THE PHYSIOLOGY OF SPORELING GROWTH IN PHYTOPHTHORA INFESTANS (MONT.) DE BARY

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by

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GENERAL INTRODUCTION

The causal organism of late blight of potatoes, Phytophthora infestans, still brings about serious economical losses in every country in the world where the potato, Solanum tuberosum, is grown, and where the climatic conditions are suitable for the pathogen. Control methods have been known for some time, yet late blight is still considered to be one of the most important plant diseases in the world (Cox and Large, 1960). Methods of control involve the expenditure of effort and capital in forecasting outbreaks and in applying protective sprays. Further, occasions arise when the cost of application of protective sprays and the mechanical damage caused to the crop, make spraying an uneconomical proposition when the cost is related to the loss in profit brought about by the disease.

It has been suggested that the most efficient method of controlling the disease would be to grow resistant varieties. Resistant varieties have not yet proved completely successful since races of P. infestans can arise which have the capacity to overcome the resistance of some new varieties of potato. Niederhauser (1959) found that no clone of S. demissum, from which most resistant genes have been introduced into the cultivated potato, was resistant to all races of the fungus.

THE GENETICS OF RESISTANCE OF S. TUBEROSUM TO P. INFESTANS

The resistance of Solanum and Lycopersicon to P. infestans has been divided by Muller (1953) into true or major gene resistance and field or minor gene resistance. True resistance, which depends upon a hypersensitive reaction to infection is found in many species of Solanum (Black and Gallegly, 1957), but it does not occur naturally in S. tuberosum. The non-allelic, dominant genes responsible for the hypersensitive reaction have been introduced into S. tuberosum mainly from S. demissum. Four of the controlling genes, designated R.1, R.2, R.3 and R.4, have been introduced singly and in all combinations. More R genes, R.5 and R.6, have been found in S. demissum and other species of Solanum by Black and Gallegly (1957) and recently Malcolmson and Black (1966) reported a further three R genes in seedlings derived from crosses between S. demissum and S. tuberosum.

An international system of nomenclature for pathogenic races and genes for resistance was formulated by Black, Mastenbroek, Mills and Petersen (1950). Each R gene confers resistance to one pathogenic race but not to others. Castronova (1954) recorded an isolate, 167 of P. infestans, from Argentina which did not fit into this system. Toxopeus (1958) found that the expression of the R genes may be modified by minor genes, and suggested that this might provide an explanation of Castronova's results.

THE PHYSIOLOGY OF RESISTANCE

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The primary means of spread of blight within a field or over a geographical region is by the aerial dissemination of sporangia. Spores from a point source have been recorded at distances of 50 Kms. and over from such a source, (Schrodter, 1960). Under conditions of high relative humidity sporangia of P. infestans may germinate in one of two ways, depending on temperature. At high temperatures (18-22°C) germination takes place directly by means of a germ tube. Infection has not been shown to occur from sporangia germinated in this way and generally a secondary sporangium is formed (Pristou and Gallegly, 1954). Low temperatures (9-15°C) favour indirect germination yielding motile zoospores. The zoospores swim for some time in the film of water on the leaf surface, settle down and encyst. Germination occurs, an appressorium is formed at the end of a short germ tube, and the epidermal cell is penetrated by an infection peg arising from this appressorium. These processes can occur in as little as two hours (De Bary, 1863, Pristou and Gallegly, 1954).

Zoospores of P. infestans can penetrate the epidermal cells of many species of non-Solanaceous families (Hori, 1935; Müller, 1950), as well as many species in the Solanaceae (Hori, 1935). In non-Solanaceous plants further development of the mycelium appeared to be restricted by a reaction of the host tissue. This reaction had strong similarities to the hypersensitive reaction of certain potato varieties possessing major gene resistance (Ferris, 1955). After a period of

2-3 days the invading hyphae are no longer detectable and only a small fleck on the surface of the leaf marks the region where penetration took place. This sequence of events prompted Pristou and Gallegly (1954) to propose that P. infestans, once it had penetrated the host cell, could not obtain sufficient nutrient for further growth and died of starvation followed by autolysis.

Chester (1933), postulated that the resistance of wheat leaves to Puccinia graminis v. tritici could be accounted for by one or both of the following reasons, assuming penetration occurs; (1) the parasite is unable to obtain sufficient nutrient, and (2) toxic substances may be present in the host prior to infection or produced in response to infection. These postulates are basically similar to the nutrition-inhibition hypothesis of Garber (1956). Garber suggests that the potential proliferation and metabolism of the parasite within a host is determined by the two environmental factors it encounters, the nutritional environment and the inhibitory environment. If the nutritional environment is inadequate for the parasite then invasion cannot take place. In this case the parasite will be avirulent. However even if the nutritional environment is adequate the parasite may still not be able to invade if the environment is inhibitory. Of the four possible combinations of these two factors, only one will favour invasion; that is when the environment is nutritionally adequate and non inhibitory.

That pre-existance of toxins may contribute to the resistance of

a host plant to an invading pathogen has been suggested by Buxton (1957) who showed that root exudates from resistant varieties of pea to Fusarium oxysporum f. pisi inhibited the germination of the spores of that organism.

Resistance in some instances has been attributed to the action of a germinating spore in stimulating the production of phytoalexins or in increasing the production of such compounds to a level at which they are toxic to the invading pathogen.

The original work on phytoalexins was described by Muller and Borger (1940). Cut tuber surfaces of several potato varieties were inoculated with sporangial suspensions of strains of P. infestans to which some varieties were susceptible and some were resistant. A second inoculation was made 24 hours later on the same tubers using a strain of P. infestans to which all the varieties were susceptible. Observations showed that where the first inoculation was made with an avirulent strain, the second inoculation failed to induce normal disease symptoms. Previous inoculation of virulent strains had no effect on the subsequent host cell reaction type. From this and further work they found that the post-infectional mechanism inhibiting fungal development in host tissue was related to the interval of time between the first and second inoculations and to the relative concentrations of the sporangia of the two strains of P. infestans and that it was associated only with the inoculated region of the tuber.

Equally, it has been shown that nutritional factors may be involved.

For example, uredospores germinated in extracts of susceptible hosts had longer germ tubes, less branching and fewer apical swellings than those germinated in extracts of resistant varieties (Hare, 1966).

Kline, Boone and Keitt (1957), working with mutants of Venturia inaequalis deficient in certain growth factors, showed that scab symptoms on host apples could not be induced unless these factors were supplied artificially and postulated that the absence of these substances in the host conferred resistance. Wheat leaves which have been subjected to treatments causing a marked increase in carbohydrate and soluble N levels become more susceptible to wheat stem rust (Forsyth and Samborski, 1958 and Johnson, 1946). If normal protein metabolism was maintained by floating detached leaves in low concentrations of kinetin or benzimidazole, carbohydrates and soluble N compounds did not accumulate and loss of resistance was prevented (Samborski, Forsyth and Person, 1958). In this case loss of resistance seemed to involve the breakdown of proteins to amino acids essential to the development of the fungus, inferring that resistant varieties normally lack these amino acids in quantities sufficient to maintain the parasite. Grainger (1956) reported that carbohydrate content in the intact plant may influence susceptibility as rapid growth periods when carbohydrates are low in relation to dry weight, are times of low disease potential for Helminthosporium leaf spot of oats and Phytophthora blight of potatoes.

Burrows (1960), however, showed that lack of nutrients was not responsible for the resistance of oat leaves to oat stem rust in

experiments using leaf sandwiches of resistant and susceptible varieties. Leaf sandwiches were prepared by removing a strip (2-3 cms long) of epidermis from the under surface of each leaf. The exposed mesophyll tissue was bathed in 0.5% water agar to prevent drying and to act as an adhesive when the two surfaces were brought together. The margins of the leaf were then cemented together with paraffin wax. He showed infection could occur from a resistant donor into a susceptible receiver part of the leaf sandwich. Normal disease symptoms arose on the surface of the susceptible receiver leaf and the resistant donor leaf showed the characteristic fleck reaction. In the reverse situation, penetration of the mesophyll of the resistant variety took place but little or no further infection occurred and certainly normal disease symptoms did not develop, except on the susceptible donor. P^{32} was shown to be transferred by the fungus from the susceptible donor section, through the bridge between the two sections into the penetrated cells of the resistant leaf. No such passage of radioactivity occurred in uninoculated controls. Burrows concluded that translocation of essential nutrients could take place within the mycelium and so resistance must be attributed to some sort of specific inhibition.

The inhibitor was however unable to exert its effect on the susceptible leaf, possibly because it was unable to diffuse in sufficient quantity into the infected sites of the susceptible section. It appears that the fungus was not killed but was able to pass from the point at which it penetrated the donor through this resistant tissue into the susceptible part. It might be suggested that a new metabolic pathway

must be established in the fungus or an existing one enhanced before proliferation could take place and that one of the intermediate products of this metabolism activated the production of the toxin *in situ* by the penetrated host cell. Growth of the hyphae through the resistant section could therefore have been due to -

(a) there was a lag phase for the production of the toxin which was of sufficient length to allow the passage of the hypha through the resistant tissue;

(b) that its metabolism did not induce toxin production;

(c) its metabolism was not affected by the toxin produced, or

(d) passage proceeded by a metabolic pathway which did not induce toxin production.

Both Chester (1933) and Garber (1956) considered the host parasite relationship at a purely physiological level. Other factors, for example morphological factors, play an important role in the resistance of *S. tuberosum* to *P. infestans*. The expression of these physiological and morphological factors is known as field resistance. Howard (1960) has divided the resistance of foliage into three principal components.

(1) Resistance to infection; that is the chance of a spore germinating and penetrating a leaf.

(2) The rate at which the mycelium spreads through the leaf tissue.

(3) The rate at which sporangia are formed and the number of sporangia formed per unit area of leaf.

These three components have been investigated by Lapwood (1961-a, b, c).

He found that ecoclimatic factors, and morphological factors such as the nature of the leaf surface infected, i.e. adaxial or abaxial surface, the growth habit of the plant, and the type of canopy produced, all contributed towards the delayed onset of the epidemic. One of the most important single factors affecting the primary spread of the infection was that of differences in sporulation. The position of the lesion on the plant was partly responsible for these differences but Lapwood also found when comparing leaves taken from similar positions in four varieties which varied in their field resistance that although the spread of the fungus through the leaf tissue was approximately the same in laboratory experiments, differences occurred in the relative number of spores produced in each variety. Lapwood (1961 b), found that the differences in sporulation could be directly attributed to the rapidity with which the infected cells of the leaf became necrotic, so diminishing the sporulation area. Subsequently, (Lapwood, 1961 c) it was shown that significant differences existed between the resistance exhibited by the four varieties to petiolar infection. The rate at which the petiole becomes infected relates to the rate of defoliation.

The growing of resistant varieties delays the initial spread of the blight epidemic compared with susceptible varieties although the rate of spread may eventually become the same (Lapwood, 1961 a). This effect is similar to the results obtained when a comparison is made between unprotected crops of a susceptible variety and crops of the same variety to which has been applied a single protective spray (Large, 1952). Delaying the epidemic by as little as one week can

have considerable economic effects as the extra growth obtained in a period of this duration at this stage in the life cycle of the potato can increase the yields substantially. Important as field resistance may be, the ultimate factor involved in the development of host-parasite relationship is almost certainly the environment in which the pathogen finds itself in the host plant.

Adequate evidence is available (Hare 1966, Cruickshank 1963) to support both the nutritional and inhibition theories, and it is therefore possible to conclude that both situations may exist in all combinations (Garber 1956). Difficulties arise in separating which of these two components may account for the resistance of a host to a particular pathogen. These two components might be resolved if the pathogen could be grown on a chemically defined artificial medium. Subsequent analysis of potential hosts might evaluate the role played by the nutritional and the inhibitory factors of the environments.

AXENIC CULTURE OF P. INFESTANS

The condition whereby one organism can grow only by securing its food from continued association with another living organism is called obligate parasitism (Gaumann, 1950). P. infestans was still considered to be an obligate parasite by De Bary in 1876, since he and an earlier worker (Berkeley, 1846) failed to grow it in axenic culture. Matruchot and Molliard in 1900 were the first definitely to succeed. However, it was not until 1912 when Jones, Giddings and Lutman found that asparagine, $MgSO_4$, KH_2PO_4 and possibly KCl. were essential for growth, that a

chemically defined medium was developed - previous workers having used plant extracts only. P. infestans was later found to have an absolute requirement for thiamine (Payette and Perrault, 1944).

Petersen (1942) obtained better growth with NH_4SO_4 than with KNO_3 or asparagine. Conversely Sakai (1955) obtained good growth with nitrate nitrogen but little growth with ammonium nitrogen, and he suggested that the inhibitory effect of the latter might have been due to a drop in the pH of the medium as the nitrogen source was utilised. Hodgson (1958) obtained evidence to show that the inhibitory effect of the nitrogen source depended on the composition of the rest of the medium. Subsequent work on P. infestans has revealed a much more complicated picture and it now appears that the different races and even different isolates of the same race, may have different nutritional requirements particularly with respect to the nitrogen source. This erratic behaviour is more pronounced on media containing different inorganic sources of nitrogen than on media containing different organic sources of nitrogen.

Good growth of an isolate of race 4 of P. infestans but not of an isolate of race 1, was obtained by French (1953) on a medium containing KH_2PO_4, $MgSO_4$, asparagine, glucose and thiamine adjusted to a pH of 5.5. Using the same medium plus $FeCl_3$, Hodgson (1958) achieved good growth of races 1.2, 1.2.3, but not of races 0 and 1. The pH of this medium was adjusted to 6.3 - 6.5. Races 0 and 1 were induced to grow by adding ascorbic acid or sodium thioglycollate to the medium. Using French's medium, Clayson (1958) obtained good growth of races 2.4, 1.4 and 4, but growth declined rapidly as the initial pH of the medium was above 6.0 or

below 5.0. She found, in an extensive survey of factors responsible for the extreme range of variation in growth rate, that one of the most important single factors was the size of the inoculum used. This varied between isolates and also with the concentration of the nitrogen in the medium. An isolate of race 4 seemed to be the least demanding with respect to the constituents of the medium. Autoclaving time and pressure, like the concentration of the nitrogen in the medium, exercised an effect. She suggested that these two factors are possibly responsible for the production of a toxin in the medium.

The media and the work described above all relate to growth from a large inoculum and until recently it has not been possible to induce isolated zoospores of P. infestans to continue growth after germination to form colonies. This is a prerequisite of any genetical, cytological, morphological, physiological or nutritional research. Clarke (1966) formulated a medium which was basically the same as that used by French (1953) but included apple pectin and yeast extract. On this medium a high proportion of isolated zoospores could be induced to form colonies within 6-8 days. Thus, it is only now that a medium is available which will support the growth of P. infestans in a similar way to the host plant, where a single zoospore can infect potato leaves (Lapwood and McKee, 1966) or tuber slices (McKee, 1964).

Since P. infestans can be grown in axenic culture, it is not classed as an obligate parasite but it is similar to them in certain other respects.

(1) It forms haustoria in S. tuberosum (Butler and Jones, 1961).

(2) It shows host specificity.

(3) The behaviour of germinating zoospores on most artificial media is very similar to that of many obligate parasites.

(4) It exhibits poor competitive saprophytic ability (Garret, 1956).

(5) In culture it has more exacting nutritional requirements than most saprophytes.

It is with the first three points that P. infestans shows closest affinity to the obligate parasites and it is with the third point that the work reported here is primarily concerned. Yarwood (1956) states, "The spores of most obligate parasites germinate well in the absence of their hosts and the germ tubes continue to elongate until the food supply is exhausted or until some other growth limiting process sets in." Therefore, although germination can be readily induced, growth never progresses farther than the germ tube stage. He then suggests that obligate parasitism could best be studied by investigating the requirements of the Peronosporales, particularly the genus Phytophthora.

Spores of Erysiphe polygoni, Uromyces phaseoli, and Peronospora destructor produce longer germ tubes when germinated in nutrient solutions than when germinated in water alone (Yarwood, 1948). Similarly, uredospores of rusts will germinate freely in moist conditions and infection structures are obtained if the spores are subjected to the proper physical conditions of light, temperature, moisture and contact with artificial membranes. Chemical factors, such as the concentration of Zn^{++}, cysteine and glutathione, also induce the formation of appressoria.

Haustoria have not been formed in axenic culture but have been initiated by placing artificial membranes on to the surface of a leaf from which the epidermis had been removed (Staples and Wynn, 1965).

Shaw (1963), like many workers, believes that obligate parasites will one day be cultured. Cutter (1959, 1960) reported the successful growth of Gymnosporangium juniperi-virginianae and Uromyces ari-triphylli in culture. The strains isolated from tissue cultures of the respective hosts were autotrophic for all vitamins except biotin and could utilise a variety of carbon and nitrogen sources. Reinfection of the host tissue showed that pathogenicity had been retained. Other workers (e.g. Turel and Ledingham, 1957) have been unable to repeat Cutter's work.

More recently, Williams, Scott and Kuhl (1966) have grown P. graminis tritici in axenic culture. The spores were plated on Czapek-Dox medium supplemented with sucrose and yeast extract in 1% agar. Germination occurred in two days, and branches were formed after a further two days. Subsequently colonies of about 1 mm. across were formed after 3-4 weeks. They were, however, unable to reinfect wheat leaves by placing the colonies under the epidermis. The results were interpreted by Williams et al. to show that physical factors were not essential for the initiation of post germ tube growth and strongly suggested that the problem is a nutritional one.

Studies on the physiology of uredospores of rusts have also indicated that a nutritional factor may be responsible. All the necessary

respiratory enzymes have been shown to be present in the spores, and yet although they were able to incorporate C^{14} labelled substrates into protein fractions no net protein synthesis occurred during the period of germination (Shaw, 1963). Adaptive enzymes can be induced but are formed at the expense of other proteins in the cell. From these results it appears that there is a requirement for an essential nutrient or for the removal of an inhibitor.

Clarke (1966) showed that if an inoculum giving 1,000 zoospores of P. infestans per Petri dish were spread evenly on agarised French's medium plus yeast extract, cultures were readily established. However, if an inoculum giving only 200 zoospores per Petri dish were inoculated so that the zoospores were widely spaced over the medium, then less than 1% of the sporelings continued growth and those that did produced a few relatively unbranched hyphae. On the same medium plus pectin he found a good proportion (10-20%) of the sporelings produced numerous highly branched hyphae forming compact colonies. The failure of sporelings to grow farther than the germ tube stage in vitro is very similar to the behaviour of the germinating spores of many obligate parasites, of which Peronospora destructor is an example (Yarwood, 1948). Thus, further work into the nature of the factors controlling the development of colonies of P. infestans may prove to be valuable in leading towards a proper understanding of the nature of obligate parasitism. Work of this type may also be informative with respect to the physiology of certain stages in the establishment of the host-parasite relationship.

The work reported in this thesis is therefore concerned with:-

(1) The morphological and physiological comparisons of spore germination and the growth of sporelings of P. infestans on media with and without pectin.

(2) An investigation of the factors in commercial apple pectin responsible for the initiation of post-sporeling growth.

II. CHOICE OF ISOLATES AND PRODUCTION OF THE INOCULUM

Standardised techniques are essential for any morphological or physiological investigations. In order to carry out the work described in this thesis which was concerned with certain aspects of the physiology of post-sporeling growth and colony formation of P. infestans, it was necessary to develop techniques which would give a plentiful supply of zoospores of which a high proportion developed into colonies. Large numbers of zoospores are essential for certain techniques such as measurements of respiration and of the synthetic capacities of the sporeling, although relatively few are required for histochemical and autoradiographic techniques.

A preliminary requirement for this type of work is:-

(ε) an isolate which grows and sporulates well, and in which the sporangia can be readily induced to form large numbers of zoospores.

(b) a high proportion of the germinated zoospores must continue growth to form colonies.

Thus an initial survey of isolates and of the conditions leading to maximum production of sporangia and germination to produce zoospores was carried out.

Isolates

A preliminary set of experiments was set up to investigate the

quantity of sporangia produced and the proportion of germinated zoospores which continued growth to form colonies in each of three isolates, R41, 91R1R and R4DS. The isolates R41 and R4DS gave consistently greater numbers of sporangia than isolate 91R1R. On a medium containing apple pectin it was found that a much greater proportion of the sporelings of isolate R4DS continued growth to form colonies than either of the other two isolates (see page 36).

As a result of these preliminary investigations the isolate used throughout the work described was the isolate of race 4, isolate 4DS, which was obtained from Prof. R. K. McKee, Queen's College, University of Belfast. The culture was maintained on chick pea agar prepared as described below and subcultured at fortnightly intervals.

Media for the production of sporangia

P. infestans has been cultured on substrates containing extracts of several non-hosts such as oats (Clinton, 1911) and pea (Dickinson and Keay, 1948) while extracts of potato have been less successful. More recently, French beans (Hall, 1959) and chick peas, Cicer arietinum (Keay, 1953) have been incorporated into media and found to support the growth and production of sporangia of P. infestans.

Preliminary experiments showed that chick pea agar was a better sporulation medium than French bean and Birds Eye pea agar, and so this medium was used throughout. The composition of the chick pea medium was:-

Dried peas	250.0 g.
Sucrose	20.0 g.
Agar (Oxoid No.2)	15.0 g.
Distilled water to	1.0 litre

The method employed in the preparation of the medium was essentially that of Keay (1953) except that the soaked peas were minced in a household mincer instead of being mashed up with a pestle and mortar. The chick peas were purchased locally and the medium was autoclaved at 10 lbs. for 15 mins.

The production of zoospore suspensions

More than a century ago De Bary (1863) showed that free water was essential for the germination of the sporangia of P. infestans. Since then several other factors have been shown to influence the germination of the sporangia of P. infestans and other species of Phytophthora. Melhus (1915) showed that the optimum range of temperature for indirect germination of the sporangia to produce zoospores was 11-13°C, while higher temperatures (around 20°C) led to direct germination. Although temperature itself affected the type of germination, other factors were also claimed to influence either the rate or the type of germination of the sporangia. More zoospores were reported to be liberated in the light than in the dark in P. faberi (Gadd, 1924) and in P. parasitica (Dastur, 1913). Blackwell and Waterhouse (1931) also reported

that the water relations of the sporangia prior to the initiation of germination could affect the germination process. McAlpine (1910) stated that the age of the sporangium of P. infestans directly affected their ability to produce zoospores, the older sporangia tending to germinate directly.

Equally as important as factors influencing the numbers of zoospores released are factors which exert an effect on the survival of the zoospores. A 2% sucrose solution was used to increase the swarming period of zoospores in P. parasitica (Gooding and Lucas, 1959) and P. cactorum (Shaw, 1965). McKee (1964) reported that zoospores of P. infestans would remain motile for periods up to 18 hrs. in a 1% boiled potato extract kept at 3°C. Although these reports indicate that the solution in which the zoospores were placed influenced the period of swarming it was however possible that similar conditions could affect survival of encysted zoospores or the germination of the sporangia or both. Experiments were therefore carried out to investigate the effect of some of these factors on the production of zoospores by the isolate 4DS.

Experimental Methods

The cultures were grown on chick pea agar which was sloped in large boiling tubes. The agar slopes were inoculated with a mycelial block and kept at room temperature for 4-5 days to allow the inoculum to become established. The slopes were then transferred to a cooled incubator held at 12°C. In the early experiments the cultures were

incubated for 12-14 days before the sporangia were washed off in 10 ml. sterile glass distilled water. The resultant sporangial suspension was then incubated in a test tube at 12°C. The zoospores which were released from the sporangia could be pipetted off from just below the surface of the water where they aggregated. In this way a zoospore suspension relatively free from sporangia was obtained since the sporangia remained at the bottom of the test-tube. Subsequent dilution of the zoospore suspension was done by pipetting 1.0 ml. spore suspension into 9.0 ml. of sterile water. These techniques were used in a series of experiments set up to investigate the effects of various factors on the numbers of zoospores produced.

Expt. 1. <u>The effect of age of the inoculum on the germination of the sporangia</u>

Eight slopes were inoculated and grown for 7 days after which a slope was removed daily and the sporangia washed off. The sporangial suspension was incubated at 12°C for 2 hrs. after which is was withdrawn, vigorously shaken and counts of the zoospores and germinated and ungerminated sporangia were made using a Thoma haemocytometer. Twenty fields were counted daily until the fifteenth day after inoculation. The experiment was repeated and the results obtained from both experiments are shown in the table below.

Fig. I. The number of zoospores, total number of sporangia and number of germinated sporangia obtained from slopes incubated for 7-15 days after inoculation

X = number of zoospores

O = total number of sporangia

O = number of germinated sporangia

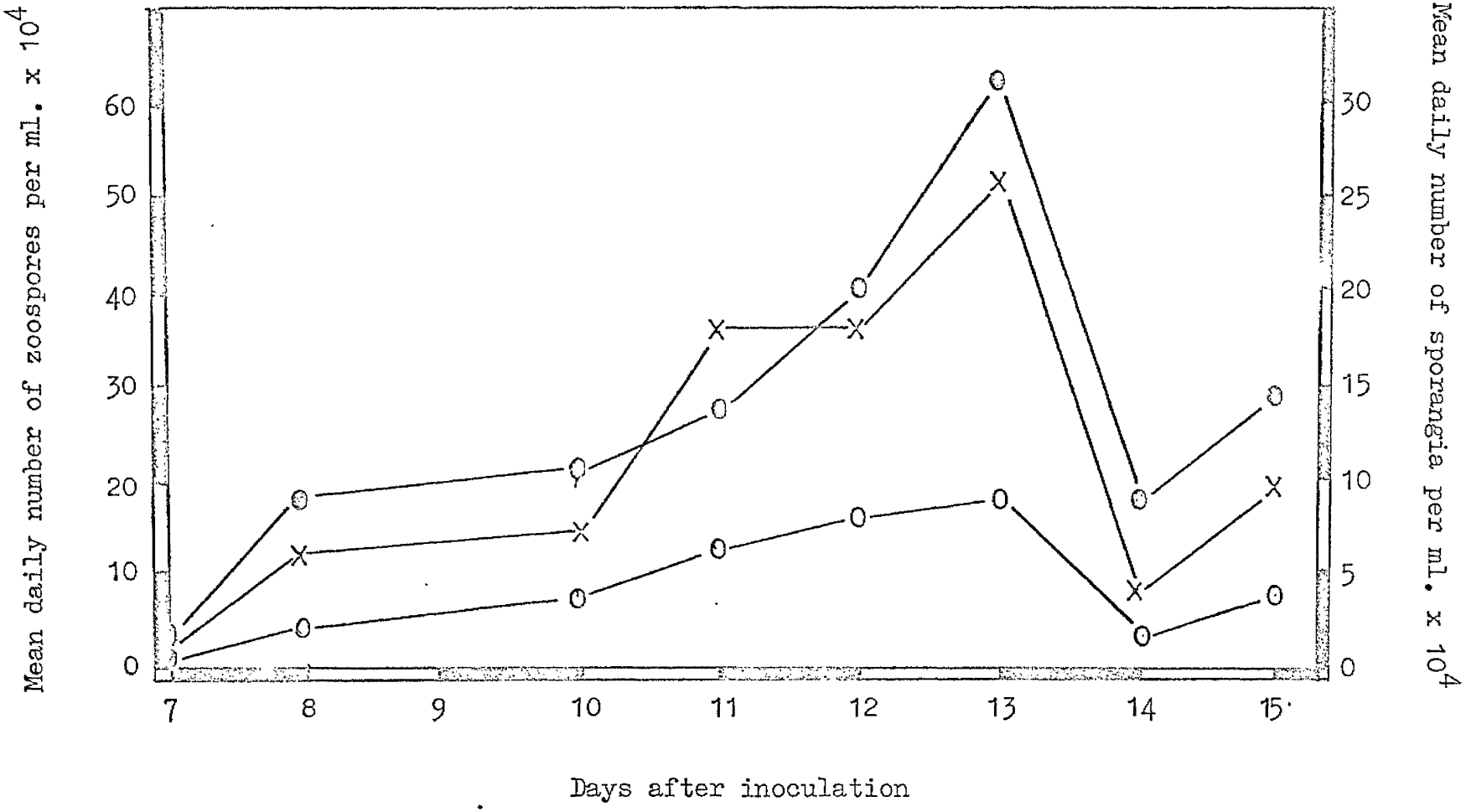


Table 1. The numbers of zoospores, and the percentage germination of the sporangia, obtained from slopes incubated for 7-15 days after inoculation

		Days after slopes were inoculated							
		7	8	10	11	12	13	14	15
Mean No. of zoospores x 10^4 per ml.	Expt. 1A	0.3	11.3	16.8	38.2	36.7	51.8	7.8	19.0
	Expt. 1B	3.3	4.5	6.8	24.7	30.7	42.7	21.3	26.7
Mean % germination of sporangia	Expt. 1A	8.3	21.2	42.3	46.4	38.1	32.7	26.1	22.2
	Expt. 1B	18.5	13.3	28.5	35.5	30.5	40.8	16.3	20.2

Figure one shows the results of experiment 1A plotted on a graph with respect to the mean daily number of germinated and total number sporangia and the number of zoospores.

