 1% acid fuchsin in 20% aqueous lactophenol. Phase contrast optics. x1200.

Plate 5. Living isolated haustorial complex in water immediately following extraction. The extrahaustorial membrane (m) has distended away from the haustorial body. Phase contrast optics. x1150.

Plate 6. Living isolated haustorium in water. The extrahaustorial membrane is not visible having been damaged or lost during the isolation. The neck of the haustorium shows two opaque bands (n). The septum (s) is located close to the haustorial body. Phase contrast optics. x1100.



Plates 1 - 12

Plate 7. Living isolated haustorium showing the neck without dense bands but a uniform thickening along almost the whole of length. As in Plate 6, although the specimen is in water, the extrahaustorial membrane cannot be seen and is presumed to have been lost during the isolation procedure. Phase contrast optics. x1200.

Plate 8. Young haustorium developing in the epidermal cell of barley (cv. Proctor) showing that even at this early stage of development at least one band (n) can be discerned on the haustorial neck between the septum (s) and the host wall. Mounted in 1% acid fuchsin in 20% aqueous lactophenol. Phase contrast optics. x950.

Plate 9. Isolated haustorium after 16 h incubation at 4°C in 0.3m sucrose, 0.1 m phosphate buffer, pH 6.8. The cytoplasm has become very granular and the haustorium appears to be degenerate. Phase contrast optics. x1100.

Plate 10. Haustorium of E. polygoni in the epidermal cell of beet. The extrahaustorial membrane (m) surrounds the haustorium and the septum (s) and two dense bands can be discerned in the neck (n) of the haustorium. Mounted in 1% acid fuchsin in 20% aqueous lactophenol. Phase contrast optics. x950.

Plate 11. Living isolated haustorial complex after 24 h in water. The complex is swollen and many lobes can be discerned within the extrahaustorial membrane (m). The neck of the haustorium (n) is overlaid by a collar of refractile material (c). Phase contrast optics. x1000.

Plate 12. Living isolated haustorium immediately after isolation and mounted in 0.3 M sucrose, phosphate buffer 0.1 m, pH 6.8. The extrahaustorial membrane (m) is in a collapsed state around the haustorium and an extensive amorphous collar surrounds the neck. Phase contrast optics. x1000.

most of the structures isolated did not reveal an extrahaustorial membrane following these treatments and it is most likely that it was damaged or removed completely during the isolation procedure.

Isolation of haustoria and haustorial complexes facilitates a thorough examination of the haustorial neck where it is difficult to resolve detail in the intact infected cell. The neck is attached to one pole of the elliptical haustorial body adjacent to the origin of one group of lobes (plates, 6, 7 and 9). Two forms of neck could be distinguished in haustoria with lobes. The neck was either uniform in width (plate 7) or had two distinctly thickened bands (plate 6) and in each type a septum was located close to the haustorial body (plates 6 and 7). Observation of haustoria developing in epidermal cells showed that at least one band (probably that near the septum and equivalent to the B band (Gil & Gay, 1977) developed before 10 h after inoculation (plate 8).

When freshly isolated young haustorial complexes (as plate 5) were suspended in nitro-blue tetrazolium, opaque crystals formed in the cytoplasm in the bodies and lobes of intact complexes after incubation for 1 to 2 h. When similar haustorial complexes were incubated at 4° for 16 h, the haustoria

seemed to degenerate and the cytoplasm became granular (plate 9). Although the granularity made observation difficult, these haustoria probably did not reduce tetrazolium salt, indicating that they had ceased respiratory activity.

ii) Sugar beet infected with Erysiphe polygoni

The method of Gil & Gay, (1977) was applied without modification since haustoria of beet and pea are similar in form and size. Comminution of the brushed infected leaves also released large quantities of intracellular crystalline inclusions. These were present in such large numbers that they interfered with the separation of haustoria from chloroplasts in the glycerol gradient and, despite their small size (1 to 2 μm), they were only partially removed by filtration. The additional filtration which was necessary to remove the inclusions also allowed many of the smaller young haustoria to pass through the 5 μm mesh and thus only large well-developed haustoria (10 to 20 × 15 μm) were recovered in the final fraction. The haustorial complexes were similar to those isolated from pea (Gil & Gay, 1977) with a compact rounded form and many lobes investing the central body. Complexes were always released with the extrahaustorial membrane intact and this distended slowly when placed in water (plate 11). Attachment of the membrane to the haustorial neck was visible. In many of the isolated haustorial complexes the collar persisted as an

amorphous refractile substance overlying the extrahaustorial membrane in the region of the neck (plate 12). Observation of the neck was confused by this material, but two neck bands were discerned and examination of younger haustoria in epidermal cells confirmed their presence (plate 10). Incubation in nitro-blue tetrazolium indicated that respiration of these isolated complexes continued after incubation up to 16 h at 4°.

iii) Apple infected with Podosphaera leucotricha

As for sugar beet, haustorial complexes were easily released. However, the thick-walled mesophyll cells of apple were not disrupted by the comminution and were also released intact. Since they are larger than the haustoria they could be removed by filtration under pressure through 10 μm mesh. However, they eventually blocked the filter and reduced the yield of haustorial complexes. Thus the numbers of haustorial complexes finally obtained were low (10^3 to 10^4 from 20 g of leaf). The haustorial complexes were small (4 to 10 μm wide) and were of the compact type. The extrahaustorial membrane was always retained intact, and swelling in water showed that it was joined to the fungus at the neck. However, the small size of the haustoria did not permit a detailed examination of the neck region.

iv) Vine infected with Uncinula necator

Haustorial complexes were released from vine leaves by comminution but further purification was not possible because of the presence of needle-shaped intracellular raphides (crystals of calcium oxalate) which were released simultaneously. Their slender shape (15 to 20 x 1 to 2 μm) made it impossible to remove them by filtration and attempted separation by centrifugation proved fruitless because they were in such large numbers that they did not all come to rest at one interface of the gradient. The size of the haustorial complexes (10 x 5 μm) observed amongst these contaminants was similar to those from apple and smaller than those from beet or pea (Gil & Gay, 1977). The complexes were of the compact type and always had the membrane intact around the body and lobes.

V) Pea infected with Erysiphe pisi

The haustorial complexes have been fully described by Gil (1976). These were isolated with a greater degree of purity than from the other combinations described above (plate 13). The haustorial complexes usually constituted 25 - 65% of the total particles isolated. Fragments of host cell walls and starch grains were the major contaminants but rhombohedral crystals, up to 15 μm in length, were also often observed in the fractions.

These have a characteristic shape of crystals of calcium oxalate (Esau, 1965), but since they were not observed in sections of leaves, they were most probably formed during comminution. Chloroplasts could be completely removed from the fraction by careful aspiration of the glycerol gradient, but when some persisted, it could be easily seen by phase contrast microscopy that they were broken and thus probably consisted of thylakoid membranes (Walker, 1965). No intact host cells were observed and fungal spores and hyphae were extremely rare.

The complexes were similar to those from other non-gramineous plants infected with powdery mildews. The bipolar arrangement of the lobes, similar to that Erysiphe graminis haustoria was clearly observed when the extrahaustorial membrane was disrupted during isolation (plate 15). Since the membrane was usually attached this was a rare observation. The haustoria did not have any refractile material obscuring the neck region and two neck bands could clearly be distinguished both in isolated complexes (plate 14) and in leaf epidermal cells (plate 16).

Plate 13. Isolated haustorial complexes of Erysiphe pisi - Pisum sativum suspended in isolation medium. The haustorial complexes (hc) are intact and there are few contaminants of host origin e.g. cell wall material (w) and chloroplasts (cp). Phase contrast optics, x 450.

Plate 14. Isolated haustorial complex of Erysiphe pisi - Pisum sativum suspended in isolation medium. The neck region shows one of the neck bands (n). The haustorium is mature and many lobes (l) surrounded by the extrahaustorial membrane (m) can be seen. Phase contrast optics, x 1300.

Plate 15. Isolated haustorium of Erysiphe pisi stained with acid fuchsin and suspended in 20% (v/v) lactophenol. The bipolar origin of the lobes (l) from the haustorial body are clearly visible. Phase contrast optics, x 450.

Plate 13.

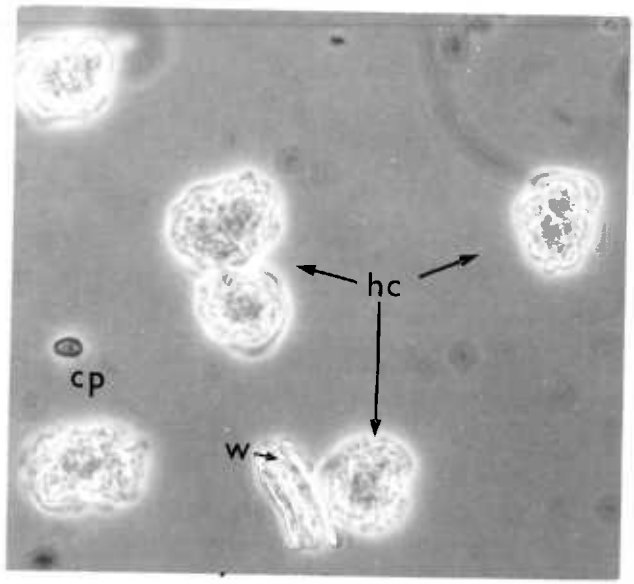


Plate 14.

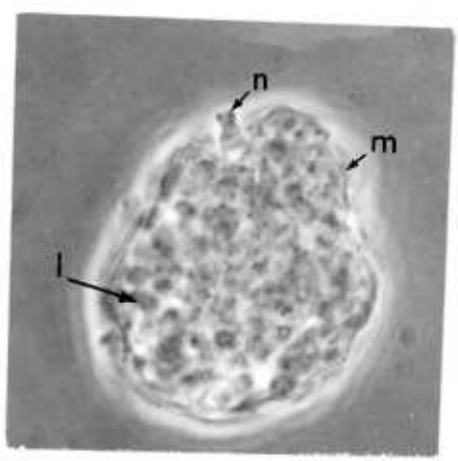
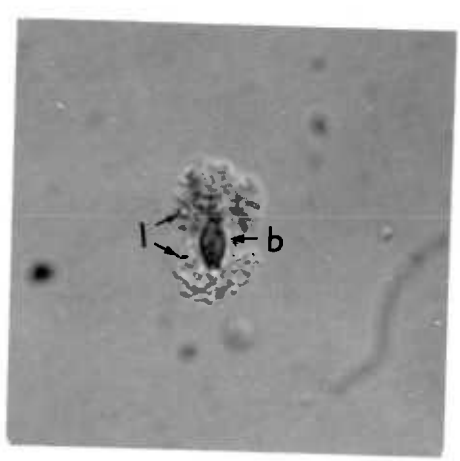


Plate 15.



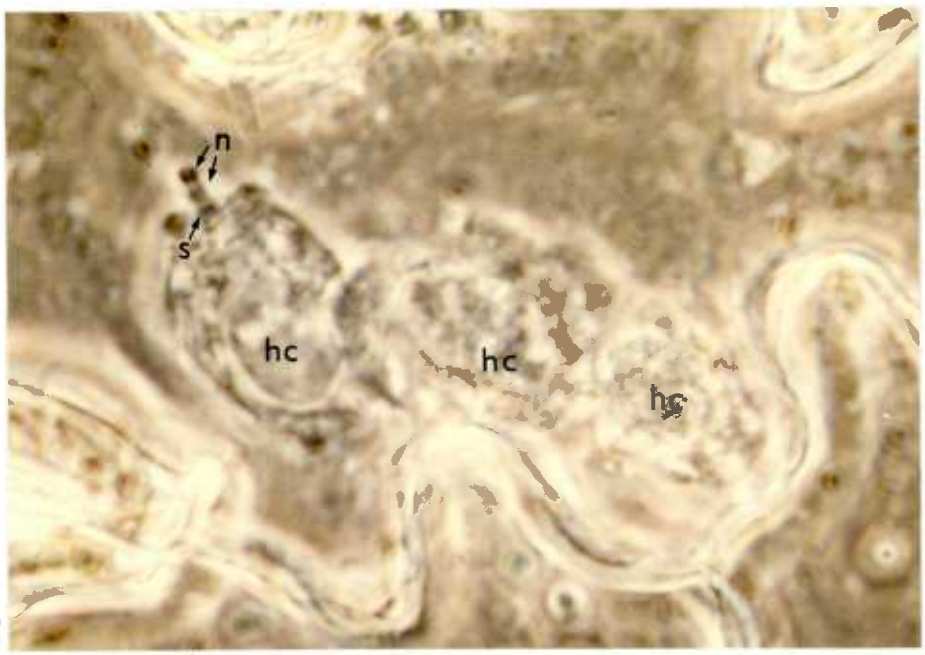


Plate 16. Three living haustorial complexes (hc) in a stripped epidermis of Pisum sativum infected with Erysiphe pisi and mounted in water. The a and b bands (n), septum (s) and extrahaustorial membrane are clearly visible on one complex. Phase contrast optics, X 950.

a) Estimation of the osmotic potential of the extrahaustorial matrix.

Swollen haustorial complexes slowly contracted in sucrose solutions of 50 mM (table 3) concentration which suggests that the extrahaustorial matrix has an osmotic potential higher than this solution. These results contrast with the conclusions of Hoskin (1969) who estimated the osmotic pressure of the matrix in incised cells of barley coleoptile epidermis infected with E. graminis and concluded that it was equal to that of 0.3 M sucrose. These discrepancies may be explained by the techniques employed. In the present study the complexes were swollen prior to estimation and this would dilute the matrical constituents. In contrast, the extrahaustorial membrane in E. graminis is thought to be adpressed closely to the haustorial surface in vivo (Bushnell, 1971) and thus the matrix would not suffer any such dilution prior to Hoskin's experiment. Moreover the incision of epidermal cells by Hoskin (1969) and the rapid volume changes recorded in the matrix around E. graminis haustoria permitted determination of the osmotic potential in situ whereas it is possible that solutes may have leaked from the matrix during the isolation of haustorial complexes of E. pisi - P. sativum. The results also suggest that the slow swelling response in these haustorial complexes may be due to small osmotic forces rather than low permeability to

Table 3

The widths of isolated haustorial complexes of Erysiphe pisi - Pisum sativum previously swollen in H_2O for 40h then transferred to either H_2O or sucrose solutions for a further 24h, all incubations were at 4°. Results are divisions of a micrometer eyepiece and are the mean of 100 measurements.

Medium	Width
H_2O	8.08 ± 0.32
0.05 M sucrose	7.07 ± 0.21
0.1 M sucrose	7.04 ± 0.14
0.5 M sucrose	6.85 ± 0.29

water or rigidity of the extrahaustorial membrane (Gil, 1976).

b) Effect of Triton X - 100 and EDTA on the extrahaustorial membrane.

Isolated haustorial complexes were suspended in 2% Triton X - 100 and replaced in water for 2h. This induced a significant increase in mean width from 8.01 micrometer units to 8.36 (significant, $p = < 0.05$). Two hours after being resuspended in 0.5M sucrose the mean width was reduced to 7.11 units, which indicates that the detergent treatment had not destroyed the semipermeability of the extrahaustorial membrane. Volume changes were induced more rapidly after detergent treatment indicating that the permeability of the membrane may have been altered. However the treatment may have also affected the solute concentration of the matrix by solubilising the cytoplasmic membranes of the haustorium or the rigidity of the membrane by removing components from it.

Gil & Gay (1977) proposed that pectins may be incorporated into the membrane since treatment with pectinase and cellulase removed polysaccharides from the membrane and this induced rapid swelling of the complexes.

Cell wall pectins are labile in solutions of the chelating agent EDTA and at a concentration of 5mM it has been used to totally remove galacturonic acid residues from the outer membrane of plant mitochondria (Mannella & Bonner, 1975). Similar treatment for 5h did not increase the swelling rate of the complexes and the final average width was 7.89 units which was not significantly different from those in water (8.01). When complexes were further treated for 24h at 4° with 50mM EDTA and examined by electron microscopy (Dr. C. Sargent, unpublished), the membrane was still intact with no observable reduction in thickness. These results suggest that the membrane does not have a large pectic component. Its composition should be investigated further using more purified enzymes than those used by Gil & Gay (1977).

II) Effect of infection on photosynthesis by the host, Pisum sativum.

The assimilation of $^{14}CO_2$ by the host is used routinely in this study and thus experiments were designed to quantify the effect of the pathogen on photosynthesis.

A) Experimental procedures

Infected pea leaves (7 days after inoculation) and healthy leaves were detached from the 5th node of four week old plants and discs (12 mm) cut from them. Four batches of 4 discs were exposed for 5 min periods in the light to an

atmosphere containing $^{14}CO_2$ (20 μCi, 60 mCi/mmol). They were subsequently treated in 80% ethanol and 1.5 N H_2SO_4 and the radioactivity of the extracts estimated by liquid scintillation counting.

B) Results

Table 4 shows that at 7 days after inoculation the mildew reduced photoassimilation of $^{14}CO_2$ by the leaf discs to 39% that of uninfected controls. These results are in direct agreement with those of Ayres (1976) for pea infected with E. pisi and with other studies of powdery mildew infections viz. Beta vulgaris - E. polygoni (Magyarosy et al,1976), Triticum aestivum - E. graminis (Allen,1942), Hordeum vulgare - E. graminis (Last,1963; Paulech,1966; Edwards,1970), Quercus robur - Microsphaera alphitoides (Hewitt & Ayres,1975).

Compared with infected leaves, healthy discs incorporated a significantly greater (probability = <0.02) proportion of label into compounds insoluble in ethanol. The ratios of label in ethanol soluble to insoluble fractions were 1: 0.14 for healthy discs and 1: 0.10 for infected discs. Exposures in the dark showed little difference in the $^{14}CO_2$ assimilated in infected and healthy discs, but since assimilation was lower in infected discs in the light dark fixation was proportionally higher in infected discs (1.5% of the amount in the light for infected and 0.5% for healthy discs). Mirocha & Rick (1967)

Table 4.

Pisum sativum - Erysiphe pisi. Assimilation of $^{14}CO_2$ by uninfected and infected leaf discs in the light and dark. The mycelium was left attached or separated by brushing prior to some exposures in the light. Results are the means of 4 replicate experiments each with 4 x 12 mm discs exposed to 20 μCi $^{14}CO_2$ for 5 min and are expressed as dpm x 10^3 mm^{-2} leaf $5\ min^{-1}$. Standard deviations are also shown.

	light		dark	
	ethanol soluble	ethanol insoluble, soluble in 1.5N H_2SO_4	ethanol soluble	ethanol insoluble, soluble in 1.5N H_2SO_4.
Uninfected, unbrushed.	20.94±3.40	2.89±0.11	0.10±.055	0.012±0.007
Uninfected, brushed.	10.70±2.15	1.30±0.21	-	-
Infected, unbrushed.	8.40±0.98	0.83±0.13	0.12±0.03	0.014±0.004
Infected, brushed.	4.66±0.91	0.40±0.06	-	-

have shown that the mycelium of E. graminis is responsible for fixation of"a substantial amount" of $^{14}CO_2$ in the dark but the present experiments suggest that this does not occur in E. pisi.

Removal of the mycelium by brushing is a routine procedure in experiments to estimate the uptake of ^{14}C photosynthates by haustorial complexes in leaves and the effect on $^{14}CO_2$ assimilation of brushing with a camel hair brush prior to $^{14}CO_2$ exposure are shown in table 4. These results show that in both uninfected and infected discs, brushing reduced the $^{14}CO_2$ assimilated by 45%. Thus removal of the mycelium by brushing has an effect on leaf physiology. No visible damage is inflicted by this treatment but closing of stomata may be partly responsible.

III) Assimilation of ^{14}C photosynthates by the mycelium

The nutritional dependance of biotrophic fungi upon their autotrophic hosts is most easily studied by supplying $^{14}CO_2$ which is assimilated by the autotroph, subsequent separation of the fungus then permits the uptake process to be followed and quantified (Smith et al, 1969). Experiments were designed to compare the kinetics of assimilation in the mycelium and host. Ethirimol (5 - n - butyl - 2 - ethylamine - 4 - hydroxy - 6 - methyl pyrimidine) is a systemic fungicide

(I.C.I. Plant Protection Ltd) active only against powdery mildews (Bebbington et al, 1969) and its effect on the assimilation of ^{14}C photosynthates by the mycelium was also studied.

A) Kinetics of assimilation.

i) Experimental procedures.

Leaf disc experiments similar to those for studying the effect of infection on photosynthesis (Results II) were set up. Discs were exposed to $^{14}CO_2$ for periods up to 1h and immediately after their removal from the chambers, the mycelia were removed with a camel hair brush and it and the disc were dropped into separate lots of boiling 80% ethanol. After refluxing for 2h the insoluble material was separated and washed twice in 80% alcohol by centrifugation (12,000 g for 10 min). The insoluble pellet was then similarly extracted in 1.5 N sulphuric acid. The supernatants were made up to known volumes and their radioactivities estimated by liquid scintillation counting (scintillant S1).

ii) Results.

Assimilation of ^{14}C per unit leaf area by the host exceeded that of the overlying mycelium as shown by ethanol and H_2SO_4 extracts respectively (fig. 3). Assimilation by the host was initially rapid with ethanol soluble compounds predominant, but the rate declined after 30 min. Ethanol soluble compounds were also predominant in the mycelium but the accumulation rate remained

Figures 3 and 4. Erysiphe pisi - Pisum sativum. Assimilation of ^{14}C compounds by the mycelium and host after exposure of infected leaf discs to $^{14}CO_2$ in the light. Each point is the mean of 4 replicate experiments, each with 4 x 12mm discs exposed to 20 μCi of $^{14}CO_2$.

Figure 3) Assimilation of ^{14}C per unit area of infected leaf. ^{14}C compounds were fractionated into compounds soluble in 80% ethanol (host -▲-, mycelium -■-) and insoluble in ethanol but soluble in 1. 5N H_2SO_4, (host -△-, mycelium -□-).

Figure 4) Total ^{14}C assimilated by leaf and mycelium per unit dry wt. Key (host -□-, mycelium -■-).

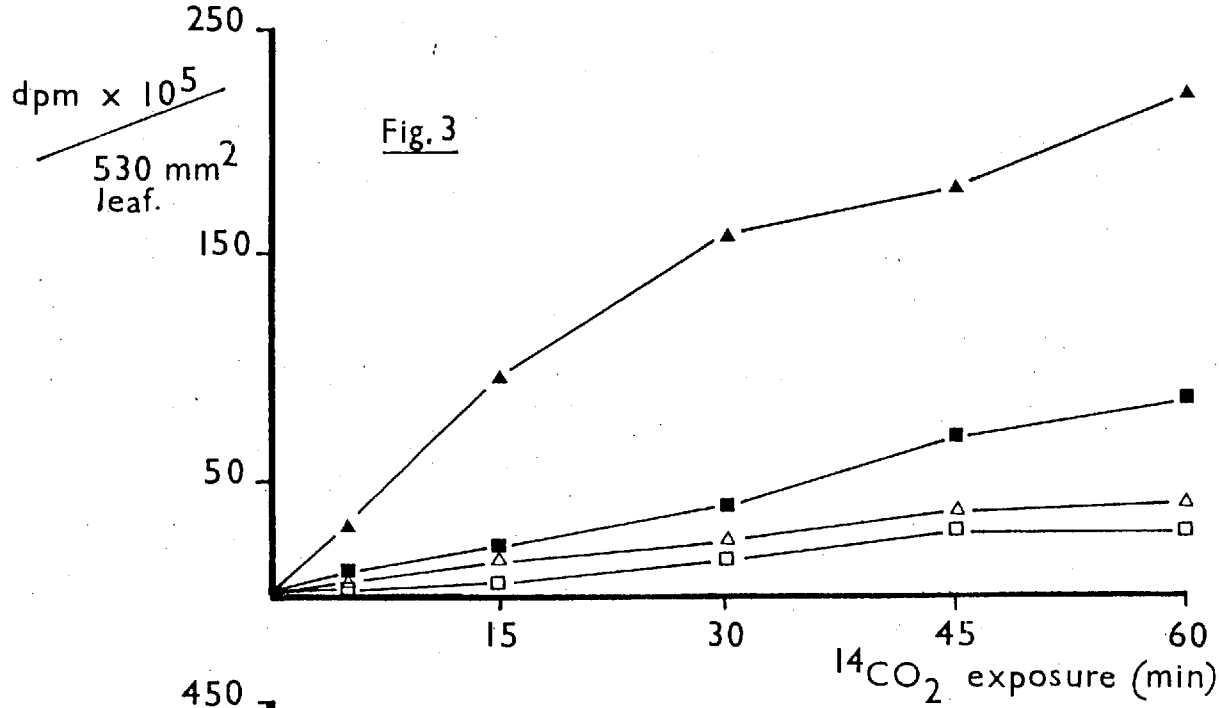

250
dpm x 10^5
530 mm^2
leaf.
150
50
15
30
45
60
14CO2 exposure (min)

Fig. 3

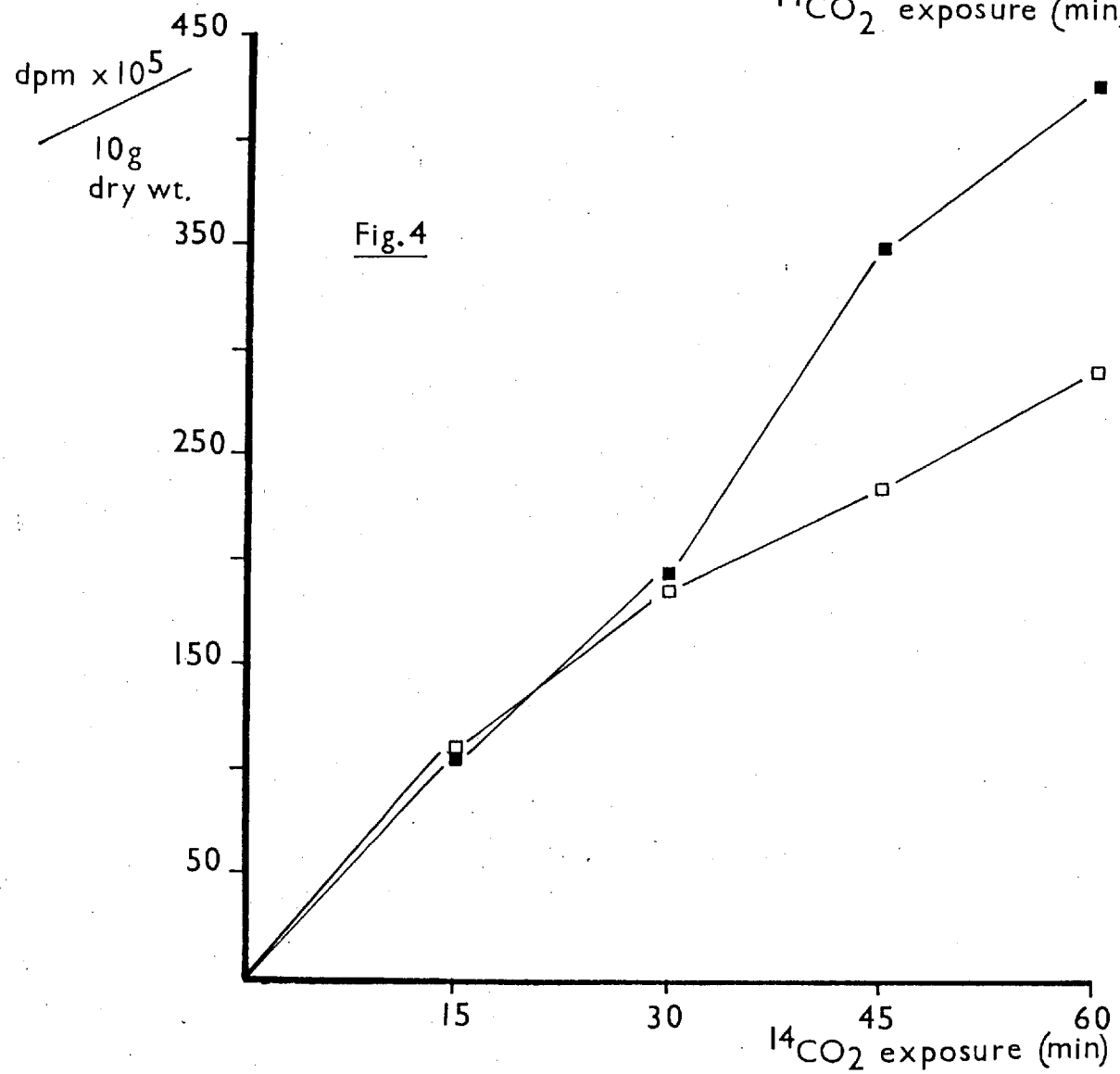

450
dpm x 10^5
10g
dry wt.
350
250
150
50
15
30
45
60
14CO2 exposure (min)

Fig. 4

steady until the experiment ended at 60 min.

The mean dry weight of 4 infected discs (530 mm^2) was 12.22 $\pm$ 1.83 mg and of 4 discs from which the mycelia had been removed by brushing was 9.50 $\pm$ 0.91. The mean dry weight of the mycelia was thus determined to be 2.72 mg and thus only 29% of the weight of unit area of the underlying host. When the data of fig. 3 is recalculated and the total assimilate plotted against dry weight (fig. 4) it is evident that the leaf and mycelium accumulate ^{14}C at the same rate for the first 30 min but thereafter accumulation by the mycelium exceeded that of the host.

The results agree with those of Edwards and Allen (1966) for E. graminis f. sp. hordei on barley except that ^{14}C assimilation by the mycelium on a dry weight basis in their experiments exceeded that of the host throughout the exposure period. It should be noted that in the present experiments and those of Edwards & Allen, (1966) brushed leaf discs still contain haustoria and thus the uptake by the mycelium described does not reflect the full extent of uptake by the parasite.

B) Effect of ethirimol on assimilation of ^{14}C photosynthates by the mycelium.

i) Experimental procedures

Thirty discs (8 mm) were removed from infected leaves and batches of 10 were incubated in 25 ml of distilled water or distilled water with the addition of ethirimol at 10 or 50 ppm for 16 h with

Table 5

Erysiphe pisi - Pisum sativum. The effect of ethirimol on assimilation of ^{14}C photosynthates by the mycelium. Infected discs were incubated in water and water with 10 ppm or 50 ppm ethirimol for 16h at 25° and subsequently exposed to $^{14}CO_2$ for 2h. The radioactivity in ethanol soluble and insoluble compounds in the mycelium was determined from the differences in activity of intact discs and those separated from the mycelium by brushing immediately after $^{14}CO_2$ exposure. The results are expressed at 10^{6} dpm disc^{-1} and each result is the mean of 5 discs.

Incubation media	Leaf Discs									
	H_2O				10ppm ethirimol				50 ppm ethirimol	
	Brushed discs		Intact discs		Brushed discs		Intact discs		Brushed discs	Intact discs
	sol	insol	sol	insol	sol	insol	sol	insol	sol insol	sol insol
	4.1	2.6	7.9	2.3	6.7	3.3	6.6	2.9	7.2 4.0	8.7 3.8
	Total		Total		Total		Total		Total	Total
	6.7 ± 1.2		10.2 ± 1.5		10.0 ± 2.7		9.5 ± 1.3		11.2 ± 1.6	12.5 ± 2.1
Mycelium (by difference)			3.5 (p= < 0.01)				0.0 (p= > 0.1)			1.3 (p= > 0.1)

gentle agitation in a shaking water bath at 25°. The discs were then removed and dried by gentle blotting between sheets of Whatman No 1 filter paper. Each batch of discs was then exposed for 2h in the light to 50 μCi of $^{14}CO_2$ (0.833 μmol) after the exposure periods the discs were removed and five from each were immediately dropped into boiling 80% ethanol. The remaining five were first brushed with a camel hair brush to remove the mycelium and before being dropped into boiling 80% ethanol. The ethanol was allowed to cool and all discs were left in it for three days and then the extract was separated by filtration through a sintered glass filter. The radioactivity of the ethanol extract was estimated by scintillation counting (scintillant mixture S2). The insoluble material was transferred to 1ml of PCS solubiliser (Amersham-Searle) in a scintillation vial and left overnight. On the following day 10 ml of scintillant (S2) was added and the activity estimated.

ii) Results

The results of these experiments are shown on table 5. The activity calculated for the mycelium from discs incubated in water was significant ($p = <0.01$) and indicated the transfer of 3.5×10^6 dpm disc^{-1} to the mycelium. This was 21% of the total assimilated by the infected discs. The differences between the mean activities of brushed and intact discs incubated in ethirimol (10 and 50 ppm) were not significant indicating that very little or no uptake by the mycelium had occurred. The results thus show

that ethirimol has an effect on the assimilation of nutrients by the mycelium. This may be due to an impairment of haustorial function.

IV) Uptake of ^{14}C photosynthates by haustorial complexes in vivo.

To study in vivo uptake by haustorial complexes infected leaves were exposed to $^{14}CO_2$ and the complexes subsequently separated from the levels by the isolation technique described in Methods (II,A). Two procedures were used and are considered separately.

A) Short term exposure to $^{14}CO_2$.

i) Experimental procedures.

Detached infected leaves (7g fresh wt) were exposed to 1 mCi of $^{14}CO_2$ by the short exposure procedure described in the methods (VI,A). Haustorial complexes (fraction - 2) were isolated immediately after the exposure and were suspended in 0.5 ml of phosphate buffer (0.1M) pH 6.8 at 4°. They were subsequently washed for 30 sec in 4 ml of 1% Triton X-100 and washed twice by centrifugation (3,000 g). The isolation and all subsequent treatments up to this stage were carried out at 4°. The supernatants were combined and an 0.2 ml aliquot removed to estimate the radioactive components solubilised by Triton X-100. The pellet was resuspended in 0.5 ml of buffer, sonicated and 0.1 ml aliquots removed for liquid scintillation counting (mixture S1) and protein estimation.

Mycelia were collected, homogenised in a Potter homogeniser. The radioactivity and protein content of the homogenate were estimated as for the haustorial complexes.

ii) Results

The isolation of haustorial complexes necessitates brushing to remove the mycelium which for 7g of leaves requires 10 - 15 min to complete. As the yield of isolated complexes from a smaller quantity of leaves would have been insufficient for analysis, it was thus not possible to investigate with precision the very early events in uptake by haustorial complexes. To partially overcome this deficiency experiments were carried out using infected leaves separated from the mycelium prior to the $^{14}CO_2$ exposure. When isolations were made from such prebrushed leaves, radioactivity was detected in the haustorial complex fractions (table 6) from 5 min and 20 min exposures to $^{14}CO_2$. Although a sucrose gradient was used to reduce starch grain contamination, microscopic examination showed that some starch grains and cell wall material persisted and it seemed likely that these had become labelled and would therefore interfere with the results. As a control on contamination, equivalent fractions were isolated from uninfected leaves similarly exposed to $^{14}CO_2$. Per unit protein, radioactivity in these fractions was only 12% of the haustorial complex fraction (table 6).

Table 6

Erysiphe pisi - Pisum sativum. Assimilation of ^{14}C by haustorial complexes and mycelia from detached infected leaves exposed to $^{14}CO_2$ (1mCi) in the light. The mycelium was either removed by brushing prior to $^{14}CO_2$ exposure or left attached. Control fractions (equivalent to the haustorial complex fraction) were isolated from uninfected leaves. Results are expressed as dpm $100\mu g$ protein^{-1}.

	Infected leaves			Uninfected leaves
	Mycelium removed	Mycelium attached		
Exposure to $^{14}CO_2$ (min)	Haustorial complex fraction	Haustorial complex fraction	Mycelium	H. C. - Equivalent fraction
5	1,691	1,936	11,232	207
20	4,410	-	-	521

When the mycelium was left attached for a five min exposure, the quantity of radioactivity detected in the haustorial complex fraction was similar to that where the leaves were separated from the mycelium prior to the $^{14}CO_2$ exposure. However, again it should be noted that 10 - 15 min is required to remove the mycelium and thus these two fractions are not directly comparable. Despite the rapid uptake of ^{14}C by the haustorial complexes the quantity of label per unit protein in the mycelium was 5.8 times greater than that of the haustorial complex fraction.

The haustorial complex and control fractions were all treated with cold 1% Triton X-100 prior to the estimation of protein and radioactivity. The purpose of this was to proportionatly reduce the effect of host contamination. It has been shown previously by swelling experiments with haustorial complexes (Gil,1976; Results I,B,V,) that the semipermeability of the extrahaustorial membrane is retained after such detergent treatments. Further evidence for this was obtained by determining the proportions of label solubilised by Triton X-100 treatment of the fractions (table 7). Triton X-100 solubilised a greater proportion of the total label from the control fraction obtained from uninfected leaves than from the haustorial complex fraction. A full consideration of contamination is made in the discussion.

Table 7

Erysiphe pisi - Pisum sativum. The proportions of radioactivity solubilised by cold 1% Triton X-100 treatment of haustorial complex fractions isolated from 7g of prebrushed infected leaves exposed to $^{14}CO_2$ and an equivalent fraction from uninfected leaves. (radioactivity is expressed as a percentage of total isotope in the fraction).

Exposure to $^{14}CO_2$ (min).	Haustorial complex fraction	Uninfected control
5	27	71
20	21	82

B) Long term exposure to $^{14}CO_2$

i) Experimental procedures

Since detached leaves are unsuitable for exposures which exceed 45-60 min as they desiccate rapidly, whole trimmed plants with their stems immersed in water were used for exposures to $^{14}CO_2$ up to 12h in duration. Plants (130g of prebrushed or uninfected plants or the equivalent weight (138g) of unbrushed infected plants) were exposed to 2 mCi of $^{14}CO_2$ in the light and dark as described in the methods (section VI,B) Afterwards, 20 leaf discs mycelial brushings and haustorial complexes (fraction 1) were obtained from the leaves, and the discs and mycelia were dropped immediately into boiling 95% ethanol. The haustorial complex fraction was suspended in 1.5 ml of 0.1M phosphate buffer pH 6.8 and their number estimated. The fraction was then divided into 3 equal parts each of approximately 0.5 ml. One part was resuspended in cold 1% Triton X-100 and washed 3 times in buffer by centrifugation (3,000g) at 4°. The supernatants were combined and the quantity of radioactivity solubilised by the Triton X-100 estimated by scintillation counting (scintillant mixture S1). The final pellet was suspended in 95% ethanol. The remaining two fractions were placed in 5 ml tubes, a disc of filter paper (Whatman No. 1) soaked in 2N KOH was suspended above each fraction and the tubes stoppered and sealed with Vaseline. One fraction was incubated in a shaking water bath at 20° for 12 h. The other was stored

overnight at 4° and incubated for 6 h at 20° during the following day. After these in vitro incubations each paper disc was removed and dropped into 10 ml of scintillant (mixture S1) and the haustorial complex fraction was treated with 1% Triton X-100, washed as described above and finally suspended in 95% (v/v) ethanol.

All samples were homogenised in a Potter homogeniser and then refluxed in boiling 95% ethanol for 3 h. The insoluble material was separated by centrifugation (12,000 g) followed by three washings in cold 95% ethanol. The supernatants were combined and reduced to a known volume at 60° under vacuum and 0.2 - 0.5 ml samples counted in scintillant mixture S1. The insoluble residues of the haustorial complexes, mycelia and leaf discs were dried at 80° to a constant weight in a preweighed scintillation vial and 0.4 ml of 95% (v/v) ethanol and 10 ml of scintillant mixture S1 added. The suspensions were thoroughly mixed and the activity estimated.

ii) Results

a) Isolation of haustorial complexes

The mean number of haustorial complexes isolated from 130 g of leaves and petioles was $2.14 \pm 0.91 \times 10^6$. Since the leaf surface area of a 30 g sample was 658 sq. cm. (upper infected surface only) and microscopic counting of haustoria in detached epidermes showed that there were 16,400 ± 4,100 haustoria / cm^2

of leaf surface, the total number of haustoria in each experiment was thus 4.7×10^7. The efficiency of extraction was therefore usually between 4 and 5%. This is lower than obtained by Dekhuijzen (1966) from infected cucumber leaves and is probably due to milder comminution of the leaves employed here. The degree of comminution used (see Methods II,A) was selected because it minimized both the period of extraction and the level of host contamination in the final fraction. The time required to complete isolation from 130g of leaves and stem segments was 2.5 - 3h.

b) Assimilation of $^{14}CO_2$ by the host and uptake by the mycelium.

The assimilation of $^{14}CO_2$ by illuminated infected leaves separated from the mycelium and spores before exposure to $^{14}CO_2$ is shown in fig 5. Assimilation was immediate and proceeded rapidly for the first 2h but then decreased, probably because the $^{14}CO_2$ became depleted. When the mycelium was attached during exposure to $^{14}CO_2$, there was considerable incorporation of label into it (30 - 50% per unit area, table 8), and (40-70%) of this was insoluble in ethanol. The total quantity of label assimilated when the mycelium was attached was less than when the mycelium had been previously removed. However, it cannot be ascertained whether the difference was directly due to the presence or absence of the mycelium or to

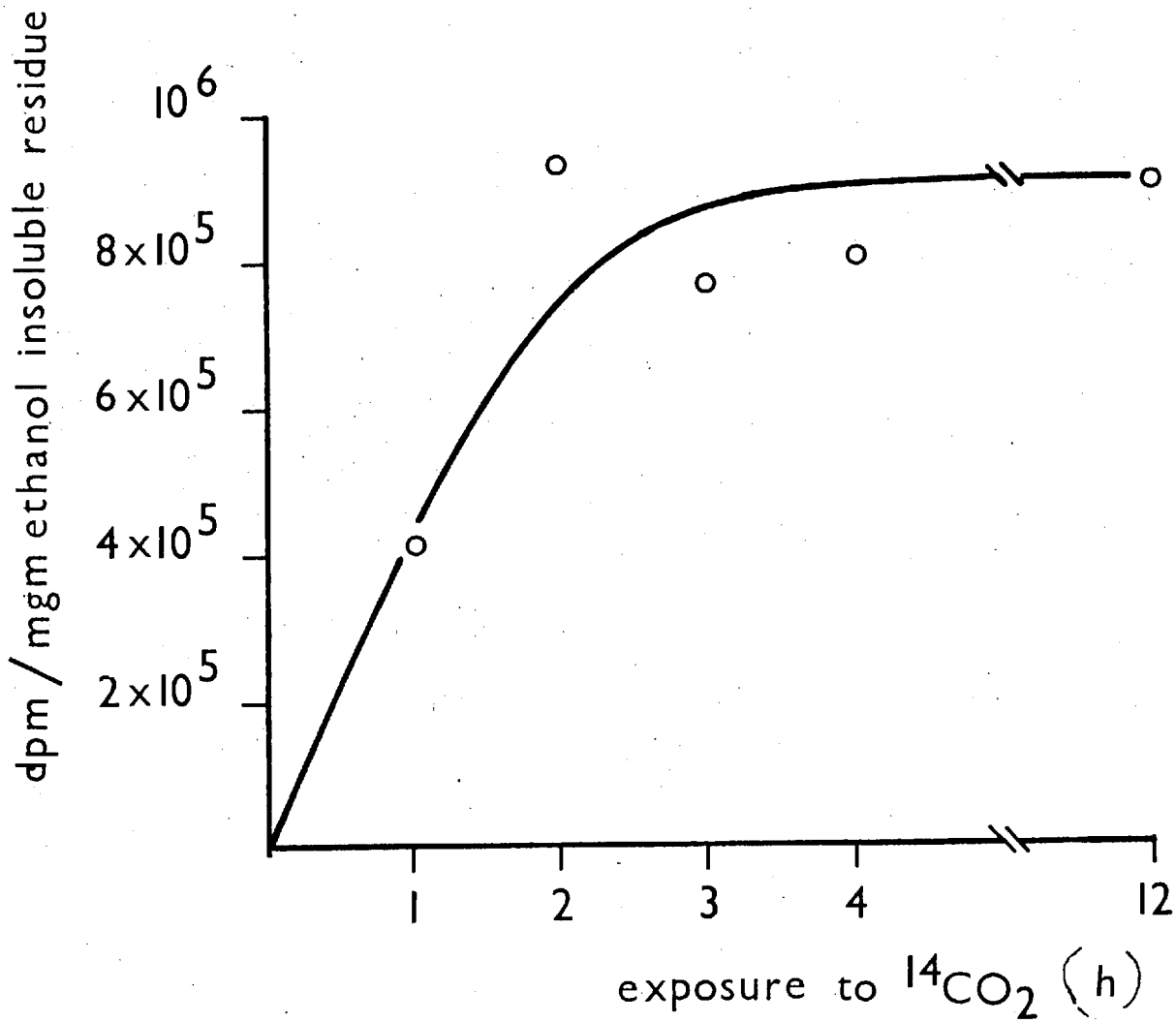


Figure 5. Erysiphe pisi - Pisum sativum. Assimilation of total ^{14}C compounds (ethanol soluble and insoluble) by infected leaves separated from the mycelium prior to exposure to $^{14}CO_2$.

Table 8

Erysiphe pisi - Pisum sativum. The distribution of ethanol soluble and insoluble label in the mycelial, host and haustorial complex fractions obtained from infected leaves exposed to $^{14}CO_2$, (10^4 dpm 100 mm^{-2} leaf).

	Mycelium removed before exposure				Mycelium attached during exposure			
Exposure to $^{14}CO_2$ (h)	2		4		2		4	
Fraction	sol	insol	sol	insol	sol	insol	sol	insol
Mycelium	-	-	-	-	8.86	10.20	10.00	22.20
Haustorial complex	0.017	0.019	0.020	0.035	0.028	0.048	0.024	0.031
Leaf	54.80	49.03	48.79	38.72	30.19	12.03	23.08	12.91
Total	103.8		87.5		61.3		68.2	

losses incurred during the period of brushing. It contrasts with data on photosynthesis of infected leaf discs where, during 5 min brushed discs assimilated less isotope than discs with the mycelium left intact (table 4). This indicates that the reduction in photosynthesis noted there was short lived and that brushed infected leaves quickly recover their full photosynthetic potential.

c) Uptake of ^{14}C assimilates by haustorial complexes and haustoria in leaves.

Isolated haustorial complexes obtained from leaves separated from the mycelium before exposure to $^{14}CO_2$ were treated to obtain three fractions, soluble in cold Triton X-100, boiling 80% ethanol and insoluble material respectively and the uptake of ^{14}C assimilates in total and into these is shown in fig. 6. Isotope was present in all fractions after only 1h exposure when insoluble label was predominant. In contrast to ^{14}C assimilation by the host, label in ethanol soluble fractions increased continuously and after 12h amounted to 62% of the total label in the fraction. In the Triton X-100 soluble and insoluble fractions label did not continue to increase after 1h and each constituted only 19% of the total label after a 12h exposure. Subsequent incubation of haustorial complexes in phosphate buffer had little effect on the quantity of ethanol soluble label per complex, (table 9) and this indicates that losses in soluble

Figure 6. Erysiphe pisi - Pisum sativum. Uptake of ^{14}C assimilates (—▲— soluble in 1% Triton X-100, —●— soluble in 95% ethanol, —■— insoluble in 95% ethanol, —○— total) by haustorial complexes isolated from infected leaves, separated from the mycelium prior to exposure to $^{14}CO_2$.

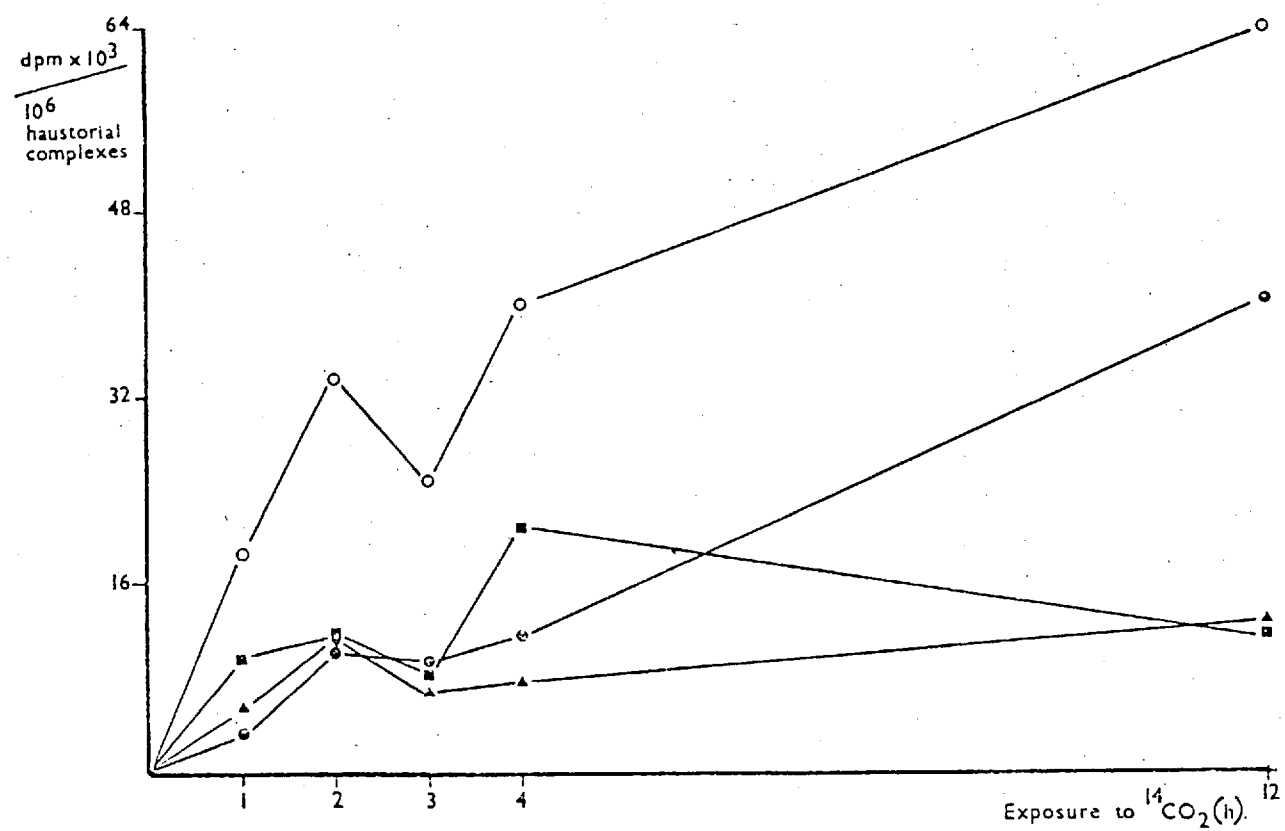


Table 9

Erysiphe pisi - *Pisum sativum*. The effect of *in vitro* incubation on ethanol soluble ^{14}C assimilates in haustorial complexes isolated from infected plants exposed to $^{14}CO_2$ (Radioactivity as a percentage of activity in the sample immediately after isolation).

	Mycelium removed prior to $^{14}CO_2$ exposure					Mycelium attached during $^{14}CO_2$ exposure
Exposure to $^{14}CO_2$ (h)	1	2	3	4	12	4
Incubation 6h	88	71	-	107	-	102
in vitro 12h	97	86	97	115	91	88

label are not incurred during the in vitro treatments of the isolation procedure. This is especially likely to be true since the latter were at 4° and the leaching experiment at 20°. It is thus likely that the quantities of label detected in the isolated complexes accurately represent that of complexes in the leaf.

In other experiments where the mycelium was left attached during 2 and 4h exposures to $^{14}CO_2$, the quantity of ethanol soluble and insoluble label detected in the isolated complexes only slightly exceeded that in haustorial complexes isolated from leaves separated from the mycelium prior to $^{14}CO_2$ feeding (table 8). Insoluble label predominated throughout these exposure periods (table 8) but it was not determined whether the amount of ethanol soluble isotope increased with extended exposure times as it did in haustorial complexes from leaves separated from the mycelium prior to $^{14}CO_2$ exposure.

