THE INFECTION OF ORANGES BY *PENICILLIUM DIGITATUM* SACCARDO

by

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ABSTRACT

The thesis is divided into two parts. The first is an examination of the extracellular enzymes produced by Penicillium digitatum in vitro and in vivo. Culture filtrates of the fungus grown on a variety of media, sound and rotted orange fruit tissues were examined for a variety of enzymes. Special attention was paid to pectic enzymes and to their possible role in tissue maceration. Macerating activity could not be identified with pectic enzyme activity. Maceration was successfully separated from α-L-arabinofuranosidase activity, by Sephadex gel filtration.

Very little pectic enzyme activity was found in rotted tissue extracts or in juice from infected fruit. A thermolabile inhibitor of the "chain splitting" and macerating enzymes was demonstrated. Paper chromatography was used to examine the breakdown products of polysaccharides from sound and rotted fruit, an albedo preparation, a protopectin preparation and potato cell wall material. Degradation products of pectic and non-pectic polysaccharides were detected. Pectolytic activity was shown in conidial extracts.

The fungus grew sparingly on hemicelluloses. Hemicelluloses, incubated with culture fluids were not degraded. Xylanase production was shown in filtrates

from cultures grown in a medium containing Esparto hemicellulose.

The second part of this thesis describes attempts to control green mould with an antimycotic - Pimaricin, and with several other chemicals. Thiabendazole was the most promising material tested.

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PART II

INTRODUCTION

This investigation attempts to elucidate some of the problems connected with the infection of Citrus fruit by Penicillium digitatum Saccardo.

This fungus causes a serious post harvest disease of Citrus fruit called 'green-mould' throughout the world. It is the major cause of Citrus wastage and responsible for large losses of fruit during transportation and storage.

Green mould has been the subject of many publications, but the more fundamental aspects on the mechanisms of infection and especially the physiology of parasitism have largely been neglected. The major part of this investigation is concerned with the physiology of infection, in particular, the role of pectic enzymes in this process. P. digitatum causes a soft rot of citrus. A characteristic of soft-rotting organisms is their ability to macerate plant tissue and one of the aims of this work was an attempt to 'isolate' a 'macerating factor'. The nature of maceration has caused considerable controversy during the past 80 years.

The second part of this investigation concerns the control of green mould. At present, diphenyl is widely used to protect citrus fruit during transportation and storage. Its use, however, has certain disadvantages and so there has been a constant search for new and better materials to prevent wastage. An antimycotic and several other chemicals have been tested for their ability to control P. digitatum on oranges.

P A R T I

REVIEW OF LITERATURE

The Fungus

The cultural characteristics of P. digitatum have been desdribed by Thom (1910), and by Raper and Thom (1949).

The Disease Symptoms

The disease symptoms caused by P. digitatum were fully described by Fawcett (1926) and have been described and compared with P. italicum, which causes blue mould of citrus fruit, in many publications subsequently.

P. digitatum is a wound parasite. Green mould first appears as a small water soaked soft area which enlarges rapidly and on which a white mycelium appears later. The development of olive green, powdery spore masses, from which the name 'green-mould' is derived begins at the focus of infection. As the lesion enlarges, a watery band encircles a white area of vegetative hyphae which in turn encircles a green central

area of sporulating hyphae. Green mould rapidly spreads through the whole fruit, usually in about six days under experimental conditions at 25°C. When kept under dry conditions, the mouldy fruit may shrink to a wrinkled dry mummy. In high humidity the fruit becomes soft and 'wet'. At this stage, other fungi and bacteria usually colonise and the fruit becomes a soft, decomposing mass.

Infection

The first studies on penetration and the mechanism of infection were by Klotz (1930), and Green (1932). These and subsequent works on spore germination, growth and infection have been reviewed by Kavanagh (1965). In a study of infection, Kavanagh found that spores suspended in water, different rind extract media, or citric acid solutions did not infect uninjured fruit. Wounds were necessary for infection, but those between the vesicles of the flavedo (the outer, compact-celled, orange-coloured layer of the fruit in which is embedded the oil vesicles or glands) were resistant to infection whereas other wounds were not. The infection of fruit through wounds into the rind tissues depended on spore

load, depth and position of wounds, number of wounds, the method of inoculation, and wound healing period. He found that in inter-vesicle wounds hyphae did not penetrate the flavedo before penetration of the albedo (the white, spongy layer). Hyphae in wounds into the oil vesicles passed down the thin walled cells of the vesicles and into the albedo before lesions were formed. In germination studies, Kavanagh showed that emanations from rind infected with P. digitatum stimulated germination of spores in water. Wounding between and into oil vesicles stimulated spore germination to the same extent.

Physiology of Parasitism

The infection of higher plant tissues by many fungi and bacteria is associated with a breakdown of the host cell walls, separation of the individual cells and a disorganization of the tissue. This phenomenon is termed maceration and is generally followed by death of the cells.

De Bary (1886) was the first to demonstrate the presence of a thermolabile substance, produced by Sclerotinia libertiana that brought about tissue

disorganization and cell death.

Early workers were divided in their opinions about the nature of the principles involved. Brown, in his now classic series of papers dealing with the physiology of parasitism of Botrytis cinerea, postulated the dependence of lethal activity on macerating activity in one of the following ways:-

(1) that both actions are due to the same substance or group of substances;

(2) that the two actions are due to different substances, but the lethal substance is unable to reach the protoplast until the permeability of the cell wall has been sufficiently increased by the action of the macerating substance.

As to the nature of the macerating substance, Brown agreed with former workers that it was enzymatic and in his first publication (1915) called it "cytase", a name which had been used by Brown and Morris (1890) for the enzyme responsible for breaking down the endosperm walls of germinating barley grains. From the supposed action of the macerating enzyme on the

pectic constituents of the cell wall, the name "pectinase" soon became used. It was replaced later by "protopectinase". This early work was summarized in two reviews by Brown in 1934 and 1936.

It is now widely believed that pectic enzymes are implicated in infection of plants by fungi and bacteria and in breakdown of plant tissue (Wood, 1955; Brown, 1965; Bateman and Millar, 1966). Many plant pathogenic fungi and bacteria have been found to produce pectic enzymes. The ability to produce these enzymes may be an important part of their pathogenic capabilities. However, it may only be one of a number of properties which are involved in pathogenesis.

In dealing with the action of pectic enzymes in tissue degradation, it is necessary to consider the nature of pectic substances. Their chemistry has been reviewed by Kertesz (1951), Deuel, Solms and Altermatt (1953), McCready and Owens (1954), Deuel and Stutz (1958). The terminology at present in use is as follows:-

Pectic substances are complex colloidal carbohydrate derivatives from plants. They are composed

mainly of anhydrogalacturonic acid residues thought to exist in chain like combination, linked through positions 1 and 4 of the pyranose ring to give a polygalacturonic acid. They are heterogeneous in that the carboxyl groups may be esterified to different degrees with methanol (pectinic acid and pectin), or be free (pectic acid). The carboxyl groups may also be neutralized with cations. In some, the hydroxyl groups are partially acetylated.

The anhydrogalacturonic acid content of pectic substances ranges from 75 to more than 90 per cent; the non-uronide fraction usually contains arabinose, galactose, rhamnose, or xylose.

Pectic materials constitute some of the more abundant polysaccharides in the cell walls of higher plants and are regarded as the principle constituents of the middle lamellae in plant tissues.

Pectinic acids are colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups.

Pectins. This term refers to water soluble pectinic acids of varying methyl ester content (usually

high) which can form gels with sugar and acid under suitable conditions.

Pectic acids are pectic substances essentially free from methyl ester groups.

Protopectin has been regarded as the water insoluble parent substance or substances which occur in plants and which upon restricted hydrolysis yield pectinic acids. Its nature is still largely unknown. In a recent review, Joslyn (1962) concludes "that it is possible that pectic substances in different plants and in different parts of the same plant may be chemically bound or physically anchored in different ways. On present evidence it appears that the most likely explanation of the relative insolubility of protopectin is that it exists as a polygalacturonide in which the hydroxyl groups on carbon atoms C4 and C5 are masked by glycosidic and ring formation and the carboxyl group on C1 is either free, esterified with methyl alcohol, or esterified with araban, galactan or other polysaccharide. The hydroxyl on carbon atoms C2 and C3 may be free, esterified with acetyl groups, or linked by ether-like linkage to

polysaccharides or lignins. It is likely that non-uronide sugars occur in the main chain itself as well as in branched chains."

The side chains may be formed by an occasional ester linkage between the terminal functional reducing group of a polysaccharide chain and the free hydroxyl of a polyuronide or by ester linkages between hydroxyls of polysaccharide and polygalacturonide.

Pectic Enzymes

There has been considerable confusion in nomenclature and classification in the history of pectic enzymes and they have been the subject of major reviews by Ehrlich (1936), Kertesz (1951), Demain and Phaff (1957), Deuel and Stutz (1958), and Bateman and Millar (1966). Up to the present, two main groups of pectic enzymes can be designated.

1. Pectin methyl esterases

Enzymes of this group catalyse the hydrolysis of the methyl ester groups in pectinic acids to yield methanol and pectinic acids of lower methoxyl content or pectic acid.

2. Pectic glycosidases and lyases

These enzymes hydrolyse the 1,4-glycosidic linkages

between adjacent anhydrogalacturonic acid residues by either a hydrolytic or a trans-eliminative mechanism, and on this basis can be divided into two groups, hydrolyases and lyases (Bateman and Millar, 1966). Within each subgroup there is a variety of enzymes exhibiting different properties with regard to substrate specificity, pH optima and co-factor requirements. These enzymes have been referred to in the past by many names including pectinase, polygalacturonase, depolymerase, endopolygalacturonase, exopolygalacturonase, depolymeric polygalacturonase, liquefying polygalacturonase, pectin transeliminase, pectate transeliminase, pectin lyase.

Based on the classification of Demain and Phaff (1957), Bateman and Millar have presented a revised classification using as major criteria the mechanism by which the α-1,4 glycosidic bond is split, substrate preference and the position in the pectic chain at which cleavage occurs.

The enzyme about which least is known and which is of greatest interest in tissue degradation is protopectinase, the enzyme which attacks protopectin and causes maceration. In this investigation, the term 'macerating factor' will be used instead of protopectinase. There is evidence that macerating activity is not caused by one enzyme only. Demain and Phaff equated maceration

with either PG or PMG, whereas Byrde and Fielding (1965) identified arabinase as a macerating factor. Phytolysin, a macerating factor distinct from pectic enzymes, has been described by Naef-Roth et al. (1961). Bateman and Millar (1966) consider endo pectic glycosidases and lyases as important in tissue maceration. In soft rot of potato caused by Erwinia aroideae, Dean and Wood (1967) suggest pectate trans-eliminase to be the cause of maceration.

Cellulases

Less is known about the cellulolytic enzymes produced by plant parasites, but they are believed to play a role in pathogenesis, especially in the development of wilt diseases (Wood, 1960; Mandels and Reese, 1965). Recent reviews dealing with cellulases generally include those of Siu (1951), Siu and Reese (1953), Reese (1956), Gascoigne and Gascoigne (1960).

In soft rots it is usually assumed that the enzymatic attack is on the middle lamella so that the cells separate, although the cell wall may remain intact. McClendon (1964) indicated that the middle lamella of the tissues concerned are mainly composed of mixtures of pectin and pectate most probably as components of the same chains. It is known that culture filtrates which macerate plant

tissue need not have any cellulase activity (Cole, 1956) and conversely filtrates may have a high cellulase but a low or no macerating activity (Reese and Levinson, 1952). It seems unlikely, therefore, that cellulases play a major role in the critical stages of tissue maceration. At later stages in soft rot diseases, however, some pathogens are actively cellulolytic (Wood, 1960).

Hemicellulases

Besides pectic substances and celluloses cell walls also contain xylans, arabans, galactans or other homoglycans and heteroglycans. Very little work has been done on these polymers and the role in maceration of the enzymes hydrolysing them. Arabans and galactans may prove to be an integral part of protopectin structure (Barrett and Northcote, 1965). Arabanases have been reported from many phytopathogenic fungi (Fuchs et al, 1965; Byrde and Fielding, 1965). Xylanase production has also been described for many fungi (Gascoigne and Gascoigne, 1960a; Strobel, 1962; Hancock and Millar, 1965).

The wide distribution of hemicellulases amongst phytopathogenic fungi, together with the possibility that arabinase or galactanase may macerate (McClendon, 1964) suggests their importance in the physiology of infection.

Proteolytic Enzymes

Northcote (1963) reported that, in addition to polysaccharides, the cell wall also contains protein. Other workers have also suggested that protein is a common constituent of the cell wall region (Ginzburg, 1961; Wallace et al.(1962). Kuć (1962) observed that proteolytic enzyme preparations increased the rates of tissue maceration by pectolytic enzymes. Proteolytic enzyme production has been described in several phytopathogenic fungi, (Kuć and Williams, 1962; Hancock and Millar, 1965) and a role for them in tissue degradation must, therefore, be considered.

Enzyme studies on Penicillium digitatum

Green (1932) first suggested that enzymes may be secreted by the fungus in rotting oranges, but she was unable to demonstrate the presence of such enzymes in extracts of rotted fruit. In 1962, Miyakawa showed that P. digitatum secreted pectin methylesterase and polygalacturonase in an asparagine-pectin or orange peel extract medium. He also showed polygalacturonase activity in infected Satsuma orange peel tissues and found its pH optimum to be between 3.6 and 5.5. Macerating activity was demonstrated by both P. digitatum

and P. italicum, but they were much lower than those of less pathogenic fungi, P. expansum and P. purpurogenum. He showed both P. digitatum and P. italicum not only decomposed pectin, but also utilized the decomposition products, di-, tri- and d-galacturonic acid, as carbon sources in media containing different nitrogen sources.

Miyakawa (1963) demonstrated more than 2 per cent of free d-galacturonic acid detectable in tissues at an early stage of infection. Since orange peel tissues were macerated in vitro with culture filtrates of P. digitatum and also with d-galacturonic acid, he suggested that d-galacturonic acid produced in the affected tissue might macerate the orange peel in association with the pectic enzymes produced, and destroy resistance to infection in the rind tissues. Garber et al. (1965) could not detect pectolytic or cellulolytic activity in extracts from healthy orange. Extracts from tissue rotted with P. digitatum and P. italicum showed both pectolytic and cellulolytic activity. They proposed that these were induced in each species of Penicillium. Using virulent and avirulent strains, they suggested that the rots of orange tissue caused by P. digitatum and P. italicum may be related to the

production of pectolytic rather than cellulolytic enzymes at the site of inoculation, since extracts from rotted tissue contained both pectolytic and cellulolytic activity whereas extracts from necrotic tissue at site of inoculation with avirulent mutants had only cellulolytic activity.

Kavanagh (1965) found active pectin methylesterase in culture filtrates of P. digitatum in vitro, but only low polygalacturonase cellulase and macerating enzyme activity. In vivo, pectin methylesterase was found to be active, macerating enzyme not very active, polygalacturonase almost inactive, and cellulase absent.

The production of pectolytic and cellulolytic enzymes by P. digitatum in vitro has thus been shown by several workers. With the exception of PME, there has been no convincing demonstration of their production in vivo.

A macerating factor has not been identified in vitro or in vivo.

MATERIALS AND METHODS

FUNGUS

An isolate of Penicillium digitatum obtained from a green-moulded Navel orange (Imperial College Culture Collection No. R.13) was used. A monospore culture was established on malt agar and its pathogenicity confirmed. Stock cultures were grown on malt agar, and on V8 vegetable juice agar in McCartney bottles, and stored at -20°C. and under sterile liquid paraffin at laboratory temperature.

Subcultures of the fungus were grown on malt agar in conical flasks at 24-26°C in the light.

FRUIT

Experimental fruit were of the Navel and Valencia varieties. They are discussed further in Part II (p. 190)

INOCULATION EXPERIMENTS

Techniques used in the preparation of spore suspensions, preparation of fruit, wounding, inoculation and incubation are described in Part II (p. 195).

CHEMICAL MATERIALS

General Chemicals

All chemicals used were of analar grade, unless

otherwise stated, and supplied by either the British Drug Houses Ltd., or Hopkins and Williams Ltd.

Sugars

Sugars were obtained from B.D.H. Ltd., Poole, Dorset, and Koch-Light Laboratories Ltd., Colnbrook.

α-D-galacturonic acid was obtained from Koch-Light Laboratories Ltd.

Cas-amino acids

Vitamin free cas-amino acids were obtained from Difco Laboratories, Detroit, U.S.A.

Yeast-Extract

Bacto-yeast extract was also supplied by Difco Laboratories.

Substrates

Pectic Substances

Pectin N.F. (P), sodium polypectate (NaPP) and polygalacturonic acid (PGA) were obtained from the Sunkist Growers Inc., California, U.S.A., and also kindly supplied by S. and S. Services Ltd., London, S.W.1.

The pectin was a high methoxyl pectin. NaPP is the sodium ammonium salt of polygalacturonic acid. It contains 1.3 per cent ammonium, 4.2 per cent sodium (Cole, 1953).

Before use, large samples were stirred into 60 per cent

(v/v) ethanol made 0.1N with HCl on a magnetic stirrer. This was followed by thorough washing on a Büchner funnel with 95 per cent (v/v) ethanol. The washed pectin and NaPP was then spread to dry at laboratory temperature and stored in screw-cap glass bottles at laboratory temperature.

Solutions of 1 per cent (w/v) concentrations were prepared by the slow addition of the powders to constantly stirred water. The pH of the solutions were adjusted to the required value by addition of N NaOH or N HCl. Whenever possible, freshly prepared solutions were used, but occasionally they were stored for a few days at 4°C. with a few drops of toluene added.

Cellulose

Soluble, high viscosity carboxymethyl cellulose (CMC) was obtained from the Hercules Powder Company, Delaware, U.S.A. It was of high chain length with a degree of substitution of 0.4. 1 per cent (w/v) solutions were prepared as for pectin and NaPP.

Hemicelluloses

Esparto hemicellulose, Jute hemicellulose (fraction B), Larch galactan, Spruce glucomannan, and Spruce xylan were kindly supplied by the Forest Products Research Laboratory, Princes Risborough, Buckinghamshire.

Phenyl α-L-arabinofuranoside was kindly supplied by Drs. R.J.W. Byrde and A.H. Fielding, Long Ashton Research Station, Bristol. This was used as the substrate for arabinosidase determinations.

o-Nitrophenyl-β-D galactropyranoside was obtained from Calbiochem Ltd., Lucerne, Switzerland. This was used as substrate for β-galactosidase determinations.

Sodium caseinate (Nutrose) was obtained from Difco Laboratories.

Potato cell wall material was kindly supplied by Mrs. C. Dean, Botany Department, Imperial College.

GROWTH MEDIA

Agar Media

a) Malt Extract Agar

30 g malt extract (Oxoid L.39)

15 g (Davis) agar

1 l water

b) V8 juice agar

200 ml V8 juice (obtained from Campbell's Soups Ltd., King's Lynn, Norfolk)

3 g $CaCO_3$

800 ml water.

All agar media used were sterilized by autoclaving for 15 minutes at 15 lbs. p.s.i. pressure. The pH was not adjusted.

Liquid Media

P. digitatum was cultured in different liquid media for studies on mycelial growth and enzyme production.

1) Medium A

This medium, having glucose as the carbon source, has proved successful in enzymatic studies for several rot causing fungi (Fuchs, 1965) and is as follows:-

10 g	KNO_3
5 g	KH_2PO_4
2.5 g	$MgSO_4.7H_2O$
1 g	Difco yeast extract
20 g	glucose
1 l	water

(pH of autoclaved medium = 4.5)

2) Medium B

This medium was a modification of medium A, with 1 g of pectin or NaPP in place of glucose.

(pH of autoclaved Pectin medium = 4.5, and NaPP medium = 4.6)

3) Medium C

(A modification of the medium described by Blackhurst, (1961)). The constituents were as follows:-

4.6 g	cas-amino acids
1.0 g	KH_2PO_4
0.5 g	glucose
0.5 g	$MgSO_4.7H_2O$
10 g	Pectin or NaPP
10 ml	trace element solution
	Distilled water to a litre.

4) Minor element solution (Klemmer and Lenney, 1965)

The stock solution contained:-

0.02 g	$FeSO_4.7H_2O$
0.1 g	$ZnSO_4.7H_2O$
0.002 g	$CuSO_4.5H_2O$
0.002 g	Na Mo $O_4.2H_2O$
0.002 g	$MnCl_2.4H_2O$
1 l	Water

Stock solution was added to give the following final concentration of microelements in the medium:-

Fe = 0.2 ppm, Zn = 1.0 ppm, Cu = 0.02 ppm, NaMo = 0.02 ppm, Mn = 0.02 ppm.

5) Orange albedo medium

100 g of albedo tissues were cut from Navel oranges

and homogenised in 500 ml distilled water in a 'Kenwood' homogeniser. 1 l of distilled water was added to the homogenate, which was then stirred on a magnetic stirrer for 20 mins, and left at 8°C for 1 hour. The tissue, after filtering through four layers of muslin, was washed thoroughly with several litres of distilled water. This washed albedo was resuspended in 1 litre of distilled water; 50 ml were dispensed into medical flats and autoclaved. (pH after autoclaving = 4.6).

CULTURAL METHODS

Culture vessels

50 ml of medium B, and washed albedo medium, were put into 10 oz medical flats. 80 ml of medium C and complete rind extract medium were put into 10 oz medical flats, or into 500 ml Pyrex conical flasks, fitted with cotton wool plugs.

Sterilization

All liquid media were sterilized by autoclaving at 15 p.s.i. for 20 minutes.

Inoculation and Incubation

Each culture vessel was inoculated with 1 ml of a dense spore suspension (approx. 12 million spores/ml). The fungus was either grown in stationary culture or shake

culture on a rotary shaker at 200 r.p.m., (5 cm throw) at 25°C in the light (fluorescent).

Collection of Culture Filtrates and Dry Weight Determinations

Culture filtrates were collected by filtration through Whatman No. 541 filter paper or several layers of muslin. The mycelium was then transferred to weighed, aluminium foil cups, which were dried at 70°C., cooled over $CaCl_2$ in a desiccator and weighed.

Culture filtrates for enzyme work were centrifuged at 26.000 g to make cell free and stored in McCartney bottles or medical flats at - 20°C. until required.

The albedo and mycelium residue of the albedo medium were filtered through four layers of muslin and then stirred with a magnetic stirrer in 0.2M NaCl (20 ml/medical flat) for 30 mins., at pH 7.5. The NaCl extract was then refiltered, centrifuged, and the filtrate stored as above.

PREPARATION OF EXTRACTS OF HEALTHY AND INFECTED FRUIT

1. Orange rind extract

Navel oranges were X wounded (see Part II, p. 196) and inoculated with a dense spore suspension of Penicillium digitatum. After three days incubation, the "white mycelium" stage had been reached. The infected rind

tissue including the water soaked zone was cut from the oranges with a scalpel. 75 g (wet wt.) were homogenised for a few minutes in a 'Kenwood' homogeniser, with a little 0.2M NaCl at pH 7.5. The homogenate was divided into several portions which were homogenised at 5,000 revs/sec. in a Sorvall Omnimix for 30 seconds. The macerate was made up to 300 ml with 0.2M NaCl and stirred on a magnetic stirrer for 30 minutes, the pH being maintained at 7.5. The homogenate was centrifuged at 2,000 g for 15 mins., and filtered through Whatman 541 filter paper to remove essential oils. Healthy extract was prepared in exactly the same way.

2. Orange juice extract

Navel oranges were X wounded and inoculated with a spore suspension. After appropriate time intervals, the juice from infected oranges and healthy controls was extracted with a 'Kenwood' juice extractor. The juice was then centrifuged for 15 mins. at 2,000 g and stored in McCartney bottles at -20°C. On removal from the deep freeze, the juice was centrifuged (26,000 g) and filtered through Whatman No. 1 filter paper to remove residual essential oils.

3. Preparation of washed albedo tissue

The albedo, from Spanish, Navel oranges, was cut with

a scalpel into distilled water. It was then homogenised in the 'Kenwood' homogeniser and resuspended in distilled water. The albedo "slurry" was stirred for 2 hours on a magnetic stirrer and then filtered through six layers of muslin. The tissue was then repeatedly washed with water until no sugar was detectable in the filtrate (by reducing group estimation, see p. 42). It was finally squeezed in the muslin, and placed in an oven overnight at 80°C. This washed albedo tissue was finally crushed in a large mortar, powdered in a Moulinex coffee grinder, and stored in screw cap bottles at laboratory temperature.

4. Preparation of orange protopectin (after Kertesz, 1951)

Albedo tissue of Spanish Navel oranges was separated from the flavedo with a scalpel. 80 g albedo were placed in 1 litre boiling 95 per cent ethanol and then cooled. It was then stirred for six hours on a magnetic stirrer, strained through four layers of muslin and squeezed dry. The albedo was resuspended in 95 per cent ethanol and left standing for three days, after which it was strained through muslin and squeezed dry, and rubbed in a large mortar until all the ethanol evaporated. These treatments removed most of the sugars. The dry

tissue was then homogenised with 95 per cent ethanol in a 'Kenwood' homogeniser, strained through muslin and rubbed dry. The ground tissues were extracted three times with three litres of cold water for one hour with each change of water. No pectin could be detected in the washings by the hydroxylamine-ferric chloride test for pectin (Gee et al., 1959).

The tissue was finally stirred in cold water for a further hour, filtered through Whatman No. 1 filter paper, resuspended in 95% ethanol, and extracted until no further solids were removed. The tissues were then filtered, rubbed dry in a mortar and the residue extracted with several changes of ether. It was finally rubbed dry in a large mortar, ground in a Moulinex coffee grinder and stored at laboratory temperature in a stoppered bottle.

pH MEASUREMENT

A Beckman 'zeromatic' pH meter was used for all pH determinations.

BUFFERS

The following buffers systems were used during the course of this investigation:-

1. Citric acid - sodium citrate (citrate)

2. Na_2HPO_4 - citric acid (McIlvaine)
3. Sodium acetate - acetic acid (acetate)
4. Na_2HPO_4 - NaH_2PO_4 (phosphate)
5. 'Tris - HCl' ('tris')
6. Glycine - HCl (glycine)

These were prepared as described by Dawson et al. (1959).

DIALYSIS

Visking tubing of $^{8}/_{32}$" and $^{24}/_{32}$" diameter, obtained from H.M.C. England, was used for dialysing culture filtrates. Dialysis was against glass distilled water, unless otherwise stated, and was always carried out in a refrigerator (4°C.).

FREEZE-DRYING

4,425 ml of a cell free culture filtrate from a four day old cas-amino pectin (medium C) culture was freeze-dried in an Edwards Vacuum freeze-drying unit (Model 30 P.I.T.). 24.1 g freeze-dried material were collected. It was stored in a polythene bag, contained in an air tight glass container, over $CaCl_2$ in a desiccator at 4°C.

The freeze-drying was kindly carried out by Twyford Laboratories Ltd., London.

Freeze-dried material was usually used at a

concentration of 0.01 g/ml distilled water (1.8 times the concentration of the original culture). After dissolving, the filtrate was centrifuged before use to remove any residual solid material.