From the results of these experiments it appears that the total number of sporangia, number of zoospores, and number of germinated sporangia all reach a maximum on the thirteenth day. Subsequently all these values, including the percentage of sporangia which germinated, decrease. Two anomalous points arise, one being that the percentage germination of the sporangia gradually increased up to the thirteenth day, and thereafter decreased. There are two factors which may account for this:-

(1) The concentration of the sporangia in suspension may influence the number of germinated sporangia so that the higher the concentration then the greater is the number of sporangia which germinate indirectly.

(2) The age of the sporangia may be important in determining the proportion of sporangia which germinate to give zoospores. Thus it may be suggested that the 13-day old slopes yield the greatest number of sporangia within the optimum age for germination.

The second anomaly is concerned with the decrease in total numbers of sporangia obtained in suspension. This may be explained by the fact that, at times greater than 13 days, the sporangia may be more difficult to get into suspension, possibly because they become more firmly attached to the sporangiophore, or because the freed sporangia are trapped in the interwoven meshwork of hyphae.

In all subsequent experiments 11-13 day old cultures were used for the production of zoospore suspensions. Since other factors have been reported to influence the germination of sporangia, the next two experiments were constructed to investigate the effects of light and the suspending solution on the production of zoospores.

Expt. 2. <u>The effect of incubating 13-day old sporangia in different solutions on the production of zoospores</u>

Six slopes were inoculated and allowed to grow for 13 days before the sporangia were washed off. 1.0 ml. samples were removed from each sporangial suspension and added to 9.0 ml. of glass distilled water, 2% sucrose solution and 1% boiled potato extract, prepared as described by McKee (1964). The tubes were then incubated for 2 hrs. at 12°C and then 20 samples were withdrawn from each and the numbers of zoospores,

and germinated and ungerminated sporangia were counted in a haemocytometer. The experiment was repeated and the mean number of zoospores and the mean percentage germination of the sporangia for each treatment is shown in table 2 for both experiments.

The results suggest that there was no marked increase in the number of zoospores produced by germinating sporangia in 2% sucrose solution or in 1% boiled potato extract and so glass distilled water was used throughout.

Table 2. The mean number of zoospores and the mean percentage germination of sporangia obtained on incubating sporangia in glass distilled water, 2% sucrose solution and 1% boiled potato extract

Incubation Solution	Mean No. of zoospores x 10^4 per ml.		Mean percentage germination	
	Expt. 2A	Expt. 2B	Expt. 2A	Expt. 2B
Glass distilled water	26.8	23.9	38.7	26.7
2% sucrose	33.1	15.8	34.1	33.2
1% boiled potato extract	24.2	27.4	35.4	23.7

Expt. 3. The effect of light on the germination of sporangia

Two 1.0 ml. samples were withdrawn from each sporangial suspension obtained from six 13-day old cultures. One 1.0 ml. sample was pipetted into 9.0 ml. of glass distilled water and placed in an incubator at 12°C in the dark as in previous experiments. The other 1.0 ml. sample was similarly treated and then placed in another incubator at 12°C at a distance of 20 cms. from two 6W fluorescent tubes approximately 23 cms. in length.

The results obtained from the two experiments which were carried out show that fewer zoospores were recorded in the tubes incubated in the light than in the dark and that this decrease could probably be attributed to fewer sporangia germinating in the light than in the dark.

Table 3. The effect of light on the proportion of germinated sporangia and on the number of zoospores obtained

Light Treatment	Mean No. of zoospores x 10^4 per ml.		Mean % germination of sporangia	
	Expt. 3A	Expt. 3B	Expt. 3A	Expt. 3B
Light	16.7	17.1	26.2	16.8
Dark	27.1	23.4	39.4	27.8

The next experiment was set up to investigate the effect of

incubation time on the number of zoospores produced by a sporangial suspension since zoospores may be capable of survival for a relatively short period and/or more sporangia may germinate with increased incubation time.

Expt. 4. The effect of incubation time on the release of zoospores from sporangia at 12°C

Sporangial suspensions were obtained from three slopes of a 13-day old culture. 1.0 ml. of each suspension was pipetted into each of 5 test tubes containing 9.0 ml. of glass distilled water. The tubes were placed in an incubator at 12°C and then after 1, 1.1/2, 2, 3 and 4 hours three tubes, one from each slope, were sampled and the number of zoospores present was estimated by counting on a haemocytometer. Ten readings were taken per tube. The experiment was carried out twice. The results are expressed as a mean of the zoospores per ml. and are shown in table 4.

Table 4. The number of zoospores produced by a sporangial suspension after various periods of incubation

Incubation Period	Mean No. of zoospores x 10^4 per ml.	
	Expt. 4A	Expt. 4B
1 hour	9.3	8.3
1.1/2 hours	14.3	14.3
2 hours	19.7	16.7
3 hours	23.3	16.7
4 hours	20.3	15.7

Since there was little increase in the number of zoospores produced after about two hours incubation, this period was used throughout all subsequent experiments. As a result of this series of preliminary experiments, the following conditions were maintained throughout the work reported in this thesis:-

(1) Sporangial suspensions were obtained from 11-13-day old cultures grown on slopes of chick pea agar.

(2) The sporangia were germinated in sterile glass distilled water for a period of 2 hours at 12°C in the dark.

III. INVESTIGATIONS ON THE STIMULATORY ACTIVITY IN APPLE PECTIN

INTRODUCTION

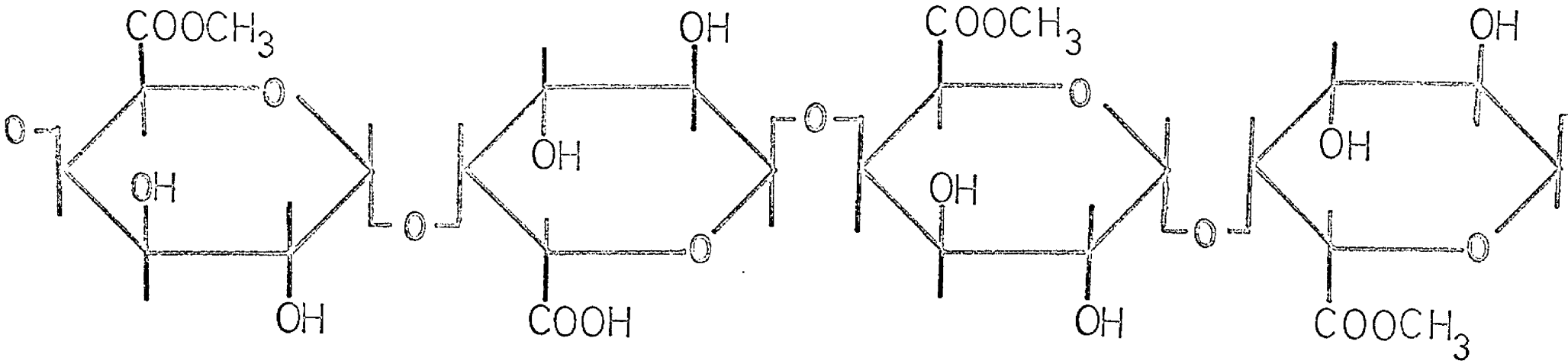
Growth of established cultures of *P. infestans* is readily obtained on an agar medium containing glucose, yeast extract, asparagine, potassium monophosphate, magnesium sulphate and thiamine, (French, 1953). However, when isolated zoospores are plated onto this medium, although germination occurs and a sporeling is produced, little morphological differentiation takes place beyond this point. Generally only a few short side branches develop on the sporelings and usually less than 0.1% continue growth to form colonies. Clarke, (1966) found that when apple pectin was added to a medium containing KH_2PO_4; $MgSO_4 7H_2O$; asparagine; glucose; thiamine HCl and solidified with agar, a proportion of the germinated zoospores produced masses of densely packed hyphae and grew to form visible colonies within 6-8 days.

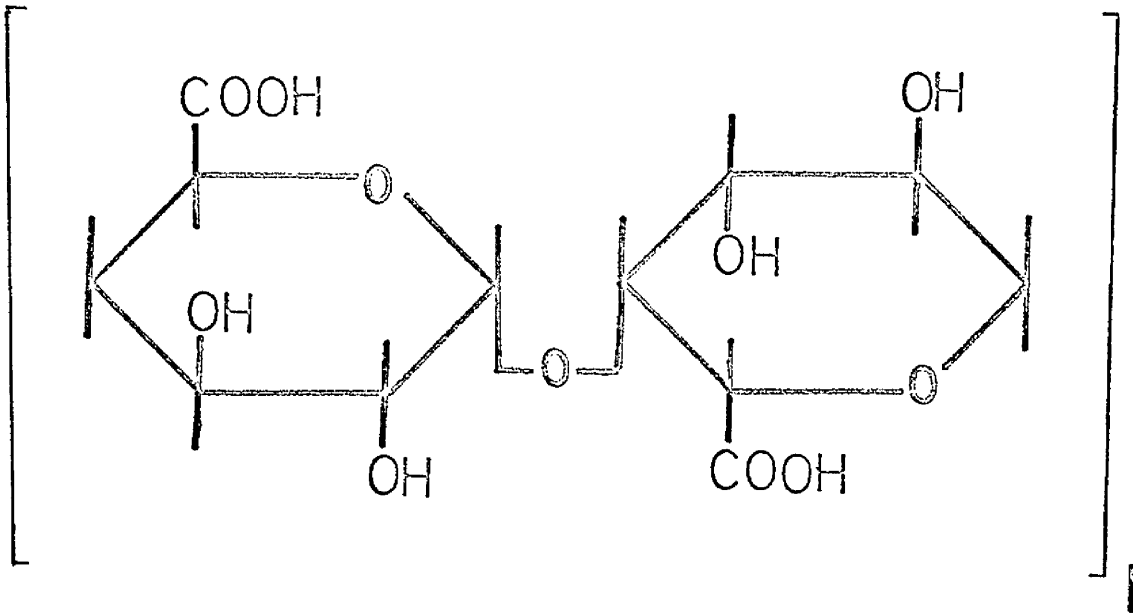
This medium then provides an environment similar to the potato plant, *S. tuberosum*, in so much as it supplies the conditions necessary for the production of masses of vegetative mycelium and reproductive structures from isolated zoospores. There are at least three ways in which this medium could initiate post-sporeling growth:-

(1) by removing an inhibitory factor which may be present in the medium or in the fungus itself. Its removal could be achieved

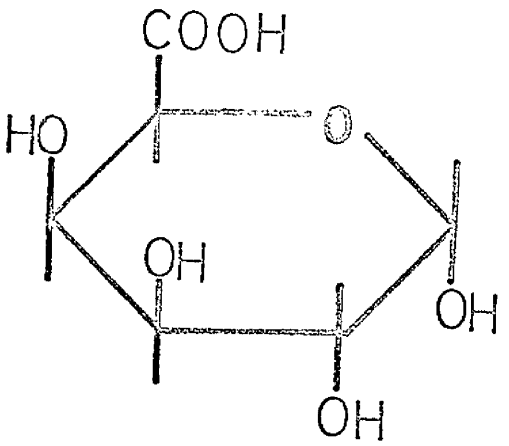
Fig. II. The structure of the pectin molecule

(a) Partially de-esterified pectin





(b) Polygalacturonic acid



(c) D-Galacturonic acid

in several ways, either by adsorption or detoxification or some other process.

(2) by supplying a substance which is necessary for the initiation of post-sporeling growth.

(3) by modifying the physical environment in some way such as providing an appropriate contact stimulus(Dickinson, 1949).

Apple pectin must be responsible for the growth effect but since commercial apple pectin, as supplied, is not a pure compound, the effect could be due to one or more of the accompanying impurities and/or the pectin itself. Commercially produced apple pectin represents a compromise to some degree between purity and economic quantity production. It is therefore desirable to discuss briefly the commercial preparation of apple pectin, but before doing so, a short account of the chemical nature of pectin will be given.

THE CHEMISTRY OF PECTIN

It is not within the scope of this introduction to deal with the chemistry of pectin in great detail, but adequate treatment of the subject has already been given by Kertesz (1951).

Pectin forms a colloidal solution in water and has the ability to form gels in the presence of sugars. This property alone has contributed greatly to its importance in the food industry. Basically pectin is a methyl ester of pectic acid which is in turn a polymer of galacturonic acid (see Fig. II). The individual galacturonic acid units are joined together by α-1-4 glycosidic ester linkages. The carboxyl

groups at the 6 position may be free or esterified with a methoxyl group. The primary structure of the pectinic acid is therefore a long chain of galacturonic acid units but a secondary structure is formed when side chains are attached. These side linkages may occur through the carboxyl groups and so a complex branched molecule is produced which in many ways resembles the general form of the amylopectin molecule. Evidence that there are other linkages besides the ester linkage has come from various work but one of the most enlightening is that of McColloch and Kertesz (1948) who used a tomato enzyme to hydrolyse pectin. They found on treating pectin solutions with this enzyme that less than 50% of the theoretical amount of hydrolysis was obtained but the addition of mould polygalacturonase completed the hydrolysis almost to the theoretical value of 100%. The interpretation of these results by the authors suggested that "limit" polygalacturonides similar to the limit dextrins of starch might be present. The break in the continuity of the polygalacturonic acid molecule might be caused by the formation of side branches, by a different type of linkage or by the presence of a different type of structural element. Acetate, phosphate, xylose, arabinose, methyl pentose and galactose are all associated with pectin in nature and may possibly enter into the composition of the molecule (Kertesz, 1951). Pectin is capable of both ionic and hydrogen bonding so that these or other compounds although being in close association with the pectin molecule, might not form an integral part of it. Much controversy has surrounded the determination of the exact constituents of pectin but it seems certain that commercial preparations are not wholly composed of

polymerised galacturonic acid units. The molecular weight has similarly caused disagreement among workers using different samples and different methods of determination but the most reliable results obtained using ultra centrifugation techniques put the moleculr weight of commercial apple pectin at approximately 67,000 and that of lemon pectin at approximately 89,000.

COMMERCIAL PREPARATION OF PECTIN

Commercially the most important raw materials for the production of pectin are the residues left over from the manufacture of citrus juices and apple juices although the residues left over from the production of sugar from sugar beet have also been used in Germany. In Britain the main source of raw material comes from the cider industry.

As the apple fruit matures, pectin continues to be synthesised and so the total weight of pectin substances per apple increases as it matures although when the weight of pectin is expressed as a percentage of the dry weight, the maximum is attained in very immature fruit (Widdowson, 1932). In the immature fruit, the pectin remains as the insoluble form known as proto-pectin and this is solubilised by the action of pectinases which exert their effect once the fruit is nearly ripe. By the time the fruit falls to the ground the proto-pectin is largely solubilised and enzymes from microbial organisms which enter the fruit through cracks and bruises further soften and degrade the pectin. Since the maximum of fermentable sugars and aroma is required for cider production, mature fruit are utilised, hence some of the pectin content

is lost in the juice which is pressed from the apples. The residue after pressing, known as apple pomace, on arrival at the pectin manufacturers contains about 66% water, besides yeasts, fungi, and bacteria which are all capable of further degrading the pectin and therefore to avoid further loss the pomace is dried as soon as possible after it arrives. Extraction and solubilisation of the proto-pectin is usually carried out by hydrolysis. Generally hydrochloric acid is used but the pH, time, temperature and ratio of water to pomace are all standardised according to the manufacturer's own ideas and to the degree of esterification which is required. The solubilised pectin is then separated from the spent pomace but as starch is extracted with the pectin - amylase is added to remove it. The weak pectin extract is then concentrated by evaporating off some of the water. Following this, as the methoxyl content of the pectin greatly affects the physical properties of the final product, the extract is subjected to one of the following processes in order to vary the degree of esterification of the final product:-

(a) controlled acid hydrolysis

(b) controlled alkaline hydrolysis

(c) enzyme action (pectin-methyl esterase)

The pectin is then precipitated out of solution using the salts of aluminium, copper or calcium depending upon the degree of esterification required. The metallic pectinate is washed with acidified alcohol after drying and grinding, to remove the metallic ions. The pectin is then washed with neutral alcohol until the washings are free of metallic ions

and then it is finally dried.

The brief description above refers to the manufacture of dry powdered apple pectin. Liquid pectin and pectin from the other raw materials mentioned earlier involve slightly different processes which have been described by Kertesz (1951) and more recently and authoratatively by Potter (1966). Both from the chemical nature of pectin and from the brief outline of the extraction procedures used it is evident that commercial apple pectin cannot be simply a polymer composed of galacturonic acid units but will certainly contain other substances. These compounds may be chemically bound within the pectin molecule or adsorbed on to it or present as free contaminants.

The experiments to be described were carried out to investigate these possibilities.

A. INVESTIGATION BASED ON UNPURIFIED PECTINS

The basal medium used throught was essentially that used by Clarke (1966) and had the following composition -

KH_2PO_4	0.500g.
$MgSO_4 7H_2O$	0.250g.
Asparagine	1.000g.
Thiamine HCl.	0.001g.
Difco Yeast Extract	5.000g.
Glucose	25.000g.
Agar	20.000g.
Water to	1.0 litre

Additions of pectins were made to this medium as described later in the text.

Analar grade chemicals were used wherever possible and all media were adjusted to pH 5.5 using NHCl. or NNaOH as required, before autoclaving at 10 lbs. for 10 mins. Clarke (1966) reported that a percentage of the sporelings developing on this medium supplemented with apple pectin (obtained from British Drug Houses) at a concentration of 10.0g. per litre, produced colonies within 6-8 days. The next experiment was carried out to determine the effect of different concentrations of B.D.H. apple pectin on the number of sporelings continuing growth to form colonies.

Expt. 5. <u>The effect of varying the concentration of pectin in the medium on the numbers of colonies produced</u>

Stock solutions of apple pectin were prepared by gradually adding the dry powdered pectin to a gently warmed volume of water which was constantly stirred. The pectin solution was added to the basal medium to give a final concentration of pectin from 0 to 20.0g. per litre in 2.5g. steps. After autoclaving the media were poured into 10 cm. petri dishes. Fifteen plates, each containing approximately 30 ml. of medium, were poured from each medium.

The plates were inoculated with 0.2 ml. of inoculum containing approximately 200 zoospores. The inoculum was then evenly distributed over the surface of the agar by means of a glass spreader and the plates were incubated at 20°C for 8 days. These methods were used throughout

*

The asterisk denotes the point at which a significant difference from the control at the .05 level is first detectable.

Analysis by the χ^2 method was carried out between the total number of colonies produced on the different media and the number produced on the apple pectin medium at 10g./litre. Thus media A, B, C, H and I, as well as medium G produced fewer total number of colonies than medium E. Medium F did not however produce a significantly greater number of colonies. This method will be used throughout the presentation of the tables in chapters III and IV.

the work. The experiment was carried out three times and similar results were obtained each time.

The results of one experiment are given in table 5, and the method of presentation employed in this table will be used throughout.

Table 5. The number of colonies produced on a basal medium supplemented with different amounts of apple pectin

Medium	Pectin conc. in g./litre	Number of colonies per plate (Mean of 15)	Percentage ø colony production
A	0	0	0
B	2.5	3.2	8.0
C	5.0	8.0	20.0
D	7.5	40.0	100.0
E	10.0	40.0	100.0
F	12.5	42.5	106.3
G	15.0	34.0	85.0*
H	17.5	33.0	82.5
I	20.0	34.0	85.0

ø These results have been calculated as a percentage value of the number of colonies produced on the medium containing apple pectin at a concentration of 10g./litre.

Most colonies developed on media containing between 7.5 and 12.5g./litre and so 10g./litre was used in all further experimental work.

Although an optimum concentration of pectin in the medium had been

* See facing page.

established for the initiation of post-sporeling growth it was, however, important that a large proportion of the zoospores plated onto this medium should develop into colonies. Haemocytometer counts of stock zoospore suspensions may prove unreliable in ascertaining this proportion as errors could arise in counting together with possible errors during pipetting and loss of spores by rupture during spreading. Other zoospores may burst or fail to germinate on the medium. Such losses would affect the results if calculated as the percentage number of colonies developing from the calculated number of zoospores plated and so it seemed more realistic to make direct measurements by taking transects with the microscope across a petri-dish which had previously been inoculated with sparce numbers of zoospores approximately one week beforehand. In observations of this type it was found that on average 92% of the germinated zoospores of isolate R4DS grew to form colonies on the medium containing apple pectin at a concentration of 10g./litre.

Approximately 20% and 5% of the sporelings of isolate 91R1R and R41 respectively continued growth to form colonies. The choice of isolate which was used for the work reported in this thesis was largely based upon these results, (see page 18).

Since a relatively high proportion of the zoospores germinated on the pectin medium produced colonies, there seemed to be no real objection to adopting these techniques as standard practice. This method provided an adequate standard for bioassaying various compounds as well as rendering a sufficient number of potential colony forming sporelings which could be studied for example histochemically.

Expt. 6. The effect of different pectins on post-sporeling growth

Pectins were obtained from a variety of sources as listed in table 6, and tested for their ability to induce post-sporeling growth. They were incorporated into the basal medium at the rate of 1.0, 5.0, 10.0 and 20.0g. per litre. Eight plates of each medium were used in each experiment and each sample of pectin was tested at least three times. The basal medium without additions was used as a control, and the plates were examined 10 days after inoculation.

A typical set of results is shown in table 7 for pectins incorporated into the medium at 10g. per litre. The effect of different concentrations of B.D.H. apple pectin in the medium on post-sporeling growth has already been tested in the previous experiment. Colonies only developed on the H. & W. apple pectin medium (medium B) when it was incorporated at a concentration of 10g. into 1 litre of medium, and no colonies developed on any of the other media. When the plates were examined microscopically it was evident that it was only on media A and B that growth beyond the sporeling stage takes place although germination occurred and growth from mass hyphal inocula took place on all media.

Table 6. Samples of commercial pectins used

Incorporated into medium	Source	Grade [A]	Fast/Slow Set [B]	Origin
A	British Drug Houses	250	-	Apples
B	Hopkins & Williams	150	Fast Set	Apples
C	Henry Manser	200	Slow Set	Apples
D	Koch Light	-	Slow Set	Citrus Fruits
E	Sigma	-	-	Citrus Fruits
F	Sunkist	-	Slow Set	Citrus Fruits
G	Sunkist	-	Fast Set	Citrus Fruits

A. The grade is a measure of the proportion of sugar which one part of solid pectin or pectin extract is capable of turning, under prescribed conditions, into a jelly with suitable characteristics.

B. Fast set pectins usually start to form a jelly at 88°C and slow set at about 54°C. The slow set pectins have a lower methoxyl content than do fast set pectins.

Table 7. Number of colonies produced on media containing different samples of pectin at a concentration of 10g./litre

Medium	No. of colonies per plate (Mean of 8)	% colony production
A	46.3	100
B	9.6	20.7*
C, D, E, F, G	0	0
Basal Medium	0	0

Two of the samples of apple pectin induced post-sporeling growth although a third sample showed no such activity. This variation could be due to differences in the pectin molecule or to the methods used in the commercial preparation. Absence of activity of the third sample of apple pectin and of the citrus pectins could be due to the presence of inhibitors. Since germination took place and vegetative growth from mass hyphal transfers occurred, the inhibitors, if present, would appear to be active only on the initiation of post-sporeling growth.

The following series of experiments was set up in an attempt to investigate whether or not substances which inhibited the activity of B.D.H. apple pectin were present in a few of the samples of pectin.

Expt. 7. The activity of purified Apple and Citrus Pectins on colony formation

25.0g. of dry apple pectin (B.D.H. and H. & W.) and citrus pectin (K-L and Sigma) were placed in a small muslin bag and extracted in a Soxhlet with 60% ethanol previously acidified to give a final solution of 0.1 N with HCl. After the pectin was washed for 24 hours, it was filtered on a Buchner funnel fitted with Whatman No.1 filter paper and washed with 60% ethanol until the washings showed no trace of chloride ions when tested with silver nitrate. The sample was then washed with 90%, 95% and 100% ethanol three times and then with ether. After drying in an oven at 60°C overnight the pectin was allowed to cool in a vacuum desiccator for 24 hours. Stock solutions were then prepared in the normal way and media were prepared.

The ethanol extracts were evaporated to dryness in a rotary evaporator under a reduced pressure and then taken up in water.

Media were prepared incorporating unpurified pectins, extracted pectins, and extracts. In the case of extracts obtained from the Koch Light citrus pectin and the H. & W. apple pectin, these were added in equivalent amounts to untreated B.D.H. apple pectin. The number of colonies produced on each medium was recorded 10 days after inoculation.

Table 8. The number of colonies produced on various media after treatment in Soxhlet Apparatus

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	B.D.H. apple pectin	38.0	100.0
C	Extracted B.D.H. apple pectin	39.6	104.2
D	K-L citrus pectin	0	0
E	Extracted K-L citrus pectin	0	0
F	B.D.H. apple pectin + K-L citrus pectin extract	37.3	98.0
G	H. & W. apple pectin	13.0	34.1
H	Extracted H. & W. apple pectin	14.2	37.3*
I	B.D.H. apple pectin + H. & W. apple pectin extract	37.0	97.4

Germination and vegetative growth from a mass hyphal inoculum occurred on all media. No colonies developed on media containing

ethanolic extracts of pectin. The experiment was carried out three times with similar results being obtained on each occasion.

If toxins in the citrus pectin are responsible for the lack of growth then they are not removed by washing for a period of 24 hours with hot acidified 60% ethanol, and so probably do no occur free.

Expt. 8. The effect of adding citrus pectin to various amounts of apple pectin in basal medium on post-sporeling growth

In order to further investigate the possibility that citrus pectin contains an inhibitory substance a further experiment was carried out in which both were incorporated at various levels into the same medium as shown in table 9.

Table 9. The concentration of apple pectin (A) and citrus pectin (C) added to the basal medium

g./litre	10A	5A	2.5A	0A
10C	10A + 10C	5A + 10C	2.5A + 10C	0A + 10C
5C	10A + 5C	5A + 5C	2.5A + 5C	0A + 5C
2.5C	10A + 2.5C	5A + 2.5C	2.5A + 2.5C	0A + 2.5C
0C	10A + 0C	5A + 0C	2.5A + 0C	0A + 0C

The experiment was repeated and results were obtained similar to those cited as follows.

Fig. III. Colony production on pectin medium to which additions of citrus and apple pectin have been made

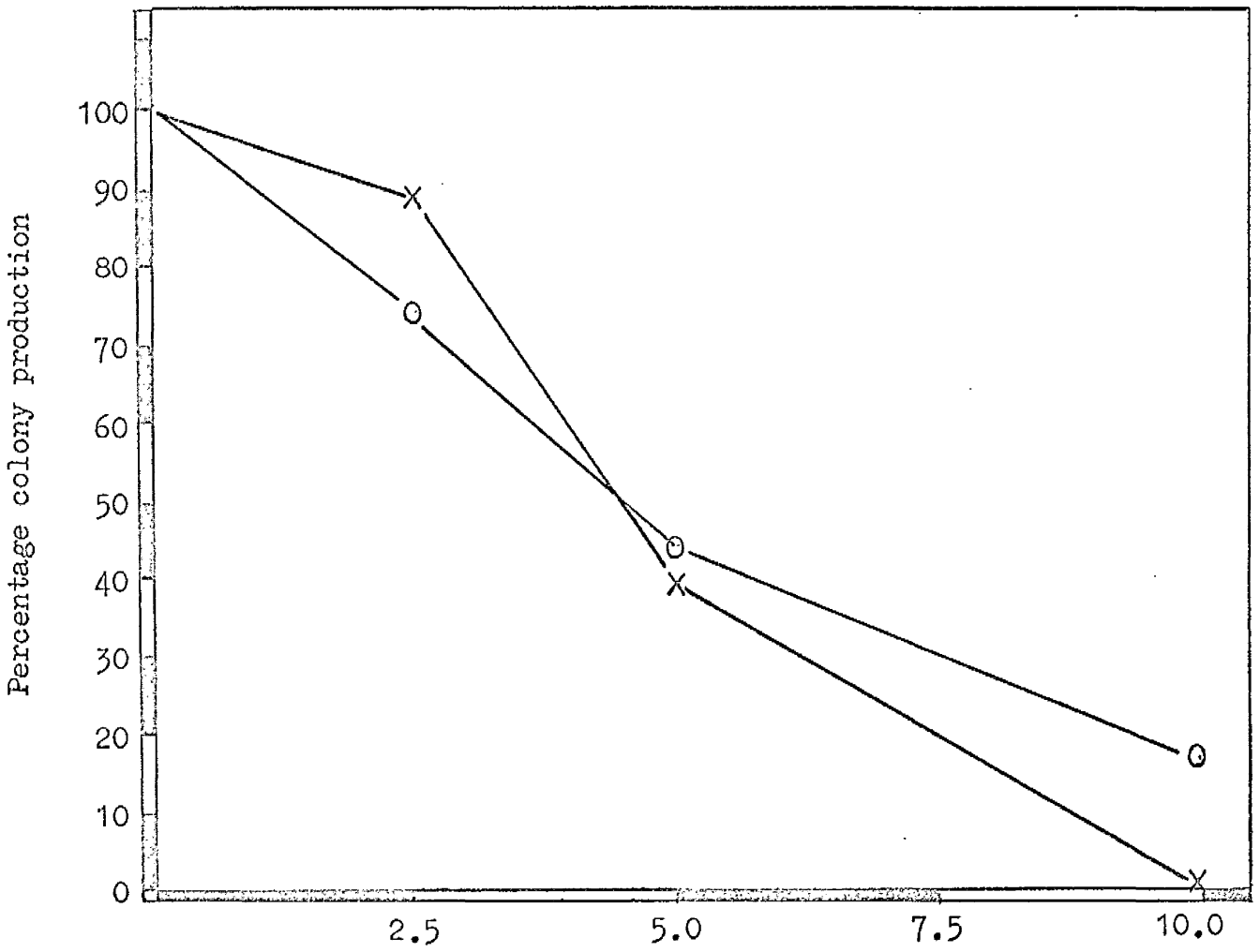


Table 10. The number of colonies produced by media containing various combinations of apple and citrus pectin

g./litre	10A		5A		2.5A		0A	
	No.	%	No.	%	No.	%	No.	%
10C	0	0	0.5	1.4	1.1	3.4	0	0
5C	12.4	39.9*	0.2	0.6	0	0	0	0
2.5C	29.4	94.4	0.1	0.2	2.1	6.8	0	0
0C	31.1	100	0	0	0	0	0	0

No. = Number of colonies per plate (mean of 8).