The contributions due to host contaminants and possible direct fixation of $^{14}CO_2$ by the fungus were also considered. As in the previous experiment, host contamination was reduced to a minimum by pretreatment of the haustorial complex fractions with 1% Triton X-100. As for the short term experiments, this solubilised a greater proportion (84%) of total soluble label from an equivalent fraction isolated from uninfected leaves than from an haustorial complex fraction isolated from prebrushed

infected leaves (48%) exposed to $^{14}CO_2$ for 4h (fig. 7). This difference in loss is again probably due to the stability of the extrahaustorial membrane and normal membrane bounded structures in detergents (Gil & Gay, 1977 and Results 1,B,v above). In fractions isolated from infected prebrushed leaves, a progressively smaller proportion of the total soluble (ethanol and Triton - X100) isotope was extracted by Triton X-100 treatment as the period of exposure to $^{14}CO_2$ increased (fig. 7) and indicates a proportionate increase of the amount of label in the haustorial complexes (fig. 6). The quantity of contamination by soluble labelled compounds of host origin was assessed by comparison with an equivalent fraction isolated from uninfected leaves. The activity in the ethanol soluble label from the haustorial complex fraction was approximately 7 times greater than this control (table 10). Although the control value was not insignificant, its contribution has a negligible effect on the kinetics of uptake of ethanol soluble label shown in fig. 6 . However because of the physiological and compositional differences between infected and uninfected plants e.g. Ayres (1976); Allen (1942); Edwards & Allen (1966); Hewitt & Ayres (1976) and Magyarosy et al (1976), this control is illustrative only and the true contribution of contamination cannot be exactly assessed. Even smaller quantities of label were detected in haustorial complex fractions from brushed leaves exposed to $^{14}CO_2$ in the dark (table 11). This shows that the incorporation of ^{14}C by the haustorial complexes

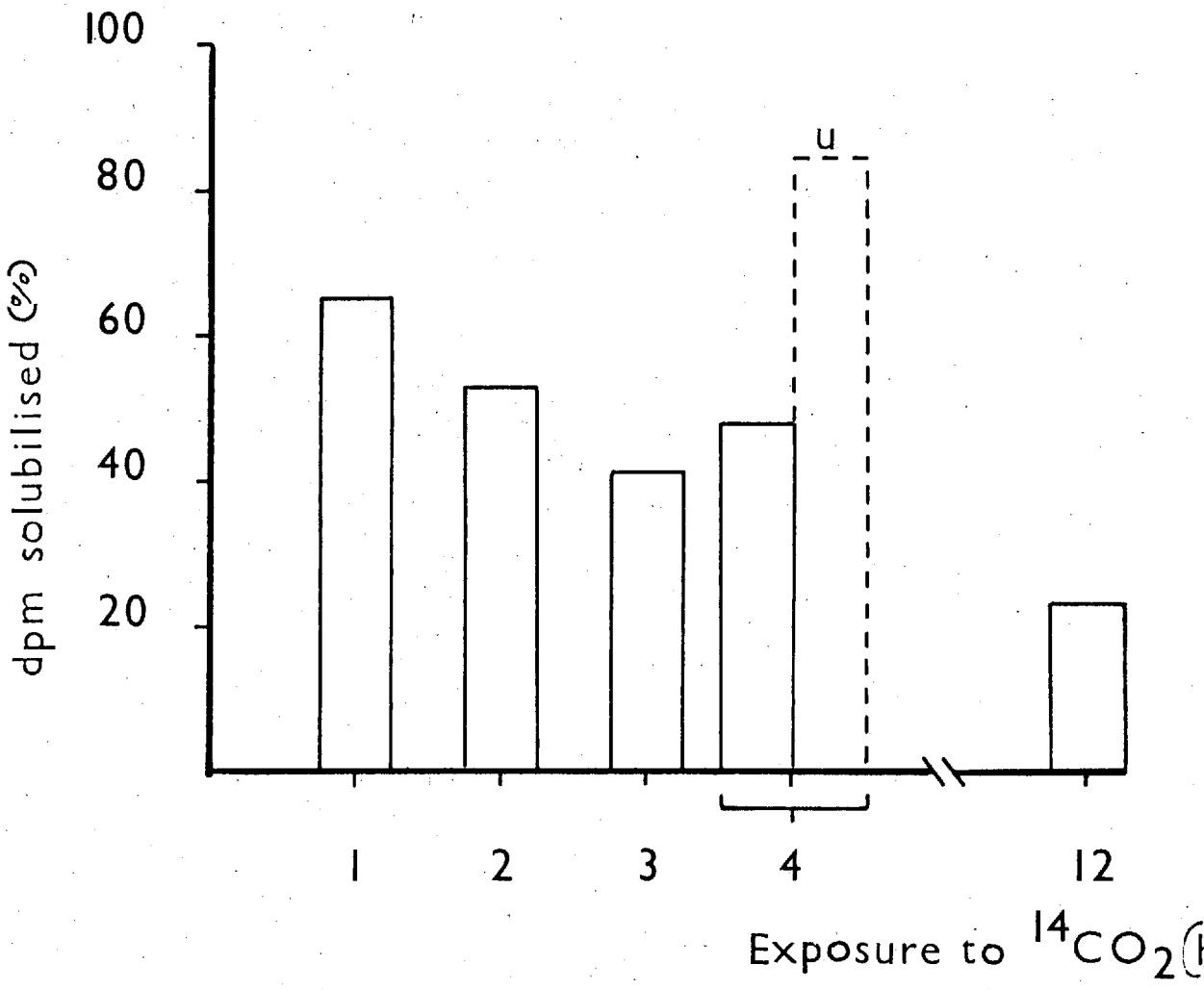


Figure 7. Erysiphe pisi - Pisum sativum. Radioactivity solubilized by 1% Triton X-100 treatment of haustorial complex fractions isolated from prebrushed infected leaves exposed to $^{14}CO_2$. A comparative value obtained from an equivalent fraction from uninfected leaves exposed to $^{14}CO_2$ for 4h is also given (u).

was not solely due to $^{14}CO_2$ fixation by the haustorium itself.

d) Release of assimilated ^{14}C by isolated haustorial complexes.

When the haustorial complex fractions isolated from prebrushed leaves illuminated in $^{14}CO_2$ were incubated *in vitro*, radioactive $^{14}CO_2$ was produced (fig.8). After a lag of nearly one hour the quantity of $^{14}CO_2$ released by the haustorial complexes was directly related to the period during which the leaves were exposed to $^{14}CO_2$. The proportion of isotope which was respired was small and only 0.23, 0.62, 1.4 and 1.35% of the initial total in the fraction were released during 6h *in vitro* incubation by fractions isolated from leaves previously exposed to $^{14}CO_2$ for 1, 2, 3 and 4h respectively. Almost identical amounts of $^{14}CO_2$ were released from haustorial complexes isolated from leaves where the mycelium was left attached and when it was separated prior to the exposure (table 11). These results suggest relatively large quantities of endogenous nutrients in the complexes and may partly explain why the respiration rate of isolated complexes is unaffected by exogenous substitutes (Dekhuijzen, 1966 and table 36). The contribution to the $^{14}CO_2$ released by contaminants of host or microbial origin was insignificant (table 11) and an haustorial complex fraction isolated from leaves exposed to $^{14}CO_2$ in the dark produced little $^{14}CO_2$ when incubated *in vitro* after isolation (table 11).

Table 10

Erysiphe pisi - *Pisum sativum*. Comparison of ethanol soluble ^{14}C compounds in haustorial complex fractions isolated from leaves separated from mycelia and exposed to $^{14}CO_2$ in light or darkness with an equivalent fraction isolated from uninfected leaves. (dpm 50 g^{-1} fresh weight of plant material exposed to $^{14}CO_2$ 4h^{-1}).

	Infected leaves		Uninfected leaves
	light	dark	light
In vitro incubation (h)			
0	7116	419	791
6	7094	-	1116
12	7654	372	988

Table 11

Erysiphe pisi - *Pisum sativum*. $^{14}CO_2$ produced *in vitro* by an haustorial complex fraction isolated from infected leaves separated from mycelia and exposed to $^{14}CO_2$ for 4 h in the light and darkness; an haustorial complex fraction isolated from leaves separated from mycelia after exposure to $^{14}CO_2$ in the light; and an equivalent fraction obtained from uninfected leaves. (dpm 50 g^{-1} fresh weight of plant material 6 h^{-1} *in vitro* incubation).

Infected leaves			Uninfected leaves
mycelium removed prior to $^{14}CO_2$ exposure		mycelium attached during $^{14}CO_2$ exposure	
light	dark	light	light
382	59	385	14

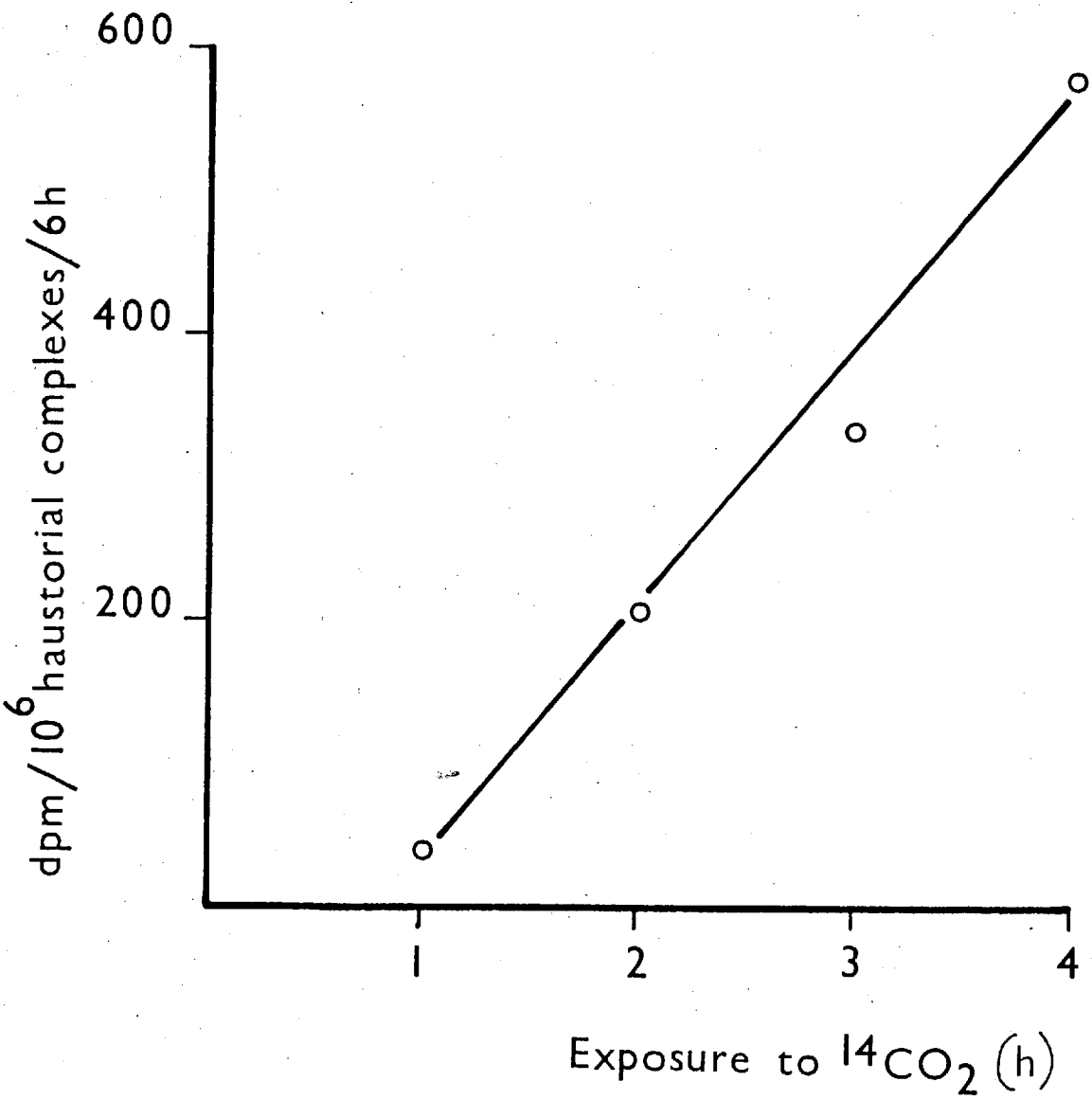


Figure 8. Erysiphe pisi - Pisum sativum. $^{14}CO_2$ released from haustorial complexes incubated in vitro following isolation from brushed leaves exposed to $^{14}CO_2$.

To test whether the $^{14}CO_2$ released was a result of decarboxylation in glycolytic or citric acid cycle reactions, haustorial complexes isolated from 3 replicate experiments with prebrushed leaves previously exposed to $^{14}CO_2$ for 3h were incubated aerobically or anaerabically in vitro. Anaerobic conditions resulted in a 33.1% reduction in the quantity of $^{14}CO_2$ released per complex in a 6h period. However, in such long incubations it is possible that a 'Pasteur effect' may have operated and increased glycolytic metabolism and thus the true difference between aerobic and anaerobic decarboxylations may be greater than this result indicates. However this demonstrates that a part of the $^{14}CO_2$ released originates from mitochondria (citric acid cycle) and is thus limited to the haustorial cytoplasm where these organelles are located (Gil, 1976).

e) The distribution of ^{14}C assimilates in host, haustorial complex and mycelial fractions.

As there were approximately 164 haustoria /mm^2 of leaf in these experiments, the quantities of labelled assimilates in the host, haustorial complexes and mycelia per unit area of infected tissue can be calculated (table 8). The label in the haustorial complexes constituted only a very small proportion of that in the other two fractions.

V) The distribution of label within the isolated haustorial complex fraction.

The distribution of radioactivity in the haustorial complex fraction after *in vivo* uptake of ^{14}C photosynthates was studied by light microscope autoradiography.

A) Experimental procedures

Plants were trimmed to provide 30g leaves with the mycelium attached, and prepared for $^{14}CO_2$ exposure as described for long exposures (Methods VI,B). They were exposed to 5mCi of $^{14}CO_2$ (60 mCi/mmol) for 2h in the light, removed from the chamber and the mycelia removed and haustorial complexes (fraction - 1) isolated. Filtrates 1 - 4 (see isolation procedure, Methods II.) were pooled during the isolation in order to increase the yield of complexes. The final fraction was divided into two approximately equal fractions. The first was washed twice with double distilled water by centrifugation (3,000g) at 4° and finally suspended in double distilled water. The density of haustorial complexes was estimated and adjusted to 10^5/ml. The second fraction was treated to remove low molecular weight compounds and lipids. It was washed successively with a series of ethanol concentrations, (once in each of 10%, 20%, 30%, 45%, 60% and twice in 70%), then once with chloroform - methanol (2:1 v/v) by centrifugation (3,000g) at 4°, suspended in ethanol (70%) and the density adjusted to 10^5/ml.

The two fractions were individually smeared on to microscope slides and covered with photographic emulsion as described in the methods. Controls included an unlabelled haustorial complex fraction.

Five days after applying the emulsion the slides were removed and developed and when dry they were observed by dark field microscopy with an anopteral lens.

B) Results

i) Labelling of haustorial complexes

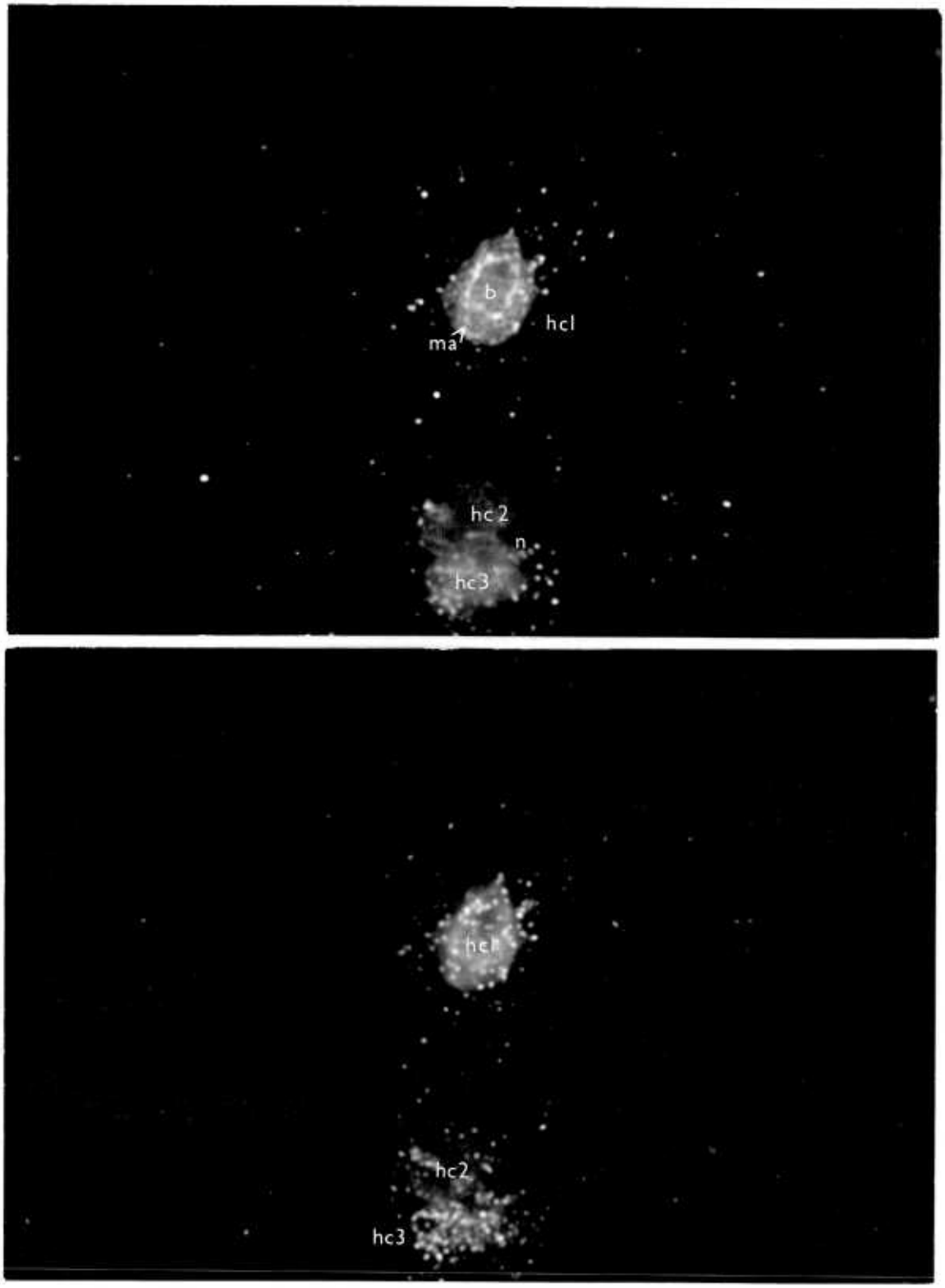
Haustorial complexes were not distinguishable on dry emulsion - coated slides, but when water and a cover glass were added the haustorial complexes swelled and were easily recognised by their characteristic shape with the extrahaustorial matrix swollen around the central haustorial body (plate 17). The annuli and septum in the neck region were also visible (plates 17 and 21). Dark field microscopy permitted simultaneous observation of silver grains and haustorial complexes although the silver grains in the emulsion were usually viewed in a different focal place (plates 17 and 18).

Silver grains were located directly over most haustorial complexes but no difference in counts ($P = > 0.1$) was observed for the complexes mounted directly and those pretreated with 70% ethanol and 2:1 chloroform - methanol to remove low molecular weight compounds and lipids (table 12). However silver grains

Plates 17 - 23. Light microscope autoradiographs of an haustorial complex fraction of Erysiphe pisi - Pisum sativum. Complexes were isolated from infected leaves (30g) which were exposed to $^{14}CO_2$ (5mCi) for 2h and autoradiographs prepared by the stripping film technique. Specimens were observed after mounting with water with a coverglass and viewed by dark field optics with an anopteral objective lens, x 480.

Plate 17. A mature haustorial complex (hc1, age group 3, see Results V,B,i) with a well developed extrahaustorial matrix (ma) and clearly distinguishable central body (b). Two other complexes (hc 2& 3) are present and neckbands (n) are evident on one (hc3).

Plate 18. The same field of view as plate 17 but at the focal plane of the emulsion. Many silver grains are visible over and around two of the ccmplexes (hc 1 & 3) but hc 2 is less labelled.



Plates 17 and 18.

Plate 19. Isolated haustorial complexes (HC) with numerous silver grains directly over them and the surrounding areas. A piece of host cell wall (w) and starch grains are also present but few silver grains associated with them.

Plate 20. A group of contaminant particles (starch grains and xylem cell walls). The absence of silver grains indicates that these had not incorporated photosynthate.

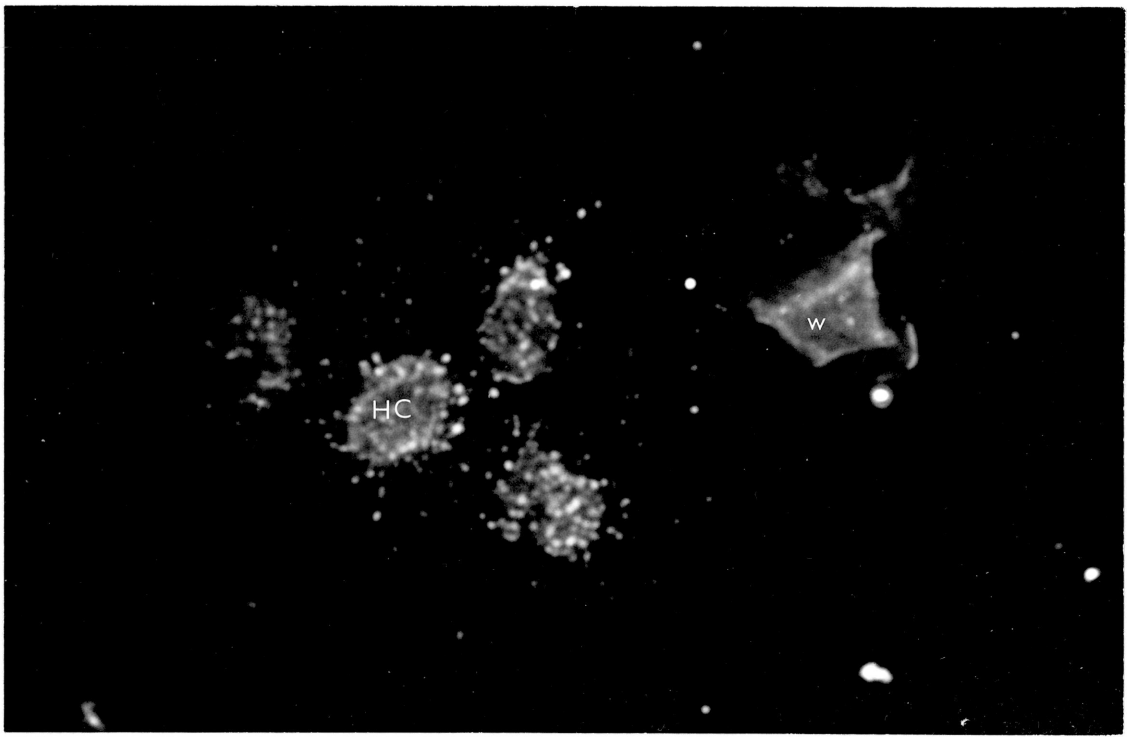


Plate 19.

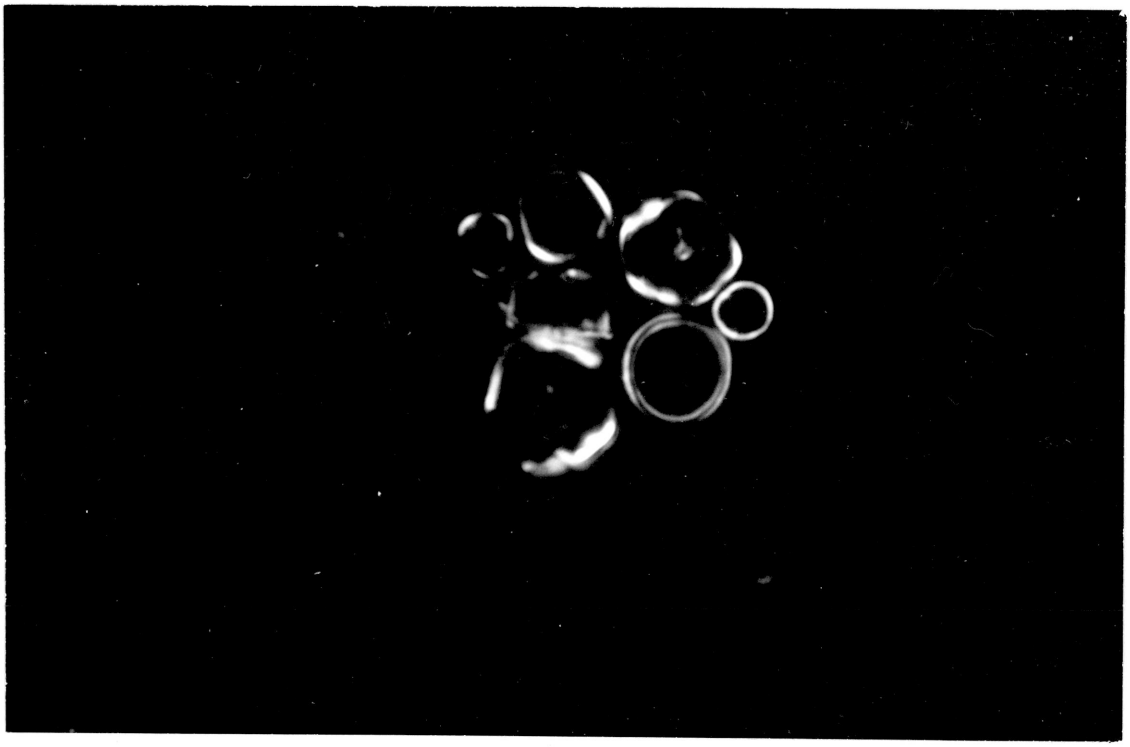
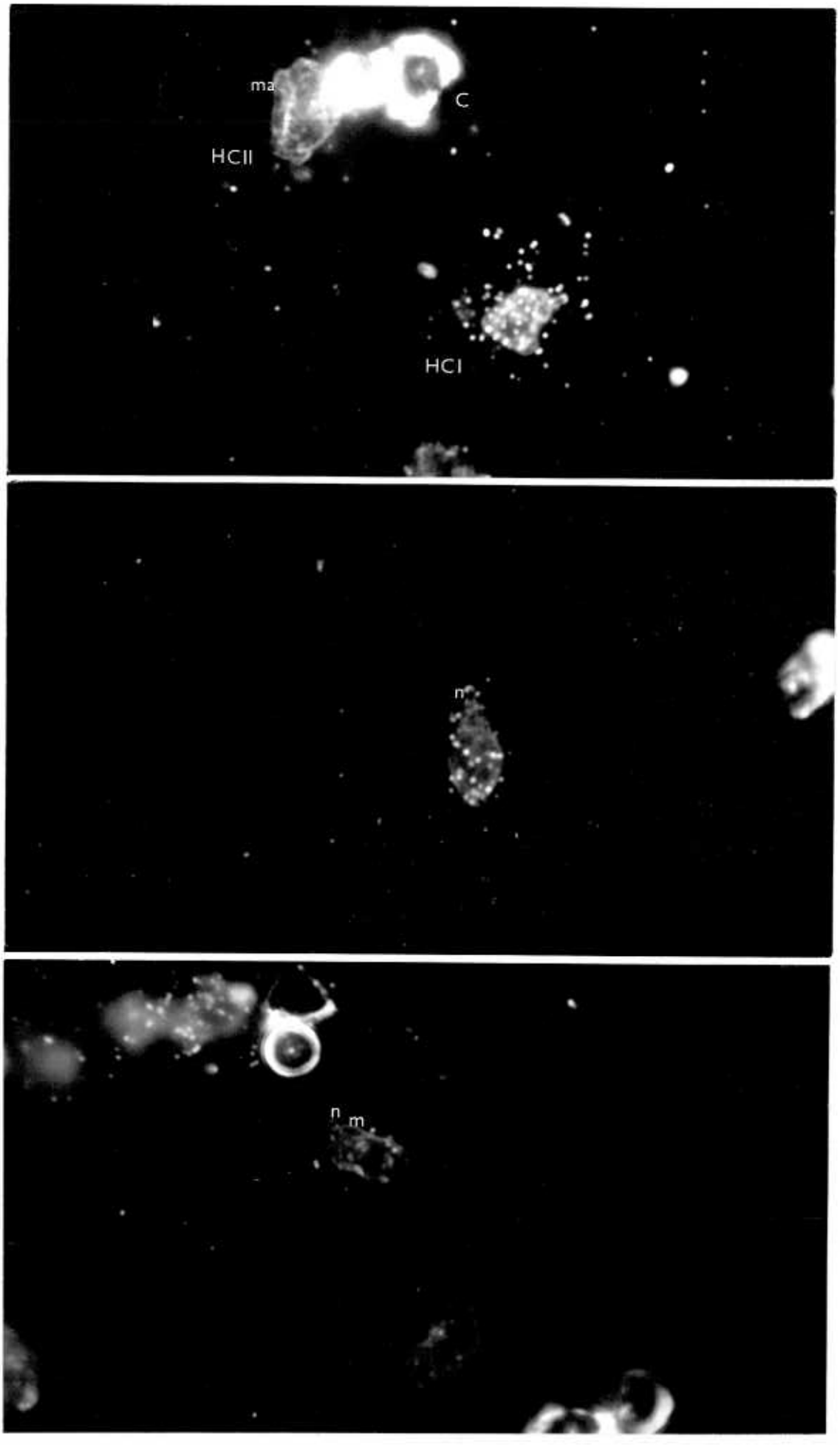


Plate 20.

Plate 21. A young haustorial complex (HC1, age group 1, see Results V, B, i) with the neck region clearly visible. Incorporation of ^{14}C is shown by the numerous silver grains over it and the surrounding area. A second haustorial complex (HC11) of intermediate age (group 2, Results V,B,i) is virtually unlabelled with few grains present; the extrahaustorial matrix (ma) is distinguishable. Very few grains are associated with the highly refractile crystalline contaminants.

Plate 22. A young haustorial complex from a fraction treated with 70% ethanol and chloroform-methanol (2:1) prior to autoradiography. The neck (n) is clearly visible but no matrix or lobes can be seen. Silver grains are evident over the complex but few are present over the surrounding area indicating that soluble compounds had been removed by the solvent treatment.

Plate 23. Haustorial complexes with few silver grains over them indicating very little incorporation of ^{14}C. The neck (n) and the attachment of the extrahaustorial membrane (m) to it, are clearly evident. A starch grain and cell wall fragment also show little incorporation of label.



Plates 21 – 23

Table 12.

Erysiphe pisi - Pisum sativum. The distribution of radioactivity between haustorial complexes and contaminant particles in an haustorial complex fraction isolated from infected leaves previously exposed to $^{14}CO_2$ for 2h shown by autoradiograms prepared by the stripping film technique. Silver grains were counted directly over haustorial complexes and within the 10 μm zone surrounding them. Results are expressed as mean numbers of silver grains haustorial complex^{-1} or particle^{-1} and each value is derived from at least 100 hcs or particles.

	Fraction washed with 70% ethanol & chloroform/methanol (2:1)		Untreated fraction	
	Haustorial complexes	Contaminants	Haustorial complexes	Contaminants
Directly above subject.	22.4 ± 15.9	2.8 ± 4.5	21.7 ± 22.9	3.2 ± 4.3
Over 10 μm peripheral band zone.	15.0 ± 13.5	1.7 ± 2.7	22.1 ± 15.5	2.7 ± 3.5
Total	37.2 ± 28.1	4.5 ± 6.1	43.8 ± 27.6	5.9 ± 6.7

were not confined to the area immediately over each haustorial complex and were also found in the surrounding zone about 10 μm wide. The number of grains counted in the zones surrounding the untreated complexes was significantly greater ($P = < 0.02$) than around the solvent treated complexes (table 12, plates 21 and 22) and suggests that they were due to water soluble compounds diffusing out of the complexes into the emulsion. The emulsion is also known to swell to 1.38 times its original area when hydrated (Rogers, 1973). Such an increase in area results in only a small increase in radius but it is possible that some grains which were originally directly above haustorial complexes may have become displaced by mounting in water. However, the width of the zone observed is greater than could be accounted for by displacement alone and some diffusion of compounds had most probably occurred.

A great deal of variation was observed in the number of silver grains associated with individual haustorial complexes and this is shown by the large standard deviations of the mean counts for the complexes (table 12). Some haustorial complexes had very few or no silver grains associated with them (plate 23). The variation could not be correlated with the morphology of the haustoria as observed by light microscopy. The complexes were divided into 3 classes according to their age as indexed by the degree of lobe and matrix development (see plates 17, 21 and 22).

The mean numbers of counts per complex for these classes were

1) Young haustoria - 41.9 ± 26.2

2) Intermediate " - 47.1 ± 27.6

3) Mature " - 43.7 ± 26.1

The differences between these means were not significant.

Controls for positive and negative chemography (see Methods IX,D) showed that these phenomena were not responsible for the labelling observed. Haustorial complexes isolated from unlabelled plants and not treated with the solvents had a mean number of 2.8 ± 2.1 silver grains per complex. This is only 6% of the value for the labelled fraction. Areas lacking silver grains were not detected on slides exposed to the light prior to development and fixation.

ii) Contaminants

Starch grains and cell wall debris were easily recognised but there were very few silver grains detected over and around these and unidentified contaminant particles (table 12 and plates 19 and 20). The ratio of the total number of silver grains counted for haustorial complexes and contaminant particles was 7.4: 1.0 for untreated fractions and 8.9 : 1.0 for ethanol and chloroform/methanol washed fractions. Since the ratio of numbers of particles to haustorial complexes in the fraction was counted as 1.67 : 1.0, the distribution of label in the fraction was calculated to be 82% associated with haustorial complexes

for untreated fractions and 84% for solvent washed fractions. Although some loss of label was likely for untreated fractions these results strongly indicate that isotope in haustorial complex fractions isolated from plants exposed to $^{14}CO_2$ is mainly associated with haustorial complexes and not with contaminants.

VI) Biochemical composition of ^{14}C assimilates in the host, haustorial complex and mycelial fractions during in vivo uptake.

The experiments described above (Results IV & V) show clearly that ^{14}C photosynthates enter the haustorial complexes. The autoradiography and other controls indicate that the ^{14}C in the isolated haustorial complex fraction was mainly in the complexes and contaminants from the host are relatively unlabelled. With this knowledge a series of experiments were designed to analyse the ^{14}C assimilates in the isolated complexes and compare them with those in the host and mycelium. In addition to this comparison the fractions were sampled after a pulse $^{14}CO_2$ feeding period (30 min) or after a further close period (20h), this was to distinguish between these compounds which are transient within the fractions and those which are sinks for the ^{14}C and are not further metabolised.

A) Experimental procedures

i) Feeding of $^{14}CO_2$ and isolation of fractions

Prior to these experiments, plants were kept for 30 h in the dark and thus were used 8 days after inoculation. Plants

trimmed to yield 80g of leaves with the mycelium attached were set up for $^{14}CO_2$ feeding by the long exposure procedure described above (Methods VI,B). They were exposed to 2mCi of $^{14}CO_2$ (60 mCi/mmol) at 25° for 30 min in the light. Complexes (fraction 1) were either isolated immediately or the leaves were left in unlabelled CO_2 for a further 20 h in the light and then used for haustorial complex isolation. These two procedures are respectively termed 'pulse' and 'chase' throughout the text.

In these experiments the leaves were kept in the light before brushing which occupied a period of 1½ - 2h. They were placed in an ice/water mixture immediately afterwards. At approximately ten min intervals during the brushing period, a disc (12 mm diameter) avoiding the midrib was removed from an unbrushed part of a leaf and the mycelium was then brushed from it. Another disc was immediately obtained from an adjacent area of the leaf and was left unbrushed. A total of 10 discs of each type were made and were immediately placed in boiling 80% alcohol. Samples of the mycelial brushing were taken at similar intervals throughout the brushing period and similarly placed in boiling 80% ethanol.

Haustorial complexes (fraction - 1) were isolated using filtrates 1 - 3 (Methods II,A). In all experiments the Triton X-100 treatment employed in previous experiments (Results IV,A & B), was omitted. After isolation the density of the haustorial complexes

was estimated and the fractions immediately placed in 80% ethanol.

ii) Biochemical fractionation of samples

The samples prepared and degree of replication was as follows.

Fractions	No. of replicate experiments	
	Chase	Pulse
Leaf discs + mycelia	2	2
Leaf discs separated from the mycelia	2	2
Mycelia	2	2
Haustorial complex fractions	3	3

The sequence used for fractionation of the samples into the major biochemical classes is shown in the flow diagram figure 9.

All samples were homogenised and refluxed in 80% ethanol for 1h. The insoluble material was separated and washed three times in cold 80% alcohol by centrifugation at 40,000g. The supernatants were combined and reduced to approximately 1ml under vacuum at 40°. The insoluble fraction was dried in a vacuum desiccator and stored at - 20°.

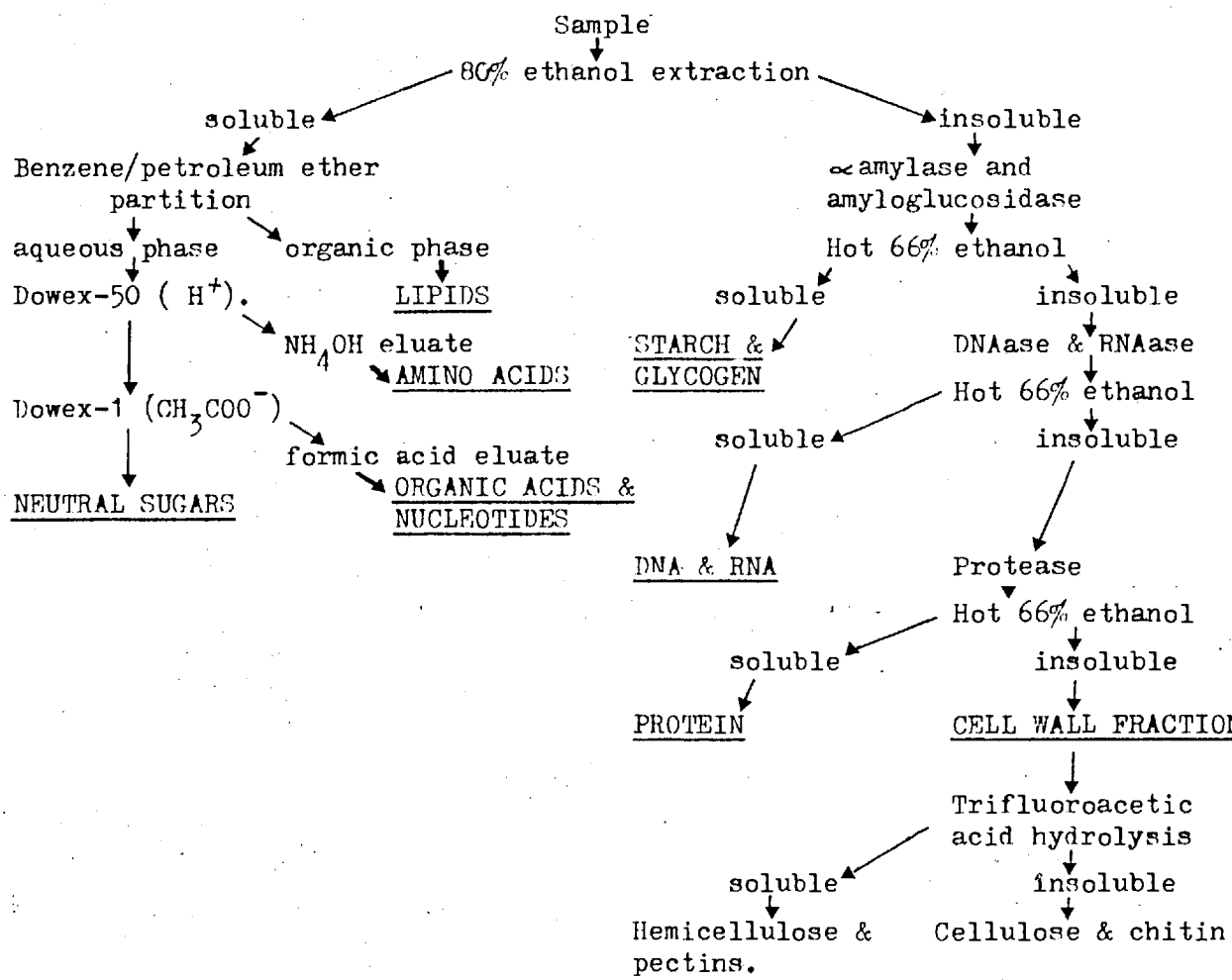
a) Fractionation and analysis of compounds soluble in hot 80% ethanol

The method of Neal and Beevers (1960) was employed.

1) Lipid extraction

The fraction was dissolved in 20 ml of water and 10 ml of benzene added. The flask was stoppered, shaken for 5 min and the

Figure 9. Fractionation scheme for ^{14}C- labelled host, haustorial complex and mycelial samples.



aqueous phase removed in a separating funnel. Petroleum ether (10 ml of 40° - 60° boiling point) was added to the aqueous phase in a stoppered flask and shaken and separated similarly. The benzene and petroleum ether fractions were combined and reduced to a known volume. This lipid fraction was stored in a vacuum desiccator at - 20° prior to further analysis by thin layer chromatography.

2) Separation of amino acids, organic acids plus nucleotides and neutral sugars

The aqueous phases from the lipid extraction (section VI, A, ii, a, 1) were pooled and passed through columns containing 3g of Dowex - 50 (x 8) H^+ form ion exchange resin. This was washed with 50 ml of deionised water and the eluates combined. Amino acids were then eluted from the column by adding 100 ml of 1N NH_4OH.

The remaining extracts were then passed through a column of 3g of Dowex - 1 (x 10) CH_3COO^- form and washed with 50 ml of deionised water. The neutral sugar fraction passed through and the absorbed organic acids and nucleotides were then eluted with 50 ml of 6N formic acid. All fractions were reduced to a known volume under vacuum at 50°. The neutral sugar fraction was further analysed by paper chromatography. The organic acids and nucleotides were not analysed further.

b) Fractionation of compounds insoluble in hot 80% ethanol.

This was fractionated into starch and glycogen, RNA and DNA, and protein by sequential treatment with enzymes which hydrolyse each

of the classes followed by separation of the unhydrolysed moieties by ethanol precipitation. Prior to each enzyme treatment the insoluble residues were dried for 16h in a vacuum desiccator, this was taken up in the enzyme solution (5ml) and homogenised in a Potter homogeniser. Using a shaking water bath, incubations were carried out under toluene to prevent bacterial contamination. The following sequence of enzyme treatments was employed.

i) Starch and glycogen

1) Incubation for 30h at 28° in a solution of ∝ amylase (1mg/ml, 4 x recrystallised, Bacillus subtilis origin, Sigma Chemicals) in 50 mM phosphate buffer at pH 6.9.

2) This was followed by incubation for 6h at 35° in a solution of amyloglucosidase (0.25 mg/ml Sigma Type III of Aspergillus oryzae origin, Sigma Chemicals) in 50 mM citrate buffer at pH5.0.

ii) RNA & DNA

Incubation for 24h at 26° in a solution of ribonuclease (0.25 mg/ml, 4× recrystallised, Miles Research Products No 1) and deoxyribonuclease (0.5 mg/ml, Miles Research Products No 1) in 0.1 M acetate buffer at pH 5.1.

iii) Protein

Incubation for 40h at 27° in a solution of 2mg/ml protease (Streptomyces griseus origin, Sigma Chemicals) 50 mM phosphate buffer pH 6.8.

At the end of each incubation a 10 ml quantity of absolute ethanol was added and the tube heated in a water bath at 100° for 5 min to inactivate the enzyme. The insoluble material was separated by centrifugation at 40,000 g and washed twice in 66% ethanol. The supernatants were combined and constituted the extracted fractions. The insoluble material was dried and taken for hydrolysis by the next enzyme in the sequence.

The insoluble residue after the final treatments was considered as the cell wall fraction. This was then subjected to acid hydrolysis by the method of Albersheim et al (1967) to solubilize the pectic and hemicellulose fractions and/or the acid labile fraction of the fungal walls and separate them from cellulose and chitin respectively. The insoluble material was resuspended in 2ml of 2N trifluoroacetic acid (TFA) in a pyrex test tube which was then sealed and autoclaved at 120° for 1h. The contents of each were then transferred to a 25ml beaker and the acid removed by blowing hot air (60°) over the surface of the solution overnight. The dried extract was then resuspended in distilled water and the insoluble material separated by centrifugation at 40,000 g and washed twice in water. The supernatants were combined and are termed the TFA soluble fraction and the residual insoluble material the cellulose and chitin fractions.

All extracts were evaporated to a known volume and samples removed for estimation of radioactivity in scintillant mixture S2. The insoluble residues were resuspended in water, homogenised in a Potter homogeniser, dried in a glass scintillation vial and counted in gelled suspension in scintillant mixture S1.

C) Analysis of biochemical classes

1) Lipid fraction

All samples were reduced to a small volume approximately 200 μl) and analysed by thin layer chromatography (silica gel) as described in Methods (X,G). Standards, (10 μg of each) were run on each side of the sample. The standards used and their Rf values are shown in table 13. After chromatography each plate was cut along its length to separate the sample and standards. The standards were detected by chlorosulphonic acid and radioactive peaks located by cutting the chromatogram into segments and directly counting these in scintillant mixture S2.

2) Neutral sugar fraction

Radioactive neutral sugar fractions were analysed by paper chromatography as described in the Methods (X, E, iv, e).

3) Trifluoroacetic acid soluble fraction

This fraction was dissolved in water and separated into acid, base and neutral fractions by ion exchange chromatography as for the ethanol soluble compounds described above, (section VI, A, ii, a).

Table 13

Standard compounds and their Rf values obtained from thin layer chromatography on silica gel (see Methods X,G).

Lipid class	Standard	Rf value	
Polar lipids	-	0	From Skipski et al (1965).
Sterols	ergosterol	.36	
Diglycerides	glycerol dipalmitate	.45	
Fatty acids	palmitic acid	.62	
Triglycerides	glycerol tripalmitate	.72	
Fatty acid methyl esters	methylpalmitate	.76	
Hydrocarbons	dodecane	.91	

The acidic fractions and neutral fractions were then individually reduced to a small volume by rotary evaporation at 45° and analysed by paper chromatography using the solvent system EAPW (see Methods X, E, iv, c). Radioactivity was detected by X-ray film and by direct counting of the chromatogram following cutting it into strips (Methods X, E, iv, e). The components were then identified by comparison with standards (galacturonic acid, galactose, glucose, mannose, arabinose, xylose, ribose, rhamnose) which were run adjacent to the samples on the chromatograms.

b) Results

i) Isolation of haustorial complexes

The mean number of haustorial complexes isolated from 80g of plants in six experiments using this experimental system was 1.81×10^6. Unlike the previous uptake experiments plants were kept in the dark for 30h before experiment and were thus used 8 days after infection and microscopic inspection of epidermal strips indicated a slightly larger mean number of haustoria per 100 mm^2 of leaf - (16,400 to 17,930). The total number of haustoria in 80g of trimmed plants was thus determined to be 28.2×10^6 and thus the efficiency of extraction - 6.5%. This is slightly more than in the uptake experiments previously described (section IV, B,ii,a) and is probably because of the increased degree of comminution (filtrates 1 - 3 were used, see Methods II,A)employed here. Since autoradiography had indicated that starch grains and cell walls of

host origin were virtually unlabelled by comparison with haustoria the slight increase in contamination by additional comminution was unimportant when considered against the improved yield.

ii) Distribution of isotope in the major biochemical classes in host, haustorial complex and mycelial fractions.

Uptake by the mycelium in these experiments was estimated from the difference in the quantity of radioactivity in infected leaf discs with the mycelium intact and the mycelium removed from the discs after the pulse and the chase periods, (table 14). These results showed that after the pulse and brushing periods, 18% of the label in infected discs was in the mycelium. After an additional chase period (20h) there was a slight increase in the proportion of label in the mycelium (22%) but a large decrease in the total quantities of label located in both mycelium and host per unit area of leaf. The losses in label are possibly the result of respiration since infected tissue respires at an appreciably higher rate than uninfected (Ayres,1976). Another possibility is through loss of spores from the mycelium. Erysiphe pisi is monoconidial (Pady et al,1969) and thus newly formed spores are easily detached. Spores are normally formed in a diurnal cycle (Yarwood,1936) and usually become detached during the light period. As the chase period was in continuous light it seems possible that spore production may have been enhanced.

Table 14

Erysiphe pisi - Pisum sativum. Determination of the uptake of ^{14}C assimilates by the mycelium from the difference in the quantities of radioactivity in infected discs and infected discs cleared of mycelia. Discs were removed from leaves immediately after a 30 min exposure to $^{14}CO_2$ in the light or after a subsequent 20h chase period in unlabelled CO_2. Results (10^3 dpm $100mm^{-2}$ of leaf) are the means of duplicate experiments, each with 10 discs.

	Infected discs	Infected discs cleared of mycelia	Mycelium by difference.
30 min $^{14}CO_2$ exposure	144	118	26
30 min $^{14}CO_2$ exposure + 20h chase.	54	42	12

The distribution of label amongst the major biochemical classes was determined by fractionation of leaf discs separated from the mycelium, mycelial brushings and the isolated haustorial complex fraction (table 15).

Immediately after the 30 min $^{14}CO_2$ pulse, the neutral sugars contained most of the radioactivity in the host (55.3%) and the mycelial (57%) fractions. Major quantities (26-31%) were also detected in the amino acid and organic acid and nucleotide fractions. All other fractions in the leaf and mycelium were labelled relatively slightly and together constituted only 13% and 17% respectively of the total quantities of label.

In contrast to the labelling patterns of the host and mycelial fractions the neutral sugar, amino acid, organic acid and nucelotide fractions of the haustorial complexes constituted only 5.7% of the total label. The major fraction (64%) was found in the cell wall fraction solubilised by hydrolysis in trifluoroacetic acid. The lipid fraction was also very significantly labelled and these two fractions made up 85% of the total radioactivity. All the other fractions contained very small quantities of isotope.

After the 20h chase period the label in the neutral sugars and amino acid fractions of the host became reduced considerably (to $\frac{1}{3}$ - $\frac{1}{2}$). The host also showed significant increases in the proportion of label in all other fractions except the organic acid and nucleotides which did not change. In the mycelium the reduction in the proportion of isotope in neutral sugar and amino

Table 15

Erysiphe pisi - Pisum sativum. The distribution of label in the leaf, haustorial complex and mycelial fractions obtained from infected plants immediately after a 30 min exposure to $^{14}CO_2$ or after a 30 min exposure and a 20 h chase in unlabelled CO_2 all exposures were in the light. (Radioactivity as % of total in each fraction. Results are the mean of at least two replicate experiments).

Fraction	30 min $^{14}CO_2$ exposure, no chase			30 min $^{14}CO_2$ exposure + 20 h chase		
	Haustorial Complex	Leaf	Mycelium	Haustorial Complex	Leaf	Mycelium
Neutral Sugars	3.0	55.3	57.0	4.8	18.8	6.0
Amino Acids	1.3	13.3	13.6	3.2	7.3	2.7
Organic Acids & Nucleotides	1.4	17.9	12.2	2.6	18.0	3.1
Lipids	21.0	0.5	5.7	29.3	3.5	2.2
Starch or Glycogen	2.3	3.5	5.7	5.7	13.3	33.7
DNA & RNA	0.7	1.4	0.5	3.5	8.2	5.0
Protein	2.9	3.4	2.6	8.7	10.5	13.5
Cell Walls -						
Soluble in TFA	64.0	4.0	1.8	38.9	18.8	20.5
Insoluble in TFA	3.1	0.6	0.7	3.4	1.5	13.2

Table 16.

Erysiphe pisi - *Pisum sativum*. The distribution of ^{14}C in leaf, haustorial complex or mycelial fractions obtained immediately or after a 20h chase period from infected plants exposed for 30 min to $^{14}CO_2$. The whole experimental period was in the light. Results are expressed as dpm $100\ mm^{-2}$ leaf.