EXTRACTION OF CONIDIAL ENZYMES

P. digitatum spores from 12 previously wounded and inoculated Spanish, Navel, oranges were collected by gentle brushing with a small, clean brush. The spores were suspended in water and centrifuged. 1 g wet weight of spores were resuspended in 10 ml sterile distilled water and put in a Mickel tube with No. 11 size ballitini. The spores were shaken with the ballitini on a Mickel shaker for six minutes. The tube and contents were kept cool by surrounding it with solid CO_2. A very high percentage of the spores were broken by this treatment. The resultant liquid was centrifuged at 26,000 g for 15 minutes and the supernatant placed in a McCartney bottle and placed overnight under toluene at $4^{o}C$. Enzyme determinations were made with the supernatant the following day.

The use of an ultrasonic probe in an attempt to break open the spores was unsuccessful. Only about 20% of the spores were broken after 25 minutes ultrasonic

disintegration, using an M.S.E. 100 watt ultrasonic disintegrator with a 3/8" titanium vibrator probe.

ESTIMATION OF ENZYME ACTIVITIES

1. PECTIN METHYLESTERASE (PME)

Estimation of liberated carboxyl groups

PME activity of the enzyme preparations was estimated by titrating the free carboxyl groups liberated during the enzymic deesterification of high methoxyl pectin against 0.01 N NaOH (Kertesz, 1951, p.362).

All reactions were performed at laboratory temperature in Pyrex glassware.

The reaction mixture consisted of 5 ml, 1% pectin, made 0.1 N with respect to NaCl and adjusted to the required pH, 4 ml water and 1 ml of culture filtrate, or multiples thereof.

The reaction mixture was continually stirred during the course of the experiments by means of a magnetic stirrer. At time 0, the culture filtrate was added and, if necessary, the pH adjusted to the required level. As the enzyme acts, the pH of the reaction mixture falls, and was constantly adjusted to the original value by dropwise addition of 0.01 N NaOH.

The quantities of NaOH added after known time

intervals were recorded. Reaction periods were normally of 15-30 minutes. Autoclaved culture filtrates were used as controls. The difference between the volumes of NaOH required to neutralize the carboxyl groups in the control and the reaction mixture is equivalent to the weight of methyl group removed.

Equivalent weight of methoxyl groups (CH_3O) = 31.

1 ml 0.01 N NaOH = 0.31 mg methoxyl group.

PME activity was expressed as the rate hydrolysis of methyl ester groups in the polymer.

$$\% \text{ hydrolysis} = \frac{\text{mg methoxyl group liberated in time t x 100}}{\text{mg methoxyl group originally present}}$$

The methoxyl group content of the pectin substrate was determined by the method of Hinton (1940) and was calculated to be 8.3%.

2. PECTIC GLYCOSIDASES AND LYASES

Pectic chain splitting enzymes were measured by:

a) Estimation of viscosity reducing activity (V.R.)

This method depends on the reduction in viscosity of solutions of pectic substrates through the activity of enzymes. Viscosity reducing activity of culture filtrates and orange extracts were measured in Cannon-Fenske size 200 viscometers. The viscometers were suspended in a water bath maintained at 25°C. The flow times for each

viscometer were measured with 10 ml water.

Reaction mixtures consisted of 5 ml of substrate (1% (w/v) P or NaPP solution adjusted to required pH with N NaOH) 2 ml buffer (normally 0.1 M citrate buffer) 2 ml distilled water and 1 ml enzyme preparation. The enzyme preparation was added to the reaction mixture at time 0. The reaction mixture was mixed by bubbling, and the flow time measured at known intervals from time zero. Autoclaved enzyme preparations were used as controls. The pH of the reaction mixture at the end of the experiment was checked. Where the reduction in viscosity was being determined over 24 hours, 0.1 ml toluene was added to the reaction mixture (1.9 ml water).

To obtain the enzyme activity, curves were drawn of flow time against corrected reaction time and the time corresponding to 50%, 25% or 10% reduction in viscosity determined. The corrected reaction time was calculated as the reaction time plus half the corresponding flow time.

The points of 50%. 25% or 10% loss in viscosity were taken as being half, quarter and tenth way respectively between the flow time for water and the control time. Activity was expressed as 100/t where t was the time in minutes for 50%, 25% or 10% reduction in viscosity of the

reaction mixtures. Measured in this way, activity is directly proportional to enzyme concentration (see p.111).

b) Estimation of reducing group liberation (RG)

The reducing groups liberated by the action of 'chain splitting enzymes' on their substrates were estimated by the Somogyi method as adapted by Nelson (1944). All reagents used were prepared as described by Nelson.

The reactions were performed at laboratory temperatures in Pyrex glassware. Reaction mixtures were the same as for the viscosity reducing estimations. To 1 ml (or less) samples of the reaction mixtures taken after the required time intervals, were added 1 ml copper reagent in test tubes (or volumes in the same ratio). 1 ml samples of appropriate controls were treated in an identical manner. The solutions were mixed, and heated for 20 minutes in a boiling water bath. At the end of this time, the tubes were cooled in cold water. 1 ml of an arsenomolybdate colour reagent was then added and thoroughly mixed with a "whirlimixer". The tubes were allowed to stand until the reaction was complete, as observed by the stoppage of CO_2 gas evolution. The mixtures were then diluted as required and colour formation read on a Gallenkamp photoelectric colorimeter using an Ilford 608 red filter (670 mμ). Water was used as the colorimeter standard.

The colorimeter readings were calibrated with standard glucose solutions (5-500 µg/ml) at different final dilutions (x 10, x 20, x 40).

The activity was calculated in µg equivalents of anhydroGA and expressed as per cent hydrolysis of the 1:4 glycosidic linkages broken during the reaction time. α-D-galacturonic acid (anhydrous mol wt = 194. anhydroGA mol wt = 176) NaPP = 94.5% anhydroGA.

$$\% \text{ Hydrolysis} = \frac{\mu g \text{ anhydroGA liberated}}{\mu g \text{ anhydroGA originally present}} \times 100$$

c) Estimation of pectic lyases

In 1960, Albersheim, Neukom and Deuel reported on an enzyme present in a commercial pectic enzyme preparation that degraded the α-1:4 glycosidic bonds by a transelimination of the proton on the 5th carbon atom of an anhydromethyl galacturonate unit with the oxygen of the adjacent glycosidic bond. Cleavage of the bonds in pectin resulted in the formation of esters of galacturonides of undetermined size with unsaturated bonds between carbon atoms 4 and 5 at the non-reducing ends of the fragments formed. These double bonds strongly absorbed light at 235 mµ. The enzymatic process was thought to occur by a reaction mechanism similar to that of the degradation of pectin in neutral or in alkaline conditions.

Eliminative cleavage of the glycosidic bonds was estimated by following the increase in absorption at 235 mμ with pectin substrate and 230 mμ for NaPP substrate (Starr and Moran, 1962) in a Beckman DB recording spectrophotometer.

The reaction mixtures consisted of:

5 ml 1% pectin/NaPP/sodium polygalacturonate (pH adjusted)

2 ml buffer

2 ml water or $CaCl_2$ solution

1 ml enzyme preparation

The enzyme preparation was added to the reaction mixture in a Pyrex test tube, quickly mixed by means of a "whirlimixer" and a portion of the reaction mixture transferred to the spectrophotometer cuvette. Controls included autoclaved enzyme preparations.

Activity was expressed at μmoles of aldehyde groups released. Edstrom and Phaff (1963) stated that during the degradation of pectin by pectin transeliminase, under standard conditions, the release of 1 μmole of aldehyde groups in a 10 ml reaction mixture causes an increase in absorbance of 0.555 at 235 mμ. The unsaturated compounds formed as the result of transeliminative cleavage react with thiobarbituric acid to form a pink coloured product which absorbs at 547 mμ (Neukom, 1960).

Samples of the reaction mixtures were treated with thiobarbituric acid according to the method of Neukom. To 1 ml samples of the reaction mixtures, 5 ml of 0.5 M HCl and 10 ml 0.01 M thiobarbituric acid were added. The solutions were placed in a boiling water bath for 30 minutes, cooled and the absorption recorded. A red colouration, with an absorption maximum at 547 mμ, confirmed pectic lyase activity.

3. CELLULASE

Estimation of cellulase activity was made by measuring the reduction in viscosity of carboxymethyl cellulose solutions. The method is the same as used for the estimation of viscosity reduction of pectin and NaPP solutions, except the reaction mixtures contained a 1% w/v solution of CMC instead of P or NaPP.

Cellulase activity was expressed as:

$$\frac{100}{\text{time for 25\% or 10\% reduction in viscosity}}$$

4. MACERATING ACTIVITY

The macerating activity of culture filtrates and extracts of rind tissue was measured by essentially the same method as described by Brown (1915). Cylinders of tissue 3 cm in diam., were cut from the medulla of a potato tuber. Discs 0.35 mm thick were cut from the

cylinder with a hand microtome and placed in distilled water. The discs were then injected with water under vacuum in a Büchner flask, for 20 minutes, followed by thorough washing with several changes of distilled water. From each large disc, 3 or 4 smaller discs, 1 cm in diam. were cut with a No. 5 cork borer, keeping the discs immersed in water.

Surplus water was removed from 10 discs before they were transferred to test solutions. The test solutions consisted of 1 ml of buffer and 1 ml of enzyme preparation (or 2 ml enzyme preparation) contained in a watch glass. All tests were carried out at laboratory temperature (19-22°C.). Autoclaved enzyme preparation was used in the controls. The maceration time was taken as the mean time (in minutes) for the potato discs to lose coherence, when tested by gently pulling between forceps. Activity was expressed as

$$\frac{100}{\text{mean maceration time}}$$

A calibration curve relating macerating activity to enzyme concentration is given on page 106.

5. TOXICITY

This was estimated by a plasmolytic method described by Tribe (1955).

Potato discs, prepared as described above, were removed from test solutions to solutions of the following composition for 20 minutes:

Molar KNO_3	8.5 ml
0.1% neutral red chloride	1.0 ml
0.2 M phosphate buffer pH 7.5	0.5 ml

The plasmolysing agent prevented further toxic action. It was possible by this method to observe living protoplasts, stained red by the vital stain and thus the proportion of dead cells could be estimated. A visual comparison was made and the results recorded by a number, referred to by Tribe as the Neutral Red Index:-

5 whole disc covered with red spots

4

3

gradation

2

1

0.5 }
} occasional spots
0.1 }

0 no spots visible

6. <u>α-L-ARABINOFURANOSIDASE</u>

Arabinosidase hydrolyses phenyl α-L-arabinoside to phenol and arabinose. Activity was measured by the colorimetric estimation of the liberated phenol.

The procedure adopted was that devised by Byrde and Fielding (Personal Communication). All reactions were performed in Pyrex glassware at 25°C. Reaction mixtures which consisted of:-

1 ml phenyl α-L-arabinofuranoside (0.5 mg/ml)

0.5 ml citrate buffer (0.1 M)

0.2 ml test enzyme preparation

0.3 ml water

were pipetted into test tubes and incubated for 30 minutes. At the end of this time, 1 ml of saturated Na_2CO_3 was added and mixed thoroughly. 5 ml distilled were then added, followed by 0.4 Folin Ciolcoteau's reagent and made up to 10 ml with distilled water. The well shaken mixture was then allowed to stand for one hour at room temperature, during which time colour development occurred. The colour was read with a Gallenkamp colorimeter, using Ilford 621 red filter (670 mμ). Substrate and enzyme blanks were treated in an identical manner.

The colorimeter readings were calibrated with a standard series of a freshly prepared aqueous phenol solution (0.50 μg) in place of the reaction mixture. The activity was expressed as percentage hydrolysis of substrate and μmoles/hr/ml of enzyme.

7. β-D-GALACTOSIDASE

The assay for β-D-galactosidase is based on the hydrolysis of the synthetic substrate o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol and galactose.

The procedure used was identical to that for estimating arabinosidase activity, except that o-nitrophenyl β-D-galactopyranoside was substituted for α-L-arabinoside.

After 30 minutes incubation of the reaction mixture, 1 ml of saturated Na_2CO_3 solution was added and the mixture made up to 10 ml with distilled water. The o-nitrophenol released was estimated in a Beckman DB spectrophotometer at 420 mμ, the absorption maximum for o-nitrophenol (Lederberg, 1950). A freshly prepared o-nitrophenol standard series (0-100 μg/ml) was run simultaneously. Activity was expressed as percentage hydrolysis of substrate and μmoles/hr/ml of enzyme.

8. PROTEOLYTIC ENZYMES

Proteolytic enzyme activity was measured by the method of Kunitz (1947). Reaction mixtures contained 1 ml enzyme preparation and 1 ml 1% soluble casein (Nutrose) in 0.1 M phosphate buffer pH 7.5. The enzyme substrate mixtures were incubated at 37°C. Residual protein was precipitated at time zero and after 40 minutes with 3% trichloracetic acid (TCA). The preparations were left to

stand for at least an hour before centrifuging and the optical density at 280 mμ measured in a Beckman DB spectrophotometer against a blank of supernatant fluid from the reaction mixture precipitated with TCA at time zero.

PROTEIN MEASUREMENT

Protein was determined by the procedure of Lowry et al. (1951) using bovine serum albumen as a standard. Samples were read in a Beckman spectrophotometer at 750 mμ.

THERMAL INACTIVATION

To determine the thermal inactivation characteristics of the culture filtrate enzymes, the following technique was used.

Six ml samples of culture filtrate were heated in thin walled glass test tubes at temperatures from 20-100°C. The test tubes were supported in a constantly stirred water bath for 10 minutes before the filtrates were added to the bottom of the tubes with a pipette. A thermometer was placed inside the tubes and the time required for the filtrate to attain the desired temperature noted. The filtrate was kept at the desired temperature for 5 minutes, then the tube was plunged into ice water immediately.

The filtrates were stored at -20°C. in McCartney bottles until required. Enzyme activities of the heat treated filtrates were measured in the manner described previously.

CHROMATOGRAPHIC TECHNIQUE

A. Fractionation of culture filtrates by column chromatography

1. Gel Filtration

Sephadex gels were used in attempts to separate a 'macerating factor' from culture filtrates. They are modified dextrans used for the fractionation of high molecular weight substances (obtained from Pharmacia Fine Chemicals, Uppsala, Sweden). Sephadex "G.75" and "G.200" were used during the investigation. "G.75" has a fractionation range of M.W. 1,000-50,000 and "G.200" from M.W. 1,000-200,000.

The gels were prepared strictly according to the manufacturers' recommendation. Glass columns of various dimensions were used and the columns were packed as described by the manufacturers. Where the flow rate was very slow, it was increased by applying pressure of 5-10 lb. sq. in. to the top of the column with compressed air. Column eluates were collected by means of an LKB 3400 B Radirac constant volume fraction collector, in glass tubes.

Eluates were stored overnight under toluene at 4°C. or longer in the deep freeze at -20°C. until enzyme determinations could be made.

2. Ion Exchangers

In attempts to absorb culture fluid enzymes onto modified celluloses, the following ion exchange celluloses were used:-

Whatman "Exteola" Cellulose ET 11 - anion exchanger

Whatman Carboxymethyl Cellulose CM 11 - cation exchanger

Whatman Cellulose Phosphate P 11 - cation exchanger.

Adsorption of activity was measured by stirring dialysed culture fluid in a test tube with the modified celluloses, equilibrated after washing with an appropriate buffer system. 0.3 g of the modified celluloses were weighed into clean tubes, shaken vigorously with 10 ml distilled water and centrifuged. The celluloses (all except cellulose phosphate) were then washed with 0.4 N NaOH, centrifuged and then washed with distilled water at least six times until the pH of the supernatant was that of the distilled water. They were then washed twice with the appropriate buffer at the required pH. 2 ml of dialysed culture fluid were then added and the tube contents stirred over a magnetic stirrer with a small magnetic flea for 15 minutes. The tubes were then centrifuged and the

supernatants collected and assayed for protein, pectolytic and macerating activity.

In the case of CMC, an elution series was made with increasing buffer molarity. With the addition of each step in the system, the tube contents were stirred in the manner described for 15 minutes and the supernatants collected and assayed as previously described.

Chromatography on cellulose phosphate

A 1 cm diameter column was packed with a suspension of 2 g cellulose phosphate in 100 ml of 0.01 M citrate buffer. 0.5 g of freeze-dried culture filtrate was dissolved in 25 ml of distilled water and dialysed against 3 litres of distilled water at 5°C. for 18 hours. 20 ml of the resultant solution was applied to the column. The following gradient elution scheme was adopted.

20 ml 0.01 M citrate pH 4.0

30 ml 0.01 M NaCl in 0.01 M citrate pH 4.0

30 ml 0.03 M NaCl in 0.01 M citrate pH 4.0

30 ml 0.05 M NaCl in 0.01 M citrate pH 4.0

Air pressure of 5 kg/cm^2 was applied to the column. 5 ml fractions were collected by an LKB fraction collector and tested for protein, macerating and pectolytic activity.

B. Paper chromatography

Ascending and descending paper chromatography by

standard techniques was used with Whatman No. 1 chromatographic paper.

The following solvent systems were used:-

1) iso-propanol-water
2) ethyl acetate-pyridine-water

} ascending chromatography

3) butanol-ethanol-water
4) n-butanol-acetic acid-water
5) n-propanol-ethyl acetate-water

} descending chromatography

Location reagents used were:-

a) naphthoresorcinol
b) aniline diphenylamine

} ascending chromatography

c) silver nitrate reagent - for descending chromatography

The solvents and location reagents were made up and used as described by Smith (1960).

Most of the analyses of reaction mixtures and fruit extracts used the butanol-acetic acid solvent. Strips of chromatographic paper 24" long x 9" wide were cut from 24" square sheets. Drip serrations were cut at the lower end of the strips. Eight spots were applied to each strip, at one inch intervals. Unknown samples for analysis were applied to the paper from melting point tubes. Approximately 0.05 ml were applied. Standard sugar solutions or a sugar mixture at 0.5% concentration were run with the unknown samples as markers. About 0.02 ml

of the markers were applied to the paper.

The chromatograms were run at laboratory temperature. Irrigation time was normally 66-72 hours. However, sometimes this time was lengthened to 120 hours for the resolution of low Rf spots. Chromatograms were developed with the silver nitrate reagent.

Using this method, the solvent drips from the end of the papers and Rf values of the spots could not be determined. R_{GA} values were calculated instead,

$$R_{GA} = \frac{\text{distance sample has travelled from origin}}{\text{distance travelled by the galacturonic acid marker.}}$$

The use of the other solvents and reagents are referred to later.

EXPERIMENTAL RESULTS

1. GROWTH AND ENZYME PRODUCTION IN VITRO

A. CONSTITUTIVE ENZYME PRODUCTION

The fungus was grown in static culture on medium A containing glucose as the carbon source (Materials and Methods, p. 30).

1. Growth and production of pectolytic and cellulolytic enzymes

Growth as increase in mycelial dry weight and pectolytic and cellulolytic enzyme production estimated as described in Materials and Methods were assessed after 3, 7, 11, 13 and 17 days incubation (Table 1).

Pectin methylesterase was measured at pH 5.0 and activity is expressed as the percentage of methyl ester groups liberated in 30 minutes. The liberation of methyl ester groups showed a linear relationship with time over the experimental period. Transeliminase activity was measured on pectin and sodium polygalacturonate at pH 5.0, the reaction mixtures containing 0.001 M $CaCl_2$. Activity was expressed as µmoles of aldehyde released in four minutes. For the first four minutes the reaction rate was approximately linear with time and the results were calculated from this part of the asymtotic curve.

The viscosity reducing enzymes were measured on a pectin solution at pH 5.5 and NaPP and CMC solutions at pH 4.6. Activity was expressed as 100/t where "t" was the time in minutes for 25 per cent reduction in viscosity of the pectin and 10 per cent reduction in viscosity of the NaPP and CMC solutions. Maceration was measured in 2 ml of culture filtrate. The results are shown in Table 1.

Pectate transeliminase activity could not be detected. In measuring pectin transeliminase activity, the absorption of the reaction products was assayed at 235 mμ. However, at the end of the reaction time each reaction mixture was scanned from 220 mμ to 280 mμ. With pectin, the peak of maximum absorption in the 3 and 9 day reaction mixtures was 235 mμ, but in the 13 day mixture the peak was at 240 mμ and in the 17 day mixture at 242 mμ. The peak became broader with increasing age of culture filtrate used. This phenomenon was experienced on many occasions. Often, a few seconds after adding the culture filtrate or column eluate to the substrate, the ultra-violet absorption maximum was in the region of 223-225 mμ. It then slowly shifted with time until the absorption peak reached 242 mμ. There was no further increase with time on this value.

Fig. 1 shows the changes in the absorption spectrum

Table 1

Growth and enzyme production in culture filtrates grown on a glucose/KNO_3/salts medium (Medium A)

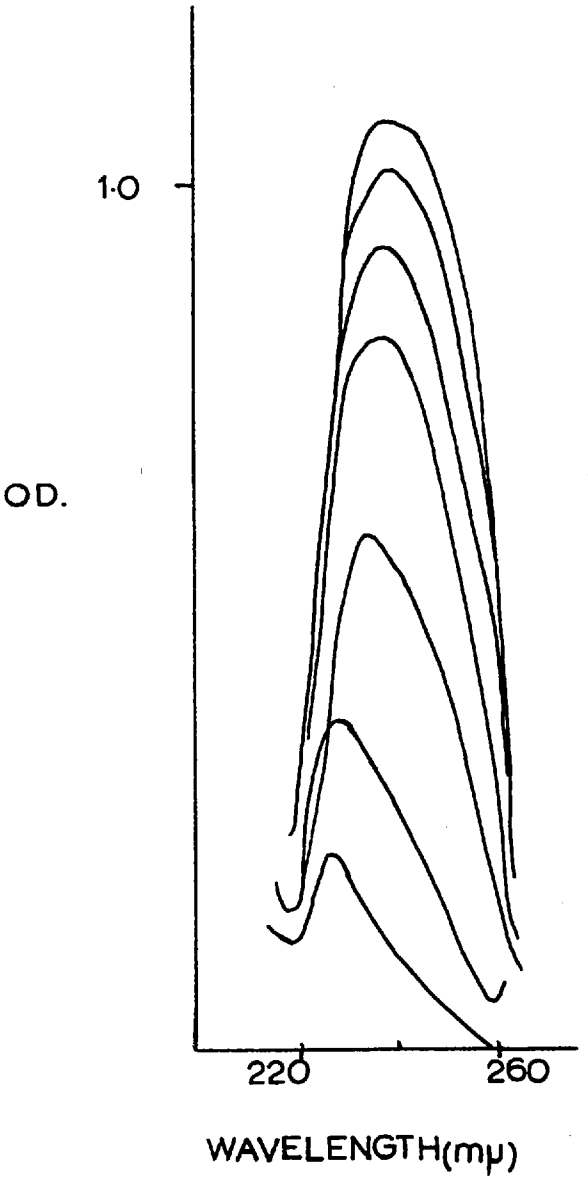
Age of Culture (days)	Mean dry wt.(mg) of mycelium (3 replicates)	PME Activity	Viscosity Reducing Activity		PTE Activity (µmoles CH_2O)	Macerating Activity	Cellulase Activity
			Pectin	NaPP			
3	216.8 (±23.0)	27.6	<3.3	11.8	0	3.5	<3.3
6	160.8 (±24.0)	20.9	15.2	15.2	0	4.6	<3.3
9	284.9 (±48.9)	17.6	57.1	16.1	-	9.1	<3.3
11	221.9 (±33.3)	-	70.4	45.5	-	11.1	<3.3
13	224.5 (±45.3)	11.4	70.0	6.0	0.12	14.3	<3.3
17	227.4 (±11.1)	8.6	76.9	3.3	0.34	14.3	25.0

(The figures in brackets represent the standard deviations)

during incubation of a Sephadex "G.75" fraction with pectin. It is probable that these changes are due to the successive appearance of unsaturated residues of different chain length which absorb at slightly different wavelengths to one another. Fuchs (1965) reported slight changes in the region between 220 and 235 mμ of various C-4,-5 unsaturated oligouronides produced by transeliminative breakdown of sodium polygalacturonate by Pseudomonas fluorescens.

From Table 1 it can be seen that the dry weight of mycelium remained relatively constant over the period 3 to 17 days. Increasing activity with increasing age of culture filtrate was seen for maceration, viscosity reduction, when tested on pectin, and pectin transeliminase. There was only a small production of cellulase in any of the culture filtrates. Maximum activity was found in the 17 day old culture. The viscosity reducing activity on NaPP was highest in the 11 day old culture filtrate and was much lower than the corresponding activity on pectin. In the culture filtrates of the fungus grown on a medium containing 1 per cent glucose as the carbon source, Kavanagh (1965) was unable to demonstrate any reduction of viscosity of a NaPP solution. Maximum PME activity was found in the 3 day old culture filtrate.

Fig.1. CHANGES IN ABSORPTION SPECTRUM DURING INCUBATION OF A PURIFIED CULTURE FILTRATE PREPARATION WITH PECTIN



The high pectin methylesterase recorded is surprising because it is such a high activity and PME of most fungi is produced inductively rather than constitutively. Clearly, Penicillium digitatum produces pectolytic and cellulolytic enzymes constitutively.

Maceration cannot, on these results, be correlated with PME, viscosity reducing activity on NaPP or cellulase aotivity. PTE activity was absent from the 3, 6 and 9 day culture filtrates, although activity was demonstrated in the 13 and 17 day old filtrates. However, it is considered unlikely that there is any correlation between maceration and pectin transeliminase activity. A much closer correlation is seen between maceration and viscosity reducing activity on pectin. Mycelial dry weight was not closely associated with any of the enzyme activities.

2. The effect of pH on the viscosity reducing enzymes

Culture filtrate from a 13 day old culture on glucose/KNO_3/salts medium was used to determine the effect of pH on the viscosity reducing enzymes, measured on pectin and NaPP solutions. The results are shown in Table 2.

Table 2

The effect of pH on the viscosity reducing enzymes

pH	Activity on Pectin $100/time$ for 25% viscosity reduction	Activity on NaPP $100/time$ for 10% viscosity reduction
3.0	3.3	gel formed
3.5	6.5	gel formed
4.0	20.0	27.8
4.5	36.4	20.4
5.0	39.5	13.2
5.5	37.0	9.9
6.0	31.9	7.5
7.0	23.0	<3.3

The optimum pH for pectin and NaPP was 5.0 and 4.0 respectively. Difficulty was encountered in measuring viscosity reducing activity on NaPP below pH 4.0, because the reaction mixture forms a gel. This limitation must, therefore, be borne in mind.

3. α-L-arabinofuranosidase activity

Arabinosidase activity was measured as described in Materials and Methods, p. 47 at pH 4.6, (acetate buffer) which may, however, not be the optimum. The results are shown in Table 3.

Table 3

Estimation of α-L-arabinofuranosidase activity

	Age of culture (days)					
	3	6	9	11	13	17
% Hydrolysis	6.3	6.6	7.0	7.5	11.9	13.7
Rate of hydrolysis µmoles/hr/ml enzyme	1.40	1.46	1.55	1.66	2.64	3.02

Activity increased with age of the culture. Penicillium digitatum produces α-L-arabinofuranosidase constitutively.