% = Percentage colony production.

The addition of citrus pectin to the standard concentration of apple pectin causes a decrease in the number of colonies produced. However, when this decrease is compared with the effect of adding additional apple pectin to the medium (Fig. III) the results are very similar. Also, in all cases when apple pectin was absent from the medium, sporeling growth was the same as that which occurred on the basal medium. On all media containing apple pectin the sporelings were larger and the hyphae more branched.

From the last two experiments it appears that the stimulatory action of apple pectin (B.D.H.) is possibly due to some chemical feature specific to this material since there was no evidence to suggest that the citrus pectin contained any inhibitory activity. However, whether the effect is due to specific contaminant or to some specific feature

of the apple pectin molecule is not known. That apple pectin contains a number of impurities was shown by the following experiment.

Expt. 9. The growth response of some biochemical mutants of Aspergillus nidulans on a medium supplemented with apple pectin

A number of biochemical mutants of Aspergillus nidulans were obtained from the Genetics Department, Glasgow University. For these tests minimal medium i.e. a minimal medium on which P. infestans would grow, was used. This minimal medium consisted of the normal basic medium without the addition of yeast extract, and was prepared in the normal way with or without the addition of apple pectin. A loopful of dry spores of A. nidulans was inoculated onto slopes of both minimal and minimal plus pectin media in test-tubes. Where growth of the mutant was supported after six days, another spore inoculum was taken from this and re-inoculated. This process was repeated three times to eliminate the possibility of nutrients being carried over with the inoculum. Where growth had not occurred, fresh inoculum was taken from the stock cultures. At the end of the third transfer Aspergillus minimal and complete media were inoculated to ensure that the strain still expressed the specific requirement. The Aspergillus minimal medium had the following composition.

Sodium nitrate	1.0g.
Pot. dihyd. phosphate	0.5g.
Mag. sulphate	0.25g.
Glucose	25.0g.
Davis Agar	15.0g.
Water to	1 litre

The medium was adjusted to pH 5.5 with NHCl. or NNaOH before autoclaving at 10 lbs. for 10 mins. Complete medium was prepared by adding the specific requirement at the following concentrations.

Amino Acids	1 g. in 10 ml.
Purines and pyrmidines	0.1g. in 10 ml.
Vitamins etc.	0.01g. in 10 ml.

0.05 ml. of the appropriate solution was added to each slope of minimal medium.

Table 11. The growth of biochemical mutants of Asp. nidulans on minimal and pectin media

Isolate Growth Requirement	M.M.	P.M.
amino-butyric acid	+	+
biotin	-	-
inositol	-	+
leucine	-	-
lysine	-	-
nicotinic acid	+	++
pantothenic acid	-	+
p-amino-benzoic acid	-	-
phenylalanine	-	-
proline	-	+
putrescine	+	++
reduced sulphur	+	+
riboflavine	-	-
thiamine	+	++

- = no growth + = growth ++ = amount of growth greater than that observed on the M.M. by the same isolate.

The pectin medium is therefore capable of supporting the growth of certain biochemical mutants of Aspergillus nidulans, and so the pectin must contain the specific requirement or a related compound. It would therefore appear that commercial apple pectin is not a pure compound. A series of experiments was therefore set up to test the hypothesis that the stimulation of post-sporeling growth was caused by a contaminant and not the pectin itself.

In Expt. 7 apple pectin was washed with acidified 60% ethanol. By so doing, free sugars and organic acids for the most part would have been largely removed, but little, if any, change should have occurred in the pectin molecule. Since the extract was inactive it must be concluded that the stimulatory fraction had not been removed in sufficient quantity, if at all, but the possibility remained that it might be removed by other solvents with different affinities for a range of compounds.

Expt. 10. The effect of washing apple pectin with various solvents on colony formation

25.0g. samples of apple pectin were extracted in a Soxhlet with diethyl ether, petroleum ether, acetone, methanol and ethanol. Methanol would also have the effect of increasing the degree of esterification of the pectin and so this change may prove important. Each preparation was extracted for 24 hrs. The extract was evaporated to dryness in a rotary evaporator under reduced pressure. The pectin was dried off in an oven at 60°C overnight and then allowed to cool in a vaccuum desiccator. Media were prepared from the pectins at normal strength (10g./litre)

and the extracted material was incorporated into media at concentrations equivalent to 10 and 50g. apple pectin per litre.

The colonies which developed on the media containing the extracted pectin, did so at approximately the same rate and were morphologically similar to each other. No colonies developed on the media containing the extracts and in every case the spores germinated but failed to grow beyond the sporeling stage. The experiment was carried out on a further two occasions and similar results were obtained.

Table 12. The number of colonies produced on pectin after treatment with various solvents and on extracted material

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	76.0	100.0
C	Pet. ether extracted A.P.	73.0	96.1
D	Pet ether extract	0	0
E	Ether extracted A.P.	65.7	86.4
F	Ether extract	0	0
G	Acetone extracted A.P.	79.8	105.0
H	Acetone extract	0	0
I	Methanol extracted A.P.	66.0	86.8*
J	Methanol extract	0	0
K	Ethanol extracted A.P.	77.0	101.3
L	Ethanol extract	0	0

The methanol extracted pectin did lose some of its ability to induce colony formation so that the probable increase in methoxyl content which occurred may have had an appreciable effect.

Thus there was no evidence to suggest that low molecular weight substances not bound to the pectin molecule were responsible for the growth effects. However it is possible that large molecular weight substances, such as cellulose, hemicellulose or protein, may be responsible and these would not be removed by any of the extraction procedures so far used.

Expt. 11. Precipitation of pectin out of aqueous solution by ethanol and acetone

Precipitation of pectin is one of the principal processes used in the manufacture of pectin to free it from other contaminating substances and ethanol has been widely used for this purpose. Acetone on the other hand, although not used commercially, is commonly used for the laboratory purification of pectin. Both solvents are reported to precipitate pectinic acids from admixed non pectic material such as hemicelluloses arabans and galactans (Kertesz, 1951). The ethanol was acidified with HCl. to give a final acid concentration of 0.05 N.HCl. as this reduces the quantity of sugars and acids co-precipitated with the pectin; aqueous solutions of 0.1% concentration were used for both precipitation procedures. The precipitate obtained was separated from the supernatant by pressing through a silk cloth, and was then washed with the appropriate solvent on a buchner funnel (until free from chloride ions

in the case of the ethanol extract) and dried by grinding in a pestle and mortar. The supernatants were evaporated to dryness in a rotary evaporator and made into media equivalent to 1.0, 10.0 and 25.0g./litre of apple pectin.

Table 13. The number of colonies produced by precipitating pectin

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0*
B	Apple pectin	26.4	100.0
C	A.P. ppted. by ethanol	25.9	98.2
D	Ethanol extract (all concs.)	0	0
E	A.P. ppted. by Acetone	24.1	91.3
F	Acetone extract (all concs.)	0	0

The morphology of the colonies and their rate of development was the same on all media containing the precipitated pectins. Germination and growth to form sporelings occurred on all media containing the acetone or ethanolic extracts.

Expt. 12. The effect of pectin dialysed against water on post-sporeling growth

Since it was conceivable that even the more polar solvents such as methanol and ethanol might not extract highly polar compounds, this final experiment was carried out to determine whether the factor would

be extracted in water.

Apple pectin solution was placed in Visking dialysis tubing (24/32") which was then placed either in 500 ml. conical flasks containing distilled water and shaken for 24 hrs. or placed in a flask through which ordinary tap water was constantly run for a period of 4 days. The undialysed pectin was then made up to give pectin at a concentration of 10g./litre in the medium and similarly the dialysate was corrected to give an equivalent volume of medium.

Table 14. The number of colonies produced by dialysed pectin solutions

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	32.5	100.0
C	Dialysed pectin (24 hrs.)	25.7	79.0*
D	Dialysate (24 hrs.)	0	0
E	C + D	22.0	67.7
F	Dialysed pectin (4 days)	24.4	75.1

Medium E which contained both dialysed pectin and dialysate consistantly supported a lower percentage number of colonies than the control medium B. It would therefore appear that a toxic substance may be originating from the dialysis tubing.

The results of these experiments enable certain conclusions to be drawn. Of several commercial preparations of citrus and apple pectin tested, only two stimulated post-sporeling growth in P. infestans. Both preparations originated from apples and the sample from B.D.H. was more active than that from Hopkins and Williams. A further sample of apple pectin was inactive and so the source of material and the method of preparation may be important. The inability of one of the citrus pectins to support post-sporeling growth was shown not to be due to the presence of an inhibitor. The optimum concentration of apple pectin (B.D.H.) which promoted post-sporeling growth was found to be 10g./litre, and in the isolate of P. infestans under test, this concentration induced 92% of the sporelings to grow to form colonies.

Attemps have been made to isolate the factor or factors present in apple pectin which is responsible for post-sporeling growth but removal from the pectin molecule has so far failed, and therefore the next series of experiments was set up.

B. INVESTIGATION OF DEGRADED PECTIN

If the active factor is the pectin molecule itself or some other compound closely bound to it, then it is possible that the fungus may obtain this factor through the activity of extracellular enzymes. Thus it may be possible to investigate this point by hydrolysing the the pectin and contaminants enzymically or chemically and investigating the break down products for the ability to stimulate post-sporeling growth.

ACID HYDROLYSIS

In the commercial preparation of apple pectin, the insoluble pectin material, the so-called protopectin, of the pomace is solubilised generally by using 1.0-2.5 N hydrochloric acid for a period determined by the degree of esterification required in the final product. The mechanism by which the protopectin is solubilised is not really known although several ways in which it could be brought about by acid have been suggested (Kertesz, 1951).

When pectin is temporarily exposed to cold dilute acids no detectable change in the molecule takes place. Demethylation of the pectinic acid does however occur on treatment with strong acids or dilute acids at 50°C or over, or to a lesser extent with prolonged periods in dilute acid. Under these conditions increased exposure brings about degradation of the pectin material. Acids bring about more or less random

cleavage of polysaccharides but those composed of uronic acids undergo more extensive hydrolysis elsewhere in the molecule than at the 1-4 glycosidic linkage (Pigman, 1957). Hydrolysis of polysaccharides using acids is a process which reaches an equilibrium - dilute acids favour the formation of monomers. Decarboxylation and further decomposition of the newly formed galacturonic acid proceeds slowly in dilute hot acids (Kertesz, 1951).

BASE HYDROLYSIS

Bases, like acids, bring about decomposition and solubilisation of crude protopectin. Their action is also similar to acids in so much as they demethylate pectinic acids. The bulk of the ester linkages may be removed in a few minutes at room temperature and this capacity has been exploited in industry for the controlled de-esterification of pectin. Glycosidic linkages have been taken as stable to the action of alkalis (Pigman, 1957). If this is strictly true, then the report by Potter (1966) that alkaline de-esterification is usually associated with some degradation of the pectin molecule may supply additional evidence for the existence of linkages other than the α 1-4 glycosidic one.

ENZYME HYDROLYSIS

Degradation of the pectin molecule by enzyme action can be achieved in several ways and a classification of pectolytic enzymes has been attempted.

Owing to various factors such as the undefined structure of the pectin, the possible interaction between enzymes, and the lack of adequate techniques which give a measure of the activity of the enzymes, the schemes put forward have largely been founded on rather poor experimental investigations. Wood (1960) follows the system put forward by Demain and Phaff (1957). This system did not however include the recently discovered group of pectolytic enzymes, which break down pectin by a transeliminative cleavage of the α 1-4 glycosidic linkage. Bateman and Millar (1966) have included this group and classify the pectolytic enzymes primarily on the type of cleavage which is effected, i.e. transeliminative or hydrolytic cleavage of the α 1-4 glycosidic linkages and subsequently on whether the cleavage occurs randomly or at terminal positions. These four groups are further subdivided on their ability to degrade pectin or pectic acid. Pectin-methylesterase brings about de-esterification of the pectinic acid, hence it can facilitate the degradation of pectin where pectolytic enzymes with a preference for pectic acid are present alone or in company with pectin attacking enzymes.

Expt. 13. Hydrolysis of pectin using commercial pectinase

A pectinase enzyme preparation was obtained from Sigma, London and 0.5g. of the dry powder was added to 5.0g. of apple pectin in 250 ml. of water which had previously been adjusted to pH 3.7 by the addition of NNaOH. The surface of the liquid was then covered with toluene to cut down contamination by other organisms and the flask incubated for 10 days

at 30°C. This is essentially the method used by Rietz and Maclay (1943) for the production of galacturonic acid from pectin. At the end of this period the hydrolysate plus toluene was evaporated to dryness in a rotary evaporator and the remaining toluene was azeotroped off with ether. As a control a pectin solution was covered with toluene and treated in the same way. Media were prepared from both samples.

Table 15. The number of colonies and vegetative growth obtained on enzyme hydrolysed pectin media

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0*
B	Apple pectin	27.8	100.0
C	Hydrolysed enzyme pectin	0	0
D	Control pectin	24.8	89.0

The experiment was carried out three times and similar results were obtained on each occasion. Commercial pectinase is reported to be only 50% electrophoretically pure. Since no purity figures were quoted for the sample, a sample was analysed by electrophoresis on a disc of acrylamide gel. This revealed the presence of three ill defined bands. Thus the failure of the hydrolysate to initiate post-sporeling growth may have been due to other enzymes degrading the active factor and so other means of degrading the pectin were sought.

Base hydrolysis of pectin brings about removal of the methyl groups without appreciable degradation of the pectin molecule and so an experiment was set up to test the activity in demethylated pectin.

Expt. 14. The effect of demethylated pectin on post-sporeling growth

A 2% apple pectin solution in N/10 NaOH was allowed to stand overnight. This was essentially the method employed by Oxford (1944) to demethylate pectin to obtain pectic acid. When the medium was prepared from this demethylated pectin, the amount of NHCl. required to adjust the pH to 5.5 was carefully noted and this amount was then added to a control medium containing untreated pectin. NNaOH was then added to adjust the pH to 5.5.

The experiment was carried out three times and similar results were obtained on each experiment. The results of one experiment are given in table 16. On each occasion the number of colonies formed on the demethylated pectin medium and on the pectin medium + HCl. showed a significant reduction. This is probably due to the NaCl. produced in the medium by the addition of HCl. or NaOH. On the preparation of this control medium every precaution was taken to ensure that even localised demethylation did not occur on the addition of the NNaOH by adding it in a dropwise fashion to the pectin while it was stirred.

Table 16. The number of colonies produced on a demethylated pectin medium

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	44.9	100.0
C	Demethylated apple pectin	37.5	83.6*
D	Apple pectin + NaCl.	35.4	78.9

In laboratory experiments NaCl. has been shown to inhibit sporangial and zoospore germination (Bollen, 1965). The reduction in colony production and rate of growth could be attributed to increase in NaCl. in the medium. Previously it was shown that apple pectin which had been treated with methanol to produce pectin with a higher degree of methylation did have an effect on its ability to induce colony formation.

The enzyme pectin-methylesterase which attacks the methyl groups of pectin has been readily detected in culture filtrates of P. infestans. Fuchs (1965) reported that rufianic acid (quinizarin-sulphonic acid) and tannic acid, among others, inhibited the action of pectin-methylesterase. Rufianic acid has also been reported to inhibit pectinase activity (Grossman, 1962), and like tannic acid it probably inhibits the action of a number of enzymes. An experiment was, however, set up to investi-

gate the effect of these inhibitors on the action of pectin-methylesterase in culture filtrates and then a further experiment was set up to test their effect at similar concentrations on colony formation.

Expt. 15. Effect of quinizarin-sulphonic acid and tannic acid on pectin methylesterase activity in vitro

The method used for the detection of pectin-methylesterase activity was essentially that of Smith (1958). The substrate had the following composition.

Apple pectin	0.50g.
Phenol	0.20g.
NaCl.	0.58g.
Bromothymol blue 0.1% soln.	2.50 ml.
Water	100 ml.

Quinizarin-sulphonic acid (rufianic acid) was added to the substrate to give final concentrations of 10^{-3}, 10^{-4}, and 10^{-5} molar solutions. Tannic acid was added at 10^{-4}, 10^{-5}, 10^{-6} M. NNaOH was added to the substrate to adjust the indicator to green. 0.6 ml. of culture filtrate of P. infestans grown in liquid French bean medium was added to two test-tubes. One tube of each pair was boiled in a water bath for 10 mins. 7.4 ml. of the substrate was pipetted into each tube, followed by enough N/20 NaOH to just turn the indicator blue. The production of an acid reaction in the mixture was taken as evidence of pectin-methylesterase activity. The results are shown in table 17.

Quinizarin-sulphonic acid at 10^{-4}M and tannic acid at 10^{-6}M failed to inhibit a change in the pH of the medium. This was interpreted as meaning that pectin-methylesterase was inhibited at concentrations of the two acids higher than these.

Table 17. Production of acid by culture filtrates of P. infestans

Treatment	Unboiled Filtrate	Boiled Filtrate
Quinizarin-sulphonic acid 10^{-2}M	-	-
Quinizarin-sulphonic acid 10^{-3}M	-	-
Quinizarin-sulphonic acid 10^{-4}M	+	-
Tannic acid 10^{-4}M	-	-
Tannic acid 10^{-5}M	-	-
Tannic acid 10^{-6}M	+	-
Untreated control	+	-

+ = acid production - = no acid production

Expt. 16. The effect of inhibitors of pectin-methylesterase activity and colony production

Pectin medium was prepared in the normal way but with the addition of quinizarin-sulphonic acid at 10^{-2}, 10^{-3} and 10^{-4}M and tannic acid 10^{-4}, 10^{-5} and 10^{-6}M. The results of the experiment which was repeated six times were somewhat inconsistent. In one experiment colonies

Table 18. The effect of inhibitors on colony formation, germination of spores, and vegetative growth

Additions to Basal Medium	Vegetative Growth		Germination		No. of colonies per plate (Mean of 8)		Percentage Colony production	
	Expt. 16A	Expt. 16B	Expt. 16A	Expt. 16B	Expt. 16A	Expt. 16B	Expt. 16A	Expt. 16B
No additions	+	+	+	+	0	0	0	0
Apple pectin	+	+	+	+	29.7	53.2	100.0	100.0
Apple pectin + Q^{-2}	-	-	-	-	0	0	0	0
Apple pectin + Q^{-3}	+	+	+	+	0	8.5	0	16.0 **
Apple pectin + Q^{-4}	+	+	+	+	30.9	52.2	104.0	98.2
Apple pectin + T^{-4}	+	+	+	+	0	0	0	0
Apple pectin + T^{-5}	+	+	+	+	0	0	0	0
Apple pectin + T^{-6}	+	+	+	+	24.9	52.7	83.7	99.1

* Colonies very variable in size and form.

developed at all concentrations of quinizarin-sulphonic acid although there was a definite decrease in rate of colony formation as the concentration increased. On another occasion none of the media containing quinizarin-sulphonic acid gave rise to colonies in 14 days. A possible reason why these anomalous results were obtained is slight variations in the time or pressure of autoclaving the medium, either producing further toxicity or reducing the toxicity of the quinizarin-sulphonic acid. Tannic acid gave consistent results throughout. The results obtained from two experiments are shown in table 18, and these include the effect of the medium on germination and vegetative growth from an inoculum of actively growing hyphae.

From the results of this experiment it appears that quinizarin-sulphonic acid and tannic acid can prevent colony formation on the pectin medium. The concentration of these inhibitors which inhibits colony formation is approximately the same as that which inhibits the action of pectin-methylesterase in culture filtrates. In view of this it might be suggested that pectin-methylesterase activity is essential for the initiation of post-sporeling growth. It would, however, appear that methanol is not responsible for this stimulation as the results of Expt. 14 showed that loss of methanol from the pectin molecule did not result in loss of activity. Clarke (1964) reported that methanol was not responsible for colony formation.

That pectic acid must first of all be formed before the active factor is released, cannot be dismissed on the strength of these results. Several workers (e.g. Wood, 1960), have failed to detect the presence of

pectolytic enzymes in P. infestans, although Grossman (1964) claims to have cultured on a complex medium a strain which produced a pectolytic enzyme with a preference for pectic acid. More recently (M. Knee, personal communication) loss of pectic material from tissue cultures infected with P. infestans has been shown to occur. This result is open to more than one interpretation. In conclusion it may be stated that if P. infestans does have the capacity to degrade pectin then the enzymes involved probably have limited activity and are closely bound to the hyphal wall.

Since quinizarin-sulphonic acid and tannic acid are non-specific inhibitors it cannot be concluded that pectin degrading enzymes are involved in the release of the active factor. However, it might be suggested that other extracellular enzymes, which are inhibited by these toxins, allow the active factor to become available to the fungus. The activity of such enzymes would appear to be only necessary for the initiation of post-sporeling growth since germination resulting in the production of a sporeling and vegetative growth from mass hyphal inoculations occurred on media which did not support the growth of colonies.

Since the results of this experiment suggest that enzyme activity and presumably degradation of a relatively high molecular weight compound is necessary for the release of the active factor, the next experiment was set up to investigate the effect of hydrolysing pectin with dilute acid on post-sporeling growth.

Expt. 17. Acid Hydrolysis

Expt. 17. Acid Hydrolysis

A solution of 50.0g. of apple pectin in 0.5N HCl. was boiled with refluxing for one hour. Shortly before the end of this period a marked change in the colour of the boiled solution was apparent. The intense brown solution became pale brown in colour. 25 ml. (the equivalent of 2.5g. of pectin) was evaporated to dryness in a rotary evaporator under vacuum. Ethanol was added to azeotrope off most of the remaining HCl. The hydrolysate was then taken up in water and 250 ml. of medium prepared from it. Since all the HCl. was probably not removed from the hydrolysed pectin a control medium was prepared which included a final concentration of NaCl. approximately equivalent to that in the medium containing the hydrolysed pectin to allow for any errors due to the presence of residual amounts of HCl. The reduction in the number of colonies produced on it and the rate of growth of the colony was slightly slower than that of the normal apple pectin medium but it was similar to that on the hydrolysed pectin medium. The experiment was carried out three times and similar results were obtained each time.

Table 19. The number of colonies formed on pectin hydrolysed in hot acid

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	35.0	100.0
C	Hydrolysed pectin	29.3	83.6
D	Apple pectin + NaCl.	30.1	86.0*

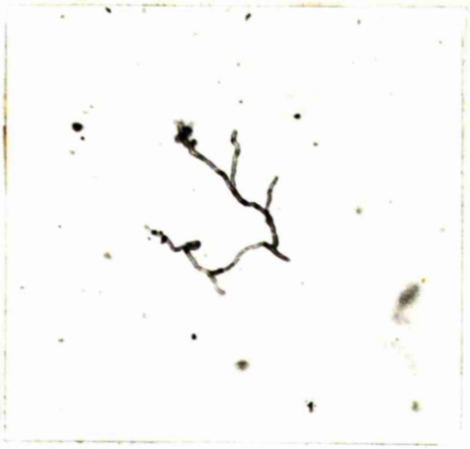
Plate 1. Sporelings growing on acid hydrolysed pectin medium three days after inoculation

All photographs were taken three days after inoculation of sporelings growing on:-

A. basal medium.

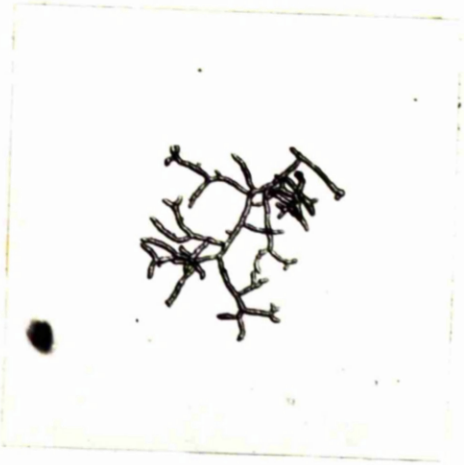
B. apple pectin medium.

C. acid hydrolysed pectin medium.



A

B



C

2·0 mm

If the hydrolysed pectin was allowed to stand overnight, it separated out into an upper brown coloured liquid of low viscosity and a lower pale brown precipitate. The activity of these two fractions was therefore investigated.

Expt. 18. Preliminary fractionation of acid hydrolysed pectin

Hydrolysed pectin was prepared as previously described. A 25.0 ml. sample was filtered through a Whatman No.1 filter paper on a Buchner funnel. The aqueous filtrate was retained and then the precipitate was washed several times with 80% ethanol until free from HCl. The ethanol extract was evaporated to dryness in a rotary evaporator. The precipitate was dried in an oven at 60°C overnight and then allowed to cool in a vacuum desiccator. Media were prepared from all three fractions. A control medium was prepared from unfiltered hydrolysate.

All media contained hydrolysed pectin or extracts equivalent to a concentration of 10g. of whole pectin per litre. No attempts were made to allow for the concentration of NaCl. present in each medium, in this or further experiments, since previous experiments had shown that this did not reduce the number of sporelings growing to form colonies by more than about 20%. Zoospore germination and growth from a mass hyphal inoculum occurred on all media. The experiment was carried out three times with similar results each time. The results of one experiment are given in table 20.

Controls

Medium	Additions to basal medium at 10g./litre	No. of colonies per plate (Mean of 8)	% colony production
Q	No additions	0	0
R	Apple pectin	49.0	100.0
S	Supernatant (Hydrolysis for 1 hr)	39.2	80.0*

Table 20. Colony formation by different fractions of the pectin hydrolysate

Medium	Additions to basal medium	No. of colonies per plate (mean of 8)	% colony production
A	No additions	0	0
B	Untreated apple pectin	38.2	100.0
C	Unfiltered hydrolysate	30.2	79.1*
D	Precipitate after hydrolysis	0	0
E	Supernatant	28.2	73.9
F	80% ethanol extract of precipitate	0	0

Thus the supernatant contained the active factor. The effect of prolonged treatment of acid on the pectin was therefore investigated to determine whether the amount of active principle in the supernatant varied according to the period of hydrolysis.

Expt. 19. The effect of prolonged boiling of pectin in 0.5N HCl.

A solution of apple pectin was boiled with refluxing for a period of 4 hours in 500 ml. of 0.5N HCl. The time at which boiling commenced was noted and 100 ml. samples were withdrawn at intervals as shown in table 21. The hydrolysate was filtered through Celite under vacuum and evaporated to dryness. The HCl. was removed by azeotroping with ethanol in the normal way. Media were prepared from the filtered hydrolysate at concentrations equivalent to 20, 10, 5 and 1.0g./litre of whole pectin.

Table 21. The number of colonies produced by various concentrations of supernatant obtained after pectin was treated for up to 4 hours in boiling acid

Medium	Treated for	Conc. equivalent to g./litre of apple pectin	No. of colonies per plate (Mean of 8)	% colony production
A	1/2 hour	20.0	2.9	6.0
B		10.0	0	0
C		5.0	0	0
D		1.0	0	0
E	1 hour	20.0	14.7	30.0
F		10.0	36.5	74.4
G		5.0	12.5	25.6
H		1.0	0	0
I	2 hours	20.0	0	0
J		10.0	43.5	88.7
K		5.0	0	0
L		1.0	0	0
M	4 hours	20.0	0	0
N		10.0	33.9	69.2
O		5.0	0	0
P		1.0	0	0

Media B, K, and O showed a greater amount of growth and branching compared with the basal medium. These plates were allowed to grow for a further week (beyond the 10 day stage) and all produced colonies

although these were of a rather open not so densely branched nature. Media I and M showed less growth and branching than the sporelings on the basal medium. From these last mentioned results it might be suggested that a toxic factor became evident with increased hydrolysis time. The results from the 1/2 hour treatment indicate that the factor responsible for the initiation of post-sporeling growth had not been released or was not synthesised in sufficient quantity. A further experiment was thus carried out to determine whether the precipitated fraction contained the activity.