Fraction	30 min ^{14}C exposure, no chase			30 min ^{14}C exposure + 20h chase		
	Haustorial Complex	Leaf	Mycelium	Haustorial Complex	Leaf	Mycelium
Neutral Sugars	7.5	65,505	14,810	22.5	7,891	738
Amino Acids	3.2	15,754	3,534	15.0	3,064	332
Organic acids & nucleotides	3.5	21,203	3,170	12.2	7,555	381
Lipids	52.3	592	1,480	137.1	1,469	270
Starch & Glycogen	5.7	4,146	1,485	26.7	5,582	4,148
DNA & RNA	1.7	1,658	129	16.4	3,442	615
Protein	7.2	4,027	675	40.7	4,407	1,660
Cell Walls -						
Soluble in TFA	159.4	4,738	468	182.0	7,898	2,521
Insoluble in TFA	7.7	711	182	15.9	630	1,623
TOTAL	248	118,334	25,933	468	41,937	12,288

acid fractions exceeded that detected in the host and after the chase those with the organic acid and nucelotide fraction comprised only 11.8% of the total where previously they had comprised 83%. However, results for the mycelium show that significant incorporation into the ethanol insoluble constituents had occurred during the chase period and the glycogen and cell wall fractions accounted for over 67% of the total isotope.

In the haustorial complex fractions, the proportions of radioactivity in the neutral sugar, amino acid, organic acid and nucleotide, starch and glycogen, protein and nucleic acid fractions were all slightly increased by the chase treatment. However, the lipid and cell wall fractions were still predominant and the proportion of label in the lipid fraction had increased slightly. That of the TFA soluble cell walls had decreased from 64 - 38.9%.

The distribution of labelled compounds in the host, haustorial complexes and mycelia per unit area of leaf was calculated as before (Results II, B, ii, e) and the results are shown in table 16. Allowance was made for the increase in haustorial frequency associated with the longer infection period and the quantity of radioactivity taken up by the mycelium was determined from the differences between infected discs and infected discs cleared of the mycelium. This was then divided by the proportions shown in table 15.

It is evident that the total quantity of radioactivity in the haustorial complexes was very small by comparison with that in the host or mycelial fractions. There were significant losses in the leaf and mycelium during the chase period but the radioactivity in haustorial complexes increased then. This suggests growth or some equivalent sink for material in established haustoria and possibly the development of new haustoria during the chase period. These would not be subject to loss as would newly matured spores on the mycelium. After the $^{14}CO_2$ pulse the proportion of radioactivity in the haustorial complexes was 0.2% of that in the host and mycelium per unit area of leaf and 0.9% after the chase period.

During the chase period there is an increase in the quantity of label in each fraction from the haustorial complexes. Even the TFA soluble cell wall fraction which decreased as a proportion of label in the haustorial complex fraction from 65% to 39% after the chase showed an increase in the quantity of radioactivity per unit area of leaf.

When the results for the host are expressed as total quantities per unit area of leaf the chase period results in considerable loss in radioactivity in neutral sugars, amino acids and organic acid fractions. With the exception of TFA insoluble cell wall fractions all others show an increase. In the mycelium all the ethanol constituents decreased but all other constituents increased with the chase.

iii) Distribution of isotope within each biochemical class of host, haustorial complex and mycelial fractions.

a) Lipids

The lipid fractions of the host, haustorial complexes and mycelia were analysed by T.L.C. and the scan profiles of the chromatograms for the pulse and chase experiments are shown on fig. 10 and 11 respectively. Since with the solvent systems (Methods X,G)used in this analysis, polar lipids remain at the origin (Skipski et al,1965), the peaks at position 1 would most probably represent phospholipids and glycolipids. These are considered to be mainly membrane constituents (Hoppe & Heitefuss, 1974c). Compounds mobile in this system are neutral lipids which may be constituents of membranes, cuticles or storage products (Martin & Juniper,1970; Losel & Lewis,1974; Brennan & Losel,1978).

Haustorial complexes:

There was little difference in the composition of the lipids of the haustorial complex fractions obtained from the pulse and the chase experiments. However, the fraction showed a 2.6 fold increase in the quantity of label with the chase period. Most of the isotope in the complexes (positions 6) co-chromatographed with the triglyceride standard (glycerol tripalmitate), an appreciable quantity was found to correspond to polar lipids, (position 1) and

traces of ^{14}C were detected in sterols, diglycerides, fatty acids and hydrocarbons. The lack of any change in composition and the increase in total counts in the lipids during the chase suggests that the ^{14}C compounds were being deposited in the lipid fraction. This is thus a sink for the ^{14}C nutrients and the triglycerides are probably contained in the osmiophilic droplets frequently seen in electron micrographs of powdery mildew haustoria (Gil,1976) and the polar lipid increase corresponds to the production and turnover of membranes. Indeed the ultrastructural observations of a highly convoluted extrahaustorial membrane and the fungal plasmalemma extending into the numerous appendages of the haustorial complexes indicate that there are abundant phospholipids in the haustorial complex.

Mycelium:

In the mycelial lipids most of the isotope was also in a broad peak (position 6) which co-chromatographed with the triglyceride standard. This band may have also contained appreciable quantities of free fatty acids which were incompletely separated from the triglycerides and a large shoulder (position 5) is evident beside the major peaks from both pulse and chase lipid experiments. This contrasts with the well defined peak for triglycerides in the haustorial complexes. Appreciable quantities of ^{14}C compounds were found in the polar lipids and hydrocarbons of the mycelium with only traces in the sterols and diglycerides. Although

glycerol esters of fatty acids have predominated, no mention has been made of them among the lipids described in powdery mildew spores, (Tulloch & Ledingham,1960; Fisher et al,1972; Johnson et al,1976). These studies have concentrated on the more facilely assayed free fatty acids, methyl esters of fatty acids and hydrocarbons and have indicated that the compounds in spores of E. graminis are constituents of lipid intracellular inclusions and some surface cuticles, both of which are present in the spores of E. pisi (M. Martin personal communication). By contrast triglycerides have previously been reported as major lipids of rust fungi and Losel & Lewis (1974) have shown that they accumulated appreciably in aecial pustules of Puccinia poarum infecting Tussilago farfara, where the direct contribution of lipids from host cells is considered to be small (Losel & Lewis ,1974). In its telial and uredinal stage on Poa pratensis triglycerides are also abundant (Losel, 1978).

During the chase period there was a decrease (1,480 to 170 dpm) in the total quantity of isotope in the mycelial lipid fractions per unit area of leaf. The analysis shows that this fall cannot be explained by a reduction in any individual component as the lipid profiles after the chase closely resembles those of the pulse experiments. The reduction of label may be due to losses by respiration, - spore dispersal or may represent turnover into other compounds. This would imply that the lipids

in the mycelium are a primary sink. It is possible that lipids synthesised in the haustorium may be transported to the mycelium whose lipid composition is strikingly similar but the persistance of label in lipids in the haustorial complexes after the chase argues against this.

Host:

The lipid constituents of the host are very different from those in the haustorial complexes and mycelia. The polar lipids fraction is the most heavily labelled after pulse $^{14}CO_2$ feeding. However, after the chase period although significant isotope is still present in the polar lipids, some other fractions are more highly labelled and in particular the diglycerides and hydrocarbons. These neutral lipids show large increases in the quantity of isotope for there is a large increase in the number of counts per unit area of leaf in the host lipids with the chase period (2.5 fold increase). Losel & Lewis (1974) showed a surprisingly high labelling of diglycerides when leaves of Tussilago farfara were exposed to $^{14}CO_2$ and chased for 24h, they attributed this to a role for diglycerides in the synthesis and breakdown of polar and neutral lipids. It should be noted that although the host fraction includes the haustorial complexes in the epidermis the ^{14}C in lipids of the complexes make up only a very small proportion (less than 10%) of that in the lipids in the host (table 16).

Figures 10 and 11. *Erysiphe pisi* - *Pisum sativum.* Scans of the radioactivity in thin layer chromatograms of lipid extracts of host, mycelial and haustorial complex fractions obtained from infected plants immediately after a 30 min exposure to $^{14}CO_2$ (fig. 10) or after a 30 min exposure and a 20h chase period in unlabelled CO_2 (fig. 11). Numbers refer to the position of lipid classes on the chromatogram as follows:

1 – Polar lipids

2 – Sterols

3 – 1:2 Diglycerides

4 – 1:3 Diglycerides

5 – Fatty acids

6 – Triglycerides

7 – Fatty acid - methyl esters

8 – Hydrocarbons

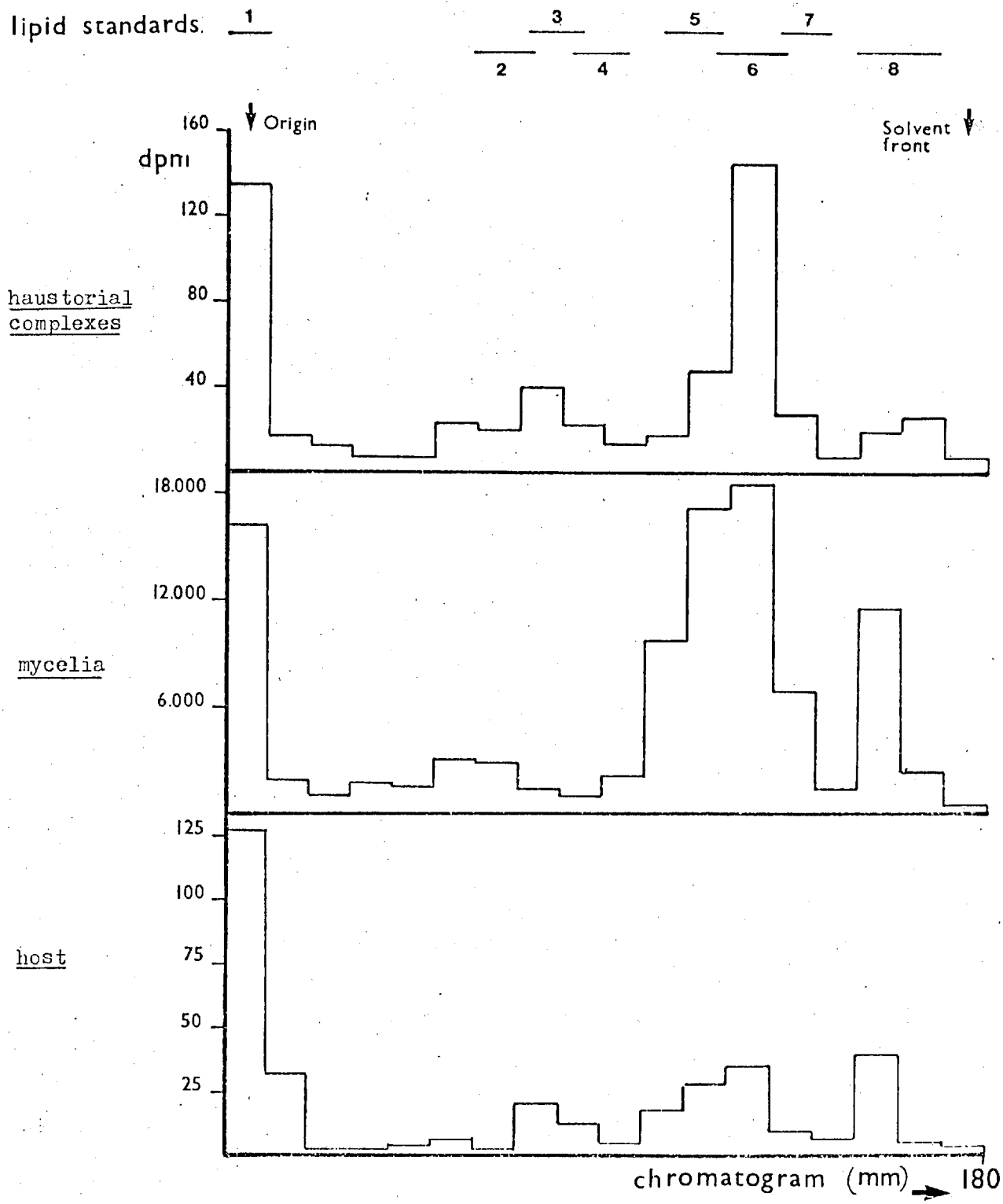


Figure 10. Lipid analyses of 'pulse' experiments.

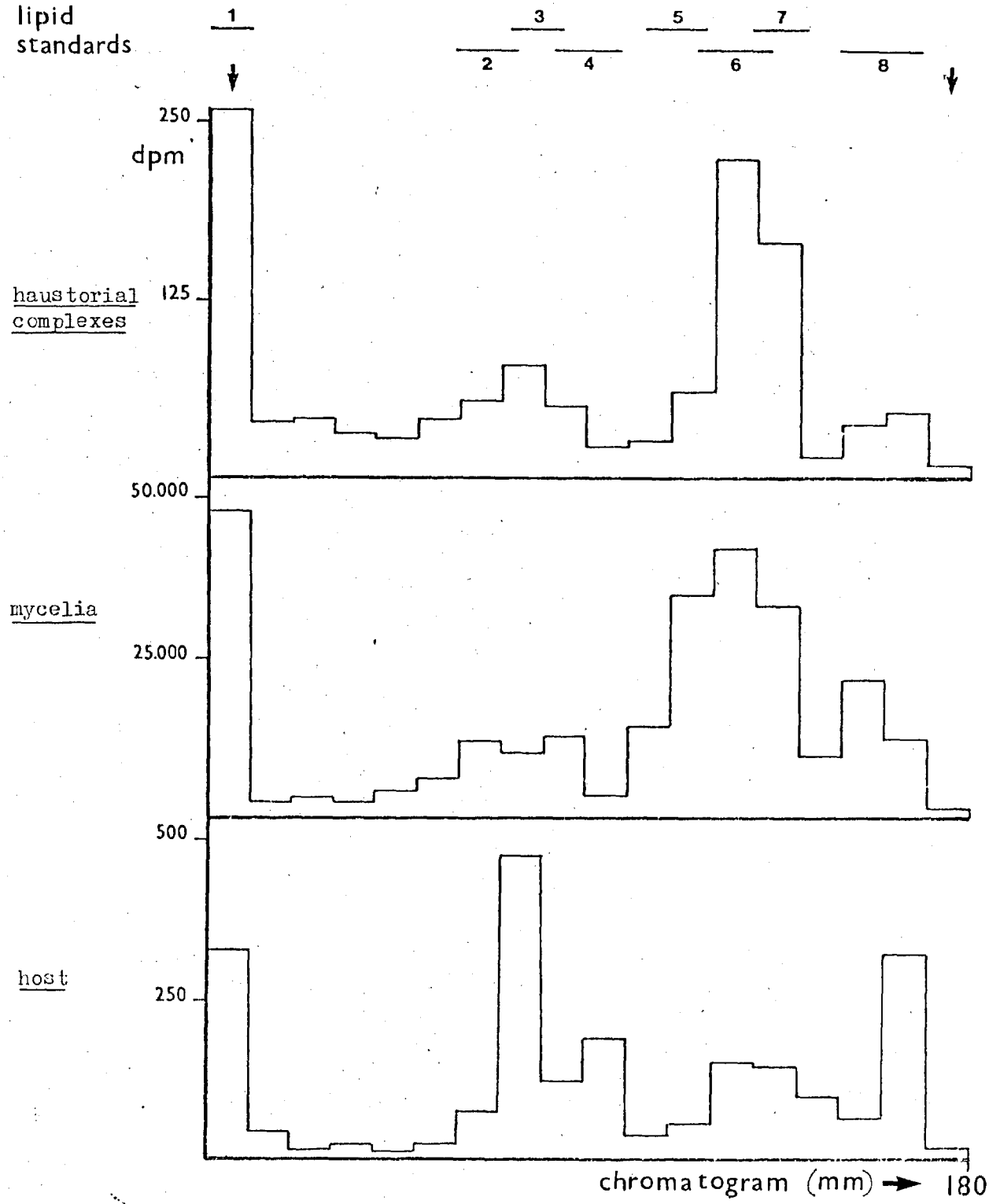


Figure 11. Lipid analyses of 'chase' experiments.

b) Trifluoroacetic acid hydrolysate (cell wall and related constituents).

The distribution of label obtained by ion exchange chromatography of the basic, acidic and neutral fractions of the hydrolysate from the host, complexes and mycelia is shown in table 17. The interpretation of the results of hydrolysis of cell walls is problematical (Jones & Albersheim, 1972) and therefore a treatment with 2N TFA at 121° for 1h was chosen as it is known to release quantitively the neutral sugar residues of the wall of plant cells but not hydrolyse cellulose (Talmadge et al, 1973). It has the disadvantage that it causes degradation by decarboxylation and lactonisation of a large proportion of the uronic acid residues. Lactonised acids behave as neutral compounds in ion exchange procedures and therefore ion exchange separations may be subject to error if all acidic compounds are considered as pectic compounds. Blake & Richards (1968) have shown that for sulphuric acid hydrolysis approximately 50% of the uronic acid residues were subject to lactonisation. A further complication is that some uronic acids in aldobiuronic acid linkages may possibly withstand this treatment (Jones & Albersheim, 1972).

Host:

The host cell wall fraction was found to contain a very small proportion of basic components (10% of the total) which may be from residual proteins not degraded by the protease

treatment. The acidic component was surprisingly large especially when the problems associated with the production of this hydrolysate are considered. However there was a slight decrease in the proportion of acid constituents after the chase. The isotope counts per unit area of leaf in the host hydrolysate increased 1.7 fold with the chase so there is a real increase in the counts in the acidic fraction.

The acidic fraction was further analysed by paper chromatography in EAPW solvent for 70h. This yielded no distinct peak and the majority of the labelled constituents being either immobile or moving very slowly (figs. 12 & 13). There was little or no sign of uronic acids in this fraction. This suggests that in the preparation of samples these may have been degraded or only partially hydrolysed. No change occurred during the chase period.

The largest proportion (64% and 70%) of the host cell wall hydrolysate was in neutral compounds. These were also analysed by paper chromatography in EAPW solvent system and an example of an autoradiogramof these is shown in fig. 16. Two spots which corresponded to galactose and glucose were present. Traces of activity were also detected in spots which corresponded to mannose, arabinose and xylose. The distribution of counts in these fractions are shown in table 18. Glucose and galactose were also the most labelled cell wall matrix compounds formed when segments of uninfected pea stems were incubated in ^{14}C - glucose (Robinson, Eisinger & Ray, 1976). Comparison of the data indicates that the

removal of starch by the amylase and amyloglucosidase treatments was nearly complete for it is unlikely that the quantity of labelled galactose would equal that of glucose if starch had persisted in the final cell wall fraction. This was supported by the two step enzymic degradation of starch (amylase and then amyloglucosidase were employed), the amyloglucosidase treatment released only 5 - 8% of the quantity of label released by the amylase (table 19).

Haustorial complexes:

The haustorial complex fraction resembled the host fraction in containing small quantities of basic compounds characteristic (table 17) of cell walls. However it contained a higher proportion of acidic compounds (41% and 33% for pulse and chase respectively). The chase period caused a reduction in the proportion of acidic compounds but as in the host fraction because of a slight increase in total counts (table 16) the total quantity of isotope in the acidic fraction changed little (65 dpm/mm^2 after the pulse and 60 dpm/mm^2 after the chase). Chromatography of this fraction indicated that composition differed from the acidic fraction of the host. Traces of label were detected in galacturonic acid but the largest proportions were either immobile or formed a peak with an R-galacturonic acid value with the EAPW solvent of 0.63 (figs.14 & 15). The composition of this is unknown although it may be an aldobiuronic acid released by partial hydrolysis of the polymer for these would

have similar mobilities on paper chromatograms with this solvent system (Talmadge et al, 1973). However, other possible identities is an acidic amino acid as found in some ascomycete fungal cell walls (Mahedaven & Tatum, 1965), also oxalate crystals in the fraction may have become labelled although the autoradiography (section V, B, ii) indicated that label was restricted to the complexes. Since both pulse and chase acidic cell wall components had a similar composition, these compounds are not further metabolised during the chase period.

The largest proportions of isotope in the TFA hydrolysate of the haustorial complexes in both pulse and chase experiments were in the neutral compounds (48% and 57% respectively). This fraction was composed mainly of glucose (table 18 and fig. 17) with small quantities of mannose and galactose. No trace of label was detected in xylose, arabinose, ribose or rhamnose which would be expected to be present after hydrolysis of plant cell walls (Talmadge et al, 1973). The ^{14}C labelled glucose is not likely to originate from starch grain contamination for the amyloglucosidase treatment released only 5% and 7% of the label released by the previous αamylase extraction, indicating that little starch persisted.

There was an increase in the radioactive counts but little change in composition (table 18) indicating that these neutral compounds constitute a sink the haustorial complexes for photosynthetic products.

Table 17

Erysiphe pisi - *Pisum sativum*. The distribution of ^{14}C in basic, acidic and neutral compounds separated by ion exchange chromatography of trifluoroacetic acid hydrolysates of the crude cell wall fraction of host, haustorial complexes and mycelia. The fractions were obtained from infected leaves either immediately after a 30 min pulse exposure to $^{14}CO_2$ or after a subsequent chase period of 20h. All treatments were in the light. (Radioactivity is expressed as a percentage of the total for each experimental fraction. Each value is the mean from at least two replicate experiments).

	Host		H.C.		Mycelium	
	Pulse	Chase	Pulse	Chase	Pulse	Chase
Basic	4.8	9.1	11.1	9.3	22.3	18.2
Acidic	30.9	21.2	41.0	33.3	6.9	7.6
Neutral	64.3	69.7	47.9	57.4	70.8	74.2

Table 18

Erysiphe pisi - *Pisum sativum*. Distribution of ^{14}C in the neutral sugars of TFA hydrolysates of the crude cell wall fractions of the host, haustorial complex and mycelia. The fractions were obtained from infected leaves either immediately after a 30 min 'pulse' exposure to $^{14}CO_2$ or after a subsequent 20 h 'chase' period. All treatments were in the light. (Radioactivity is expressed as percentage of the total in each experimental fraction; each value is the mean from at least 2 replicate experiments).

Fraction	Host		Haustorial complex		Mycelium	
	Pulse	Chase	Pulse	Chase	Pulse	Chase
Sugars						
Galactose	40.6	45.3	6.5	7.4	18.9	22.3
Glucose	55.9	53.0	88.5	87.6	52.3	61.0
Mannose	1.8	0.8	4.8	5.0	28.3	16.6
Arabinose	0.8	0.6	0.0	0.0	0.4	0.1
Xylose	0.3	0.3	0.0	0.0	0.0	0.0

Table 19

Erysiphe pisi - Pisum sativum. The sequential hydrolysis by ∝ amylase and amyloglucosidase of ethanol insoluble compounds from host, haustorial complex and mycelial fractions obtained from infected leaves either immediately after a 30 min 'pulse' exposure to $^{14}CO_2$ or after a subsequent 'chase' period of 20h. All treatments were in the light, details of enzyme incubations are described in Results: VI, A, ii, b. Results are expressed as percentages of the total radioactivity in the fractions hydrolysed to ethanol scluble constituents by each enzyme treatment.

		∝ amylase	amyloglucosidase
Haustorial complex			
	pulse	2.18	0.12
	chase	5.32	0.38
Host			
	pulse	3.22	0.28
	chase	12.63	0.67
Mycelia			
	pulse	5.24	0.45
	chase	30.66	3.04

Figures 12, 13, 14 and 15. Erysiphe pisi - Pisum sativum. The distribution of radioactivity in paper chromatograms, of the acidic fraction of the trifluoracetic acid hydrolysate of infected leaves cleared of mycelia (figs. 12 & 13) and haustorial complexes (figs. 14 & 15). Fractions were obtained either immediately after a 30 min (pulse) exposure to $^{14}CO_2$ or after a further (chase) period of 20h in unlabelled $^{14}CO_2$; both treatments were in the light. The position of a galacturonic acid standard is shown, chromatograms were developed in solvent (ethyl acetate: acetic acid: pyridine: water, 50:12:18:10) for 70h.

Fig.12

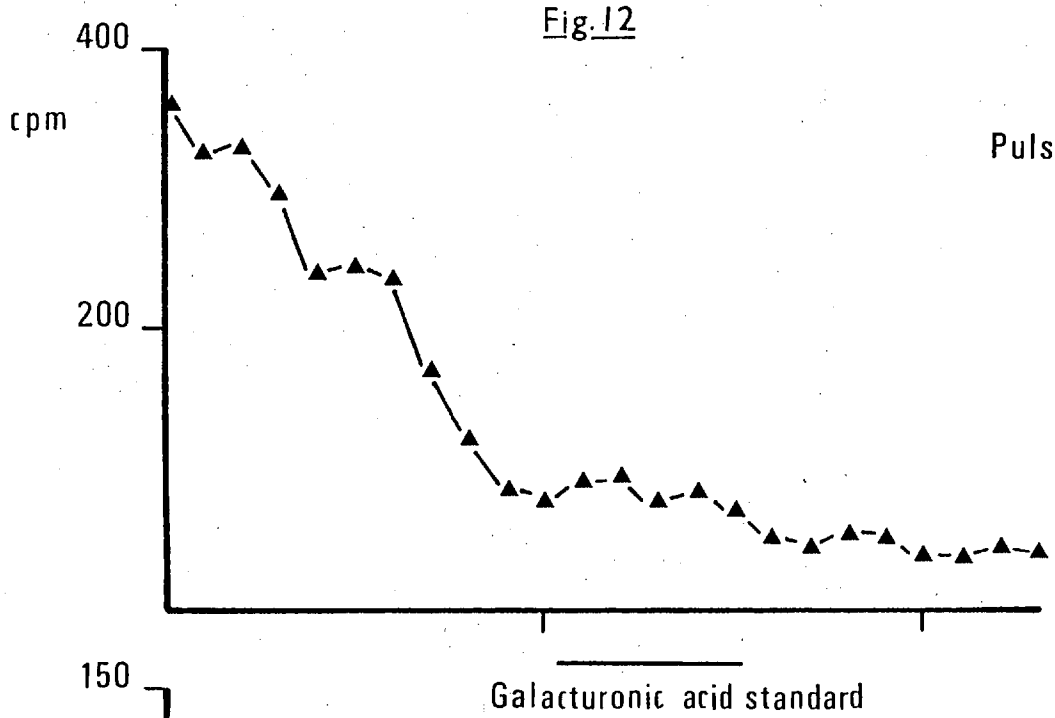
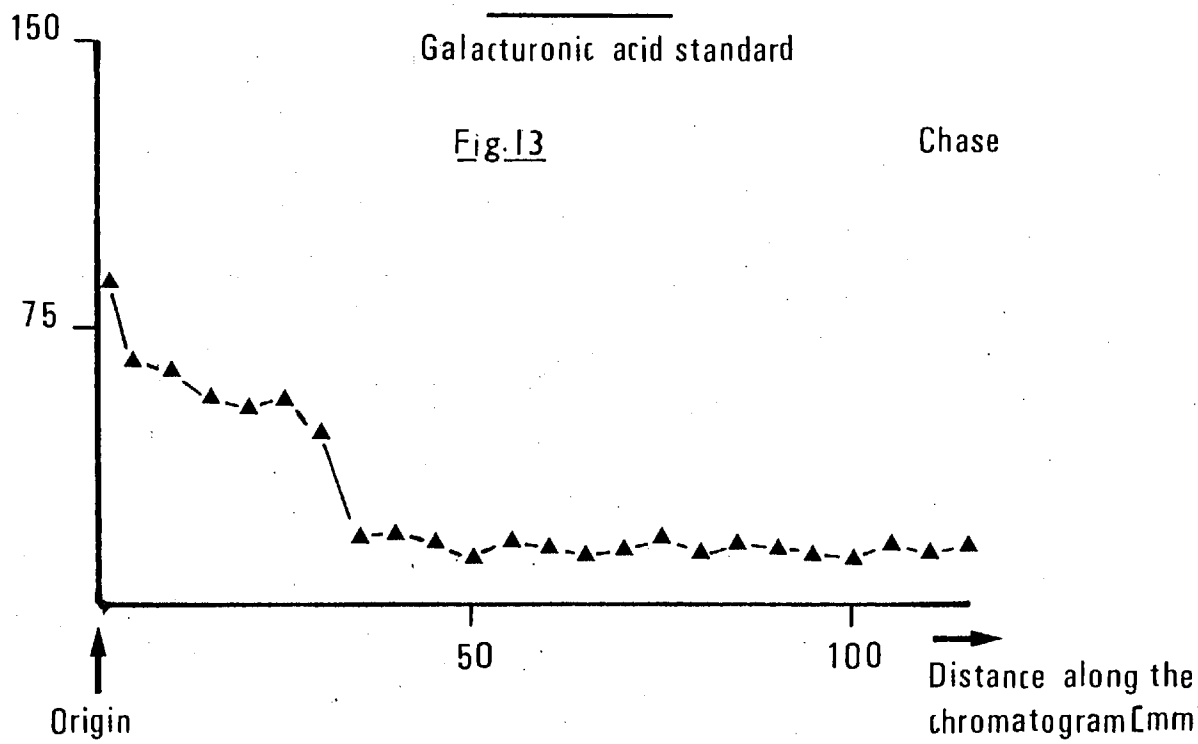

400
cpm
200
Pulse
Galacturonic acid standard

Fig.13


150
75
Chase
50
100
Origin
Distance along the chromatogram [mm]

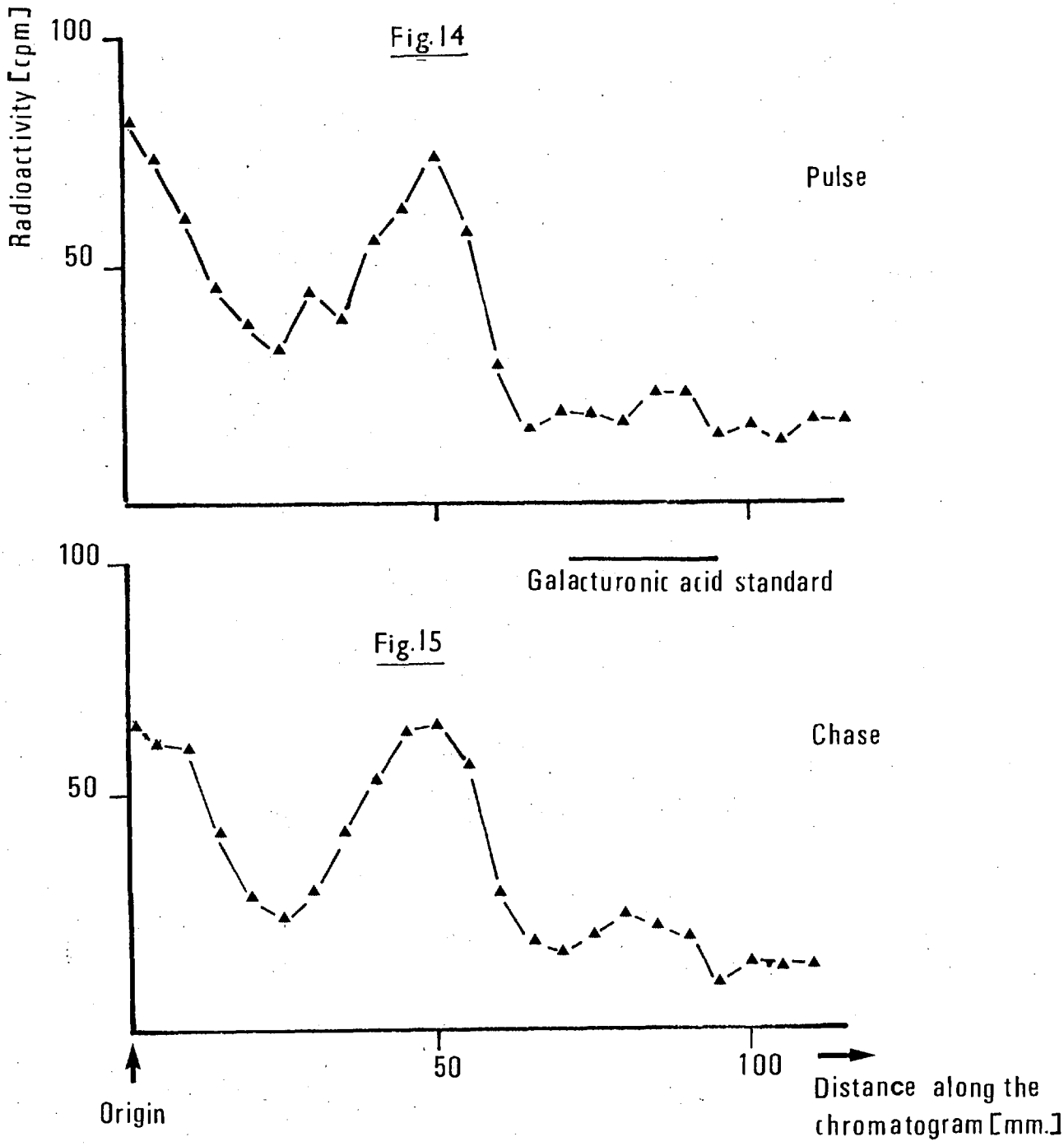

Radioactivity [cpm]
100
50
Pulse
Galacturonic acid standard
100
50
Chase
Origin
50
100
Distance along the chromatogram [mm.]

Fig.14

Fig.15

Figures 16 and 17. *Erysiphe pisi* - *Pisum sativum*.

Autoradiograms of paper chromatograms (solvent system, ethyl acetate: pyridine: water: acetic acid, 50:18:10:12) of the neutral fractions of trifluoracetic acid hydrolysates of infected leaves cleared of mycelia (fig. 16), and haustorial complexes (fig. 17). Fractions were obtained either immediately after a 30 min (pulse) exposure to $^{14}CO_2$ or after a further (chase) period of 20h in unlabelled $^{14}CO_2$; both treatments were in the light. The positions of standard compounds are also shown, (see Key below).

Key to abbreviations for standard carbohydrates

A =	arabitol	In =	myo-inositol
As=	arabinose	M =	mannitol
E =	erythritol	Ms =	mannose
F =	fructose	O =	origin, position probably corresponding to oligosaccarides.
G =	glucose	P =	pentose
Ga=	galactose	R =	ribose
GL=	glycerol	S =	sucrose
H =	hexose	T =	trehalose
		X =	xylose

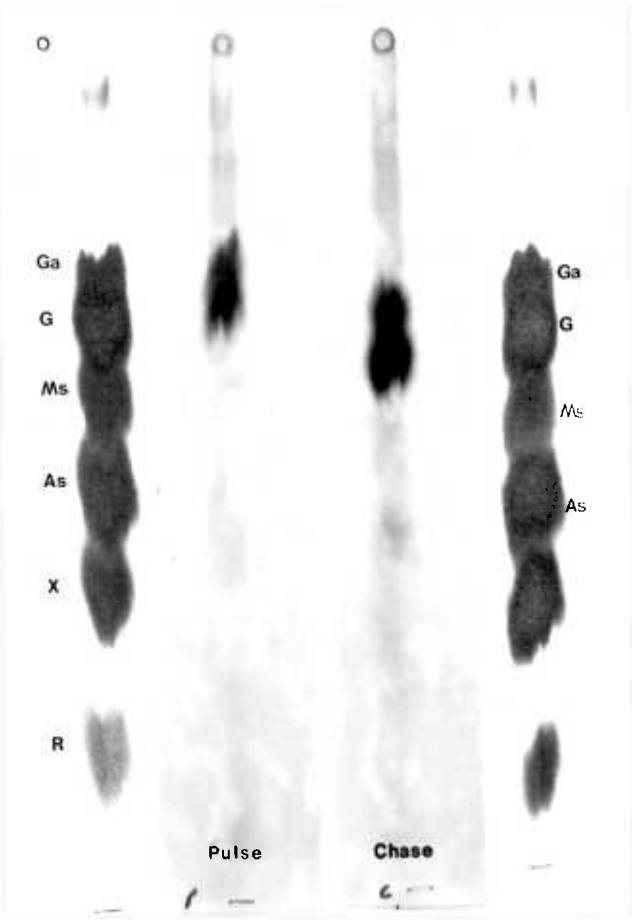


Figure 16.

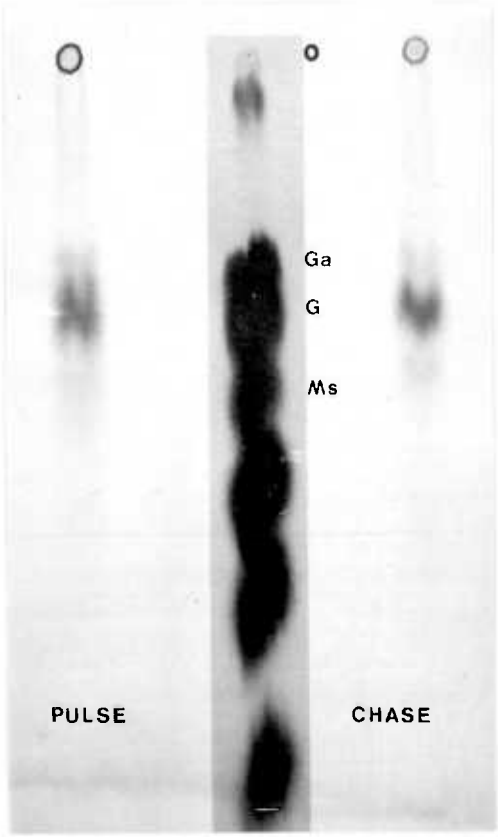


Figure 17.

Mycelium:

The hydrolysate from the mycelium had a larger proportion of label in the basic fraction (18 - 22%) than in either the host or the haustorial complex fractions. This may be due to the protein content of fungal walls being higher than in plant cell walls or to the release of n-acetyl glucosamine by the acid hydrolysis of short chain polymers of chitin demonstrated by McMurrough et al (1971). Components released by either of these mechanisms would behave as basic compounds in the ion exchange chromatography. Neither the basic fraction or small acidic fractions were analysed further. The largest proportion of the label from both pulse and chase experiments were in neutral compounds which were identified as galactose, glucose and mannose (table 18 & fig. 18). Their relative proportions were approximately 1: 2.7 : 1 with little change upon chase treatment. There was a large increase in the total number of counts per unit area in the mycelial cell wall hydrolysate 5.4 fold during the chase period and thus it is evident that these compounds represent a large sink in the fungus for the products of host photosynthesis. The sink probably represents new hyphal growth and spore development.

C) Neutral Sugars

The neutral sugar fractions of host, haustorial complexes and mycelia were separated by single dimensional paper chromatography in each of the three solvent systems, EAW, PEW and MAB. These solvents were used as they individually separate the disaccharides,

Figure 18. Erysiphe pisi Autoradiograms of paper chromatograms (solvent system, ethyl acetate: pyridine: water: acetic acid, 50:18:10:12) of the neutral fractions of trifluoracetic acid hydrolysates of mycelia. The fractions were obtained from infected leaves either immediately after a 30 min (pulse) exposure to $^{14}CO_2$ or after a further (chase) period of 20h in unlabelled $^{14}CO_2$; both treatments were in the light. The positions of standard compounds are also shown, (for Key see page 149).

Figure 19. Erysiphe pisi - Pisum sativum. Autoradiogram of a paper chromatogram (solvent system, n-propanol: ethyl acetate: water, 7:1:2) of the neutral sugar fraction of an ethanol extract from infected leaves cleared of mycelia. The fraction was obtained after a 30 min exposure (pulse) to $^{14}CO_2$ in the light. The position of standard compounds are also shown (for key see page 149).

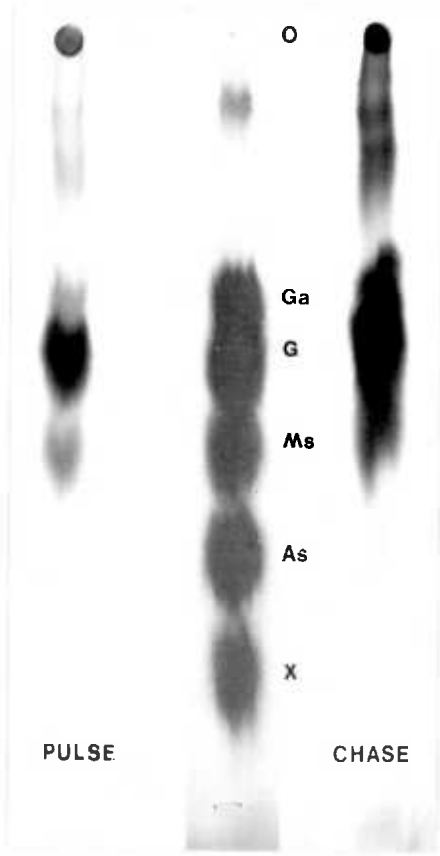


Figure 18.

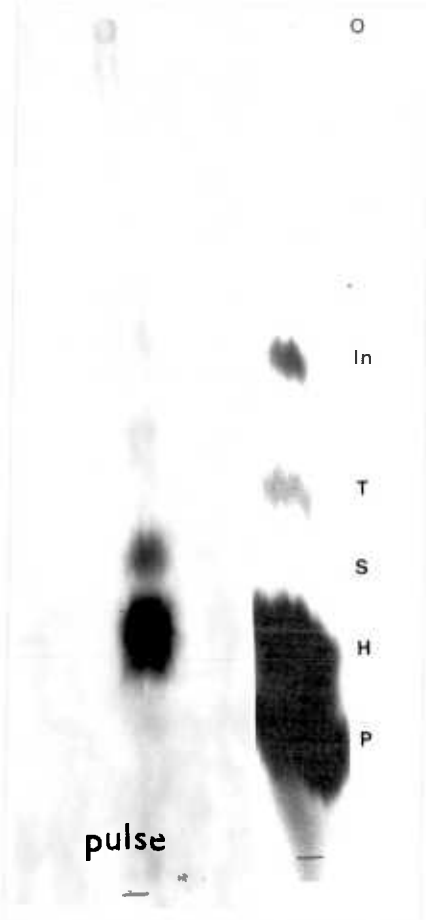


Figure 19.

monosaccharides and sugar alcohols to different extents (see Methods X, E, iv, C). Analysis of the chromatograms together thus permits a quantitative assessment of each constituent and avoids some of the identification problems which would have arisen following development in two dimensions.

Host:

The distribution of ^{14}C in the neutral sugars of the host fractions from both pulse and chase experiments are shown in table 19 and some examples of the autoradiograms are reproduced in figs. 19 & 20. After the pulse $^{14}CO_2$ exposure the largest proportion of ^{14}C was in glucose (43.8% and fructose (38.2%) with a smaller proportion (13.2%) in sucrose. Mannitol, arabitol and glycerol were detected in trace quantities but neither erythritol or trehalose was detected. Myo-inositol was the only sugar alcohol found at greater than trace quantities. A significant number of counts also occurred at the origin in compounds presumed to be oligosacchorides.

After the chase period the same compounds were labelled and all contained considerably smaller quantities of label than previously. Most of the isotope was still in glucose (40.9%) but the number of counts in this pool had been reduced to only 11% of that after the pulse. Fructose was reduced to a greater degree, only 6% of the label remaining after the chase. There was a complementary increase in labelled sucrose, (13.2% to 30.3%) but even so there was a 3.6 fold reduction in the total number of counts.

Table 20

Erysiphe pisi - *Pisum sativum*. Distribution of ^{14}C in the neutral sugar fraction obtained from infected leaf discs. Infected leaves were sampled after a 30 min 'pulse' exposure to $^{14}CO_2$ or a further 20h 'chase' period and mycelia were removed prior tc extraction and analysis of the sugars. All treatments were in the light. Each result is the mean of 2 experiments. Values are expressed as percentages of the total radioactivity in fraction and as dpm mm^{-2} infected leaf.

	Pulse		Chase	
Sugar	%	dpm $100mm^{-2}$	%	dpm $100mm^{-2}$
Origin	2.0	1,310	4.3	340
Myo-inositol	1.7	1,110	3.8	300
Sucrose	13.2	8,650	30.3	2,390
Trehalose	0.0	0	0	0
Glucose	43.8	28,690	40.9	3,230
Mannitol	0.3	200	0.8	70
Fructose	38.2	25,020	19.4	1,530
Arabitol	0.1	70	0.1	10
Erythritol	0.0	0	0	0
Glycerol	0.2	130	0	0

Figure 20. Erysiphe pisi - Pisum sativum. Autoradiograms of paper chromatograms (solvent system, ethyl-methyl ketone: acetic acid: saturated boric acid, 9:1:1) of the neutral sugar fractions of ethanol extracts from infected leaves cleared of mycelia. The fractions were obtained either immediately after a 30 min exposure (pulse) to $^{14}CO_2$ or after a further (chase) period of 20h in unlabelled $^{14}CO_2$; both treatments were in the light. The positions of standard compounds are also shown (see page 149 for key).

Figure 24. Erysiphe pisi. Autoradiograms of paper chromatograms (solvent system, ethyl acetate: acetic acid: water, 14:3:3) of the neutral sugar fractions of ethanol extracts of mycelia. Fractions were obtained either immediately after a 30 min (pulse) exposure to $^{14}CO_2$ or after a further (chase) period of 20h in unlabelled $^{14}CO_2$; both treatments were in the light. The positions of corresponding standard compounds are also indicated by their initials, (see page 149 for key).

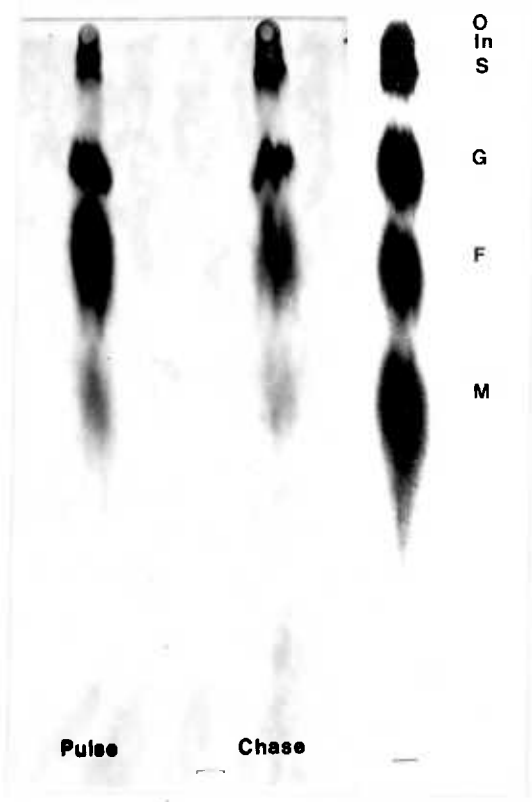


Figure 20.

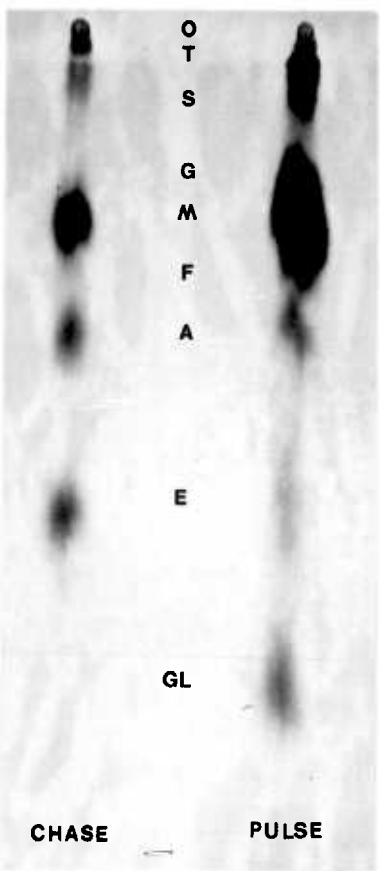


Figure 24.

The preferential fixation of $^{14}CO_2$ into hexoses by the host has been used as an indicator of enhanced invertase activity (Long & Cooke, 1974) but the proportionate increase in sucrose in the host fraction with chase treatment indicates that invertase activity was not enhanced in this tissue. It is thought that sucrose is the compound translocated in phloem of Pisum sativum (Warmer, 1952) and in intercellular transport in beet, (Geiger, 1975; Giaquinta, 1978) and squash (Hendrix, 1978) and thus the proportionate increase of label in sucrose may be due to its mobilisation in connection with intercellular transport of this compound in the leaf.

Haustorial complexes:

The number of counts obtained from neutral sugars in haustorial complexes was very low and thus it was impossible to obtain autoradiograms of the chromatograms indicating the presence of ^{14}C. The chromatograms were thus cut into strips and these counted directly in scintillant. The results obtained are shown in figs. 21, 21, 23. Although the numbers of counts recovered were small definite peaks and some significant trends after the chase period could be detected. The distribution of label in areas corresponding to standard sugars are shown in table 20.

A large proportion (14 & 26% for pulse and chase experiments respectively) of the ^{14}C in the haustorial complexes remained at the origin during chromatography, and as was suggested for the host sugars it probably represents oligosaccharides. The relatively large proportion of ^{14}C in these compounds may be an accurate representation

or may be due to their preferential retention, being lost at a slower rate than lower molecular weight sugars during the isolation procedure. The loss of compounds during isolation is discussed below.

After the pulse treatment the predominantly labelled compounds were sucrose (28%), mannitol (14.5%) and glycerol (22.1%). The labelling of glycerol is noteworthy as it occurred in only trace quantities in host and mycelial samples. The predominance in the haustorial complex fraction of sucrose and glycerol over glucose and fructose which predominated in the host fractions is a good indication that host contamination was very small. Arabitol and trehalose also contained significant quantities of label and with mannitol which was present, are metabolites characteristic of fungi (Smith et al, 1969).

After the chase there were increases in the number of counts per haustorial complex for oligosaccharides (material at the origin), mannitol, trehalose, glucose and arabinose (table 20). The largest increase was in mannitol which then constituted 45% of the ^{14}C in the fraction. There were large decreases in the proportion of radioactivity in sucrose and glycerol but little change in that of fructose. These decreases indicate that these compounds had been either metabolized by the haustorium or translocated to the mycelium during the chase period. The occurrance of glycerol has not been reported in higher plant/ obligate parasite associations in such proportions before.

Table 21

Erysiphe pisi - *Pisum sativum*. Distribution of ^{14}C in the neutral sugar fraction from isolated haustorial complexes. Complexes were isolated from infected leaves after a 30 min 'pulse' exposure to $^{14}CO_2$ or a subsequent 20h 'chase' period. All treatments were in the light. (Each result is the mean of 3 experiments. Values are expressed as percentages of the total radioactivity in the fraction and as dpm 2×10^{-6} HCs).

	Pulse		Chase	
Sugar	%	dpm 2×10^{-6} HCs	%	dpm 2×10^{-6} HCs
Origin	13.9	117	25.8	325
Myo-inositol	0.0	0.0	0.0	0
Sucrose	28.3	238	4.7	59
Trehalose	3.3	28	4.0	50
Glucose	9.1	76	9.8	124
Mannitol	14.5	122	45.2	569
Fructose	5.8	49	3.4	43
Arabitol	2.9	24	4.5	57
Erythritol	0.0	0.0	0.0	0.0
Glycerol	22.1	185	2.5	32

Figures 21, 22, 23. *Erysiphe pisi* - *Pisum sativum*. Scans of paper chromatograms of neutral sugar extracts of haustorial complexes isolated from infected leaves either immediately after a 30 min (pulse) exposure to $^{14}CO_2$ or after a subsequent 20h (chase) period. All treatments were in the light, solvent systems are shown with each figure.

Figure 21. Solvent system, ethyl acetate: acetic acid: water, 14:3:3.