4. Effect of pH on arabinosidase activity

The arabinofuranosidase activity of a 13 day old culture filtrate was measured at different pH's (0.1 M citrate buffer) at 25°C. The results are shown in Table 4.

The pH optimum of α-L-arabinofuranosidase, produced by Penicillium digitatum growing on a medium containing glucose as the carbon source, was 3.0 - 5.0. There was, however, not much effect of pH over the range tested 3.0 - 7.0.

Table 4

Effect of pH on arabinosidase activity

pH	% Hydrolysis	Rate of Hydrolysis (μmoles/hr/ml enzyme)
3.0	13.4	2.97
3.5	14.2	3.14
4.0	12.4	2.73
4.5	13.1	2.90
5.0	14.2	3.14
5.5	9.8	2.17
6.0	10.4	2.31
6.5	7.0	1.54
7.0	6.9	1.52

5. β-D-galactopyranosidase activity

β-D-galactopyranosidase activity was estimated as described in Materials and Methods, p. 49 at pH 5.4 which may not be the optimal pH. The results are shown in Table 5.

β-D-galactosidase activity was not detected in the 3 day old culture filtrate and only small amounts were detected in 6, 9 and 11 day old filtrates, with most in the 17 day old filtrate.

Table 5

Estimation of β-D-galactosidase activity

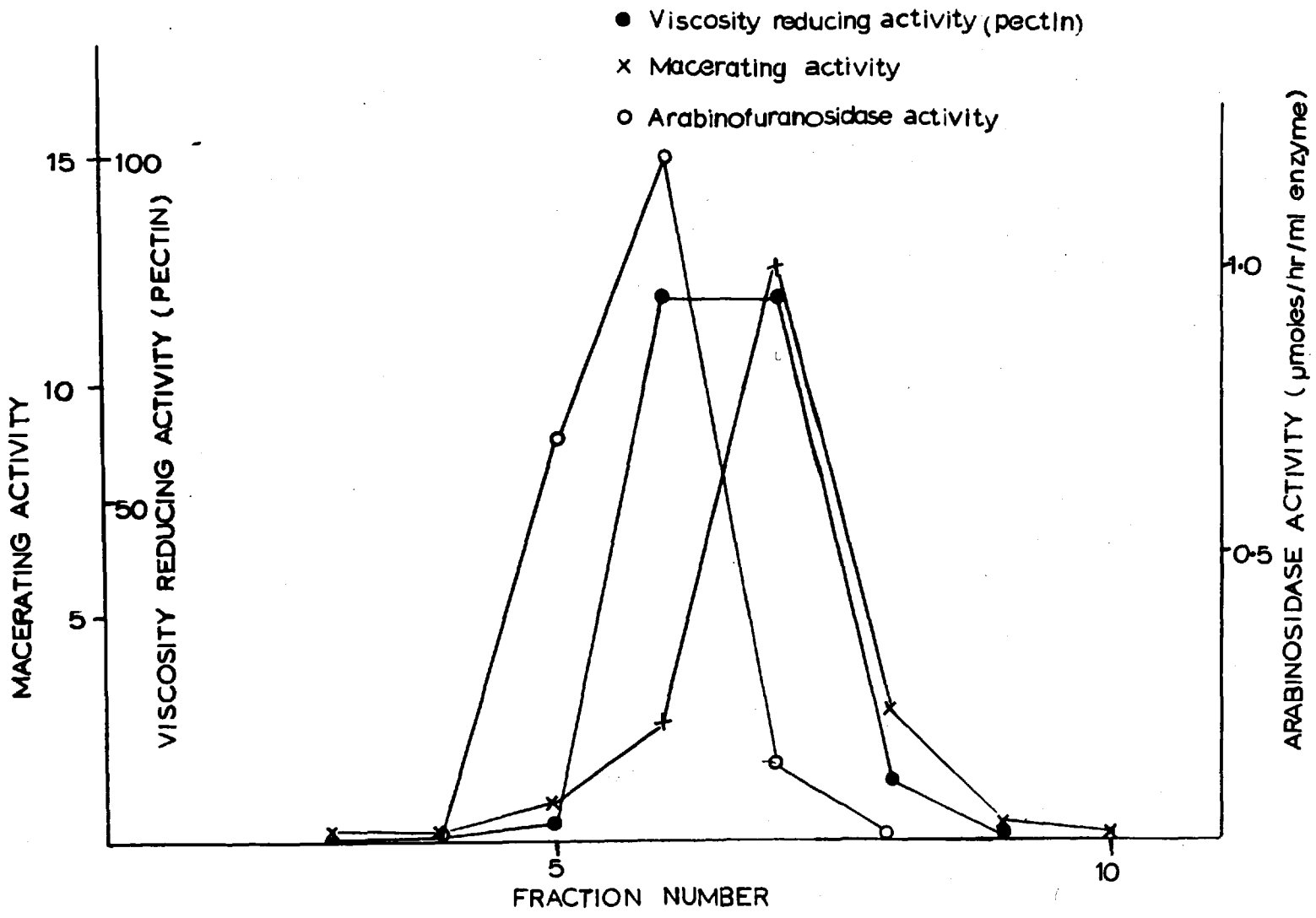
Age of Culture (days)	% Hydrolysis in 30 mins.	Rate of Hydrolysis (μmoles/hr/ml enzyme)
3	0	0
6	0.5	0.04
9	0.5	0.04
11	0.5	0.04
13	1.0	0.07
17	3.5	0.25

6. Fractionation of culture filtrate on Sephadex "G.75"

In an attempt to separate a 'macerating factor', 20 ml of a 17 day old crude filtrate from a culture of *P. digitatum* grown on a medium containing glucose as the carbon source, were applied to a Sephadex "G.75" column (22 cm x 2 cm). The column was eluted with water and 10 ml fractions collected. The fractions were assayed for enzyme activity in the usual manner at pH 5.0, except that viscosity reducing activity on NaPP was measured at pH 4.0. The results can be seen in Fig. 2.

A single peak of macerating activity was found, closely associated with viscosity reducing activity on pectin.

Fig. 2. FRACTIONATION OF CULTURE FILTRATE ON SEPHADEX G.75. (1)



Viscosity reducing activity on NaPP was not detected, neither was PME, when tested for at pH 5.0. The α-L-arabinofuranosidase peak was one fraction removed from that for maceration. The fractionation was, therefore, repeated with a longer column.

22.5 ml of the same culture filtrate were applied to a Sephadex "G.75" column (33 cm x 2 cm). The column was eluted and the fractions collected and assayed as before. The results are shown in Fig. 3. A single peak of macerating activity was obtained, coincident with the viscosity reducing activity when tested on pectin and pectin transeliminase activity. The peak for α-L-arabinofuranosidase activity was two fractions removed from that for maceration.

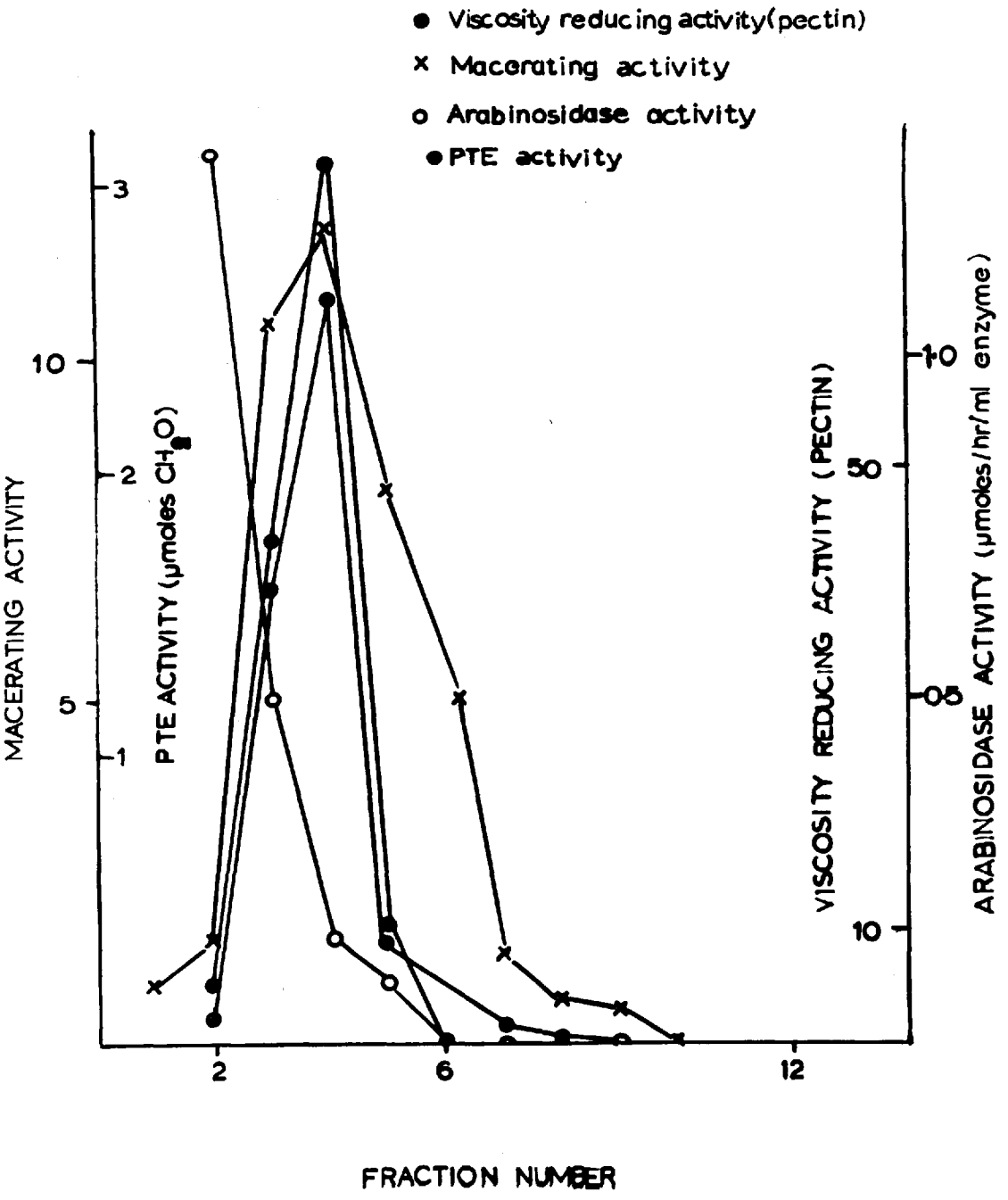
B. THE PRODUCTION OF EXTRACELLULAR ENZYMES ON PECTIN AND NaPP SUBSTRATES

P. digitatum was grown in stationary culture on medium B, containing either 0.1 per cent pectin or 0.1 per cent NaPP as the main carbon source, KNO_3 as the nitrogen source, and salts. Growth and enzyme production were measured after 3, 7, 10, 13 and 17 days.

1. Growth and production of pectolytic enzymes

Growth was measured by the increase in mycelial dry

Fig.3. FRACTIONATION OF CULTURE FILTRATE ON SEPHADEX G.75 (2)



weight as previously described. PME activity was measured at pH 5.0. The viscosity reducing enzymes were measured on pectin at pH 5.5 and NaPP at pH 4.0. Activity was expressed as $^{100}/_{time}$ for a 50 per cent reduction in viscosity of the solutions. Pectin transeliminase was measured at pH 5.5 and the activity expressed as µmoles aldehyde released in 2 minutes. Maceration was measured in 2 ml of culture fluid. The results of these assays are shown in Table 6.

Growth on both types of media was small with a maximum after three days. The dry weight of the mycelia then decreased until after 17 days when the dry weight of the mycelium from the pectin cultures was approximately half that of the three day value. There was more growth in the NaPP cultures; here the 17 day value was about two-thirds the three day value. Growth of cultures on medium A, containing glucose, was at least ten times as great as growth on medium B, containing pectin or NaPP. The viscosity reducing activities of the filtrates from both types of media were much higher than those of the glucose filtrates. NaPP filtrates, tested on pectin and NaPP, showed optimal activity after 7 days. Optimal activity of the pectin filtrates, tested on NaPP and pectin, was found after 3 and 13 days respectively.

Table 6

Growth and enzyme production in culture filtrates grown on pectin or NaPP/KNO_3/salts medium (Medium B)

Age of Culture (days)	Mean dry wt. (mg) of mycelium (3 replicates)		PME Activity % hydrolysis in 30 mins		Viscosity Reducing Activity				PTE Activity (µmoles CH_2O)		M.A		pH of culture fluid	
	P	NaPP	P	NaPP	P	(NaPP)	(P)	NaPP (NaPP)	P	NaPP	P	NaPP	P	NaPP
3	17.1 (±4.8)	27.2 (±2.5)	12.5	4.3	21.3	34.5	20.8	7.7	0.2	0.3	18.5	13.3	5.1	5.4
7	14.0 (±3.0)	22.5 (±1.4)	12.5	4.3	30.3	23.8	45.5	55.5	0.9	0.8	22.2	18.8	5.6	5.6
10	11.9 (±3.2)	20.2 (±2.8)	16.1	4.7	26.3	27.0	45.5	34.5	0.8	0.7	20.4	20.0	5.7	5.7
13	9.9 (±1.5)	26.0 (±3.0)	8.7	8.1	41.7	25.0	38.5	15.2	0.5	0.6	19.2	25.0	5.7	5.8
17	8.7 (±0.4)	18.1 (±3.5)	9.5	4.3	27.0	23.3	-	-	0.3	0.4	15.0	15.9	5.7	5.8

MA = Macerating Activity

The figures in brackets represent the standard deviations

The viscosity reducing activity of the glucose filtrates (medium A) was higher when tested on pectin than on NaPP. The viscosity reducing activities of the pectin and NaPP filtrates tested on pectin and NaPP were similar except the 3 and 13 day NaPP filtrates and 13 day pectin filtrate, where activity was much greater on pectin than on NaPP.

PME activity was higher from the pectin than the NaPP filtrates. Maximum activity was found after 10 days in the pectin cultures and after 13 days in the NaPP cultures. PME activity was lower from these filtrates than the corresponding filtrates of cultures grown on medium A containing glucose.

Macerating activity of both sets of culture filtrates was high. Maximum activity was recorded from the 13 day NaPP filtrate. Activity was relatively constant over the experimental period. Greater macerating activity was found in filtrates from both media than in filtrates from cultures grown on medium A.

PTE activity was recorded from all the culture filtrates. Maximum activity of both sets of filtrates was found after 7 days. Activity was high, and greater than the activity recorded for the glucose culture filtrates.

The mycelial dry weight from the glucose cultures was

very much greater than that from the pectin and NaPP cultures. However, with the exception of PME activity, all the enzyme activities measured were higher from filtrates of cultures grown on medium B than on medium A. Although the mycelial dry weight of cultures on the pectin and NaPP media decreased after 3 days, no corresponding decrease in any of the enzyme activities, except the viscosity reducing activity of the NaPP filtrates tested on NaPP, was found. The results from filtrates of cultures grown on medium A and medium B would indicate that enzymic activity is independent of mycelial growth.

The pH of both media increased over the experimental period. In the case of the pectin medium, it increased from 4.5 to 5.7 after 10 days. The original pH of the NaPP medium was 4.6, increasing to 5.8 after 13 days. This increase of pH is interesting because a similar increase is not seen when the fungus was grown on an orange albedo medium or *in vivo* in the juice from infected oranges. In both these cases, the pH falls with time.

2. Effect of pH on the viscosity reducing enzymes

The viscosity reducing activity of 7 day old filtrates from cultures grown on media containing pectin and NaPP. was measured at different pH values. Activity is expressed as $^{100}/_{\text{time}}$, in minutes, for a 50 per cent

reduction in viscosity of the test pectin and NaPP solutions. The results are shown in Table 7.

Table 7

Effect of pH on viscosity reducing enzymes

pH	Pectin medium		NaPP medium	
	Activity(P)	Activity(NaPP)	Activity(P)	Activity(NaPP)
4.0	31.3	55.5	15.6	23.8
4.5	38.5	55.5	19.6	20.0
5.0	45.5	<10	30.3	<10
5.5	45.5	<10	32.3	<10
6.0	38.5	<10	15.6	<10
6.5	25.0	<10	15.6	<10

The optimum pH for viscosity reduction of the pectin and pectate solutions was 5.0 - 5.5 and 4.0 - 4.5 respectively. This confirms the result obtained from filtrates of cultures grown on medium A.

3. Fractionation of culture filtrate on Sephadex "G.75"

In a further attempt to separate the 'macerating factor' from α-L-arabinofuranosidase and other enzymes present in culture fluids, a 10 day old pectin culture filtrate was fractionated by Sephadex gel filtration.

40 ml of the culture flitrate were applied to a Sephadex "G.75" column (44 x 3 cm); 10 ml fractions were collected and assayed in the normal way at pH 5.5. The results of the assays are shown in Fig. 4.

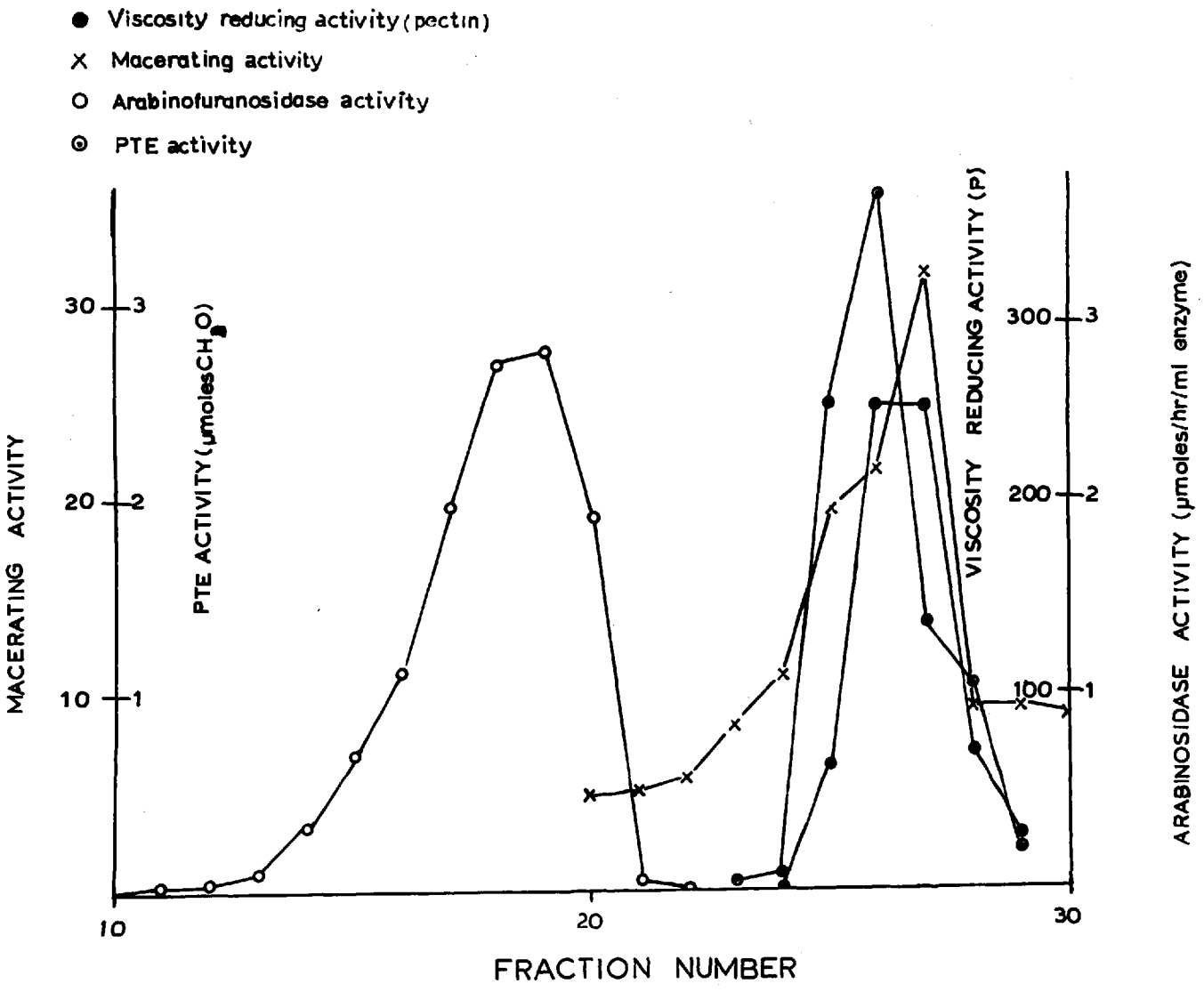
A single peak of macerating activity was obtained coircident with the peaks of viscosity reducing activity with pectin as substrate and with pectin transeliminase activity. The α-L-arabinofuranosidase and maceration peaks were well separated. There was no pectin methylesterase activity in column eluates 20-40, which contained macerating, viscosity reducing and pectin transeliminase activity.

This shows conclusively that arabinofuranosidase is not responsible for maceration.

4. Fractionation of culture filtrate on Sephadex "G.200"

To try and separate maceration from the viscosity reducing enzymes, 2 ml samples from fractions 21-30 of the Sephadex "G.75" column were pooled and applied to a Sephadex "G.200" column (22 x 2 cm); 5 ml fractions were collected and assayed as before. Only one peak of maceration occurred in the eluates. This coincided with the enzymes reducing the viscosity of pectin and with pectin transeliminase.

Fig.4. FRACTIONATION OF CULTURE FILTRATE ON SEPHADEX G.75 (3)



C. GROWTH AND PRODUCTION OF EXTRACELLULAR ENZYMES ON MEDIUM C

80 ml of the medium containing either 1 per cent pectin or 1 per cent NaPP as the carbon source and cas-amino acids as the nitrogen source were dispensed into 500 ml conical flasks and inoculated with 1 ml containing 5 million spores. The flasks were incubated at 25°C. in static and shake culture for 1, 2, 3, 5, 7 and 9 days. At the end of each time period, three shake and stationary cultures were taken and the mycelium and culture filtrates were collected for growth and enzyme estimations.

1. Comparison of growth in static and shake cultures

Growth, as measured by the increase in mycelial dry weight, was estimated in static and shake culture on media containing pectin and NaPP. The results are given in Table 8.

Initially growth in shake culture was greater than in static culture on both pectin and NaPP media. In shake culture, mycelial growth reached a maximum after 5 days on the medium containing pectin and after 2 days on the NaPP medium. The mycelial dry weight then decreased. In static culture, maximum growth was attained

after 9 days on the pectin medium and after 5 days on the NaPP medium. A similar decrease in weight as observed in shake culture was seen in static cultures grown on the medium containing NaPP.

Table 8

Comparison of growth in static and shake cultures

Age of Culture (days)	Mean dry weight of mycelium (mg)			
	Pectin medium		NaPP medium	
	Static	Shake	Static	Shake
1	3.9 (±0.1)	11.3 (±1.9)	10.6 (±2.0)	59.1 (±3.5)
2	15.7 (±0.8)	56.8 (±7.4)	79.6 (±14.8)	218.5 (±4.4)
3	50.5 (±19.1)	156.0 (±26.3)	180.3 (±16.2)	183.9 (±22.9)
5	193.5 (±16.1)	177.8 (±6.1)	185.7 (±3.3)	122.4 (±0)
7	209.4 (±4.9)	139.1 (±2.3)	149.6 (±6.7)	90.3 (±5.1)
9	215.0 (±11.4)	124.2 (±7.8)	101.7 (±10.8)	83.7 (±6.5)

The figures in brackets represent the standard deviations

2. Viscosity reducing activity

Activities of the culture filtrates were measured on pectin and NaPP solutions at pH 5.5. Activity was expressed as ^{100}/time taken for a 10 per cent reduction

in the viscosity of the test solutions. The results are shown in Table 9.

Table 9

Viscosity reducing activity of static and shake filtrates of cultures grown on medium C

Age of Culture (days)	Pectin culture filtrates				NaPP culture filtrates			
	Static		Shake		Static		Shake	
	Tested on				Tested on			
	P	NaPP	P	NaPP	P	NaPP	P	NaPP
1	<3.3	<3.3	<3.3	<3.3	6.3	20.8	10.5	25.0
2	<3.3	<3.3	<3.3	<3.3	14.9	18.1	20.8	25.0
3	<3.3	<3.3	<3.3	<3.3	14.9	27.0	13.3	4.0
5	4.3	<3.3	<3.3	<3.3	9.5	13.3	7.1	<3.3
7	7.1	<3.3	<3.3	<3.3	22.0	19.2	10.8	<3.3
9	6.6	<3.3	<3.3	<3.3	10.0	<3.3	11.1	<3.3

The viscosity reducing activities were small from both static and shake culture filtrates, when tested on either pectin or NaPP. Greater activity was obtained from the NaPP filtrates than the pectin filtrates. This is a reversal of the situation found in cultures grown on media A and B. The filtrates from NaPP cultures showed a sharp fall in activity when tested on NaPP after 7 days in static culture and after 3 days in shake culture. No

sharp decrease in activity was observed when the filtrates were tested on a pectin solution. Maximum activity from the static culture filtrates tested on pectin and NaPP was in the 7 and 3 day filtrates respectively. In shake culture, maximum activity was found in the 2 day old filtrate for activity on pectin and in the 1 day old filtrate for activity on NaPP. Filtrates from pectin static cultures and NaPP shake cultures showed only a very slight viscosity reducing activity when tested on pectin or NaPP.

There was no apparent correlation between mycelial growth and viscosity reducing activity.

3. Estimation of reducing group liberation

Using culture filtrates of the fungus grown in static culture, the release of reducing groups was measured over 1, 3, 6, 10 and 24 hours at 25°C. in a reaction mixture containing the following:

5 ml 1 per cent pectin (pH 5.5)

2 ml citrate buffer (pH 5.5)

2 ml water

1 ml dialysed enzyme preparation.

Autoclaved dialysed culture filtrates were used for the controls.

The results are shown in Table 10.

Table 10

Estimation of reducing group liberation

Age of Culture (days)	% hydrolysis of 1:4 glycosidic bonds									
	Pectin culture filtrates					NaPP culture filtrates				
	incubation time (hr)									
	1	3	6	10	24	1	3	6	10	24
1	0.5	2.2	2.9	2.9	2.9	2.0	1.8	3.2	3.6	3.9
2	0.3	1.4	2.8	2.9	2.8	2.2	2.5	3.4	4.1	4.1
3	0.2	1.4	2.6	2.9	2.8	2.3	3.5	5.4	5.1	5.0
5	0	2.3	4.1	3.8	3.6	2.2	3.2	3.6	4.5	5.0
7	0	3.0	3.6	3.8	4.2	2.2	3.5	4.2	5.1	5.1
9	0.3	3.0	4.1	3.5	3.2	2.3	4.1	4.2	5.7	5.7

There was a very slow release of reducing groups over twenty-four hours. Very little increase was observed after 6 hours in pectin filtrates or after 10 hours in the NaPP filtrates.