Expt. 20. Stimulation of colony production by digested and undigested pectin

The stock solution was prepared in the normal way and boiled with refluxing for 1/2 hour. The hydrolysate was then filtered through a Buchner funnel fitted with a Whatman No.1 filter paper. The filtrate was withdrawn and the precipitate washed several times with 80% ethanol. The washings were retained and evaporated to dryness and then resuspended in water. The precipitate was dried in an oven at 60°C overnight and then allowed to cool in a vacuum desiccator. A medium was prepared from this equivalent to a concentration of 10g. of apple pectin per litre.

Table 22. The number of colonies produced by various fractions of pectin hydrolysed for 30 minutes

Medium	Additions to basal medium	Conc. equivalent to g./litre of whole pectin	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	-	0	0
B	Apple pectin	10.0	24.0	100.0
C	Supernatant	20.0	2.2	9.1
D	Supernatant	10.0	0	0
E	Supernatant	5.0	0	0
F	Supernatant	1.0	0	0
G	Precipitate	10.0	13.8	57.3*
H	80% ethanol washings of precipitate	20.0	0	0
I	80% ethanol washings of precipitate	10.0	0	0
J	B + G	10.0	19.6	81.7

Medium G also produces significantly fewer colonies than medium J. The experiment was carried out three times and similar results were obtained each time. From these results it appears that the stimulatory fraction is not released from the pectin by hydrolysis for only 1/2 hour, although it is still possible that a new stimulatory compound is being produced as fast as the original one in pectin is being destroyed. Further experiments were carried out to investigate the possibility of breakdown products being converted to new and stimulatory compounds and these are reported elsewhere in this thesis (Part IV).

Expt. 21. Dialysis of hydrolysates of pectin

The filtered pectin hydrolysate was formed in the usual way and excess HCl. was driven off. 50 ml. of the stock solution, equivalent to 5.0g. of apple pectin, were pipetted into 24/32" Visking dialysis tubing. The closed tubing was then placed in a 500 ml. conical flask followed by 100 ml. glass distilled water. The flask was stoppered and shaken for 24 hours. The dialysate was incorporated into 250 ml. of medium as was the material inside the tubing. Controls were prepared from the stock hydrolysate and by adding the two fractions back together again after dialysis.

Table 23. Number of colonies produced by dialysed fractions of pectin hydrolysates

Medium	Additions to basal medium	Conc. equivalent to g./litre of whole pectin	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	-	0	0
B	Apple pectin	10.0	46.3	100.0
C	Supernatant	10.0	33.5	72.3*
D	Dialysate	-	21.2	45.7
E	Dialysed supernatant	-	17.4	37.5
F	D + E	20.0	11.6	25.0
G	D + E	10.0	29.2	63.1

The experiment was repeated three times and similar results were obtained in each case. Again when the fractions were added together

there was a decrease in the number of colonies produced; this is in accordance with the results obtained on bulking the solutions obtained after dialysis of whole pectin and can probably be attributed to toxins in the tubing. It is evident from these results, however, that the stimulatory fraction is dialysable and therefore of relatively low molecular weight.

Expt. 22. Separation of stimulatory fraction using liquid/ liquid fractionation

Stock filtered hydrolysates were obtained as previously described. 50 ml. of the solution, containing the equivalent of 5.0g. of apple pectin, were pipetted into a separating funnel with a slightly greater volume of solvent. The separating funnel was vigorously shaken, the solvent drained off and the whole procedure was carried out another twice. Both samples were evaporated to dryness and taken up in water. Media (250 ml.) were then prepared from each sample.

Table 24. Number of colonies produced by various solvent extracts

Table 24. Number of colonies produced by various solvent extracts

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	24.1	100.0
C	Supernatant	21.9	91.0
D	Supernatant extracted with ether	19.6	81.2
E	Ether extract	0	0
F	Supernatant extracted with pet. ether	18.4	76.3*
G	Pet. ether extract	0	0
H	Supernatant extracted with chloroform	0.5	2.1
I	Chloroform extract	0	0
J	Supernatant extracted with ethyl acetate	0	0
K	Ethyl acetate extract	0	0

Bulking fractions of media H and I, and J and K gave no increase in colony formation or amount of sporeling growth in the two subsequent experiments. Since no stimulatory action appeared to be removed at pH 2.2 at which the extractions were carried out it was possible that separation may be accomplished at a neutral pH.

Expt. 23. Fractionation of hydrolysate by liquid/liquid separation at pH 7.0

The experiment was repeated in the same manner as Expt. 22 with the exception that the hydrolysate was first of all adjusted to pH 7.0 by the addition of NNaOH. When media were prepared from the different fractions, the pH was adjusted to 5.5.

Table 25. Number of colonies produced by various fractions obtained at pH 7.0

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	26.5	100.0
C	Supernatant adjusted to pH 7.0	21.2	80.0*
D	Supernatant extracted with ether	19.9	74.9
E	Ether extract	0	0
F	Supernatant extracted with pet. ether	0	0
G	Pet. ether extract	0	0
H	Supernatant extracted with chloroform	12.2	46.1
I	Chloroform extract	0	0
J	Supernatant extracted with ethyl acetate	6.6	24.8
K	Ethyl acetate extract	0	0

Similar results were obtained on two more occasions. Again the pet. ether fractions did not support the growth of the sporelings beyond the stage reached on the basal medium. Germination occurred on all media tested.

Expt. 24. Removal of stimulatory fraction by trituration

A sample of stock supernatant was evaporated to dryness and the residual HCl. removed. The dry hydrolysate was then triturated with one of the solvents tested. Hot ethanol and hot acetic acid were both used because they were capable of dissolving a greater amount of organic material at higher temperatures ($75^{\circ}C$). Media were prepared (250 ml.) from the equivalent of 5.0g. pectin.

Table 26. Colony production by fractions obtained by trituration with various solvents

Table 26. Colony production by fractions obtained by trituration with various solvents

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	47.4	100.0
C	Supernatant	32.7	68.9*
D	Cold (20°C) ethanol ext.	0	0
E	Cold (20°C) 60% ethanol extract	22.8	48.0
F	Remaining solids	0	0
G	Hot (75°C) ethanol ext.	0	0
H	Remaining solids	0	0
I	Hot (75°C) glacial acetic acid extract	0	0
J	Remaining solids	0	0
K	Acetone (20°C) extract	26.6	56.2
L	Remaining solids	0	0

The experiment was carried out three times and similar results were obtained. Germination and vegetative growth from a mycelial inoculum took place on all the media. Colony growth on the acetone and 60% ethanol was slightly slower than the stock hydrolysate and consequently markedly slower than the apple pectin medium. Colonies were however visible in 12 days. (Colonies on apple pectin took 8 days to appear).

DISCUSSION

The optimum concentration of apple pectin which induced colony formation from 92% of the sporelings was 10g. per litre of medium. Of several other samples of pectin tested only one, another commercial preparation of apple pectin, induced colony production in 6-8 days, and this was to a lesser extent. Another sample of apple pectin and all samples of citrus pectin did not support growth further than the sporeling stage. The inability to initiate colony growth of one of the pectins was tested and found not to be due to the presence of a toxin or toxins in sufficient quantities to suppress the initiation of growth of the sporelings by the most active apple pectin. The active factor present in the pectins which induced post-sporeling growth therefore appears to be a chemical contaminant. It is not, however, a free contaminant in the pectin but appears to be bound to some high molecular weight complex since various solvents and dialysis against water failed to remove it. This high molecular weight complex is probably pectin since after associated high molecular weight substances such as cellulose and arabans were removed by precipitating the pectin out of aqueous solution with acetone and ethanol, the active factor remained within the precipitated pectin.

Acid hydrolysis appears to release the active factor from the complex but it is not extractable from the supernatant hydrolysate with pet. ether or ether at pH 2.2 and not with ether, chloroform, or ethyl

acetate at pH 7.0. The activity was lost when the aqueous supernatant was extracted with chloroform and ethyl acetate at pH 2.2 and with pet. ether at pH 7.0. The activity may have been destroyed by a chemical reaction between the active compound and the extracting solvent or to some other process such as the production of toxins. Dialysis of the supernatant against water shows the active factor to be of relatively low molecular weight. It is soluble in acetone, aqueous solutions of ethanol but it is insoluble in absolute ethanol. Ethanol and glacial acetic acid destroys the activity at a temperature of 75°C.

Since the active factor appears to be bound to a high molecular weight complex, the problem arises how it is made available to P. infestans. It is probable that extracellular enzymes are involved since the enzyme inhibitors quinizarin-sulphonic acid and tannic acid suppress colony formation on the pectin medium. Germination of the zoospore and vegetative growth can occur on media which contain these inhibitors at concentrations that inhibit colony formation and so it appears that, like the active factor itself, the action of these extracellular enzymes is necessary only on the initiation of post-sporeling growth. If the complex to which the active factor is bound is pectin, then pectin degrading enzymes may be involved. The evidence for these enzymes, other than pectin-methylesterase, in P. infestans is inconclusive.

It has been found that the active factor becomes detectable approximately as quickly as it disappears from the pectin residue on acid

hydrolysis. This is not conclusive evidence that the active factor in the supernatant hydrolysate is identical with the active factor in the untreated apple pectin and so the possibility that a new substance is formed should be investigated.

IV. BIOASSAY OF VARIOUS COMPOUNDS FOR THE ABILITY TO INITIATE COLONY FORMATION

The results obtained so far indicate that the stimulation of post-sporeling growth by B.D.H. apple pectin may be due to an impurity rather than to the pectin itself. Many compounds may be present as impurities in the apple pectin and the work reported here is concerned with the effect of such compounds on sporeling growth.

The substances examined fell broadly into three categories:-

(1) compounds reported to be present in commercial preparations of pectin;

(2) compounds which might readily be expected to be present in the apple fruits from which the pectin material had been extracted, and

(3) compounds known to exert morphogenetic effects on micro-organisms.

The second group of compounds may be present in pectin either because they become chemically bonded or strongly adsorbed to the apple pectin during extraction, or because their properties are similar to the pectin and so are extracted along with it. Also tested in this section are compounds which are themselves inactive but which on treatment with HCl. may give rise to compounds which are active.

CARBOHYDRATES

The compounds tested in this group are subdivided according to whether they have been reported to be present in commercial apple pectin or in whole apple fruits.

Many of the carbohydrates found in apple fruits and associated with apple pectin can supply the sole carbon source for a number of micro-organisms. Although the basal medium can support the growth of mature hyphae of P. infestans it may be that the glucose in the medium is not readily utilised by the sporelings. Lindberg (1967) found that Ophiostoma multiannulatum was able to respire on galactose but was unable to grow on it. However when xylose was introduced into the medium at low concentrations the fungus was able to grow on the galactose. Thus it may be that some of the carbohydrates present in the apple pectin are operating in a similar manner so enabling the sporelings to utilise the glucose present.

A. CARBOHYDRATES PRESENT IN COMMERCIAL APPLE PECTIN

Generally between 75% and 90% of commercial pectin samples are composed of uronic acids. Naturally, of the uronide content galacturonic acid is by far the most common and Kertesz (1951) believes that the uronide fraction is almost completely composed of galacturonic acid since glucuronic and mannuronic acids are very uncommon in the plant world.

Of the non-uronide fraction of pectin, arabinose, galactose,

rhamnose and xylose are reported to be generally present (Bateman and Millar, 1966). Cellulose, the main structural polysaccharide of plants may itself be present because of its close association with pectin in the cell wall (Setterfield and Bayley, 1961) or its breakdown product cellobiose may be present.

B. CARBOHYDRATES PRESENT IN WHOLE APPLE FRUITS

Some of the sugars reported in section A also occur in abundance in apple fruits, e.g. galacturonic acid and xylose (Long, 1961). D-mannose is reported (Guichard, 1954) to exist in the free state in the fruits of peach, apple and orange. The trisaccharide raffinose, like fructose and sucrose, is widely distributed in the plant kingdom and it can yield galactose and sucrose or fructose and melibiose depending on which linkage is attacked. In apple fruits sorbitol has been detected (Barker, 1955) and although its corresponding sugar, sorbose has not been detected (Long, 1961), on account of the morphological effect it has on some organisms it was also tested.

Neurospora crassa normally produces diffuse colonies composed of hyphae which spread widely over an agar plate but in the presence of sorbose small compact colonies composed of densely branched hyphae are produced (Tatum, Barrat and Cutter, 1949). Sorbose appears to have a toxic effect as Lilly and Barnett (1953) found that the hyphal tips were first of all killed and this was followed by branching of the mycelium behind the killed portion. Except for its toxicity sorbose

shows similar effects on Neurospora crassa as pectin does on developing colonies and mature hyphae of P. infestans.

In the commercial preparation of apple pectin amylase is added to the extract before precipitation to solubilise the starch present, thus if the action of the amylase is incomplete starch may be precipitated out of solution with the pectin. Intermediates of starch hydrolysis, including maltose may thus he present.

Trehalose, dulcitol, adonitol and lactose were also tested because they were available although they have not been reported to be present in apple fruits.

AMINO ACIDS

Most of the nitrogen of plants is present in the form of amino acids or amides either existing free or combined in proteins. At least eighteen amino acids plus the amides asparagine and glutamine have been reported in the acid soluble nitrogen fraction although there are probably many more present. Alanine, arginine, serine, glutamic and aspartic acids, and their amides are often present in major proportions whilst tyrosine, tryptophan, cysteine, cystine, histidine, methionine and phenylalanine are rarely found other than in trace amounts.

Stimulatory and inhibitory effects of amino acids in vitro are dependent on the concentration of the amino acid concerned and the composition of the rest of the medium particularly with regard to the

presence of other amino acids, trace elements and vitamins (Cochrane, 1958). Samborski and Forsyth (1960) claimed that an amino acid may stimulate growth at one concentration and be inhibitory at another. Specific requirements have only been shown for artificially induced mutants (Tuveson and Garber, 1960), although amino acids have been shown to stimulate certain phases of the life cycle of certain fungi. D-serine was found to inhibit mycelial growth of Cladosporium cucumerinum at high concentrations but its effect was reversed by L-serine (van Andel, 1966). Proline has been found to specifically promote germination in spores of Rhizopus arrhizus (Weber, 1965). Leucine, cysteine and glutathione stimulated not only the growth of hyphae of germinating rust spores but also the production of vesicles (van Andel, 1966). Therefore amino acids have been shown to exert an effect on the metabolism of some fungi which sometimes results in morphological differentiation being accomplished.

VITAMINS

Almost any natural product is contaminated with significant amounts of vitamins and that apple pectin probably contains some of these growth factors has been shown previously in Expt. 9 using mutants of A. nidulans. Thiamine is incorporated into the basal medium since it was shown to be an absolute requirement for P. infestans (Payette and Perrault, 1944). All the vitamins tested have been shown to be required by or stimulatory towards the growth of micro-organisms.

PHENOLIC COMPOUNDS

According to Potter (personal communication) the main difference between apple pectin and citrus pectin is the presence of anthocyanin pigments in the former. Also, an analysis of apple pectin given by one manufacturer (Hopkins and Williams) showed that phenolic compounds were present in their sample of apple pectin.

Sando (1937) reported that the principal anthocyanins in apples were quercetin and cyanidin galactosides. In a comprehensive study of the phenolic compounds present in cider apples, Williams, (1958, 1960), found that chlorogenic acid, 3-glycosides of quercetin in which the sugar moiety was either galactose, arabinose, glucose, xylose or rhamnose, and cyanidin galactoside were all present in detectable amounts in the flesh or skin of the fruit. Phloridzin was found in the seed but not in the rest of the fruit, and a few other related compounds such as catechins and leuco-anthocyanins were also present in the flesh. Williams also states that the amount and proportion of phenolics can vary greatly according to variety and even within varieties grown on different sites or on the same site in different seasons. A number of glycosides have been shown to reduce permeability in animal tissue (Pigman, 1957) and phloridzin has been used to study the transfer of glucose across animal membranes (Harborne, 1964). If these factors are responsible in some way for the inability of the sporelings of P. infestans to continue growth, then possibly incorporating glycosides into the medium may promote sporeling growth.

HYDROLYSATES OF PECTINS AND SUGARS

Since hydrolysed pectin is active it is likely that the active fraction was released from the pectin to which it had been chemically bonded in some way. However, it may be that additional compounds with similar effects were produced *de novo* during the acid treatment. Treatment with HCl. is an essential process in the preparation of commercial apple pectin and so it is possible that the active factors present in commercial apple pectin have arisen during this procedure and they may not be constituents of apple fruits. Thus the two major carbohydrate components of apple pectin glucose and galacturonic acid and citrus pectin were treated with HCl. prior to testing for their effects on post-sporeling growth.

A number of aldohexoses having a pyranose structure tend to produce 1-6 anhydro-β-D-glycopyranoses upon heating with dilute HCl. Armstrong, England, Morton and Webb (1963) found that anhydroglucose in the presence of Ca^{++} stimulated the sporulation of *Penicillium griseofulvum*, and so it is worth investigating the effect of this and other anhydro-sugars on the post-sporeling growth of *P. infestans*.

METHODS

Additions were made to the basal medium at concentrations given in the text. The concentrations used were such that they covered the range normally expected to occur in apple pectin and in some cases were

much greater than that. Since many of the compounds tested are derived from natural sources it was thought that other growth stimulatory factors might be discovered associated with them when they were incorporated at the higher concentrations.

A volume of 250 ml. of each medium was prepared and eight plates were poured from this volume. The adjustment of the pH and autoclaving of the media were carried out as previously described. Pectin and basal media were included as controls in all experiments. A zoospore suspension was used to inoculate seven of these plates and the eighth was inoculated with a mass hyphal inoculum. Visual estimations of growth were made with particular attention to morphogenetic effects similar to pectin. The plates were incubated at 20°-22°C for 14 days.

EXPERIMENTAL RESULTS

Expt. 25. Bioassay of possible contaminants in pectin for the induction of colony growth

The sugars, xylose, rhamnose, galactose, cellobiose, arabinose and the polysaccharide cellulose were added to the basal medium at three different concentrations, 10.0, 1.0 and 0.1g. per litre. Galacturonic and glucuronic acids were tested at the same concentrations and also at a concentration of 0.01g. per litre.

Vegetative growth from mycelial inocula occurred on all media. Only on the media containing 10.0 and 1.0g. per litre of glucuronic or

galacturonic acid was there less growth than that which occurred on the control media. Spore germination occurred on all media but colonies only developed on the pectin medium. Sporelings on the media containing xylose at all concentrations were slightly larger and more branched than those on the basal medium, but there were no visible differences between the sporelings produced on the different concentrations of xylose.

Expt. 26. The activity of constituent carbohydrates of apple fruits

The additions made to the basal medium were:- sorbose, sorbitol, maltose, starch, mannose, fructose, sucrose, raffinose, lactose, dulcitol, adonitol and trehalose. The starch was dissolved up by the same method as that used for dissolving apple pectin. All the sugars were tested at 10, 1.0 and 0.1g. per litre except adonitol and trehalose which were only tested at 1.0 and 0.1g./litre.

Vegetative growth from the hyphal inoculum occurred on all media. No compound had any morphological effect on the hyphae. On none of the media except the apple pectin were colonies formed although germination occurred on all media. Sporelings on the sorbose and sorbitol media did however grow slightly better than those on the basal medium. The growth produced on those two media was of a relatively unbranched attenuated nature and so the branching effect which was pronounced in Neurospora crassa was not evident in the germinated spores of P. infestans. The results detailed above were consistent in all three experiments which were carried out.

SUMMARY

The carbohydrates tested were adonitol, arabinose, cellobiose, cellulose, dulcitol, fructose, galactose, galacturonic acid, glucuronic acid, lactose, maltose, mannose, raffinose, rhamnose, starch, sorbitol, sorbose, sucrose, trehalose and xylose. None of these substances were capable of inducing colony formation under the conditions of the experiment and only three (xylose, sorbose and sorbitol) produced slightly more growth than the basal medium alone. Galacturonic acid, which is the basic building unit of the pectin molecule was inactive in respect of colony production and this infers additional proof that the pectin itself is not the factor involved.

Expt. 27. The effect of amino acids on post-sporeling growth

A preliminary experiment was carried out by preparing amino acid solutions which were added to the basal medium (without agar) to give final concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. The DL forms were incorporated at twice the concentration of the L forms. The method used was that described by Holliday (1956) which was developed for the rapid determination of the growth requirements of mutant strains in Ustilago maydis and involved the use of a number of amino acid pools. The construction of these pools is such that it is possible to determine the amino acid responsible should colonies develop on one or other of the pools. The pools were formulated as shown in table 27. Thus if colonies only develop in medium A, DL-Citrulline may be the amino acid

responsible and if colonies developed on media A and D then the amino acid L-Aspartic may be the active constituent since it is common to both. DL-Tryptophan was not tested in this experiment due to its extreme insolubility.

Table 27. The amino acid pools incorporated into the basal medium

Medium	
A	DL-Citrulline, DL-Alanine, L-Arginine, L-Aspartic, L-Glutamic, L-Cysteine.
B	DL-Cystine*, DL-Alanine, L-Histidine, L-Leucine, L-Lysine, DL-Methionine.
C	DL-Tryosine, L-Arginine, L-Histidine, DL-Ornithine, DL-β-Phenyl-alanine, L-Proline.
D	DL-Serine, L-Aspartic, L-Leucine, DL-Ornithine, DL-Threonine, DL-Valine.
E	DL-Glutamine, L-Glutamic, L-Lysine, DL-β-Phenyl-alanine, DL-Threonine, DL-Amino-n-butyric.
F	Glycine, L-Cysteine, DL-Methionine, L-Proline, DL-Valine, DL-Amino-n-butyric.

* DL-Cystine was insoluble at 10^{-3} and 10^{-4} M and was therefore in suspension in the medium.

Each medium therefore contained six different amino acids, and ten flasks (100 ml.) containing 25 ml. were prepared from each medium. Control flasks of pectin and basal media were also included.

The number of colonies visible after 14 days incubation at 20°C

are recorded in table 28. Three flasks of each medium were then taken and the contents filtered through an 8μ Millipore filter. After air drying overnight the sporelings were observed in situ by flooding the filter disc with xylol to make it transparent. In this way a visual comparison of the amount and type of growth made by the sporelings in the different media could be made.

Table 28. The number of colonies produced on media containing amino acid pools at 10^{-3} M

Medium	No. of colonies/flask (Mean of 10)	Percentage colony production
A	0	0
B	4.8	36.1*
C	0	0
D	0.1	0.8
E	0.2	1.5
F	1.3	9.8
Basal medium	0	0
Basal medium + A.P.	13.3	100.0

The sporelings on media A, C, D, E, F containing amino acids at 10^{-3} M were longer and more branched than those on the basal medium, although there were no obvious differences between the sporelings on the different media despite the production of an occasional colony.

The colonies formed on medium B were diffuse compared with the densely branched compact colonies formed on the pectin medium. All media containing amino acids at 10^{-4} and 10^{-5} M produced sporelings similar to those on the basal medium and so it appears that DL-Cystine or possibly DL-Methionine (being common to media B and F) at 2×10^{-3} M have the ability to initiate post-sporeling growth. The next experiment was therefore set up to test these two amino acids separately in an agar medium.

Expt. 28. The effect of DL-Cystine, DL-Methionine and DL-Tryptophan on post-sporeling growth

Three concentrations of the three amino acids were tested; these were 10^{-3}, 10^{-4} and 10^{-5} M. Where the amino acid under test was insoluble it was suspended in the medium prior to pouring into the petri dishes. The experiment was carried out three times and results similar to those shown in table 29 were obtained each time. The sporelings on media D and I were consistently longer and more branched than those on the basal medium. The colonies formed on the medium containing the greatest concentration of DL-Cystine were variable in size but were generally smaller and more diffuse than those which developed on the pectin medium. Germination and vegetative growth from a mass hyphal inoculum occurred on all media.

Table 29. Number of colonies produced on agar media containing DL-Cystine, DL-Methionine and DL-Tryptophan

Medium	Additions to basal medium	Concentration	No. of colonies produced/plate (Mean of 8)	% colony production
A	No additions	-	0	0
B	Apple pectin	10g./l.	30.2	100
C	DL-Cystine	2×10^{-3} M	19.4	64.2*
D	DL-Cystine	2×10^{-4} M	1.2	4.0
E	DL-Cystine	2×10^{-5} M	0	0
F	DL-Methionine	2×10^{-3} M	0	0
G	DL-Methionine	2×10^{-4} M	0	0
H	DL-Methionine	2×10^{-5} M	0	0
I	DL-Tryptophan	2×10^{-3} M	1.0	3.3
J	DL-Tryptophan	2×10^{-4} M	0	0
K	DL-Tryptophan	2×10^{-5} M	0	0

DL-Cystine was insoluble in the basal medium at concentrations of 2×10^{-3} and 2×10^{-4} M. Since the 2×10^{-4} M medium had little effect on post-sporeling growth it would appear that the activity of the 2×10^{-3} M solution could not be attributed to a dissociation constant effect although the 2×10^{-4} M solution should, according to the solubility figures presented, have completely dissolved. The next experiment was therefore set up to further investigate the solubility factor.

Expt. 29. The effect of completely dissolved DL-Cystine on post-sporeling growth

A known weight of DL-Cystine was dissolved in 0.5N HCl. The HCl. was then evaporated off and the newly formed DL-Cystine hydrochloride was then dissolved in water and made up to a given volume. Media were then prepared from this solution to give final concentrations of 2×10^{-3}, 2×10^{-4} and 2×10^{-5} M DL-Cystine. The cystine HCl. was soluble at all concentrations. Pectin, basal and untreated DL-Cystine media were prepared and used as controls.

Colonies developed on the untreated cystine only at the 2×10^{-3} M and similarly the cystine HCl. medium at 2×10^{-3} M was the only concentration to induce colony formation. Media prepared from all other concentrations of the treated and untreated cystine supported germination and the production of sporelings but did not induce any further growth. The experiment was carried out three times and results were obtained similar to those shown in table 30.

Table 30. The number of colonies produced on media containing dissolved and undissolved DL-Cystine

Medium	Additions to basal medium	Conc.	No. of colonies/ plate (Mean of 8)	% colony production
A	No additions	-	0	0
B	Apple pectin	10g./l.	24.0	100
C	DL-Cystine (untreated)	2×10^{-3} M	7.2	30.0*
D	" "	2×10^{-4} M	0	0
E	" "	2×10^{-5} M	0	0
F	DL-Cystine HCl.	2×10^{-3} M	5.8	24.2
G	" "	2×10^{-4} M	0	0
H	" "	2×10^{-5} M	0	0

From the results of this experiment it appears that the solubility of cystine has little or no effect on post-sporeling growth. The conclusions reached so far might suggest that there are at least two possible explanations for the activity of DL-Cystine:-

(1) that the activity is associated with a chemical impurity in the cystine. Since cystine is generally obtained from natural sources, this is a reasonable possibility.

(2) that the activity is due to some particular feature of the cystine molecule such as the sulphydryl reducing group.

The next experiment was set up to investigate the effect of cystines derived from different sources on post-sporeling growth.

Expt. 30. The effect of three cystine preparations on post-sporeling growth

The preparation of cystine which had previously been used was DL-Cystine obtained from British Drug Houses. L-Cystine was obtained from the same manufacturer and another sample of L-Cystine was obtained from Hopkins and Williams. Media were prepared incorporating the different samples of cystine at concentrations of 10^{-2}, 10^{-3} and 10^{-4} M.

Colonies developed on all the media containing cystine at a concentration of 10^{-3} M and there were no visible morphological differences between the colonies on these media although they were all variable in size and generally smaller and slower growing than the colonies growing on the pectin medium. Germination occurred and sporelings were produced.

Three experiments were carried out on the media containing other concentrations of cystine, and the results were similar on each occasion. A typical set of results is given in table 31.

Table 31. The number of colonies produced on media containing three cystine preparations

Medium	Additions to basal medium	Concentration	No. of colonies (Mean of 8)	% colony production
A	No additions	-	0	0
B	Apple pectin	10g./l.	34.0	100
C	B.D.H. DL-Cystine	2×10^{-2} M	0	0
D	" "	2×10^{-3} M	20.6	60.6*
E	" "	2×10^{-4} M	0	0
F	B.D.H. L-Cystine	1×10^{-2} M	0	0
G	" "	1×10^{-3} M	14.8	43.5
H	" "	1×10^{-4} M	0	0
I	H. & W. L-Cystine	1×10^{-2} M	0	0
J	" "	1×10^{-3} M	18.3	53.8
K	" "	1×10^{-4} M	0	0

Thus it appears that if the activity is due to a contaminant in cystine it must be present in all three samples. A further set of experiments was set up in the hope that the contaminant, if present, could be extracted in water.

Expt. 31. Purification of Cystine by dialysis, filtration and chromatographic separation

Cystine was washed on a Whatman No.1 filter paper several times with water, and then the cystine was resuspended and incorporated into a medium. The aqueous extracts were also incorporated into medium equivalent to 10^{-3} M cystine. The results indicated that the active factor could not be extracted from cystine using water. Similar results were obtained by incorporating cystine which had been dialysed against water for 24 hours into the basal medium and so preliminary purification of the cystine was unable to remove the active factor from the cystine. Thus an experiment was set up to investigate the effect of purifying the cystine chromatographically.