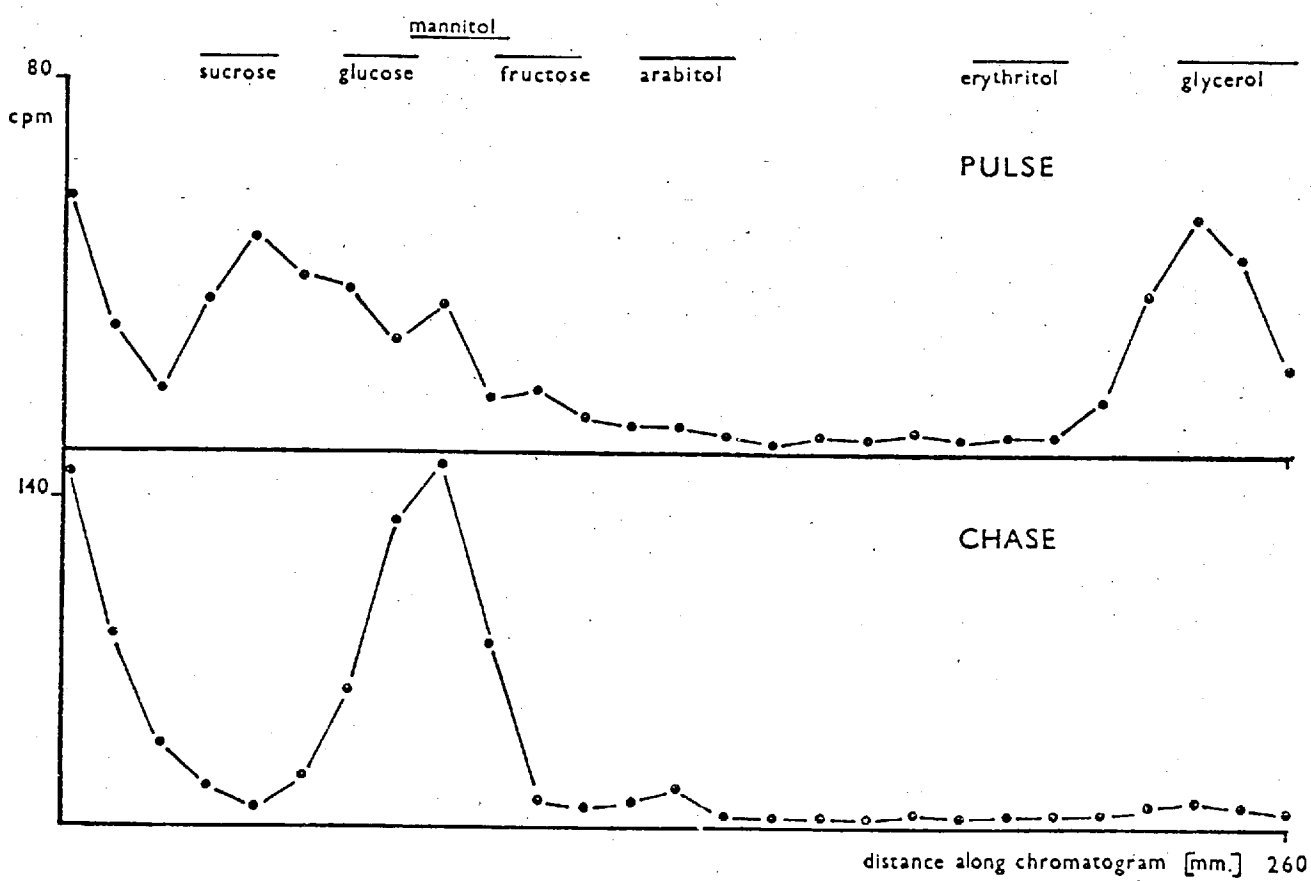


Figure 22. Solvent system, n-propanol: ethyl acetate: water, 7:1:2.

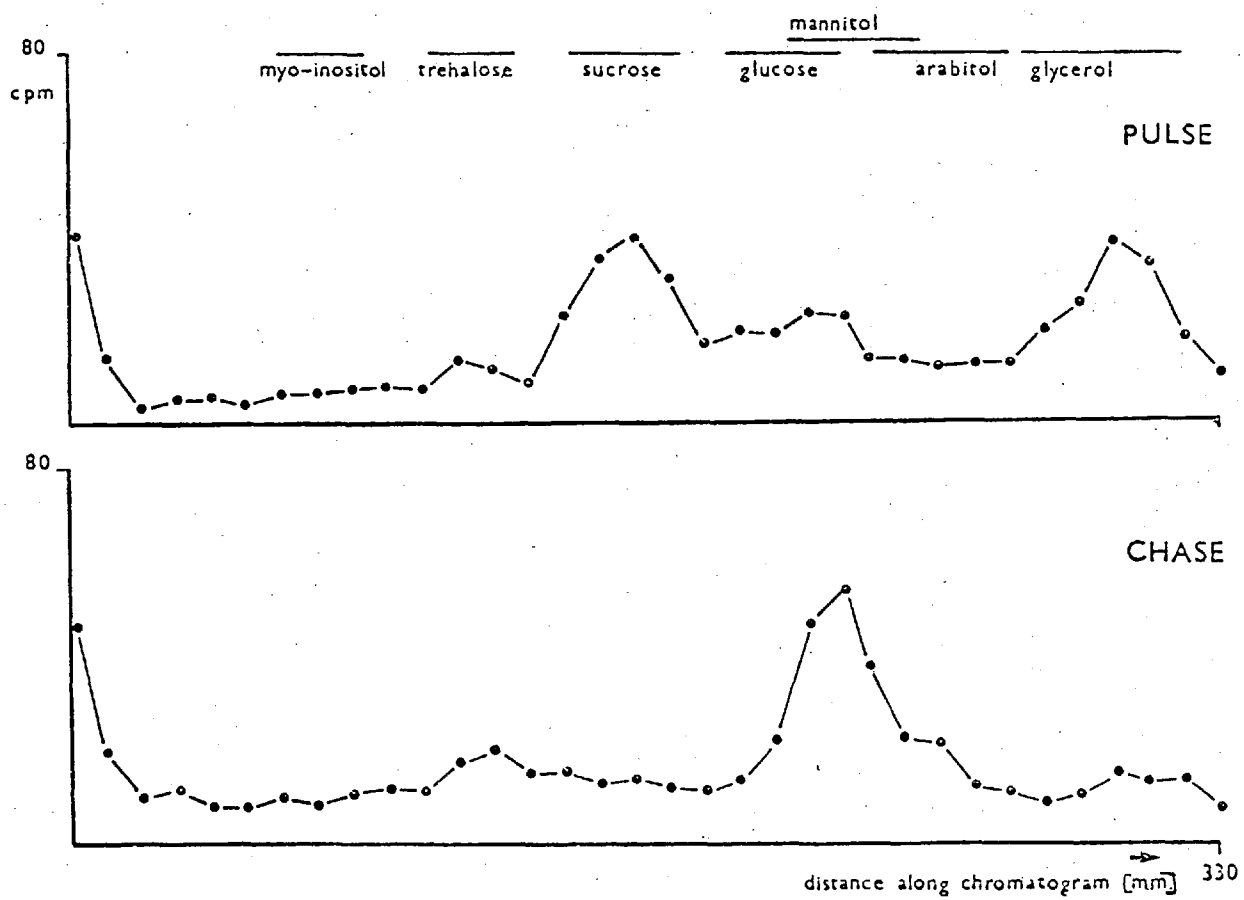
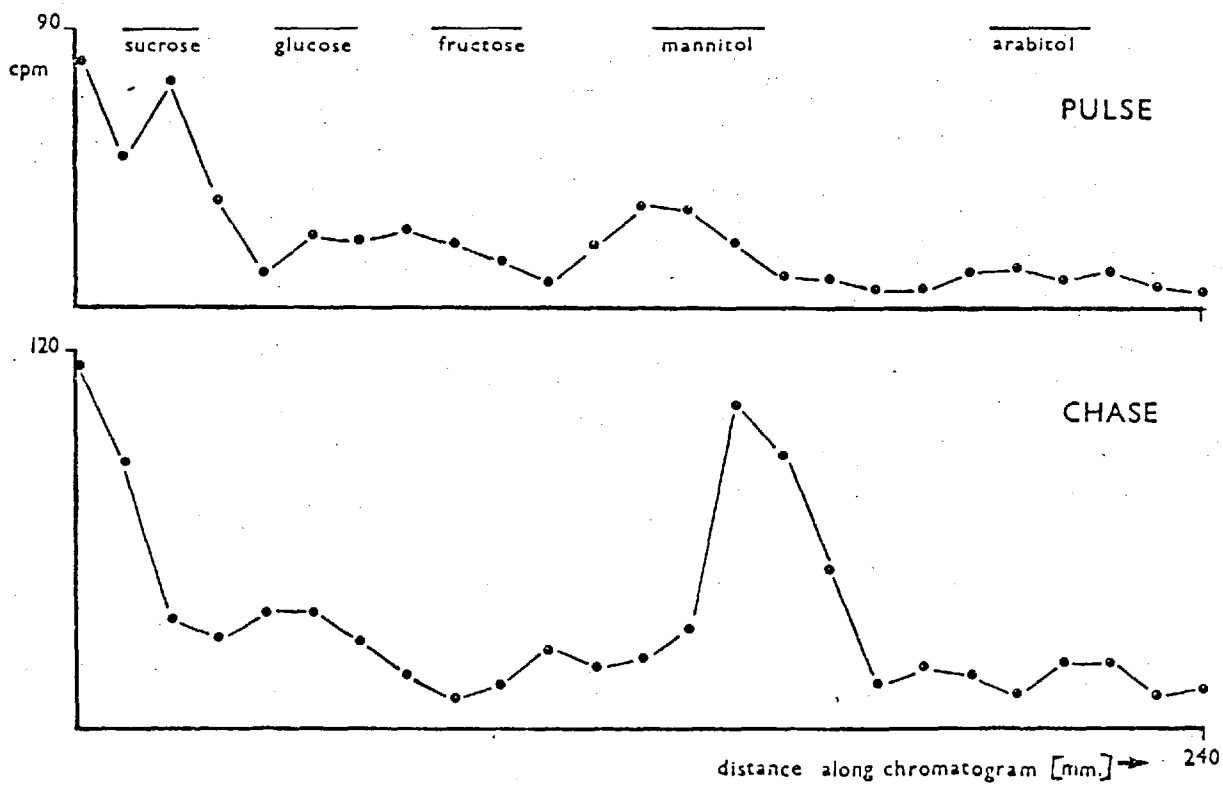


Figure 23. Solvent system, ethyl-methyl ketone: acetic acid: saturated boric acid, 9:1:1.



Mycelia:

Analysis of mycelial fractions showed that mannitol was very rapidly labelled and amounted to 82% of the ^{14}C in the neutral sugars after the pulse feeding, (table 21 and figs. 24, 25, 26). Significant quantities of label were also found in trehalose, sucrose (fig. 27), glucose, fructose, arabitol and glycerol with traces in erythritol and compounds immobile in the solvent systems used. There were substantial decreases in the number of counts in most of these compounds during the chase period. Mannitol was reduced so that only 3% of the pulse level persisted. There were increases in the quantity of ^{14}C in erythritol (4.7 fold increase) and in compounds remaining at the origin (2 fold increase). Arabitol and erythritol contained large proportions of the isotope (13.8% and 18.7% respectively) and may represent processes of spore development since arabitol was predominant in the spores of Erysiphe graminis (Edwards & Allen, 1966). These authors also showed that mannitol predominated in the mycelium. There is a clear reduction in both the proportions and numbers of counts in glucose, fructose and sucrose which are characteristic of the host. Similar kinetics were described in E. graminis (Edwards & Allen, 1966) where during one hour a proportionate reduction in labelled sucrose was detected.

Table 22

Erysiphe pisi. Distribution of ^{14}C in the neutral sugar fraction of the mycelium obtained from infected pea leaves either immediately after a 30 min 'pulse' exposure to $^{14}CO_2$ or a subsequent 20h 'chase' period. All treatments were in the light. (Each result is the mean of 2 separate experiments and is expressed as a percentage of the total radioactivity in each fraction or as dpm $100\ mm^2$ of infected leaf).

Sugar	Pulse		Chase	
	%	dpm $100mm^{-2}$	%	dpm $100mm^{-2}$
Origin	0.2	30	8.2	60
Myo-inositol	0.0	0.0	0.1	trace
Sucrose	4.4	650	0.8	trace
Trehalose	6.4	950	6.8	50
Glucose	2.5	370	0.4	trace
Mannitol	81.7	12,100	51.0	376
Fructose	2.0	300	0.2	trace
Arabitol	1.1	160	13.8	100
Erythritol	0.2	30	18.7	140
Glycerol	1.5	220	0.0	0

Figures 25, 26 and 27. Erysiphe pisi. Autoradiograms of paper chromatograms of the neutral sugar fractions of ethanol extracts of mycelia. Fractions were obtained either immediately after a 30 min (pulse) exposure to $^{14}CO_2$ or after a further period (chase) of 20h in unlabelled $^{14}CO_2$; both treatments were in the light. The positions of standard compounds are also shown, (see page 149 for key).

Figure 25. Solvent system, ethyl-methyl ketone: acetic acid: saturated boric acid, 9:1:1.

Figure 26. Solvent system, ethyl acetate: acetic acid: pyridine: water, 50:12:18:10.

Figure 27. The radioactive areas corresponding to sucrose and trehalose in the 'pulse' fraction from fig. 25 were eluted from the chromatogram and rechromatographed with solvent system n-propanol: ethyl acetate: water, 7:1:2, showing the absence of labelled myo-inositol.

Figure 25.

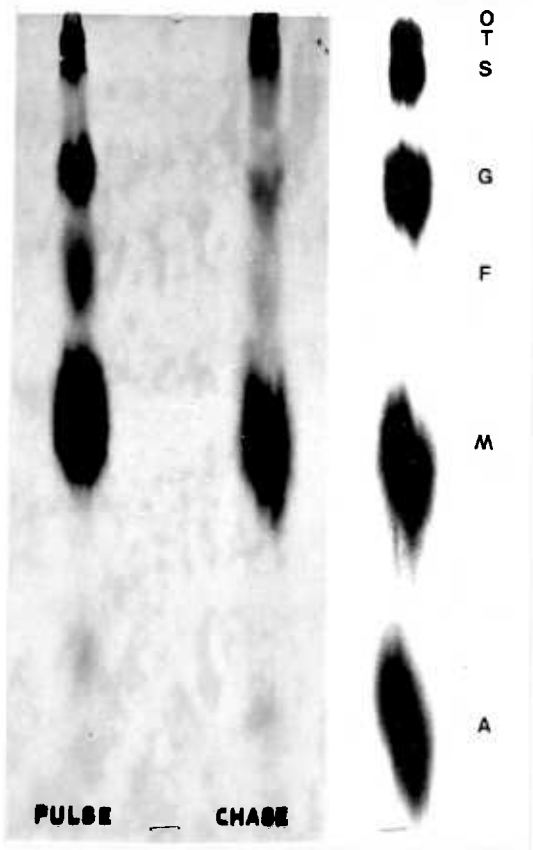


Figure 26.

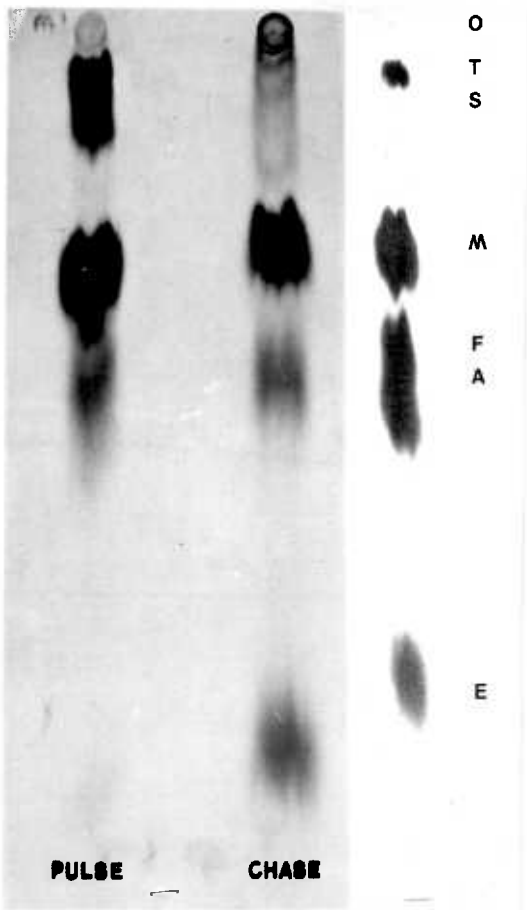
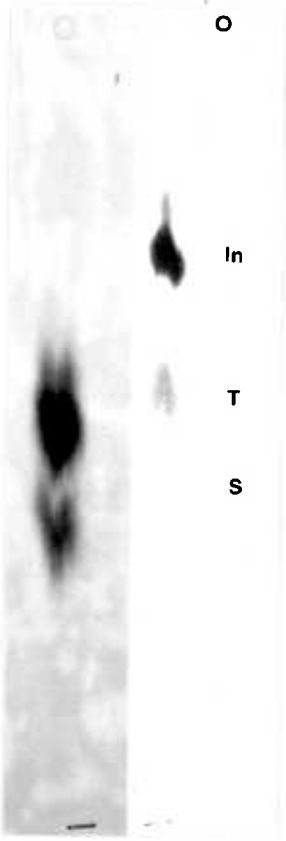


Figure 27.



VII) Identification and quantification of ethanol soluble carbohydrates in the host, haustorial complexes and mycelia.

The previous section described the distribution of ^{14}C in the neutral sugars and sugar alcohols of host, haustorial complexes and mycelia during and after transfer of ^{14}C photosynthates from host to parasite. It is evident that these compounds maybe important in the transfer of photosynthates from host to parasite. The specific activities of the labelled carbohydrates were not estimated as the samples were used extensively for estimation of the distribution of radioactivity and further analysis was not possible. The following experiments were designed to identify and quantify the neutral sugars in extracts of infected host, haustorial complexes, mycelia and healthy host without employing isotopic carbon dioxide.

A) Experimental procedures.

Discs (5g fresh weight), from uninfected leaves and infected leaves separated from the mycelium and mycelial brushings (0.5g fresh weight) were treated in 80% (v/v) ethanol. Haustorial complexes were isolated in an ionic isolation medium substituting 0.1 M KCl for the sucrose and $7 - 9 \times 10^6$/λ HCl were treated similarly. The ethanol soluble extracts were deionised, cleared and the final extract was analysed either by paper chromatography or gas-liquid chromatography as described in the Methods section (X, E).

Sugars and sugar alcohols were identified by their mobilities and reaction with specific detection agents on paper chromatograms, and their elution temperatures and retention times on the G.L.C. They were quantified by comparison of the peak heights on the gas-liquid chromatograms with that of known standards (Holligan,1971).

B) Results

1) Uninfected host:

Examples of the paper chromatograms of the extracts from uninfected pea leaves are illustrated in figs. 28 and 29. The mobilities of the spots obtained from the extracts and corresponding reference compounds relative to that of glucose (Rg value) are shown in table 23. Reactions with p-anisidine and bromocresol purple and Rg values, indicate that the spots A, B, C were most probably myo-inositol, sucrose and glucose respectively. Spot D gave a faint yellow with bromocresol purple after development in the PEW solvent. This indicates that it was a polyol (Lewis & Smith,1967b) but its reaction with p-anisidine and mobility in EAW indicates a ketohexose, probably fructose. However the reaction with bromocresol purple may have been due to traces of mannitol.

Two other spots were obtained for which no corresponding standard could be found. They probably correspond to the unknown compounds X and Y described in an investigation of the sugars in pea seedlings by Vitek (1964) who reported two compounds with

Figures 28 and 29. *Erysiphe pisi* - *Pisum sativum.* Paper chromatographic separation of the ethanol soluble carbohydrates of uninfected host (H), infected host (I), infected host separated from the mycelium (IH), mycelia (M) and haustorial complex fractions. The solvent systems used were n-propanol: ethyl acetate: water, 7:1:2 (fig. 28) and ethyl acetate; acetic acid: water, 14:3:3 (fig. 29) and carbohydrates were detected by alkaline silver nitrate treatment. The colour reactions of the spots, mobilities relative to glucose and possible identities are shown in tables, 23, 27, 29 and 31.

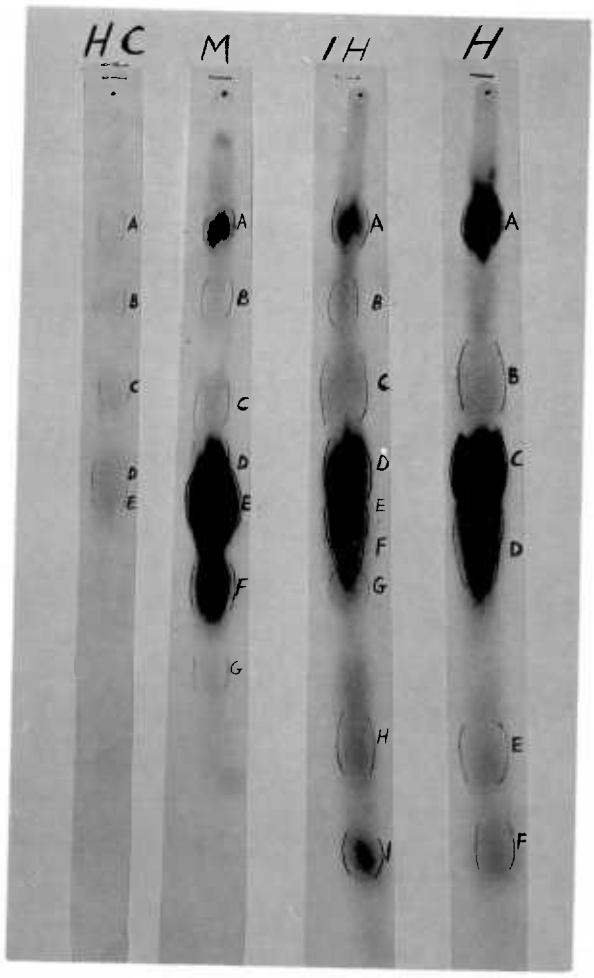


Figure 28.

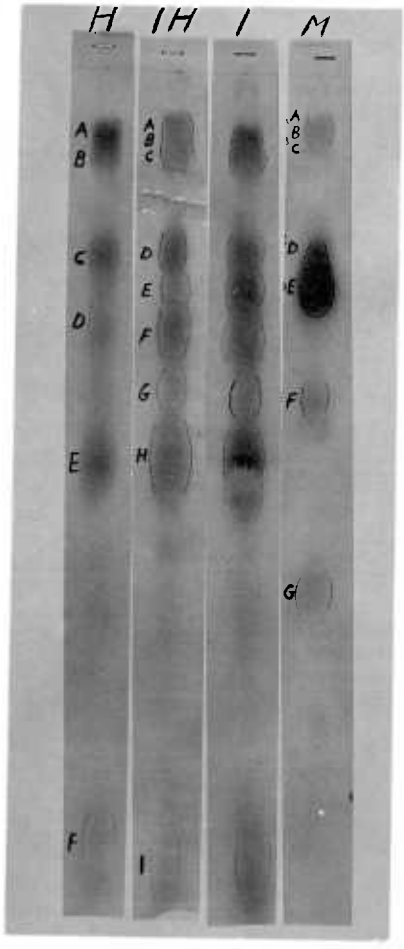


Figure 29.

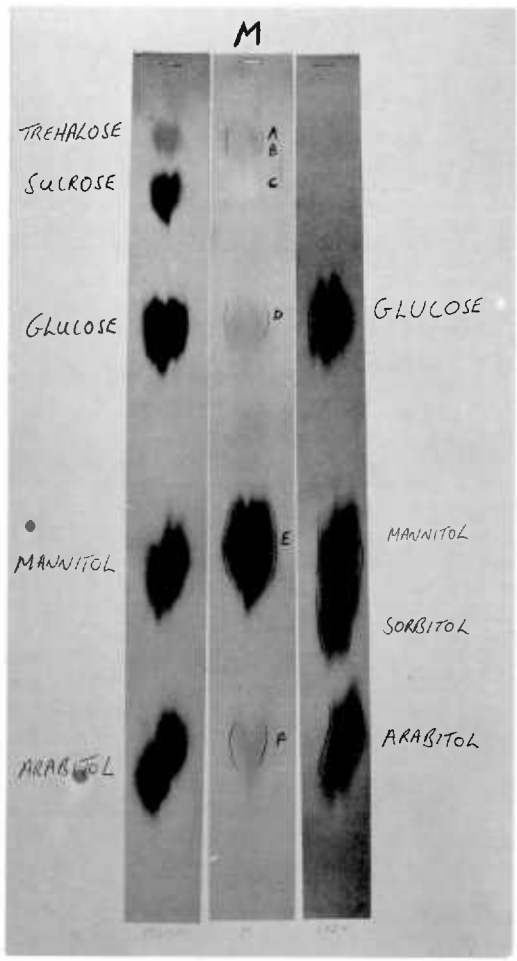


Figure 30. Erysiphe pisi. Paper chromatographic separation of ethanol soluble carbohydrates from the mycelium (M). The solvent system was ethyl-methyl ketone: acetic acid: saturated boric acid, 9:1:1 and carbohydrates were detected by alkaline silver nitrate treatment. Standard compounds are also shown.

mobilities and reactivities with p-anisidine similar to those described here. However, Vitek did not obtain reactions with silver nitrate reagent. He suggested that the compounds were probably complex as hexoses, amino acids and phosphate were released after acid hydrolysis (Vitek, 1964).

Analysis of the extracts of uninfected leaves by gas-liquid chromatography after silylation confirmed the identifications made on paper chromatography. The elution temperatures and retention times of the methylsilyl derivatives of the standards used throughout this investigation are shown in table 24. Six distinct peaks were obtained from the extract of healthy leaves (table 25) and these coincided with fructose, glucose, hexitol, myo-inositol and sucrose. The detection of hexitol in small quantities may explain the faint yellow spot obtained with bromocresol purple on paper. This was confirmed on the paper chromatogram with fructose from which it is poorly separated by the PEW solvent. The unidentified compounds from the paper chromatograms did not appear on the gas chromatograms.

Sugar alcohols occur in a wide range of higher plants and are usually mobile in the phloem (Lewis & Smith, 1967a). Thus it is likely that those detected here occurred in the vascular elements in the leaf. The ethanol soluble carbohydrates from uninfected leaves were quantitatively assayed by comparisons of peak heights with those of standard compounds. The results are shown in table 26

Table 23

Pisum sativum. Paper chromatographic identification of ethanol soluble carbohydrates of uninfected leaves by comparison of their mobilities and colour reactions with those of standard compounds.

1) Mobilities

Spots as in figs. 28 & 29	Rg value		Corresponding Standard
	PEW	EAW	
A	40	33	Myo-inositol
B	71	45	Sucrose
C	100	100	Glucose
D	114	136	Fructose
E	168	219	Unknown
F	204	450	Unknown

2) Colour reactions.

Spots as in figs. 28 & 29	Colour rdaction. p-anisidine-Ac	bromocresol	Possible Identity
	60° & 100°	purple	
A	-	-	Non Ketose containing disaccharide
B	Yellow	-	Ketose-containing disaccharide
C	brown	-	aldohexose
D	yellow	faint-yellow	Ketohexose/polyol
E	yellow	-	Ketohexose
F	brown	-	aldohexose

Table 24

Retention times and elution temperatures for gas-liquid chromatography of trimethylsilyl derivatives of standard compounds.

	Elution temperature	Retention time (min)
Glycerol	134°	2½
Erythritol	134°	4
Xylose	144° & 152°	7 and 8½
Arabitol	148°	7¾
Fructose	158°	10
Glucose	169° & 182°	12 and 14½
Mannitol	174°	13
Myo-inositol	188°	15¾
Sucrose	235°	25
Trehalose	242°	26

Table 25

Pisum sativum. Gas-liquid chromatographic identification of ethanol soluble carbohydrates from uninfected pea leaves. Comparison of the elution temperatures and retention times of their trimethylsilyl derivatives with those of standard compounds.

Peak.	Elution temp.	Retention time (min).	Corresponding standard.
A	158°	10	fructose
B	169°	12	glucose
C	174°	13½	hexitol
D	182°	14½	glucose
E	189°	16	myo-inositol
F	235°	25	sucrose

Table 26.

Erysiphe pisi - Pisum sativum. Components of the ethanol soluble carbohydrates of uninfected leaf, infected leaf separated from the mycelium, mycelium and haustorial complex fractions. Carbohydrates were quantified by gas-liquid chromatography of their trimethylsilyl derivatives. Values (mg ethanol soluble carbohydrate $100mg^{-1}$ dry wt) are the mean of at least 3 samples where standard deviations are shown. * Mean of 2 replicate samples.

Carbohydrate	Uninfected leaf	Infected leaf separated from the mycelium.	Mycelium	Haustorial complex fraction.
Glycerol	-	-	-	0.061 ± 0.039
Erythritol	-	-	3.31 ± 0.45	-
Arabitol	-	0.04 ± 0.05	1.34 ± 0.17	0.078 ± 0.052
Xylose	-	0.11 ± 0.10	-	0.158 ± 0.104
Fructose	2.13*	0.87 ± 0.24	0.56 ± 0.16	0.054 ± 0.031
Glucose	3.79*	2.16 ± 0.61	0.63 ± 0.20	0.158 ± 0.089
Mannitol	0.11*	0.11 ± 0.14	7.60 ± 1.29	0.061 ± 0.031
Myo-Inositol	0.44*	0.12 ± 0.09	0.26 ± 0.11	0.042 ± 0.025
Sucrose	1.95*	0.94 ± 0.16	0.19 ± 0.21	0.447 ± 0.147
Trehalose	-	0.06 ± 0.09	0.33 ± 0.21	0.093 ± 0.066
TOTAL	8.42	4.37	14.3	1.15

and it is clear that glucose which comprised 45% of the total sugar content was the major compound present in these leaves. Fructose and sucrose were present in near equal quantities (25% and 23% of the total sugars respectively). Myo-inositol and mannitol were minor constituents. Together the sugars constituted 8.5% of the dry weight of the leaves. However the sugar content of leaves varies, ranging from approximately 1 - 2% of the dry weight as reported for Senecio squalidus by (Long & Cooke, 1974) to 28% for Tulipa gesneriana (Rutter, Johnston & Willmer, 1977). Presumably the age and photosynthetic capability of the leaves also affects the sugar content.

ii) Infected host separated from the mycelium

Paper chromatography of infected leaves separated from the mycelium showed all the compounds in extracts from the healthy host (figs. 28 & 29 and table 27) and three additional spots were detected. From their mobilities and colour reactions these were identified as trehalose, a hexitol and pentitol. These were probably of fungal origin as they were not detected in the uninfected leaves and at least trehalose is not thought to occur in higher plants (Gussin, 1972). These compounds together with the increase in intensity of the colour yellow with bromocresol purple in the hexitol region in PEW solvent indicate the presence of characteristically fungal carbohydrates (Smith et al, 1969) in the infected leaf. It seems likely that they are constituents of the haustorium.

Gas chromatography confirmed all the identifications made by paper chromatography (table 28) and additional compounds were detected. One of these (peak A) was eluted from the column at 134° after 5 min and no corresponding standard could be found. Peaks B and D corresponded to those of D-xylose. However D-xylose should have been detected on the paper chromatograms by p-anisidine and thus only a tentative identification can be concluded. Peak A was present in only trace quantities and was ignored in the quantitative analysis (table 26), peaks B and D were quantified by comparison with D-xylose. This revealed reductions in the quantities of glucose, fructose, sucrose and myo-inositol when compared to uninfected leaves and only slight increases in quantities of hexitol (table 25). Pentitols and hexitols were present in only trace quantities (less than 1% of the total sugars) and the putative xylose constituted only 2.5% of the total soluble carbohydrate in the extract. The soluble carbohydrate fraction made up only 4.4% of the dry weight of the infected leaf which is half that of the uninfected leaf. The dry weight of healthy and infected leaf discs cleaned by mycelia were 0.020 ± 0.003 mg/mm^2 and 0.018 ± 0.002 mg/mm^2 respectively so these results also indicated differences in the quantities of soluble sugars per unit leaf area. They contrast the results of other investigations with Erysiphe graminis (Allen, 1942) and rusts (Lewis, 1976) where accumulations of soluble carbohydrates have been reported.

Table 27

Erysiphe pisi - *Pisum sativum*. Paper chromatographic identification of the ethanol soluble carbohydrates of infected pea leaves separated from the mycelium. Carbohydrates were identified by comparison of their mobilities and colour reactions with those of standard compounds.

1) Mobilities.

Spots as in figs. 28-29	Rg value PEW	Rg value EAW	Corresponding Standard
A	35	32	Myo-inositol
B	52	26	Trehalose
C	71	43	Sucrose
D	100	100	Glucose
E	106	120	Mannitol
F	113	136	Fructose
G	126	181	Arabitol
H	172	221	Unknown
I	206	455	Unknown

2) Colour reactions.

Spots as in figs. 28 & 29	Colour reaction: p-anisidine-Ac 60° & 100°	Colour reaction: bromocresol purple	Possible identity
A	-	-	Non-ketose disaccharides
B	-	-	
C	Yellow	-	Ketose-disaccharide
D	Brown	-	aldohexose
E	-	Yellow	polyol
F	Yellow	Yellow	polyol/ketose
G	-	Yellow	polyol
H	Yellow	-	Ketohexose
I	Brown	-	aldohexose

Table 28

Erysiphe pisi - *Pisum sativum*. Gas liquid chromatographic identification of ethanol soluble carbohydrates of infected leaves separated from the mycelium. Compounds were identified by comparison of the elution temperatures and retention times of their trimethylsilyl derivatives with those of standard compounds.

Peak	Elution Temperature	Retention Time (min)	Corresponding Standard
A	134°	5	Unknown
B	142°	6⅔	Xylose
C	148°	7½	Arabitol
D	152°	8	Xylose
E	155°	8⅔	Unknown
F	160°	10	Fructose
G	169°	11½	Glucose
H	175°	12¾	Mannitol
I	182°	14	Glucose
J	190°	15¾	*Myo*-Inositol
K	234°	24	Sucrose
L	240°	25½	Trehalose

These biotrophs form distinct lesions and thus may permit accumulation from surrounding healthy leaf tissue. Alternatively the differences may be due to different intensities of infection. In the present work carbohydrates were measured seven days after inoculation when the whole of the leaf surface was covered with mycelium. Hewitt & Ayres (1976) investigated the soluble carbohydrates of mildewed oak leaves, like pea mildew confluent colonies were formed, but they measured an increase in soluble carbohydrates, but from data available it is not possible to compare the infection intensity.

iii) The mycelium.

The results of paper chromatography of the ethanol soluble carbohydrates of the mycelium are shown on table 29 and some examples of the chromatograms in figs 28, 29 and 30. Seven spots were detected. Trehalose and sucrose were present in only trace amounts (figs. 28 & 30) but myo-inositol was more obvious. The major compound (E) detected on the chromatograms was mannitol and another region (spot F) which gave a yellow colour with the bromocresol purple reagent was also detected. This probably contained a mixture of arabitol (spot F) and erythritol (spot G) which are poorly separated on the PEW solvent but clearly distinguished with EAW solvent (table 29). The MAB solvent system was also used for the mycelial extracts (fig.30.) as this separated isomeric hexitols. This showed that no

Table 29

Erysiphe pisi. Paper chromatographic identification of ethanol soluble carbohydrates of mycelia. Carbohydrates were identified by comparison of their mobilities and colour reaction with those of standard compounds.

1) Mobilities

Spots as in Figs. 28, 29, 30.	Rg value PEW	EAW	MAB	Corresponding standard
A	40	35	15	Myo-Inositol
B	56	30	15	Trehalose
C	78	48	30	Sucrose
D	100	100	100	Glucose
E	106	120	210	Mannitol
F	124	182	283	Arabitol
G	146	304	-	Erythritol

2) Colour reactions

Spots as in Figs 28, 29, 30,	p-anisidine-Ac⁻ 60° & 100°	Bromocresol purple	Possible Identity
A	-	-	Nonketose disaccharides
B	-	-	
C	Yellow	-	Ketose containing disaccharide
D	Red/Brown	-	Aldohexose
E	-	Yellow	polyol
F	-	Yellow	polyol
G	-	-	-

Table 30

Gas-liquid chromatographic identification of ethanol soluble carbohydrates of mycelia by comparison of the elution temperatures and retention times of their trimethysilyl derivatives with those of standard compounds.

Peak *	Elution Temperature	Retention time (min)	Corresponding standard
A	136°	4¾	Erythritol
B	148°	7½	Arabitol
C	175°	13	Mannitol
D	190°	15½	Myo-inositol

* Trace peaks coincident with glucose, fructose, sucrose and trehalose were detected at machine sensitivities 10 fold greater than those for the above compounds and are not listed.

significant quantities of sorbitol, as indicated for some rust fungi, (Fung, 1975; Maclean & Scott, 1976) were present. MAB also gave a clear separation of glucose from mannitol which was confirmed by p-anisidine detection (table 29). Fructose was not detected and glucose and sucrose were present in only trace quantities thus demonstrating an unequivocal difference between the composition of the soluble carbohydrates in fungus and host.

These identifications were confirmed by G. L. C. analysis (table 30) except that fructose, glucose, trehalose and sucrose were not detected unless a high attenuation level was used.

Quantification of the sugars indicated a greater concentration of soluble carbohydrate in the mycelium than in either healthy or infected host tissue (table 26). It constituted 14.3% of the total dry weight of the fungus. Most of this comprised mannitol (7.6% w/w) and significant proportions of the fungal dry weight were found in erythritol and arabitol (3.3% and 1.3% respectively). The remainder was composed of trace quantities of glucose, fructose, trehalose, myo-inositol and sucrose.

The high proportion of soluble carbohydrate in the mycelium is in accord with the observations of Thatcher (1942) that the mycelium has an osmotic pressure greater than the host cells.

iv) Haustorial complexes.

Considerable difficulty was encountered in paper chromatographic analysis of ethanol soluble carbohydrates from the

isolated haustorial complexes. The small quantities obtained gave spots which were barely detectable with every detection reagent (fig.28) and thus are probably only representative of the major carbohydrates present. The results of the paper chromatographic analysis are shown on table 31. Only five compounds were detected with the silver nitrate method. Myo-inositol, trehalose and sucrose were clearly separated with PEW (fig.28). Glucose and mannitol were confirmed from their mobilities in EAW and their reaction with p-anisidine and bromocresol purple.

When analysed by G.L.C. peaks corresponding with all of the sugars identified by paper chromatography were present (table 32). Six other peaks were also detected, one of which was barely distinct from the solvent front and co-chromatographed with glycerol. This may have originated from the glycerol gradient used to prepare haustorial complexes. This compound has been shown to readily penetrate the plasma membrane of yeasts (Niedermeyer,1977). Alternatively glycerol may be a metabolite present in the complexes as indicated in the radiotracer experiments described above (Results V1, B, iii, c).

The two peaks coincident with, and tentatively identified as xylose in extracts of infected leaf tissue (Results VII, B, ii) were also obtained. These constituted 1% of the total soluble carbohydrate and the putative xylose was present in a quantity similar to that of glucose. However, glucose but no xylose was

identified on the paper chromatograms with both p-anisidine or silver nitrate. Thus the identification of xylose here similarly remains tentative, and the bulking of samples, G.L.C. of acetate derivatives and the use of other detection techniques on paper will be necessary to clearly identify the two peaks. G.L.C. also indicated significant quantities of arabitol, fructose and another peak of unknown composition (E).

Sucrose was the predominant sugar in the ethanol extracts of the isolated haustorial complexes constituting 39% of the total. Glucose and the putative xylose were the next most abundant each constituting 14%. As expected, mannitol and arabitol were found in this fungal fraction. Although arabitol and trehalose constituted larger proportions of the carbohydrate than mannitol the differences were not significant ($P = > 0.1$). This apparently contradicts the results of radiotracer experiments (Results VI, B, iii, c) which indicated a larger proportion of label in mannitol in the complexes. These probably reflect differences in the turnover of compounds in the complexes or different experimental conditions (labelling experiments employed dark pre-treated plants and extended illumination during experiments). Unlike the mycelium the haustorial complex fraction did not contain any detectable erythritol.

Soluble carbohydrates accounted for only 1.15% of the dry weight of the isolated haustorial fraction as estimated by G.L.C.

The mean dry weight of 10^6 haustorial complexes from three independent isolations was 2.7 mg, thus the soluble carbohydrate content was 0.031 mg per 10^6 haustorial complexes. Anthrone assays of ethanol extracts of an haustorial complex fraction gave good agreement with this and indicated a soluble carbohydrate content of 0.025 mg per 10^6 haustorial complexes. On a cell basis this indicates a carbohydrate content of 31 pg per complex, this is much lower than the quantities of sugar detected in plant cells e.g. palisade cells of Vicia faba containing about 700 pg of sucrose, (Jones et al,1977), but haustorial complexes are a smaller size than leaf cells. As no data is available on the cellular content of carbohydrates of pea leaf cells direct comparisons with the host cannot be made.

The carbohydrate content of the haustorial complex fraction was much lower than in the other fractions analysed and suggests a lower concentration of soluble carbohydrates in the complexes. However inevitable contamination by starch grains and host cell wall fragments accounted for part of the dry weight of the fraction and thus reduced the proportion of ethanol soluble carbohydrates. Also it is likely that loss from the haustorial complexes of low molecular weight solutes occurred during the isolation procedure.

Table 31

Erysiphe pisi - *Pisum sativum*. Paper chromatographic identification of ethanol soluble carbohydrates of the isolated haustorial complex fraction. Carbohydrates were identified by comparison of their mobilities and colour reactions with those of standard compounds.

1) Mobilities

Spots as in fig. 28	Rg value		Corresponding standard
	PEW	EAW	
A	40	35	Myo-Inositol
B	56	30	Trehalose
C	78	45	Sucrose
D	100	100	Glucose
E	160	120	Mannitol

2) Colour reactions

Spots as in fig. 28	Colour reaction		Possible identity
	p-anisidine-Ac 60° & 100°	bromocresol purple	
A	-	-	non ketose containing disaccharides
B	-	-	
C	Yellow	-	Ketose containing disaccharide
D	brown	-	Aldohexose
E	-	Yellow	Polyol

Table 32

Erysiphe pisi - *Pisum sativum*. Gas-liquid chromatographic identification of ethanol soluble carbohydrates of the isolated haustorial complex fraction by comparison of the elution temperatures and retention times of their trimethylsilyl derivatives with those of standard compounds.

Peak	Elution Temperature	Retention Time	Corresponding Standard
A	134°	2 $\frac{2}{3}$	Glycerol
B	142°	6 $\frac{1}{2}$	Xylose
C	149°	7 $\frac{1}{2}$	Arabitol
D	151°	8	Xylose
E	155°	8 $\frac{1}{2}$	Unknown
F	159°	9 $\frac{1}{4}$	Fructose
G	168°	11	Glucose
H	173°	12 $\frac{1}{4}$	Mannitol
I	180°	13 $\frac{1}{2}$	Glucose
J	188°	15	Myo-Inositol
K	232°	23	Sucrose
L	240°	24 $\frac{1}{2}$	Trehalose

VIII) Invertase, pectinase and glucan synthetase in mycelial, haustorial complex and host fractions.

A) Invertase

The experiments described above (Results VI & VII) on the analysis of labelled and unlabelled ethanol soluble carbohydrates demonstrate that these compounds are important in the nutrient transfer between pea and the parasite, Erysiphe pisi. The quantities of sucrose in leaf tissue and haustorial complexes in both ^{14}C labelled and non-labelled experiments indicates that it is transferred. The enzyme β-fructofuranosidase is essential for the further metabolism of this compound, particularly for its conversion to sugar alcohols. This is thought to occur by way of fructose or fructose-phosphate precursor for mannitol, (Lewis & Smith, 1967a). In infections by rust fungi there is usually an increase in the activity of invertase in the infected host tissue. Long et al (1975) suggested that this represents cell wall bound invertase which hydrolyses sucrose prior to uptake by the parasite. No similar experiments have been described for a powdery mildew and it was thus important to investigate the activity and location of invertase in the present study and to relate the results to the analyses of ethanol soluble carbohydrate.

i) Experimental procedures

a) Mycelial and haustorial complex fractions.

Mycelial brushings (50 mg fresh weight) and isolated haustorial complexes ($1 - 3 \times 10^6$) were homogenised in a Potter homogeniser at 4° in small volumes of cold distilled water, (2 - 3 ml for the mycelium, and 0.5 ml for the haustorial complexes). The suspensions were then centrifuged at 40,000 g for 20 min at 4° and their supernatants removed and their volumes measured. The pellets were suspended in distilled water to equal the volumes of the supernatants. Samples (0.5 ml) of the 40,000 g supernatants and pellet suspensions were then removed and dried at 80° for 48h to determine the dry weights. The protein content of both fractions were estimated by the Lowry method (Lowry *et al*, 1951). From these estimates direct relationships between protein content and dry weight or number of isolated complexes for the extracts were calculated. The supernatants and suspensions were then dialysed against 1 litre of distilled water at 4° for 24h. For each fraction, three replicate tubes were then set up as follows.

Mycelium	Haustorial complexes
0.2 ml enzyme solution or suspension	0.25 ml enzyme solution or suspension
0.3 ml H_2O	0.25 ml 0.5M sucrose
0.5 ml 0.5M sucrose	0.75 ml 0.1M Phosphate buffer pH 6.8.
1.5 ml 0.1M phosphate buffer pH 6.8.	

The mixtures were incubated at 32° for either 30 min (mycelial extracts) or extended until 3h to improve the sensitivity for haustorial complexes. Control tubes containing boiled enzyme were also included. After the incubation, reducing sugars were estimated by either the dinitrosalycilic acid or Nelson-Somogyi assays. When pH optima were to be estimated the buffer between pH4 and 6 was 0.1M citrate and between 6 and 8, 0.1M phosphate buffer.

b) Leaf fractions.

In order to assay the invertase of the healthy and infected host the methods of Bacon, MacDonald & Knight (1965) and Long et al (1975) were employed. Discs (5mm diameter) were removed from uninfected leaves and infected leaves separated from the mycelium and incubated for 30 min in ice cold ethyl acetate. They were then washed for 30 min in distilled water at 4°. Twenty disc samples were then incubated in 10ml of a mixture containing 0.08M citrate-phosphate buffer at pH values ranging from

4.5 - 7 and 0.1M sucrose. After 3h incubation, 1 ml samples were removed and reducing sugar estimated by the dinitrosalycilic acid assay or Nelson-Somogyi assay. Controls without any discs or without the sucrose substrate were included to estimate the spontaneous hydrolysis of sucrose and the reducing sugar in the discs. Dry weights were determined by blotting and drying discs for 48h at 80°.

ii) Results

a) Mycelial invertase.

The pH profiles of the invertase activity from the 40,000g supernatant and residue from the mycelium are shown in fig. 31. The activity in the supernatant was far higher than that in the pellet. Both fractions had a pH optimum of pH 6.5 - 6.8. Results are quoted as μg glucose equivalents mg protein^{-1} 30 min^{-1} but since 1mg of soluble protein was equivalent to 13.7 mg dry weight of fungus the activities at optimal pH in μg glucose equivalents mg^{-1} dry wt mycelium 30 min^{-1} were 174.4 and 9.5 for the supernatant and residue respectively. Thus 95% of the invertase activity was associated with the supernatant fraction, which strongly suggests an intracellular rather than a wall related location for this enzyme.

The Michaelis constant for the soluble enzyme was estimated from its activity in sucrose concentrations between 10 and 100 mM. From the Lineweaver-Burke plot shown on fig 32, the

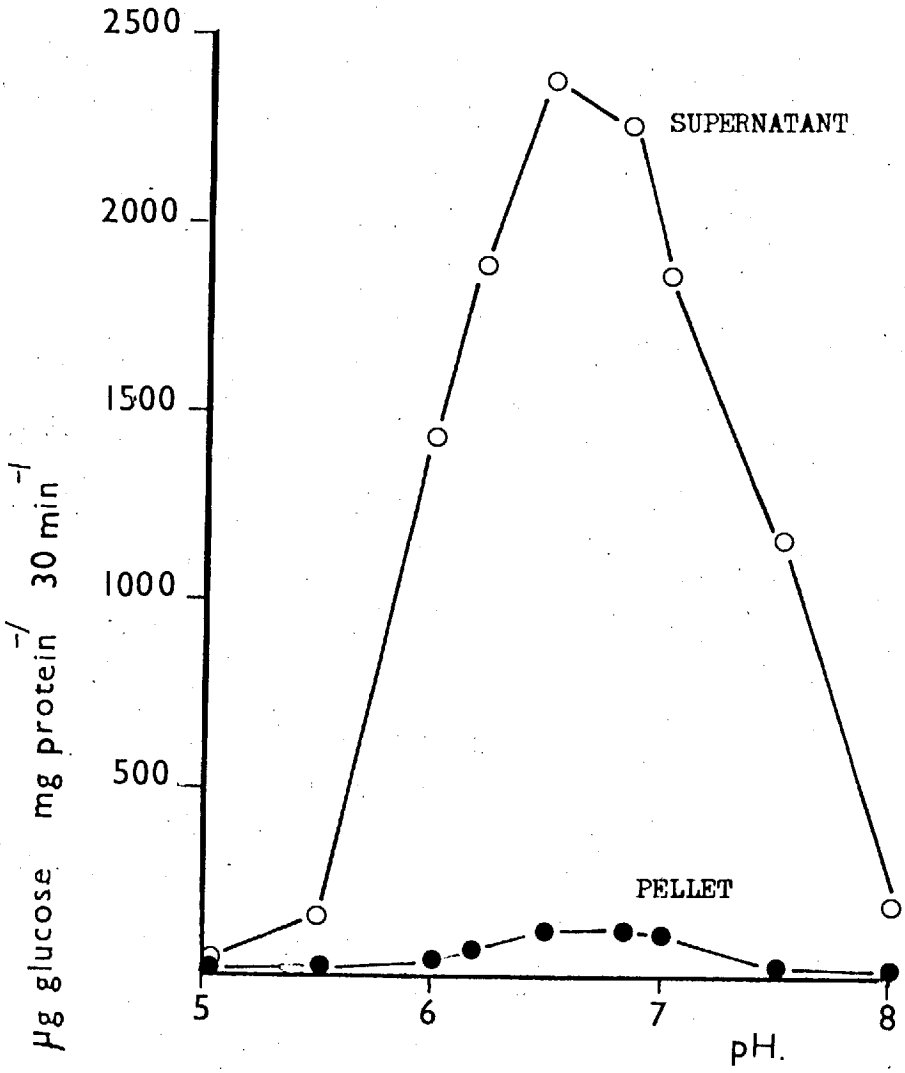


Figure 31. Erysiphe pisi, pH profile of activity of invertase from the mycelium. Two fractions, separated by centrifugation at 40,000 g, were prepared from an homogenate and incubated with sucrose (0.1M) for 30 min at 32°.

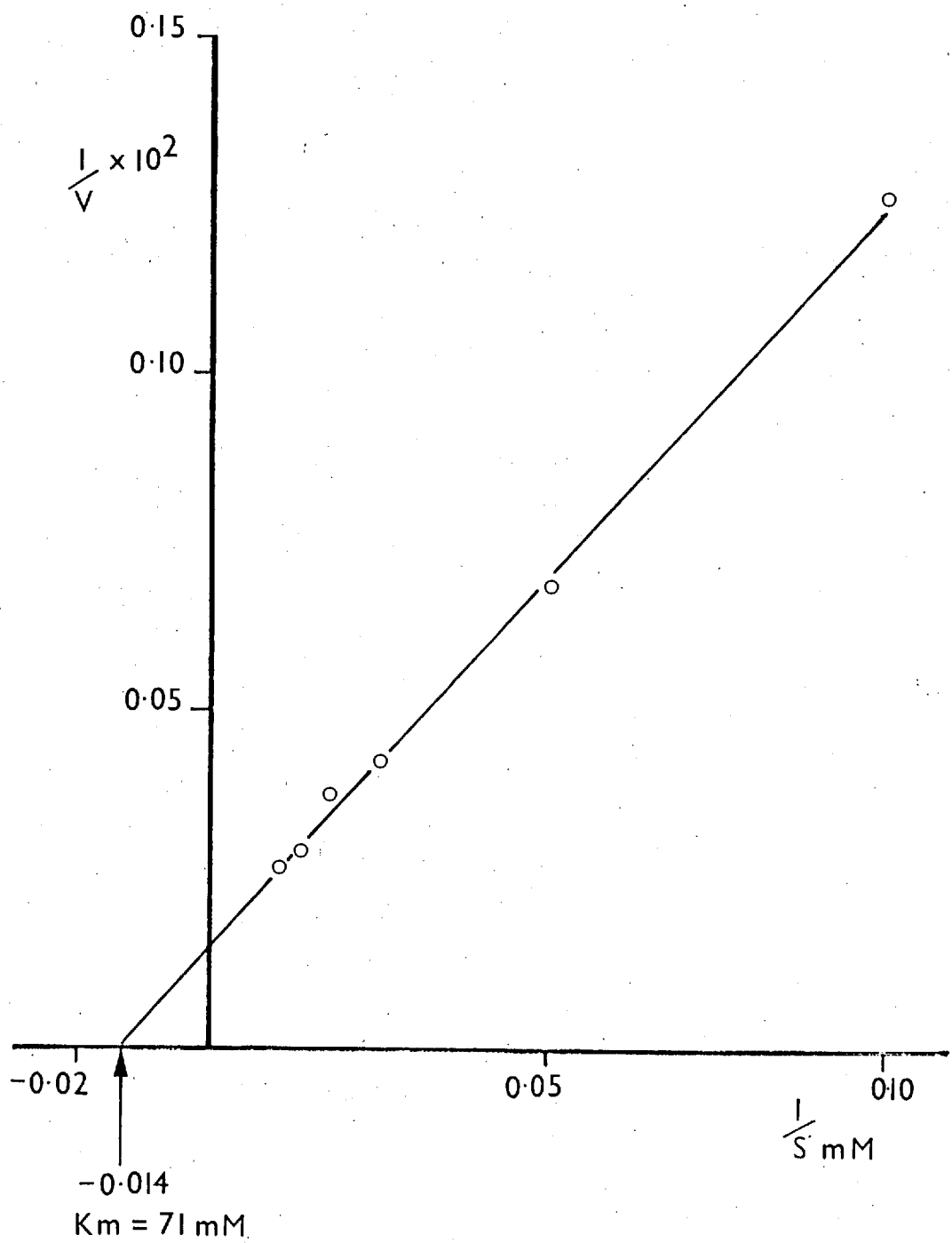


Figure 32. Erysiphe pisi. Lineweaver - Burke plot of soluble invertase activity from the mycelium incubated with sucrose for 30 min at 32°. S= sucrose conc, V= activity as μg reducing sugar mg^{-1} protein $30\ min^{-1}$.