Reducing group liberation was higher from filtrates of cultures grown on NaPP than pectin. There was only a small variation between filtrates from cultures on either the pectin or NaPP media. Maximum activity was found in the 7 day pectin filtrate and in the 10 day NaPP filtrate.

The hydrolysis of 1:4 glycosidic bonds of the substrate may be terminal or random. A rapid decrease

in viscosity accompanied by a slow rate of release of reducing groups indicates a random splitting of the substrate, whereas a rapid release of reducing groups with a slow decrease in viscosity indicates a terminal attack on the substrate. Although the viscosity reducing activity by these culture filtrates was relatively slow, reducing group liberation was very slow so that the activity probably represents a random mechanism of attack.

4. Macerating activity

The macerating activities of static and shake filtrates from cultures grown on pectin and NaPP media were measured at pH 5.5. Activity was expressed as $^{100}/_{\text{mean maceration time}}$. The results are shown in Table 11.

Macerating activity was high in all the culture filtrates. The activities of the static culture filtrates were very similar to those of the shake filtrates. Filtrates from cultures grown on NaPP medium showed greater macerating activity than the corresponding filtrates from pectin cultures. After an initial 2 day period, the macerating activity remained relatively constant at a high level, in all the sets of filtrates.

Table 11

Macerating activity of shake and static filtrates of cultures grown on medium C

Age of Culture (days)	Pectin culture filtrates		NaPP culture filtrates	
	Static	Shake	Static	Shake
1	9.0	11.5	9.9	11.8
2	10.6	14.1	20.4	20.4
3	12.3	11.9	16.5	16.7
5	15.6	13.7	20.0	21.3
7	19.6	14.1	22.2	25.6
9	25.0	12.5	21.7	23.8

Macerating activities were very high, yet viscosity reducing activities were very low. This contrasts markedly with filtrates from cultures grown on medium B, containing pectin or NaPP, where both macerating activities and viscosity reducing activities were high. Therefore, it is unlikely that maceration can be correlated with the viscosity reducing enzymes.

5. Pectin lyase activity

Pectic lyase activity was estimated using dialysed culture fluid, because crude culture fluid had a large background absorbance at 235 mμ. Activity was measured

for the static culture flitrates at pH 5.5, the optimum pH for PTE activity (see Table 13) and expressed as μmoles aldehyde released in 15 minutes.

Pectate transeliminase was not detected in filtrates of static cultures grown on the pectin medium. Of the filtrates of cultures grown on the NaPP medium, activity was only detected in the 1 day old culture filtrate. The activity was low (0.07 μmole/15 minutes). Pectin transeliminase activity is given in Table 12.

Table 12

PTE activity of filtrates grown in static culture on medium C

Age of Culture (days)	Pectin Culture filtrate	NaPP Culture filtrate
1	0	0.10
2	0	0.18
3	0	0.18
5	0.24	0.47
7	0.32	0.48
9	0.30	1.00

Maximum activity of the pectin cultures was found in the 7 day filtrate, and in the NaPP cultures in the 9 day filtrate. Activities were higher from the NaPP

cultures than the pectin cultures. PTE was not detected in the 1, 2 or 3 day pectin filtrates. These filtrates, however, showed high macerating activity. No close correlation between macerating activity and pectin transeliminase activity is shown.

6. Effect of pH on pectin transeliminase activity

The activity of a 7 day pectin, static, dialysed culture filtrate was measured at different pH values. The results are shown in Table 13.

Table 13

Effect of pH on pectin transeliminase activity

pH	PTE Activity (µmoles CH_2O/20 mins)
3.5	0
4.0	0.08
4.5	0.24
5.0	0.23
5.5	0.32
6.0	0.20

The optimum pH for PTE activity was near 5.5.

7. Effect of pH on macerating activity

The macerating activity of a 7 day pectin, static culture filtrate was measured at different pH values.

The results are given in Table 14.

Table 14

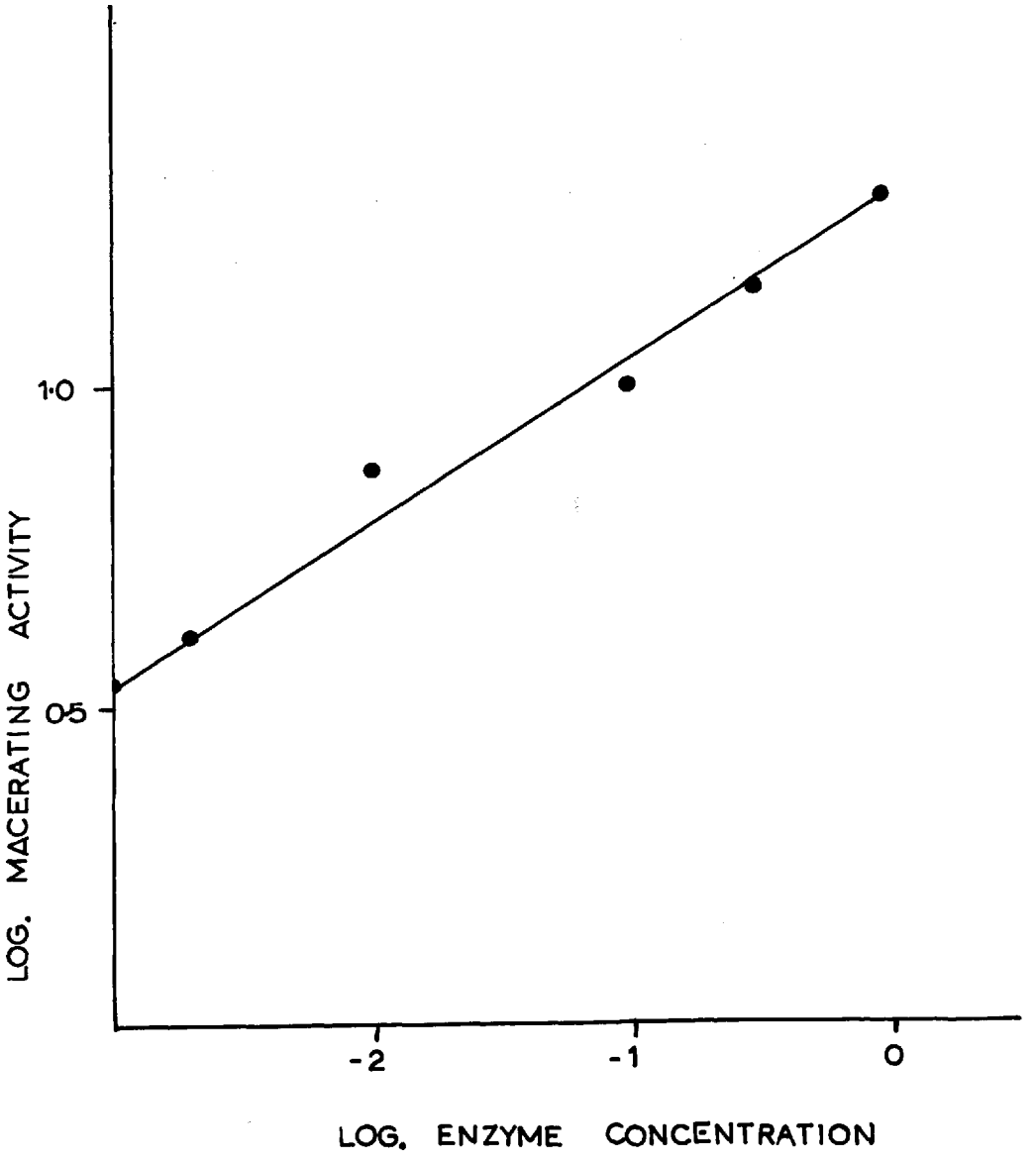
Effect of pH on maceration

pH	Macerating activity	Macerating activity ($^1/_{10}$ dilution)
3.5	8.3	4.4
4.0	10.9	4.9
4.5	14.3	6.9
5.0	18.5	9.3
5.5	20.4	10.3
6.0	13.9	7.5

The optimum pH for macerating activity was again near 5.5.

The effect of dilution on macerating activity shown in Table 14 is interesting because a 10 fold dilution reduced the activity by only one half. In a further experiment to test the effect of dilution upon macerating activity, a 7 day static, pectin filtrate was diluted with water to give samples of the following dilutions: undiluted, $^1/_5$, $^1/_{10}$, $^1/_{50}$, $^1/_{100}$, $^1/_{500}$, $^1/_{1000}$. The result as shown in Fig. 5 indicates a linear relationship between log. macerating activity and log. enzyme concentration. The

Fig.5. RELATIONSHIP BETWEEN LOG. ENZYME CONCENTRATION AND LOG. MACERATING ACTIVITY



relationship between macerating activity and enzyme concentration is non-linear. This dilution experiment indicates that macerating activity is very high from filtrates grown on medium C. Some discrepancy exists between the results obtained from the experiment on the effect of pH on maceration, which showed that a 10 fold dilution of the filtrate reduced the macerating activity by one half, and the dilution experiment, which showed only a slight reduction in macerating activity, with a 10 fold dilution. However, the activity at a 10 fold dilution is approximately twice that of the 100 fold dilution, which in turn is approximately twice that of the 1000 fold dilution.

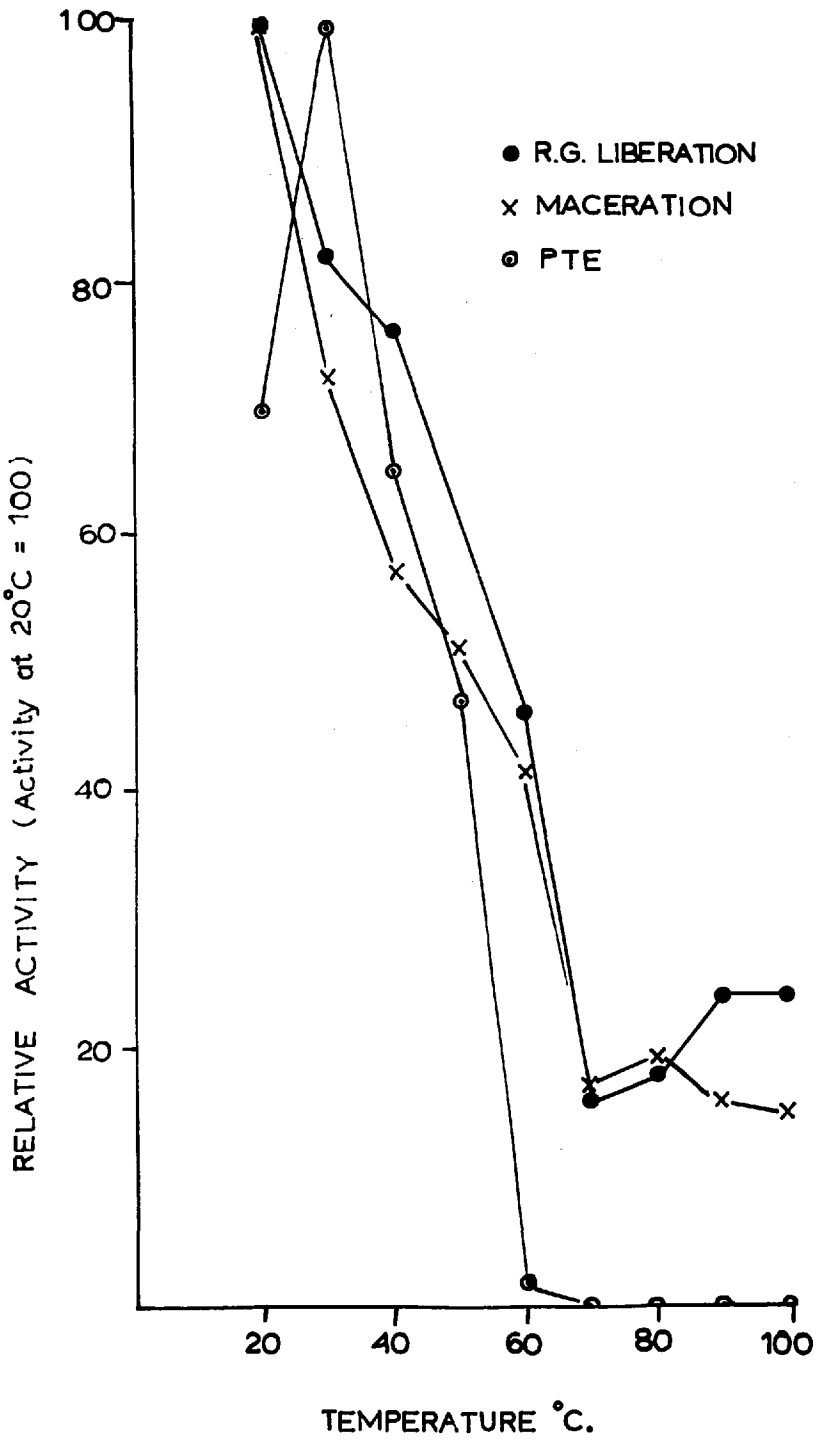
8. Thermal inactivation

Six ml samples of a 7 day old static filtrate from a pectin culture were treated as described on page 50.

Fig. 6 shows the effect of heating on macerating activity, pectin transeliminase activity and on reducing group release in a reaction mixture consisting of:-

5 ml	1 per cent pectin	(pH 5.5)
2 ml	citrate buffer	(0.1 M, pH 5.5)
2 ml	water	
1 ml	culture filtrate.	

Fig.6. THERMAL INACTIVATION OF CULTURE FLUID ENZYMES



There was a steady loss of macerating activity and reducing group liberating activity from 20° to 70°C. At 80°C., reducing group liberation increased slightly and further increased at 90°C. and maintained at 100°C. Macerating activity remained steady from 70° - 100°C. PTE activity was greater at 30°C. than at 20°C. The activity then fell steadily to 60°C. At 70° - 100°C. no activity was detected.

Jarvis (1953) reported that macerating activity could be distinguished from PG by its differential heat stability. My results do not confirm his findings, because there was no material difference in the heat sensitivity of the enzymes measured.

D. EXTRACELLULAR ENZYME PRODUCTION ON MEDIUM D

Because the viscosity reducing and reducing group activity of filtrates of cultures grown on medium C, containing pectin, was very low, but macerating activity was high, this medium would appear to be useful for attempts to separate macerating activity from viscosity reducing and reducing group liberating activity. Therefore a large volume of medium C was prepared for freeze-drying as described on page 37. The constituents of the medium were identical, except in the constituents of the minor

element solution which was composed as follows:-

$FeSO_4.7H_2O$ = 0.125 g

$ZnSO_4.7H_2O$ = 0.11 g

$CuSO_4.5H_2O$ = 0.02 g

$MnSO_4.4H_2O$ = 0.02 g

$Na_2MoO_4.2H_2O$ = 0.025 g

Water to 500 ml.

1 ml of this trace element solution was added to 99 ml of media.

80 ml of the media were put in each of 72 conical 500 ml flasks with 1 ml of a dense spore suspension. The flasks were shaken on a rotary shaker for 4 days at 25°C., and then harvested and bulked. The pH of the culture filtrate was 4.3. 200 ml of the filtrate were assayed immediately for enzyme activity; the remainder was freeze-dried as described on page 37.

1. Enzyme activities of filtrates of cultures grown on medium D

The filtrate, from cultures on medium D, was tested for a number of enzyme activities.

a) Viscosity reducing activity

The viscosity reducing activity of a culture filtrate grown on medium D, containing pectin, was

compared at pH 3.5 - 6.0 (citrate buffer) and pH 6.0 - 8.0 (phosphate buffer). The results, as shown in Table 16, are expressed as ^{100}/time for a 25 per cent reduction in viscosity of the test pectin solution and a 50 per cent reduction in viscosity of the NaPP solution.

Table 16

Effect of pH on viscosity reducing activity

pH	Activity on Pectin	Activity on NaPP
3.5	16.6	-
4.0	25.0	13.3
4.5	28.0	20.0
5.0	≤6.7	≤6.7
5.5	≤6.7	≤6.7
6.0	≤6.7	≤6.7
6.5	≤6.7	≤6.7
7.0	≤6.7	≤6.7
8.0	-	≤6.7

The pH optimum was near pH 4.5 for activity on pectin and NaPP. This confirms work by Kavanagh for the activity of culture filtrates grown in a basal medium containing 1 per cent pectin, when tested on NaPP, The viscosity reducing activity of the filtrate of the culture

grown on medium D fell off markedly near pH 5.0. This is in disagreement with the results of Kavanagh, who showed the pH optimum, when tested on pectin, to be 5.0.

b) Macerating activity

The effect of pH on macerating activity of culture filtrates of the fungus grown on medium D is shown in Table 17.

Table 17

Effect of pH on macerating activity

pH	3.0	3.5	4.0	4.5	5.0	5.5	6.0	7.0	8.0
Activity	16.7	20.0	17.9	9.5	7.9	7.6	5.9	4.6	1.7

The optimum pH for maceration was near pH 3.5. Activity was high at pH 3.0 - 4.0. The pH optimum for maceration does not coincide with that for the viscosity reducing activity (pectin and NaPP). It has usually been found that the pH optima for degradation of substrates by α-1,4 glycosidases in culture filtrates and the pH optima for maceration by the same preparations are closely similar (Echandi and Walker, 1957; Bateman, 1963). This is a further indication that macerating activity is not synonomous with viscosity reducing activity.

c) Pectic lyase activity

The activity of culture filtrate on pectin at pH 3.0, 4.0, 5.0 and 6.0; on NaPP at pH 3.0, 4.0, 5.0 and 6.0 and 3.0, and on sodium polygalacturonate at pH 4.5, 6.0 and 8.0 was measured by following the rate of increase of absorbance at 235 mμ for pectin substrate and at 230 mμ for NaPP and sodium polygalacturonate substrates.

Pectin and pectate transeliminases were not detected at any of the pH values tested.

d) Comparison of enzyme activities between filtrates from cultures grown on medium C and medium D

There was a distinct difference in the enzyme pattern obtained from medium C and medium D. Greater viscosity reducing activity was observed in filtrates of cultures grown on medium D than on medium C. The pH optimum for macerating activity from medium C filtrates was 5.5, but in medium D filtrates, the optimum pH was 3.5. The pH optima of macerating preparations of Erwinia aroideae and Pseudomonas sp. 169 have been shown to depend upon the medium into which they were secreted (Murant and Wood, 1957) Medium D was identical to medium C, except in the constituents of the trace element solution.

Bateman (1966) considered that pH may be responsible for the variable patterns of enzyme activity obtained in

different media. The pH of medium C was 5.0 and that of D was 4.3. It is considered unlikely that such a small difference would have such a pronounced effect. The differences obtained were, therefore, probably caused by the different proportions of trace elements incorporated in the media.

2. Freeze-dried culture filtrate

4,425 ml of fluid from cultures grown on medium D, yielded 24.1 g on freeze-drying. 0.01 g freeze-dried culture filtrate material when dissolved in 1.84 ml water gave a solution of the same concentration as the original culture filtrate. The viscosity reducing activity of this solution, when tested on pectin at pH 4.5, was 8.2 (for a 25 per cent reduction of viscosity). The corresponding activity of the original culture fluid was 28.0, hence there was a considerable loss of activity on freeze-drying. The macerating activity of the solution, prepared from freeze-dried material, at pH 4.5 was 20. The macerating activity of the original filtrate was also 20, at pH 4.5. Thus macerating activity had not been reduced on freeze-drying. (Macerating activity was shown to have a linear relationship with enzyme concentration over the range 0.05 - 1.0 enzyme concentration (undiluted = 1) see page 106).

It was decided to continue the use of freeze-dried material at a concentration of 0.01 g/ml water. This, when tested by the viscometric method on pectin and NaPP at pH 4.5, gave activities of 28.5 (25 per cent reduction in viscosity (P)) and 50.0 (50 per cent reduction in viscosity (NaPP)). The macerating activity at pH 4.5 was 20. The viscosity reducing activity (NaPP) was over twice that of the original filtrate and, therefore, had not been seriously affected by the freeze-drying process. There was, therefore, a differential stability of enzymatic activity during the freeze-drying process. It would appear from these results that macerating activity cannot be identified with viscosity reducing activity on pectin.

Freeze-drying might well provide a useful technique in distinguishing maceration from other enzyme activities.

a) Effect of Dialysis

50 ml of a solution of the freeze-dried culture filtrate preparation (0.01 g/ml) were dialysed against distilled water for 24 and 48 hours at 4°C. A little toluene was added to the culture solution to prevent bacterial contamination.

The viscosity reducing activities of the dialysed culture solution were measured on pectin at pH 4.5. The results are given in Table 18.

Table 18

Effect of dialysis on viscosity reducing activity (pectin)

Time of dialysis (hours)	Viscosity reducing activity (^{100}/time for 25% reduction in viscosity)
0	28.6
24	10.0
48	$\leq$3.3

After 24 hours, the viscosity reducing activity had been reduced to about one-third of the original value. After a further 24 hours dialysis, the activity was reduced further to a very low level. This inactivation of viscosity reducing enzymes has been reported by many workers and has usually been attributed to the loss of certain cations from culture fluids, in particular Ca^{++}. Calcium is a well-known activator of polygalacturonase.

b) Effect of calcium

The effect of adding different concentrations of calcium chloride to 24 and 48 hour dialysed culture solutions (freeze-dried preparations, 0.01 g/ml) were measured viscometrically on pectin at pH 4.5 in reaction mixtures containing: 5 ml 1 per cent pectin (pH 4.5), 2 ml 0.1 M citrate buffer (pH 4.5), 2 ml calcium chloride

solution and 1 ml culture solution preparation.

The activity of the 24 hour dialysed solution was expressed as ^{100}/time for a 25 per cent reduction in viscosity and of the 48 hour dialysed solution, as ^{100}/time for a 10 per cent reduction in viscosity. The results are shown in Table 19.

Table 19

Effect of calcium addition on the viscosity reducing activity (pectin) of dialysed culture filtrate

Final $CaCl_2$ concentration in reaction mixture	Viscosity reducing activity	
	100/time for 25% reduction in viscosity 24 hr dialysed	100/time for 10% reduction in viscosity 48 hr dialysed
0	10.0	<3.3
5×10^{-5} M	11.4	<3.3
5×10^{-4} M	12.1	<3.3
5×10^{-3} M	9.5	<3.3
5×10^{-2} M	9.5	<3.3

There was no significant recovery of activity on the addition of calcium to the reaction mixtures.

The next experiment was done to see if the addition of various salts to the water used for dialysis would reduce this loss of activity. 10 ml of a culture solution

prepared from freeze-dried material (0.01 g/ml) were dialysed against the following solutions:-

Water (Control)

0.01 M citrate buffer (pH 4.5)

0.01 M $CaCl_2$

0.01 M NaCl

0.001 M $MgSO_4$

0.01 M $CaCl_2$ + 0.01 M NaCl + 0.001 M $MgSO_4$.

After dialysis a volume correction was made to allow for osmotic effects and the viscosity reducing activities of the dialysed filtrate solutions were measured on pectin and NaPP at pH 4.5. The results are shown in Table 20.

Table 20

Effect of dialysis against water and salt solutions on viscosity reducing activity

Solution	Viscosity reducing activity (pectin) (100/time for 25% viscosity loss)		Viscosity reducing activity (naPP) (100/time for 50% viscosity loss)	
	Time of dialysis			
	24 hr	48 hr	24 hr	48 hr
water	13.3	10.8	16.6	13.3
0.01M citrate	12.5	10.0	18.2	16.0
0.01M $CaCl_2$	22.2	25.0	gel	gel
0.01M NaCl	13.3	7.1	14.3	<10.0
0.001M $MgSO_4$	22.2	15.4	22.2	25.0
0.01M $CaCl_2$ + 0.01M NaCl + 0.001M $MgSO_4$	23.5	22.5	gel	gel

Undialysed culture solution

Viscosity reducing activity (pectin) = 28.5 (for a 25% viscosity loss)

Viscosity reducing activity (NaPP) = 50.0 (for a 50% viscosity loss)

The loss in activity was reduced by half when the culture solution was dialysed against $CaCl_2$, $MgSO_4$ and the mixture of salts. Dialysis against NaCl and citrate buffer caused a similar loss in activity as dialysis against water. This may mean that Ca^{++} and Mg^{++} have some stabilizing effect.

Effect of dialysis against water and salt solutions on macerating activity

The effect of dialysing a culture solution (freeze-dried preparation (0.01 g/ml)) against water and salt solutions as described above, was measured on macerating activity at pH 4.5. The results are shown in Table 21.

There was a slight reduction in macerating activity after 24 hours dialysis. After 48 hours dialysis, activity had been reduced by approximately half. There was no significant difference, in contrast with the result for viscosity reducing activity, in macerating activity between enzyme solutions dialysed against water or salt solutions.

Table 21

Effect of dialysis against water and salt solutions on macerating activity

Solutions	Macerating Activity	
	24 hr dialysed	48 hr dialysed
water	15.9	9.4
0.01M citrate	14.0	10.3
0.01M $CaCl_2$	15.9	10.5
0.01M NaCl	12.8	8.2
0.001M $MgSO_4$	12.5	10.4
0.01M $CaCl_2$ + 0.01M NaCl + 0.001M $MgSO_4$	11.1	8.4

(macerating activity of undialysed preparation = 20)

From these results it would appear that Ca^{++} was not important for the activity of the viscosity reducing enzymes, although their stability on dialysis may be increased by its addition. To confirm this result, the effect of ethylenediaminetetra-acetic acid (EDTA), a sequestering agent, was measured on the reduction of viscosity of pectin and NaPP solutions in reaction mixtures of the following composition:-

5 ml 1 per cent pectin (pH 4.5)

2 ml 0.1 M citrate buffer (pH 4.5)

2 ml EDTA

1 ml culture filtrate (0.01 g/ml)

The results are shown in Table 22.

Table 22

Effect of EDTA on viscosity reducing activity

Final EDTA concentration in reaction mixture	Activity on pectin (100/time 25% viscosity reduction)	Activity on NaPP (100/time 50% viscosity reduction)
0	15.4	15.4
1×10^{-5}	14.3	16.0
1×10^{-4}	14.7	16.7
1×10^{-3}	15.4	18.2
1×10^{-2}	10.5	14.3
2×10^{-2}	10.0	14.8
5×10^{-2}	5.5	10.0
1×10^{-1}	gel	gel

EDTA had no effect on the viscosity reducing activity up to 1×10^{-3} M final concentration. At higher concentrations, the activity was slightly reduced until at 10^{-1} M gelling of the reaction mixture occurred. This result confirms the lack of activation by Ca^{++}.