Preliminary experiments were carried out in an attempt to identify any possible impurities in cystine. Thin layer chromatograms were run in two solvents, butanol : acetic acid : water (80:20:20) and 96% ethanol : water (70:30). The plates were sprayed with seric sulphate, ninhydrin, and 2,4-Dinitrophenylhydrazine before viewing under an ultra violet lamp. All three reagents located the position of the cystine but gave no indication of the presence of any other compounds. Since the butanol : acetic acid : water gave the most compact spot this was used as the solvent for a preparative chromatogram.

The plates were pre-run in the solvent and then streaked with a known weight of cystine. Bands of silica gel were removed from the plate after drying in an oven at 100°-110°C to remove the solvent. One

band contained the cystine fraction and another equivalent in size was scraped from the plate at a distance from the cystine but over which the solvent had travelled. The latter amount of gel was added to apple pectin as a control.

The number of colonies produced on the pectin and silica gel medium was considerably less than that produced on the pectin medium alone. Similarly fewer colonies were produced on the medium prepared from the cystine band although the sporelings on media C and E were longer and more branched than those on the basal medium. The experiment was repeated and similar results were obtained. The results of one experiment are illustrated in table 32.

Table 32. The number of colonies produced on medium containing chromatographically purified cystine

Medium	Additions to basal medium	No. of colonies per plate (Mean of 8)	% colony production
A	No additions	0	0
B	Apple pectin	58.4	100.0
C	Apple pectin + gel	6.1	10.4
D	DL-Cystine (2×10^{-3} M)	22.8	39.0*
E	DL-Cystine + gel (2×10^{-3} M)	0.3	0.4

Thus, although there were fewer colonies formed on cystine after partial purification chromatographically (medium E), it appeared that the active factor had not been removed from it. The decrease in activity

could probably be attributed to the presence of small amounts of the solvent in the gel or to impurities within the gel itself. From these experiments it appears that the activity of the cystine cannot be due to an impurity in the preparation but may be due to some specific feature of the cystine molecule itself. The next experiment was therefore set up in order to test the activity of other sulphur containing reducing compounds.

Expt. 32. The effect of sulphur containing compounds on post-sporeling growth

Cystine can act either as a reducing agent by oxidation of the sulphur atoms or as an oxidising agent by the addition of hydrogen to the sulphur atoms.

Similarly glutathione can either be oxidised or reduced. α-lipoic acid (thioctic acid) is a sulphur containing oxidising agent and ascorbic acid is a non-sulphur containing reducing agent. Cystine is composed of two cysteine molecules joined together by the S-S bond, and like cystine and glutathione, cysteine can either be oxidised or reduced.

Each compound was tested at 10^{-3}, 10^{-4} and 10^{-5} M in the first experiment but due to the inhibition of germination by α-lipoic acid at 10^{-3} and 10^{-4} M, in the next two experiments this compound was tested at 10^{-5}, 10^{-6} and 10^{-7} M.

Spore germination and vegetative growth from a mass hyphal inoculum occurred on all media (except 10^{-3} and 10^{-4} M thioctic acid); no

further development of the sporelings occurred on any media except the cystine (10^{-3} M) and the apple pectin media, although there was a slight increase in growth and branching of sporelings on the ascorbic acid medium at 10^{-3} M.

SUMMARY

Of twenty five amino acids tested only cystine was found to induce post-sporeling growth to form colonies. These colonies were generally smaller and composed of hyphae which were less branched compared with those developing on the pectin medium. The activity could not be attributed to some chemical impurity in the cystine as other samples also showed the activity and the active factor could not be removed by dialysis against water, filtration or chromatographic separation. A few other sulphur containing compounds with similar properties were tested and found to be inactive. Although cystine was active at a concentration at which it was insoluble, when the solubility was increased the activity remained at the same concentration so that dissociation factors would appear to be inoperative. The possibility of a chemical contaminant has not been completely eliminated.

Expt. 33. The effect of vitamins on post-sporeling growth

The following vitamins were incorporated individually at .01, .001, and .0001g./litre into the basal medium:- p-aminobenzoic acid, biotin, folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxine and

riboflavine. The pectin medium and basal medium were included as controls in the three experiments. Colonies developed on the pectin medium in 6-8 days on all three occasions and after 14 days incubation in every case there were still no visible differences between the sporelings growing on the basal medium and those growing on any of the media containing vitamins at any concentration. Thus vitamins appear to have no effect on post-sporeling growth.

Expt. 34. The effect of phenolic compounds on post-sporeling growth

Quercetin, cyanidin chloride, phloridzin and chlorogenic acid were tested along with trans- and cis-cinnamic acids which are precursors of anthocyanins. Arbutin, orcinol, caffeic acid and phloroglucinol being available were also tested. All but cyanidin were incorporated into the basal medium at 10^{-3}, 10^{-4} and 10^{-5} M. Owing to the limited quantity of cyanidin chloride available this was tested at 10^{-5} and 10^{-6} M. The basal medium and pectin medium were included as controls.

The experiment was carried out three times and on all occasions colonies developed only on the pectin medium. Although germination and some vegetative growth occurred on all media no growth further than the sporeling stage occurred.

Thus none of the phenolic compounds tested promoted post-sporeling growth.

Expt. 35. Acid treatment of citrus pectin, galacturonic acid and glucose

Solutions containing 10g./100 ml. of 0.5N HCl. of citrus pectin,

galacturonic acid and glucose were heated with refluxing for 1 hour. The methods used for hydrolysis and for removal of the HCl. were identical with those used in the hydrolysis of apple pectin.

Media were prepared from the hydrolysates equivalent to 10, 1.0 and 0.1 g./litre of untreated compound. The citrus pectin was also filtered and the supernatant and precipitate were separately incorporated into media also at these concentrations.

A sample of 1-6 anhydroglucose was tested at 5×10^{-4}, 5×10^{-5} and 5×10^{-6} g./litre.

Colonies developed only on the pectin medium. None of the media containing the hydrolysates supported the growth of the sporelings although germination and growth from a mycelial inoculum took place on the three occasions when the experiment was carried out. Thus it appears that none of the most likely reversion products stimulated the growth of sporelings although further work may be valuable in similarly testing other sugars commonly found in pectin such as xylose and arabinose. When considering the other evidence obtained on the release of the active compound from apple pectin it seems unlikely that the hydrolysis procedure results in the production of an active factor.

DISCUSSION

In any bioassay system the ability of a given compound or compounds to bring about some discernible change in the growth of an organism is limited by the conditions under which the experiment is carried out. By testing the compounds as far as possible to the same conditions in

which sporelings of P. infestans continue growth to form colonies on the apple pectin medium, an attempt has been made to overcome some of these difficulties. However, the work is limited in part by the concentrations at which the compounds were tested and other factors such as a reaction between the compounds and other contituents of the medium may change their activity and so limit the value of this work. Such reactions may not occur when the compound is present along with pectin due to some protective effects.

The compounds tested were reported either to be present in commercial apple pectin or could be expected to occur in apple pectin because they were found in apple fruits from which the pectin was derived. Of several carbohydrates tested only xylose, sorbose and sorbitol supported slightly more growth of the sporelings when incorporated into the basal medium than did the basal medium alone. In all three cases colonies were not formed and the effect of these compounds was merely to slightly increase the size of the sporeling without inducing further branching.

Cystine appeared to be the only amino acid of 25 tested which initiated post-sporeling growth. The colonies formed were slower growing, composed of hyphae which were less branched, and fewer in number than those developed on the pectin medium. No evidence was obtained to suggest that the activity of cystine was due to an associated contaminant but the effects obtained with different concentrations of cystine make it difficult to envisage that the activity is due to the cystine alone.

None of the common vitamins had any effect on sporeling growth nor

did any of the phenolic compounds tested. There is certain circumstantial evidence that the active factors in apple pectin may be phenolic or have similar properties.

(1) The active factor has similar solubilities since it is extracted in 80% ethanol.

(2) Marked variation of the activity between different samples of apple pectin might be explained by the variation in quantity and proportion of phenolic compounds in apple fruits in relation to environmental conditions of growth.

(3) That some of these compounds are able to become linked to the sugars which commonly are found in association with the pectin and may in fact form an integral part of it (Kertesz, 1951) would explain the difficulty with which the stimulator is removed from the pectin.

The effect of hydrolysing citrus pectin, galacturonic acid and glucose, all of which did not promote sporeling growth in the untreated condition, was examined. There was no detectable increase in the amount of sporeling growth which occurred on media prepared from these hydrolysates over that which occurred on the basal medium. Other sugars commonly found in association with apple pectin can form reversion products by treatment with dilute HCl. and since this method is commonly used in the preparation of the commercial product it follows that anhydro-sugars may be present in the sample. It may therefore be valuable to test the effect of this treatment on the ability of other sugars to induce post-sporeling growth.

The evidence so far suggests that the active factor in apple pectin is a contaminant but it is not one of the compounds which have been detected by chemical analysis and so is probably a compound which is present in very small quantities in certain preparations only.

V. MORPHOLOGICAL AND PHYSIOLOGICAL INVESTIGATIONS

In the preceding chapters of this thesis it was reported that single zoospore colonies develop on the pectin medium to a size visible to the naked eye within 6-8 days of inoculation. At this stage the sporelings on the basal medium consist only of a few relatively unbranched hyphae. In order to gain a better understanding of how pectin promotes post-sporeling growth in *P. infestans* it was thought necessary to compare the growth of the sporelings on the two media to determine if possible the stage of growth at which pectin exerts its effect. It may then be possible to obtain information on whether the growth promoting effect of pectin is expressed morphologically as soon as germination takes place or if it becomes apparent only after a period of growth has occurred. The time at which the morphological differences first become manifest may be reasonably assumed to have resulted from certain physiological changes which occurred prior to the change in morphology. Studies on the sporelings to determine the nature of the physiological changes associated with the initiation of post-sporeling growth could then be carried out prior to the occurrence of any morphological changes.

The work reported in this section is thus divided into two parts:-

A. Studies on the growth of the sporelings on the pectin medium and on the basal medium in order to determine the time after germination at which the first morphological differences occur, and

B. Physiological studies on the sporelings covering the period from germination until the morphological changes become fully manifest.

A. MORPHOLOGICAL STUDIES

The spores of P. infestans and many obligate parasites readily germinate in vitro. Pristou and Gallegly (1954) found that when zoospores of P. infestans were placed in petri dishes containing a little water they germinated and produced an appressorium at the distal end of the germ tube. A membrane separated the protoplasm in the appressorium from the empty spore and germ tube. The formation of an appressorium on potato leaves was first illustrated by de Bary (1863) and confirmed more recently by Ferris (1955).

Generally in axenic culture obligate parasites germinate well but give rise only to a germ tube with little or no branching. Even under the best nutrient conditions available Yarwood (1948) found that spores of Peronospora destructor gave rise only to slightly larger and occasionally branched germ tubes. Similarly Dickinson (1949) found that when spores of Puccinia triticiana were germinated in certain conditions germ tubes of limited length were produced and these sometimes formed a "gnarled" type of growth reminiscent of a series of unextended branch initials.

Although rust spores germinate under a wide range of conditions to produce a long sparingly branched germ tube in vitro they do not often differentiate further into infection structures. However, under

certain environmental conditions infection structures are formed. Dickinson (1949) claims that a contact stimulus is essential although other workers have shown that certain nutrients and mineral salts are all that is required (Hurd-Karrer and Rodenheiser, 1947, and Sharp and Smith, 1952).

The following experiments were carried out to investigate the growth of sporelings on the pectin and basal media and also to investigate the morphological changes associated with the change over from sporeling to post-sporeling growth.

Expt. 36. The morphology of sporeling and post-sporeling growth on pectin and basal media

Approximately 24 plates of each medium were inoculated and at 24, 48 and 72 hours after inoculation measurements were made of the total hyphal length of the sporelings and of the number of branches. Twenty readings from each of five plates of each medium were taken daily. The observations were made by cutting a slice of agar, approximately 1 cm. wide, across the diameter of the petri dish. The agar block was mounted on a microscope slide and a drop of water was placed on the surface before covering with a large coverslip. The measurements were then made by using a microscope fitted with a squared eyepiece graticule calibrated to measure to the nearest 10μ. Protrusions from the main hyphae were classified as branches if they projected more than the diameter of the subtending hypha.

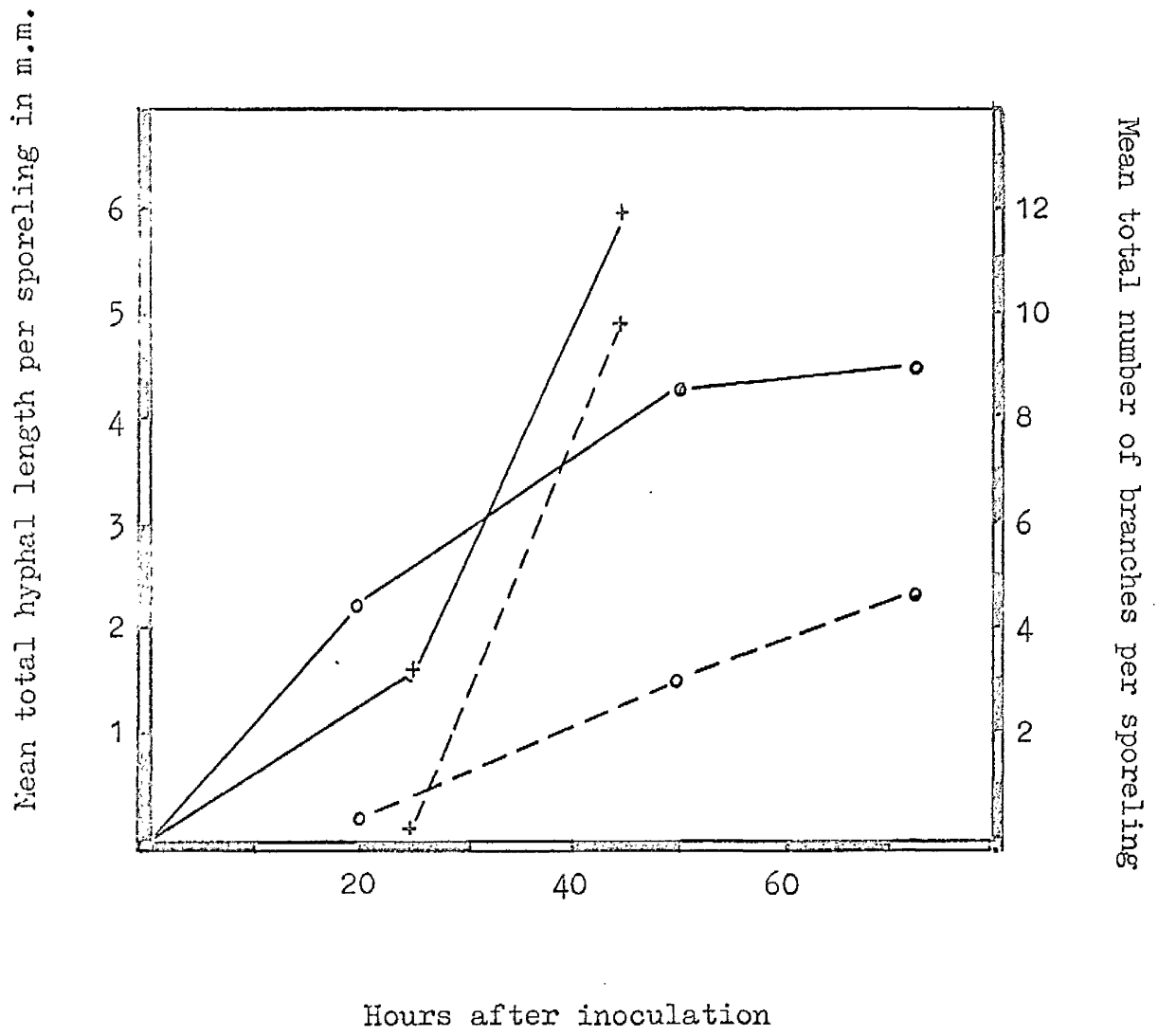
Fig. IV. The growth of sporelings on pectin and basal media

o = basal medium

\+ = pectin medium

___ = mean total hyphal length per sporeling

--- = mean total number of branches per sporeling



A preliminary experiment was carried out to determine the appropriate periods of time to investigate the sporeling growth in more detail. The results of this experiment are shown in table 33, and graphically in Fig. IV. Although generally all the spores germinated within 2 hrs. of inoculation they did not do so uniformly within that period and so the time of inoculation was chosen as the arbitrary time from which all observations were related.

Table 33. The growth of sporelings on pectin and basal media

Medium	Hours after inoculation	Value per sporeling (Mean of 100)	
		Total hyphal length m.m.	Total No. of branches
Basal	20.0	2.05	0.30
	50.0	4.21	1.50
	72.5	4.43	2.29
Pectin	25.5	1.63	0.22
	45.0	5.92	9.86
	No measurements		possible

On no occasion were any structures similar to appressoria or haustoria observed. The total hyphal length and the number of branches per sporeling were slightly less on the pectin medium than on the basal medium at the reading taken at 24 hours, but by 48 hours the total hyphal length was greater on the pectin medium. A marked change occurred in the number of branches produced as the number per sporeling was much greater on the pectin medium than on the basal medium. No measurements

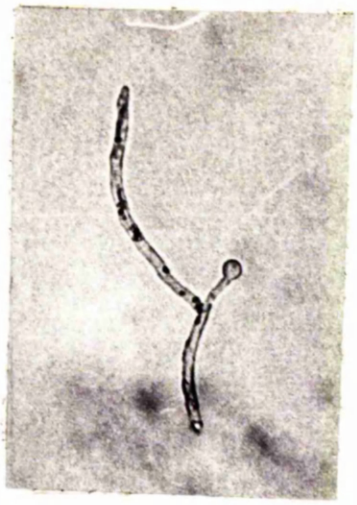
Plate 2A. Growth of sporelings on the basal medium

The photographs on the following page show typical sporelings at -

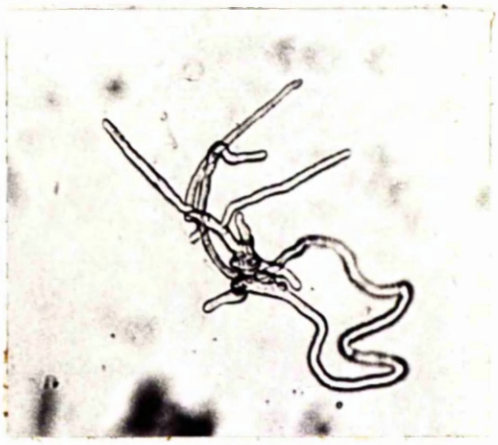
1. 20 hours after inoculation

2. 50 hours after inoculation

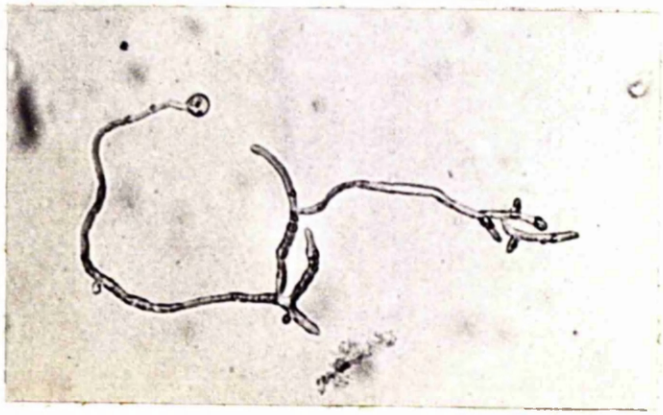
3. 72.5 hours after inoculation



1



2



3

1·0 mm.

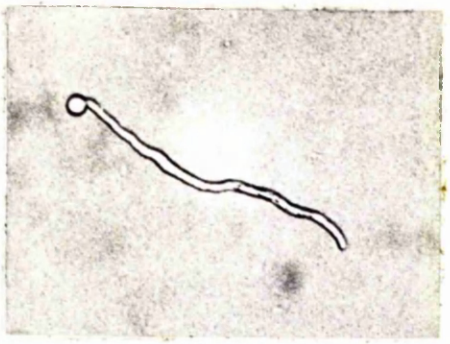
Plate 2B. Growth of sporelings on the pectin medium

The photographs on the following page show typical sporelings at -

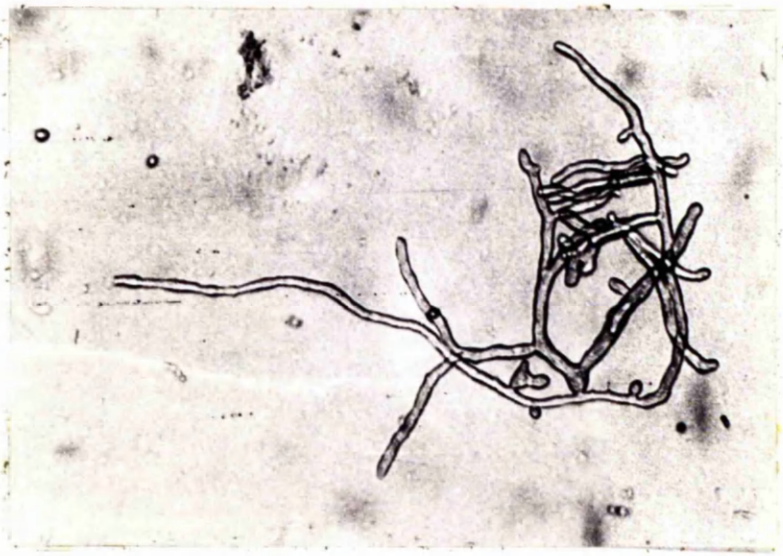
4. 25.5 hours after inoculation

5. 45 hours after inoculation

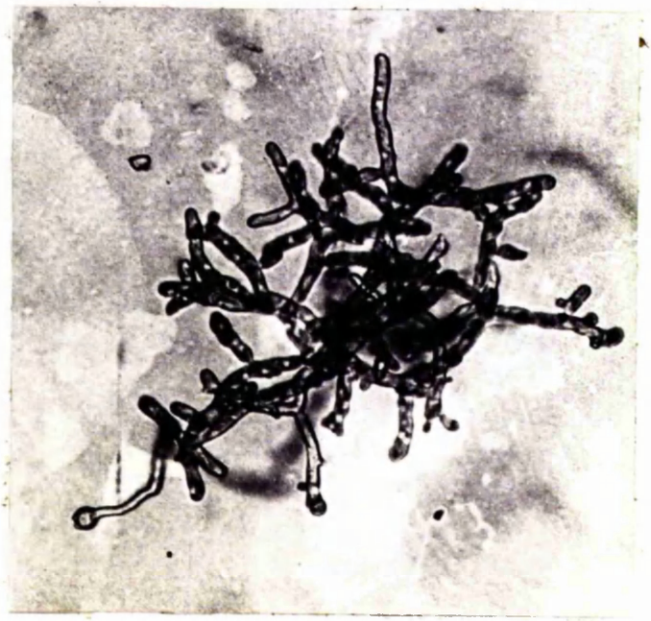
6. 75 hours after inoculation



4



5

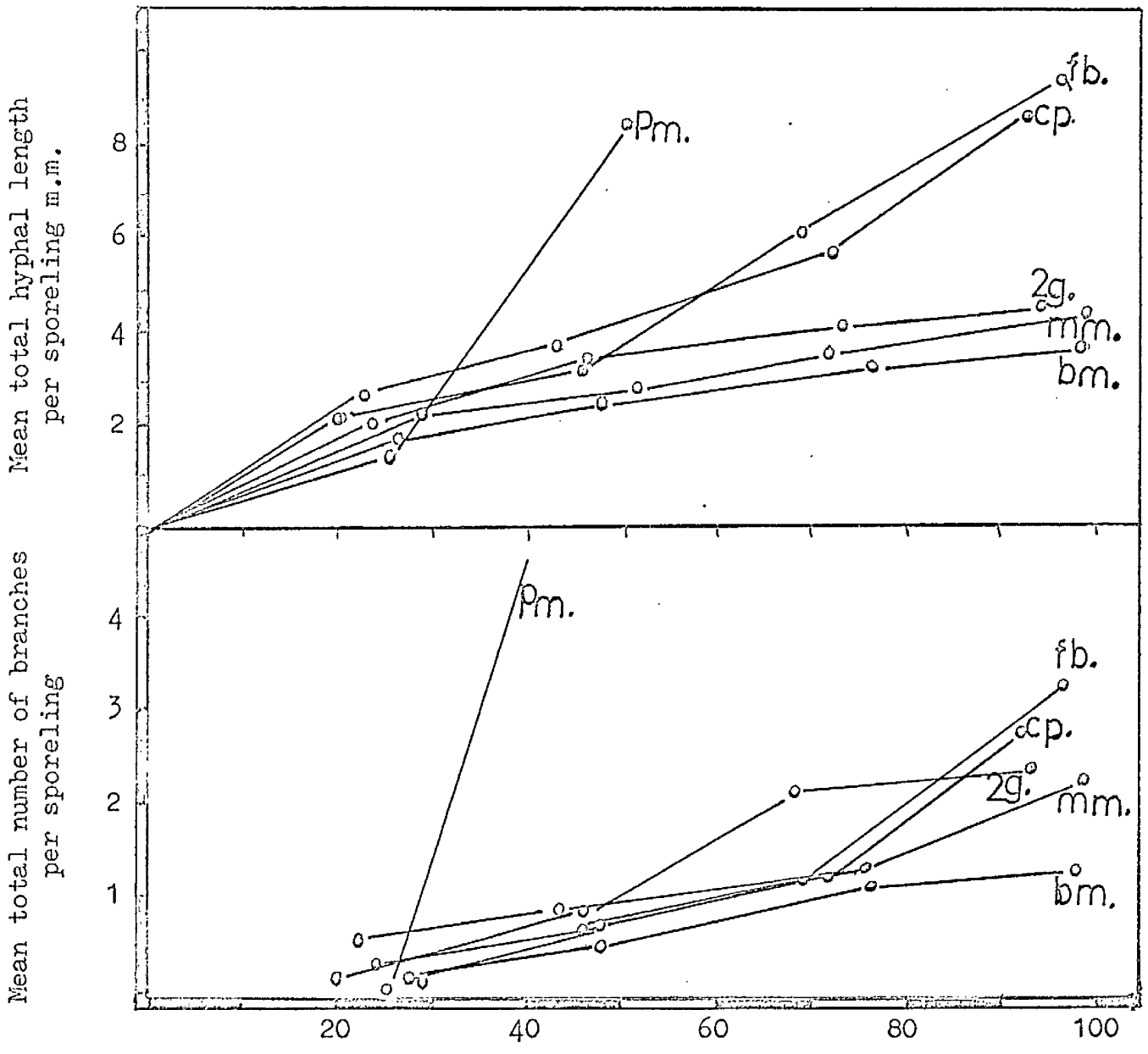


1·0 mm.

6

Fig. V. The growth of sporelings on media containing different nutrients

pm = pectin medium
bm = basal medium
mm = basal medium without yeast extract
2g = basal medium plus twice the normal conc. of glucose
fb = French bean medium
cp = chick pea medium



were capable of being made after 48 hours on the pectin medium since a network of densely branched hyphae was produced.

Thus this preliminary experiment has revealed that the main morphological changes become apparent in the period between 24 and 48 hours after inoculation. More precise information would be obtained by making more frequent observations but, before reporting this work a series of experiments will be described which were concerned with investigating more precisely the specificity of the apple pectin effect. For this purpose a variety of media were used and measurements were made of sporeling growth at timed intervals and comparisons made with growth on the pectin and basal media.

Expt. 37. The morphology of sporeling and post-sporeling growth on various natural and synthetic media

The media used in this experiment are listed in table 34. They were prepared as described previously except that Davis agar at 20g./litre was used in all cases and they were all autoclaved at 10 lbs. for 10 mins. One hundred sporelings were measured daily on each medium and the experiment was carried out twice. A typical set of results from one experiment is shown in table 34 and graphically in Fig. V.

None of the media (except the pectin medium) produced sporelings of significantly greater hyphal length or with a significantly greater number of branches than the sporelings on the basal medium, at approximately 100 hours after inoculation. The French bean and chick pea media did however consistently produce sporelings of slightly greater hyphal length than those on the basal medium.