Km was 71 mM. This indicates that the enzyme has a relatively low affinity for sucrose. However this is a greater substrate affinity than for the β-fructofuranosidase enzyme of another parasitic ascomycete Claviceps purporea for which a Km of 138 mM was obtained (Dickerson, 1972).

b) Invertase of haustorial complexes

A comparison was made of the activity of invertase at pH 5 and 6.8 in the mycelium and haustorial complexes and the results are shown on table 33. Invertase activity in haustorial complexes was detected only in the 40,000 g supernatant fraction at pH 6.8. The activity per unit soluble protein was only 11% of that of the equivalent fraction from the mycelium. No activity was obtained in the haustorial complex residue or in either fraction at pH 5. This indicates a much lower invertase activity in the complexes, which may be due to smaller quantities of enzyme or to a larger proportion of other proteins. Haustoria have been shown to have denser cytoplasm with smaller volumes of cytoplasmic vesicles than the mycelium and the larger proportion of protein may effectively dilute the activity in the fraction.

c) Host invertase.

The pH profile of invertase activity obtained from the assay of discs of uninfected and infected leaves separated from the mycelium is shown in fig. 33. The optimal pH was 5 and therefore differed from the mycelial enzyme whose optimal pH was 6.8. The assay procedure employed has not been considered to distinguish

Table 33

Erysiphe pisi - *Pisum sativum*. A comparison of invertase activities in homogenates of mycelia and haustorial complexes incubated with sucrose (100 mM) at 32° for 3h. Values represent μg reducing sugar mg protein $^{-1}$ 30 min^{-1}.

	pH	
Mycelium	6.8	5.0
40,000g-Pellet	130	0.0
-Supernatant	2,401	32.0
Haustorial complexes		
40,000g- Pellet	0.0	0.0
- Supernatant	268	0.0

between the activities of insoluble and soluble invertase thus allowing total activity to be assessed (Long et al, 1975). However, a recent paper by Billett, Billett & Burnett (1977) has criticized this method since their data indicated that only insoluble invertase activity was estimated. No further experiments were carried out here to investigate these contradictions and the results must therefore be interpreted with caution. Lyne & Ap Rees (1971) described an acid invertase in pea root tissue with an optimum of pH 5. Using a similar assay technique they found that both soluble and insoluble invertase was assayed and that most of the invertase (between 50 and 80%) was soluble and cytoplasmically located. Similarly, Maclachlan et al (1970) also found an acid invertase in the epicotyl of pea, and it also was cytoplasmically located. The activities estimated in both these studies were much greater than in this present investigation of mature leaves. Maclachlan et al (1970) for example, obtained activities of 210 μg reducing sugar mg fresh wt^{-1} 3h^{-1} incubation, compared with 12 μg reducing sugar mg dry wt^{-1} 3h^{-1} for healthy host leaves used in the present experiments. These results indicate that invertase levels in the mature leaf are very low when compared with actively developing tissue in pea.

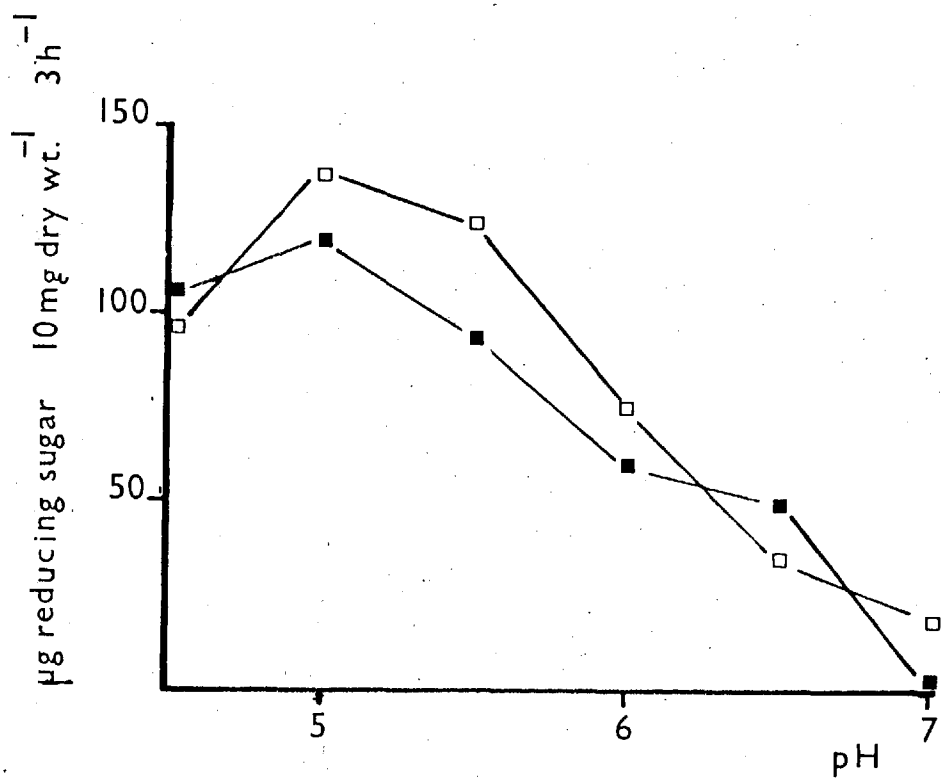


Figure 33. Erysiphe pisi - Pisum sativum. A comparison of invertase activity in healthy (■) and infected (□) leaf discs cleared of mycelia and incubated in sucrose (100 mM) at 32° for 3h.

Figure 33 shows that there were no significant differences between the invertase activities of infected and uninfected leaf discs over a range of pH values although it should be emphasised that host invertase levels were low. In other investigations, infection by biotrophic fungi has brought about large increases in the invertase activity. (Long et al, 1975; Mitchell & Cooke, 1976; Mitchell et al, 1978; Billett et al, 1977). Billett et al (1977) have shown this to be of host origin in Zea mays infected with Ustilago maydis.

A comparison of invertase activities shows that the mycelium is more active than the host. Total mycelial invertase was capable of generating 183.9 μg of glucose equivalents mg^{-1} dry weight of mycelium $30\ min^{-1}$ incubation, whereas the host activity was only 2 μg of reducing sugar mg^{-1} dry weight of infected leaf tissue $30\ min^{-1}$ incubation. Thus infected leaves showed greatly increased activity of invertase but it was located almost exclusively in the mycelium.

B) Pectolytic enzymes in haustorial complexes.

It has been suggested by Gil, (1976) that the host secretes pectic and hemicellulosic cell wall constituents into the extra-haustorial matrix and that these are subsequently hydrolysed by enzymes of fungal origin. Finally the resultant low molecular weight carbohydrates are absorbed by the haustorium. In order to

test part of this hypothesis attempts were made to detect pectolytic enzymes in isolated haustorial complexes.

i) Experimental procedures

Assays for pectolytic enzymes were made only on the isolated haustorial complexes. Extracts were prepared by disintegrating 3-4 x 10^6 haustorial complexes with 0.85% NaCl (0.5 - 1ml) in a Potter homogeniser at 0° (Stevens, 1974). After centrifugation and then dialysis as for the invertase assays, the protein concentration in the final extract was 300-700 μg per ml. Samples of between 0.2 and 0.5 ml were assayed for pectin methyl esterase, polygalacturonase (pH 5.0) and pectintranseliminase (pH9) as described in the methods.

ii) Results

No activity was detected in any of the assays. This may be due to a low concentration of enzyme although assay tubes were incubated for periods of up to 6 hours. It is thus unlikely that any pectinase operates in haustorial complexes.

(C) Glucan synthetase (UDP - glucosyl transferase).

The <u>in vivo</u> $^{14}CO_2$ feeding experiments (Results VI, B, iii, c) indicated that a glucan (not α1-4 linked) is rapidly produced within the complexes. Experiments were thus set up to investigate the glucan synthetase activity of intact and disintegrated complexes. The rationale of using intact as well as disintegrated complexes was to provide the UDP ^{14}C - Glucose

substrate with ready access to the outer surface of the extra-haustorial membrane, whereas in assays using sonicated complexes the substrate would also be exposed to any synthetase enzymes within the haustorium.

i) Experimental procedures

Haustorial complexes (0.1ml, quantities of a suspension containing 2 - 4 x 10^6 haustorial complexes / ml) were each suspended in a 5ml tube and then assayed immediately or sonicated for three periods of 10 sec. The tubes were kept on ice throughout the sonication to prevent heating. Assays for glucan synthetase were then performed with UDP ^{14}C - glucose substrate (312 mCi/mmol sp. act.) at a concentration of 0.4 μM (50,000 dpm/0.2 ml) incubated for 30 min at 25°. Activity was detected by scintillation counting after precipitation of glucans by 70% ethanol and 3 subsequent washes in 70% ethanol at 12,000g.

ii) Results

The glucan synthetic activity of the intact and sonicated complexes is shown on table 34. Activity was greater in the intact complexes. This may be due to direct impairment of activity by the sonication or inhibitory substances released from within the complexes. β-glucan synthetase activity of pea stem segments has been shown to be labile upon tissue homogenisation, (Ray, 1973; Raymond et al 1978). The activities per unit protein were of a similar order to that obtained by Ray(1973)for particulate

Table 34

Erysiphe pisi - Pisum sativum. Glucan synthetase activity in suspensions of intact or sonicated haustorial complexes.

Incorporation of isotope into ethanol insoluble material from 0.4 μM UDP ^{14}C-glucose (312 mCi/mmol) substrate at 25° for 30 min. Each result is the mean of 2 replicate assays.

	pmol ^{14}C-Glucose mg protein^{-1} 30min^{-1}
Intact HC's	12.0
Sonicated HC's	4.2

	pmol ^{14}C-Glucose 10^{-6} HC's 30min^{-1}
Intact HC's	14.8
Sonicated HC's	5.2

β glucan synthetase in elongating pea stem homogenates. He obtained rates of 10 - 50 picomoles mg particulate protein^{-1} 10 min^{-1}. In the present work, assays included both soluble and particulate protein so if the synthetase is all membrane bound, activities equivalent to those in very active plant tissue are present. A more critical attempt to locate and quantify this enzyme activity is required, but these preliminary results indicate significant activity, which is possibly located on the surface of the haustorial complex i.e. on the extrahaustorial membrane. In future work the reaction products should also be analysed to define precisely the enzymic properties of the UDP-glucosyltransferase activity.

IX) In vitro uptake of sugars & other metabolites by haustorial complexes.

Experiments described above have clearly shown that photosynthetic products of host origin will enter the haustorial complex *in vivo*. The isolated haustorial complex permits a determination of the transport properties of the parasitic interface and thus compounds which are thought to traverse the interface *in vivo* can be tested *in vitro*. The surface of the extrahaustorial membrane which is exposed to the ambient medium *in vitro* is the same surface which subtends the cytoplasm of the epidermal cell, *in vivo*. Experiments were thus designed to study the uptake of ^{14}C - labelled sugars by isolated haustorial complexes.

A) Experimental procedures.

The procedure for the study of the uptake of ^{14}C - solutes by isolated haustorial complexes was employed as described in the Methods (VII,A).

Universally ^{14}C labelled glucose (specific activity 130 - 350 mCi/mmol) and sucrose (350-400 mCi/mmol) were used for uptake studies. A suspension of haustorial complexes (5-10 x 10^5 ml) in 0.2 ml of buffer at the required pH. [0.1M phosphate buffer for pH 6-8, 0.1M citrate buffer for pH 4-6 or 0.1M Tris-HCl buffer for pH 8 - 9] . A labelled sugar solution was added (0.1 ml) so that the final buffer concentration was 67 mM and the final sugar concentration between 0.02 and 1 mM. Tubes were incubated at 25° for periods of up to 2h on a shaking waterbath. Samples were removed, washed 3 times in ice cold buffer and the final pellet assayed for protein and radioactivity as described in the methods section.

Uptake of sugars was compared to seven other ^{14}C - labelled compounds which were tested similarly, their specific activities were as follows.

Compound	Specific activity (mCi/mmol)
Ethirimol	25. 1
Ethanolamine	50. 0
Uracil	59. 0
SCN^-	25. 0
Glycine	59. 1
Inulin	9. 8
UDP - ^{14}C-glucose	312. 0

B) Results

i) Kinetics of uptake

When haustorial complexes were suspended in 60 µM glucose or sucrose in 67 mM phosphate buffer pH 6.8 these compounds were detected in the complexes. The kinetics of uptake per unit protein are shown in fig. 34. The uptake rate of glucose exceeded that of sucrose. From the mean result of three independent estimates, in these experiments 50 µg of protein was found to be equivalent to 4.4×10^4 haustorial complexes. The uptake of glucose was characterised further. The effect of different concentrations of glucose on the uptake is shown on fig. 35. Uptake was clearly related to concentration up to a glucose concentration of 1 mM.

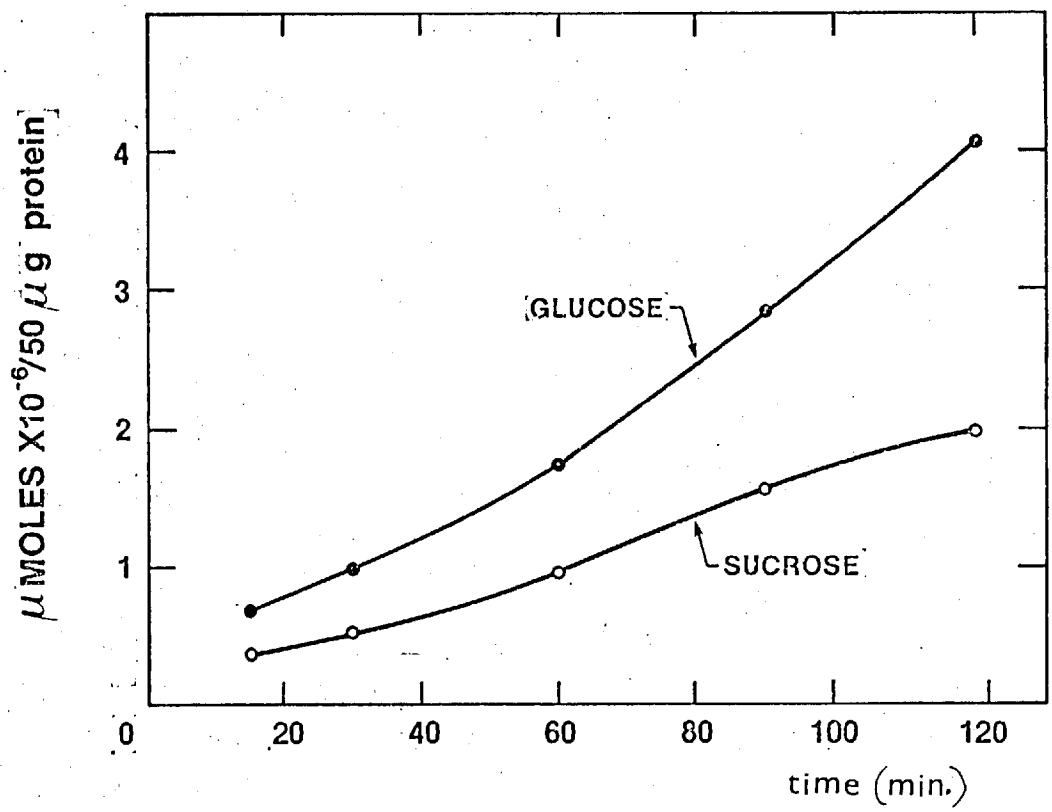


Figure 34. Erysiphe pisi - Pisum sativum. The uptake of glucose and sucrose by the haustorial complex fraction suspended in 60 μM ^{14}C - sugar and 67 mM phosphate buffer at 25°, pH 6.8.

2) Contribution by contamination.

In order to investigate the contribution of microbial or host contaminants to the glucose uptake the haustorial complex isolation procedure was used to obtain a fraction from uninfected leaves and its uptake of ^{14}C-glucose (60μM in 67 mM phosphate buffer pH 6.8) was compared with that of an haustorial complex fraction isolated from brushed infected leaves of equivalent dry weight. The results of the comparison are shown on fig. 36 and indicate that contaminants contributed approximately one half of the uptake measured.

Microscopic examination of both fractions failed to reveal any contamination by host cells. Bacterial contamination of the two fractions after the 2h incubation period was assessed by 1000 fold dilution of 0.2 ml of the isolated fractions with sterile distilled water, and then 0.2 ml quantities were plated on nutrient agar in a plastic petri dish and incubated at 38° for 24h. Colony counts showed no significant difference in the bacterial content of the two fractions and indicated bacterial concentrations of $1.32 \pm 0.51 \times 10^4$ /ml and $1.59 \pm 0.86 \times 10^4$/ml in the haustorial complex and equivalent uninfected leaf fractions respectively. The frequency of bacteria was between 50 - 100 times lower than that of haustorial complexes in the fraction, further their individual dry weights are considerably smaller. Despite this, the small numbers of active microbial contaminants seemed to contribute considerably to the uptake and thus it was concluded that although

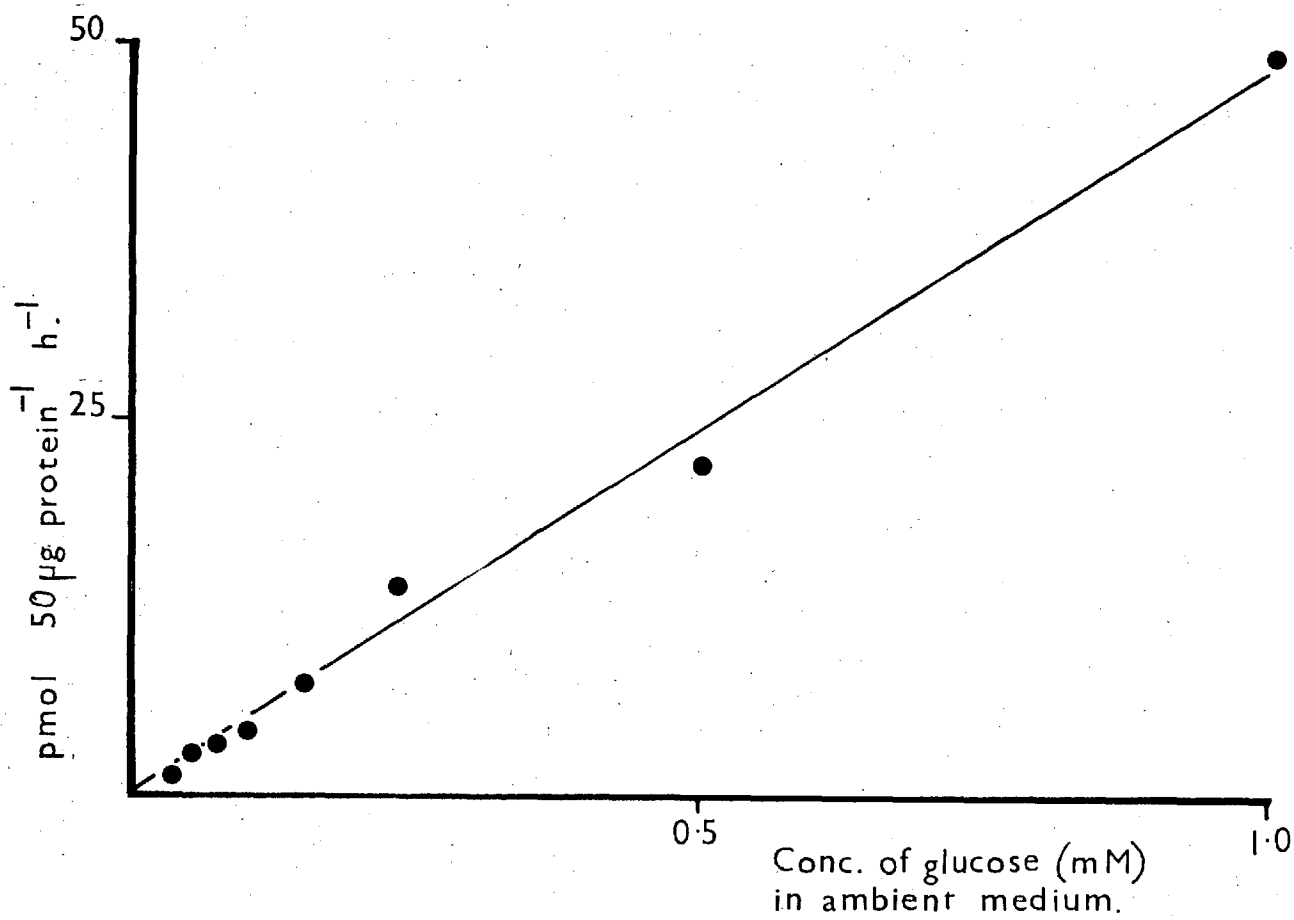


Figure 35. Erysiphe pisi - Pisum sativum. The effect of ambient glucose concentration on the rate of uptake of ^{14}C-glucose by the haustorial complex fraction from a medium containing 67 mM phosphate buffer at pH 6.8 at 25°.

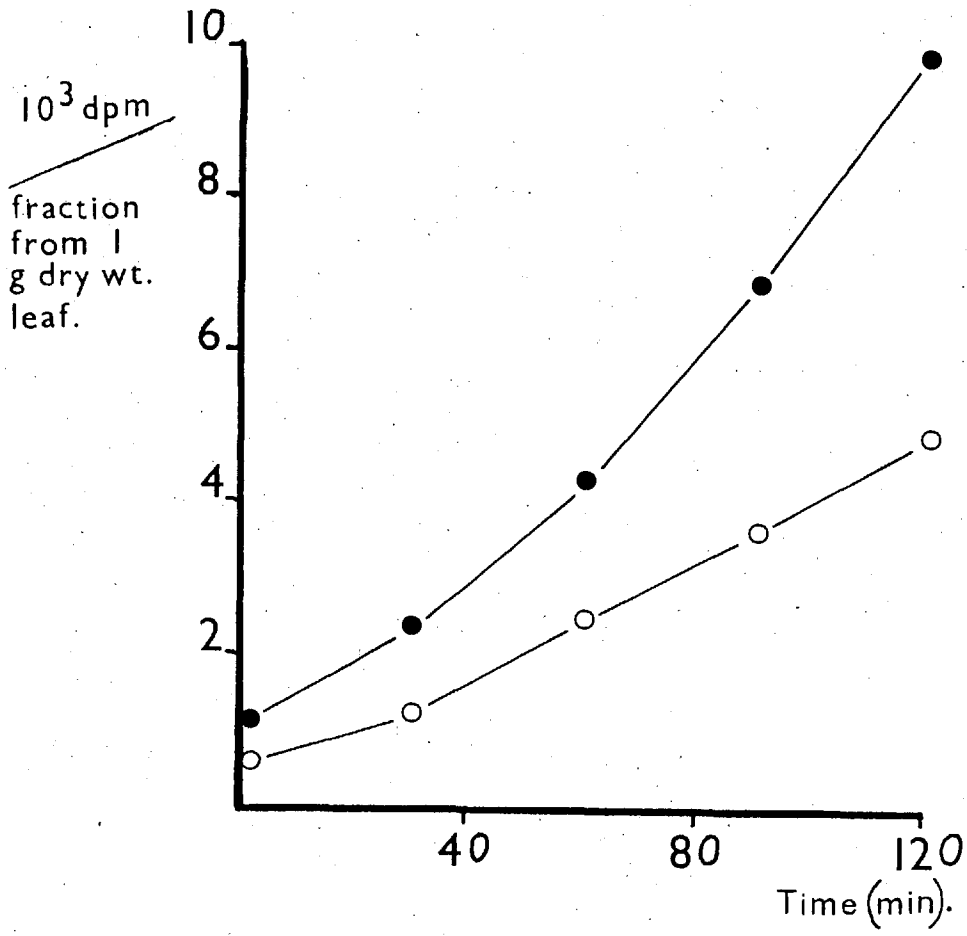


Figure 36. Erysiphe pisi - Pisum sativum. A comparison of glucose uptake by the haustorial complex fraction, (●) and an equivalent fraction isolated from uninfected leaves, (O) in vitro. Similar viable counts of bacteria were obtained for both fractions.

sucrose and glucose may enter the haustorial complexes in vitro the process is extremely slow.

iii) Comparison with yeast

A comparison between uptake of ^{14}C-glucose by haustorial complexes and a yeast suspension was also made. Bakers' yeast (Saccharomyces cerevisiae) was obtained commercially and resuspended in 0.1M phosphate buffer pH 6.5 with 1mM glucose for 30 min at 20°. The cells were then washed three times in buffer and the pellet resuspended (3.5×10^6 yeast cells /ml) in 21 µM ^{14}C-glucose (131 mci/mmol) in 50 mM phosphate buffer pH 6.5 for 40 min at 25°. The uptake was estimated as for haustorial complexes which were also tested. During 40 min periods the yeasts absorbed 122,030 dpm/50 µg protein and the haustorial complexes only 1,735 dpm /50 µg protein. This 70 fold greater uptake indicates that any contaminant yeast cells present in a haustorial complex fraction would substantially contribute to the measured uptake.

iv) Uptake of glucose and sucrose by isolated haustorial complexes compared with uptake of other compounds.

The uptake experiments used for glucose and sucrose were extended using seven other radio labelled compounds and results are given in table 35. Inulin (Mol wt 5,000) and UDP glucose which are unlikely to permeate cell membranes did not enter haustorial complexes at rates different from that for sucrose. These results are consistent with the haustorial complex swelling experiments by

Table 35. Comparison of uptake of nine compounds by isolated haustorial complexes of Erysiphe pisi - Pisum sativum. Concentrations (n moles/2 x 10^5 haustorial complexes) after 15 min incubation in test compound (40 μM) in phosphate buffer (50 mM) at 25°C.

Compound	Concentration
Ethirimol	0.1312
Ethanolamine	0.0161
Uracil	0.0058
$S^{14}CN^-$	0.0051
Glucose	0.0034
Glycine	0.0031
Sucrose	0.0023
Inulin	0.0022
Uridine diphosphate glucose	0.0018

Gil & Gay (1977) who concluded that the extrahaustorial membrane was differentially permeable to sucrose solutions. Glucose influx was only slightly faster than those of inulin or UDP glucose but as approximately 50% of the glucose uptake can be attributed to the activity of contaminants it is unlikely that much weight can be placed on these differences. Substantial uptake was shown by more lipid soluble compounds e.g. ethanolamine and ethirimol. Collander (1954) has shown that the diffusion of non electrolytes into Nitella cells is related to their lipid solubilities.

v) Effect of dinitrophenol, Triton X-100, pH, sequential sonication and heat treatment on the uptake of glucose by haustorial complexes in vitro.

Isolated haustorial complexes were subjected to the following pretreatments prior to estimation of uptake.

1) Washing for 1 min with solutions containing 0.01 - 10% (v/v) Triton X-100, at 4°.

2) Incubation for 20 min in 10^{-4} - 10^{-3} M dinitrophenol (DNP) at 25°.

3) Sonication at 4° for 2 - 40 sec.

4) Heating at 100° for 10 mins.

The complexes were then washed twice by centrifugation at 4° in 0.1M phosphate buffer and the uptake of ^{14}C-glucose compared with that of untreated controls, (table 36). The effect of pH on the uptake of untreated fractions was estimated by substitution of a

buffer of the required pH for the usual phosphate buffer pH 6.8. The results are also shown in table 36.

Triton X-100 and DNP had little effect on the uptake of glucose reducing it by only 10 - 20% and 15 - 18% respectively. The first result is consistent with the stability of the extra-haustorial membrane in detergents (Gil & Gay, 1977). Further gram negative bacteria, which may comprise some of the contaminants are resistant to such detergent treatments (Nixdorff et al, 1978). The small effect (15-20% inhibition) of DNP is consistent with diffusive uptake by the complexes and a proportionally larger inhibition of the microbial contaminants.

The pH profile (table 36) shows no particular optimum and uptake was reduced significantly only in the more acid solutions, (pH 4 and 4.5). This relative insensitivity to pH difference is also consistent with uptake being independent of physiological processes. Although a more specific optima might be expected from what is probably an active uptake by contaminant microbes, this component may be masked by a passive uptake by the haustorial complexes or due to the physiological heterogeneity of the contaminant population.

The contribution by contaminants was also confirmed by sonication of the haustorial complex fraction. After 40 seconds sonication, when all the haustorial complexes were disrupted 50% of the uptake activity remained. All the activity was removed by

Table 36.

Erysiphe pisi - Pisum sativum. The effect of detergent, dinitrophenol, pH, sequential sonication, and boiling treatments on the in vitro uptake of glucose by the haustorial complex fraction.

Uptake values are given as the percentages of that for an untreated fraction after incubation in ^{14}C-glucose (20 μM), pH 6.5, 0.05M phosphate buffer, 25° for 40 min.

Treatment of haustorial complex fraction	uptake (%)
10% Triton X-100	84
1.0% " "	92
0.1% " "	79
0.01% " "	83
10^{-4}M dinitrophenol	85
10^{-3}M "	82
pH 4.0	75
4.5	79
5.0	114
6.0	105
7.0	100
8.0	104
8.5	111
9.0	95
2 sec sonication	100
5 " "	71
10 " "	61
20 " "	65
30 " "	64
40 " "	50
100° for 10 min	?

heating at 100° for 10 min and this also is consistent with the non-haustorial complex uptake being due to small microorganisms and probably bacteria.

In summary the experiments investigating the uptake of sugars by haustorial complexes indicate that glucose and sucrose are not actively absorbed by the haustorial complexes although they may penetrate by diffusion. Since this is a slow process the few active microbes in the fraction contribute disproportionately to the results and confuse any attempts to quantify uptake by the complexes *in vitro*. These results have also recently been confirmed by light microscope autoradiography (P. Spencer-Phillips personal communication). It has been demonstrated that microbial contaminants, particularly those of fungal origin, are heavily labelled *in vitro*. Haustorial complexes are also labelled but only slightly above background, indicating some incorporation.

The contribution of contaminants similarly confused experiments by Keen *et al* (1969) with isolated plasmodia of *Plasmodiophora brassicae*. Although the fractions incorporated substrates into protein and lipid the bacteria present (1 - 5 bacteria to each plasmodium) may have been entirely responsible and thus the *in vitro* activity of their isolated cells was questionable.

X) Oxygen uptake by isolated haustorial complexes

Previous experiments using tetrazolium dyes have demonstrated that oxido-reductive metabolic processes occur in isolated haustorial complexes of Sphaerotheca fuliginea - Cucuminis sativus (Dekhuijzen, 1966), Erysiphe polygoni - Beta vulgaris, E. pisi - Pisum sativum, E. graminis - Hordeum vulgare (Results section I, B, i, ii, v). Experiments were designed to quantify the respiratory uptake and reduction of molecular oxygen by isolated complexes of E. pisi - P. sativum and to test the effect of exogenously supplied substrates and an antimildew fungicide.

A) Experimental procedures.

Haustorial complexes ($2 - 4 \times 10^6$) were isolated and suspended in either 0.5 ml of 0.1M phosphate buffer pH 6.8, or in the buffer with the substrates, 0.05 - 0.4M glucose, 0.4M sucrose, 0.4M mannitol in a Clark oxygen electrode chamber and stirred at 25°. After 20 min oxygen uptake measurements were begun and continued for a period 15 min.

The effect of the systemic pyrimidine fungicide ethirimol (I.C.I. Ltd) was studied by preliminary incubation of complexes in 5-50 ppm ethirimol in 50mM phosphate buffer for 30 min at 25° and the oxygen uptake was measured for a further 3h. The oxygen uptake of a yeast suspension was measured under identical conditions.

At the end of each estimation the haustorial complex or cell concentration was estimated.

B) Results:

The results (table 37) show that isolated haustorial complexes took up oxygen at a rate of 2.71×10^{-13} mmol O_2 haustorial complex^{-1} min^{-1}. The rate was unaffected by incubation in 0.05, 0.1, 0.4M glucose, 0.4M sucrose or 0.4M mannitol. Similar results were obtained by Dekhuijzen (1966) from whose data it is calculated that for Sphaerotheca fuliginea - Cucumis sativus haustorial complexes the uptake rate was 1.8×10^{-13} mmol O_2 haustorial complex^{-1} min^{-1}.

Based on numbers of individuals the yeast cells took up oxygen at only one fifth the rate of the haustorial complexes in phosphate buffer, but in 0.4M glucose the rate was almost three times that for haustorial complexes. These experiments show clearly that isolated haustorial complexes have significant respiratory activity which is of the same order of magnitude as that of a yeast cell. Dekhuijzen (1966) showed that, on a dry weight basis the respiration rate of the isolated haustorial complexes of S. fuliginea - C. sativus exceeded that of the mycelium, but was less than that of the spores. Dekhuijzen's haustorial complexes were from older infections than those isolated here, (10 day as opposed to 7 day infections) and were subjected to detergent and sonication treatment during the isolation. These factors may explain the slightly lower

respiration rates observed.

The rate of respiration of isolated haustorial complexes is comparable with that of other infection structures isolated from biotrophic fungal associations with higher plants. From the data of Keen et al (1969) a rate of 10^{-15} mmol O_2 plasmodium^{-1} min^{-1} is calculated for plasmodia isolated mechanically from clubroot galls. This rate was of the same order as that of hyphae of Uromyces phaseoli isolated from infected french bean leaves (Dekhuijzen et al, 1967) and much lower (100 fold) than rates observed for haustorial complexes of S. fuliginea - Cucuminis sativus and E. pisi - Pisum sativum. However, Dekhuijzen (1975) observed much higher rates of respiration from plasmodia of Plasmodiophora brassicae isolated both mechanically and enzymatically from turnip callus tissue (10^{-12} and 2.7×10^{-11} mmol O_2 plasmodium^{-1} min^{-1} respectively). The differences (approximately 1,000 fold) in these rates and those of Keen et al (1969) are impossible to interpret, but these intracellular biotrophic structures respire at a rate 10 - 100 times that of powdery mildew haustorial complexes from infected pea and cucumber.

It is also significant that the oxygen uptake of the other isolated infection structures were also unaffected by exogenous substrates (Dekhuijzen et al 1967; Dekhuijzen 1975). With isolated haustorial complexes of S. fuliginea - C. sativus Dekhuijzen (1966) used pyruvate, succinate and acetate in addition to the sucrose,

Table 37.

Erysiphe pisi - Pisum sativum. Comparison of oxygen uptake rates of suspensions of isolated haustorial complexes and a baker's yeast at 25° in phosphate buffer 0.1M, pH 6.8 with carbohydrate additions. Each value (mmol O_2 haustorial complex $^{-1}$ or yeast cell $^{-1}$ min^{-1}) is the mean of two separate experiments.

Incubation medium. Based on phosphate buffer (0.1M pH 6.8).	Haustorial Complex Fraction			Yeast
	Immediately after extraction	20h after extraction	44h after extraction	
No addition	2.71×10^{-13}	2.24×10^{-13}	2.44×10^{-13}	6.19×10^{-14}
0.05M glucose	-	2.82×10^{-13}	-	4.34×10^{-13}
0.1M glucose	-	2.65×10^{-13}	-	6.52×10^{-13}
0.4M glucose	-	2.34×10^{-13}	-	6.91×10^{-13}
0.4M mannitol	-	3.10×10^{-13}	2.9×10^{-13}	-
0.4M sucrose	-	-	2.88×10^{-13}	-

glucose and mannitol used in the present work. The reason for the insensitivity may be due to a low uptake rate, as suggested by the in vitro uptake experiments described above or to high concentrations of endogenous nutrients. The latter is unlikely as the G.L.C. analysis has showed that the concentration of sugars per unit dry weight of haustorial complexes is lower than in the host or mycelium.

When haustorial complexes were incubated in 5, 10 and 50 ppm ethirimol there was no decrease in the respiration rate of the haustorial complexes (table 38). This indicates no effect of ethirimol on the uptake of oxygen by haustorial complexes at concentrations which would greatly exceed those likely to be present in the leaf (usually between 1 - 5 ppm, K. Bent personal communication). Bent (1970) showed that 50 ppm is sufficient to completely suppress spore germination of barley mildew spores. Fisher (1977) found that similar concentrations of ethirimol (20 ppm) increased the oxygen uptake of Actinomucor elegans (a zygomyceteous fungus). He also observed an increase in the respiratory quotient and ethanol production and suggested that this compound altered metabolism in favour of fermentative pathways but did not decrease oxygen uptake. However this fungicide is specific to powdery mildews and an action on a general metabolic process such as respiration, is unlikely.

Table 38

Erysiphe pisi - Pisum sativum. Effect of ethirimol on the endogenous respiration rate of isolated haustorial complexes at 25°, in phosphate buffer 0.1M, pH 6.8. Each value is the result of a single experiment.

Incubation medium based on 0.1 M phosphate buffer at pH 6.8.	Oxygen uptake 10^{-13} mmol O_2 HC^{-1} min^{-1}
No addition	3.4
5 ppm ethirimol	3.2
10 ppm "	3.5
50 ppm "	3.8

XI) The influx of ethirimol into isolated haustorial complexes.

A) Experimental Procedures

The method described for the uptake of ^{14}C-solutes by isolated haustorial complexes (Methods VII,A) was directly adapted. The incubation medium consisted of ^{14}C- ethirimol (25.1 mCi/mmol, 5-400 μM , supplied by I.C.I. Ltd, Plant Protection Division, Jealotts Hill Research Station) 50mM phosphate buffer at pH 6.8 (except where indicated), 2% methanol (in order to solubilise the ethirimol) with between 5 - 10 x 10^5 haustorial complexes per millilitre. This was incubated in a shaking waterbath at 25° for periods up to 23h and samples (usually 0.2ml) removed at intervals. The haustorial complexes were quickly separated from the medium and washed three times in 4ml of cold buffer by centrifugation at 3,000g at 4°C. The final pellet was resuspended in 1ml of 95% methanol, sonicated for 1 min and the radioactivity determined in 8ml of scintillant mixture S2.

To study the efflux of ^{14}C - ethirimol, haustorial complexes were incubated for 2h in 20 μM ethirimol, separated from the medium and washed, all as described above. The final fraction was resuspended in 1 ml of 0.1M phosphate buffer at pH 6.8 in a millipore filtration tower (25ml) with a .22 μm pore size filter. Phosphate buffer (room temperature) was then allowed to drip into the tower maintaining a constant volume of 1ml

above the filter. The eluate was collected and sampled for estimation of radioactivity at intervals over 3h. The millipore filter was then dropped into 1ml of 95% methanol and left for 1h when 8 ml of S2 scintillant mixture was added and the radioactivity remaining on the filter estimated.

To investigate the possibility that ethirimol was metabolised, haustorial complexes were incubated in 40 μM ^{14}C-ethirimol for 2h and separated from the medium and washed as described in other ethirimol experiments. Then it was separated into methanol soluble and insoluble fractions by centrifugation at 40,000 g, followed by 3 centrifugation washes in 4ml of 95% methanol. Radioactivities of the pellets and supernatants were then estimated. The supernatants were reduced to small volumes at 35° under vacuum and spotted onto a 200 x 200mm T.L.C. plate (silica base used for lipid chromatography, see Methods, X.G,). This was developed in one dimension in solvent chloroform; methanol; ethyl acetate (6:3:1). This procedure has been shown to separate most of the metabolites of bupirimate (5 - butyl - 2 - ethylamino - 6 - methyl pyrimidin - 4 - yl dimethylsulphamate, I.C.I. Ltd) and dimethirimol (5 - n - butyl - 2 - dimethylamino - 4 - hydroxy - 6 - methyl pyrimidine, I.C.I. Ltd) both of which are close analogues of ethirimol, in extracts from apple leaves (Teal & Cavell, 1974). The chromatogram was then cut parallel to the solvent front into 10 mm strips which were immersed in 10 ml of scintillant mixture S2 for estimation of radioactivity.

B) Results

i) Kinetics of influx of ethirimol into haustorial complexes.

The kinetics of uptake of ^{14}C- ethirimol from a medium containing 40 μM ethirimol is shown on Fig. 37. Uptake was rapid and equilibrium was reached after approximately 30 min when there was no change in the quantity of ethirimol absorbed during a further 23h (fig. 38). Similar uptake kinetics of ethirimol were obtained by Peterson & Edgington (1976) for the influx into potato tuber cells; equilibrium was reached in their system after 30 minutes. If after 3h in 40 μM ^{14}C- ethirimol (25 mCi/mmol sp. activity) unlabelled ethirimol was added to increase the external concentration to 100 μM but thereby decreasing the specific activity to 5 mCi/mmol a rapid efflux of ^{14}C ethirimol occurred. This indicates that ethirimol taken up by haustorial complexes was relatively freely exchangeable with that in the ambient medium. The uptake observed is unlikely to represent binding of ethirimol as such a physical process would reach equilibrium more quickly.

ii) Contribution of contaminants to result of influx of ethirimol into haustorial complexes.

The contribution of contaminants to the results was estimated by a method similar to that of ^{14}C - glucose uptake. A fraction was isolated from 50 g fresh weight of uninfected and brushed infected leaves and the uptake from a solution containing

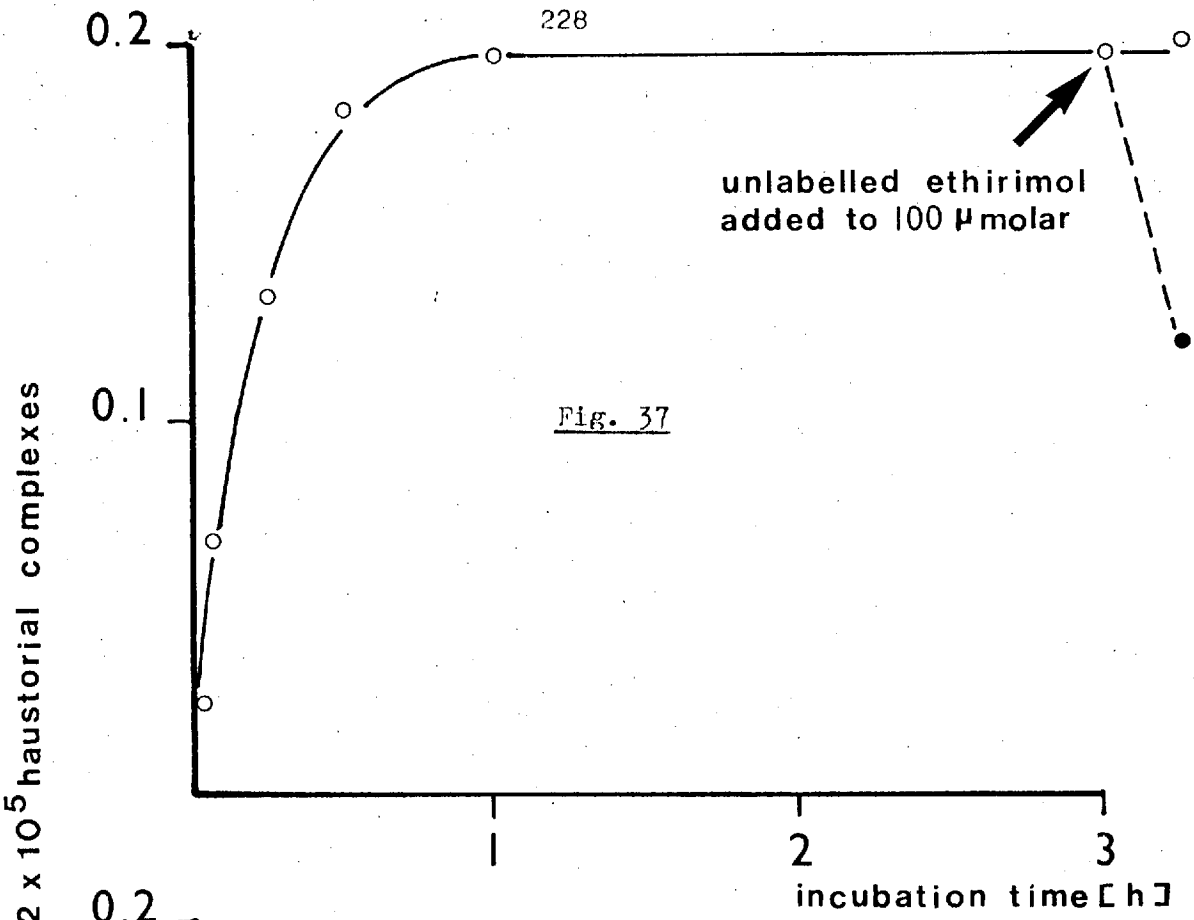


Fig. 37

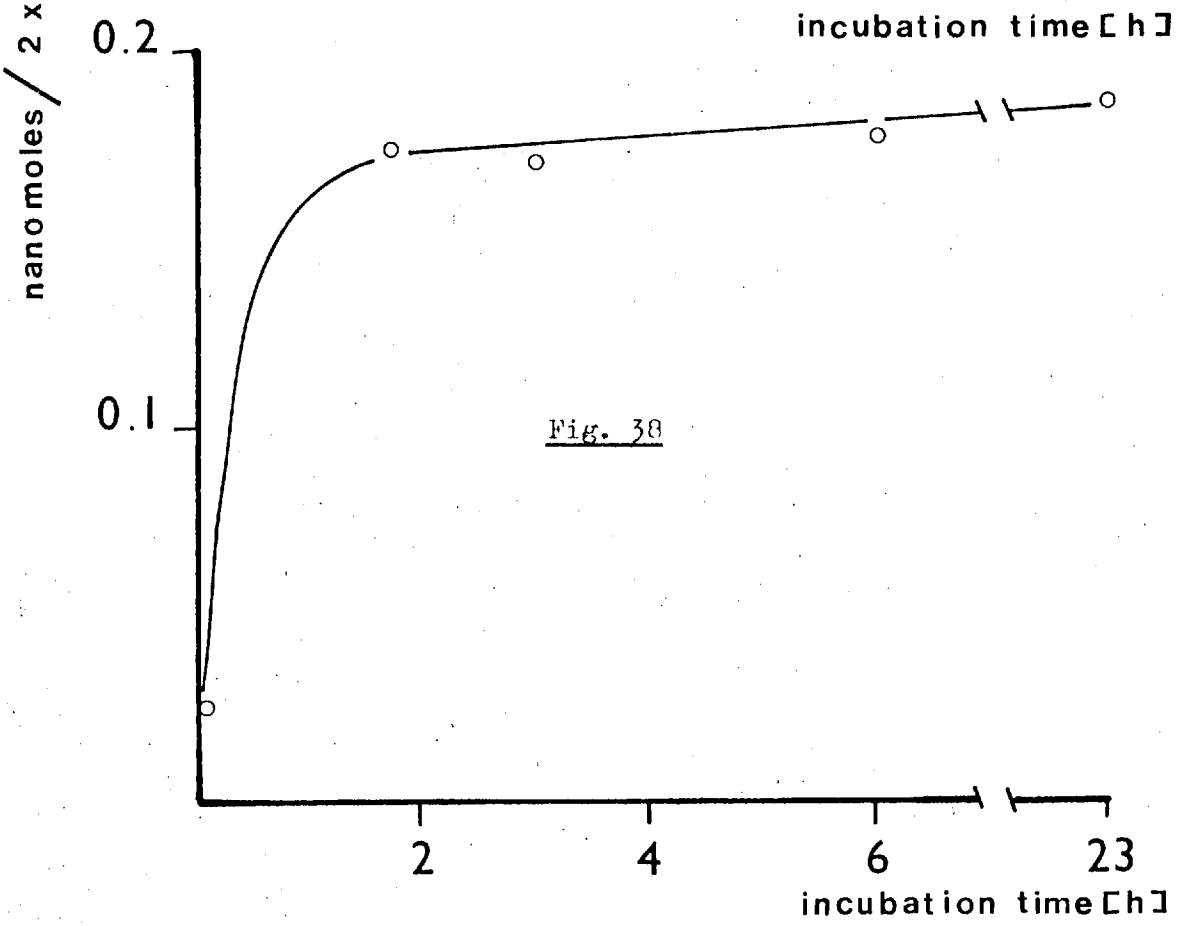


Fig. 38

Figures 37 and 38. Erysiphe pisi - Pisum sativum. Uptake of ^{14}C - ethirimol by isolated haustorial complexes in a medium containing 40 µM ^{14}C - ethirimol, 50 mM phosphate buffer pH 6.8 and 2% methanol at 25°.

50 μM ethirimol was measured after incubation for 1h at 25° at pH 6.8. The control fraction incorporated 0.065 nanomoles of ethirimol and the haustorial complex fraction incorporated 1.45 nanomoles. This indicates that the contribution by microbial or host contaminants to the uptake result is minimal. This contrasts with the results for uptake by sugars by haustorial complexes *in vitro*.

iii) Influx of ethirimol into haustorial complexes in various concentrations of ethirimol.

The quantity of ethirimol absorbed by haustorial complexes after incubation for 1h in concentrations ranging from 40 - 400 μM is shown in fig. 39. There is a direct relationship between the quantity of radioactivity in the complexes at equilibrium and that in the external solution. A second experiment was set up to study this relationship over the lower concentration range of 0.4 - 40 μM. The results (fig. 40) show a non-linear relationship for 0.4 μM where a slightly larger quantity than expected was absorbed. This may have been due to some binding which was more readily detected at lower concentrations where the quantity of ethirimol permeating into the complexes is reduced relative to that which binds.