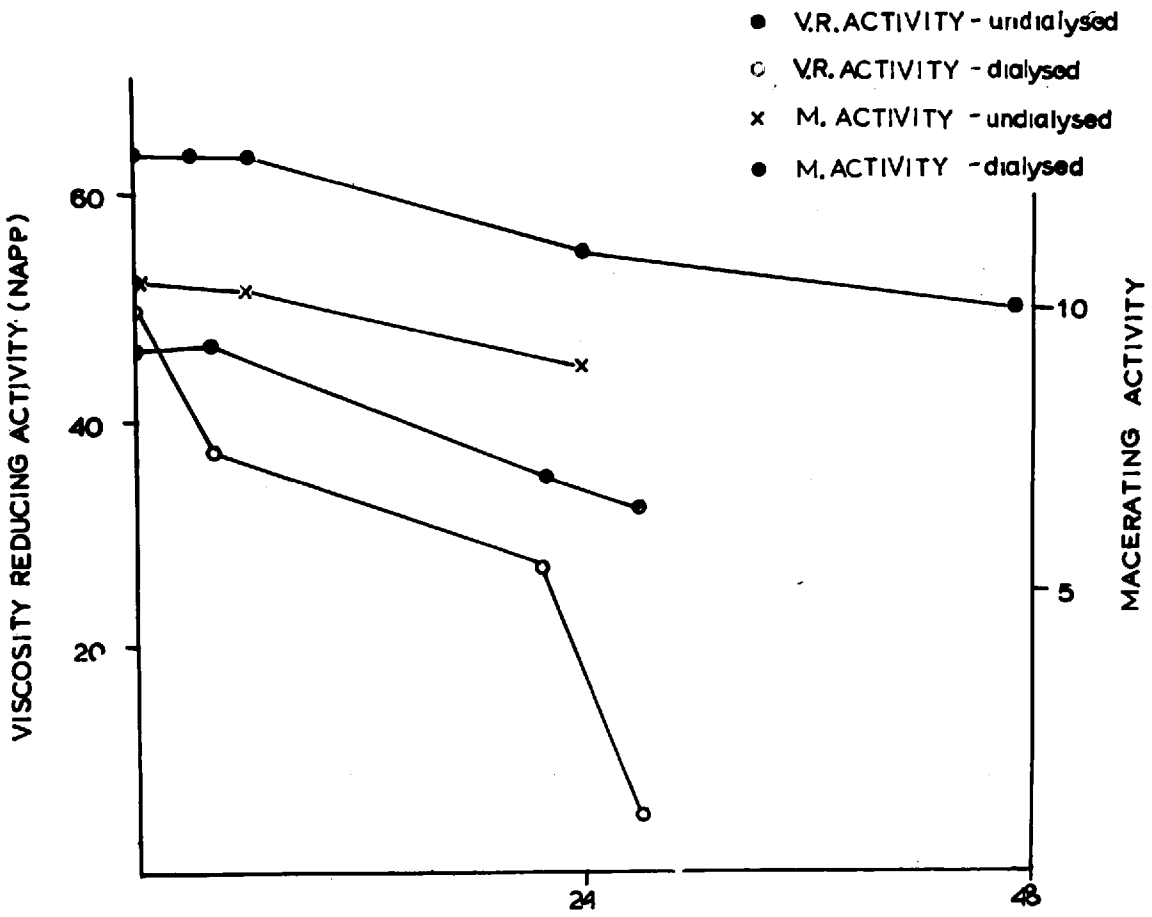
The loss in viscosity reducing activity upon dialysis could not, therefore, be explained by loss of calcium.

It is possible that these losses of activity may have resulted from an 'inherent' loss on standing. To test this hypothesis, an experiment was conducted to determine the possible loss of viscosity reducing and macerating activity of dialysed and undialysed culture fluid with time.

A culture filtrate solution was prepared from freeze-dried material (0.01 g/ml). Half the solution was dialysed against distilled water for 24 hours at 4°C. The remainder was kept, as prepared, at 4°C. for 24 hours. At the end of this time both the dialysed and undialysed preparations were measured for viscosity reducing activity (NaPP) and macerating activity at pH 4.5. These preparations were then left at laboratory temperature and their enzyme activities were measured after standing for different time periods. The results are shown in Fig. 7.

In the dialysed preparation there was a considerable reduction in viscosity reducing activity (NaPP) over a period of 24 hours standing. In the non-dialysed preparation only a slight reduction in activity was seen. Macerating activity was lower in the dialysed preparation after 24 hours than in the non-dialysed, although both activities showed a reduction of activity with time.

Fig. 7. EFFECT OF STANDING ON VISCOSITY REDUCING AND MACERATING ACTIVITY OF UNDIALYSED AND DIALYSED CULTURE FILTRATE



The macerating and viscosity reducing activity of the dialysed preparation was lower at time zero than the undialysed preparation, indicating some loss of activity upon dialysis.

The reduction in macerating and viscosity reducing activity in dialysed culture solutions is presumably caused by the loss of 'stabilizing agents'.

c) Effect of pH on the liberation of reducing groups

The liberation of reducing groups upon the addition of a dialysed culture solution prepared from freeze-dried material (0.01 g/ml) was measured in the following reaction mixture:

5 ml 1 per cent pectin/NaPP

2 ml citrate buffer

2 ml water

1 ml culture solution (0.01 g/ml)

at pH 3.0, -6.0 and 6.0. Reducing groups were measured after 30 minutes incubation, at 25°C. The results are given in Table 23.

The optimal pH was near 5.0 on pectin and 4.0 on NaPP.

Table 23

Effect of pH on reducing group liberation by a dialysed culture solution (freeze-dried preparation (0.01 g/ml))

pH	% Hydrolysis	
	Pectin substrate	NaPP substrate
3.5	0.05	-
4.0	0.13	0.20
4.5	0.16	0.18
5.0	0.18	0.16
5.5	0.14	0.16
6.0	0.09	0.08

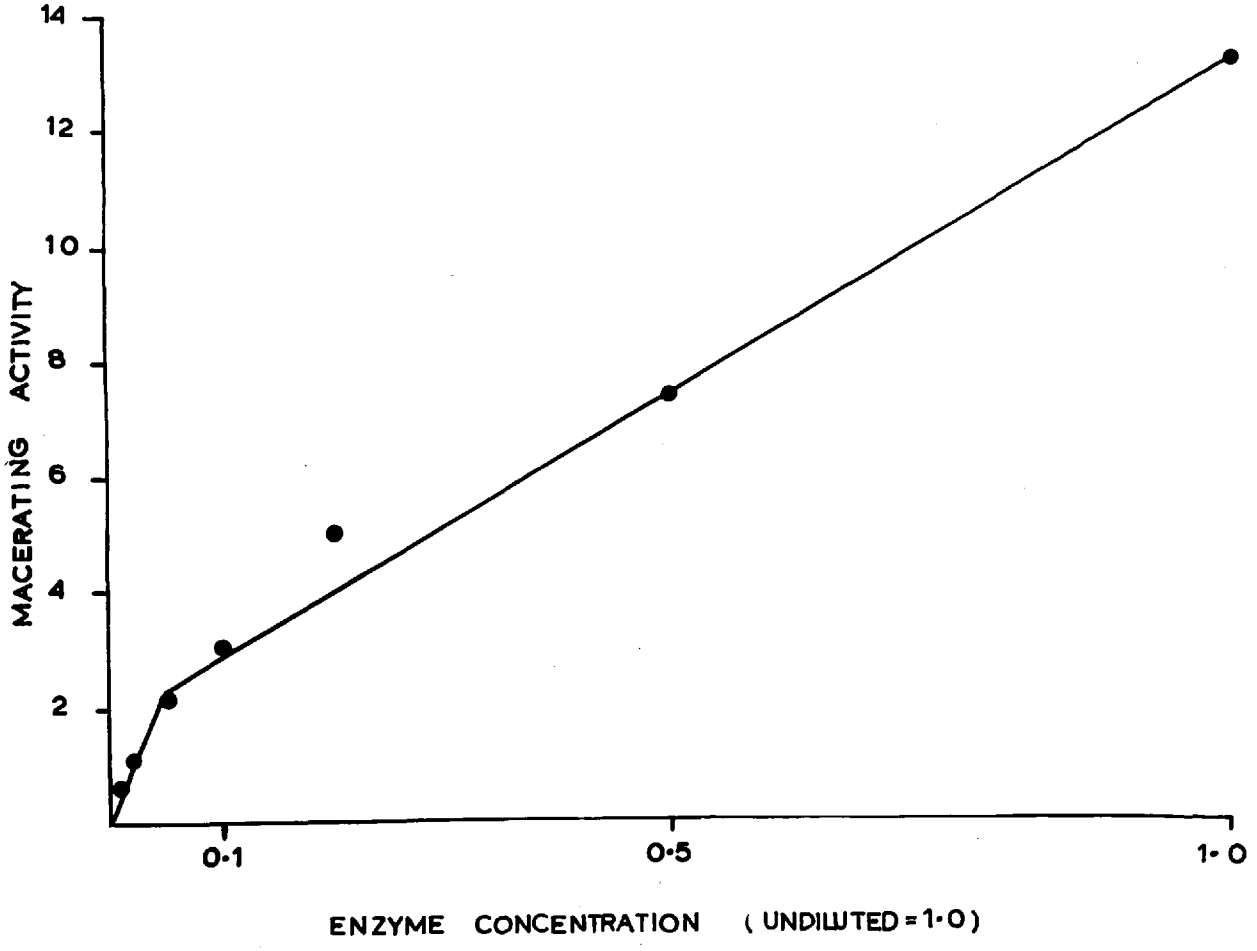
d) Effect of dilution on macerating activity

A culture solution prepared from a freeze-dried culture filtrate preparation (0.01 g/ml) was diluted with water to give samples of the following dilutions: undiluted, $^{1}/_{2}$, $^{1}/_{5}$, $^{1}/_{10}$, $^{1}/_{20}$, $^{1}/_{50}$ and $^{1}/_{100}$. These were assayed at pH 4.5. The results, shown in Fig. 8, indicate a linear relationship between enzyme concentration and macerating activity down to a concentration of 0.05 (undiluted = 1.0). Below this concentration activity fell off slightly more sharply.

e) Effect of dilution on the liberation of reducing groups

Fifty ml of a culture solution prepared from freeze-

Fig.8. RELATIONSHIP BETWEEN ENZYME CONCENTRATION AND MACERATING ACTIVITY



dried material (0.01 g/ml) were dialysed, for 18 hours, at 4°C. against distilled water. The dialysed solution was diluted with water to give samples of appropriate dilutions. One ml samples of the following enzyme concentrations:- undiluted, 1/5, 1/10, 1/50 and 1/100, were added to 5 ml 1 per cent pectin/NaPP (pH 4.5), 2 ml citrate buffer and 2 ml water. The reaction mixtures were incubated with a drop of toluene, to prevent bacterial contamination, at 25°C. At known time intervals up to 24 hours, 1 ml samples of the reaction mixtures were removed and the reducing groups estimated. Autoclaved, dialysed culture solution was used in the control reaction mixtures. The results are shown in Tables 24 and 25.

A very low activity, even in the undiluted culture solution was recorded for activity on both pectin and NaPP. This confirms the production of endo-enzymes, since the viscosity reducing activity on pectin and NaPP is high. In Fig. 9, the percentage hydrolysis of substrates after 24 hours incubation is plotted against enzyme concentration (undiluted culture solution = 1). A linear relationship between enzyme concentration and reducing group liberation activity was found when pectin was used as the substrate. For the NaPP substrate a non-linear relationship was seen. After 4 hours incubation

Table 24

Effect of dilution on the liberation of reducing groups from a pectin substrate over 24 hours

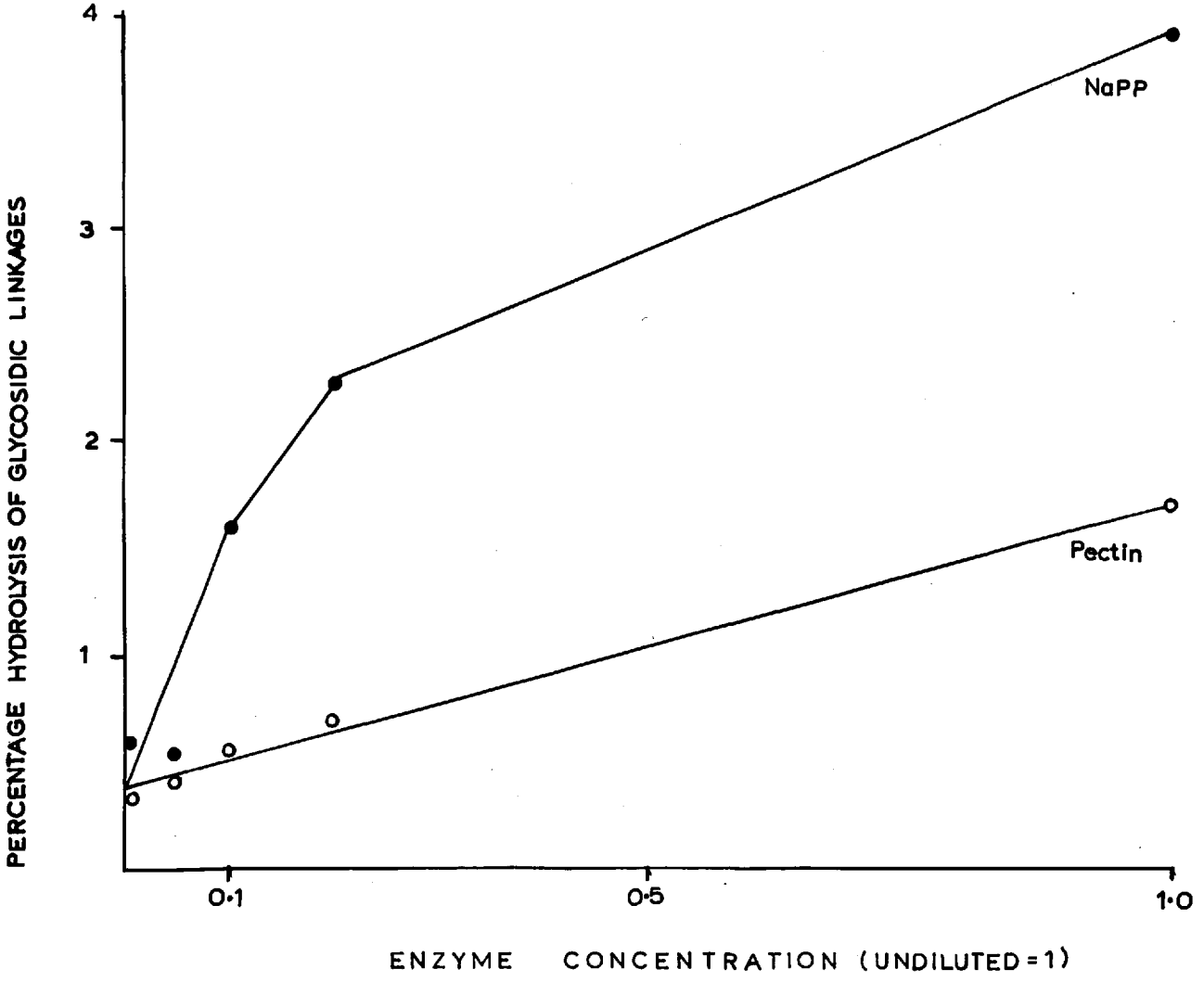
Dilution	% hydrolysis of 1,4 glycosidic linkages incubation time (hr)						
	0.5	1	2	4	6	18	24
1/1	0.12	0.18	0.18	0.38	0.36	1.14	1.70
1/5	<0.10	<0.10	0.12	0.14	0.20	0.32	0.68
1/10	"	"	<0.10	<0.10	0.10	0.10	0.55
1/50	"	"	"	"	<0.10	<0.10	0.40
1/100	"	"	"	"	"	"	0.30
1/500	"	"	"	"	"	"	0.15

Table 25

Effect of dilution on the liberation of reducing groups from a NaPP substrate over 24 hours

Dilution	% hydrolysis of 1,4 glycosidic linkages incubation time (hr)						
	0.5	1	2	4	6	18	24
1/1	0.33	0.67	0.72	1.60	2.20	3.30	3.90
1/5	0	0.13	0.13	0.56	0.86	1.67	2.29
1/10	0	0	0.05	0.21	0.47	0.99	1.57
1/50	0	0	0	0.09	0.09	0.19	0.54
1/100	0	0	0	0.05	0.05	0.17	0.60
1/500	0	0	0	0	0	0	0.15

Fig.9. RELATIONSHIP BETWEEN ENZYME CONCENTRATION AND REDUCING GROUP ACTIVITY



of culture fluid with NaPP the relationship is linear, but thereafter it becomes non-linear. This is presumably caused by enzyme inactivation.

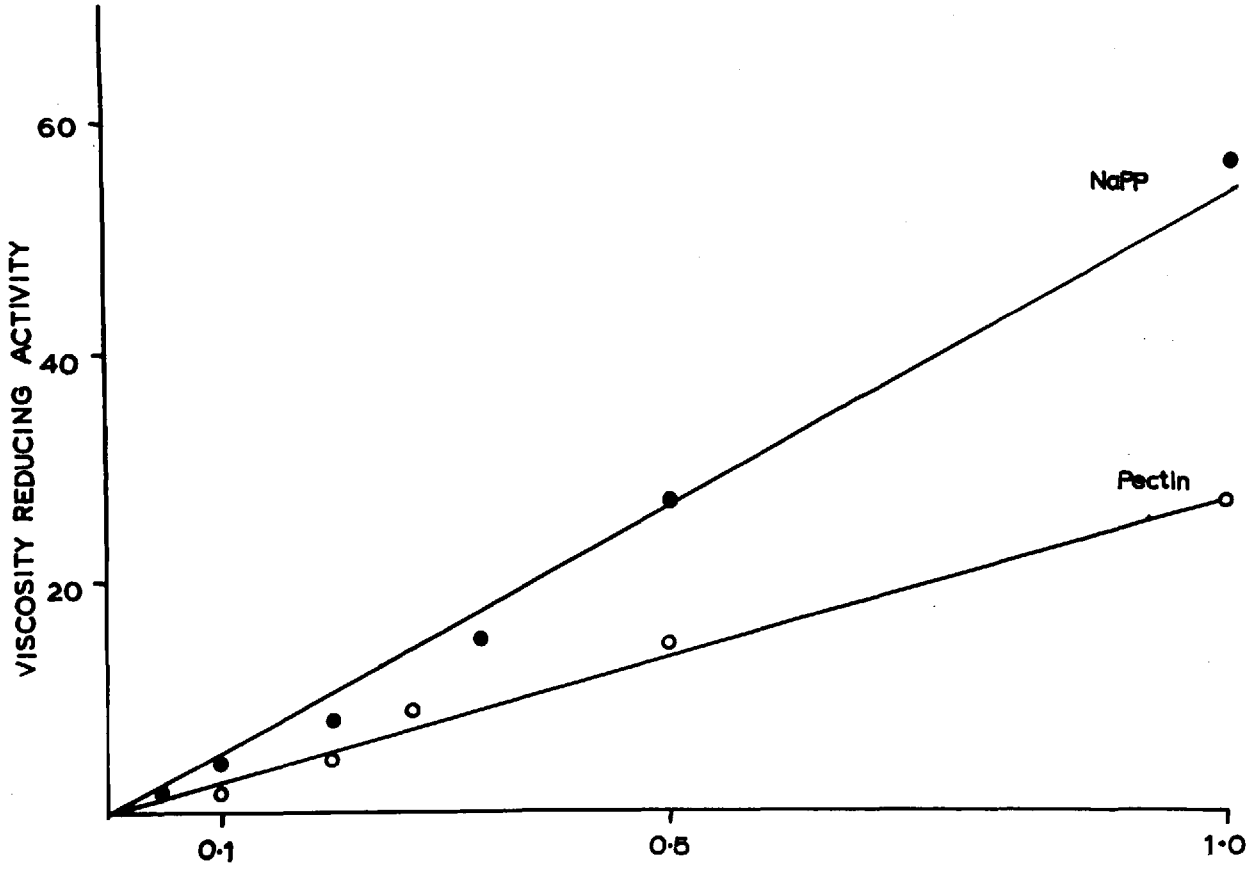
f) Effect of dilution on viscosity reducing activity

A culture solution was prepared from freeze-dried material (0.01 g/ml) and samples of the following dilutions made:- undiluted, $^{1}/_{2}$, $^{1}/_{3}$, $^{1}/_{5}$, $^{1}/_{10}$, $^{1}/_{20}$. The viscosity reducing activity of each sample was measured in the usual way on pectin at pH 4.5 and on NaPP at pH 4.0. Activity was calculated as $^{100}/_{\text{time}}$ for a 25 per cent reduction in viscosity of the pectin and NaPP solutions. When these values were plotted against enzyme concentration, as shown in Fig. 10 , a linear relationship was obtained between enzyme concentration and viscosity reducing activity (P) and (NaPP).

g) Maceration in relation to toxicity

Maceration was measured in the usual way on potato discs at pH 4.5. Toxicity was estimated by the plasmolytic method described in Materials and Methods, p. 46. A culture solution prepared from freeze-dried culture filtrate (0.01 g/ml) was used as the macerating solution. Potato discs were placed in this macerating solution for different time periods. At the end of each period, the observations as recorded in Table 26 were made, and four

Fig.10 RELATIONSHIP BETWEEN ENZYME CONCENTRATION AND VISCOSITY REDUCING ACTIVITY



discs were treated with neutral red. The Neutral Red Index (see p. 47) was recorded, as shown in Table 26.

Fig. 11 shows the appearance of the discs after immersion in the macerating solution and treatment with neutral red. The letters refer to the times of immersion as given in Table 26.

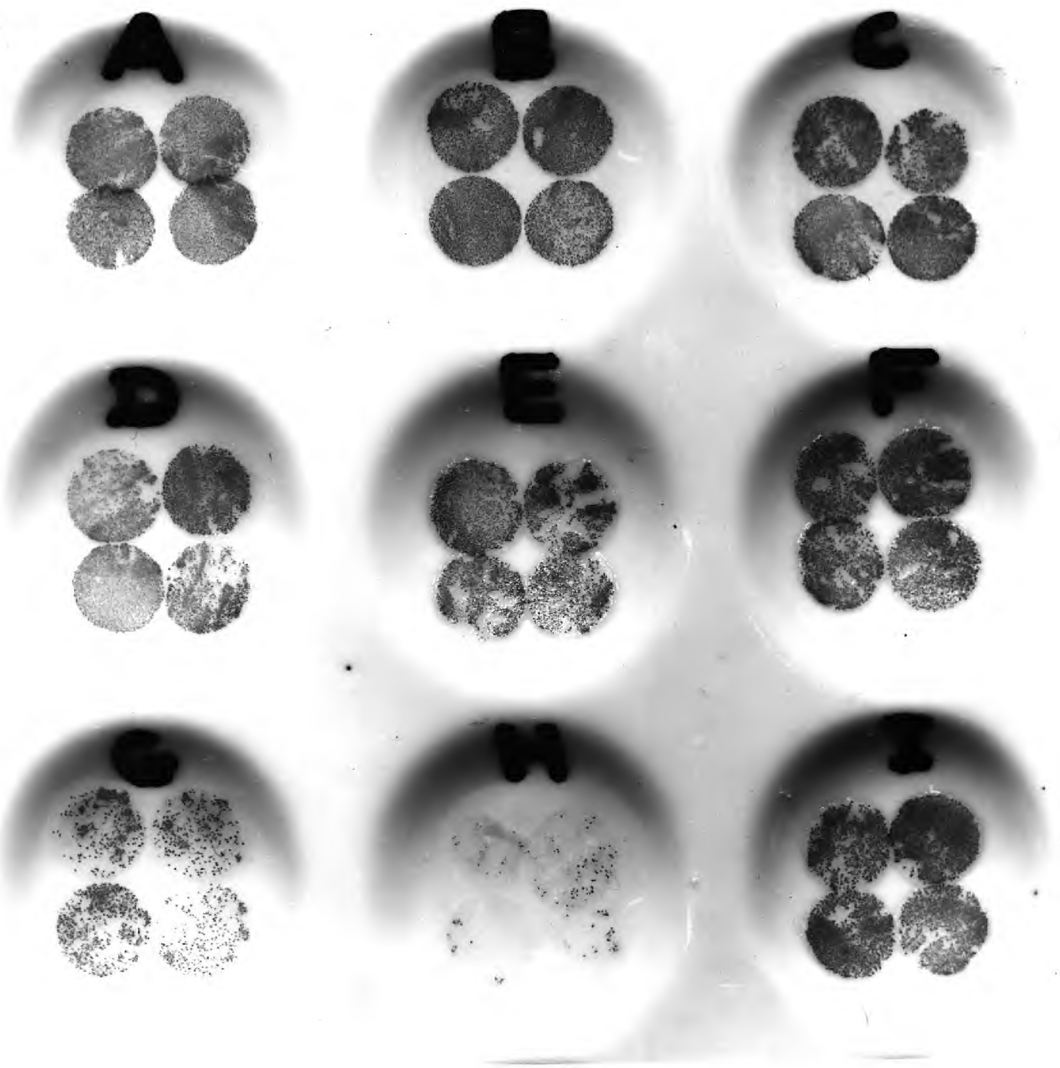
Table 26

Maceration in Relation to toxicity

Time of Immersion in macerating fluid	Observations on discs	Neutral Red Index	Ref. Fig. 11
0 min	Tissue firm	5	A
5 min	Slight loss of crispness	5	B
10 min	Tissue macerated, retaining rigidity	4	C
15 min	Tissue still retaining some rigidity	4	D
30 min	Some loss in tissue rigidity	3	E
1 hr	Tissue losing rigidity	3	F
2 hr	Tissue limp, breaking on picking up	1	G
3 hr	Tissue very limp	0.5	H
3 hr	Autoclaved control, tissue firm	5	I

The tissue began to lose coherence after 6 minutes, and after 10 minutes the tissues were fully macerated.

Fig. 11 APPEARANCE OF POTATO DISCS AFTER IMMERSION IN A MACERATING SOLUTION AND TREATMENT WITH NEUTRAL RED



A - I SEE TABLE 26

The majority of the cells were still alive at this time. There was a considerable delay after the tissues were fully macerated before most of the cells had been killed. After 2 hours in the macerating solution, a considerable number of cells were still alive. This phenomenon was also recorded by Tribe (1955) in observing the killing of turnip and cucumber cells by a filtrate from a culture of Botrytis cinerea.

h) Cell wall effect

Potato discs were immersed in a macerating solution at pH 4.5 in the normal way. Maceration occurred in 6 to 8 minutes. After intervals of 20 minutes, 30 minutes, 1 hour, 2 hours and 3 hours, discs were removed from the macerating solution and placed in a $^{1}/_{5000}$ solution of ruthenium red for 20 minutes. Ruthenium red is a stain which is fairly specific for pectic substances. The observations given in Table 27 were made.

After 20 minutes, although the potato discs were fully macerated, a large amount of red staining was present in the cell wall region. The stain slowly disappeared with increasing time of immersion in the macerating solution. After 3 hours, no red staining was seen. At this stage, the cells were tending to separate, and become rounded off, presumably by the complete

dissolution of the middle lamella. These results indicate that toxicity may be correlated with the disappearance of ruthenium red staining materials from the cell walls.

Table 27

Observations on macerated tissue stained with ruthenium red

Time in macerating solution	Observations
20 min	Tissue fully macerated but a large amount of red staining of cell walls seen (N.R. Index = 4)
30 min	as above (N.R. Index = 3)
1 hr	Large proportion of cell walls stained red (N.R. Index = 3)
2 hr	Red wall stain decreasing (N.R. Index = 3)
3 hr	No red staining of the walls evident (N.R. Index = 0.1)

i) Adsorption of culture filtrate enzymes on modified celluloses

Cellulose ion exchange adsorbents have proved useful in fractionating pectolytic enzymes present in culture filtrates (McClendon and Kreisher, 1962; Dean and Wood, 1967).