Table 34. The growth of sporelings on media containing different nutrients

Medium	Hours after inoculation	Value per sporeling (Mean of 100)	
		Total hyphal length m.m.	Total No. of branches
Pectin medium	25.5	1.42	0.02
	50.0	8.60	8.01
Basal medium	27.0	1.91	0.08
	48.0	2.68	0.54
	76.0	3.33	1.13
	99.0	3.88	1.32
Basal medium without yeast extract	29.0	2.42	0.10
	52.0	2.97	0.62
	72.0	3.52	1.39
	99.5	4.40	2.33
Basal medium plus twice the normal concentration of glucose	20.0	2.15	0.21
	47.0	3.53	0.96
	68.0	4.08	2.03
	94.5	4.38	2.40
French bean medium	24.0	2.05	0.32
	45.5	3.31	0.62
	68.5	6.04	1.10
	96.0	9.67	3.37
Chick pea medium	22.5	2.77	0.55
	44.0	3.94	0.85
	72.5	5.91	1.24
	92.5	8.80	2.93

Fig. VI The growth of sporelings on citrus pectin medium

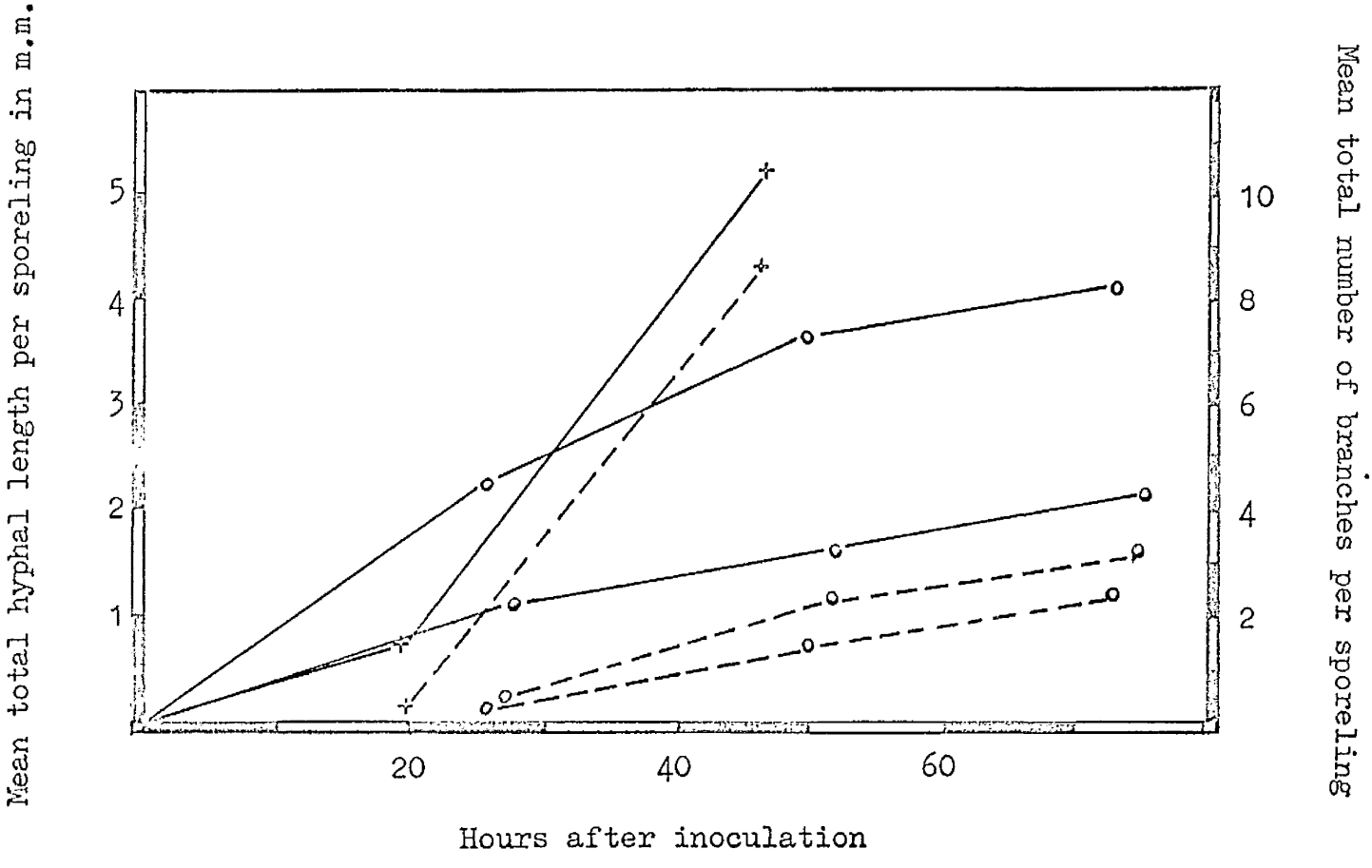
o = basal medium

\+ = apple pectin medium

o = citrus pectin medium

____ = mean total hyphal length per sporeling

---- = mean total number of branches per sporeling



The results show that different nutrient levels other than that provided by apple pectin have very little effect on the growth of the sporelings. Although the total hyphal length per sporeling was greater on the chick pea and French bean media than on the synthetic media the number of branches produced was similar on all media except the pectin medium. Observations made 8 days after inoculation indicated that the sporelings had grown and branched very little after the fourth day. The final experiment in this series was set up to investigate the effect of citrus pectin on the initial stages of sporeling growth.

Expt. 38. The effect of citrus pectin on the development of the sporeling

A sample of methoxylated citrus pectin was obtained from Sigma, London. The medium used in this experiment had the same composition as the pectin medium except that the apple pectin was replaced by citrus pectin at the rate of 10g./litre. Twenty-five sporelings were measured on each of four plates of each medium giving a total of 100 sporelings. The results are given in table 35 and expressed graphically in Fig. VI. The experiment was repeated and essentially the same results were obtained. The results show that the growth of the sporelings on the citrus pectin medium was essentially the same as that on the basal medium and so it can be concluded that citrus pectin does not contain any of the growth factors present in apple pectin.

Table 35. The growth of sporelings on citrus pectin medium

Medium	Hours after inoculation	Value per sporeling (Mean of 100)	
		Total hyphal length m.m.	Total No. of branches
Basal Medium	25.5	2.35	0.24
	50.0	3.63	1.46
	72.5	4.09	2.45
Apple pectin medium	20.0	1.63	0.14
	45.0	5.21	8.61
Citrus pectin medium	28.5	1.13	0.43
	51.5	1.68	2.31
	75.0	2.17	3.36

The results of these experiments show that the rate of growth of the sporelings on the basal medium is slightly greater than that on the pectin medium for the first 24 hours after inoculation. At this time both the number of branches and the amount of hyphal extension produced begin to increase on the pectin medium but to decrease on the basal medium. Increasing or varying the supply of nutrients has very little effect other than slightly increasing the rate of hyphal extension in some cases, e.g. chick pea medium, but has no effect on the number of branches produced. The rate of extension was slightly greater on the basal medium without yeast extract than on the basal medium during the period of observation (72 hours). Clarke (1964) found that fewer

sporelings continued growth to form colonies on a pectin medium without yeast extract than on a pectin medium with yeast extract. Thus yeast extract may be inhibitory to the initial stages of sporeling growth but beneficial for the later stages in the formation of colonies. Citrus pectin had no effect on the initiation of post-sporeling growth.

B. PHYSIOLOGICAL INVESTIGATION

Previous work showed that the physiological changes associated with the initiation of post-sporeling growth occurred within approximately 30 hours of germination. Thus it is obvious that studies designed to investigate these changes should be carried out over this period. Since mass zoospore suspensions will continue growth to form colonies presumably due to synergistic interactions, conventional physiological studies on large masses of fungal tissue are liable to give misleading information. It is only when zoospores germinate and grow in isolation that the growth effects occur. Therefore the main emphasis of the physiological work has been concerned with histochemical investigations on isolated sporelings. The essential aim of these studies is to link changes in metabolism with morphogenetic changes.

Most of the work on histochemical techniques has been done with animal tissues, (reviewed by Pearse, 1961) although some have been adapted for use with plant material (e.g. Jensen, 1962). Histochemical reactions have been used to locate many compounds but they are usually qualitative and very few techniques lend themselves to exact quantitative

estimations without more complicated work. There are many difficulties in detecting water soluble materials such as amino acids and free sugars, although these techniques can locate polymers such as proteins, polysaccharides, nucleic acids, lipids etc.

Zalokar (1959 a,b, 1960) made extensive use of histochemical techniques to study the distribution of certain materials and enzymes within the hyphae of Neurospora crassa. In this way he was able to locate for example nuclei by staining with Azure A, fat with Sudan IV, protein by various methods including the Sakaguchi reaction, and RNA with acridene orange. Using spectrophotometry and certain modified histochemical reactions Zalokar (1959 b) was also able to derive quantitative estimations of the activity of the enzymes aldolase, β-galactosidase, succinic dehydrogenase and tryptophan synthetase. By feeding tritiated leucine and uridene and applying autoradiographic techniques he (Zalokar, 1960) obtained information on the active sites of protein and RNA synthesis respectively.

The widest use of histochemistry has undoubtedly been in the study of nuclear cytology and a large number of stains have been employed to stain the chromatin material of the cell. Very little use has been made of this technique in investigations of the nuclear phenomena of sporeling growth in fungi although some workers (e.g. Rice, 1927) stained the nuclei of the host/parasite complex but this has mainly been to look for nuclear abnormalities in the former. Savile (1939) working with Uromyces fabae, Puccinia maydis, P. malvacearum and five other rusts

and Craigie (1959) working with P. helianthi both found that when rust spores were germinated in vitro each nucleus divided once so that a germ tube of limited growth was produced which contained four nuclei. However Dickinson (1949) found that germinating spores of P. triticina, P. graminis and P. glumarum formed appressoria, substomatal vesicles and infection hyphae after coming in contact with certain membranes, and that this same stimulus also initiated nuclear division. More recently Maheshwari, Hildebrandt and Allen (1967) have found that nuclear division in Uromyces phaseoli occurs in the germ tubes when grown under conditions where appressoria are formed but prior to their formation. Similarly they found that nuclear division occurs prior to the formation of substomatal vesicles.

The nuclear cytology of P. infestans has already been discussed by Marks (1965) who investigated the morphology and division of the nuclei in hyphae. He appears to have found that germ tubes mostly contain two nuclei 24 hours after inoculation but he does not record further details on the rate and number of divisions completed in any one time. There is therefore a remarkable lack of knowledge concerning these factors in P. infestans or for that matter any other fungal parasites and so the original aims of the investigations reported here were to study the differences in the metabolism of DNA, RNA and proteins.

METHODS

In order that sporelings growing on the pectin and basal media may be compared it was essential that a method be developed which would allow

representative samples to be taken and stained in sufficient numbers for microscopic examination. A relatively simple method for the recovery of the sporelings which was tried first was to grow them in liquid culture and subsequently concentrate by filtration on a Millipore filter which could be made transparent by flooding with toluene after the sporelings were stained in situ on the filter membrane. Although this method worked reasonably well for sporelings growing in the basal medium it could not be applied successfully to the pectin medium as the filter membrane soon became blocked because of the colloidal nature of pectin.

A second method which was tried was to grow the sporelings on pieces of cellophane placed on top of the agar surface according to the method devised by Carmichael (1956) but this was unsuccessful as further growth beyond the sporeling stage did not take place. This would be understandable if the factor in apple pectin were bound in some way and only released by extracellular enzyme activity, since such enzymes would not traverse the cellophane. However this point was not investigated. A third method tried was to strip the sporelings from the agar surface using "Sellotape" but it would not withstand the treatment of the fixing solutions.

The method which was finally found satisfactory and was adopted was to wash the sporelings off the agar media and concentrate them by centrifugation. The sporelings were washed off the agar surface by flooding the plate with 5-10 ml. of water and agitating with a glass spreader.

This method usually removed about 50% of the sporelings since they were quite firmly attached to the surface of the agar. The remaining sporelings could not be loosened by subjecting them to treatment with 2.5N HCl., H_2SO_4, NaOH and NH_4OH or a variety of organic solvents. This adhesive property may be important in the attachment of the sporeling to the leaf surface prior to infection. No morphological or size differences were however found to exist between the freed and attached sporelings and so it was assumed that a representative sample was obtained by this technique.

After centrifuging the sporeling suspension obtained by washing the plates with water, the supernatant was withdrawn and a drop of the remaining concentrated sporeling suspension was pipetted onto a glass microscope slide. Haupt's adhesive (Jensen, 1962) was used to attach sporelings to the slide but it was found that some of the procedures, particularly acid hydrolysis, included in certain staining techniques released the sporelings from the slide. Egg albumen adhesive (McLean and Cook, 1941) was however found to withstand the various treatments and so this was used throughout the histochemical investigations apart from the occasions when sporelings were stained with acridene orange or when autoradiographs were required. Under these circumstances when the egg albumen may interfere with results of these experiments, a drop of the sporeling suspension was allowed to evaporate to dryness on the surface of a slide which had been thoroughly cleaned in 95% alcohol. By this method the sporelings became firmly attached to the glass slide without an adhesive.

The sporelings were chemically fixed by covering them with a few drops of Carnoy's fixing solution (Johansen, 1940) and allowing it to evaporate off. The composition of the Carnoy's solution which was used throughout the histochemical procedures was as follows:-

Absolute alcohol	30 ml.
Glacial acetic acid	5 ml.
Chloroform	15 ml.

Neotetrazolium chloride has been used to detect succinic dehydrogenase activity and tetrazolium blue has been used to stain protein bound sulphydryl groups. Both of these tetrazolium salts (obtained from E. Gurr, London) were employed according to the methods described by Farber and Louviere (1956) and Barrnett and Seligman (1958) respectively. On no occasion did any of the sporelings stained by either of these methods give the characteristic colour reaction. However, when whole colonies were stained the characteristic reddish-purple colour developed towards the centre of the colony. On further examination it was found that the actively growing hyphae of the colony had not stained but the colour apparently was present in the older evacuated hyphae. Thus it may be that the permeability of actively growing hyphae of P. infestans is such that these salts are unable to react with the appropriate compounds in the cell. An attempt was also made to detect protein containing arginine by the Sakaguchi reaction (McLeish and Sherrat, 1958) but this also failed to give positive results.

A number of nuclear stains were tried and of those visible in the light microscope, Delafield's haematoxylin (Johansen, 1940), Azure A, (Huebschman, 1952) and propionic orcein (Marks, 1965) were most successful. However, it was found that the most consistent and easily obtained results were achieved using acridene orange. The sporelings were examined using a blue light fluorescence microscope which was a Reichert Neopan with a quartz-iodine lamp and fitted with the appropriate excitation and barrier filters. The DNA of stained sporelings fluoresced green while the RNA fluoresced red. The procedure employed was as follows.

(1) The sporelings were fixed in situ on the slide with Carnoy's solution.

(2) The slide was transferred to the staining solution for 1/2 - 5 minutes. The exact time varied and was determined by experiment. The reagent contained 50 mg. acridene orange N (Michrome No.87 obtained from E. Gurr, London) per litre of phosphate buffer at pH 7.0.

(3) The slides were then rinsed for 1 min. and mounted in phosphate buffer and examined immediately since the fluorescence tended to fade with time.

An experiment was set up employing these techniques to investigate the changes in the nuclear complement in relation to changes in the morphology of sporelings growing on pectin and basal media.

Expt. 39. Nuclear changes associated with morphological changes in sporelings growing on pectin and basal media

Two batches of approximately 75 petri dishes of each medium were inoculated on two consecutive days, the second batch being inoculated exactly 24 hours after the first. A random sample of 8 petri dishes of each medium and from each inoculation were taken at 4 hourly intervals. Thus the first samples taken 4 hours after the second inoculation was made included a set of petri dishes which had been inoculated 4 hours previously and another set inoculated 28 hours previously. In this way readings from 0-48 hours at 4 hourly intervals were obtained over a period of 24 hours. Both batches were examined at approximately 24 hours and at 28 hours after inoculation to provide an overlap between the two inoculations.

The sporelings from each sample were sampled and taken through to the end of the fixing stage using the procedures described previously. They were then stored until required for staining. Measurements of total hyphal length, and counts of the number of branches and of the total number of nuclei per sporeling were made on 50 sporelings in each sample. The results are given in table 36.

Table 36 (contd)

B. PECTIN MEDIUM

Hours after inoculation	Value per sporeling (Mean of 50)		
	Total hyphal length m.m.	Total number of branches	Total number of nuclei
4	0.26	0	1.02
8	0.52	0.06	1.92
12	0.74	0.14	2.78
16	0.86	0.14	3.42
20	1.12	0.58	5.32
24	1.45	0.94	8.64
25	1.61	1.14	7.36
28	1.77	1.18	10.64
28	2.08	1.82	10.72
32	2.30	2.12	9.44
36	3.34	3.84	16.42
44	6.17	5.44	30.18
48	5.38	4.90	26.26

Table 36. The total length, number of branches and number of nuclei obtained from sporelings grown on pectin and basal media

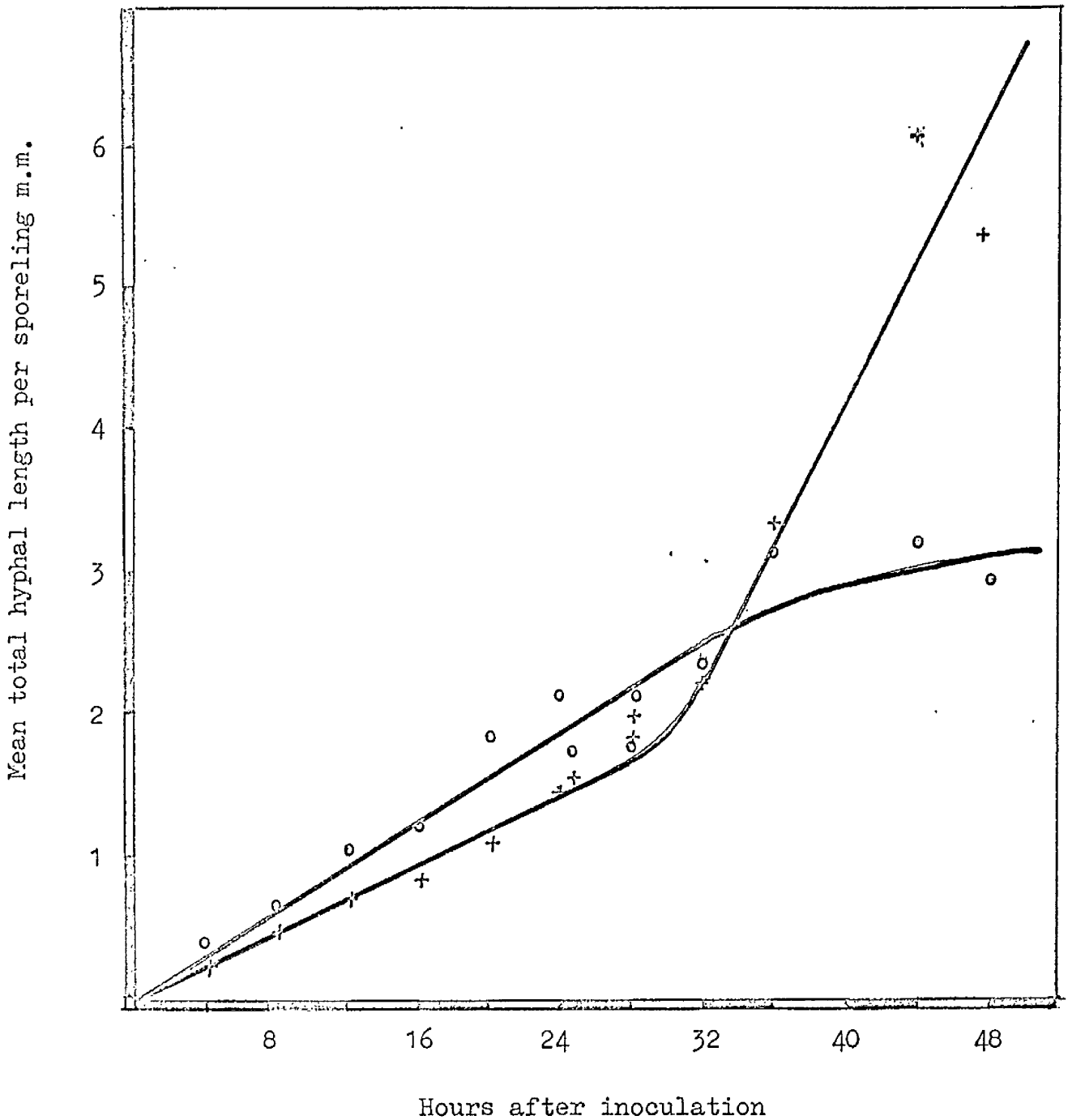
A. BASAL MEDIUM

Hours after inoculation	Value per sporeling (Mean of 50)		
	Total hyphal length m.m.	Total number of branches	Total number of nuclei
4	0.43	0	1.30
8	0.68	0.16	1.96
12	1.04	0.26	2.90
16	1.47	0.68	4.04
20	1.86	1.00	5.32
24	2.15	1.06	5.78
25	1.72	0.94	4.90
28	2.11	1.16	8.86
28	1.71	0.88	5.40
32	2.35	1.48	6.70
36	3.14	1.88	8.74
44	3.23	1.46	9.18
48	2.98	1.40	9.16

Fig. VII. The total hyphal length of sporelings growing on pectin and basal media

o = basal medium

\+ = pectin medium



The growth rate, as measured by total hyphal length, is relatively constant on both media until 24 hours after inoculation (see Fig. VII). After 24 hours the rate of increase of total hyphal length on the basal medium begins to fall off at the same time as it begins to increase on the pectin medium. The total hyphal length of the sporelings is, however, almost linearly related to time after inoculation on the two media up to 24 hours after inoculation. The regression equations of growth rate (as measured by total hyphal length) over this period are:-

			S.E. of regression coefficient
(i)	basal medium	$y = 0.0898x - 0.016$	$\pm$ 0.0054
(ii)	pectin medium	$y = 0.07078x - 0.167$	$\pm$ 0.0352

where y = total hyphal length per sporeling in m.m.

and x = time in hours after inoculation

The linear regressions are significantly different at the 0.001 level and so the growth rate of the sporelings on the pectin medium is significantly less than that of the sporelings on the basal medium up to 24 hours after inoculation.

Similar results are obtained when the number of branches produced by the sporelings growing on the two media is compared (Fig. VIII) except that the rate of production of branches on the pectin medium begins much earlier, between 16 and 20 hours after inoculation. This is approximately 8-12 hours before the increase in the rate of increase

Fig. VIII. The total number of branches produced on sporelings growing on pectin and basal media

o = basal medium

\+ = pectin medium

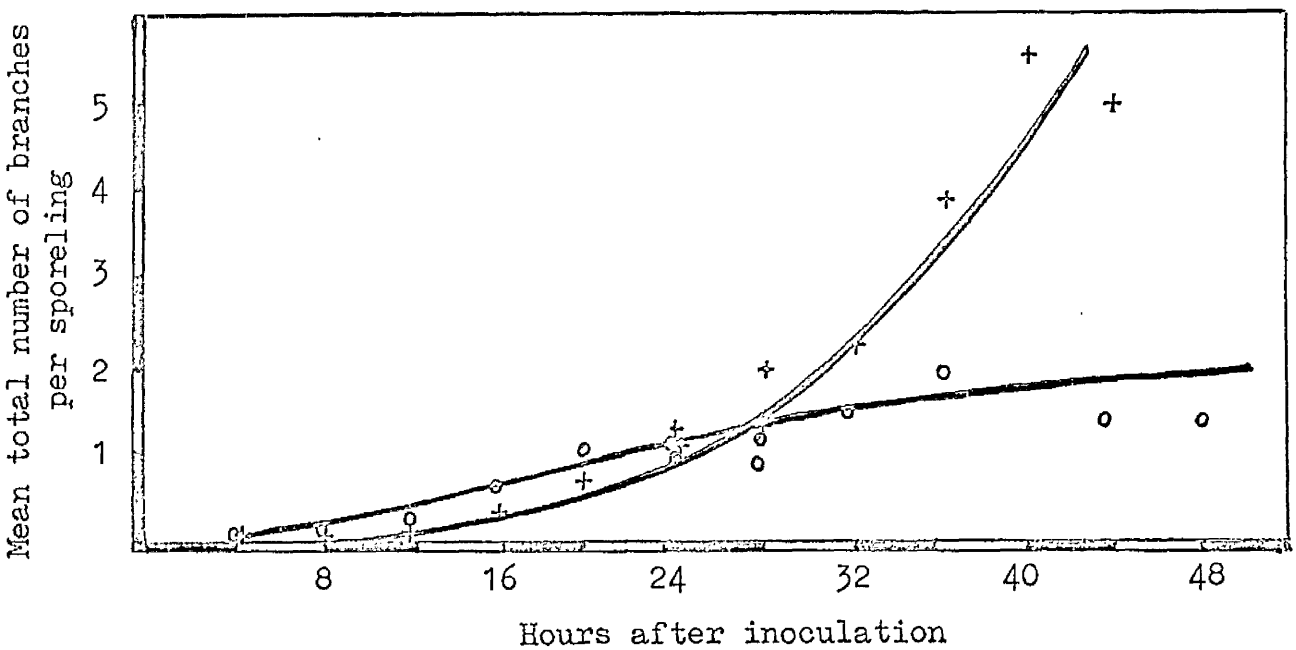
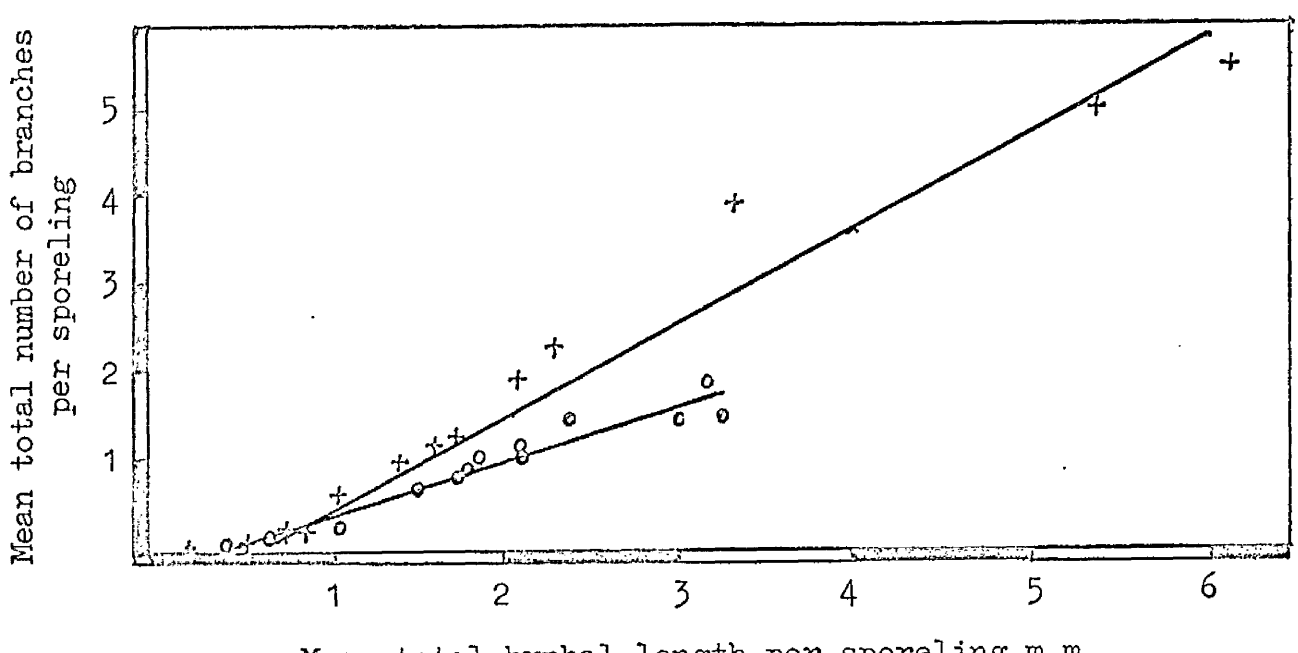


Fig. IX. Relation between number of branches and total hyphal length

o = basal medium

\+ = pectin medium



of total hyphal length. The regression equations for number of branches produced on the pectin medium and on the basal medium on time after inoculation up to 16 hours are -

			S.E. of regression coefficient
(i)	basal medium	$y = 0.0535x - 0.260$	$\pm$ 0.011
(ii)	pectin medium	$y = 0.0125x - 0.040$	$\pm$ 0.003

where x = number of hours after inoculation (up to 16 hours)

and y = number of branches per sporeling

These two regressions are significantly different at the .05 level and so it appears that significantly fewer branches are produced on the pectin medium than on the basal medium up to 16 hours after inoculation. The delay in branching of the sporelings on the pectin medium has already been shown in the previous experiments in section A of this chapter.

The mean number of branches per sporeling were plotted against the mean total hyphal length per sporeling (Fig. IX) and the linear regressions calculated.