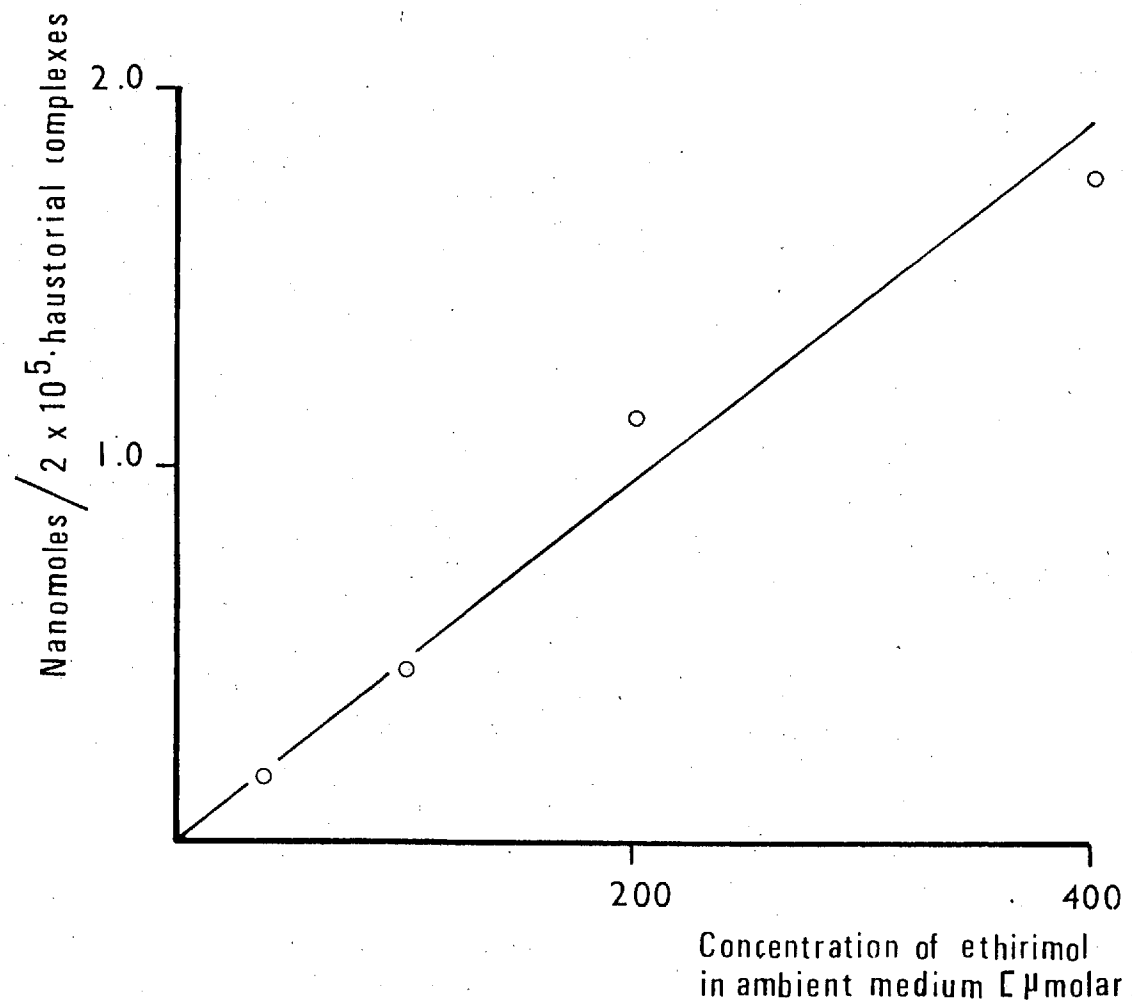


Figure 39. Erysiphe pisi - Pisum sativum. Effect of ethirimol concentration on its uptake by isolated haustorial complexes incubated at 25° for 1h in 67 mM phosphate buffer pH 6.8 and 2% methanol for all ethirimol concentrations except 400 μM where 5% was used.

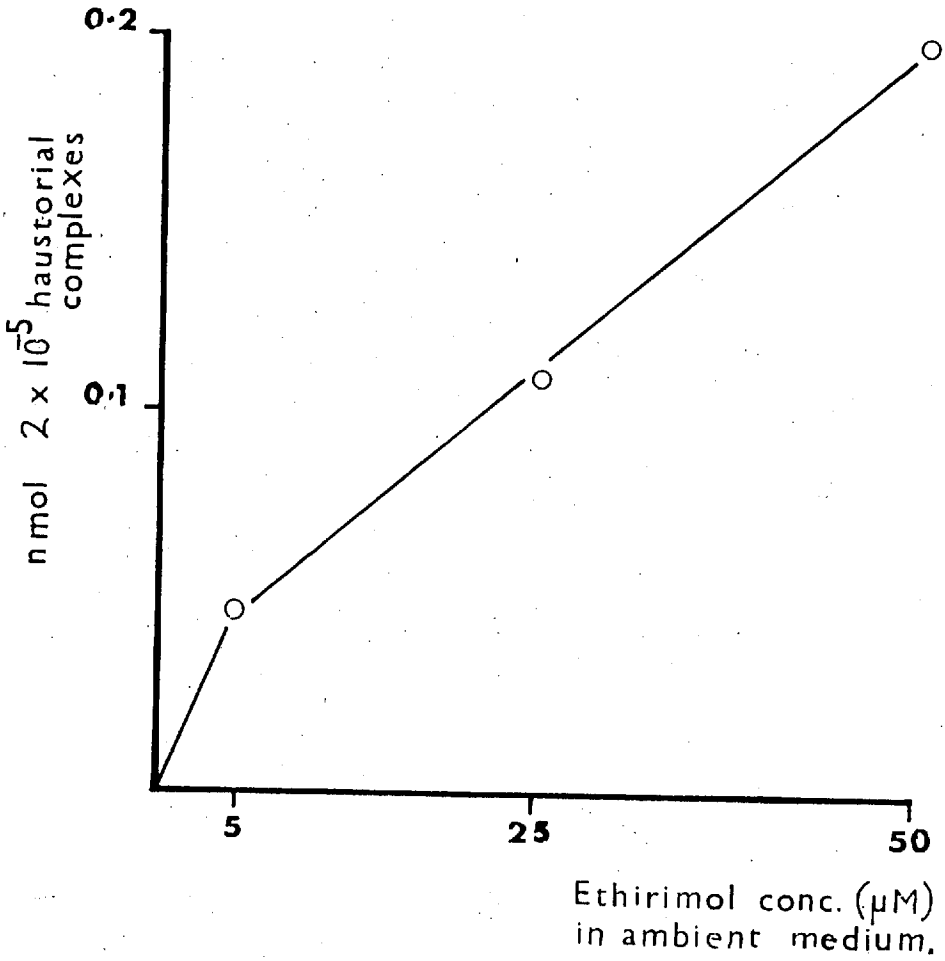


Figure 40. Erysiphe pisi - Pisum sativum. Effect of ethirimol concentration on its uptake by isolated haustorial complexes incubated at 25° for 1h in 67 mM phosphate buffer pH 6.8 and 2% methanol.

iv) The efflux of ethirimol from haustorial complexes.

The kinetics of efflux of ethirimol from haustorial complexes which had been previously loaded with ^{14}C-ethirimol by incubation in a 20 μM solution for 2h as described previously, is shown in fig. 41. Ethirimol is rapidly lost from the complexes, one third of the total being eluted after only 5 minutes. However, thereafter the efflux slowed and after 1 and 3h one third and one sixth respectively remained in the haustorial complexes. It is instructive to compare the efflux kinetics with those of influx where equilibrium was reached in 30 minutes. The efflux kinetics from haustorial complexes also contrasts that with potato tuber cells where for total influx and efflux both periods were 30 min (Peterson & Edgington, 1976). Efflux from these cells closely resembled that from dead tissue and was therefore a process of diffusion. The efflux from haustorial complexes is clearly a restricted process and more closely resembles the elution from apple roots where ethirimol was accumulated and released only slowly unless the tissue was killed (Shephard, 1973).

Analysis of efflux kinetics:

The kinetics of ion efflux from plant cells and tissues is routinely used to study intracellular-ion compartmentation, (MacRobbie, 1971; Walker & Pitman, 1975).

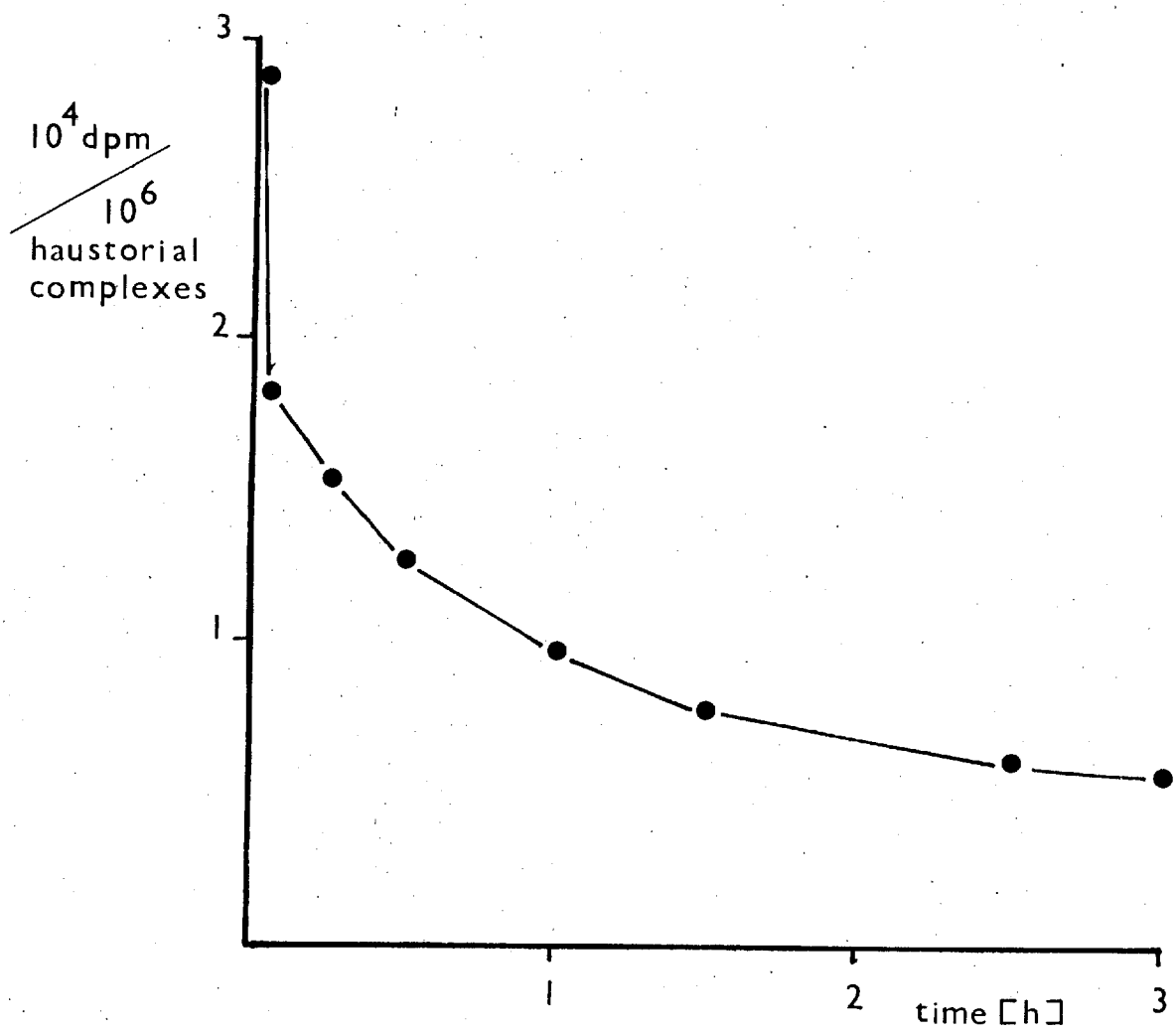


Figure 41. Erysiphe pisi - Pisum sativum. Efflux of ^{14}C - ethirimol from isolated haustorial complexes. Complexes were incubated in 20 µM ethirimol, 67 mM phosphate buffer at pH 6.8 and 2% methanol for 2h at 25°. Samples were first washed by centrifugation at 4° and then continually washed on a millipore filter in phosphate buffer pH 6.8 at 25°. Each point represents the mean from two experiments.

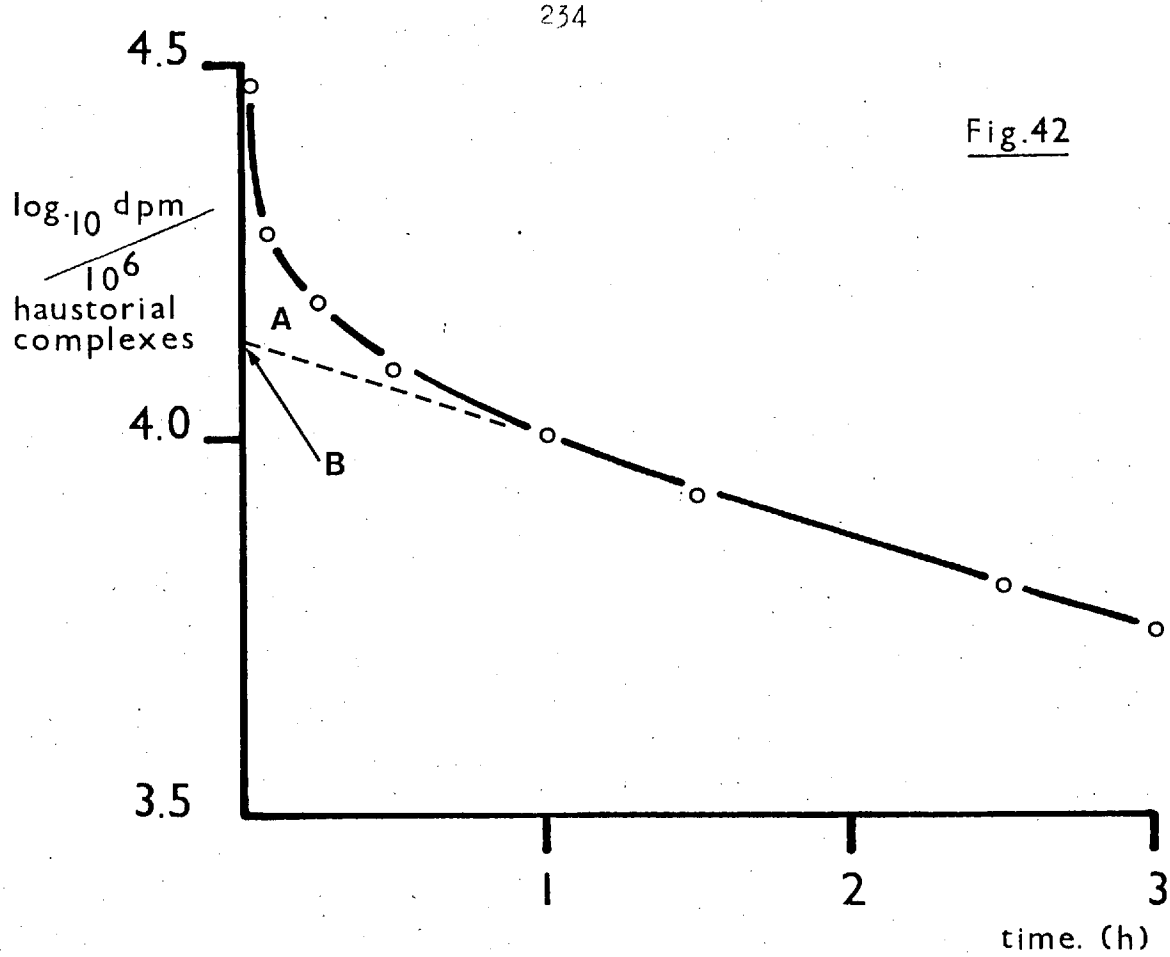


Fig.42

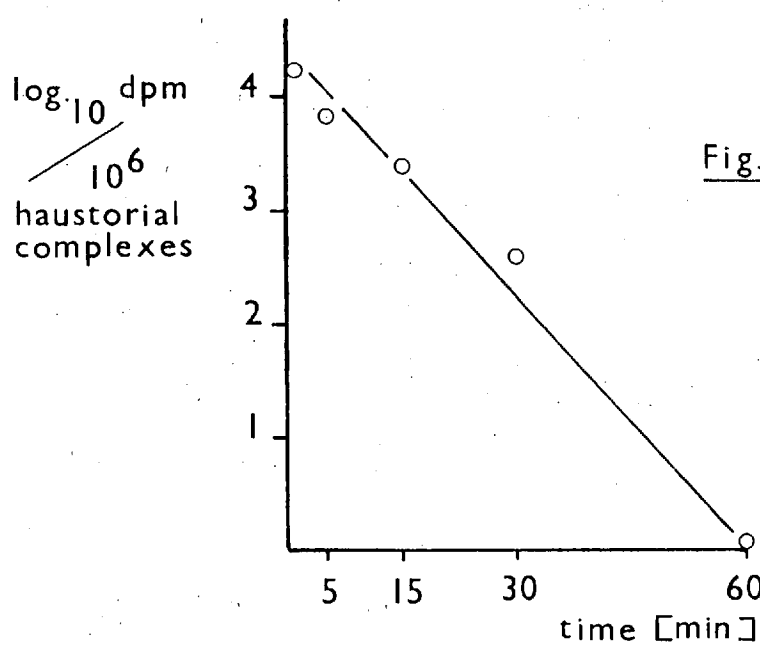


Fig.43

Figures 42 and 43. Erysiphe pisi - Pisum sativum. Efflux of ^{14}C - ethirimol from isolated haustorial complexes. The data used in fig. 41 is plotted semi-logarithmically in fig. 42. The area A is replotted in fig. 43 by subtracting the anti-logs of the extrapolated linear component (--------) of fig. 42 from the experimental data obtained during the first hour of efflux.

In these systems the kinetics commonly follow a course indicating three compartments exchanging in series. These are described as the vacuole, cytoplasm and ambient medium which is in continuum with the free space (apoplast) (MacRobbie, 1971). Pitman (1963) has shown that exchange between each of these compartments has a single exponential time course and can thus be described by a semi-logarithmic plot of the compartmental concentration against time, and efflux measurements are preferred because the concentration in the ambient compartment (zero) can be exactly defined (Walker & Pitman, 1975). Such a plot for ethirimol is presented on fig. 42. Exponential loss is obtained only after one hour and when extrapolated the intercept at B represents the concentration of ethirimol in this more slowly exchanging compartment in the complexes prior to efflux treatment. This constituted 30% of the total ethirimol absorbed.

The component responsible for the greater efflux during the first 60 minutes is further resolved by subtracting the slower component from the total efflux and replotting the result. This represents area A on fig. 42 and is shown on fig. 43. A second linear relationship is obtained and thus, throughout the whole period of efflux, ethirimol was released from two distinct compartments within the complexes. The compound was distributed in the compartments in the ratio of 7:3 with the larger proportion freely exchangeable with an ambient medium after only 1 hour. Correction for free space is not necessary as the washings made by centrifugation

would have removed all the ambient label and the single cell system used here is not subject to a diffusion component arising from the free space which is encountered in ion efflux experiments with tissues of higher plants (Walker & Pitman, 1975).

The conclusion that ethirimol entered more than one compartment within the complex provides good evidence that the compound had entered the haustorial cytoplasm and was not restricted to the extrahaustorial matrix.

v) Effect of pH on ethirimol uptake

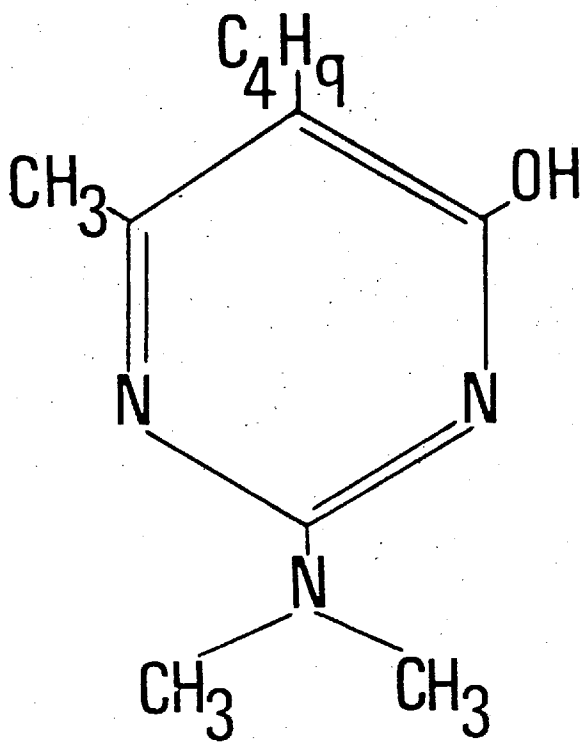
Ethirimol has a pK value of 4.8 (Shone & Wood, 1974) and its lipid solubility is decreased appreciably at pH values lower than this, (Shone & Wood, 1974). Since Collander (1954) showed a relation between the permeability of non-electrolytes and their lipid solubility, uptake of (40 μM) ethirimol was studied at pH 4.2 and pH 6.2, (both in 50mM citric acid buffer). In this experiment, there was no significant difference between the uptake measured at the two pH values after 15 min incubation at 25°. The results for 2 x 10^5 haustorial complexes were 7,397 dpm at pH 4.2 and 6,975 at pH 6.2. This indicates that lipid solubility is not important for uptake. However, it may be that at pH 4.2 the pH within the complexes was sufficient to lower the ionization of ethirimol and thereby permit progressive accumulation. Shone & Wood (1974) found that in barley roots ethirimol was accumulated to different extents at pH 4 and 6, but since the concentration was

higher at pH 4 it seems unlikely that the property of lipid solubility was an important factor. It should be noted that Shone & Wood (1974) considered ethirimol to be confined within the free space.

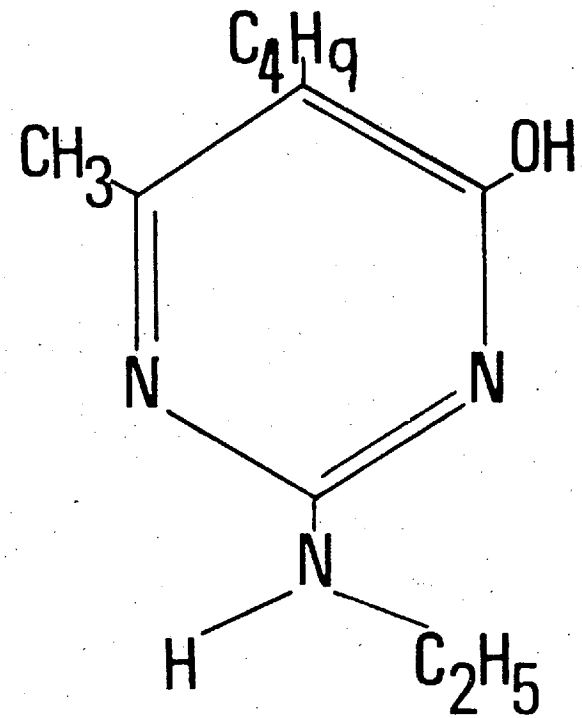
vi) Comparisons of influx into haustorial complexes of ethirimol and other solutes.

The uptake of ethirimol was compared with that of eight other compounds each at a concentration of 40 μM. Ethirimol uptake greatly exceeded that of all other compounds studied (table 35) indicating that haustorial complexes have a high affinity for the compound. It is significant that the rate for uracil, which is also a pyrimidine is 23 times slower than that for ethirimol and the only compound tested which was taken up at a rate comparable with that for ethirimol was ethanolamine. However, even here the rate was only one eighth. Thus the accumulation is very specific to the fungicides structure.

The pyrimidine fungicide Dimethirimol is a dimethylamino isomer of ethirimol (structures shown on fig. 44). and is also specifically active against powdery mildews, particularly mildew of cucurbits, (Elias, Shepherd, Snell & Stubbs, 1968). This was also tested in preliminary experiments. An uptake 50% of that of ethirimol was found but the result was unreliable because the specific activity of dimethirimol was only 3 mCi/mmol and the numbers of counts recovered proportionately small. As it was supplied in a



Dimethirimol



Ethirimol

Figure 44. The molecular structure of dimethirimol and ethirimol.

solution in methanol, when a concentration of 5 μM was used the methanol in the ambient solution amounted to 4%. However, these preliminary results indicate that this pyrimidine fungicide is taken up at least to the same order of magnitude as ethirimol and clearly much faster than the other solutes tested. This has established a need to study a larger spectrum of compounds to obtain a clearer correlation between their physico/chemical features and uptake by haustorial complexes.

vii) Effect of temperature on influx of ethirimol into haustorial complexes.

Haustorial complexes were incubated at 0° in ^{14}C-ethirimol for 15 min as described in table 35. The uptake was 0.0623 nanomoles 2×10^{-5} haustorial complexes, this was 47% that at 25°. This suggests that an active process was involved in the accumulation or lower temperature may affect the physical state of an important component of the system. Intracellular lipids represent a likely candidate for such an effect, temperature reducing the proportion of ethirimol partitioned into them.

viii) Analysis of ethirimol taken into haustorial complexes.

Since ethirimol is metabolised and several derivatives formed in plants (Cavell, Hemingway & Teal 1971) haustorial complexes were incubated in 40 μM ^{14}C-ethirimol for 2h at 25° when their content was analysed by thin layer chromatography. In preparation for the analyses the complexes were subjected to 95% methanol when

94% of the radioactivity was extracted. This suggests that 6% of the ethirimol was irreversibly bound to the complexes. This observation is consistent with the relationship between uptake and ambient concentration of ethirimol fig 40 and also with the small quantity of ethirimol (5 - 10% of the total absorbed) which was always detected at zero time (fig. 37).

The methanol soluble compounds were analysed by one dimensional chromatography on silica gel plates in the solvent $CHCl_3$: MeOH: EtAc , 6:3:1. The scan of radioactivity in the T.L.C. plate is shown on fig. 45. The major porportion (80%) of the label extracted co-chromatographed with ethirimol standard. There was also evidence of some metabolism as a proportion (10%) of the label remained or moved only slightly from the origin. The mobility of this compound resembles those of ethyl guanidine which is found in apple leaves after degradation of bupirimate or ethirimol (Teal & Cavell, 1971) and ethirimol glucoside which is formed from ethirimol by barley leaves (Cavell, Hemingway, Teal, 1971). However the degradation by leaf tissue took place during several days and contrasts with the rapid conversion of ethirimol possibly to a glucoside detected here. However this may offer little protection to the fungus as this compound is also fungicidal, (Cavell, Hemingway & Teal, 1971) and the data indicates that most of the label detected in the haustorial complexes was still a constituent of ethirimol.

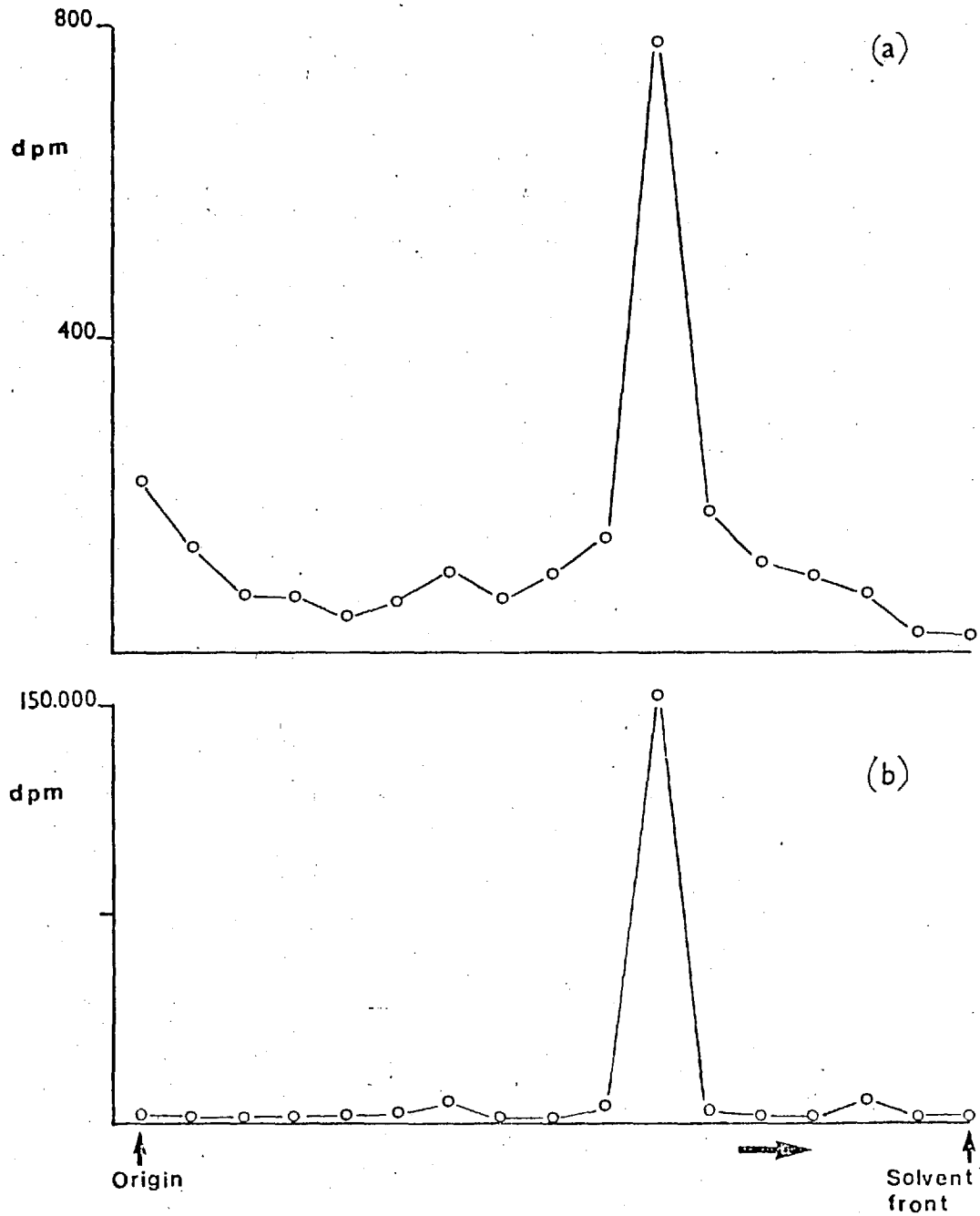


Figure 45. Erysiphe pisi - Pisum sativum. Radioactivity in a thin layer chromatograph of a 95% methanol extract of haustorial complexes incubated for 2h at 25° in 40 μM ^{14}C - ethirimol, 67 mM phosphate buffer pH 6.8 and 2% methanol.

(a) Haustorial complex extract
(b) Ethirimol standard.

Plates were developed in chloroform: methanol: ethyl acetate (6:3:1).

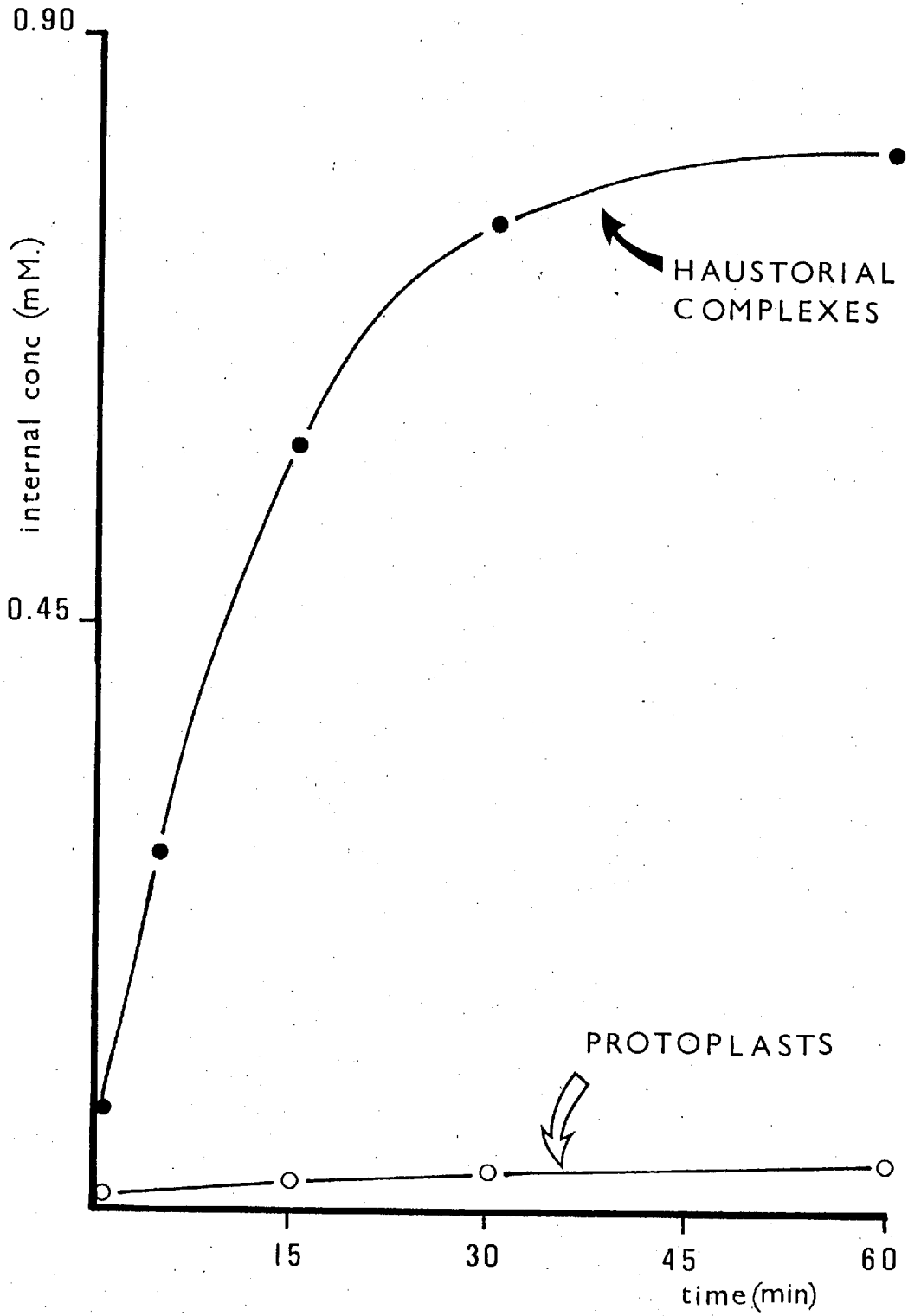


Figure 46. Erysiphe pisi - Pisum sativum. A comparison of uptake of ^{14}C - ethirimol by haustorial complexes and mesophyll protoplasts from an uninfected plant in a medium containing 40 µM ^{14}C - ethirimol, 50 mM phosphate buffer at pH 6.8, 2.5% KCl, 1% $MgSO_4 7H_2O$ at 25°.

ix) Estimation of ethirimol concentration within haustorial complexes.

In order to estimate the concentration of ethirimol within the complexes the mean volume of the complexes was estimated. The number of complexes isolated was insufficient to use the packed cell volume technique (Barber, 1968), and thus a microscopic method was used. The mean width (w) of 100 complexes was $10.8 \pm 1.9\ \mu M$ and the average length excluding the neck $16.13 \pm 2.5\ \mu M$. The volume was calculated from the formulae described in the Methods (IV,8).

The average volume of 1 haustorium was calculated to be $1,146\ \mu M^3$ and that of 2×10^5 haustorial complexes $0.23\ \mu l$. The uptake by haustorial complexes described in fig. 37 thus results in a final average internal concentration of $830\ \mu M$. This is 21 times greater than the concentration in the ambient medium. The kinetics of uptake expressed in terms of internal concentration are shown in fig. 46.

XII) Uptake of ethirimol by isolated mesophyll protoplasts of Pisum sativum.

Ethirimol is thought to move in the apoplast and in herbaceous plants it accumulates at the leaf margin, a region of high evapotranspiration (Cavell, Hemingway & Teal, 1971). In woody plants, (vine and apple) it accumulated in the vascular tissue, (Cavell, Hemingway & Teal, 1971). However, Peterson & Edgington (1976) proposed that most pesticides, although transported

in the apoplast also penetrate the symplast, and they termed such compounds, including ethirimol, pseudoapoplastic.

The *in vitro* uptake experiments with ^{14}C- ethirimol described above show that haustorial complexes have a high affinity for the compound. Gil & Gay (1977) have shown that the haustorium is isolated from the apoplast of the leaf. The haustorial complex *in vivo* has intimate contact with the epidermal cytoplasm and thus for the accumulation by haustorial complexes *in vitro* to have any relevance *in vivo*, it is important to show that ethirimol will penetrate the plant symplast.

The experiments by Peterson & Edgington (1976) were performed on tissue and thus cell wall binding may have confused their conclusions. Isolated plant protoplasts offer a system to study uptake by the plant cell without such interference, and it has found recent use in studying the uptake of exogenous DNA (Suzuki & Takebe, 1976; Hoffmann & Hess, 1973), nucleosides (Coutts, Barnett & Wood, 1975), hormones (Dekhuijzen *et al*, 1978; Sonka *et al*, 1977), sugars and amino acids (Guy, Rheinhold, Laties, 1978), herbicides (Boulware & Camper, 1973) and ions (Morris, 1977).

A) Experimental procedures.

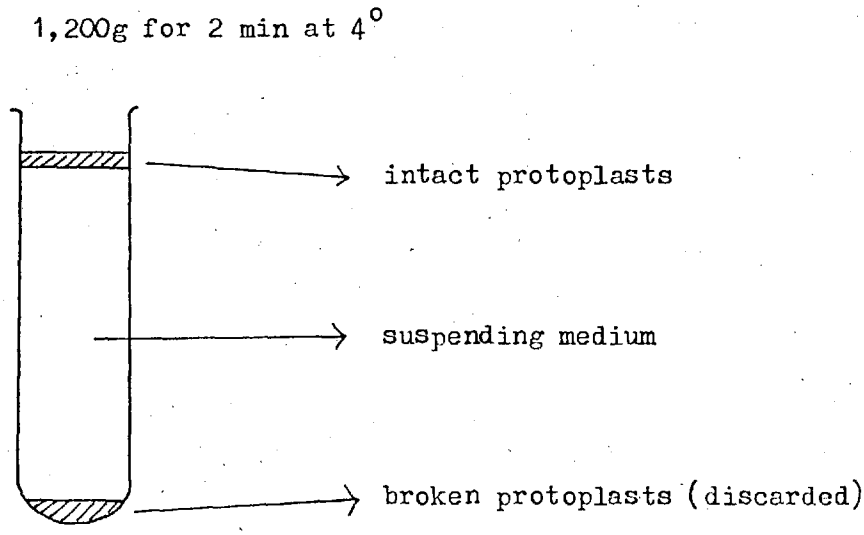
Protoplasts, isolated enzymatically from leaves of *Pisum sativum* as described in the Methods (III) were resuspended ($1 - 2 \times 10^6$/ml) in 50 mM phosphate buffer at pH 6.8 with either 2.5%

(w/v) KCl, 1% (w/v) $MgSO_4$ $7H_2O$ or 20% (w/v) sucrose. Four volumes of the protoplast suspension were added to 1 volume of 250 μM ^{14}C- ethirimol (25 mCi/mmol) and incubated with very gentle agitation at 25° for up to 18h. The incubated flasks were illuminated by a reflector lamp (9,000 lux).

Samples (0.5 - 2 ml) were removed at intervals and added to 20 ml of the ionic medium at 4° and washed twice in this medium at 4° at 500g, each centrifugation lasting 1½ - 2 min. The pellet was then resuspended in 5 ml of 20% sucrose and centrifuged at 1,200g for 2 min. This step was particularly important as it separated intact protoplasts, which floated on the sucrose solution, from broken protoplasts and debris (Galbraith & Northcote, 1977). It has recently been used in transport studies to study both ion (Morris, 1977) and DNA uptake (Hughes, White & Smith, 1978). The last study showed that previous experiments where such separation was omitted resulted in considerable errors.

The intact protoplasts were removed from the surface of the sucrose (fig. 47), and the cell density estimated, radioactivity therein was determined in scintillant mixture S2. The time required to completely separate the protoplasts from the radioactive medium was 12 min. The radioactivity in the supernatants was also estimated (fig. 47) and this was used in the formula below (fig. 47) as a background count to estimate the radioactivity in the protoplasts.

Fig. 47. Diagram illustrating purification of isolated protoplasts of Pisum sativum by centrifugation in a 20% sucrose solution before estimation of ethirimol uptake, and formula used in computing results.



Formula:

$$\text{cpm in protoplasts} = \left(\text{cpm 50 } \mu\text{l protoplast suspensions} - \text{cpm 50 } \mu\text{l suspending medium}\right) \left[\frac{50\ \mu\text{l} - \text{volume of protoplasts in 50 } \mu\text{l of suspension}}{50\ \mu\text{l}}\right]$$

Aliquots (50µl) of the protoplast suspension and suspending medium were counted. The volumes of the protoplasts were estimated from microscopic measurements, each using 50 - 100 protoplasts. Since protoplasts are spherical, accurate estimation of volume was facilitated. Radioactive compounds in the protoplasts were analysed by T.L.C. after extraction in 95% methanol. The

procedure was the same as used for isolated haustorial complexes, (section X1, B, viii).

B) Results

Microscopic examination showed that the protoplast preparations were almost pure and only 5 - 10% of the total numbers were vacuoles.

The results of ethirimol uptake by isolated mesophyll protoplasts shown in fig. 48 indicate biphasic kinetics. Considerable uptake occurred in the first hour and then there was a change to a steady slower influx which continued for the remaining 18h of the incubation period. Peterson & Edgington's (1976) experiments with potato slices indicated that ethirimol occupied 75% of the tissue volume, and probably penetrated the symplast. Uptake was followed for only 3h and this may have been insufficient to resolve the two phases detected here. A similar biphasic uptake kinetic was obtained by Guy et al (1978) for glucose and α amino-butyric acid uptake by pea mesophyll protoplasts and they interpreted this as a penetration of two compartments within the protoplast. It is possible that ethirimol has a similar intracellular distribution.

Radioactive compounds located in the protoplasts after 2h were analysed by T.L.C. and the scan of the chromatogram is shown in fig. 49. There had been very little degradation and 85% of the label was still in ethirimol. Very minor, but significant quantities of ethirimol were found at Rethirimol values of 0.3 and 0.7.

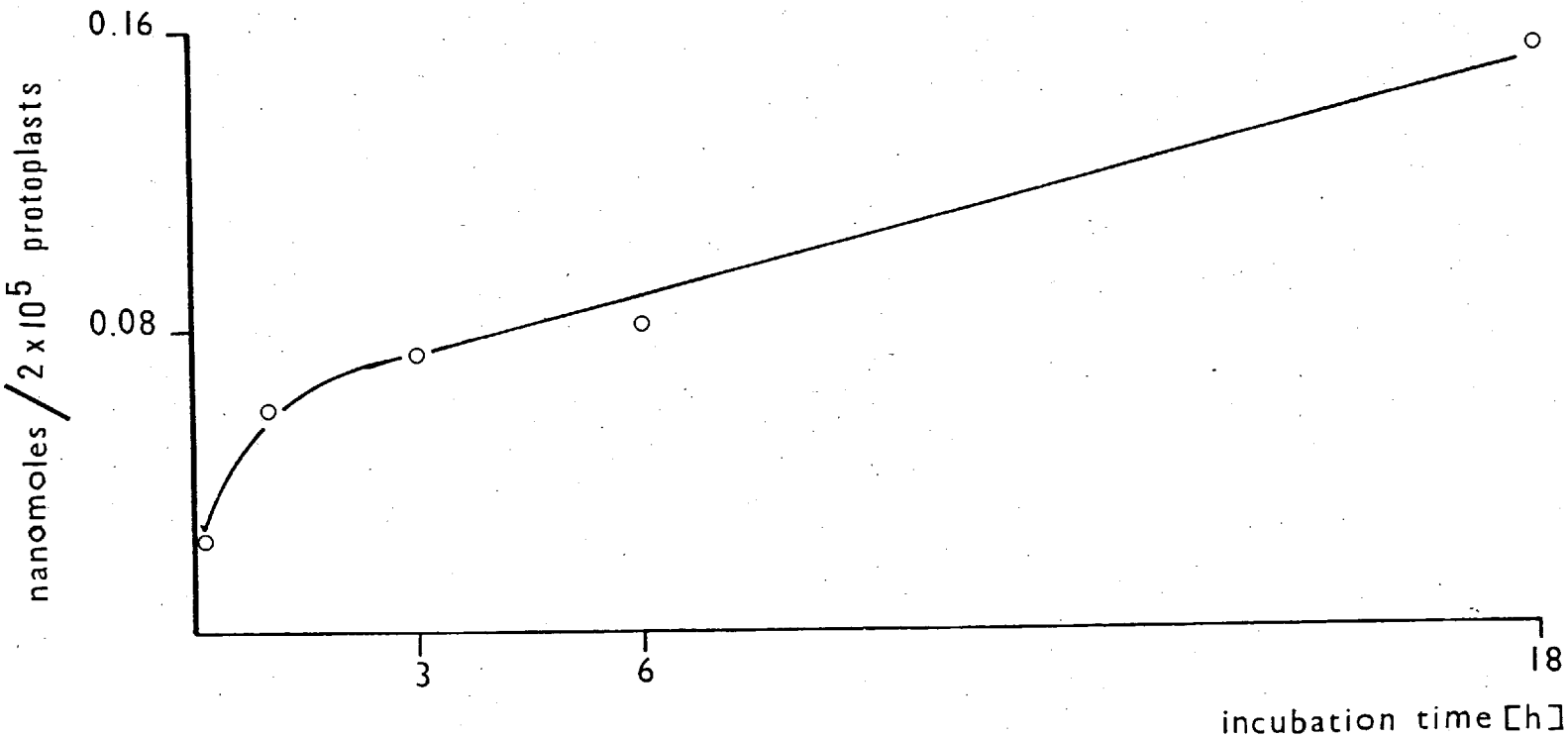


Figure 48. Pisum sativum. Uptake of ^{14}C - ethirimol by isolated mesophyll protoplasts in a medium containing 50 μM ^{14}C - ethirimol, 50 mM phosphate buffer at pH 6.8, 2.5% KCl, 1% $MgSO_4 \cdot 7H_2O$ at 25°. Each point is the mean from 2 replicate experiments.

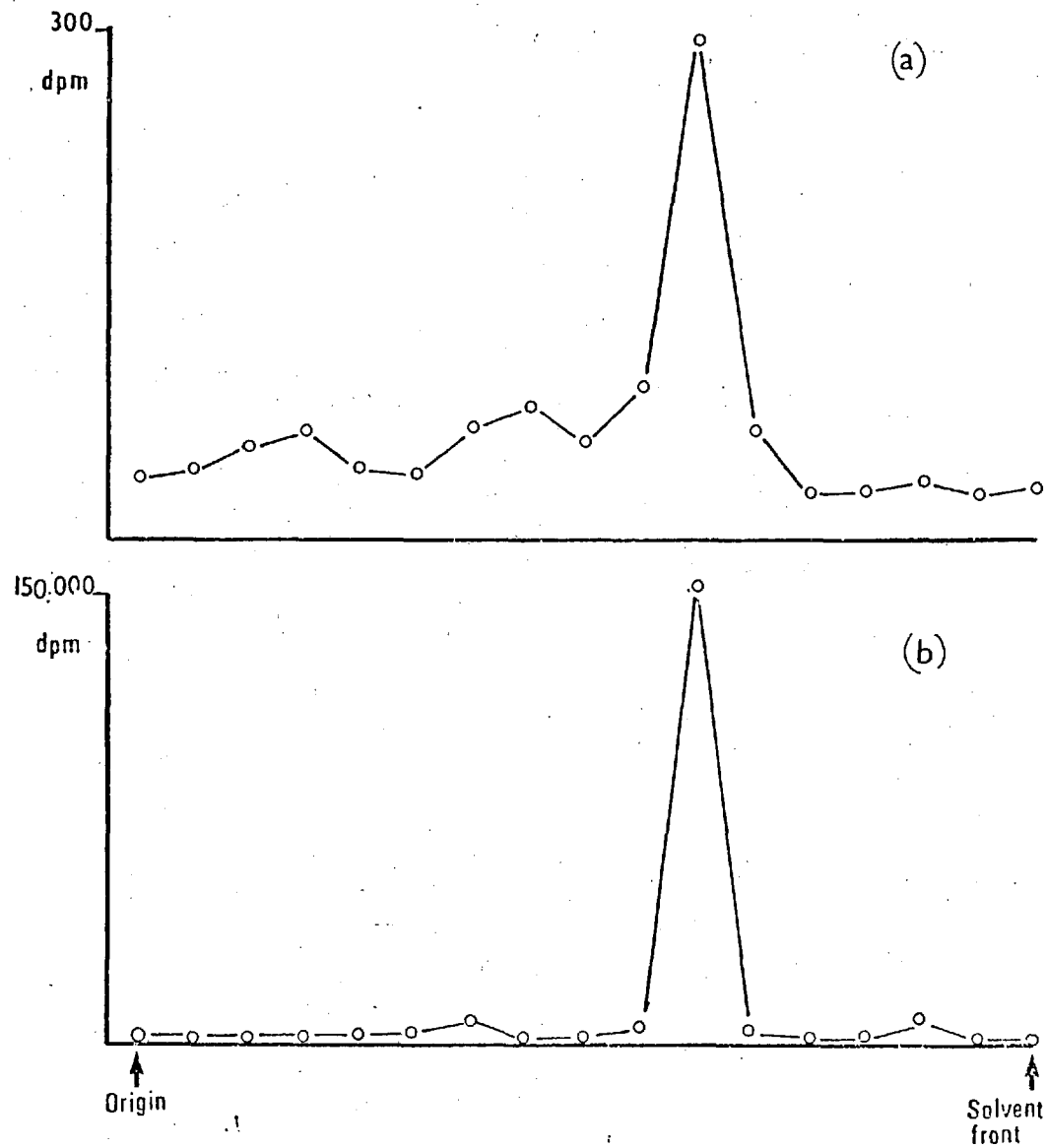


Figure 49. Pisum sativum. Radioactivity in a thin layer chromatogram of a 95% methanol extract of mesophyll protoplasts incubated for 2h at 25° in 50 μM ^{14}C - ethirimol, 40 mM phosphate buffer pH 6.8, 2.5% KCl and 1% $MgSO_4\ 7H_2O$.

a) Protoplast extract.
b) Ethirimol standard.

Chromatograms were developed in chloroform: methanol: ethyl acetate (6:3:1).

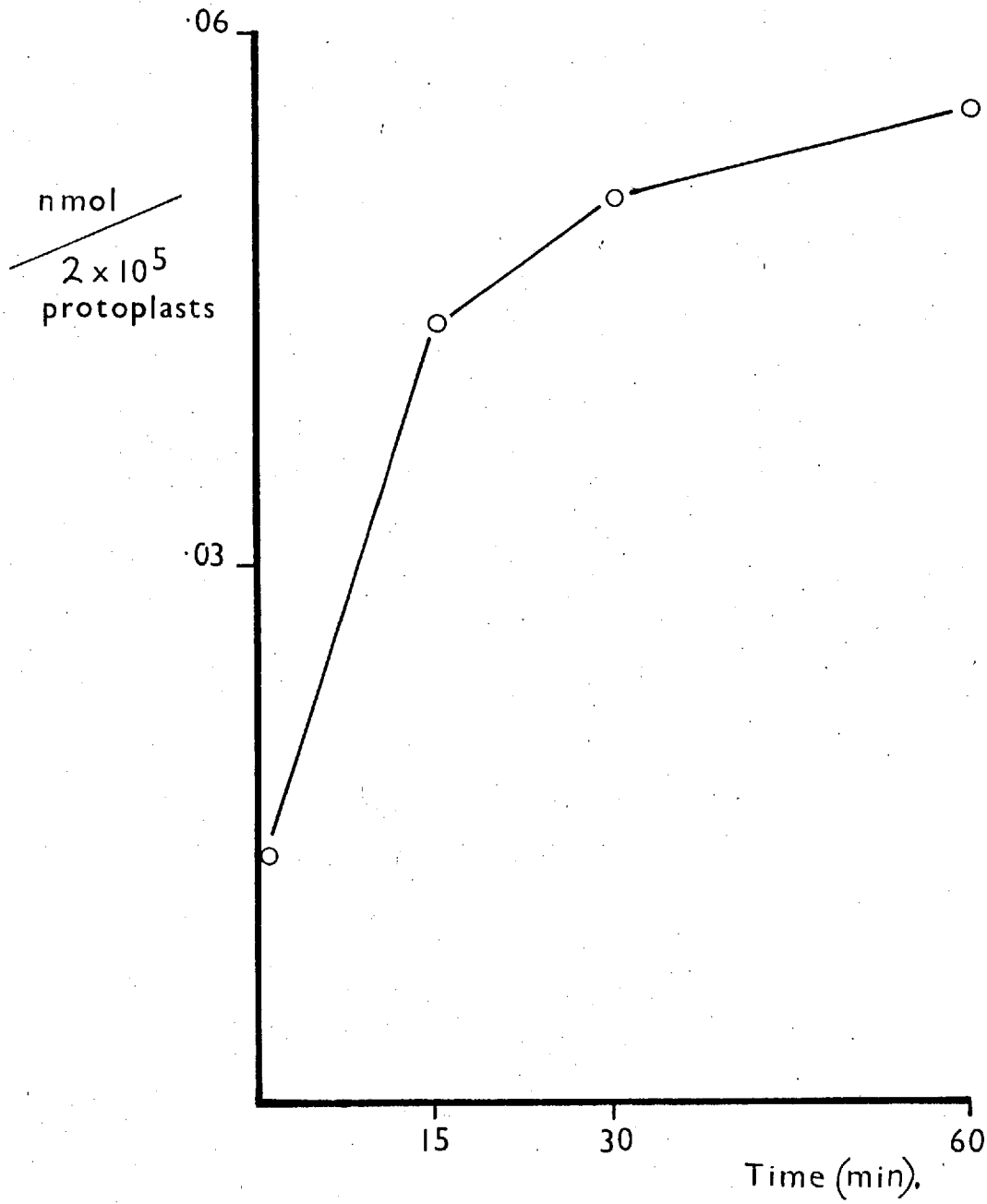


Figure 50. Pisum sativum. Uptake of ^{14}C - ethirimol by isolated mesophyll protoplasts in a medium containing 50 μM ethirimol, 40 mM phosphate buffer at pH 6.8, 2.5% KCl and 1% $MgSO_4 . 7 H_2O$ at 25^0. Each point is the mean of two replicate samples from a single experiment.