Attempts were made to adsorb enzymes in filtrates from cultures of P. digitatum on three modified celluloses, Ecteola cellulose, which is an anion exchanger, carboxymethylcellulose (CMC), and cellulose phosphate, which are cation exchangers (Materials and Methods, p. 52).

A culture solution, prepared from freeze-dried culture filtrate (0.01 g/ml) was dialysed at 4°C., against water overnight.

i) Adsorption on Ecteola cellulose

In preliminary experiments there was no significant adsorption of protein, macerating activity or reducing group liberating activity and viscosity reducing activity (NaPP) with 0.01 M citrate buffer, at pH 3.5, 4.5, 5.0 or with 0.01 M phosphate buffer at pH 6.0, 7.0 and 8.0. It was interesting to note at this stage that the culture filtrates, tested for adsorption at pH 6.0, 7.0 and 8.0 by viscometry at pH 4.5, on NaPP, showed a reduced activity. Activity was low from the filtrate at pH 6.0 and no activity was found in filtrates at pH 7.0 or 8.0. This was not due to adsorption of the enzyme, but to inactivation at these pH levels.

ii) Adsorption on carboxymethylcellulose

Adsorption on CMC was tested with the following buffer systems:

0.02 M	acetate buffer	pH 4.6, 5.4
0.02 M	citrate buffer	pH 4.0
0.01 M	citrate buffer	pH 4.0, 5.0, 6.0
0.005M	glycine buffer	pH 3.4
water		pH 5.2

Macerating activity and reducing group liberating activity was measured for each supernatant. There was some adsorption of macerating activity with all buffer systems. There was also a slight adsorption of reducing group activity (NaPP as substrate). There was most adsorption with the citrate buffer system. The adsorption on CMC was repeated with the following buffer systems:

0.01 M citrate buffer pH 3.0, 4.0, 5.0, 6.0

0.01 M McIlvaine's buffer pH 3.0, 4.0, 5.0, 6.0

2 ml of dialysed culture fluid was added to 0.3 g CMC in buffer and stirred for 15 minutes, centrifuged and the supernatants kept. The CMC samples were then washed with:

I 0.02 M citrate or McIlvaine's buffer

II 0.05 M citrate or McIlvaine's buffer

III 0.1 M citrate or McIlvaine's buffer

IV 0.05 M NaCl in 0.01 M citrate or McIlvaine's buffer.

Protein, macerating activity and reducing group activity (NaPP substrate) was measured for the original supernatant and the washings.

In all cases, much of the protein remained in the original supernatants and the amount removed by successive washings decreased. With the citrate buffer system at pH 4.0, 5.0 and 6.0, and McIlvaine's buffer system at pH 5.0

and 6.0, some of the reducing group activity remained unadsorbed and was recovered in the original supernatant but more activity was recovered in subsequent elution. Little activity was recovered in any of the supernatants from the citrate or McIlvaine's buffer system at pH 3.0. Highest macerating activity was seen in the original supernatant and very little was found in any of the successive washings. From these results, adsorption on CMC was not considered sufficiently promising to warrant further investigation by column chromatography.

iii) Adsorption on cellulose phosphate

Adsorption on cellulose phosphate was attempted in the same manner described for CMC, using the following buffer systems:

0.01 M citrate pH 4.0, 5.0, 6.0

0.01 M McIlvaine pH 3.0, 4.0

0.02 M acetate pH 4.6, 5.4.

After the first supernatant had been collected, the cellulose samples were washed with a) 0.02 M buffer, b) 0.05 M buffer and c) 0.1 M buffer.

Protein, macerating activity and reducing group activity on a NaPP substrate, was measured for each supernatant, and the results are shown in Table 28.

Table 28

Adsorption of culture fluid enzymes on cellulose phosphate

Buffer pH	Original fluid + CMC			1st wash			2nd wash			3rd wash		
citrate	P	RG	M	P	RG	M	P	RG	M	P	RG	M
4.0	40	120	3.7	17	20	4.9	13	85	4.3	25	85	3.9
5.0	70	205	3.6	32	140	5.9	9	65	5.9	2	42	1.3
6.0	105	245	3.3	36	132	5.0	9	72	4.2	13	25	1.8
McIlvaine												
3.0	55	60	3.3	17	33	6.1	13	18	2.4	21	7	1.3
5.0	70	113	3.9	28	132	4.2	21	72	2.9	9	55	1.3
acetate												
4.6	51	113	4.6	70	150	4.3	25	83	1.3	25	122	1.3
5.4	82	200	4.0	44	140	3.6	28	75	1.3	28	40	5.0
water	70	370	5.1	28	125	4.6	21	75	2.2	17	46	2.9

P = protein (mg/ml)

RG = reducing group activity (µg glucose equivalents/ml)

M = macerating activity

Activity of original fluid

protein = 140 mg/ml

reducing groups = 400 µg/ml

macerating activity = 15.9

In the samples, most of the protein remained unadsorbed and the amount removed by successive washings decreased. Most reducing group activity, similarly, was not adsorbed and was recovered in the original supernatant, decreasing with each wash. With the exception of McIlvaine's buffer at pH 5.0, more activity was recovered in the first wash supernatant than in the original supernatant. Little reducing group activity was recovered from any of the supernatants, with McIlvaine's buffer at pH 3.0. This is similar to what was found for adsorption on CMC at pH 3.0. Whilst some macerating activity was recovered in the original supernatants, more was recovered on subsequent elution with some of the buffer systems used.

Greater recovery of macerating activity was achieved using cellulose phosphate as adsorbent than when CMC was used.

Of the cellulose adsorbents tested, cellulose phosphate was the only one to appear sufficiently promising to be worthwhile investigating by column chromatography.

iv) Column chromatography of culture filtrate on cellulose phosphate

Two grams cellulose phosphate was stirred into 100 ml of 0.01 M citrate starting buffer (pH 4) and packed into a

column 12 cm long, 1 cm diameter.

0.5 g freeze-dried culture filtrate was dissolved in 25 ml water and dialysed in 'visking' tubing ($^{8}/_{32}$") against 3 litres of distilled water at 5°C. for 18 hours (final volume = 29 ml).

20 ml of the dialysed culture fluid was applied to the column and air pressure of 0.5 kg/cm^2 applied to the top. The column was eluted with the following solutions:

1) 20 ml 0.01 M citrate pH 4
2) 30 ml 0.01 M NaCl in 0.01 M citrate pH 4
3) 30 ml 0.02 M " " " " "
4) 30 ml 0.03 M " " " " "
5) 30 ml 0.05 M " " " " "

Thirty-two 5.0 ml fractions were collected and assayed for protein, maceration and liberation of reducing groups.

There were protein peaks at fractions 5 and 25. Reducing group activity on sodium polypectate at pH 4.5 also showed peaks at fractions 4 and 25. However, macerating activity higher than 0.5, which is a low value, could not be detected in any of the fractions collected. Maceration can, therefore, not be correlated with reducing group activity on pectate. It is possible that the enzyme or enzymes responsible for maceration were inactivated during the exchange reactions as has been reported for polygalacturonase by Swinburne and Corden (1967).

E. PRODUCTION OF EXTRACELLULAR ENZYMES ON AN ORANGE ALBEDO MEDIUM

Penicillium digitatum is a wound parasite. Kavanagh (1965) showed that the albedo tissue, unlike the flavedo tissue, when exposed on wounding, yielded readily to fungal invasion. The albedo tissue wound, therefore, seem a good substrate for investigating the initial stages of enzyme production *in vitro*. The fungus was grown on an albedo extract medium as described on p. 31. After 6, 12 and 24 hours incubation, culture filtrates and sodium chloride extracts of the residual material were collected (p. 33).

The filtrates and extracts were assayed for macerating and pectic enzyme activity, at pH 5.0, and examined chromatographically for degradation products on Whatman No. 1 paper. The papers were irrigated for 72 hours in butanol-acetic acid solvent and developed with $AgNO_3$ reagent. The results of the enzyme assays and chromatographic analyses are shown in Table 29.

A number of enzyme activities could be detected in the filtrates and extracts from the 6 hour cultures. The values obtained were low, but easily detectable. The "chain splitting enzyme" was active on pectin but not on NaPP because there was no reduction in viscosity of the

Table 29

Activity of filtrates and NaCl extracts of mycelium from cultures on an orange albedo medium

Age of culture (hr)	pH of filtrate	Sugars detected in filtrates by chromatography	Viscosity reducing activity (100/time for 10% viscosity loss) Pectin	NaPP	Reducing group activity (P) (% hydrolysis /24 hr)	PTE activity (µmoles CH_3O/ 15 min)	PME activity (% hydrolysis /30 min)	Macerating activity
6	4.8	glucose++++ fructose++++ ? (R_{GA}0.57)+	3.3 (3.3)	0 (0)	0.5 (1.0)	0.04 (0.05)	0 (0)	3.8 (3.2)
12	4.1	glucose++++ fructose++++ monoGA+ ? (R_{GA}0.57)	6.7 (4.5)	0 (0)	1.9 (4.2)	0.11 (0.13)	0 (0)	5.5 (4.3)
24	3.5	glucose++++ fructose++++ galactose+++ monoGA++++ ? (R_{GA}0.57)+	6.7 (4.4)	0 (0)	2.6 (4.2)	0.34 (0.22)	0 (0)	7.6 (5.6)
Healthy	4.6	glucose++++ fructose++++ ? (R_{GA}0.57)+	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The enzyme activities of the NaCl extract are given in brackets

+ sign indicates size of spot

test NaPP solution by the filtrates or extracts at pH 4.0 or 5.0. Macerating activity was detected in all the filtrates and extracts. Macerating activity cannot, therefore, be equated with the viscosity reducing activities, even after 6 hours, was surprisingly high. Pectin transeliminase was detected in all the filtrates and extracts. Pectin methylesterase activity was not detected. The pH of the culture fluid fell quite rapidly to 3.5 after 24 hours incubation. Galacturonic acid was detectable in the culture fluid, by chromatography after 12 hours incubation, and after 24 hours incubation both galacturonic acid and galactose were present.

F. DEGRADATION OF WASHED ORANGE ALBEDO AND ORANGE PROTOPECTIN

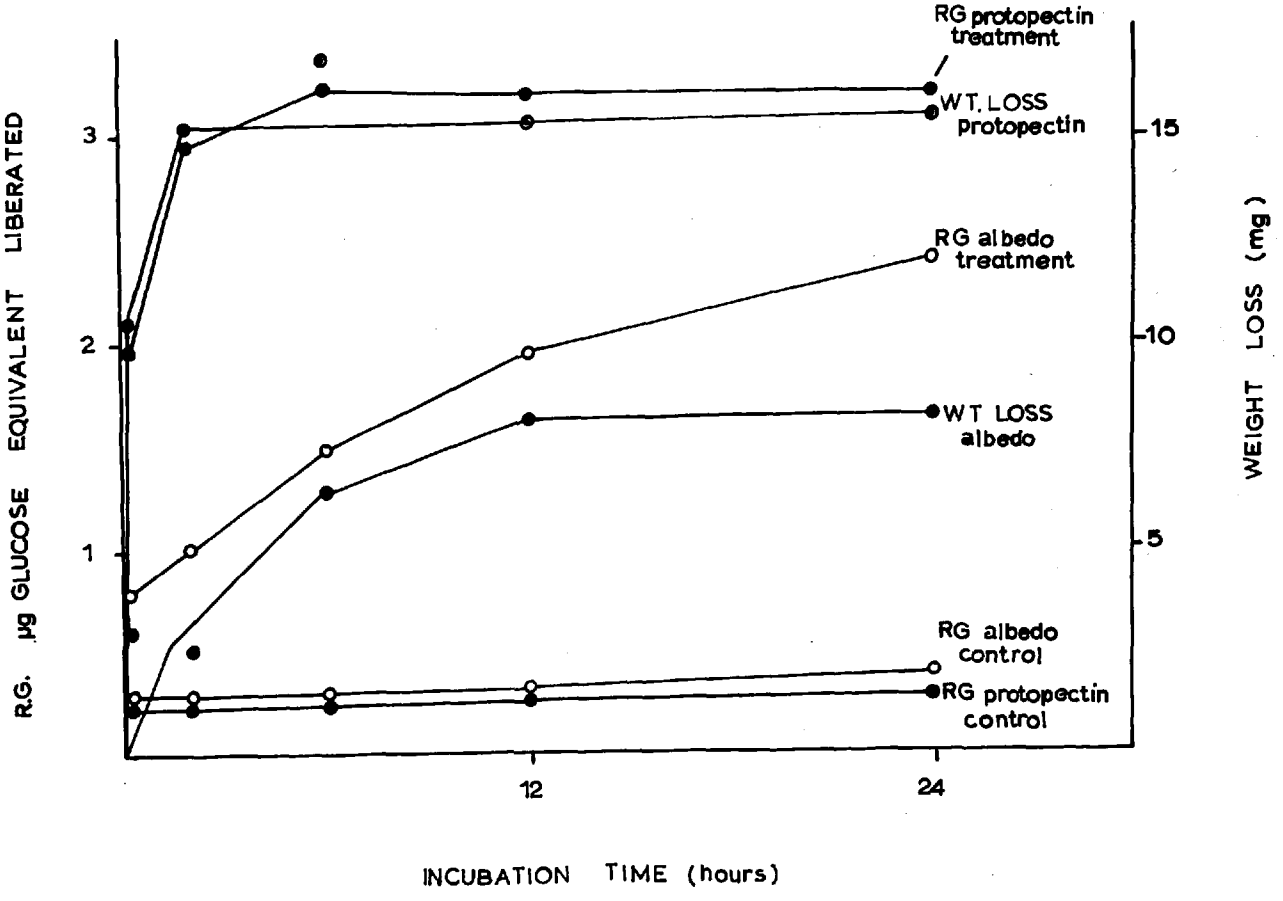
Washed orange albedo and orange protopectin was prepared as described in Materials and Methods, p. 34-35.

0.15 g albedo tissue and 0.10 g protopectin samples were weighed into clean, weighed test tubes. These samples were then incubated with 4 ml of a dialysed (freeze-dried) enzyme preparation (0.01 g/ml) adjusted to pH 4.5. An autoclaved enzyme preparation was used for the controls. A drop of toluene was added to each tube to prevent bacterial contamination over the course of the experiment.

Supernatants were collected at the sampling times of 30 minutes, 2, 6, 12 and 24 hours. The test tubes containing the insoluble residues were plunged into boiling water for 10 minutes to inactivate any enzymes and then placed in an 80°C. oven until the residues were dry when the test tubes were finally reweighed. Reducing group determinations and chromatography analyses were made on the supernatants.

Fig. 12 shows the loss in weight of the albedo and protopectin samples and increase of reducing groups in the supernatants upon incubation, and that there was quite a close correlation between the two over the course of the

Fig. 12. WEIGHT LOSS AND REDUCING GROUP LIBERATION FROM WASHED ALBEDO AND PROTOPECTIN AFTER INCUBATION WITH CULTURE FLUID



experiment.

The results of the chromatographic analyses of the supernatants are shown in Table 30.

Table 30

Paper chromatographic analysis of supernatants

(butanol-acetic acid solvent - 72 hr irrigation)

Spots	Washed albedo				Protopectin			
	Incubation time (hr)							
	0.5	6	24	Control	0.5	6	24	Control
monoGA	++	++	+++	+	+++	+++	++++	+
galactose	++	++	+++	tr	+++	+++	++++	-
arabinose	++	+++	++++	tr	++	+++	+++	-
unknown (R_{GA} 0.55/0.61)	-	-	tr	tr	tr	tr	tr	-

+ sign indicates size and intensity of spot

- sign indicates absence of spot

tr indicates a trace.

Galactose and arabinose spots were confirmed by ascending chromatography on Whatman No. 1 paper using a pyridine-ethyl acetate solvent. The papers were irrigated for 24 hours at 25°C. and papers after drying sprayed with the aniline diphenylamine reagent. A third solvent useful for resolving oligossacharides was also used for

analysing the washed albedo supernatants. Whatman No. 1 papers were irrigated for 24 hours in n-propanol-ethyl acetate-water solvent at 25°C. and developed with $AgNO_3$ reagent. The 12 and 24 hour supernatants were analysed and the results are shown in Table 31.

Table 31

Chromatographic analysis of washed albedo supernatants

Spot	Incubation time 12 hour	Incubation time 24 hour	Control (24 hr)	RGA
A	+	+	-	3.08
xylose	-	-	-	2.39
fructose arabinose	+++	+++	+	2.18
glucose galactose	+++	+++	+	1.98 1.84
B	++	++	-	1.48
monoGA	++++	++++	++	1.00
C	tr	tr	-	0.63
D	tr	tr	-	0.31

+ sign indicates size and intensity of spot

- sign indicates absence of spot

tr indicates a trace.

With the active filtrate, using this solvent the GA spot was elongated and showed the double lactone-uronic acid form. The above R_{GA}'s are based on the smaller slower moving part. In the autoclaved control treatments, the GA spot did not have the double form. Arabinose and fructose, glucose and galactose were insufficiently resolved to distinguish between them. In consequence, they are bracketed in the above table.

Considering weight loss in the protopectin preparation, it may be seen that approximately 13 per cent of the material was solubilized in 1 hour's treatment with active culture filtrate. At this time, about 10 per cent of the protopectin was found as reducing groups in the soluble fraction. It would appear, therefore, that the majority of the soluble products were monosaccharides. From paper chromatography, these monosaccharides were shown to be "galacturonic acid", galactose and arabinase.

The absorption spectra of each supernatant after 24 hours incubation was examined. No absorption peaks at 230 or 235 mμ were detected in the washed albedo or protopectin supernatants. No red colouration was obtained on reaction of the supernatants with thiobarbituric acid. There was, therefore, no evidence of pectic lyase activity. The enzymes responsible for the breakdown of the protopectin

would, therefore, appear to be pectic hydrolase, arabanase and galactanase. The relatively low percentage weight loss during treatment showed that a great proportion of the protopectin constituents were not solubilised. This was possibly caused by the inactivation of the enzymes. It would be interesting to determine the position at a later stage, after the addition of fresh enzyme solution.

G. GROWTH AND ENZYME PRODUCTION ON HEMICELLULOSES

In addition to pectin and cellulose, plant cell walls are known to contain hemicelluloses. Few studies have been made of the extracellular fungal enzymes catalysing the hydrolysis of hemicelluloses or of their significance in pathological processes. Only a few fungi have been studied for their ability to produce hemicellulases. There has been some evidence that hemicellulases may play a role in tissue maceration. The growth and enzyme production of Penicillium digitatum grown on a medium containing hemicelluloses was investigated.

The following medium was prepared:

Cas-amino acids (Difco vitamin free)	4.6 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Hemicellulose	2.5 g

Trace element solution (see p. 31) 2.5 g

Water to 1 litre.

Three hemicelluloses were incorporated into the medium:

1) Jute hemicellulose (Fraction B, water soluble)

2) Larch (arabino-galactan, water soluble)

3) Spruce (mixture of mannan + xylan, water soluble).

50 ml media were dispensed into 150 ml flasks inoculated with 1 ml of a 10 million/ml spore suspension and placed on a Baird and Tatlock horizontal shaker at 25°C. for 6 days.

At the end of this period, the culture filtrates were collected, the mycelium harvested and dry weight estimated. The results can be seen in Table 32.

Table 32

Growth of P. digitatum in media containing hemicelluloses

hemicellulose	pH of medium	Mean dry wt. of mycelium (mg) (3 replicates)
jute	6.8	22.7 (±4.0)
larch	6.9	19.3 (±2.6)
spruce	6.6	18.8 (±7.7)

Figures in brackets represent the standard deviations.

Growth was sparse in the three media. The culture filtrates were tested for macerating activity at pH 5.5. The activities for jute, larch and spruce culture filtrates respectively were 6.9, 6.3 and 5.1.

The jute hemicellulose culture filtrate and a 7 day old culture filtrate grown on medium B containing pectin were used to examine the degradation of hemicelluloses.

The culture filtrates were added to the following substrates:

spruce glucommannan (water insoluble)

spruce xylan and mannan (water soluble)

larch galactan (water soluble)

esparto hemicellulcse (insoluble)

jute hemicellulose (soluble)

potato cell wall preparation (insoluble)

in reaction mixtures containing:

0.05 g hemicellulose (0.02 g potato cell wall)

1.0 ml culture fluid

1.0 ml citrate buffer (pH 5.5)

The reaction mixtures were incubated for 21 hr in test tubes at 25°C. with a drop of toluene added.

After this period, the supernatants of the reaction mixtures containing insoluble substrates were obtained by centrifuging at 2,000 g for 10 minutes. These

supernatants and the whole reaction mixtures of the soluble substrates were assayed chromatographically. 25 µl samples were applied to Whatman No. 1 papers. The papers were developed by ascending technique in iso-propanol-water solvent for 18 hours. Arabinose, galactose, xylose, glucose, mannose and galacturonic acid were used as markers. Concentrations of reaction mixtures applied to the paper were such that a 20 per cent breakdown of the substrates to monosaccharide would give spots of the same strength as the marker spots. A concentration of 5 to 10 times less than the marker spots would be expected to be detectable. The papers were developed with aniline diphenylamine reagent. Autoclaved culture filtrate was used in the control reaction mixtures.

None of the hemicelluloses were degraded by the culture fluids under the experimental conditions employed. The action of pectin culture filtrate on potato cell wall yielded a trace of galacturonic acid and a large amount of galactose (approx. 75 per cent that of the marker spot). Jute culture filtrate on potato cell wall yielded a strong galactose spot but galacturonic acid was not detectable.

The activity of crude jute hemicellulose culture filtrate on pectin, NaPP, orange protopectin and potato cell wall was also examined chromatographically using the

descending technique. Whatman No. 1 papers were irrigated with butanol-acetic acid solvent for 66 hours.

0.05 g orange protopectin and 0.01 g potato cell wall was incubated with 2 ml culture fluid and 2 ml 0.1 M citrate buffer at pH 4.5. Incubation of culture filtrate with NaPP and pectin was in the proportions of the normal reaction mixture. Reaction mixtures were incubated for 24 hr at 25°C. Autoclaved culture filtrate was used for the controls.

The results of the chromatographic analysis are shown in Table 33.

Table 33

Chromatographic analysis of reaction mixtures containing jute hemicellulose culture filtrate

Spots	Substrates							
	Pectin		NaPP		Protopectin		Potato cell wall	
	RM	C	RM	C	RM	C	RM	C
monoGA	++	-	++	-	-	-	-	-
glucose	-	-	-	-	-	-	-	-
galactose	++	-	++	-	+++	-	+++	-
arabinose	-	-	+	-	++	-	++	-
xylose	-	-	+	-	++	-	-	-
R_{GA} 1.64/1.85	-	-	+	-	++	-	tr	-
base-line	-	-	-	-	tr	-	tr	tr

RM = reaction mixture, C = Control containing autoclaved filtrate. + indicates size and intensity of spot, - indicates absence of spot, tr indicates a trace.

Galacturonic acid and galactose were detected in reaction mixtures containing pectin. In those containing NaPP, five products were found: galacturonic acid, galactose, arabinose, xylose and an unknown product with an R_{GA} of 1.64 - 1.85.

Galacturonic acid could not be detected from reaction mixtures containing protopectin or potato cell wall. Galactose was present in large amounts from both reaction mixtures and arabinose to a slightly less degree. Xylose was present in reaction mixtures containing protopectin but not in those containing potato cell wall.

The unknown spot was present in reaction mixtures containing protopectin and a trace was detected in those containing potato cell wall.

Xylanase production by P. digitatum was confirmed by growing it on medium B containing 1 per cent esparto hemicellulose. After 15 days incubation, at 25°C., in static culture, the culture filtrate was collected and examined chromatographically. The ascending technique was used with n-butanol-ethanol-water solvent. Reducing sugars were detected with the aniline diphenylamine reagent.

Xylose was detected in the culture filtrate after fungal growth, but could not be detected in uninoculated

medium.

Although degradation products from the activity of a jute hemicellulose and a pectin culture filtrate on five hemicelluloses were not detected, the activity of these filtrates on a potato cell wall preparation yielded a strong galactose spot. Further chromatographic analyses of the products of the activity of jute hemicellulose culture filtrate on a protopectin and a potato cell wall preparation yielded galactose, arabinose and xylose, the breakdown products of non-pectic substances. Galacturonic acid or polymers of galacturonic acid were not detected, although galacturonic acid was found in reaction mixtures containing pectin and NaPP. These culture filtrates showed high macerating activity and, therefore, the possibility that hemicellulases, such as arabanase, galactanase and xylanase, play a role in tissue maceration must be considered.

2. ENZYME PRODUCTION IN VIVO

Kavanagh (1965), in a study of inoculated orange fruits, showed that fruit wounded into the outer flavedo layer showed a resistance to infection, whereas when deeper wounds into the albedo layer were made, this resistance was overcome. It seems that the "spongy" albedo layer yields more readily to the fungus. Also the hyphae in wounds into the albedo do not penetrate the flavedo cells around the wounds, but grow down into the albedo layer. The albedo layer has a high pectin content and the ability of the fungus to secrete enzymes *in vivo* to degrade this pectin might have some bearing on its pathogenicity.

Extracts of sound and infected orange rind tissue and orange juice from sound and infected fruit were examined for enzyme activity.

A. DETERMINATION OF ENZYME ACTIVITY IN THE RIND OF SOUND AND INFECTED ORANGES

Extracts of healthy and diseased fruit were prepared as described in Materials and Methods, p. 33. The extracts were assayed for pectin methylesterase, viscosity reducing, macerating and pectin transeliminase activity, and analysed by paper chromatography. Orange rind has long

been used for the preparation of PME. Polygalacturonase was shown to be absent from orange flavedo by MacDonnell et al. (1945), and the only other reference to pectic enzymes, other than PME, present in citrus fruit was by Pratt and Powers (1953) who, on the basis of a decrease in the viscosity of pectin solutions reported the presence of a depolymerizing enzyme in grapefruit juice which was more heat resistant than PME.

1. Pectin methylesterase

The PME activity of healthy and diseased orange rind extracts was measured over a wide pH range. The results are shown in Table 34. Activity is expressed as the percentage hydrolysis of methyl ester groups of the substrate in 15 minutes.

Table 34

Effect of pH on PME activity of sound and infected orange rind tissues

	% hydrolysis/15 min							
pH	3.8	4.5	5.0	5.5	6.0	6.5	7.1	7.7
Healthy	0	-	-	3.0	-	9.0	11.2	16.4
Infected	0	5.6	6.7	6.7	3.7	4.5	5.2	7.5
Control	0	0	0	0	0	0	0.2	0.4

The occurrence of PME in healthy citrus rind was demonstrated by MacDonnell et al. (1945) who found the pH optimum for activity depended upon concentrations and kind of cations present in the reaction mixture. In the presence of 0.05M $CaCl_2$, for example, the pH activity curve formed an almost level plateau from pH 5.0 to 8.0. In the results given in Table 34, activity rises with pH to a maximum at the highest pH value measured.