			S.E. of regression coefficient
(i)	basal medium	$y = 0.606x - 0.286$	$\pm$ 0.0162
(ii)	pectin medium	$y = 1.0089x - 0.426$	$\pm$ 0.0334

where x = mean total hyphal length in m.m. per sporeling

and y = mean number of branches per sporeling

Fig. X. Mean total number of nuclei produced by sporelings growing on pectin and basal media

o = basal medium

\+ = pectin medium

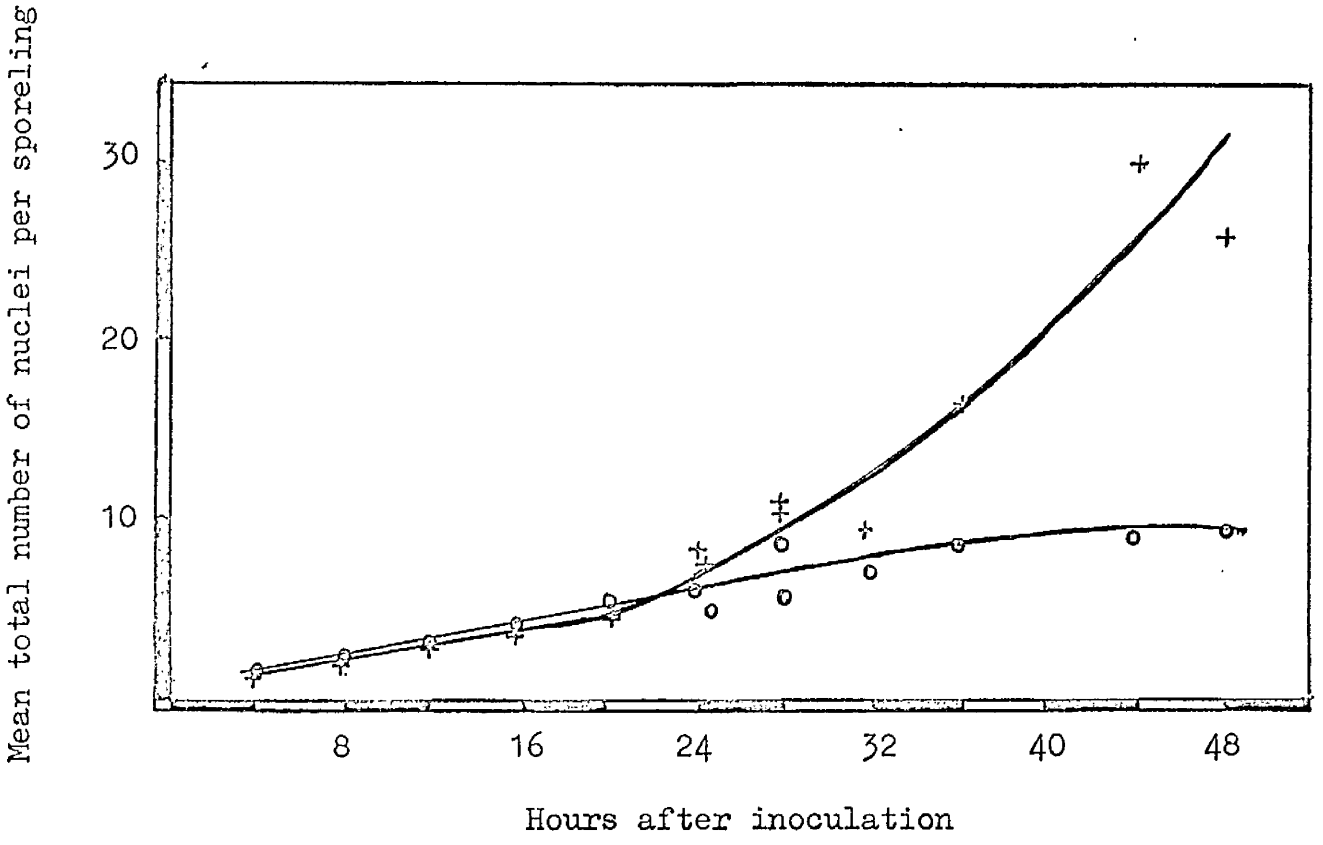


Fig. XI. The distribution of sporelings with given numbers of nuclei

= number of sporelings on basal medium

= number of sporelings on pectin medium

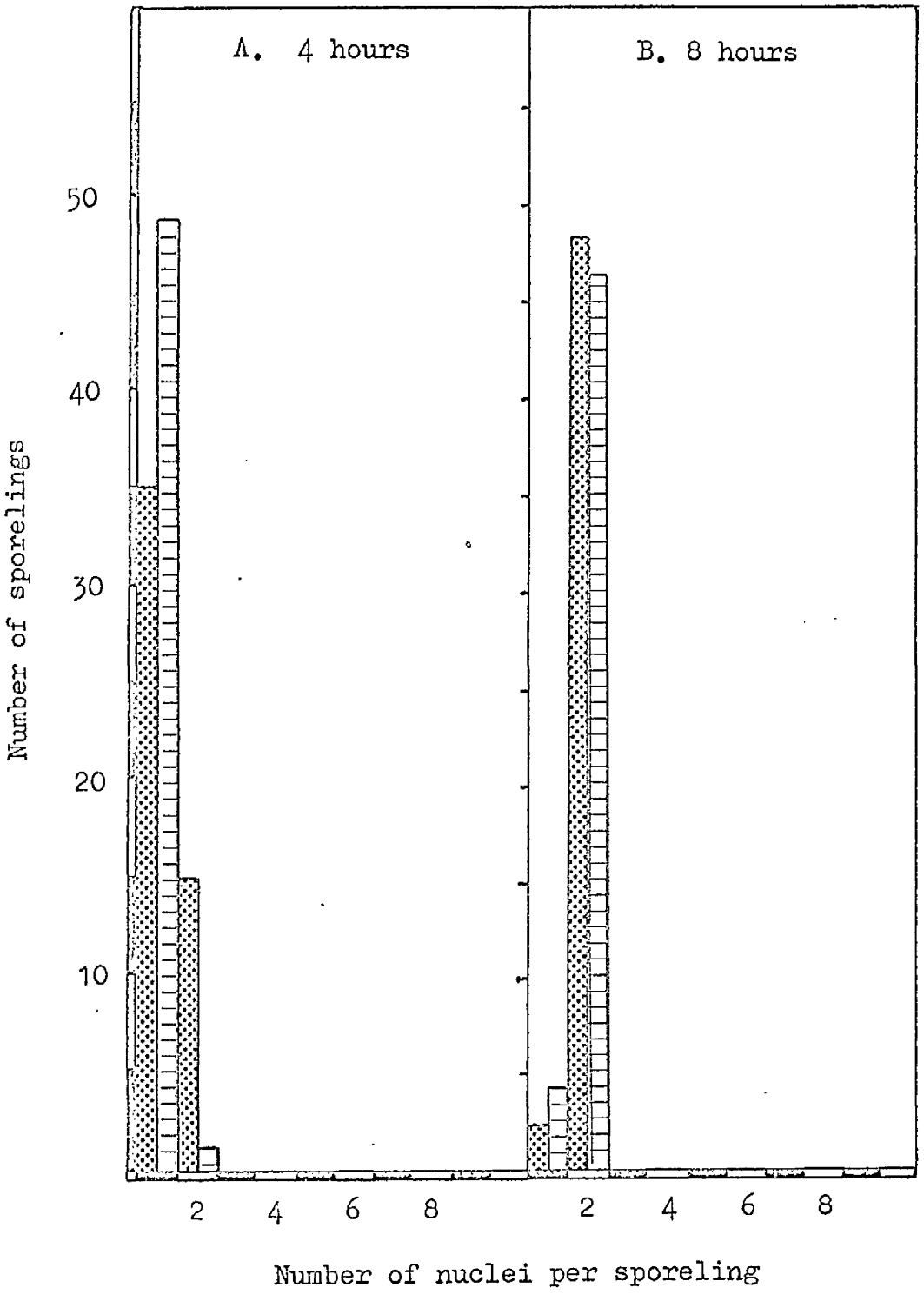


Fig. XI (contd)

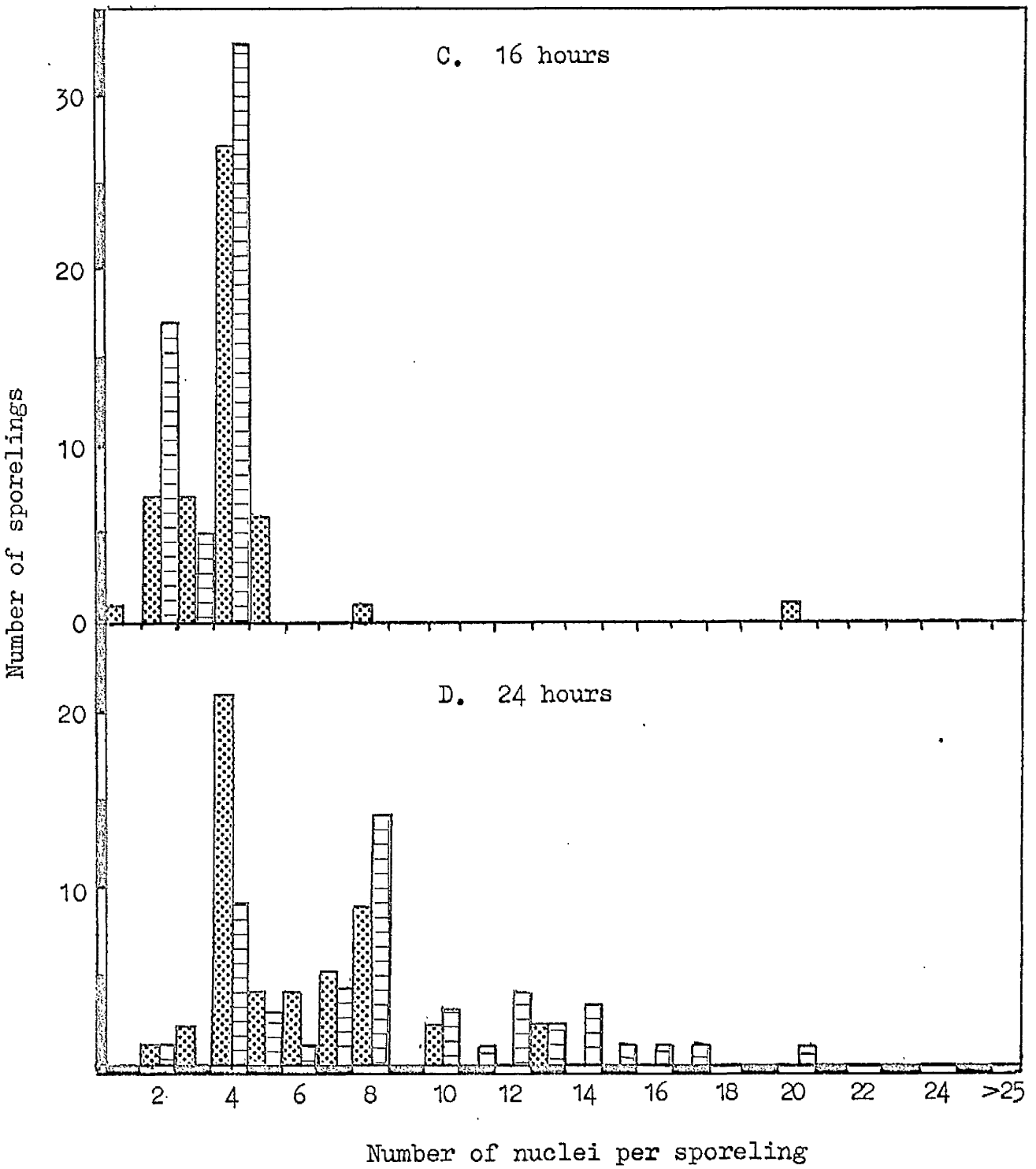
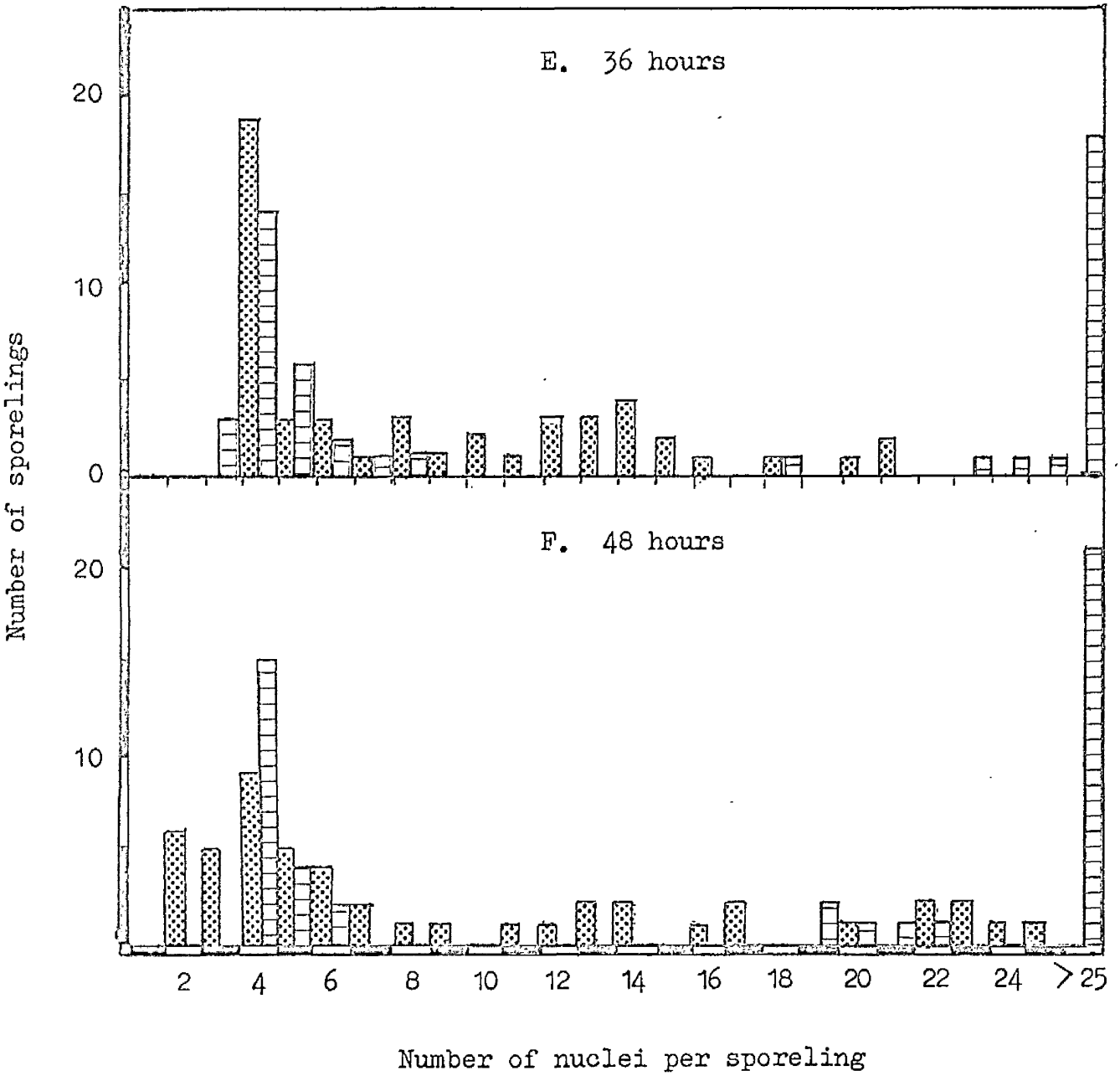

C. 16 hours
D. 24 hours
Number of sporelings
30
20
10
0
20
10
2
4
6
8
10
12
14
16
18
20
22
24
>25
Number of nuclei per sporeling

Fig. XI (contd)


E. 36 hours
F. 48 hours
Number of sporelings
20
10
0
20
10
2
4
6
8
10
12
14
16
18
20
22
24
>25
Number of nuclei per sporeling

Thus it appears that a constant increase in the number of branches per unit length is maintained throughout the growth of the sporelings up to 48 hours after inoculation on both media. However, the number of branches produced per unit length of hypha is greater on the pectin medium than on the basal medium ($P = .001$).

The mean number of nuclei per sporeling is plotted against time after inoculation in Fig. X. The distribution of the sporelings with given numbers of nuclei in each sample for different sampling times up to 48 hours are also given in the histograms (Fig. XI) The histograms show that the first few divisions of the nuclei up to 16 hours are approximately synchronous since most sporelings have either 2 or 4 nuclei and relatively few have 3 or 5 or more.

The mean nuclear generation time for the two media can be derived from Fig. X and this is given in table 37.

Table 37. Mean nuclear generation time of sporelings growing on pectin and basal media

Generation	Basal Medium	Pectin Medium
1 (1n→ 2n)	8 hours	8 hours
2 (2n→ 4n)	8 hours	8 hours
3 (4n→ 8n)	14 hours	9 hours
4 (8n→16n)	≏74 hours	15 hours

These figures suggest that the rate of nuclear division is the same on

Fig. XII. Relation between number of nuclei and total hyphal length

o = basal medium

\+ = pectin medium

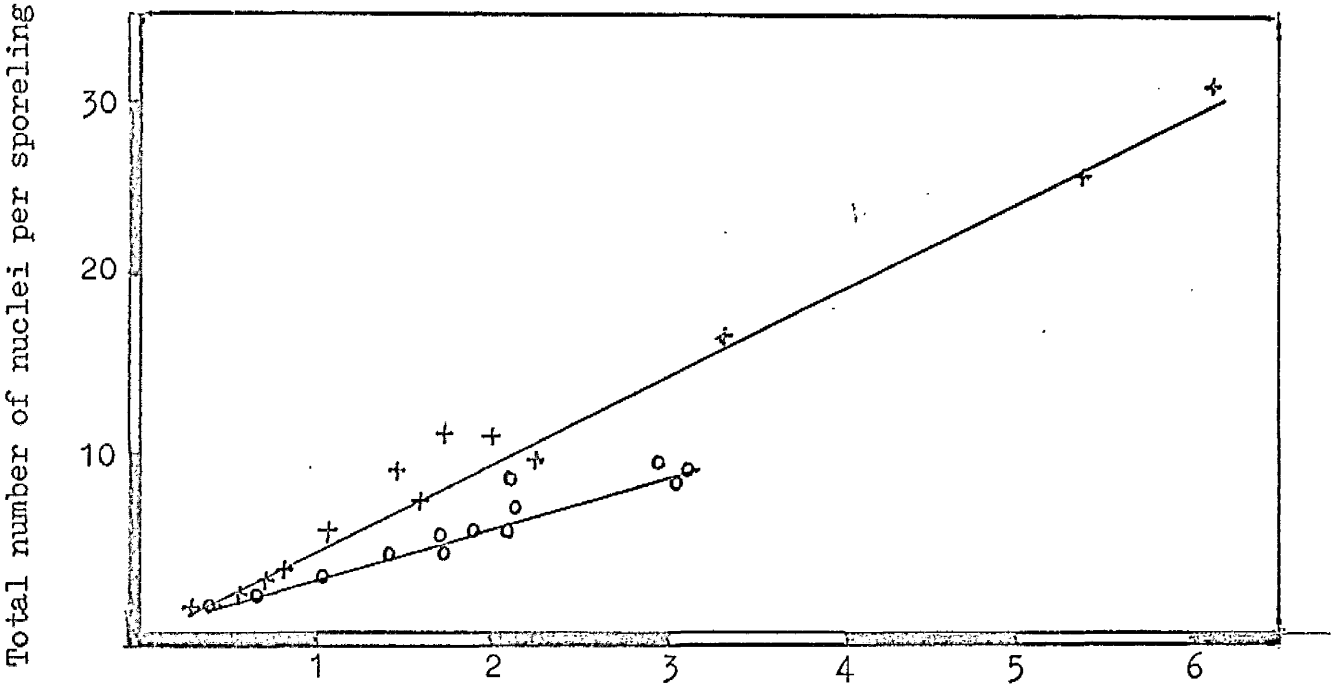
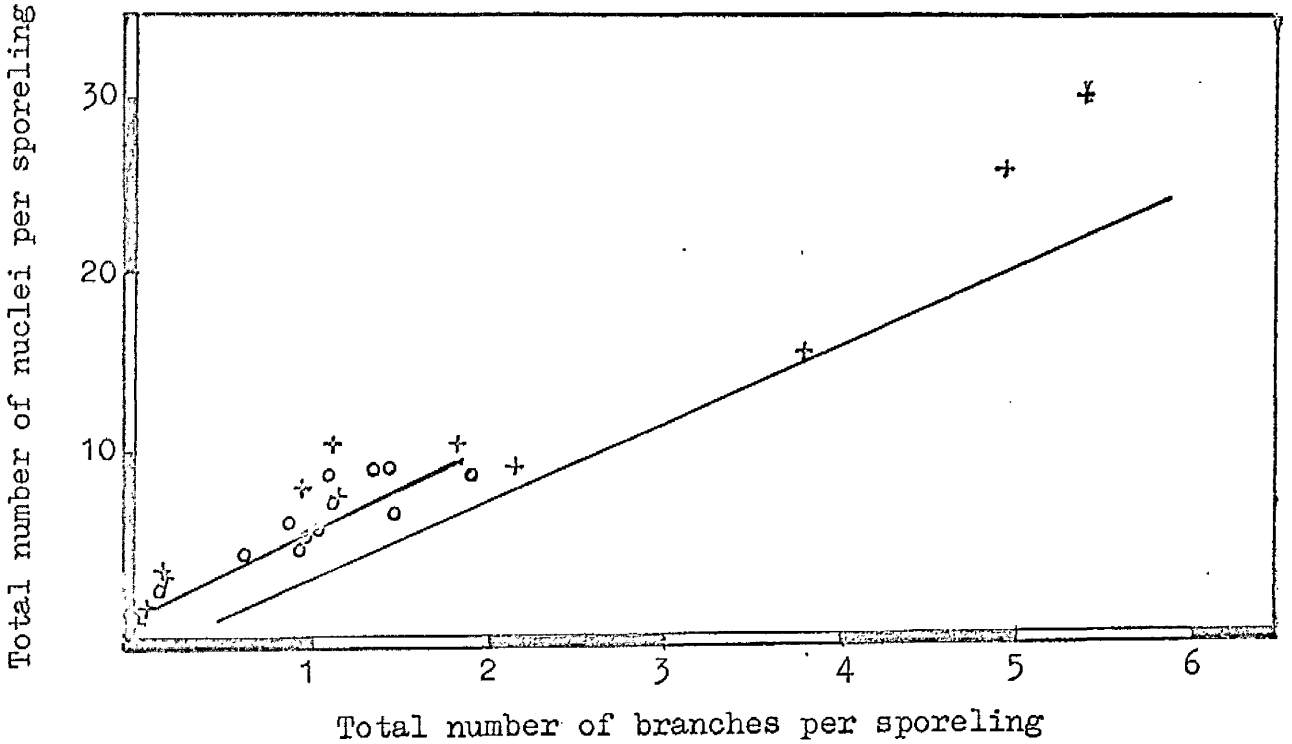


Fig. XIII. Relation between number of nuclei and number of branches

o = basal medium

\+ = pectin medium



both media up until the second division, i.e. the 4 nucleate stage at 16 hours. After this the rate of nuclear division decreases markedly on the basal medium but the rate is maintained almost at the original rate on the pectin medium. Since the figures quoted in table 37 have been derived from the mean of the total population and a proportion of these tend to stop growing after the 4 nucleate stage is reached, the actual rate of nuclear division of the sporelings which eventually grow on to form colonies is certainly higher and is probably maintained around the 8 hour period.

The mean number of nuclei per sporeling was plotted against the total hyphal length for both media (Fig. XII) and the linear regressions were calculated.

		S.E. of regression coefficient
(i)	basal medium $y = 2.935x + 0.094$	$\pm$ 0.86
(ii)	pectin medium $y = 4.936x - 0.1602$	$\pm$ 1.043

where x = mean total hyphal length in m.m. per sporeling

and y = mean number of nuclei per sporeling

There is a linear correlation between total numbers of nuclei and total hyphal length in both cases, but there is a greater number of nuclei per unit length of hypha of the sporelings growing on the pectin medium than those growing on the basal medium (P = greater than .01).

Thus it has been shown that the sporelings on the pectin medium have a significantly greater number of branches and nuclei per unit of

hyphal length than those on the basal medium. The number of nuclei per sporeling was plotted against the number of branches produced to see whether there was any relationship between the total number of nuclei per sporeling and the ability of branch initials to continue growth (Fig. XIII). The linear regressions were also calculated.

		S.E. of regression coefficient
(i)	basal medium $y = 4.5x + 1.435$	$\pm$ 1.09
(ii)	pectin medium $y = 4.74x - 2.182$	$\pm$ 1.99

where x = mean number of branches per sporeling

and y = mean number of nuclei per sporeling

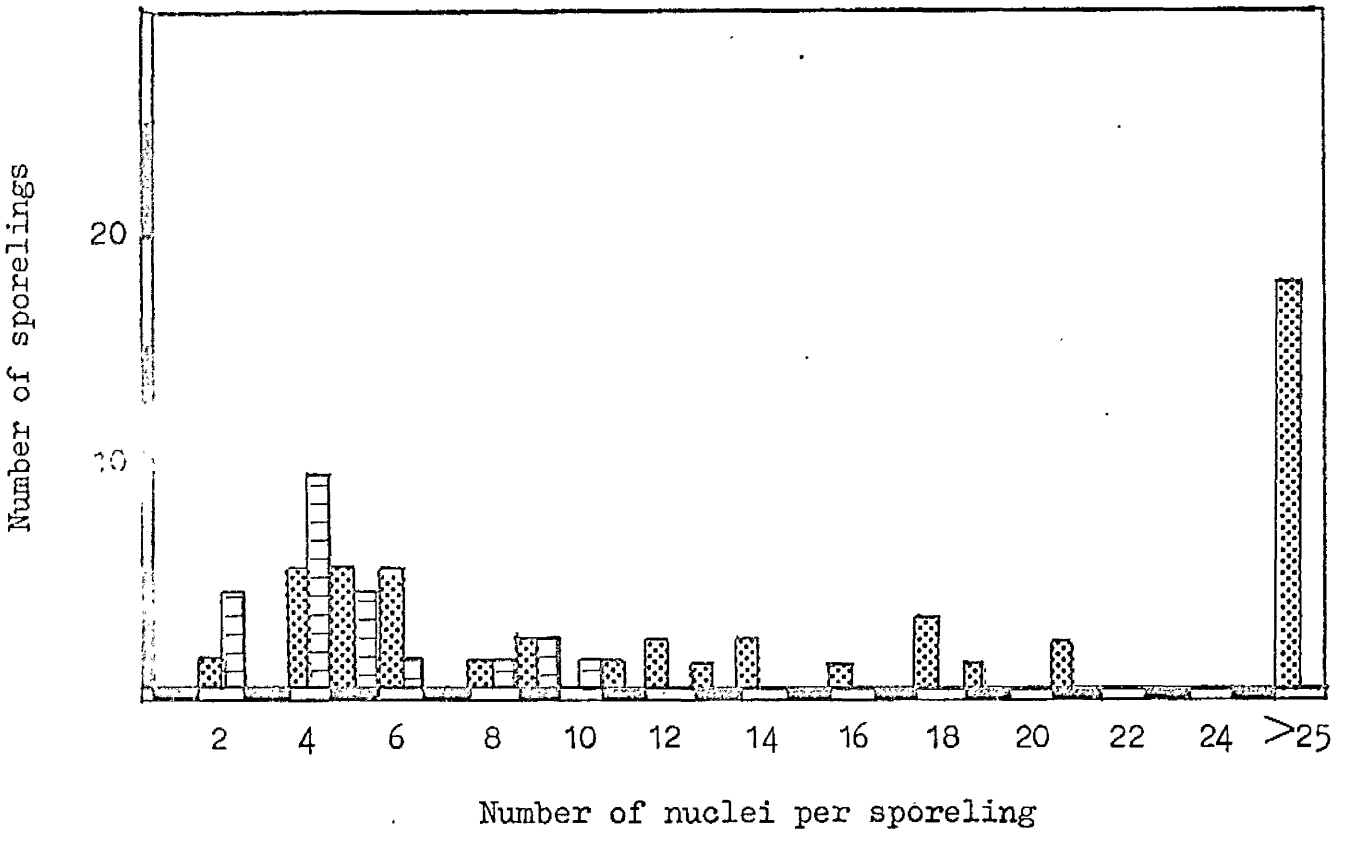
Thus it appears that the total number of nuclei per unit number of branches per sporeling tends to remain constant throughout the growth of the sporelings on both media and that there is no significant difference between the sporelings growing on the two media since the probability is less than 0.05.

From the measurements taken at 48 hours after inoculation the maximum number of nuclei contained by any of the sporelings sampled on the basal medium was 25. However on the pectin medium at the same stage 21 sporelings out of the sample of 50 contained more than 25 nuclei and the maximum recorded was 72 nuclei per sporeling. Although at 48 hours many of the sporelings contained relatively large numbers of nuclei a number of the sporelings, approximately 40%, on the pectin medium had less than 8 nuclei. It was therefore of interest to determine whether

Fig. XIV. The distribution of sporelings with given numbers of nuclei eight days after inoculation

= number of sporelings on basal medium

= number of non-colony forming sporelings on pectin medium



any of this group continued growth to form colonies.

After eight days incubation colonies became visible on the pectin medium but none were observed on the basal medium - a number of plates from the two batches had been retained and incubated for a longer period to ensure that colonies developed only on the pectin medium. The petri dishes containing the pectin medium were examined under the microscope and the percentage number of sporelings which had not formed colonies was calculated. The number was found to be somewhat higher than was usual in that 31.2% of the sporelings failed to continue growth, whereas in previous experiments 90% or over formed colonies. The reason for this difference is not known. A finely divided piece of bamboo was found to be the only satisfactory method by which the sporelings could be removed from the agar surface of the pectin medium. Samples were also taken from the basal medium in the usual way. The usual measurements were made and are recorded in table 38, and the number of sporelings with given numbers of nuclei in the samples is given in the form of a histogram in Fig. XIV.