Although these are termed metabolites the possibility that they may have been formed in the ambient medium during the incubation period and subsequently absorbed was not investigated.(Teal & Cavell (1975), have reported the breakdown of ethirimol in aqueous solution exposed to sunlight). It is thus possible that the second phase of uptake (1 - 18h) may be due to metabolism or uptake of degradation products.

The initial uptake period was studied in more detail and the kinetics during the first hour are shown in fig. 50. A significant proportion of the ethirimol was absorbed immediately and this may have been due to binding at the cell surface. Most of the remaining uptake occurred during the first 30 min of incubation.

The internal concentrations can be calculated from this data and the protoplast volumes. The mean diameter of 100 protoplasts was 26.21 $\pm$ 3.61 μM and thus the volume of 2 x 10^5 protoplasts was 1.74 μl. This is 7.5 times greater than the volume of the same number of haustorial complexes. Thus after 1h incubation the internal concentration of ethirimol reached 32 μM which is 64% of that of the ambient medium. This is less than would be expected from an equilibration across the plasma membranes of the protoplasts and it cannot be ruled out that losses were not incurred during the washing period. After 18h incubation the concentration within the protoplasts was 87 μM which is 1.74 times that of the ambient medium. Neither the 1h or 18h samples were analysed by T.L.C. and thus the

purity of the radioactive component, especially after the longer period, is in doubt. However these results do clearly show that ethirimol penetrates the protoplast plasmamembrane and is in agreement with Peterson & Edgington (1976) who used potato tissue.

A direct comparison of uptake in haustorial complexes and protoplasts is shown on fig. 46 where internal concentrations are shown. It is clear that the isolated haustorial complex has a far greater affinity for the fungicide than the isolated host protoplast. After one hour the haustorial complexes contained ethirimol at a concentration 28 times greater than that in the host protoplasts.

XII) Leaching of ^{14}C photosynthates from infected pea leaf discs.

It has been shown that during assimilation of $^{14}CO_2$ a considerable quantity of the photosynthate is rapidly transported to the mycelium (section III, A, ii). The pathway of photosynthates from the chloroplasts of mesophyll cells and sieve tubes to the haustoria and thence to the mycelium is not clearly understood (Bushnell & Gay, 1978). Current literature suggests that in both mildewed and rusted tissue the cell membranes of the mesophyll cells become more permeable and allow the loss of ions and non electrolytes (Thatcher 1942; Hoppe & Heitefuss, 1974abc; Ayres, 1977; Bushnell & Gay, 1978). This process would increase the concentration of nutrients in the apoplast. Nutrients could then be absorbed by infected epidermal cells and thence carried to the haustoria. The quantities of solutes

released may be determined by changes in membrane permeability (including transport characteristics) or concentration gradient. Thus where possible results of leaf disc experiments carried out are presented with a view to interpretation in this framework.

A major disadvantage in using leaf discs for permeability studies is the small diffusive area in contact with the ambient medium (Smith & Epstein, 1964). Morrod (1974) showed that this could be overcome by removal of the epidermes, and he obtained reliable fluxes of various solutes with epidermis-free discs of tobacco leaves. It was found to be impractical to remove both epidermes from the pea leaf discs, but the lower epidermis could be removed easily with forceps and in one experiment the leakage with and without the lower epidermis was studied.

A) Experimental procedures.

Three experiments were performed and slightly different procedures were used in each. Discs were removed from uninfected leaves and from infected leaves separated from mycelia. In experiments 1 and 2 the discs were 6mm in diameter. In experiment 3 3mm discs were used and additionally the lower (infected) epidermis was removed with forceps from half of the uninfected and infected discs. In all experiments the discs were then washed in distilled water, blotted gently and exposed to $^{14}CO_2$ (100 μCi for 10 min) at 20° in the light (10,000 lux). For experiments 1 & 2 two replicate

experiments were performed with 20 uninfected and infected leaf discs but for experiment 3, 18 discs were used in each set. The discs were then removed from the exposure chamber and transferred to a conical flask containing distilled water (15 ml in experiment 1 and 10 ml in experiments 2 and 3). The electrical conductivity of the water was measured immediately and the flasks gently agitated at 25°. At intervals samples were removed and the conductivities remeasured. The discs were dropped into boiling 80% ethanol and after ethanol extraction for 1h they were extracted in boiling 1.5N H_2SO_4 for 2h, the discs were then discarded and the acid neutralised by addition of $BaCO_3$. The incubation medium, ethanol and sulphuric acid extracts were then assayed (1 ml) for radioactivity in scintillant mixture S2.

B) Results.

In experiment 1 the discs were incubated for 2h and ambient conductivities and radioactivities leached from these discs are shown in tables 39and 40 respectively. In order to compensate for the difference in $^{14}CO_2$ fixation rates in healthy and infected discs (section II,8) the results are expressed as fractions of the residual counts. The ethanol insoluble label was usually less than 10% of the insoluble label and it is unlikely to be directly involved in leaching it was ignored in the expression of results.

The results from experiments 1 & 2 indicated that the uninfected leaf discs lost a larger proportion of photosynthate to

Table 39

Erysiphe pisi - Pisum sativum. The leakage of ions from infected leaf discs separated from the mycelium and uninfected leaf discs. Samples of 20 (6mm) were incubated in distilled water (15 ml expt. 1. 10 ml expt. 2.). The conductivity measurements (μmhos) are averages of 2 replicates in each experiment.

Expt. 1.	Hours of incubation		
	1	2	3
Uninfected	-	60	-
Infected	-	57	-
Expt. 2.			
Uninfected	32	36	50
Infected	17	30	52

Table 40

Erysiphe pisi - Pisum sativum. The loss of ^{14}C photosynthates from infected leaf discs separated from the mycelium and uninfected discs. Washed discs were illuminated in $^{14}CO_2$ for 20 min, incubated in water at 25° and dropped in 80% ethanol. Results (dpm x 10^5 ambient dpm x 10^{-6} ethanol soluble remaining in discs) are the mean of 2 replicates in each experiment.

	Hours of incubation		
Expt. 1.	1	2	3
Uninfected	-	4.10	-
Infected	-	1.13	-
Expt. 2.			
Uninfected	0.49	0.64	0.92
Infected	0.06	0.12	0.31
Expt. 3.			
Uninfected	-	1.05	-
(Lower epidermis removed)	-	3.50	-
Infected	-	0.64	-
(Lower infected epidermis removed.)	-	1.56	-

thd medium than infected discs (table 40) Both showed the same changes in conductivities indicating equality of ion leakage but as no compensation for the ion content of the tissue was made the gradient from tissue to medium for electrolytes was not defined.

In the experiment (3) where the lower epidermis was removed the infected discs were cut from leaves with abaxial infections only. Thus the haustoria and infected host cells were removed and this enabled a direct comparison between the two types of disc. Results of the quantities of isotope leached during 2h (table 40) show that removal of the epidermis increased the proportion of label leaked from both uninfected and infected discs. Further as in experiments 1 and 2 infection decreased the proportion of label released into the incubation medium.

Discussion

I) Light microscopic observations on isolated haustorial complexes of apple, barley, beet, pea and vine infected with powdery mildews.

The experiments described above and those of other workers (Hirata & Kojima, 1962; Dekhuijzen, 1966; Bracker, 1968; Gil & Gay, 1977) show that the haustorium or haustorial complex of powdery mildew fungi is easily released mechanically from infected epidermal cells. For those host species whose mildew haustoria are of the smaller compact form, e.g. cucumber (Hirata & Kojima, 1962), pea (Gil & Gay, 1977), apple, beet and grape vine, the haustoria are usually released intact and almost always in association with the extrahaustorial membrane and other composite structures of the host-parasite interface which together comprise the haustorial complex (Gil & Gay, 1977). However, further purification to obtain fractions of haustorial complexes suitable for biochemical studies as is possible for pea mildew (Gil, 1976) is impaired by either the simultaneous release of intact host cells (apple), or intracellular inclusions (sugar beet and vine). These contaminants are difficult to separate from the haustorial complexes and also impede other purification stages. In the elongate haustoria of Erysiphe graminis only the young

haustoria are released without damage. Consequently only small numbers can be isolated and even these are not always associated with the extrahaustorial membrane. Thus the final fraction is a heterogeneous mixture of haustoria, haustorial complexes and major fragments.

It is thus obvious that the host-parasite combinations other than E. pisi - Pisum sativum tested in the present work are not suitable for studies on the biochemistry of haustorial function, but valuable new microscopic observations have been made, particularly on the haustoria of E. graminis. In no combination did the purity approach that of fractions of haustorial complexes from P. sativum infected with E. pisi. In these preparations the haustorial complexes are almost all intact and they are free from other fungal structures. Only starch grains and cell wall fragments from the host are present as contaminants and all studies of the physiology and biochemistry of powdery mildew haustoria in this investigation employed this experimental system and results are discussed below.

Although the overall morphology of the haustorium of E. graminis is very different from that of compact haustoria of non-gramineous hosts, careful observation shows that in their essential structure they have much in common. In both, the extrahaustorial membrane is retained around the haustorium although the degree of retention differs

in the two types. In E. graminis it only occasionally remains intact when it distends without rupture in hypotonic media. When it distends it separates from the surface of the haustorial body but remains attached at the neck and extremities of the lobes. Similarly, the extrahaustorial membrane remains around the compact haustoria in non-gramineous hosts. It also has mechanical strength and is attached at the neck and lobe tips. Similarity is also shown in the neck regions. In E. graminis the two annular structures correspond to those seen in haustoria of E. pisi (Gil , 1976) and E. polygoni. The band nearest to the septum probably has a similar function in sealing the extrahaustorial membrane to the haustorial neck as shown in the B band of E. pisi (Gil , 1976) and thus confines the extrahaustorial matrix in a discrete compartment. The other band is presumably identical to the A band in E. pisi (Gil , 1976). These bands are formed at a very early stage of haustorium development in E. graminis and the B band is already present while the haustorial body is still expanding. Similar early development of the neck band in the rust fungus Melampsora lini has been reported by Littlefield, (1972) and although the equivalence of the structures in the two fungal groups is not clear (Heath, 1976), diffusion of marker substances has demonstrated the barrier at the membrane attachment in a rust fungus (Heath, 1976). In powdery mildews, the neck in both haustorial types is joined to the haustorial body

at one pole and the haustoria are similar in having bipolar lobe origins clearly shown here.

Despite all these similarities, the isolated haustorial complex of E. graminis does differ from that of the compact type in some of its physiological reactions. In water, the distension of the extrahaustorial membrane of undamaged haustorial complexes is rapid and thus in accord with observations made on infected detached epidermis of barley coleoptile where the complex was exposed to a hypotonic ambient medium by microdissection of the host cell (Hoskin, 1969). The slow swelling of haustorial complexes isolated from sugar beet and previously from pea (Gil & Gay, 1977) infected with powdery mildew is now comparable under identical conditions of examination. The difference in rates of swelling may reflect differences in osmotic potential of the extrahaustorial matrix, or in the permeability or rigidity of the membrane. Differences in membrane characteristics may be due to differences in polysaccharide content or rigidity of the folding noted by Gil & Gay, (1977). Loss of the membrane during isolation is probably due to the shape of the haustorium of E. graminis, but since it is also often lost by young haustoria it is probably mechanically weaker than its counterpart around the compact haustoria which is usually retained regardless of the age of the haustorium. (In

these species age is indexed both by the size of the haustorial complex and the degree of lobe development).

It has been noted that the haustorium of E. graminis retains physiological activity and may even develop within the epidermal cell after removal of the superficial mycelium (Hirata, 1967). Evidence has been obtained that the isolated haustorial complex retains physiological activity at 4° in vitro for several days in Sphaerotheca fuliginea (Gil, 1976), (Dekhuijzen, 1966), E. pisi (Gil, 1976) and now at least overnight in E. polygoni. However, in E. graminis incubation at 4° for 16 h had a deleterious effect on the haustorial cytoplasm and this appeared to be independent of the integrity of the extrahaustorial membrane. Degeneration of haustoria of E. graminis in epidermis detached from barley coleoptile has also been observed after incision of host cells (Bushnell, 1971). Thus, although differing in form, the haustoria in E. graminis and in powdery mildews of non-gramineous hosts are structurally similar. However, the two types differ in certain aspects of their physiology.

II) A critical appraisal of the permeability of the extrahaustorial membrane in powdery mildew infections.

Previous workers (Hirata & Kojima, 1962; Hirata, 1967; Bushnell, 1971 and Gil & Gay, 1977) have concluded that the changes in volume of the extrahaustorial matrix depend on the semi-permeability of the extrahaustorial membrane. However, the results of their experiments to monitor semi-permeability of this membrane are not unequivocal and require critical reappraisal.

Early work on this subject (Hirata, 1967) indicated that the extrahaustorial membrane would distend and contract after haustoria in stripped infected epidermes of barley were treated with mixtures of formalin, acetic acid and ethanol. This treatment usually destroys the semi-permeability of biological membranes and suggests that the membrane is extraordinary and, as Wheeler (1976) has proposed, that other factors, such as volume changes in a gel in varying ambient tonicity, may contribute to swelling. Thus the combination of a gel in the extrahaustorial matrix and an extrahaustorial membrane permeable to the solutes may be expected to produce the same results as a matrix containing solutes enclosed by a semi-permeable extrahaustorial membrane.

Semi-permeability of the extrahaustorial membrane in rust and downy mildew infections seems in little doubt. Thatcher (1943) and Fraymouth (1956) have shown that when the infected host

cell is plasmolysed, the extrahaustorial membrane retracts from the haustorium. This indicates that the extrahaustorial membrane is semipermeable, but because it is part of the host plasmalemma (Littlefield & Bracker, 1970) the same effect could occur where the extrahaustorial membrane were completely impermeable and water moving entirely through the uninvaginated portion of the membrane. A similar experiment with barley infected by E. graminis (Hoskin, 1969) led to retraction of the protoplast from the epidermal wall, but not from the haustorial surface. From comparative evidence (Gil & Gay, 1977) and results described above, this occurs because the extrahaustorial membrane is firmly attached to the neck of the haustorium by the neck bands. The rust fungi have a single neck band where the membrane adheres to the neck, but this is much less differentiated than that of the powdery mildews and the plasmolysis experiments of Thatcher (1943) indicate that the membrane is not firmly adherent to the neck. However, results from rusts and downy mildews cannot be directly extrapolated to the extrahaustorial membrane in powdery mildew infections where its structure with polysaccharide thickening is so distinctive.

In powdery mildew infections, experiments and thorough examination of the extrahaustorial membrane have helped to resolve the problem (Gil & Gay, 1977). In isolated haustorial complexes they defined the boundary of the matrical compartment which swells as the extrahaustorial membrane, the fungal plasmalemma and a portion

of the neck between the B beckband and the fungal septum where the fungal plasma membrane can be seen lining the wall. The contribution by the cast region can probably be ignored in in vitro swelling experiments because similar responses have been shown after incision of infected host cells with minimal disturbance to the fungus and where presumably the region is still lined by a functional plasma membrane. Thus the effective boundaries of the matrix are the fungal plasmalemma and extrahaustorial membrane.

The retention of the reversible swelling responses and indeed of the extrahaustorial membrane itself after detergent treatment is unusual and as Gil & Gay, (1977) have indicated the presence of large quantities of polysaccharide on the membrane may protect the membrane lipids from emulsification. This addition may have a parallel in experiments incorporating lipopolysaccharide from gram-negative bacteria into artificial phospho-lipid vesicles which subsequently protected them from disruption by detergent (Nixdorff et al, 1978). In haustorial complexes detergent treatment also increased the rate of swelling suggesting an increase in the permeability of the membrane to water or disruption of the haustorial plasma membrane. The latter presumably released the haustorial contents thereby lowering the osmotic potential of the matrix. However, polysaccharide degrading enzymes have also been shown to increase the swelling and contraction rates and these also substantially reduce the membrane thickness (Gil & Gay, 1977). This suggests that

it is the structural properties of the extrahaustorial membrane and not the nature of the matrix alone which controls swelling. Further, recent experiments by J. Gay (personal communication) also suggest that swelling depends on the semipermeability of the membrane. He observed that when unswollen haustorial complexes of E. pisi - P. sativum were gently crushed in water with a micromanipulator pieces of the extrahaustorial membrane remained approximately in the position of the intact membrane and did not move during the following 20 min. period. However, the slow swelling responses of haustorial complexes of E. pisi - P. sativum make longer term observations desirable before a definitive conclusion can be reached. The low incorporation rates of sugars and amino acids in vitro and lack of respiratory response to exogenous substrates by the haustorial complexes are also consistent with their exclusion by the extrahaustorial membrane although another, but a less likely alternative, is that certain physiological activities of haustoria are impaired during the isolation procedure.

Further evidence against haustorial complex enlargement being due to gel swelling is provided by the effect of ambient solutions. If swelling and shrinkage responses were solely due to hydration and dehydration of a gel in the extrahaustorial matrix then a more direct relationship between the width eventually obtained by the complexes and the water activity of the ambient medium is to

be expected. On the other hand, osmotic responses resulting from the presence of a semipermeable membrane would result in the same widths at equilibrium although the rates of swelling and shrinkage would be related to the osmotic potential of the ambient medium. Since there were no significant differences in the ultimate widths of complexes after incubation in 50 mM, 100 mM or 500 mM sucrose it is most probable that the extra-haustorial membrane is semipermeable.

Gil (1976) demonstrated that despite the large quantities of polysaccharide which impregnate this membrane a bilayer structure could nonetheless be distinguished. The presence of a lipid bilayer does not necessarily indicate its semipermeability. For instance, the outer membrane of the chloroplast despite structural intactness, is freely permeable to low molecular weight solutes e.g. ATP, sucrose, sorbitol, inorganic phosphate, glucose-6-phosphate, malate, aspartate (Heldt & Sauer, 1971) and similarly the outer mitochondrial membrane is permeable to small molecules (Pfaff et al, 1968). In this context it may be significant that the outermitochondrial membrane contains significant quantities of carbohydrates (Manella & Bonner, 1975) but the EDTA extractions of haustorial complexes attempted indicate these are not equivalent.

In conclusion, it is not yet possible to unequivocably state that the extrahaustorial membrane is permeable to water and not to low molecular weight solutes. Although on balance the evidence favours that conclusion, it may be that membrane semi-permeability and gel swelling both contribute to swelling. Application of apoplastic markers in an electron microscope examination of isolated haustorial complexes should help to clarify the swelling responses and determine the extent of the continuum between the host cytoplasm and extrahaustorial matrix.

The status of the boundary provided by the extrahaustorial membrane is important in assessing biochemical analyses of isolated haustorial complexes and in light of this discussion it is at present prudent to consider it as a semipermeable membrane.

III) Uptake of ^{14}C photosynthates by haustoria in vivo.

A) Proof of uptake.

It has previously been shown that nutrients move from the host to the surface mycelium of Erysiphe graminis DC. f. sp hordei (Edwards & Allen, 1966), E. graminis DC. f. sp. tritici (Mount & Ellingboe, 1969; Slesinski & Ellingboe, 1971) and Microsphaera alphitoides Griff & Maubl (Hewitt & Ayres, 1976). Evidence of entry into mycelia has been extended to E. pisi in the present work.

Previous studies have not provided any direct evidence for the role of haustoria in this transport process. The results presented in this thesis (Results IV,V,VI) show that the products of photosynthesis of Pisum sativum enter haustoria of E. pisi.

It should be noted that the isolated haustorial complex comprises two distinct compartments, viz. the haustorium with its cytoplasm and the extrahaustorial matrix which lacks organelles (Gil & Gay, 1977). Thus, in addition to the detection of labelled assimilates in the complexes (Results V, VI), the subsequent respiratory liberation of $^{14}CO_2$ by them in vitro (fig. 8) constitutes invaluable evidence that the assimilates had entered the haustorium itself. It should also be noted that adequate controls were provided, particularly by comparative fractions from uninfected plants, detergent treatment to solubilize normal membranes and avoiding starch contamination by confining some observations to assimilates soluble in ethanol.

Studies using high resolution autoradiography have shown that radio-isotopes, initially fed to the host are incorporated into both haustoria and mycelia of rust fungi (Onoe, Tani & Naito, 1973; Manocha, 1975; Mendgen, 1977) and downy mildews (Andrews, 1975; Takahashi, Inaba & Kajiwara, 1977). However, it cannot be concluded from these studies that the isotope in the haustorium entered directly from the host or whether entry was indirect through intercellular hyphae. Since results presented here show that

nutrients were absorbed by haustoria in the absence of the superficial mycelium (figs. 6 & 8), this study has provided the first demonstration that nutrients traverse the host - haustorium interface *in vivo*.

B) Uptake activity of individual haustoria.

Considerable variation in the labelling of haustorial complexes was observed in light microscope autoradiograms (plates 21 and 23). This was not correlated with haustorial age, results indicating that both young and old haustoria are capable of absorbing nutrients. This finding agrees with the results of Hirata (1971) who estimated the activity of the primary haustorium of *Erysiphe graminis* by measuring hyphal extension on the leaf surface. He concluded that the primary haustorium remained active for at least four days.

Some haustoria in each age group were unlabelled (plates 21 and 23). This may have been due to a low photosynthetic potential of the particular leaves from which they were isolated but it seems more likely that these haustoria were unable to take up and incorporate photosynthate. This is particularly relevant in view of the work on *E. graminis* from Ellingboe's laboratory (Slesinski & Ellingboe, 1971; Martin & Ellingboe, 1978) and from Mendgen (1977) and Manocha (1975) with rust haustoria. These workers have shown that reduced incorporation

of label was associated with host resistance reactions. Mr. P. Spencer-Phillips (personal communication), has made a preliminary investigation of E. pisi which parallels the autoradiography described in this thesis but employing high resolution autoradiography. He has observed that some isolated complexes have necrotic host cytoplasm adhering to the extrahaustorial membrane and this is associated with reduced incorporation of host photosynthates by the haustoria. Thus, although the host appears susceptible to this fungus there may be individual or groups of infected cells showing resistant reactions. The problem of heterogeneity of the isolated complexes is a problem for future investigations.

There are difficulties in fully interpreting both light and electron microscope autoradiograms of ^{14}C labelled haustorial complexes for uptake studies. Although label is associated with the complex the low resolution obtained does not readily permit localisation of the label to either the matrical or haustorial cytoplasmic compartments. Also there may be losses of soluble label from the complexes both during the isolation procedure and preparation of autoradiograms, but recent techniques have shown some promise in overcoming these problems by using freeze substitution (Fisher & Housley, 1972) or freeze drying (Hessler & Peveling, 1978) during preparation. These would be useful if applied to host-parasite combinations.

C) Contamination and controls.

The ^{14}C labelling of infected leaves and the fractionation technique applied in this study has permitted the quantitative analysis of both ethanol soluble and insoluble labelled compounds in haustorial complexes. Several controls have been used to monitor contamination and they indicate that between 80-90% of the isotope in the fraction was in the complexes. Microscopic examination of the fractions showed that intact host cells and hyphae were absent, removal of the host chloroplasts was almost complete (plate 13) and any residual chloroplasts were broken and thus had lost their soluble constituents. However starch grains (plate 20), cell walls (plate 19) and other unidentified rhombohedral crystals (plate 21) possibly composed of calcium oxalate were present in the fraction. Numerically, the contaminant particles exceeded the haustorial complexes but individually they were usually smaller. Thus microscopy indicates that contamination by water soluble compounds retained by intact membranes was probably very low whilst estimation of insoluble compounds may have been considerably affected by contamination. Consequently in experiments where unlabelled carbohydrates were examined analysis was limited to those soluble in 80% ethanol and most probably the results represent only those present within the complexes.

Comparison of the ^{14}C in fractions isolated from uninfected leaves previously exposed to $^{14}CO_2$ was valuable in estimating host contamination of the haustorial complex fraction. Such estimates indicate contamination between 10% and 15% (tables 6 and 10). However, this is probably an over-estimate since uninfected leaves photosynthesize faster and convert proportionately more $^{14}CO_2$ into insoluble compounds than infected leaves (table 4). Thus such controls are not exactly comparable. The effect of contamination of host origin in the haustorial complex fraction was apparently reduced by treatment with cold Triton X-100 which preferentially solubilises label from control fractions (table 7 and fig. 7). The preferential solubilisation may have been due to differences in the composition of assimilate in the two components or more probably to the resistance shown by the extra-haustorial membrane to detergent treatment in swelling experiments with haustorial complexes (Gil & Gay, 1977). The detergent treatment increases the rates of swelling and shrinkage of isolated complexes most probably by increasing the membrane permeability (Gil, 1976). Thus some labelled compounds were also probably lost from the treated complexes. However, further and more direct controls using autoradiography and biochemical markers with fractions not treated with detergent indicated that the detergent treatment was an unnecessary precaution. These

showed that the ^{14}C contributed by host contaminants did not significantly interfere in estimations of that of the complexes.

Light microscope autoradiography demonstrated that although host cell walls and starch grains were present in the fraction obtained from leaves exposed to $^{14}CO_2$, the complexes were preferentially labelled. Here also contaminant particles accounted for approximately 15% of the isotope. Soluble compounds were probably lost from the complexes during the preparation of autoradiograms and thus this method only provides a reliable estimate of radioactivity in high molecular compounds. Since analysis of ^{14}C labelled fractions showed that between 60 - 70% of the label in the fractions was ethanol insoluble (tables 8 and 15) the autoradiography has made a valuable contribution to the evidence on uptake by haustoria.

Further evidence that contamination did not unduly influence the results of analyses of haustorial complexes was obtained by comparing them with results of host and mycelial analyses. In several instances the biochemical content of particular fractions was highly characteristic. For example isolation of haustorial complexes from brushed infected leaves lead to considerable enrichment of triglycerides (fig. 10), sucrose, glycerol and mannitol (table 21), and the uncharacterised glucan (not α 1-4 linked) (table 18), these were present in

only trace quantities in the host. Furthermore by comparison with the mycelium the triglycerides and mannitol are good marker compounds for the fungus. Low levels of host contamination is also indicated by compositional changes which occurred during pulse-chase $^{14}CO_2$ experiments. Host lipid analyses showed large increases in diglycerides (fig. 11) which were barely detectible in the isolated haustorial fraction. Similarly in the neutral sugar fraction proportions of labelled sucrose increased in the host but showed a large decrease in the haustorial complex fraction with chase. Similarly glycerol, which was virtually absent from the host and mannitol were major components of neutral sugar fractions in the haustorial complexes during pulse and chase treatments respectively.

D) Evidence for the role of haustoria in nutrient translocation.

Since a period of at least ten minutes is necessary to brush enough leaves for isolation of haustorial complexes, the technique does not allow studies of uptake over very short time periods. Thus no kinetic evidence could be obtained to demonstrate that ^{14}C assimilates enter haustoria before entering the mycelium. The mycelium contained six times as much label per unit protein as the complexes after 5 min $^{14}CO_2$ exposure. Unlike the mycelium which is highly vacuolate, the haustorium has very few vacuoles and dense cytoplasm and thus on a cell volume basis the haustoria were probably comparatively more heavily labelled. This is

clearly an area for further application of autoradiographic techniques. In view of the absence of such kinetic data prior to considering transport from host to parasite it is necessary to summarise the evidence which indicates that the powdery mildew haustorium has a major role in this process.

1) ^{14}C host assimilates are detected in similar quantities in haustoria of E. pisi in both the presence and absence of the superficial mycelium (table 8).

2) Radiolabelled sucrose is present in haustorial complexes isolated immediately following exposure of infected leaves to $^{14}CO_2$ but is virtually unlabelled when isolated after a 20h chase period in unlabelled CO_2 (table 21).

3) Excision of the primary haustorium of Erysiphe graminis by micromanipulation arrests growth of the surface hyphae (Bushnell, 1967).

4) During primary infection, uptake of ^{35}S and ^{32}P by the superficial fungal structures and development of secondary hyphae are dependant on the formation of the haustorium, (Masri & Ellingboe, 1966; Mount & Ellingboe, 1969; Slesinski & Ellingboe, 1971).

5) Resistance to powdery mildew infection is associated with reduced haustorium development and the colony size is related to haustorium size (Carver & Carr, 1978).

6) Fungicidal action of some anti-mildew compounds is associated with abnormal haustorium development (Dekker & Van der Hoek-Scheur, 1964; Bent, 1978).

Thus although there is no unequivocal proof that haustoria transport solutes from the host to the superficial mycelia there is considerable evidence that they are essential to the process

IV) Metabolites: Transport and translocation.

A) Introduction

Translocation is defined as the 'transport of dry weight' (Canny, 1975a). Biotrophic associations usually result in accumulation of metabolites (Lewis, 1970; Bushnell & Gay, 1978). Pea mildew is no exception but the increase in dry weight of the infected leaves is confined to the parasite. It is thus clear that transport of organic material occurs from host to parasite and further experimental demonstration of this is the assimilation of ^{14}C photosynthates by the mycelium (fig. 3). In other powdery mildew and rust infections the dry weight of the host tissue also increases (Millerd & Scott, 1963; Bushnell, 1967; Holligan, Chen & Lewis, 1973). However, this accumulated material disappears during the dark period and this process is probably closely related to the nutrition of the fungus (Lewis, 1970).

In the normal physiology of the healthy plant, organic matter is translocated from sites of synthesis and storage to regions of growth. The parasite competes with these functions and redirects transport for its own needs, (Smith et al, 1969; Lewis, 1970; Bushnell & Gay, 1978). Implicit in the biotrophic mode of nutrition is the ability of the parasite to collect nutrients without degrading the host tissue (Lewis, 1973). Haustoria are formed within living host cells and as discussed above have a role in this interchange of substances between host and fungus.

The following discussion considers the dynamics of the transport system in terms of its major components i.e. the host tissue, the interface and the mycelium. Since the transport system maybe as dependant upon the quantities and composition of nutrients in these components as much as on the specific mechanisms involved, prior to discussing the pathways and mechanisms of transport it is important to consider the distribution of metabolites and the biochemical transformations associated with transport.

B) Metabolites.

i) Nutrient source: the host.

The sites where new organic compounds are synthesized or remobilised from storage products are termed sources (Canny,

1975a). The mesophyll cells are the most immediate source in the infected pea leaf, but their photosynthetic products may be augmented by material from the vascular tissue transported from other regions of the plant. In powdery mildew infections, redirection of translocates to infected leaves is well documented (Bushnell & Gay, 1978) and without such redirection it is unlikely that metabolites would accumulate in infected areas since there is a marked decrease in the rate of photosynthesis, (table 4) and the respiration rate of the host tissue increases several fold, (Scott, 1965; Ayres, 1976).

The host fraction which was analysed in this study contained haustoria in the epidermal cells and it is to be expected that they may confuse the results of the analysis of host assimilates. The contribution of the haustorial complexes to the assimilates of the infected leaf can be computed from the data obtained for isolated haustorial complexes. There were approximately 16,400 haustoria per 100 mm^2 of leaf and these had a dry weight of 44 μg (Results, VII, B, iv). Since the dry weight of the leaf was 1.8 mg/100 mm^2 the complexes represented about 2% of the whole fraction. The true figure is even lower because the haustorial complex fraction contained host contaminants. Thus the contribution of the fungal component to the host analyses can be ignored. This conclusion is supported by isotope experiments from which it can be computed that the complexes contributed only 0.2 - 1 % of the label in a unit area of leaf (table 16).

The carbohydrate metabolism of the host is a key function in biotrophic associations (Smith et al, 1969; Lewis, 1976) and this will now be examined for the infected pea leaf.

a) Low molecular weight carbohydrates.

The total quantities of low molecular weight carbohydrates were greatly reduced upon infection. These results from pea agree with those of Allen (1942) for mildewed wheat at the time of sporulation and although he detected greater quantities of soluble carbohydrates prior to sporulation they indicate that levels of these compounds in the leaf are reduced when the nutritional demands of the fungus were at their greatest. That soluble carbohydrates are transported to the parasite was also indicated by the rapid incorporation of $^{14}CO_2$ into glucose, fructose and sucrose (table 20) during uptake by the mycelium.

The relative amounts of hexose and sucrose in healthy leaves are regulated by the enzymes invertase and sucrose synthetase (Maclachlan et al, 1970). Infection did not appreciably alter the proportion of these sugars in the host and this is consistent with the similar invertase activities in healthy and diseased leaves.

b) Starch metabolism.

Analysis of ^{14}C assimilates (table 4) indicated that starch synthesis was repressed in infected pea and similar results have been obtained for mildewed oak (Hewitt & Ayres, 1976). These results contrast with findings for wheat infected with E. graminis

where starch accumulated in and around lesions, (Allen, 1942); similar results have been obtained for rust lesions, (Wang, 1961; Thrower, 1965). E. graminis and rusts form distinct lesions and these starch accumulations are mobilised in the dark and then absorbed by the fungus (Thrower, 1965). The repression of starch synthesis in leaves infected with Microsphaera alphitoides and E. pisi, which form extensive confluent colonies permits a larger proportion of photosynthate to be mobilised; this would be an advantage to the parasite.

ii) The conduit; the haustorial complex.

Analysis of the isolated haustorial complex provides a means of directly detecting compounds which may be in transit at the interface. This is directly comparable with experimental studies of translocation in a green plant where the best evidence that sucrose, raffinose-based oligosaccharides and polyols are the translocated compounds in phloem is from direct sampling and analysis of the sieve tube exudates (Ziegler, 1975). As the haustorial complex may be considered to be a conduit for compounds from host to the mycelium, the detection therein of compounds specific to the host suggest that they are translocates. However, such conclusions are subject to qualification since the haustorial complex contains both an interfacial and fungal component and the compounds cannot be specifically assigned to either the extra-

haustorial matrix or the haustorial cytoplasm. Also there may be loss of compounds from the complexes during their isolation. The isolation procedure is lengthy and involves cold osmotic stress in a glycerol gradient. Similar treatment has been shown to rapidly increase the efflux of ions and amino acids through oat coleoptile plasma-membranes (Rubinstein et al, 1977), the membranes of complexes may undergo similar changes during isolation. The complexes were shown to have a lower proportion of soluble sugars than the host and mycelial fractions and although this component may be underestimated by host contaminants contributing to the dry weight of the fraction, it indicates that some sugars may be lost. Similarly in the ^{14}C labelling experiments only a very small proportion of label in the complexes was detected in low molecular weight compounds. On the other hand there is no a priori reason for a direct relationship between the mass of a vehicle and its translocate. Further, as discussed above, there is evidence to suggest that the extrahaustorial membrane is semipermeable and this would indicate that leakage is quite small.

In conclusion the problem of leakage during experimentation is difficult to assess and although it probably has little effect on the qualitative identification of compounds in the complexes it may possibly affect the the quantitative arguments relating to mechanisms of transfer which are discussed below.

As well as acting as a conduit for nutrients from host to mycelium the haustorial complexes are actively growing entities where compounds are being deposited as structural components. The analysis of complexes from plants after both pulse and chase labelling experiments was essential to resolve compounds which were transient in the complexes during nutrient transfer from those constitutive compounds which represent nutrient sinks in the complex. When the quantity of radioactivity in a specific compound decreased with chase treatment then this compound is termed transient. Unfortunately insufficient time was available to analyse all biochemical classes to their specific compounds viz. amino acids, organic acids and nucleotides, proteins, RNA and DNA. Although the number of counts in these classes of compounds all increased with chase and thus they appear as non-transient but as for the neutral sugars this may mask changes in the labelling of their constituents and they may contain transient compounds.

a) Transient compounds.

1) Sucrose.

Analyses of the ethanol soluble carbohydrates (table 26) and ^{14}C labelled assimilates (table 21, pulse labelling experiment) in isolated haustorial complexes show that sucrose was the predominant sugar with glucose and fructose present in significant

quantities. The subsequent reduction in labelling of sucrose after the 20h chase period indicates that it was metabolised *in situ* or translocated to the mycelium. Since the haustorial complex comprises a fungal compartment and one which, lying between host and parasite is essentially intercellular, the interpretation of mechanisms of transport depends on the exact position of the assimilates. This point is discussed further in section IV, C, ii, c.

2) Glycerol.

The labelling of glycerol in the haustorial complexes showed a similar pattern to that of sucrose and its occurrence there indicates a role for this compound in host-parasite transport. Since it is present in only trace quantities in the host and mycelial fractions it is likely that its transport requires specialised processes at the interface. An analagous system may be that of the symbiosis of zooxanthellae and several animal hosts (Muscatine, 1967; Smith *et al*, 1969), where glycerol constitutes a small proportion of the soluble compounds of the algae when free living but it is the principal compound released by them to its animal host. The animal cells are rich in lipid (Smith *et al*, 1969) and glycerol is presumably incorporated into them after forming esters with fatty acids. Thus it may be significant that lipids and in particular triglycerides are rapidly formed in haustorial complexes and further suggests that a selective transfer of glycerol

occurs across the host-parasite interface of pea powdery mildew. An exciting experiment would be to reconstruct the release as achieved for the zooxanthellae by Muscatine (1967) by adding haustorial extracts to pea cells.

An alternative proposal is that glycerol is formed within the haustorium from other compounds of host origin as a prelude to the synthesis of triglycerides. However, the immediate precursor of glyceride esters is glycerol-3-phosphate (Brennan & Losel, 1978) which is formed directly from the glycolytic pathways, and the formation of free glycerol is not obligatory for lipid metabolism. A further explanation for the labelling is its formation by the activity of an haustorial lipase on triglycerides formed from other translocates. Lipolytic activity has been detected in the primary vesicle, an intracellular feeding structure (Andrews, 1975) of Bremia lactucae, (Dudderidge & Sargent, 1978). However, subsequent loss of labelled glycerol but the retention of labelled triglycerides with chase would indicate that the lipids formed are not further metabolized. Some glycerol may be translocated to the mycelium where it is only detected in small quantities after the pulse $^{14}CO_2$ exposure.

In conclusion, the evidence suggests that sucrose and glycerol are transported at the host-parasite interface. The proportion of

^{14}C in these compounds in the complexes was less than 3% of the total radioactivity in the haustorial complexes after the pulse exposure to $^{14}CO_2$. This indicates that they may be transported so rapidly that accumulation does not occur. There is good evidence for high fluxes through the complexes and this is discussed below (section IV,C,ii).

b) Non-transient compounds.

'The places of disposal of the body substances (translocates) for the formation of new organs or the laying down of reserves' are termed sinks, (Canny, 1975a). This definition is used for studies in the whole plant translocatory system and is equally applicable to non-transient compounds in the host-parasite transport system studied here. The sinks of prime interest are in the fungus, these have been evaluated in the mycelial and haustorial complex fractions. The interfacial structures viz; the extra-haustorial membrane and matrix also represent potential sinks and as they are isolated as part of the haustorial complex the isotopic labelling experiments have permitted resolution of some possible constituents of these structures.

1) Trifluoroacetic acid hydrolysate.

The residual material hydrolysed by 2N TFA in the haustorial complexes (table 15) is the largest sink within the complexes. It was composed mainly of glucose, with an unidentified acidic component present in significant quantities. The glucan was

most likely to have been either part of the extrahaustorial membrane or the extrahaustorial matrix. It is unlikely to have been part of the fungal cell wall because analysis of the mycelial cell wall showed very different ratios of glucose, mannose and galactose. Further, Gil (1976) has shown that the extrahaustorial membrane and matrix are especially rich in polysaccharide and are labile in cell wall degrading enzymes and sodium hydroxide. He failed to detect any fibrils which might have represented cellulose. Further, since cellulose would have resisted sodium hydroxide he concluded that pectins and hemicelluloses were its constituents. It is probable that these polysaccharides were the glucans detected in the ^{14}C pulse-chase labelling experiments. Non-cellulosic β-linked glucans are a constituent of the xyloglucans of pea cell walls, (Gilkes & Hall, 1977) and are common in many forms in the cell walls of many different plant species, (see survey of Robinson, 1977). There is also evidence that polysaccharides are constituents of a host-parasite interface from studies of Piptocephalis virgineana (Manocha, 1978). Using autoradiography and biochemical analysis combined with the specific chitin synthetase inhibitor polyoxin-D, he has demonstrated that chitin was a constituent of the layer which surrounds the haustorium of the mycoparasite in its mucoralean host Choenophora cucurbitarum. However, this region named the sheath by Manocha (1978) is equivalent to an encasement (Bracker & Littlefield, 1973)

which in fungal-higher plant interactions has been thought to play a part in host resistance (Heath, 1971; Heath & Heath, 1971; Manocha, 1975). Thus it is unlikely that the carbohydrate around the haustoria of Piptocephalis virgineana can be directly compared with the extra-haustorial matrix formed in compatible interactions between plants and powdery mildews.

No evidence has been obtained that callose is a constituent of the interface. Staining with aniline blue shows strong fluorescence by the papillae which are sometimes present around the haustorial neck in vivo but no fluorescence around the haustorial body or lobes (P. Spencer-Phillips, personal communication). However papillae are not isolated with the haustorial complexes and it is thus unlikely that the glucans detected here originated in callose.

Incorporation of ^{14}C - glucose from UDP - ^{14}C-glucose into ethanol insoluble compounds by intact complexes suggests that the glucan synthesis may also occur by isolated haustorial complexes. Synthesis of non-cellulosic β-linked glucans by isolated plant membrane fractions has been investigated by many workers (Robinson, 1977). This activity is restricted to the Golgi apparatus and plasma membrane. The Golgi enzyme has a higher substrate affinity and is though to be concerned with the synthesis of β-linked glucans which are alkali soluble (Van der Woude et al, 1974; Raymond et al, 1978).

Chao et al (1978) have shown that activity of β-glucan synthetase in pea stem is regulated by a soluble cytoplasmic factor, it is possible that the parasite interferes with such a regulator and induces the host to produce large quantities of glucans which become associated with the membrane. The extrahaustorial membrane is unique in the normal pea plant or indeed in other uninfected plants. Its thickness, structure and polysaccharide content is not found in any other cellular membrane of plants and it is likely that highly specific mechanisms are involved in its biogenesis.

The results of the experiments with UDP ^{14}C-glucose which indicated low levels of uptake and incorporation (table 35) are seemingly inconsistent with the relatively high glucan synthetase activities found in haustorial complexes. However, the UDP - ^{14}C-glucose concentration was 100 fold greater than in the enzyme assays. As alkali soluble glucan synthetases usually have a Km in the low μM range (Van der Woude et al, 1974) enzymic incorporation would have been near to its maximal velocity during the enzyme assays, thus it is not surprising that the 100 fold increase in substrate concentration used for uptake studies was followed by an increase of only 1.7 in ^{14}C incorporation, (cf. tables 34 & 35).

The second most abundant component of the TFA hydrolysate was an acidic compound but it was not identified further. No evidence for the pectic compounds indicated by the staining reaction of the matrix with ruthenium red (Caporali, 1960; Dr. C. Sargent, unpublished) was found. Nor could the lability of the extrahaustorial

membrane in cell wall degrading enzymes be further explained. The absence of any affect on it by EDTA suggests that polyuronides were not present in it. However it must be recognised that acid hydrolysis degrades most uronic acid residues and it is thus unlikely that these could have been detected in the labelling experiments. However none of the neutral sugar components characteristic of pectins in plant cell walls, eg. arabinose, ribose, rhamnose, galactose were detected in quantities to suggest their deposition during the 20h period of the experiments. The acidic component should be further analysed as it is an important component of the complexes.

2) Lipids.

Lipids in the haustorial complexes became rapidly labelled after the pulse ^{14}C exposure and as there was a further increase with chase and no change in composition there are clearly sinks in the haustorial complex.

The triglycerides which were the major lipid component probably correspond to the osmiophilic droplets frequently observed in haustoria of E. pisi by electron microscopy, (Gil, 1976). Lipids have also been reported in haustoria of other powdery mildews viz. E. graminis, (Bracker, 1968); E. cichoracearum, (Mckeen et al, 1966); rusts, (Hardwick et al, 1970; Coffey et al, 1972; Losel, 1978); downy mildews (Berlin & Bowen, 1964). Moreover their high frequency in the intracellular hyphae of vesicular

arbuscular mycorrhizas (Cox & Sanders, 1974) and plasmodia of Plasmodiophora brassicae (Brennan & Losel, 1978) suggests that a specialised lipid metabolism is a common feature of biotrophic associations which have a higher degree of intracellular association and the uptake of lipid precursors may occur at these interfaces.

The lipid droplets observed in micrographs occupy a very small volume of the complex by ^{14}C labelling indicated that lipids constituted up to 30% of the total assimilate. This discrepancy may be reconciled because the droplets are non-aqueous organic solutions in which ^{14}C is highly concentrated whereas most of the other regions of the complexes are aqueous solutions or hydrated polymers. The lipids are probably produced as storage compounds but their formation may also be especially important because it removes carbon compounds from the aqueous phase while permitting their retention in a structure whose function is to translocate compounds to the mycelium. The rapid synthesis of lipids thus provides a copious sink close to the interface thus maintaining a concentration gradient for its precursors. This is most likely since results have indicated that glycerol is possibly transported from the host. The positions of the label in the triglycerides and polar lipid molecules were not investigated and although it may have been confined to the glycerol backbone it seems likely during the 20h chase period incorporation into the acyl chains of these compounds would have occurred.

The dependence of biotrophic fungi on their hosts for the necessary precursors of lipid metabolism is not known. Some dependence has been demonstrated for the haustorial mycoparasite Piptocephalis virginiana on its host Choenophora cucurbitarum, (Manocha, 1975). The parasite is unable to synthesize linolenic acid in spores produced during its restricted growth in vitro but assimilated an abundance of it in spores formed during the parasitic phase, despite this apparant dependence addition of linolenic acid to the medium did not promote axenic growth of the parasite, (Manocha & Campbell, 1978). Similarities in the sterols of spores of Plasmodiophora brassicae and those of the host from which they were isolated similarly has suggested some dependance on the host, (Knights, 1970). However work on rust fungi has shown that hexoses lead to higher rates of fatty acid synthesis than acetate by the fungus in infected leaves (Ziegler, 1971) and this has prompted Reisner (1976) to suggest that these may be derived from acetyl phosphate from the pentose phosphate pathway, these mechanisms would obviate the necessity of transferring fatty acids from host to parasite. These aspects of lipid metabolism need to be investigated in powdery mildew infections before any further conclusions on the dependence of the fungus on the host can be concluded.

The polar lipids of the haustorial complex may also be of considerable interest. These are probably located in the extra-

haustorial membrane and haustorial plasma membrane. In view of their proposed roles in nutrient transport (section IV,C) these merit further analysis.

3) Mannitol.

This compound was significantly labelled during the pulse exposure, afterwards the level increased considerably so that by the end of the 20h chase period most of the label in the neutral sugar fraction in the complexes was in mannitol. Unlike sucrose the mannitol present was almost certainly located in the haustorial cytoplasmic compartment of the complex since this polyol was the major soluble carbohydrate of the mycelium and was barely detected in the host fractions. Since the chase was performed in the light, unlabelled carbon compounds were presumably being transferred to the mycelium while the complex retains a pool of ^{14}C - mannitol absorbed earlier. This suggests a separation of transient transport pools from a more slowly turned over (storage) pool of low molecular weight carbohydrates within the complexes.

The labelling of mannitol in the complex is also significant in view of the hypothesis of Smith et al (1969) that a conversion from host sugars to fungal polyols maintains the concentration gradient of host assimilates to the fungus. Polyols are not readily utilizable by many higher plants (Lewis & Smith, 1967a) and thus the host cannot compete with the fungus and reverse the flow of assimilates. It is probable that there are specific

permeability barriers to prevent a reciprocal flow of polyol to the host. Moreover, there is evidence that mannitol disturbs the functions of parasitic associations. Applied to infected epidermal strips of lettuce, mannitol inhibited development of E.cichoracearum and similarly rust lesions became necrotic when fed with sugar alcohol solutions, (Samborski & Forsyth, 1960). Thus although transformation of host metabolites to sugar alcohols may provide a valuable mechanism in maintaining the concentration gradient of the translocates, it is essential that counterflow of the polyol does not occur and in this respect it is significant that the concentration of mannitol in the haustorial complex is much lower than in the mycelium.

iii) The Mycelium.

Although some nutrient sinks were detected in the haustorial complexes most of the assimilates absorbed were translocated to the surface mycelium and spores with only a small percentage ($< 1\%$) of the label residing in the complexes. The mycelium is thus the major sink and analysis of pulse-chase labelled compounds has shown that a sequence of conversions of assimilates occur in the mycelium and thus, as for the complexes transient and non-transient compounds were recognised. As before the distinguishing criteria were decreasing quantities of

radioactivity in transient compounds and increasing levels in non transients.

a) Transient compounds.

1) Neutral sugars.

The most clearly marked group of transient compounds was the neutral sugar fraction. The largest decrease (30 fold) in labelling was in mannitol and significant reductions in the number of counts in sucrose, glucose, fructose, trehalose and glycerol, (table 22).

Sucrose, glucose & fructose:

These sugars are characteristic of the host and are probably absorbed from it by the haustorium and then translocated to the mycelium. The small quantities of label detected in these compounds at this location suggests that they are turned over very rapidly. A more precise description of the kinetics of the transformation has been given by Edwards & Allen (1966). In E. graminis mycelia, sucrose initially contained a larger proportion of the label than in mycelia of E. pisi and its level declined rapidly with continued exposure. In E. pisi detection of these sugars in the mycelium after the pulse is consistent with their presence in the haustorial complex and further evidence that some transfer occurs by way of the haustorium.

Glycerol:

As for the host sugars this is detected in the mycelium after the pulse and since it comprises a major component of the neutral sugar fraction in the complexes, it also is probably translocated from the haustorium. The lipid fraction of the mycelium which was mainly triglycerides was also a transient component and it seems likely that glycerol is incorporated into the lipids synthesised and that these serve as primary sinks in the mycelium for this compound and are then either further metabolised or are incorporated into spores. The latter effectively leave the mycelial fraction at spore release.

Mannitol & trehalose:

These compounds, and especially mannitol, were highly labelled after the pulse $^{14}CO_2$ exposure. Mannitol was an abundant constituent of the mycelia and constituted 50% of the label after the pulse and 7% of the total dry weight. There is no clear evidence as to whether the mannitol in the mycelium was translocated from the small amounts formed in the haustorium or if it was formed in the mycelium from host sugars imported from the haustorium. However regardless of the site of transformation the rapid conversion of host sugars to mannitol and trehalose is a feature common to many biotrophic fungi associated with green plants, (Smith et al, 1969). Ultimately (after chase) the mannitol and

trehalose showed large decreases in labelling. These observations suggest that they act as primary sinks in the fungus but are subsequently further metabolised to constituents of other compounds eg. glycogen, cell walls, protein and nucleic acids. The metabolism of sugar alcohols and glycogen is though to be interrelated in many fungi: (Lewis & Smith, 1967a). A direct stoichometry between mannitol and glycogen synthesis has been shown to exist in Dendryphiella salina and this is critical to osmotic regulation during the active uptake of sugars by the fungus (Jennings & Austin, 1973). It seems likely that a similar relationship exists in E. pisi with sugar alcohols acting as an intermediate metabolite between host sugars absorbed and glycogen which is the ultimate major storage product. The other possible physiological roles of sugar alcohols in fungi have been discovered in detail by Lewis & Smith, (1967a); Smith et al, (1969) and Brown, (1978) and are considered important in providing;

1) an energy store.