The extract of diseased rind tissue showed two peaks of activity, one at pH 5.0 - 5.5 and the other at 7.1 - 7.7. There was only a very slight demethylation in the control (autoclaved enzyme preparation) at pH 7.1 - 7.7, which would not account for the second peak of activity. It is suggested that the peak at 5.0 - 5.5 is caused by fungal PME and that at 7.1 - 7.7 by the orange PME. The PME activity of the orange rind tissue was reduced by about one half on infection.

2. Maceration

The macerating activities of the extracts of sound and infected orange rind were measured over a wide range of pH. The activities are shown in Table 35.

Table 35

Effect of pH on maceration

pH	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
Macerating activity	4.8	5.6	5.9	6.3	7.2	7.4	7.1	6.0	5.4	4.2	3.9

Macerating activity was not detected at pH 3.0 - 8.0 in healthy rind extract or autoclaved extract from infected rind. The optimal pH for macerating activity of the infected rind extract was pH 5.0 - 6.0. However, the enzyme was active over a wide pH range.

3. Viscosity reducing enzymes

Extracts of healthy and infected rind did not reduce the viscosity of CMC solutions at pH 3.0, 4.0, 5.0 or 6.0. Cellulase was, therefore, absent for these extracts. Very slight viscosity reducing activity was detected on pectin at pH 5.0, and on NaPP at pH 4.0 (less than 5 per cent viscosity loss in 30 min) in extracts of infected rind tissue. Healthy rind extracts were inactive.

4. Pectin transeliminase

PTE activity was not detected from extracts of healthy or infected rind tissues at pH 3.5, 4.5, 5.5 and 6.0.

5. Proteolytic activity

Proteolytic activity could not be detected in healthy or infected rind extracts at pH 7.0, using the method of Kunitz (1947).

6. Analysis of extracts by paper chromatography

Evidence for the action of pectic and possibly other enzymes during the rotting of oranges was obtained by

paper chromatography of reducing compounds in extracts of infected rind in comparison with those extracts of sound rind, as described under Materials and Methods. Galacturonic acid, xylose, arabinose, fructose and glucose were identified by comparing the R_{GA} values obtained when juices and authentic substances were run on the same chromatogram. Measurement of R_{GA} values of other spots were made in an attempt to identify them. Chromatograms were irrigated for 114 hours. Table 36 shows the distribution of spots of known and unknown compounds in extracts of infected and sound orange rind.

Spots A, C and D and monogalacturonic acid (monoGA) were detected in extracts of infected tissue but not in extracts of healthy tissue.

Spots B, E and base line were present in extracts of both sound and diseased tissue. It was thought possible that spots GA, A, C, D might be members of a series, A, C and D being polymers of galacturonic acid. Jermyn and Tomkins (1950) stated that a log function of the Rf values of breakdown products of pectic substances increased regularly with increasing molecular complexity. For their results an almost straight line was obtained when the theoretical number of αGA units per polymer molecule was plotted against $\log_{10} R_{GA}$ for the corresponding spot.

Table 36

Paper chromatographic analyses of extracts of healthy and infected orange rind

Spots	Infected	Healthy	R_{GA}
xylose	++	++	2.28
arabinose fructose	+++	+++	2.07 2.02
glucose galactose	++++	++++	1.40 1.20
monoGA	+++	-	1.00
A	tr	-	0.80
B	++	++	0.52
C	+	-	0.38
D	+	-	0.24
E	+	+	0.10
base line	tr	tr	-

+ indicates size and density of spot

- indicates absence of spot

tr indicates a trace.

Arabinose almost coincided with fructose, and galactose with glucose, so arabinose and galactose were insufficiently resolved for positive detection in these extracts, and, therefore, have been bracketed together in Table 36.

Spots A, C and D were assumed to be members of a series containing 2, 3 and 4 αGA residues respectively. The log R_{GA} of these spots when plotted against the number of residues gave a straight line with a gradient of -0.21. This gives a ratio of movement by each successive spot in the series of 1.6. The R_{GA} values of spots GA, A, C and D are 1.00, 0.8, 0.38 and 0.24 respectively. Spot A is the only member of the series which does not conform to a ratio of movement of 1.6. However, spot A is closely associated with a well defined, colourless spot on the chromatogram and spot A appears to have run in front of this spot giving an increased R_{GA} value. It is, therefore, considered likely that spots A, C and D are di-, tri- and tetra-galacturonic acid respectively.

B. DETERMINATION OF ENZYME ACTIVITY IN THE JUICE OF SOUND AND INFECTED ORANGES

The juice from 6 oranges in the following stages of infection was collected as described in Materials and Methods, p. 34 :-

a) white mycelium stage, $3\frac{1}{2}$ days after inoculation. At this stage, fungal hyphae can be detected ramifying in the pulp of the orange beneath the lesion.

b) where a quarter of the orange was covered with green spores and the white mycelium had reached the equator of the fruit, 6 days after inoculation. Extensive mycelial ramification in the pulp is seen at this stage of infection.

c) where the fruit was 3/4 covered with green spores and the white mycelium had covered the remaining portion of the fruit, 7 days after inoculation.

d) healthy fruit, after 7 days.

The juice from the whole orange was collected.

The volumes and pH of the juice collected were:

	Volume ml	pH
a)	198	3.4
b)	224	3.4
c)	220	3.4
d)	240	3.6

In the case of treatment (c) the fruit were very soft and tended to disintegrate in the juice extractor. When this occurred, the juice was collected from the residue by squeezing through several layers of muslin.

The juice from sound and infected orange fruit were assayed for cellulase and pectic enzyme activity.

1) Pectin methylesterase activity

PME activity could not be detected in the juice of healthy or infected fruit at pH 5.0.

2) Macerating activity

Macerating activity was measured at pH 4.5, and the results are shown in Table 37.

Table 37

Macerating activity of juice from sound and infected oranges

Days after inoculation	Macerating activity
a) $3\frac{1}{2}$ days	1.7
b) 6 days	2.3
c) 7 days	2.3
d) Healthy (7 days)	0

Macerating activity was detected in the $3\frac{1}{2}$, 6 and 7 day juice of infected fruit. Activity was not detected in the juice of healthy fruit or in autoclaved juice from infected fruit.

3) Viscosity reducing activity

Very slight activity (a reduction in flow time of 1 or 2 seconds over 30 minutes) could be detected in the $3\frac{1}{2}$, 6 and 7 day juice from infected fruit when tested on pectin. No activity could be detected when tested on NaPP or CMC at pH 3.5, 4.5 and 5.5 or in healthy juice when tested on pectin and NaPP. The macerating activity was relatively high compared with the viscosity reducing activity which was negligible. It is difficult to

envisage in these circumstances that maceration is caused by the viscosity reducing enzymes.

4) Pectin transeliminase activity

PTE activity was not detected in the juice of sound and infected fruit at pH 4.5.

5) Paper chromatographic analyses of the juice of sound and infected fruit

Chromatographic analysis of the juice from healthy and infected fruit indicated increasing concentrations of galacturonic acid from the 3 day to the 7 day samples of infected fruit. It was, therefore, apparent that the enzymes responsible for the production of galacturonic acid were being inactivated in some way, since no activity could be detected in the viscosity reducing tests. To test this hypothesis, juice of healthy fruit was added to a dialysed culture filtrate preparation (freeze-dried filtrate 0.01 g/ml) and the viscosity reducing activity determined in the normal way.

The following treatments were investigated:

(1) 0.5 ml culture filtrate was added to:

a) 0.5 ml juice of healthy fruit and

b) 0.5 ml distilled water.

The resultant mixtures were used as the enzyme preparations in viscosity reducing tests on sodium

polypectate at pH 4.5. It was found that both preparations reduced the viscosity of sodium polypectate by 10 per cent in 1.25 minutes.

(2) 0.25 ml culture filtrate was added to:

a) 0.75 ml juice of healthy fruit and

b) 0.75 ml distilled water

and left to stand at laboratory temperature for 1 hour before assaying on NaPP as previously described. No viscosity reducing activity could be detected for the culture filtrate with added orange juice. The culture filtrate + water gave a 10 per cent reduction in viscosity of NaPP in 2.25 minutes.

(3) 0.25 ml culture filtrate was added to

a) 0.75 ml undialysed juice of healthy fruit and

b) 0.75 ml dialysed juice of healthy fruit

(33 ml orange juice was dialysed overnight in 5 litres of distilled water (final volume = 48 ml).

c) 0.75 ml autoclaved undialysed orange juice

d) 0.75 ml water

at laboratory temperatures before testing as previously. Only very slight activity could be detected in treatments (a) and (b) (a reduction in flow time of 2 - 3 seconds in 10 minutes). Treatments (c) and (d) showed a 10 per cent reduction of viscosity in 7 minutes.

The difference between treatment (b) in the previous experiment and treatment (d) can be explained by the fact that the culture filtrate used had been kept in a refrigerator for 48 hours and there had been some reduction in activity, as experienced in earlier experiments on standing.

It can be concluded from these experiments that unautoclaved orange juice inactivates in some way the viscosity reducing enzymes. Autoclaved orange juice does not inactivate these enzymes.

6. The effect of orange juice on macerating activity

0.5 ml of a culture solution prepared from freeze-dried culture filtrate (0.01 g/ml) was added to

a) unautoclaved orange juice from healthy fruit

b) autoclaved orange juice from healthy fruit

c) water

and allowed to stand for 5 minutes at laboratory temperature. Maceration was measured in the normal way at pH 4.5. The results are shown in Table 38.

Table 38

Effect of orange juice on macerating activity

Addition	Average time for maceration (min)
orange juice	approx. 180
autoclaved orange juice	12
water	12

Macerating activity was greatly inhibited by the addition of unautoclaved orange juice. The inhibition was not evident when autoclaved orange juice was used.

C. CONIDIAL ENZYMES

Van Sumere et al. (1957) showed that uredospores of Puccinia graminis var tritici contained enzymes capable of hydrolysing plant polysaccharides. These enzymes were considered to play an important role both during the germination of the rust spores and during infection of the stem and leaf of the host. An attempt was made to show the existence of cellulase, pectic hydrolase and macerating activity in the conidia of P. digitatum.

An extract of spores was prepared as described on page 38. The ability of this extract to reduce the viscosity of pectin, NaPP and CMC solutions at pH 4.5 was determined in the usual manner.

Table 39

Viscosity reducing activity of a conidial extract

Activity is expressed as $\frac{1000}{\text{time}}$ for a 25 per cent reduction in viscosity.

Substrate	Treatment	Activity Autoclaved control
Pectin	6.0	0
NaPP	4.8	0
CMC	0	0

Both pectin and sodium polypectate were degraded, CMC was not. The spores in this experiment had been taken from diseased oranges and, therefore, were probably bacterially contaminated. Toluene was added to the reaction mixture to prevent bacterial growth during the experiment.

Macerating activity of the spore extract at pH 4.5 was 2.3.

It is possible that some of this activity recorded may have been derived from contaminating bacteria, but this is considered unlikely, especially as the broken spores viewed under a microscope showed little evidence of bacterial contamination. Pectic enzyme constituents of spores has also been demonstrated in Colletotrichum orbiculare (Porter, 1963) and Puccinia graminis var tritici (van Sumere et al., 1957).

The presence of pectinases in ungerminated spores, and their detection in culture filtrates a short time after germination, may indicate their potential involvement in the establishment of infection.

DISCUSSION

In this investigation, a study has been made of the extracellular enzymes produced by *Penicillium digitatum* which may play a part in its pathogenicity. The ultimate aim of such studies is the possible development of a control mechanism for green mould of citrus fruit. An attempt has been made to relate the enzyme activity of the fungus to the process of tissue maceration. The main approach to this problem has been the examination of crude culture filtrates from a variety of media to test for a number of enzyme activities which might be correlated with macerating activity. Attempts were made to fractionate the enzymes in these culture filtrates to separate macerating from other activities.

The ability to produce cell wall degrading enzymes *in vitro* does not always reflect the position *in vivo*. Harter and Weimer (1921) showed that the macerating activity of culture solutions in which various species of *Rhizopus* had been grown bore no relation to the capacity of the organisms themselves to cause disease.

Other workers have also failed to correlate the activity of enzymes produced *in vitro* and the activities of extracts of infected host tissues (Cole, 1956;

Bateman, 1963a; de Silva, 1963). An examination of the enzymes produced *in vivo* was, therefore, also undertaken to determine any correlation between *in vivo* and *in vitro* studies.

In most plant pathogenic fungi, pectic enzymes are induced rather than constitutive. In culture filtrates of *P. digitatum* grown on a 2 per cent glucose medium (medium A) high PME, viscosity reducing and macerating activities were recorded. In addition, the constitutive production of cellulase, α-L-arabinofuranosidase and β-D-galactopyranosidase was observed. All these enzymes were also produced when the parasite was grown on media containing pectin or NaPP. Enzyme production on these substrates was found to be independent of growth. Both pectin and NaPP were degraded in the absence of pectin methylesterase. The very slow release of reducing groups from pectin or NaPP substrate, but high viscosity reducing activity, indicates the production of endo-enzymes. Pectic hydrolase activity was demonstrated in the absence of pectic lyase activity in filtrates from cultures grown on medium D containing cas-amino acids and pectin or NaPP.

Pectin methylesterase and macerating activity was shown in extracts of infected orange rind, but pectin and pectate transeliminase and cellulase activity was not

found. Only a very slight viscosity reducing activity on pectin and NaPP was detected. In juice from infected fruit, viscosity reducing activity was not found, although some macerating activity was detected.

The production of pectic enzymes by the fungus during the infection of oranges would be a reasonable assumption because of the high pectin content of the rind. Approximately 30-40 per cent of the dry weight of the albedo tissue is pectic in nature (Sinclair, 1961). Chromatographic analyses of healthy and infected orange rind tissues indicated a high concentration of galacturonic acid in the diseased tissues. If pectic enzymes are produced during the production of the disease lesions, then since their activity cannot be detected but the products of their activity can, they must be inactivated in some way.

Orange juice was found to inhibit the activity of the viscosity reducing and macerating enzymes. By autoclaving the orange juice, this inhibition was overcome. A thermolabile inactivating substance or substances must be postulated as operative after the enzymes have produced their effects. Phenolic compounds, and in particular their oxidation products, have been shown to inactivate pectinases (Byrde et al., 1960; Deverall

and Wood, 1961). Phenolic compounds are found in great abundance in citrus fruit and it is possible they may have an inactivating effect, although polyphenol oxidase has never been detected.

Although orange juice was shown to inhibit the macerating activity of a culture filtrate, macerating activity was found in the juice from infected oranges. It appears, therefore, that the inactivating substance (or substances) is not operating *in vivo*. It is possible some interaction between the constituents of the culture filtrate and those of the orange juice might occur, in which a toxic product is formed, inhibiting enzyme activity. Another possible explanation is that the quantities of macerating enzyme produced *in vivo* are so large that the amount of inactivator present is inadequate. A third possibility is that the macerating factor produced *in vivo* is not the same as that produced *in vitro*. In orange rind, however, when culture filtrate is injected into the rind tissue, a typical water soaked lesion, identical to that produced at the beginning of infection is seen. This effect is not seen if water or autoclaved filtrate is injected. Thus, even if the enzymes are different, they still produce the same pathological effect in rind tissue. It is possible that a separate macerating enzyme is produced in the

infection of the pulp.

Maceration has been attributed to the activity of many enzymes, including cellulase, pectin methylesterase, polygalacturonase and pectic lyase.

Cellulase is not regarded as of great importance in tissue maceration. In many studies *in vitro* and *in vivo* cellulase activity has been absent or very low when macerating activity has been reported high. Cellulase activity from filtrates of cultures grown on medium A was absent, yet macerating activity was high. Cellulase was not found in extracts of diseased rind tissue, where macerating activity was detected. Glucose was not detected during paper chromatographic examination of the degradation products of washed albedo tissue, orange protopectin or potato cell wall.

Similarly pectin methylesterase is generally not considered significant in maceration, although synergistic effects have been reported between PME and the activity of endo-PG (Jansen *et al.*, 1945), and tissue degradation (Yamazaki, 1957). Culture filtrates of many fungi grown *in vitro* have shown macerating ability without any corresponding PME activity (Wood, 1955; Bateman, 1963a; Spalding, 1963; Dean and Wood, 1967). Garber *et al.* (1965) could not detect PME in culture filtrates of

virulent strains of P. digitatum. In this study the macerating culture filtrates of the fungus grown on an albedo extract medium did not possess PME activity. PME could not be detected in any of the fractions possessing macerating activity after Sephadex gel filtration of crude culture fluid.

Macerating activity was attributed to the activity of endo-PG and endo-PGTE by Bateman (1964) and pectate transeliminase by Dean and Wood (1967). My results have not confirmed their findings. From cultural studies on different media, high macerating activity has been found with little or no pectic hydrolase or lyase activity. In vivo, macerating activity has been detected without any corresponding pectic enzyme activity. Where macerating activity has been found with pectic enzyme activity, there has been a marked discrepancy between their activities. Davison and Willaman (1927), Gupta (1958) and Jarvis (1953) reported that macerating activity may be distinguished from polygalacturonase, as it was then defined. Gupta and Jarvis found that certain properties of "protopectinase" parallelled a "depolymerase". However, Jarvis stated that protopectinase was distinct from polygalacturonase,

depolymerase and pectinmethylesterase. The conclusions of these workers were based upon a similarity or disimilarity of properties between the activity of the macerating enzyme and the activity of the respective pectic enzyme. These properties include pH optima for substrate degradation thermal inactivation characteristics, the action of stimulatory or inactivating agents and the dependence of activity upon certain cations, especially calcium.

Endo-transeliminases were reported by Edstrom and Phaff (1964) to be stimulated by Ca^{++} and some were shown to be dependent upon Ca^{++} (Bateman, 1966). Starr and Moran (1962) and Bateman (1965) found Ca^{++} was inhibitory to endo-PG activity on pectic substrates.

The viscosity reducing enzymes of P. digitatum were not dependent upon calcium. The addition of $CaCl_2$ to reaction mixtures did not stimulate or inhibit activity on pectin or NaPP. EDTA, which sequestres calcium did not have any effect except at very high concentrations when activity was slightly inhibited. Calcium and magnesium, however, appeared to have some stabilizing effect on dialysis.

Joslyn (1962) and McClendon and Somers (1960) have discussed the possibility that maceration may result from

the chelation of calcium. Carr and Ng (1959) determined that calcium was removed from wheat coleoptile cell walls and filter paper by citric-phosphate buffer at pH 5. During maceration tests in this investigation citrate buffer was nearly always used (0.1 M) and it would, therefore, appear that the exchangeable calcium would be removed from the potato discs. However, since no maceration occurred in control solutions containing citrate buffer, the strength of slices must be due to the organic structure and maceration to the enzymatic components of the solutions used.

The pH optimum for degradation by pectic glycosidases in culture filtrates and extracts of diseased tissues and the pH optimum for maceration has often been found to be closely similar (Talboys, 1950; Tribe, 1955; Echandi and Walker, 1957; Bateman, 1963). In culture filtrates from medium D the pH optimum for maceration was 3.5, whereas the optimum for viscosity reducing activity on pectin and NaPP was 4.5. This difference in pH optima, although small, may indicate that macerating activity cannot be correlated with viscosity reducing activity.

Culture filtrates of the fungus grown on medium C had a pH optimum for maceration of 5.5, yet in medium D it was 3.5. The constituents of medium D were different to C

only in the composition of the micro-element solution. The pattern of enzyme activity has also been shown to depend upon the pH of the culture medium (Hancock, 1965; Bateman, 1966). The pH of medium C was 5.0 and that of D 4.3. It is considered unlikely that this small pH change could have such a profound effect on the pH optima for maceration. If this is so, then the micro-element constituents of the medium have a marked effect on the enzymatic pattern.

The fluid from cultures grown on an orange albedo medium showed macerating activity, and reduced the viscosity of a pectin solution, but not a NaPP solution. In maceration, the cells of a tissue are thought to separate along the middle lamella which is often considered to consist of calcium and magnesium pectates. If maceration occurs from the breakdown of pectates, it would be expected that a macerating solution would degrade NaPP. This was not so in the filtrates from the orange albedo medium or in eluates from a cellulose phosphate column. The middle lamella may, therefore, contain methylated pectic substances or other substances such as protein or hemicelluloses or the degradation of other layers than the middle lamella results in maceration.

In addition to cellulose and pectin, plant cell walls

contain large quantities of hemicelluloses, the detailed structures of which are still largely unknown. It is known that most represent mixtures of two types of polysaccharide: one composed of pentose or hexose sugar units, and the other of polyuronides containing one or more glucuronic acid units joined in the polysaccharide molecule. Xylan, composed of anhydroxylose units, is the major component of this mixture in most plants. Other hemicelluloses contain mannans, arabans and galactans.

Many microrganisms produce hemicellulose degrading enzymes, but their role in tissue maceration, if any, is unknown.

None of the hemicelluloses examined (larch arabo-galactan, spruce glucomannan, spruce xylan and mannan, esparto grass hemicellulose and jute hemicellulose) were degraded by a 7 day old pectin culture filtrate or a culture filtrate from a medium containing jute hemicellulose under the experimental conditions employed. However, xylanase activity was shown in culture filtrates of the fungus grown on esparto hemicellulose. Further evidence of hemicellulase activity has been shown by chromatographic analysis of the degradation products from the activity of culture filtrates on orange protopectin, washed albedo, and potato cell wall material.

One of the possible explanations given for the relative insolubility of pectin by Joslyn (1962) was its esterification with araban or galactan. Byrde and Fielding (1962) separated endopolygalacturonase from a macerating factor in culture filtrates of Sclerotinia fructigena by means of dextran gel filtration and Ecteola cellulose chromatography. McClendon (1964) demonstrated on an ultrafiltered and freeze-dried sample of Byrde and Fielding's preparation that a PG peak was eluted at pH 5.4 but macerating activity was eluted in two peaks, one corresponding to the PG peak and the other to a minor PG peak, with indications that arabanase or galactanase may macerate. Galactans and arabans consist of chains of α-L-arabinofuranoside (Hirst and Jones, 1947), and β-D-galactopyranoside (Hirst et al., 1947). Byrde and Fielding (1965) examined the activity of a purified preparation of their factor, in hydrolysing these substances. Arabinofuranoside was attacked by this preparation and it was considered that the Sclerotinia fructigena macerating factor might be identical with α-L-arabinofuranoside. Filtrates from cultures of P. digitatum grown on a medium containing pectin as the carbon source were subjected to gel filtration on Sephadex 'G.75'. The single peaks of macerating activity

obtained were not coincident with the peak of arabinofuranosidase activity. In the case of the filtrate from a culture grown on pectin (medium B), the peak of arabinase activity was completely removed from the macerating peak and no arabinase activity could be detected in any of the fractions possessing good macerating activity. The peak of macerating activity in each case was coincident with the peak of viscosity reducing activity.

A great deal of information concerning the nature of the enzymatic characteristics of plant pathogenic organisms can be obtained using substrates which have been prepared as extracts of plant cell wall material such as pectin and sodium polypectate. In studying the activity of 'protopectinase', however, it would also be advantageous to use 'protopectin' as a substrate. Protopectin was prepared from orange albedo tissue and the activity of culture filtrates on it examined. In maceration tests, potato tuber tissue was used for convenience, because of the difficulty in obtaining standard orange rind discs and in determining maceration. Culture filtrates macerating potato tissue also macerated orange rind tissue.

Intact, purified potato cell wall material was also used to investigate the activity of culture filtrates during the degradative processes.

The activity of culture fluid on orange albedo tissue and protopectin showed that a large proportion of this material was not rendered soluble within the incubation time by the culture preparation. However, the reducing groups liberated were closely correlated with the loss in weight of the substrates. From chromatographic analyses of the soluble fractions, the major products were shown to be galacturonic acid, arabinose and galactose.

Culture filtrates of the fungus grown on pectin and jute hemicellulose were incubated with a potato cell wall preparation. The major product of the activity of the pectin culture filtrate was galactose, with a slight trace of galacturonic acid. Jute hemicellulose culture filtrate on potato cell wall yielded a strong galactose spot but galacturonic acid was absent. The jute hemicellulose culture filtrate was further tested on pectin, NaPP, protopectin and potato cell wall. Neither galacturonic acid nor higher polyuronides were detected in either the protopectin or potato cell wall supernatants, although galacturonic acid was detected in the pectin and sodium polypectate reaction mixtures. Galactose and arabinose were liberated in the protopectin and potato cell wall supernatants in substantial amounts. That pectic substances are not the only cell wall materials degraded

during fungal invasion of orange tissue is borne out by the fact that galactose, arabinose and xylose are detectable breakdown products.

Arabanase is not thought to be solely responsible for maceration.

Galactose - the product of galactanase activity has been noted in many investigations on the fungal invasion of plant tissues, and there has been an indication by McClendon (1964) that galactanase may macerate. Galactose was detected in all chromatograms of reaction mixtures and degradation products of host tissue during the course of this investigation, and galactanase may well be implicated in the maceration process. Certain results, however, do not support the view that galactanase is solely responsible for maceration. Larch galactan, as the other hemicelluloses, was not degraded under the experimental conditions employed, and β-D-galactosidase activity was very slight from filtrates of cultures from medium A, containing glucose, at a pH where macerating activity was high. Dean and Wood (1967) discarded the theory that galactanase was responsible for maceration because galactose oligomer production from NaPP was not influenced by Ca^{++} addition, whereas maceration was slightly stimulated by this ion and strongly inhibited by EDTA.

Maceration may be caused by the degradation of pectic or other substances in such a way that the breakdown products are not detected by experiments as described above. This method of chromatography may not be suitable for such a purpose. In this context, it is interesting to note that Dean (1967)(personal communication) found that polymers with a minimum molecular weight of 10,000, that is anhydrogalacturonic acid polymers consisting of approximately 50 units, would not migrate on chromatography paper using the butanol-acetic acid solvent. She found that more than half the uronide liberated from potato cell wall remained on the origin of chromatograms developed with butanol-acetic acid. Maceration may result from the degradation of only a very small part or layer of the middle lamellar structure, thus making detection of enzyme activity and breakdown products difficult.

Proteolytic activity was only examined in vivo and none was found. Protein has recently been found in plant cell walls and proteolytic enzymes may well be involved in tissue maceration. Further studies on the production and role of these enzymes would be desirable.

Many workers have indicated that the establishment and continued pathogenicity of plant pathogenic fungi is correlated with their ability to produce pectolytic enzymes

in vitro and *in vivo*. Conidial extracts of *P. digitatum* were shown to have pectic enzyme and macerating activity. The products of pectic enzyme activity could be detected a short time after spores were "sown" in an albedo extract medium. This would suggest that pectic enzymes are involved in the establishment of "green mould". However, Green (1932) and Kavanagh (1965) showed that *Penicillium digitatum* more readily infected oranges if the infection drop contained orange juice. Orange juice was shown in this study to inhibit viscosity reducing pectic enzyme activity and, therefore, the role of these enzymes in the establishment of infection must be questioned. Certainly, pectic enzymes could be detected *in vitro* on several types of media. Soft rot pathogens have been shown to destroy up to 48 per cent of the pectic substance of apple tissue (Cole and Wood, 1961). Pectic degradation of potato tissue by culture filtrates of *P. digitatum* was shown by staining with ruthenium red.