Table 38. The total length, number of nuclei and number of branches in sporelings on basal and pectin medium 8 days after inoculation

Medium	Total length	Total No. of branches	Total No. of nuclei
Basal Medium	5.32	3.58	27.06
Pectin Medium (Non colony producing sporelings)	1.438	1.36	3.20

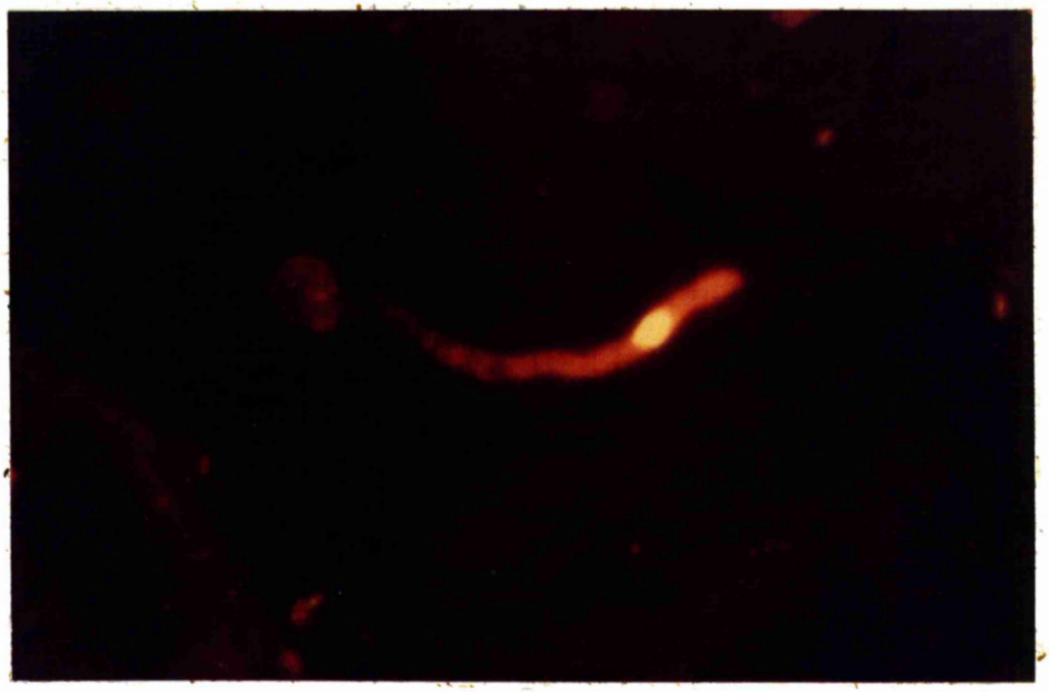
Plate 3. Nuclei of sporelings grown on pectin medium stained with acridene orange and viewed in blue light

A. Uninucleate sporeling four hours after inoculation.

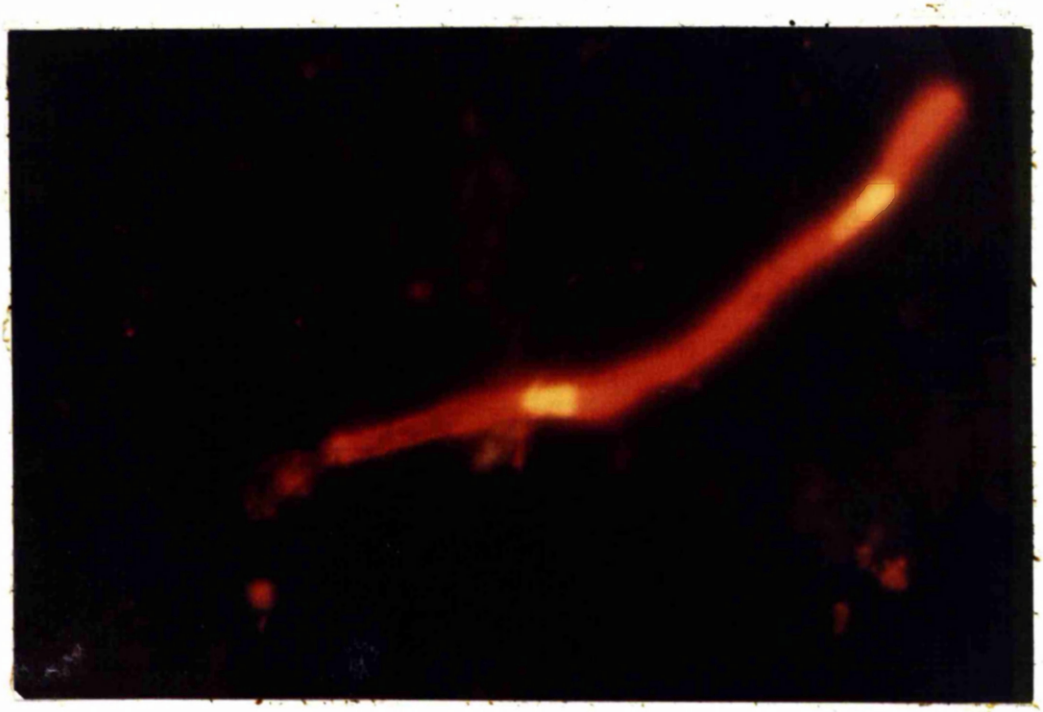
B. Binucleate sporeling eight hours after inoculation.

C. 4-nucleate sporeling sixteen hours after inoculation.

D. Typical non-colony forming sporeling eight days after inoculation.

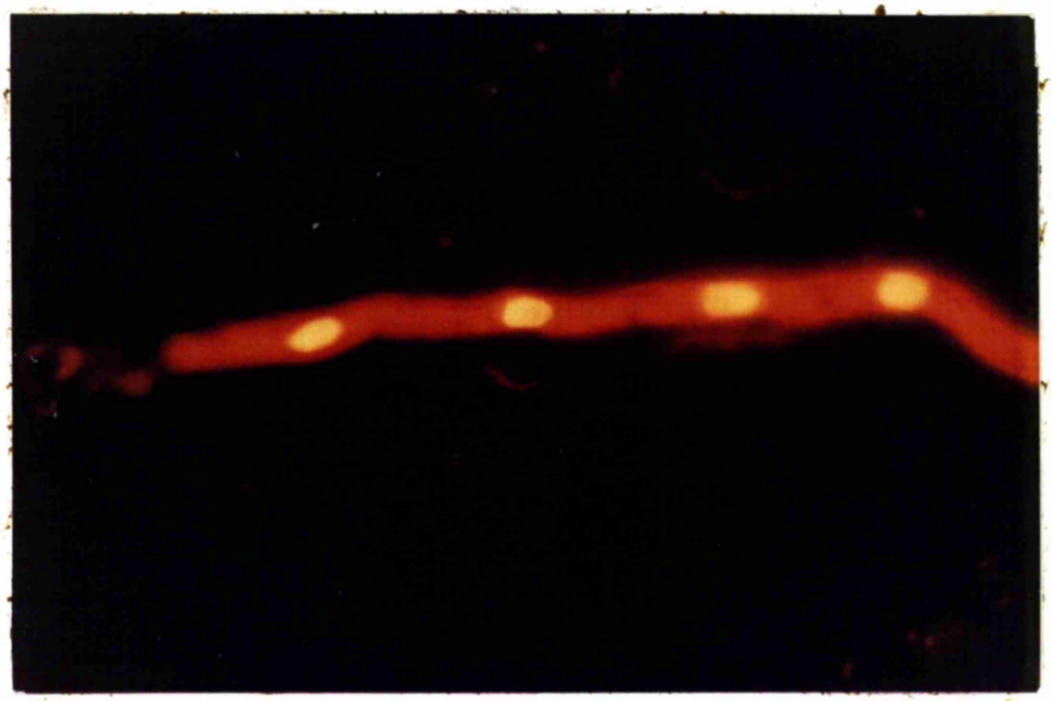


A

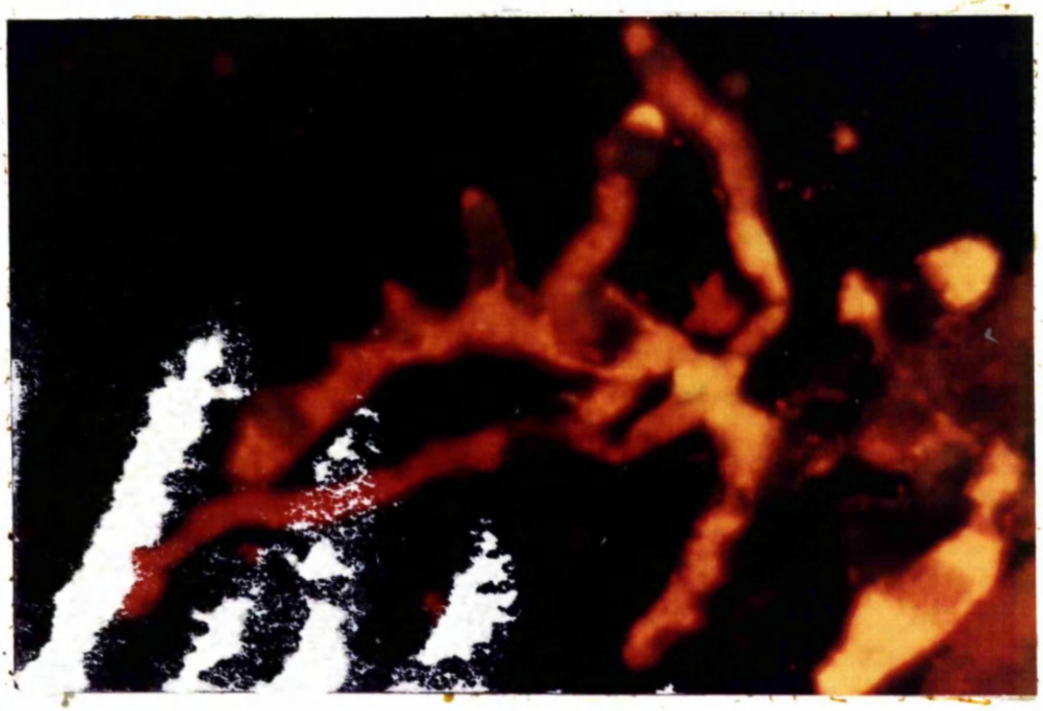


B

0·1mm.



C



D

0·1 mm.

The first point to be made is that the sporelings on the basal medium have continued to grow and the nuclei to divide but at a much slower rate than those on the pectin medium, cf. Fig. XI. Of the 50 sporelings sampled on the basal medium 18 contained more than 25 nuclei and the maximum reached was 112 in one sporeling. The second and most remarkable point is that every sporeling which failed to grow further on the pectin medium was found to stain much less intensely than previously and the protoplasm appeared generally disorganised and highly vacuolated. These sporelings were almost certainly dead. When the non-colony forming sporelings were examined from a number of other experiments in every case they all appeared to be "dead". However, never on any occasion were any sporelings growing on the basal medium recorded which appeared to be similar to this group. Thus the majority of the sporelings on the pectin medium, which 48 hours after inoculation contained less than 8 nuclei, did not initiate post-sporeling growth.

The sporelings growing on the basal medium were capable of growth although at a much reduced rate compared with those on the pectin medium. In order to obtain a better understanding of the metabolic processes which might be limiting the rate of growth of the sporelings on the basal medium, the mean hyphal length of the sporelings containing 4 nuclei was calculated for all the sampling times. The 4 nucleate sporelings were arbitrarily chosen since they represented a group which was present in comparatively high numbers throughout the samples taken after 8 hours. The results are given in table 39.

Table 39. Mean total hyphal length of sporelings with 4 nuclei

Hours after inoculation	No. of sporelings with 4 nuclei	Mean total hyphal length in m.m. per sporeling
12	10	12.3
16	27	15.26
20	14	16.71
24	20	18.5
25	25	15.12
28	16	18.38
28	23	15.35
32	21	18.38
36	19	23.16
44	16	24.56
48	9	32.22
192	5	22.40

It is evident that nuclear division stops or slows down before growth in length (i.e. protein synthesis), and so an attempt was made to investigate the synthesis of DNA and protein by autoradiography.

Expt. 40. Autoradiography

Sporelings which had been grown on the pectin and basal media were sampled at intervals using the methods described previously with the exception that instead of washing the sporelings off the agar

surface in water and concentrating by certifugation, they were filtered through a Millipore filter and placed into 3.0 ml. of a medium containing the labelled compound. Zoospore suspensions were also inoculated directly into the medium containing the labelled compound and allowed to germinate and grow for up to 24 hours before sampling. On several occasions one week old colonies were removed from the pectin medium and grown in the labelled medium for periods of either 1/2, 1, 2, 8, 12 or 24 hours. The medium contained -

Glucose	25.0 g.
Asparagine	1.0 g.
$MgSO_4.7H_2O$	0.25 g.
KH_2PO_4	0.50 g.
Thiamine	0.001 g.
Water to	1.0 litre

The medium was adjusted to pH 5.5 before autoclaving at 10 lbs. for 10 minutes. ^{3}H-thymidine and ^{3}H-leucine were added to this medium as required. The ^{3}H-thymidine which was labelled in the 6 position had a specific activity of 68 mc./mg. was used to investigate DNA synthesis. The ^{3}H-leucine which was generally labelled had a specific activity of 1.1 mc./mg., and was used to investigate protein synthesis.

After the sporelings had been grown in the presence of the labelled compound for a given period they were again filtered off, resuspended in water, and placed on ordinary glass microscope slides. The sporelings were fixed <u>in situ</u> on the slide using Carnoy's fixative as des-

cribed previously. The slides were then washed for 5 minutes in three solutions of 5% trichloroacetic acid (TCA) and finally washed for one hour in running tap water. This treatment should remove all soluble low molecular weight compounds such as sugars, organic acids, free short chain peptides as well as unincorporated labelled compounds.

The slides were then dipped in a dilute emulsion of NTB 2 nuclear track emulsion obtained from Kodak Limited, London, and left to expose in a light-tight container for a period varying from a few days to six weeks. The slides were developed in Kodak D19b developer for 2 mins. and rapidly washed for 5 mins. in running tap water, before fixing in Kodak acid fixer. They were then washed for one hour in running water before drying and staining with Delafield's haematoxylin, which stained the nuclei through the emulsion.

RESULTS

In every case when the autoradiographs were developed it was found that deposition of silver grains as a result of disintegration of labelled compounds did not take place. Silver grains were however deposited on the emulsion covering a slide on which a drop of the labelled medium had been placed. This slide was not rinsed in 5% TCA. Thus the failure to detect incorporation of the labelled compound was not due to a fault in the emulsion. It may therefore be concluded that the ^{3}H-thymidine was not incorporated into DNA and the ^{3}H-leucine

was not incorporated into protein at a sufficient level of radioactivity to be detected by autoradiographic techniques.

That ^{3}H-thymidine is not readily incorporated into DNA has been reported by a number of workers studying DNA synthesis in fungi. Thus Baer and St. Lawrence (1964) found that the failure of this technique in Neurospora crassa was probably due to the fact that the fungus produces enzymes which degrade thymidine to yield uridine which appeared then to be incorporated into both DNA and RNA at a very low level. No such incorporation was observed however in the work carried out here with P. infestans.

Williams (1966) however found when studying the parasitic relationship of Plasmodiophora brassicae on cabbage that ^{3}H-thymidine was incorporated into the nuclei of both host and parasite. ^{3}H-thymidine was incorporated into the nuclei of rusted bean leaves but no label was found in the fungal cells of Uromyces phaseoli (Staples and Ledbetter, 1960). However Heitetuss (1966) reports that the nuclei of both organisms have been labelled by C^{14} orotic acid which is a precursor of the nucleotides present in DNA and RNA. This method in conjunction with DNAase and RNAase may prove to be the only suitable method for the labelling of nucleotides in P. infestans as Page (1965) found that it had the ability to degrade exogenously supplied RNA to hypoxanthine and uracil.

Although ^{3}H-leucine has been used to study protein synthesis in a number of fungi, e.g. N. crassa (Zalokar, 1960), it has been shown that

it can be broken down by Phytophthora heveae to yield valeric, propionic, and butyric acids which are toxic (Leal and Gomez-Miranda, 1967). Deamination of some exogenously supplied amino acids has also been reported for P. infestans by Sakai (1962). Thus other amino acids may prove more useful in the study of protein synthesis by autoradiography.

DISCUSSION

The growth of sporelings on the pectin medium has been compared with that of sporelings growing on the basal medium. On germination the zoospore produces a germ tube which grows across the medium sooner or later producing branches. A significantly greater number of branches are produced by the sporelings on the basal medium during the first 16 hours after inoculation than by the sporelings on the pectin medium. After 16 hours however the rate of production of branches by the sporelings on the pectin medium increases while that by the sporelings on the basal medium begins to decrease. Most of the branches produced by the sporelings on the pectin medium continue growth and ultimately develop further branches while those produced by the sporelings on the basal medium often protrude no further than approximately one hyphal diameter from the main hyphae (Plate 2). Thus each branch initial produced by the sporelings on the pectin medium represents an active site of growth whereas most branch initials on the sporelings on the basal medium do not. This is one of the main differences between the sporelings growing on the two media.

Measurements of total hyphal length made at timed intervals after inoculation showed that the rate of growth for the first 24 hours on the pectin medium was significantly lower than that on the basal medium. After 24 hours the rate of growth on the pectin medium begins to increase and enters the log phase while on the basal medium the growth rate decreases to a rather low level. However growth at this low level may continue on the basal medium for periods up to 8 days.

The number of branches produced per unit length of hypha by sporelings on both media is linearly related over the period of 48 hours, but there is a significantly greater number of branches produced per unit length of hypha by the sporelings growing on the pectin medium than by those on the basal medium. Thus the greater number of branches produced by the sporelings on the basal medium during the first 16 hours is clearly related to the greater rate of growth of the germ tubes. The greater density of branches of the sporelings on the pectin medium and the fact that each branch initial is an active site of growth are responsible for the differences in growth rates on the two media after 24 hours. Thus the sporelings on the pectin medium continue to develop more actively growing branches and so the exponential increase in the total hyphal length is obtained with time.

Observations on the number of nuclei per sporeling made at intervals after inoculation showed that the rates of nuclear division were almost exactly the same in sporelings on both media for the first 20 hours. Clearly then the greater rate of hyphal growth on the basal medium over that on the pectin medium was not matched by a greater rate of nuclear

division. During this stage of growth and indeed during all stages of growth the number of nuclei per unit length of hypha was constant for both media but there were significantly more nuclei per unit of hyphal length in the pectin medium sporelings than in the basal medium sporelings. The mean generation time of nuclei in the sporelings on the pectin medium was in the region of 8 hours while that on the basal medium was initially 8 hours but tended to increase with time so that the third generation time (i.e. the time taken for the mean number of nuclei per sporeling to increase from four to eight) was 15 hours. The fourth generation time on the basal medium was of a substantially greater period being approximately 74 hours. Thus the rate of production of nuclear material in the sporelings on the basal medium decreases after 20 hours.

Measurements of total hyphal length made on 4 nucleate sporelings growing on the basal medium showed that the mean total hyphal length increased with time after inoculation so that the synthesis of nuclear materials would appear to slow down before the synthesis of extra-nuclear material. Since the nuclei of 4 nucleate sporelings did not appear to be larger at the end of the period of observation (48 hours) than they were at the beginning (12 hours), it is assumed that synthesis of nuclear materials was slowed down and so resulted in a decrease in the rate of nuclear division.

The ratio of mean number of nuclei to mean number of branches per sporeling remained constant on both media through the period of observation. The ratio of nuclei to branches for the sporelings on the pectin

medium was not significantly different from that for the sporelings on the basal medium. Fewer branches per unit of hyphal length of the sporelings on the basal medium is related then to the fewer nuclei per unit of hyphal length on the basal medium but the causal relationship is not clear since the rate of production of both nuclear material and branches changes at approximately the same time (16-20 hours).

An attempt was made to investigate the synthesis of protein and DNA using labelled leucine and thymidine respectively for the production of autoradiographs. However, neither of these compounds was incorporated into the hyphae at a sufficient level in these experiments to be detected by this technique. The reasons for this are not clear but a permeability factor may be operative in that the precursor does not enter the hyphae or reach the sites of syntheses. Also, the labelled compounds may be enzymatically broken down resulting in the loss of the isotopic fraction. The use of other labelled compounds as precursors may help in the further elucidation of the problem of the synthesis of protein, DNA and possibly other compounds by autoradiography.

Growth of a hypha is largely a matter of elongation, differentiation, and branching of single filamentous cells and it has been established that elongation occurs only at the extreme tips of the hyphae (Hawker, 1965). The rate of elongation, i.e. the increase in hyphal length, is dependent on food supply and the accumulation of growth accelerating and growth retarding substances. Banbury (1952) was able to show that elongation of the sporangiophores of Phycomyces could be induced by griseofulvin but not by indolylacetic acid.

Although the rate and form of branching is under genetic control environmental factors have been shown to have a marked effect on the intensity of branching. Thus Hawker, Harrison, Nicholls and Ham (1957) found that increasing the concentration of glucose or thiamine increased the branching of Pythium ultimum. However, although greater numbers of branches are formed per unit length they may not contribute greatly to the growth rate as measured in hyphal length since they may not elongate to any great extent. In this case where the main hypha has greatest vigour and so exhibits apical dominance over the lateral branches, colony formation would be impossible if these conditions were maintained (Robertson, 1965). At this point it is interesting to consider the growth of sporelings of P. infestans on the basal medium since the elongation of the lateral branches would appear to be influenced by the apical dominance of the main hypha, so preventing the formation of colonies. The branches produced by the sporelings growing on the pectin medium on the other hand grow freely.

The method by which apical dominance is maintained is not known but a few methods have been suggested. Butler (1961) showed that the primary branch hyphae are narrower than the main hyphae in Coprinus disseminatus and that there is a significant correlation between rate of elongation and hyphal diameter. This, she postulated, could be due to the competition for nutrients between the apex and side branches since the food material was transported from a food base some distance behind the advancing edge of the colony and so the hyphae with the greater cross-sectional area would receive the greatest proportion of nutrients.

However when main hyphae and lateral branches of equal cross-sectional areas were compared it was found that the rate of elongation was significantly greater in the former suggesting that the difference between main and branch hyphae is not simply one of hyphal diameter. Robertson (1965) suggests that apical dominance may be controlled by internal hormones for which there is no evidence except the analogy with the auxin-gibberellin system in higher plants. Robertson also suggests that the dominance could be controlled on the agar plate by the secretion of a toxin by the fungus. The only parts of the fungus which would not be growing in this toxin would be the main hyphal apices.

The initiation of antheridial branching in Achlya ambisexualis and A. bisexualis has been shown to be controlled by Hormone A which is secreted by the female plant, (Raper, 1952). More recent work has shown that this hormone acts by inducing a rise in the amount of cellulase in the hyphae (Thomas and Mullins, 1967). Vegetative branching induced by substrates such as casein hydrolysate, is also accompanied by a rise in cellulase. Thomas and Mullins conclude that wall softening may prove to be a fundamental morphogenetic process in a wide variety of plant materials. The induction of colonial growth in Neurospora by snail digestive juice appears to involve the creation of "weak spots" in the cell wall (de Terra and Tatum, 1963). Pectin does, however, appear to play a double role in that it not only brings about a greater branching intensity but also appears to act by causing the cessation of apical dominance of the sporelings of P. infestans.

Nutritional factors have been shown to influence the number of

nuclei within a cell. Thus Huebschman (1952) was able to vary the number of nuclei within the conidia of Neurospora crassa by altering the nutritional status of the medium. The nutritional factors responsible appeared to be, at least partly, due to dicarboxylic acids. He found that the nuclear to cystoplasmic ratio remained fairly constant as there was an increase in the volume of the conidia with greater numbers of nuclei. This does not appear to be the case in P. infestans where pectin brings about an increase in the number of nuclei per unit of hyphal length and so no change in the volume is evident.

Nutritional factors therefore have been shown to influence certain aspects of the growth of a number of fungi in vitro. These factors may bring about an alteration in the rate of elongation, the branching intensity, and the number of nuclei per cell. Since the ratio of the number of branches and the number of nuclei per unit length of hypha of the sporelings of P. infestans growing on the pectin medium are more or less constant from the time of germination and both of these ratios are greater than those on the basal medium it appears that the sporelings on the pectin medium are growing in a more suitable nutritional environment and that its effect is manifest at or soon after germination. Although the increase in branching intensity may be important it would appear that the ability of pectin to overcome the apical dominance of the main hyphae of the sporelings so allowing the elongation of the lateral branches is probably more important.

Axenic culture of P. infestans and its relationship to that of obligate parasites.

Many obligate parasites germinate and form branch initials which do not develop further. Dickinson (1949) reported that spores of Puccinia triticina germinated under conditions not conducive to the formation of appressoria and other infection structures, produced a germ tube with many unextended branch initials thus giving a "gnarled" appearance to the whole germ tube. Here again it may be suggested that apical dominance persisted.

Cell wall material is synthesised during germination by Puccinia graminis tritici and Uromyces phaseoli and this is accompanied by a marked decrease in the lipid content (Shu, Tanner and Ledingham, 1954). Staples, Syamananda, Kao and Block (1962) found however that the nucleic acid levels did not change during the germination of uredospores of Uromyces phaseoli, although this may have been due to the fact that they were not germinated in the right conditions. Dickinson (1949) was the first to show that the formation of infection structures in P. triticina, P. graminis and P. glumarum could be induced by the appropriate contact stimulus and that these processes were accompanied by nuclear division. Further work has shown that the nuclear division in the same species is induced by the same contact stimulus as that which induces the formation of infection structures since if this contact stimulus is absent then nuclear division does not occur (Maheshwari, Hildebrandt and Allen, 1967). These workers suggest that DNA synthesis

probably occurs during the development of the infection structures and is triggered by the same stimulus as induces them to develop. This net synthesis could be accomplished at the expense of the rich pool of metabolites in the spore.

Uredospores of the rusts that have been investigated are unique in that they germinate in the absence of a net gain in protein but the spores do not lack a protein synthetic apparatus nor do they lack the ability to synthesise a wide range of enzymes. The absence of net protein gain is supported cytologically as the protoplasm travels along in the apical region of the germ tube and there is no obvious increase in volume. Sporelings of P. infestans grown on the basal medium do not lack the ability to synthesise cytoplasmic and cell wall material but do appear to be unable to maintain DNA synthesis at the normal rate. The growth of sporelings of P. infestans on pectin and basal media is compared with that of obligate parasites, exemplified by the rusts in table 40 below.

Table 40. Comparison of the physiological processes of rust spore germination with those of sporeling growth of P. infestans on pectin and basal media

Physiological process	Rusts	P. infestans	
		Basal Medium	Pectin Medium
Cell wall synthesis	+	+	+
Protein synthesis	-	+	+
Nucleic acid synthesis	+	-	+
Extension of lateral branches (Absence of apical dominance)	-	-	+

Thus it is with the last point that the growth of isolated sporelings of P. infestans shows the greatest similarity to that of the obligate parasites. Further work into the mechanisms controlling the extension of lateral hyphal branches may provide some insight into not only the establishment of host parasite relations in P. infestans and possibly obligate parasites but also the biological phenomenom of differentiation.

VI. GENERAL CONCLUSIONS

The work reported in this thesis has been concerned with the identification of the active factor in apple pectin and the investigation of the physiological processes involved in the post-sporeling growth of *P. infestans*.

The optimum concentration of apple pectin which induced colony formation from, on average, over 90% of the sporelings was 10g./litre. It is not however the pectin which induces post-sporeling growth but a contaminant which is chemically bonded or adsorbed onto the apple pectin molecule. The active factor can be released by acid treatment of the apple pectin and can be extracted from the hydrolysate in acetone, 80% ethanol, and water. The sporelings of *P. infestans* normally acquire it by the action of extracellular enzymes. These enzymes are only required on the initiation of post-sporeling growth.

Many compounds have been reported to be present in commercial preparations of apple pectin but none were found to induce post-sporeling growth. Other compounds, which may be present in apple pectin since they are found in apple fruits from which the pectin was extracted, were tested and all were found to lack the ability to initiate post-sporeling growth except the amino acid, cystine which induced significantly fewer colonies to develop than the apple pectin. The active factor in apple pectin is a contaminant but it is not one of the com-

pounds which have been detected by chemical analysis and so is probably a compound which is present in very small quantities in certain preparations only.

Observations were made of sporelings growing on the pectin and on the basal media as a means of comparing those sporelings which continued growth with those that did not. Morphologically the main differences between the sporelings growing on the pectin medium and those growing on the basal medium are that in the former the branching intensity is significantly greater and that each branch initial represents a site of active growth whereas those on the latter do not. As a result, growth, as measured as total hyphal length, passes into the exponential phase after an initial lag phase on the pectin medium.

Physiologically the initial rate of DNA synthesis is maintained on the pectin medium and decreases markedly on the basal medium without an equivalent decrease in the synthesis of protein and cell wall material. All three processes do however continue at a much reduced rate on the basal medium. Pectin acts by supplying a nutritional environment which enables the initial rate of DNA synthesis to be maintained. The synthesis of DNA is not only related to branching intensity but also to the cessation of the apical dominance of the main hypha thus allowing colony formation to proceed.

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