2) osmo regulation.

3) compatible solute to maintain enzyme activity at low intracellular water activity.

4) non-host metabolite to maintain concentration gradient in biotrophic fungi.

Arabitol and erythritol were also detected in the mycelium and as they showed very little change in labelling after the chase period (slight decrease of arabitol and slight increase of erythritol) they should probably be considered as secondary sinks rather than transient compounds. They are most probably located in the spores, a conclusion drawn by analogy with the results of Edwards & Allen (1966) who found that arabitol was predominant in E. graminis conidia, and mannitol was most abundant in hyphae.

Amino acids, organic acids & nucleotides:

These classes of compounds were also transient but as they were not further analysed here. Mount & Ellingboe (1969) have shown that ^{35}S and ^{32}P are readily transported to the mycelium and it is most likely that these were constituents of some amino acids and nucleotides. Clearly further experimentation should include a full analysis of these components.

b) Non transient compounds.

1) Glycogen, cell walls, protein, RNA & DNA:

These are the ultimate and major sinks in the fungus and the large increases in label detected in them are indicative of the very extensive development and sporulation of the pathogen on the leaf surface. Glycogen is a major constituent of the spores of

E. pisi with the electron microscope and has been seen in the spores of other powdery mildews (Perera, 1972). It is only very rarely observed in the haustorium and this is consistent with the small quantities of label detected in the glycogen fraction of the complexes.

c) Transport and translocation.

1) Host intercellular transport.

The primary sources of nutrients in an infected leaf are the phloem sieve tubes and the host chloroplasts. Nutrients thus originate in the host symplast. Movement of substances is diffusive following concentration gradients but within cells it may be assisted by cytoplasmic streaming or possibly by electrical potentials, (Crafts & Crisp, 1971). Cell to cell transport in the host may not require transfer across plasma membranes the pathway probably being achieved by plasmadesmata (Gunning & Robards, 1976). However substances cannot move directly to the haustorium via the symplast since the extrahaustorial membrane separates it from the infected cell cytoplasm. Further, it has recently been reported that in pea leaves, although plasmadesmata are frequent between adjacent epidermal cells they rarely occur between infected epidermal cells and palisade cells (Bushnell & Gay, 1978). Thus substances moving from their source to the infected cells must at some stage pass

through the leaf apoplast. Direct access from the leaf apoplast to the haustorium surface is interupted by the bands around the haustorial neck (Gil & Gay, 1977). Thus nutrients are directed from the apoplast through the epidermal cytoplasm and are then transported across the extrahaustorial membrane to the haustorium.

From this discussion, it follows that the quantities of solutes in the apoplast may be expected to influence powdery mildew development. Thus Thatcher (1942) measured the time required for plasmolysis to determine the permeability of mesophyll cells in Brassica rutabaga infected with Erysiphe polygoni. He deduced that the permeability increased upon infection and proposed that this facilitated transport of nutrients to the fungus. Williams (1958) attempted to repeat Thatcher's experiments on mesophyll cells of mildewed barley but found considerable variation in the rates of plasmolysis and no significant differences in the permeability of cells of infected or uninfected tissue could be detected. Thatcher's experiments with rusted tissue are more persuasive as deplasmolysis in urea solutions was used in this instance and these experiments clearly indicated an increase in membrane permeability to this non-electrolyte.

Bushnell (see, Bushnell & Gay, 1978) found that for barley infected with Erysiphe graminis, leached nitrogen compounds represented a larger proportion of the total nitrogen in infected

leaf segments. It is likely that much of the nitrogen was in protein and that lower molecular weight, amino acids and peptides were leached. The differences in loss may therefore be due to a larger proportion of the nitrogenous compounds being in the form of amino acids and ammonium ions in mildewed tissue as reported by Sadler & Scott, (1974) rather than an alteration in membrane permeability. Similarly Ayres (1977) and Hoppe & Heitefuss (1974a) measured the leakage of ions from mildewed pea leaves and ions, sugars and amino acids from rusted bean leaves respectively. They only estimated the solutes in the ambient medium and thus did not consider the concentration gradient of the particular solutes leaked. Solute concentrations vary considerably with infection but usually the concentrations of ions, (Shaw et al, 1954; Shaw & Samborski, 1956), sugars (Lewis, 1971; 1976 and references therein) and amino acids (Sadler & Scott, 1974) increased upon infection. Thus again, increased quantities of leached solute are not directly indicative of increased membrane permeability. Thus although in biotrophic systems there are well authenticated reports that solute leakage increased after infection there is no unequivocal evidence which demonstrates that it is due to increases in host membrane permeability. Indeed leakage of ^{14}C photosynthates from infected pea leaf discs measured here were related to the soluble label in the leaf discs and it was evident that there was a slight decrease

in the membrane permeability, leakage experiments after the removal of the infected epidermis showed that this reduction was not due to enhanced nutrient scavenging by the infected epidermis but truly reflected the membrane permeability of the mesophyll cells.

However when the pathogen is present it is most likely that a concentration gradient is set up between the mesophyll cells and the infected epidermal cells and that despite a reduced permeability solutes pass rapidly between these cells and suggests that a process analagous to phloem loading in healthy leaves is occurring. In a comprehensive assessment of phloem loading in higher plants Geiger (1975) suggested that substances move through the symplast to a point near the minor veins and after entering the apoplast, are actively absorbed by the sieve element companion cell complex. Changes in the translocation pattern observed following infection (Hewitt & Ayres, 1976) indicated that these processes were impaired and this may also lead to less solute leached from the apoplast of the diseased leaves and to a greater level of solutes in these leaves. Thus, the translocation pattern in the leaf would be dominated by new gradients caused directly or indirectly by the fungus.

Support for these hypotheses is provided by observations indicating apoplastic transport of sugars from mesophyll to

epidermal cells in Commelina communis. Labelled sugars appeared in stripped epidermes replaced upon the mesophyll which had previously been exposed to $^{14}CO_2$ (Dittrich & Raschke, 1977). That epidermal cells are heterotrophic prior to infection suggests that systems capable of maintaining high flux rates to haustoria (see below) may already exist in the epidermal plasmamembrane. Intercellular transport of specific compounds and its relationship to phloem loading in infected leaves has received little attention at the cell membrane level. Pea mildew which permits separation of the parasite, infected cells and mesophyll cells offers a valuable experimental system for such studies.

ii) Host-haustorium interface.

The interface is a multicomponent structure and from ultrastructural studies two pathways across it can be defined, (Gil, 1976). As the haustorium is physiologically isolated from the apoplast of the leaf solutes must cross the extrahaustorial membrane and may then either enter the fungus directly where the walls of the haustorial lobes contact it, (Gil, 1976) or diffuse across the matrix and then be absorbed by the fungus. Comparison of fig. 6 and fig. 8 shows that although transfer of label to the complexes is initially rapid, respiration of these nutrients is significantly delayed. Although this may be due to a lengthy conversion to a respirable substrate it seems likely that assimilates in the complex

reside first in the matrix and that they enter the haustorial cytoplasm in a second stage. No evidence for or against the more direct pathway at lobe tip connections has been obtained.

a) Fluxes.

Prior to a discussion of the mechanisms of transport at the interface it is imperative to obtain estimates of the rates of nutrient flux through the haustorial complexes and across the interfacial membranes. Table 4, shows that infected, illuminated leaf discs assimilated 9.23×10^3 dpm mm^{-2} leaf $5\ min^{-1}$ from an atmosphere containing $^{14}CO_2$ (0.03% v/v). As the specific activity of the $^{14}CO_2$ was 60 mCi/mmol the rate of fixation was 6.93 nmol $100\ mm^{-2}$ $5\ min^{-1}$. Figure 3 shows that during photoassimilation, 20-30% of the assimilated $^{14}CO_2$ was transported to the mycelium and thus the rate of assimilation by the fungus was 2.08 nmol $100\ mm^2$ leaf $5\ min^{-1}$. Since fixation of 1 mole of CO_2 results in 1/6 mol of $C_6H_{12}O_6$ this is equivalent to 0.21 ng of labelled carbohydrate $100\ mm^2$ sec^{-1} being transferred to the mycelium. There was no attempt to remove the unlabelled atmospheric CO_2 prior to feeding $^{14}CO_2$ and therefore the actual specific activity was probably lower than this and the flux to the mycelium proportionately greater. Servaites & Geiger, (1974) recorded a sucrose flux of 10 ng $100\ mm^{-2}$ leaf sec^{-1} for active phloem loading in sugar beet leaves at similar light intensities. In view of the greatly reduced rate of photosynthesis (table 4) and

increased rates of respiration (Ayres, 1976) in mildewed pea leaves these figures may be more closely comparable than they first appear. Additionally this estimate for flux to the mycelium takes into account only labelled ^{14}C. If one assumes that most of the $^{14}CO_2$ is assimilated to soluble carbohydrate considerable dilution of the ^{14}C by unlabelled solutes already present would occur. The soluble carbohydrate content of the infected leaf was 65 μg 100 mm^{-2} leaf. Thus if all the fixed ^{14}C were mixed immediately with the whole soluble carbohydrate pool of the leaf and 30% of this pool was <u>in transit</u>, the flux would be considerable (19 μg 100 mm^{-2} sec^{-1}. This is three orders of magnitude greater than that for phloem loading in beet and as the CO_2 fixation rate here was only 0.693 ng 100 mm^{-2} leaf sec^{-1} the proposition is clearly untenable. This indicates that in the leaf that only a part of the assimilated $^{14}CO_2$ enters the 'transport pool' which is transferred to the mycelium and there is little mixing with soluble carbohydrates resident in the leaf. Such a compartmentalization of soluble storage and transport pools have previously been proposed for plant cells (Nelson, 1962; Garrard & Humphreys, 1971). Humphreys & Garrard (1971 a & b) expanded upon this possibility after experiments with maize scutellum, storage sucrose, postulated to be in the vacuoles, leaked only slightly whereas sucrose in the transport pool, postulated to be in the vascular tissue, leaked

readily. On the other hand, in beet Geiger & Swanson (1965) showed kinetic evidence indicating that almost all the leaf sucrose was available for translocation, but Sovonick et al, (1974) showed that 80% of the leaf sucrose was already contained in the sieve element - companion cell complex and were thus already compartmentalized. The location of the transport and storage pools in mildewed pea are not known. These experiments also indicate that in the light, the major translocate may be newly synthesised assimilates and that nutrients redirected from healthy parts of the plant (Edwards, 1971; Hewitt & Ayres, 1976) contribute significantly to the translocate only during the dark period.

The flux of carbohydrate through the haustorial complexes may also be computed by extending the calculations made above. In single surface infections the haustorial frequency was 16,400 100 mm^2 of leaf and thus the flux of ^{14}C - labelled compounds through each complex was, 0.013 pg haustorial complex $^{-1}$ sec^{-1}.

These estimates are obtained from experiments and calculations which involve very few assumptions although it is presumed that all the label was transported by the haustoria and that carbohydrates are the major translocates, these assumptions are not unreasonable in view of the evidence presented in this thesis, (sections, III & IV). They provide minimal estimates of the transfer of photosynthates through the complexes to the

mycelium. Other, but less certain estimates may be obtained from the transit time of compounds in the haustorium calculated from the number of counts in the complexes and mycelia per unit area of leaf. Absolute values may then be obtained from knowledge of the soluble carbohydrate or dry weight of the complexes. Unfortunately the long period required for brushing prior to isolation which causes some transport to cease prior to sampling and the lack of precise identities and specific activities of translocates makes the estimates unreliable. Thus all further discussion of membrane fluxes is based on data obtained from the disc experiments.

The extrahaustorial membranes & haustorial plasmalemma:

In order to discuss possible mechanisms of nutrient transport across this membrane it is important to quantify the flux. The idealized model of the haustorial complex used for light microscopic estimation of volume also allows the area of the extrahaustorial membrane to be calculated. The surface area of the volume included by 1.82×10^5 haustorial complexes was 100 mm^2. The minimal flux of carbohydrate across this membrane was thus 2.4 ng 100 mm^{-2} sec^{-1}. Since Gil (1976) has shown that the extrahaustorial membrane is convoluted with many microvilli which are not recognisable with a light microscope, this rate is an

upper estimate but provides a clear indication of the order of magnitude. The flux is compared to membrane fluxes of carbohydrate across other plant membranes in table 41. It greatly exceeds (10^2 - 10^6 fold) the slow diffusion down large concentration gradients estimated for algal cells, carrot callus cells and isolated chloroplasts. It is approximately one seventh of the flux observed for phloem loading in sugar beet leaves and glucose uptake by Neurospora crassa. The last two are coupled to energy input, (Sovonick et al, 1974; Scarborough, 1971, a & b). The rate for the haustorial complexes is close to that obtained for sucrose uptake by maize scutellum tissue (Humphreys, 1973) where its transport has also been shown to be ATP coupled, (Humphreys, 1978). A recent survey by Komor, (1977) has shown that scutella show higher rates of sucrose uptake than many plant tissues. From these comparisons it is likely that carbohydrate transfer across the extrahaustorial membrane is not mediated entirely by simple diffusion. This estimate of a transmembrane flux is the first obtained for a biotrophic association of a higher plant and a fungus.

Light microscopy does not permit estimation of the area of the fungal (haustorial) plasmamembrane but from electron micrographs, Perera (1972) has estimated that in Sphaerotheca pannosa it was 2-3 fold that of the extrahaustorial membrane

(which was not villous as in pea). Thus carbohydrate fluxes of a similar order to those across the extrahaustorial membrane are likely and are well within the rate published for a free living fungus (Neurospora crassa, Jennings, 1976). However, because the extrahaustorial matrix and fungal wall probably represent a compartment sealed to diffusion continuous balance is not essential for efficient operation.

Membrane fluxes have been estimated in the lichen Xanthoria parietina where ribitol is transported between algal cells and intercellular hyphae (Collins & Farrar, 1978). Exchanges were not limited by diffusion between the two organisms but probably by transmembrane fluxes. The maximal uptake rate for the fungal plasmamembrane was very small and only one tenth of the efflux from the alga, (table, 41). This led Collins & Farrar, (1978) to suggest that carriers of ribitol on the fungal plasmamembrane might be restricted to regions proximal to the algal cells. Efflux across the algal plasmalemma in X. parietina was only one third of that calculated for the extrahaustorial membrane and if the convolutions noted above are taken into account, the fluxes are probably closely similar. The mechanism of transport across the algal plasmamembrane is not known but studies of glucose efflux from the alga in the lichen Peltigera polydactyla indicate that a specific carrier facilitated diffusion process operates. The rate of flux across the extrahaustorial membrane is too rapid

to be explained by simple diffusion and thus other similar facilitated transport systems must be considered.

Table 41. Membrane fluxes of carbohydrates in plant and fungal cells.

Organism (& membrane)	Method	Compound	Flux	Comments	Reference
			$ng/100/mm^2/sec^{-1}$		
Plagiothecium denticulatum (Plasmalemma)	influx	sucrose	0.03	down a 1M conc. gradient.	Spector, (1961)
Curcuma rubricaulis (Plasmalemma)	influx	"	"	"	"
Saccharum officinarum (chloroplast envelope)	efflux	"	0.05	"	Edelman et al, (1971).
Daucus carota, callus. (plasmalemma)	influx	"	0.02	from 0.1M medium.	"
	efflux	"	0.01×10^{-4}	to zero conc.	
Allium cepa, epidermis. (plasmalemma)	efflux	glucose	0.5	to zero conc.	Steinbrecher & Luttge, (1969).
Zea mays, scutellum.					
(plasmalemma)	influx	sucrose	4.2	ATP coupled.	Humphreys, (1973).
(tonoplast)	efflux	sucrose	0.08	From 0.1M to zero conc.	

Beta vulgaris, (plasmalemma of companion cell-sieve element complex, phloem loading).	influx	sucrose	18.0	Up 1:40 conc. gradient.	Sovonick et al, (1974)
Neurospora crassa (plasmalemma).	influx	glucose	16.2	ATP coupled.	Jennings, (1976).
BIOTROPHY					
Xanthoria parietina (algal plasmalemma)	efflux	ribitol	0.8		Collins & Farrar, (1978).
(Fungal plasmalemma)	influx	"	0.08	Vmax	
Erysithe pisi - Pisum sativum, (extrahaustorial membrane).	efflux	probably sucrose.	2.4		

b) Mechanisms.

Active transport or facilitated diffusion?:

Current research on plant cells has shown that sugar transport against a concentration gradient is associated with membrane depolarisation usually generated by ATP dependant proton pumps (Komor, 1977; Malek & Baker, 1977; Racusen & Galston, 1977; Humphreys, 1978). The extrahaustorial membrane has not been shown to have any physiological property although the balance of evidence discussed earlier strongly suggests that it is a semipermeable barrier.

The best indication of active transport is movement against a concentration gradient (Geiger, 1975). This distinguishes it from facilitated diffusion which is thermodynamically impossible against a gradient. It is difficult to quantify the concentration difference at the interface. Analyses of complexes together with the volume estimates indicate concentrations of 30 mM and 20 mM for sucrose and glucose respectively. These are lower than the cellular concentrations of these sugars commonly found in plants (Kriedemann & Beevers, 1967; Humphreys, 1973; Geiger, 1975; Fisher, 1978) but if they were restricted to the extrahaustorial matrix their concentration would be proportionately higher. Swelling experiments have indicated that the osmotic pressure in the matrix is less than that of 50 mM sucrose, but these experiments

were performed with already swollen complexes where the matrical solutes would have been greatly diluted. Hoskin, (1969) estimated that the osmotic pressure of the matrix of E. graminis - Hordeum vulgare was equal to that of the host cytoplasm, i.e. approximately equal to 0.3M sucrose thus indicating little or no gradient for transport. Sugars are unlikely to accumulate in the matrix because there they could be actively absorbed by the fungus. Thus until the compartmentation of host sugars in the complex is fully elucidated there is no evidence of transport against a concentration gradient across the extrahaustorial membrane of E. pisi - P. sativum. Because of the many cell components of both fungal and host which are involved in the transport system it is impossible to use criteria such as saturation kinetics or energy metabolism inhibitors for experimental investigation of active transport. Specific inhibitors active against the fungus or host as used in studies on a lichen may provide potentially powerful tools. In Peltigera polydactyla, using digitonin and deoxyglucose to interfere with uptake by the fungus and sorbose to inhibit glucose efflux from the alga, Chambers et al (1976) concluded that efflux depended upon a carrier specific facilitated diffusion process. However because the parasitic interface in powdery mildew infections is enclosed in a sealed

compartment, access to inhibitors may be precluded. The predominance of sucrose and glycerol in the neutral sugars of the complexes whilst glucose and fructose were dominant in the host fraction suggests specificity in transport across the membrane and specific carriers could be involved in either an active or facilitated diffusion mechanism.

Although it has not been possible to obtain evidence of concentration gradients experimental evidence for active energy coupled transport has been obtained from microdissection studies of Sullivan et al (1974) on barley infected with E. graminis. By incising infected cells, these workers obtained a correlation between the quantity of host cytoplasm which remained over the extrahaustorial membrane and the extension of the surface hyphae. That the host cytoplasm was essential for transport was also concluded by Gil, (1976) who used the fluorogenic ester, fluoroscein diacetate (FD_A)as a permeability probe. This method is commonly used as a viability test in plant and animal cells (Rotman & Papermaster, 1966; Heslop-Harrison & Heslop-Harrison, 1970) and depends upon the penetration of the ester into the cell and liberation of free fluoroscein by cellular esterases. As this molecule is charged, it accumulates if the plasma-membranes are functional and then can be seen by light microscopy. Gil (1976) observed fluorescence in haustoria of E. pisi in stripped epidermes of P. sativum but not in haustorial complexes isolated

from the host by the method used in this study. This observation is also in agreement with the very low levels of glucose and sucrose incorporation observed in fractions of isolated complexes and the lack of any respiratory response of isolated complexes to exogenous substrates. All these suggest that when the host cytoplasm has been washed from the extrahaustorial membrane the compounds are not accessible to the haustorium. However, these experiments raise the question of the viability of the isolated complexes and this requires a more detailed assessment.

The best evidence that isolated haustorial complexes are viable is that they still take up oxygen and liberate $^{14}CO_2$ absorbed from the host; thus their respiratory metabolism is apparently intact. The addition of glucose does not directly stimulate oxygen consumption, but similar results were obtained by supplying succinate to isolated complexes of Sphaerotheca fuliginea - Cucumis sativus, (Dekhuijzen, 1966). This compound would be directly metabolised by the haustorial mitochondria if it were taken up. The results of Gil (1976) in which fluorescein accumulated in complexes in stripped epidermes show that removal of the mycelium does not kill the haustoria and the haustorial cytoplasm is retained by a functional plasma membrane. The only treatment subsequent to removal of the mycelium which is likely to damage the haustoria is centrifugation into 80% glycerol. This must result in a considerable osmotic stress to the complexes. However,

the cytoplasm in the final fraction is still intact and turgid and there is no evidence to suggest that this treatment destroys the integrity of the haustorial membranes. Thus this discussion indicates that it is the permeability barrier of the extra-haustorial membrane in the absence of epidermal cytoplasm which is responsible for the lack of accumulation of FDA or exogenous sugars and not the viability of the isolated haustoria.

A mechanism in which the host cytoplasm may participate was proposed by Gil (1976). He suggested that reverse pinocytosis of Golgi vesicles may contribute to transport across the extra-haustorial membrane. This process would be active, (i.e. consume energy), (Robinson & Ray, 1977). Atkinson & Shaw (1955) demonstrated acid phosphatase activity around the haustorium of E. graminis but resolution with the light microscope was inadequate to specifically locate it. Similar activity is often found in large quantities in plant cells, strategically situated where a large amount of short-distance sugar transport occurs e.g. nectaries (Luttge, 1975). However, it is not a single specific enzyme and it is not known if the enzymes at these sites are involved in sugar or carrier phosphorylation or enhanced intermediary metabolism possibly concerned with polysaccharide synthesis and secretion. There are no estimates of the nature of the transmembrane solute fluxes that could be maintained by this process although rates of vesicle deposition can be prolific, (Gunning & Steer, 1975). Estimates of

the transit time for vesicles from the Golgi to the plasmamebrane vary between 2.5 - 30 min and it is thus difficult to conceive that this process could maintain the transport observed here where considerable transfer from the host chloroplasts to the mycelium had occurred in less than 5 minutes. Also no evidence was obtained here for the involvement of metabolites such as pectins and hemicelluloses which are characteristic of Golgi apparatus.

A mechanism which does not require the participation of the host cytoplasm to drive transport was proposed by Smith (1974) for biotrophic transport in lichens. He proposed that the higher membrane potential of fungi (cf. data in Slayman et al, 1970 and Barber, 1968) may depolarise that of the host membrane by either physical contact or by ion extrusion in regions of close proximity. Significantly, Gil (1976) has shown that the lobe tips of the haustorium intimately contact the extrahaustorial membrane and took up cation specific stains. It is not clear how removal of the host cytoplasm could impair this mechanism.

The extrahaustorial membrane is very different from the normal host plasmamembrane and the high flux rates observed in vivo may be directly related to a structural feature of this specialised membrane. It contains large quantities of polysaccharide and if these polymers traverse the lipid bilayer then pores may be formed permitting passage of nutrients which would normally be restricted.

Also, more subtle changes in membrane composition e.g. fatty acid chain length and saturation, substantially alter the permeability of plant membranes (Wade & Bishop, 1978). Such changes in the passive permeability are not consistent with the evidence for semipermeability presented earlier. Gil & Gay, (1977) proposed that the special properties of the extrahaustorial membrane were restricted specifically to this part of the epidermal plasmalemma by the neckbands which attach it to the haustorial neck. As nutrients enter and leave the epidermal cell this topographical separation of properties is important. The structurally unmodified epidermal plasmamembrane facilitates an influx of nutrients to the cell whilst the extrahaustorial membrane an efflux to the extra-haustorial matrix. Thus the structural polarity maybe essential to maintain the passage of nutrients through the cell.

It is evident from these studies than an understanding of the altered permeability of the extrahaustorial membrane is hindered by our ignorance of its composition, both enzymic and structural. Future research in this field should be directed at characterisation of its properties by cytochemistry and if possible, sub-fractionation of the isolated complex to obtain purified fractions of membranes for more complete biochemical analysis. In many associations of both parasitic and mutualistic types, intra-cellulity invariably involves envelopment of the invading structure by a host membrane (Bracker & Littlefield, 1973). Of these only the

membrane surrounding the bacteroid in the nodules of legumes has been isolated and characterised (Robertson et al, 1978; Verma et al, 1978). The peribacteroid membrane has significant ATP-ase activity and a very high lipid to protein ratio and both of these properties probably greatly influence transport between the symbionts. The extrahaustorial membrane is of host origin, (Gil & Gay, 1977) and both its production and the maintenance of high flux rates across it indicate that the pathogen has some control of the host metabolism. Clearly a regulation of its permeability greatly influences host susceptibility and is a further reason for fully understanding its properties.

c) Uptake by the haustorium.

Solutes passed out of the epidermal cell either enter the sealed compartment of the extrahaustorial matrix or approach the fungal plasmamembrane directly where the haustorial lobes are attached to the extrahaustorial membrane. The special circumstances of the second event have already been discussed and here special attention will be given to passage across the haustorial plasmalemma and the physiological significance of the matrix.

The first report bearing on the physiology of the extrahaustorial matrix in Hordeum vulgare infected with Erysiphe graminis

by Hirata (1967) who described it as an expansible buffering zone which would protect the host cell from excessive and potentially lethal absorption by the haustorium. Hirata (1967) also noted that an expanded matrix was associated with poor growth and therefore concluded that the condition impaired absorption by the haustorium. He was unsure whether the expanded matrix was the result or cause of this reduced absorption. Sullivan et al (1974) showed that when the matrix around the haustorium of E. graminis was swollen by hypotonic media after incision of the infected host cell, the haustorium degenerated rapidly. This observation and those of Hirata (1967) indicate that the nutritional and osmotic environment provided by the matrix constituents influence the efficient functioning of the haustorium. Expansion of the matrix would both dilute the nutrients therein and increase the distance through which nutrients must diffuse from the extra-haustorial membrane to the fungal plasmalemma. Both of these effects would hinder transport across the interface.

After transfer across the extrahaustorial matrix, nutrients are presumably absorbed by the fungus. Evidence has been discussed that this is an active mechanism and the large numbers of mitochondria present in haustoria indicate it is is well equiped for such a process which is documented in fungi (Jennings, 1976).

Analysis of haustorial complexes suggests that sucrose is the major compound released by the host. Unfortunately it is not at

present possible to locate this compound specifically to the matrix or haustorial cytoplasm. Thus less direct evidence must be interpreted to decide whether sucrose is absorbed directly into the haustorium or hydrolysed in the matrix prior to uptake.

Sucrose hydrolysis prior to uptake?

There is an impressive experimental history which supports the thesis that sucrose is only absorbed after hydrolysis to its constitutive hexoses. In biotrophic associations this has been discussed in detail by Lewis (1976) and Long et al (1975) and evidence is summarised in table 42.

Table 42. Evidence for hydrolysis of sucrose prior to uptake by biotrophic fungi.

	References
1) Saprophytic fungi invert sucrose prior to uptake.	Jennings, (1974) Walsh & Harley, (1963).
2) Biotrophic fungi invert sucrose prior to uptake in axenic culture.	Maclean, (1966). Dickerson, (1972).
3) Intact biotrophic associations hydrolyse sucrose prior to uptake.	Harley & Jennings, (1958). Harley & Smith, (1956).
4) Exogenous glucose and fructose compete with ^{14}C transfer from host to parasite (Inhibition technique).	Lewis, (1976).

5) Invertase activity is enhanced in biotrophic associations. Long et al, (1975) Mitchell et al (1978).

So far, all the biotrophic associations examined have large unspecialised areas of intercellular contact.

Evidence for the direct uptake of sucrose or following its hydrolysis in powdery mildew infections is available from several types of investigation. Edwards & Allen (1966) sampled the mycelium of E. graminis at intervals after exposing infected barley leaf sections to $^{14}CO_2$. After a 15 min exposure 57% of the isotope in the mycelium was in sucrose, and afterwards the proportion declined, thus indicating assimilation of this compound into fungal metabolites. Assimilated sucrose was also detected in the mycelium of E. pisi in the present study (table 22). In isotopic CO_2 experiments the labelled sucrose present after a 20h chase period fell to only 8% of that initially present. These experiments indicate that sucrose enters the mycelium and the haustorium may therefore absorb it intact. However, Long et al (1975) have criticized the results of Edwards & Allen (1966), and contended that the sucrose detected came from the leaf surface and was located outside the hyphae. A more careful consideration of Edwards & Allen's data shows that in a 15 min period if all the labelled sucrose in the mycelial sample had passed through the leaf surface 13% of the total label assimilated by the host would have penetrated the cuticle. This seems unlikely. The data presented

here for E. pisi is more equivocal as, based on leaf areas sucrose represented only 2.5% of the total assimilate in the mycelial fraction after a 30 min $^{14}CO_2$ exposure. A larger number of counts was detected in sucrose than in glucose or fructose in the mycelium but these hexoses were predominantly labelled in the host. Differences in these ratios argue against a leaf surface pathway, but possible preferential uptake of hexoses by the mycelium from the leaf surface would confuse the estimations.

The invertase activities of haustorial complex and mycelial fractions also provide indirect evidence to support the hypothesis of direct uptake of sucrose. The fungal invertase was almost totally soluble and thus apparantly has an intracellular location in the mycelium. Although it had a lower activity per unit protein the invertase in the haustorial complexes had similar pH characteristics and was also soluble. It is thus probable that the same enzyme exists in the haustorial complex and the mycelium. An intercellular location of invertase would necessitate uptake of sucrose intact. Unfortunately the localisation of an invertase by its solubility during homogenisation and centrifugation may be misleading because the pH and ionic strength of the extraction medium influence the dissociation of plant invertases from cell wall preparations, (Ricardo & ap Rees, 1970; Little & Edelman, 1973).

The total activity of invertase in the mycelium was higher than that in haustorial complexes. This suggests that most hydrolysis of sucrose occurs in the mycelium and it follows that as observed, sucrose predominated over its constituent hexoses in the haustorial complexes. Thus it could be absorbed in that form. However, it is likely that hydrolysis occurs in haustorial complexes and if the invertase present was in the matrix the constituent hexoses rather than sucrose may be absorbed. To test this possibility it is necessary to calculate the expected hydrolysis rate in haustorial complexes and compare it with the estimated flux of assimilate.

The activity of invertase in isolated haustorial complexes at 32° in 100 mM sucrose was 268 μg reducing sugar mg^{-1} protein $30\ min^{-1}$, and the activity per complex was thus 1.74×10^{-4} pmol sucrose haustorial $complex^{-1}$ sec^{-1}. The flux of carbohydrate per 100 mm^2 of infected leaf was 0.61 pmol sucrose $100\ mm^{-2}$ sec^{-1} and as there were 16,400 haustorial complexes per 100 mm^{-2} of leaf, the permitted rate of hydrolysis at 32°, 100 mM sucrose was 2.9 pmol sucrose $100\ mm^{-2}$ leaf sec^{-1}. Haustoria <u>in vivo</u> were at a lower temperature (25°) and probably in contact with lower concentrations of sucrose (30 mM) but even considering these differences it seems likely that the invertase of the complexes could hydrolyse a large proportion of the observed flux even if sucrose were the only mobile organic

compound. However, it should be noted that the estimate of flux used was a minimal figure and that larger values are likely.

It is probably significant that the invertase activity of the mycelium was considerably greater (77 pmol sucrose $100\ mm^{-2}\ sec^{-1}$) than that in the associated haustorial complexes and thus sucrose which was not inverted in the complex would be rapidly inverted after entering the mycelium. The relative activities explain why sucrose was detected in such small quantities in mycelia during the labelling experiments.

Thus there is no evidence whether the haustorium of powdery mildews absorbs sucrose intact or after hydrolysis in the matrix. The 'inhibition technique' which has been successfully applied to study nutrient transfer in lichens, (Drew & Smith, 1967b; Chambers et al, 1976) has been used by Hewitt & Ayres (1976) for oak infected with Microsphaera alphitoides and exogenous glucose inhibited the transfer of ^{14}C photosynthates from host to parasite to a greater extent than sucrose. However, because the haustorial interface is sealed from the leaf apoplast this inhibition probably reflects an impairments of host intercellular transport and not transport events at the interface. In conclusion therefore, indirect evidence suggests that it is probably absorbed intact. There is no objection a priori that sucrose cannot be transported intact across cell membranes and this is known to occur between many plant cells, (Porter & May, 1955; Hatch & Glasziou, 1964;

Kriedemann & Beevers, 1967; Humphreys & Garrard, 1968; Giaquinta, 1977). Evidence here does not preclude concomitant uptake of hexoses but as sucrose is the major soluble carbohydrate in the complexes this is probably the major compound traversing the extrahaustorial membrane. The possibility that it is absorbed intact would be a useful adaption to biotrophy. If this is a general feature of powdery mildews it would permit rapid assimilation by these fungi of a compound which is usually mobile in higher plants.

d) Translocation to the mycelium.

After absorption by the haustorium assimilates are translocated to the surface hyphae and eventually to the spores. Translocation in filamentous fungi has received little critical attention and there are few estimates of the nutrient fluxes involved, (Jennings, 1976). Thus prior to a discussion of mechanisms it is important to quantify the nutrient flow through the haustorial neck.

The neck is extremely narrow, and in E. pisi is usually between 1 - 1.5 μm in diameter. The septal pore is even narrower with an average diameter of 0.1 μm, (M. Martin, personal communication). Assuming that the structures are cylinders, the transectional areas can be calculated and an estimate of the fluxes obtained. The sectional areas of the neck and pore are thus 1.77 μm^{-2}

and 0.03 μm^2 respectively. As the flux through each haustorial complex was calculated to be 0.013 pg carbohydrate haustorial complex^{-1} sec^{-1} the mass transfer through the neck and septal pore are 0.75 and 41 mg . 100 mm^{-2} sec^{-1} respectively. The estimate for the neck is close to the only published estimates of mass transfer in fungal hyphae. Jennings (1976) estimated this as 2.4 mg cm^{-2} sec^{-1} in Serpula lachrymans which can grow for large distances over inert surfaces and also has septal pores (dolipores). The E. pisi value is also close to estimated rates of mass transfer in the phloem. These vary considerably but Canny (1975a) considered a value of 1.2 mg cm^{-2} sec^{-1} as the most acceptable mean of a number of plant species.

Mechanisms:

1) Diffusion

The length of the neck is approximately 3-4 μm, (estimated from micrographs in Gil & Gay, 1977) and thus diffusion is likely to have a greater role than in Serpula lachrymans or plant sieve tubes where the distances over which translocation is maintained are much greater. The G.L.C. analysis suggests that, on a dry weight basis a concentration gradient which would permit diffusive transport towards the mycelium only exists for sucrose. The concentration in the complexes was 30 mM and thus only very

small sucrose gradients (less than 30 mM) exist along the neck. To test whether diffusion could explain the rates of transfer observed the following form of Ficks' law can be used to obtain the required concentration gradient which would maintain the flux observed.

mass transfer = diffusion coefficient (concentration gradient)

($mg\ cm^{-2}\ sec^{-1}$) ($cm^2\ sec^{-1}$) ($mg\ cm^{-3}$)

Substituting the coefficient of diffusion for sucrose in water as 2×10^{-6} $cm^2\ sec^{-1}$ (Canny 1975a) and the observed mass transfer of 0.75 mg $cm^{-2}\ sec^{-1}$ a concentration difference of 3.75×10^5 mg cm^{-3} is obtained. However, the length of the neck is only 3.5×10^{-4} cm and thus the required gradient along the neck would be 132 mg cm^{-3} (or 0.4M). This is far greater than the gradient obtained by the G.L.C. analysis and also takes no account of the diffusional resistance of the pore, it is clear that alternative mechanisms must be considered. Jennings (1976) also concluded that the translocation rates measured in Serpula lachrymans could not be maintained by diffusion.

2) Protoplasmic streaming.

This seems an unlikely mechanism as there have been no reports of cytoplasmic streaming in haustoria. It has been shown

that protoplasmic streaming could just account for the mass transfer observed in the phloem of higher plants, but according to Canny (1975b) streaming has not been observed in sieve tubes either.

3) Pressure flow.

Jennings (1976) has proposed that a pressure driven flow of solution could account for the fluxes observed in Serpula lachrymans and this seems a most likely mechanism in haustoria. Jennings noted that such a process may result in the bulk flow of cytoplasm which should not be confused with cytoplasmic streaming which is generated by shearing forces which are metabolically actuated by the organism.

By the pressure flow mechanism, it is postulated that a solution would flow from the haustorium to the mycelium. The hydrostatic pressure required to drive such a process would be maintained by the osmotic pressure of the haustorial cytoplasm being higher than that of the extrahaustorial matrix and the cytoplasm of the epidermal cell. Thatcher (1942) has convincingly shown by plasmolysis experiments that the osmotic pressure of the fungal cytoplasm is much greater than that of the host cells and thus a potential to promote water flow to the haustorium exists. This would be continuously maintained by active uptake of solutes by the haustorium. The mechanism requires that the flow be accommodated by water loss or volume increases by the mycelium. In Serpula

lachrymans, flow is maintained by water exudation in droplets from the hyphal tips but these have not been observed in powdery mildews. However, there are three possible mechanisms by which the mycelium might accommodate and therefore promote a flow of solution from the haustorium.

2.1) Evapo-transpiration.

In pea mildew this is an unlikely mechanism for infection reduces transpiration from the leaves and thus little evaporation occurs from the fungus (Ayres, 1976). This is consistent with the hydrophobic fungal surfaces and the cuticular layer observed around spores and hyphae of E. pisi, (M. Martin, personal communication). Similar transpiration data have been obtained with Microsphaera alphitoides, (Hewitt & Ayres, 1975) and M. diffusa, (Mignucci & Boyer, 1977). On the other hand Majernick(1965) has shown that a rapid rise in evapotranspiration occurs in E. graminis infected barley plants during sporulation and thus this may provide a force for this mechanism in mildew on cereals.

2.2) Circular flow.

When solutes reach sites of deposition (sinks) they are converted to compounds which are osmotically less active and the osmotic potential of these regions is thus raised. If the solution

in these regions was at a higher osmotic potential than and in continuum with the host solutions osmotically driven water flow would occur towards the host. This mechanism is unlikely while hydrophobic cuticular layers separate the water phases of host and fungus. However, such a process could possibly operate during the early stages of haustorial development when the cuticle is penetrated and continual growth of the haustorium would raise its osmotic potential. Evidence of such a flow may be the nuclear migration into haustoria which occurs early in haustorium formation. Mount & Ellingboe, (1968; 1969) have shown that the nucleus moves just prior to the onset of nutrient transport from the haustorium to the surface structures. This suggests a distinct switch in the direction of flow through the neck during haustorial formation. Thus as fungal development progresses the process of forming haustoria may promote a circular flow of water. This could not explain translocation through conidiophores but could promote the exit of solution through the necks of established haustoria.

2.3) Balanced growth.

The volume of solution flowing from the haustorium may also be accommodated by volume increases during the growth and sporulation of the surface mycelium. These processes provide major eventual sinks for the nutrients and it is possible that the

volume increases and weights used in synthetic processes are balanced with quantities of solutes and water passing from the haustorium. The packaging of both solutes and water in spores and their loss at absiscion provides the most likely mechanism aiding solution flow from the haustorium. The high water content of powdery mildew spores (Yarwood, 1950) is well established and although it is thought to be important in germination at low humidities it is clearly advantageous for the parasite to lose large quantities of water during sporulation to promote nutrient flow. The volume increases and losses could be estimated from the relationship between hyphal development, sporulation and haustorial frequency in E. pisi as carried out by Hirata, (1967) for E. graminis; he estimated that each haustorium was responsible for approximately 1,500 μm^3 of spore volume per hour. If there is the same passage of water through each haustorium of E. pisi then the mass transfer observed could be maintained by the flow of a 3% solution of carbohydrate through the haustorial neck.

The efficient removal of solutes from the haustorium is also essential to maintain steady state transport from the host to the mycelium. Direct evidence for this role of the mycelium has also been obtained. Table 8 shows that when the mycelium is attached during exposure to $^{14}CO_2$, the quantity of label retained in the haustorial complexes was similar to that in complexes isolated from leaves where the mycelium was removed before an

equivalent exposure. Thus in the absence of the mycelium transport across the whole host-parasite interface is limited. The control of transport across the membranes of the interface must thus be maintained by some form of transinhibition (Jennings, 1976) where excessive concentrations of solutes in the matrix or in haustorial cytoplasm prevent further transport across the extrahaustorial membrane and haustorial plasmamembrane. It is unlikely that the conversion of solutes to insoluble compounds in the mycelium is a major factor in maintaining the high fluxes across these membranes and from the haustorium to the surface hyphae because mechanisms driven solely by concentration gradients are untenable. Where these conversions in the mycelium result in a volume increase they would promote solution flow from the haustorium.

The ultrastructure of the haustorial neck is also in accord with the proposed pressure flow mechanism. Its cytoplasm is different from that of the haustorium or hyphae and contains no organelles or vesicular components which would restrict flow (Bracker, 1968; Gil, 1976). There are no cytoplasmic inclusions in close proximity suggesting a specialised translocation mechanism. The only evidence against solution flow promoted by volume increases in the mycelium is that during studies of primary infection of barley by E. graminis, the transfer of ^{32}P and ^{35}S from the

haustorium to the surface structures occurs prior to emergence of the secondary hyphae. However, no flux data are available for this stage of development and thus diffusion may be more important there.

V) Ethirimol at the host-haustorial interface.

Results of the present study indicate that ethirimol was readily absorbed by isolated haustorial complexes of E. pisi - P. sativum and isolated mesophyll protoplasts of P. sativum. It was rapidly concentrated in the complexes to an internal concentration 20 times greater than that of the ambient medium but did not accumulate significantly against a concentration gradient in mesophyll protoplasts. These findings strongly suggest preferential accumulation by powdery mildew haustoria in infected plants. They are pertinent to the distribution of ethirimol in the host, absorption by the parasite and to the mode of action of this fungicide and are discussed in detail under those headings. A critical discussion of the method employed is also included.

A) Distribution and movement of ethirimol.

Ethirimol and the closely related dimethirimol are thought to be transported in the apoplast of the leaf (Cavell,

Hemingway & Teal, 1971). They follow the transpiration stream moving acropetally accumulating at the leaf margins which are regions of high evapotranspiration. There is no evidence for basipetal translocation which is characteristic of compounds transported in the symplast (Price, 1973). Movement is further restricted in woody plants where these fungicides do not readily move from the leaf veins and here the systemic activity is reduced.

Peterson & Edgington (1976)studied the uptake by potato slices of a large spectrum of systemic pesticides, including ethirimol and concluded that the property of a pesticide to be transported in the symplast depends upon its retention in the symplast. They obtained no evidence for the retention of ethirimol. The uptake of ethirimol by isolated protoplasts indicates that absence of symplastic transport is not due to its inability to penetrate the plasmamembrane of the host. However, efflux kinetics from protoplasts were not studied and thus the degree of retention by pea mesophyll cells was not elucidated and Peterson & Edgington's hypothesis not fully tested.

Peterson & Edgington (1976) questioned their data on the uptake of ethirimol. It penetrated more slowly than other pesticides and they suggested that this was because it was more lipophobic than other compounds tested. They also thought that a reversible binding process may occur either intra - or

intercellularly. The advantage of studying influxes with isolated protoplasts is that results are not confused by cell wall binding and since a similar kinetic of uptake was obtained here as for potato tissue it is more likely that penetration is limiting and that if binding occurs, it is at intracellular sites.

Protoplasts have previously been used for studies of pesticide uptake (Boulware & Camper, 1973) but no attempt was made to maintain protoplast integrity. The method (Methods, VII, B) described here measured uptake into intact protoplasts only and avoided the effects of damaged cells. No checks were made on the viability of the protoplasts, but Ruesink & Thimann (1966) have shown that protoplasts from Avena coleoptiles remained viable in a similar ionic isolation medium for several days.

B) Absorption by the parasite.

The absorption of systemic fungicides by powdery mildew fungi is little understood (Bent, 1978). From the apoplast of the host, fungicides can move to the fungal cytoplasm either by passing through the cuticle of the host and then traverse the cuticular layer, cell wall and plasmamembrane of the fungus or through the epidermal cytoplasm and thence to the haustorium by way of the pathways discussed in detail above for nutrients.

The transcuticular pathway.

Ethirimol may penetrate the cuticle more readily than nutrients since it is a relatively lipophilic compound (Shone & Wood, 1973) although less so than many pesticides. Proof that ethirimol is absorbed by spores of E. graminis through the plant cuticle was obtained by Bent, (1970) who observed marked reductions in spore germination on leaves after root treatmdnt at a concentration of 5 ppm. Thus it is unlikely that the protectant activity of ethirimol is totally dependant on its absorption by the haustorium. Transcuticular transport may be aided by removal of the surface waxes by the surface spores and hyphae of E. graminis (Day & Scott, 1973). However, recent observations by M. Martin (personal communication) have shown fusion of the surface waxes of spores of E. pisi and F. sativum occurs, thus the tracks free of wax platelets observed by Day & Scott (1973) may have resulted from detachment of waxes with the hyphae rather than their degradation on the surface. There is no evidence that other antimildew systemic fungicides cross the cuticle and when spores alight on a leaf treated systemically with benomyl, tridemorph, triforine, triarimol or triamiphos, they germinate and produce normal growth tubes and appresoria (Bent, 1978). Fungicidal action is evident only when the cuticle and epidermis are penetrated.

The haustorial pathway.

Where transcuticular movement is restricted, absorption by the haustorium is essential for an anti-mildew fungicide to be effective. Where transcuticular movement of ethirimol does occur absorption by the haustorium may facilitate the control of established colonies. Furthermore as concentrations of systemic fungicides in plants are generally low (Bent, 1978) and these compounds are also being continually degraded by the plant, such preferential accumulation by the haustorium may be very important particularly where the fungicide concentration becomes critically depleted.

The neck bands which attach the extrahaustorial membrane to the neck of the haustorium are a barrier to the movement of solutes (Gil & Gay, 1977) but it is not known whether fungicides are also restricted. The influx of fungicide into the isolated complexes most probably represents transport across the extrahaustorial membrane but the possibility of influx through this neck region cannot be totally excluded. Thereafter it accumulated in two compartments within the complexes which have been resolved by analysis of efflux kinetics (figs. 42 & 43). The extra-haustorial matrix is an amorphous region with little internal complexity (Gil, 1976) and thus it is most likely that at least

one of these compartments is within the haustorial cytoplasm. The ethirimol is distributed in a ratio of 7:3 in compartments which show fast and slow efflux kinetics and from the influx data both of these accumulate against a concentration gradient. This may under estimate the extent of the accumulation for in experiments using both protoplasts and isolated haustorial complexes the washing procedures necessary to remove the radioactive ambient medium from the cells may have allowed the efflux of ethirimol. Cram (1969) has shown that to measure the ion flux across the plasmamembrane of Nitella a short uptake period (less than 1 minute) followed by a rapid wash is necessary. Future studies using isolated protoplasts and haustorial complexes should concentrate on rapid washing. Guy et al (1978) and Heldt & Sauer (1970) have described promising methods using n-butyl phthalate and silicone oils as non-aqueous traps which permit rapid separation by centrifugation of fragile cells and organelles from an incubation medium. This method destroys the integrity of the sample, Dekhuijzen et al, (1978) has used a gentle non-destructive method to measure uptake of nucleotides by isolated plant protoplasts by sampling the loss of these compounds from the ambient medium during uptake. These methods warrant further trials particularly because they would permit experiments over

shorter periods and enable accurate estimation of fluxes and permeability coefficients of the extrahaustorial membrane for particular solutes. The shorter washing would thus avoid influx into intracellular compartments which have slower efflux kinetics. In Nitella cells and Cl^- ions this is the vacuole. However, despite these technical imperfections, this approach has clearly demonstrated that haustoria accumulate this fungicide and these experiments have given the first indication of the cellular distribution of a systemic fungicide in the infected leaf.

The mechanism of accumulation by the haustorial complexes has not been fully elucidated. At ambient concentrations exceeding 50 μM the concentration within the complexes exceeded the aqueous solubility of ethirimol (approximately 850 μM) and suggests that some intracellular binding or partitioning into lipids may occur. Such processes would explain the relatively slow second stage of efflux which was observed when a significant proportion of the ethirimol was retained after a three hour washing period. Easy removal of most of the ethirimol with methanol also suggests that partitioning into lipid may be more important than true binding in the complexes. The biochemical fractionation after $^{14}CO_2$ feeding indicates that lipids and in particular triglycerides are relatively abundant in the haustorium.

An alternative mechanism of accumulation to be considered is that ethirimol follows an electrochemical gradient. The negative potential inside plant or fungal cells (Barber 1968 ; Slayman et al, 1970) selectively favours the accumulation of cations (Dainty, 1962). The pK of ethirimol is 4.8 (Shone & Wood, 1973) and thus at pH 6.5 which was used for most uptake studies very few charged moieties would be expected and rapid accumulation by such a mechanism seems unlikely. This was supported by similar uptake rates at ambient pH 4.2 and 6.2. Similarly, absence of any pH effect is uncharacteristic of an active transport process, but for a lipid partition it would be the intracellular pH which is responsible for the extent of accumulation. The linear relationship of ethirimol uptake with concentration also suggests a passive process, but concentrations higher than 400 µM were not tested because of the poor water solubility of ethirimol.

C) Mode of action.

The haustorium is a possible site for the toxic action of systemic fungicides. Several systemically active compounds have been shown to affect the morphology of powdery mildew haustoria (Dekker & Van der Hoek-Scheur, 1964; Sijpesteijn, 1972; Paul & Scheinpflug, 1978) and as mentioned above for some

fungicides, primary infection and the commencement of haustorial formation often coincides with the first signs of fungicidal activity. Similarly using the fungicide oxycarboxin to control Uromyces phaseoli on french bean, Fring & Richmond (1973) observed that necrosis occurred first in the haustorium and that intercellular hyphae were disrupted in a later stage. Results such as these indicate that the fungicide either affects metabolic processes which are specific to the haustorium or the fungicide is specifically located or accumulated in the haustorium.

The $^{14}CO_2$ labelling experiments discussed above indicated that the metabolism of the haustorium is quite distinct from the host plant and mycelium and such studies provide a basis for further studies of the mode of action where the haustorium is a suspected fungicide target. Although ethirimol is active at spore germination, other sites, possibly in the haustorium may be affected. The biochemical mode of action of ethirimol is not yet fully understood; Bent (1970), on the basis of reversal reactions, suggested that folic acid C-1 transfer reactions may be inhibited. Recently Hollomann (1978) has shown that metabolism of adenine, but not its synthesis, may also be specifically inhibited. The intracellular concentrations at which ethirimol is active in spores are not

precisely known but on a dry weight basis they are not thought to exceed those occurring in the host viz. a few parts per million (K. Bent, personal communication). Thus fungicidal action of ethirimol may be more marked in haustoria where comparison of absorption by isolated haustorial complexes and isolated protoplasts clearly indicates a preferential concentration by the haustorium. The enhancement of fungicidal activity by concentration of a compound in the pathogen is common. Yarwood & Jacobsen (1950) have shown that sulphur is accumulated in lesions of E. graminis. Results showed that transfer of ^{14}C photosynthates from the host to the mycelium was inhibited by ethirimol. This suggests haustorial malfunction although it is possible that the effect may also result from impairment in the nutrient sink of the mycelium.

VI) Concluding statement

The techniques used in this study have permitted the fullest characterisation of the physiology of the haustoria of any fungus so far achieved. The major findings are;

1) haustoria absorb nutrients from the host;

2) identified translocates and sinks occur at the haustorial interface and in the mycelium;

3) transport across the interfacial membranes and translocation to the mycelium are achieved by specialised processes.

4) haustoria readily accumulate an anti-mildew systemic fungicide.

Although the haustorium has a degree of autonomy and absorbs nutrients from the host, its efficient functioning in host-parasite transport also depends upon active participation by the host and mycelium. However key processes appear to occur at the interface between the haustorium and the infected cell and this study has laid a basis for their full elucidation.

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