The ability of spores suspended in pectin to infect oranges wounded into the inter-vesicle flavedo tissues, whereas spores in other carbohydrates did not (Kavanagh, 1965) is interesting, especially since pectin is one of the major components of orange rind. The greater part of this pectic material is found in the albedo tissue and it

is noteworthy that fruit were resistant to infection when wounded into the flavedo tissue but not if wounded into the albedo layer. The mechanical barrier provided by the compact flavedo cells may play a part in this resistance. On the other hand, the non-availability of a suitable substrate for pectic enzymes might provide this apparent resistance to infection.

In an attempt to relate macerating activity with toxicity, it was shown that there was a considerable delay after potato discs were macerated by a culture solution before the cells were killed. The killing of the cells, however, was correlated with the disappearance of ruthenium red stain from the cell walls. It would, therefore, appear that death of the cells does not occur until the cell wall had been sufficiently degraded. The mere removal of wall substance from contact with the outer protoplasmic layers may lead to death of the cell, but this is generally considered unlikely. The removal of wall material, containing pectin substances, would enable the "lethal principle" to reach the protoplastic surface and cause death. The nature of the lethal principle is unknown. It may be the "macerating factor" itself, or possibly a lipolytic or proteolytic enzyme as suggested by Brown (1965).

There is, therefore, strong circumstantial evidence that pectic enzymes play a role in pathogenesis. It is not yet possible to attribute pathogenicity to the activity of any single enzyme. Similarly the process of maceration cannot be attributed to the activity of any one enzyme. It is, of course, possible that several enzymes have the ability to macerate tissue, or maceration may occur by the combination of a number of activities. There was a distinct lack of correlation between pectic enzyme activity and maceration in this investigation. The breakdown of non-pectic polysaccharides during degradation processes may well indicate the involvement of hemicellulases. Further work on the role of hemicellulases, proteolytic and perhaps lipolytic enzymes in phytopathological processes should prove rewarding.

SUMMARY

PART I

1. GROWTH AND ENZYME PRODUCTION IN VITRO

A. CONSTITUTIVE ENZYME PRODUCTION

1) The constitutive production of cellulase, pectin methylesterase, viscosity reducing (pectin and NaPP) enzymes, pectin transeliminase, α-L-arabinofuranosidase, β-D-galactopyranosidase and macerating enzyme was demonstrated in a medium containing 2 per cent glucose as the carbon source. Mycelial growth was good. High PME, viscosity reducing, macerating and α-L-arabinofuranosidase activities were shown. Cellulase and galactosidase activity was slight. On measuring PTE activity, the peak of maximum absorption of the reaction products was shown to shift with time.

2) The optimum pH for viscosity reducing activity on pectin was near 5.0 and for activity on NaPP near pH 4.0.

3) The pH optimum for α-L-arabinosidase activity was 3.0 - 5.0. There was, however, not much effect of pH over the range tested, 3.0 - 7.0.

4) Fractionation of a 17 day culture filtrate on Sephadex G.75 showed a single peak of macerating activity, coincident with the peak for viscosity reducing activity

on pectin and PTE activity. The peak of α-L-arabinosidase activity was slightly removed from that of macerating activity.

B. THE PRODUCTION OF EXTRACELLULAR ENZYMES ON PECTIN AND NAPP SUBSTRATES

1) P. digitatum was grown in stationary culture on medium B, containing either 0.1 per cent pectin or NaPP as the main carbon source. Mycelial growth was small. The enzymes produced on medium A were also produced on medium B. Growth was not correlated with enzyme production. PME, viscosity reducing (pectin and NaPP), PTE and macerating activities were high.

2) The pH optimum for viscosity reduction of the pectin and pectate solutions was near 5.0-5.5 and 4.0-4.5. for filtrates from the pectin and NaPP cultures.

3) Fractionation of a 10 day culture filtrate on Sephadex G.75 showed a single peak of macerating activity coincident with peaks of viscosity reducing activity (pectin) and with PTE activity. The α-L-arabinofuranosidase and maceration peaks were well separated.

C. GROWTH AND PRODUCTION OF EXTRACELLULAR ENZYMES ON MEDIUM C

1) Growth was good in both static and shake culture. Initially growth in shake culture was greater than in static

culture on both pectin and NaPP media. Growth was similar on pectin and NaPP media. All but the static pectic cultures showed a decrease in mycelial dry weight after varying time periods.

2) Viscosity reducing activity was small from all filtrates. Higher activity was seen in the NaPP than in the pectin culture filtrates.

3) Reducing group liberation from a pectin substrate by static culture filtrates was very low over a 24 hr incubation period. Slightly higher activity was recorded from the NaPP culture filtrates.

4) Macerating activity was high from all the sets of filtrates.

5) PTE activity was higher from the NaPP filtrates than the pectin filtrates. PTE was not detected in 1, 2 or 3 day filtrates from pectin cultures. Pectate transeliminase was not detected.

6) The optimum pH for PTE activity was near 5.5.

7) The optimum pH for macerating activity was again near 5.5.

8) A non-linear relationship between enzyme concentration and macerating activity was shown.

9) Macerating activity could not be distinguished from pectic hydrolase or lyase activity by thermal

inactivation.

D. EXTRACELLULAR ENZYME PRODUCTION ON MEDIUM D

1) Viscosity reducing activity was high when tested on pectin and NaPP solutions. The pH optimum was near 4.5. At pH 5.0, activity fell off markedly.

2) Macerating activity was high. The optimum pH for maceration was near pH 3.5.

3) Pectic lyase activity was not detected.

4) There was a considerable reduction in viscosity reducing activity on pectin on freeze-drying the culture filtrate. Viscosity reducing activity on NaPP and macerating activity were not affected.

5) The viscosity reducing activity (pectin) of a culture solution was severely reduced by dialysis against water for 24 hr and further reduced after 48 hr dialysis.

6) There was no significant recovery of viscosity reducing activity by the addition of calcium to dialysed culture solutions.

7) Dialysis of a culture solution against salts did not prevent a reduction in viscosity reducing activity. However, the loss in activity was reduced by dialysing against $CaCl_2$ and $MgSO_4$.

8) A reduction in macerating activity of a culture solution on dialysis against water was not affected by

dialysis against salt solutions.

9) EDTA addition to reaction mixtures had no effect on the viscosity reducing activity (pectin) of a culture solution at levels of up to 1 x 10^{-3}M.

10) A considerable reduction in viscosity reducing activity (NaPP) over 24 hr was shown for a dialysed culture solution. Only a slight reduction in activity was seen for the non-dialysed preparation. Macerating activity was lower in the dialysed preparation after 24 hr standing than in the non-dialysed.

11) The optimal pH for reducing group liberation from a pectin substrate was near 5.0 and from a NaPP substrate near 4.0.

12) A linear relationship was found between enzyme concentration and macerating activity, down to $^{1}/_{20}$ dilution, where activity fell off more steeply.

13) A linear relationship was found between enzyme concentration and reducing group activity (pectin) after a 24 hr incubation time. Up to 4 hr incubation, enzyme concentration showed a linear relationship with reducing group activity (NaPP). However, after 4 hr, a non-linear relationship was found.

14) A linear relationship between enzyme concentration and viscosity reducing activity (pectin and NaPP) was shown.

15) There was a considerable delay after potato discs were macerated before the majority of the cells were killed.

16) Toxicity was correlated with the disappearance of ruthenium red staining materials from the cell walls of potato tissue.

17) Adsorption of culture fluid enzymes on Ecteola cellulose, and CMC was not thought sufficiently promising to attempt column chromatography with these materials. Better adsorption was achieved using cellulose phosphate. Fractionation of a culture solution on cellulose phosphate showed protein peaks, coincident with peaks of reducing group activity (NaPP). Very little macerating activity was detected from the column eluates and it was thought the macerating enzymes may have been inactivated by the exchange reactions.

E. PRODUCTION OF EXTRACELLULAR ENZYMES ON AN ORANGE ALBEDO MEDIUM

P. digitatum was grown in static culture on an orange albedo medium for 6, 12 and 24 hr. Culture filtrates and sodium chloride extracts of the residue were assayed for enzyme activity and examined chromatographically for degradation products.

Viscosity reducing activity on pectin was detected,

but not on NaPP. Pectin transeliminase and macerating activities were easily detectable. PME activity was not found. Paper chromatography revealed presence of galacturonic acid after 6 hr incubation and galacturonic acid and galactose after 24 hr incubation.

F. DEGRADATION OF WASHED ALBEDO AND ORANGE PROTOPECTIN

When washed, albedo and orange protopectin were incubated with a culture solution, a close correlation between the loss in weight of the substrates and reducing group release was found. The products of pectic lyase activity were not detected spectrophotometrically or by the thiobarbituric acid test. Detectable breakdown products were galacturonic acid, galactose, arabinose and unknown substances of R_{GA} values 1.48, 0.63 and 0.31.

G. GROWTH AND ENZYME PRODUCTION ON HEMICELLULOSES

Growth on media containing jute, larch and spruce hemicellulose was sparse. Macerating activity was detected in all the culture filtrates. A number of hemicellulose were not degraded by a jute hemicellulose or a 7 day pectin culture filtrate. Incubation of jute culture filtrate with pectin yielded galacturonic acid and galactose, arabinose, xylose and an unknown spot, R_{GA} 1.64 - 1.85; with protopectin, galactose, arabinose,

xylose and R_{GA} 1.64 - 1.85; with potato cell wall galactose, arabinose and a trace of R_{GA} 1.64 - 1.85. Xylanase production was confirmed by growing P. digitatum on a medium containing esparto grass hemicellulose.

2. ENZYME PRODUCTION IN VIVO

A. DETERMINATION OF ENZYME ACTIVITY IN THE RIND OF SOUND AND INFECTED ORANGES

Extracts from sound and infected oranges were examined for PME, macerating, viscosity reducing, PTE and proteolytic activity, and evidence of breakdown products determined by paper chromatography.

1) PME was detected in extracts of healthy and diseased rind. Extract of diseased rind tissue showed two pH optima for activity, one at 5.5, and the other at 7.7. The healthy showed maximum activity at pH 7.7. The peak at pH 5.5 is presumably caused by fungal PME.

2) Macerating activity was shown in the rind of infected fruit, with a pH optimum of 5.0 - 6.0. However, the enzyme was active over a wide range.

3) Extracts of healthy and infected orange rind did not reduce the viscosity of pectin or NaPP solutions to any extent (less than 5 per cent viscosity loss in 30 min). Activity on CMC was not found.

4) PTE activity was not detected in extracts of healthy and infected rind tissues at pH 3.5 - 6.0.

5) Proteolytic activity could not be demonstrated in extracts of sound and rotted rind tissue.

6) Analysis of sound and infected fruit by paper chromatography revealed in infected tissue monogalacturonic acid and a series of unknown spots of R_{GA} values 0.8, 0.38 and 0.24. These are thought to be di-, tri- and tetra- galacturonic acid respectively.

B. DETERMINATION OF ENZYME ACTIVITY IN THE JUICE OF SOUND AND INFECTED ORANGES

The juice from healthy and infected oranges, $3\frac{1}{2}$, 6 and 7 days after inoculation, was collected and assayed for enzyme activity.

1) PME could not be detected in the juice from healthy or infected fruit.

2) Macerating activity was detected in the inoculated but not in the healthy fruit.

3) Very slight viscosity reducing activity on pectin was detected in the juice from infected fruit. No activity on NaPP or CMC was found.

4) PTE activity was not detected in the juice from sound or infected fruit.

5) Paper chromatographic analysis of the juice from sound and infected fruit indicated galacturonic acid in juice from infected fruit. Thus the products of enzyme activity were detected, without any corresponding enzyme activity. Orange juice was shown to inhibit the viscosity reducing activity and macerating activity of a culture solution. Autoclaved juice did not inhibit enzyme activity.

C. CONIDIAL ENZYMES

An extract of P. digitatum spores showed viscosity reducing activity when tested on pectin and NaPP solutions at pH 4.5. Cellulase activity was not detected.

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P A R T II

INTRODUCTION

Most of the post harvest wastage in South African citrus fruit is caused by Penicillium digitatum. The control of this fungus has been the subject of many investigations over the past fifty years. In 1935, Tompkins discovered that diphenyl (biphenyl, phenyl benzene) vapour had a fungistatic effect on moulds such as P. digitatum. From that time, the use of diphenyl for protecting fruit from post harvest wastage has rapidly increased so that today it is used to preserve a large proportion of citrus exports all over the world. Diphenyl is usually applied by means of impregnated tissue wrappers or pads in the packing box. The diphenyl sublimates and creates a fungistatic atmosphere around the fruits. Although diphenyl has been a most successful fungistat, there are certain objections to its continued use. The main disadvantage is that it is a fungistat and not a fungicide, and the amount of protection achieved often leaves room for improvement. Other disadvantages are its unpleasant odour, its ineffectiveness against other citrus pathogens, and the fact that certain strains of

P. digitatum have recently been found to be resistant to diphenyl (Harding, 1961, Duran and Norman, 1961).

Part of the diphenyl applied to the fruit is absorbed, accumulating mainly in the oil cells of the peel, but the peeled fruit or the fruit juice may also contain small amounts. This, therefore, represents a possible toxicological hazard to the consumer. In the last five years, prompted by the tightening of Food Additive laws, the search for an effective alternative to diphenyl has been intensified. Many chemicals have been screened to find a suitable replacement. Some of the more promising are dibromotetrachloroethane, (Eckert and Kolbezen, 1963); dichloro-4-nitroaniline (Houck, 1965); 2-aminobutane salts (Eckert and Kolbezen, 1963); 2-(4-thiazolyl) benzimidazole, and an antimycotic - Pimaricin.

In this investigation, attempts have been made to control green mould of oranges by the use of:

a) Pimaricin

b) Dichloran

c) "Deciquam"

d) "Panacide"

e) Thiabendazole

f) Orthophenyl phenol acetate.

MATERIALS AND METHODS

FUNGUS

The same isolate of *Penicillium digitatum* was used in all experiments as used in Part I (see p. 26). Subcultures of the fungus were prepared as described previously.

FRUIT

Experimental fruit were of the Navel and Valencia varieties imported from South Africa, and Navel and Blanca oranges from Spain. Fruit were usually of sizes described in the trade as count 88 or 100, i.e. there are 88 or 100 fruit in a carton, measuring $15^{7}/_{8}$ x $10^{3}/_{8}$ x $10^{1}/_{4}$.

Usually all fruit imported has been waxed, and wrapped in diphenyl impregnated wrappers. All fruit used in investigating Pimaricin for control for green mould had neither been waxed nor treated with diphenyl. When some of the other materials were tested, it was not possible to obtain unwaxed fruit, but none of the fruit used in these investigations had been treated with diphenyl.

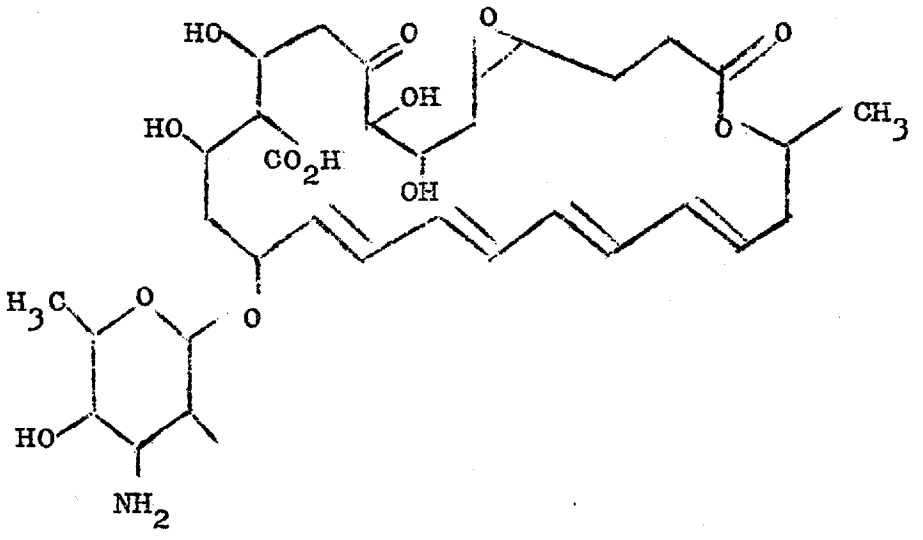
The fruit were stored in a cold-room at 5°C. until

used. All fruit were washed in tap water, dried and carefully examined before use. Fruit with injuries or bad blemishes were rejected. Fruit were surface sterilized by swabbing with 95 per cent alcohol which was allowed to dry without flaming.

FUNGITOXIC MATERIALS

a) Pimaricin

Pimaricin (Delvocid; A-5283; Myprozine) is the name for the polyene antimycotic agent produced by Streptomyces natalensis. It was derived from a culture originally isolated and named after a soil sample obtained near Pietermaritzburg, South Africa (Struyk et al., 1957). It belongs to the tetraene group of antibiotics which includes amphotericin, candicidin, nystatin and rimocidin. The structural formula of Pimaricin is as follows:-



It is a creamy white, odourless crystalline amphoteric powder, very slightly soluble in water and slightly soluble in some low molecular weight alcohols, but increased sclubility is obtained in certain other solvents, such as dimethylsulphoxide, dimethylformamide and piperidine. The molecule is unsaturated and is easily inactivated by oxidising agents, SO_2, sunlight and ultraviolet light. It is inactivated at a low pH or at pH values higher than 8. Metallic ions will also inactivate Pimaricin.

At room temperatures, Pimaricin dry powder is very stable when protected from direct light and moisture. Its stability in solution or suspension depends upon storage conditions.

A 5 per cent suspension of Pimaricin was kindly supplied by the Royal Netherlands Fermentation Industries Ltd., Delft, The Netherlands. This suspension was kept in a stoppered bottle in a refrigerator at 5^{o}C. in the dark.

b) Dichloran

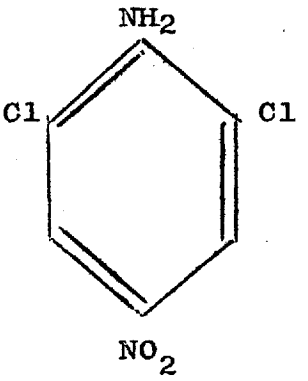
(2,6,dichloro-4-nitroaniline)(DCNA)

Technical DCNA was kindly supplied by Boots Pure Drug Co. Ltd., Nottingham.

DCNA was developed for the control of soil borne,

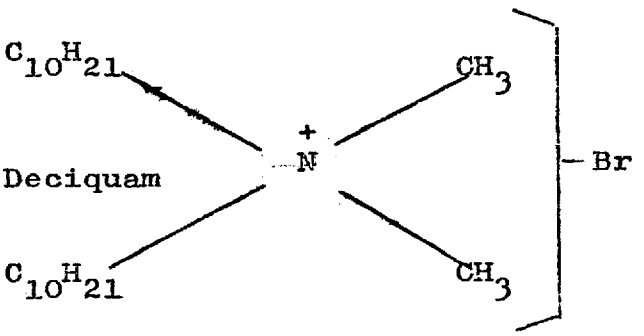
sclerotium-forming fungi. When pure, dichloran is a yellow, crystalline compound. It is almost insoluble in water and was used as a suspension in water. It is stable in all orthodox types of formulation.

Structural formula:



c) "Deciquam"

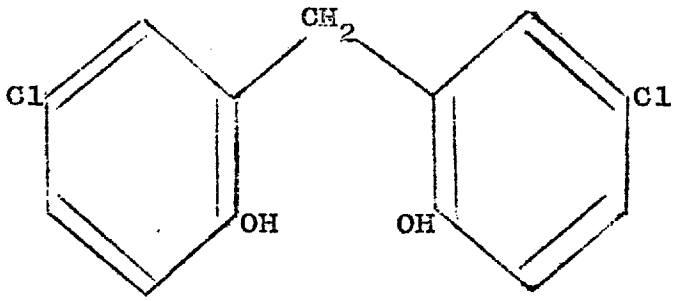
"Deciquam", a twin Quaternary ammonium compound, soluble in water (di-decyl di-methyl-ammonium bromide) is a product of the British Hydrological Corporation who kindly supplied a preparation of their "Deciquam 222", which is a liquid commercial preparation of 50 per cent strength.



d) "Panacide"

"Panacide" is a 40 per cent water miscible solution of Dichlorophen (5:5'-dichloro-2:2'-dihydroxy-diphenylmethane) which was kindly supplied by British Drug Houses Ltd. The impregnation grade supplied was a straw coloured liquid (sp. gr. 1.2), miscible with water at the concentration used.

Structural formula:

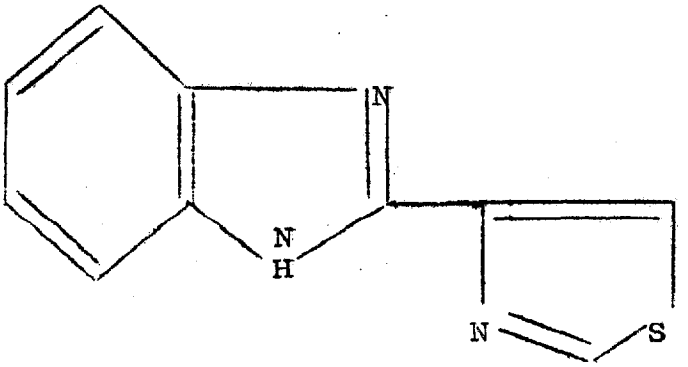


e) Thiabendazole (TBZ)

(2-4-thiazolyl benzimidazole)

A solution of thiabendazole lactate (TBZL) was kindly supplied by Merck Sharp and Dohme Ltd., Hertfordshire, containing 25 per cent TBZ. This soluble form is a solution of TBZL in excess lactic acid, which can be diluted with water to any concentration without precipitation of the active base.

The chemical structure of thiabendazole is:



f) Ortho-phenyl-phenol acetate

Borax Consolidated Ltd., London, supplied wrappers impregnated with 25 and 50 mg ortho-phenyl-phenol acetate for transport experiments from South Africa to the United Kingdom. These experiments were designed by Dr. R.A. Christ of the South African Co-operative Citrus Exchange Ltd. who supervised the treatments. All wastage inspections were made by me in London. Residue analysis of the fruit after shipment was undertaken by Borax Consolidated Ltd., London.

INOCULATION EXPERIMENTS

1. Preparation of spore suspensions

Subcultures of the fungus were grown in 150 ml and 250 ml conical flasks containing a layer of malt agar in the bottom at 25°C. in light. When the agar layer was completely covered with green spores, a small volume of

sterile distilled water was poured into the flasks and the flasks gently shaken. The spore suspension was transferred to a sterile McCartney bottle and violently shaken for several minutes. The resulting spore suspension contained a negligible amount of mycelial fragments and it was not necessary to filter.

A sample of the spore suspension was subjected to 30 seconds ultrasonic disintegration to break up any spore clumps. The resultant suspension was then counted using a haemocytometer and the spore suspension adjusted to the required concentration with sterile distilled water.

2. Wounding technique

The type of wound varied in different experiments. Wounds in the form of pricks were made using No. 1 entomological pins sealed into glass tubes with "Araldite", so that different lengths of pin projected. The instrument was sterilized by flaming after dipping in alcohol. Wounds in the form of an X were made using a sterile scalpel blade. These wounds were $^{1}/8"$ x $^{1}/8"$ and 1 or 2 mm in depth. Wounds were made at the equator of the fruit.

3. Inoculation of spore suspension

Spore suspension (0.01 ml) was applied to the inoculum site from the hypodermic needle of an "Agla" micrometer

syringe, or fruit were dipped into a spore suspension.

4. Experimental design

Fruit of one variety and of the same consignment were used in each experiment. One inoculation site per fruit was used and 12 fruit per treatment.

5. Application of fungitoxic materials

Pimaricin was applied either by means of an "Agla" syringe or fruit were dipped into suspensions or solutions as described later.

6. Incubation

After treatments with spore suspension or fungitoxic materials, each fruit was placed on an aluminium ring or plastic cup and placed in a plastic box which had a tightly fitting transparent lid. A 95-100 per cent humidity was maintained in the box by covering the bottom with a layer of water. Boxes held either 6 or 12 fruit. The boxes were incubated in a constant temperature room at 25°C.

7. Assessment of results

After incubation, the number of infected fruit were counted. A fruit was described as infected on observing the soft, watery spot produced at the site of inoculation.

EXPERIMENTAL RESULTS

Attempts to control green mould of oranges were made with the following substances.

A. PIMARICIN

The antimycotic, Pimaricin, has been shown to possess a strong inhibitory action against a large variety of fungi and pathogenic yeasts in both clinical and laboratory tests. Experimentally, perishable food and agricultural products have been protected against fungal spoilage by the application of Pimaricin at a level of between 10-20 mcg/ml Pimaricin. The first use of Pimaricin to control wastage in citrus fruit was in experiments conducted by L.J. Klotz in the U.S.A. Further tests were carried out by Dr. v. Eek and his colleagues in Delft on the control of blue mould of citrus, caused by the fungus P. italicum. The author working in co-operation with Dr. v. Eeek carried out preliminary investigations on the effectiveness of Pimaricin to control green mould.

1. Effect of using Pimaricin with dimethyl sulphoxide on the control of green mould

Pimaricin is very easily inactivated and one of the major difficulties was to find a suitable solvent for

Pimaricin. Attempts to incorporate Pimaricin into the wax coatings of oranges failed. Pimaricin was found to be highly soluble in 75 per cent dimethyl sulphoxide which is noted for its penetrating power and it was tested as a possible carrier for Pimaricin.

Oranges were wounded in three ways:

a) 3 x 3 mm deep closely adjacent pin pricks

b) 3 x 5 mm deep closely adjacent pin pricks

c) 2 mm deep X wound

0.01 ml of Pimaricin suspension was applied to cover the wound. Water was applied for control fruit. The results can be seen in Table 1.

Table 1

Effect of Pimaricin and DMSO on the control of green mould

Wound technique	3x3 mm pricks		3x5 mm pricks		2 mm X wound	
Days after treatment	7	14	7	14	7	14
Treatment						
5000 ppm Pim.	11	11	4	8	4	5
2000 ppm Pim.	7	11	7	7	10	10
5000 ppm Pim. in 75% DMSO	6	9	3	8	5	5
2000 ppm Pim. in 75% DMSO	8	12	10	11	8	9
Control	11	12	11	11	12	12
	no. of infections out of 12					

From these results, it can be seen that no advantage was gained by using Pimaricin incorporated into DMSO than by using Pimaricin alone. Control was far from satisfactory in any of the treatments.

In further experiments, the volume of Pimaricin solution or suspension applied to wounds was increased to 0.02 ml.

2. The effect of varying the application time of Pimaricin

In two experiments, Pimaricin (0.02 ml) was applied to 1 mm deep X wounds at a concentration of 2000 ppm, immediately, 24 hours before, and 24 hours after wounding and incoulation. The results are shown in Table 2.

Table 2

Effect of varying the time of Pimaricin application

	Pimaricin Application		
	Immediate	24h before	24h after
Experiment 1			
2000 ppm Pimaricin	2	1	2
Control (water)	12	12	12
Experiment 2			
2000 ppm Pimaricin	1	0	2
Control (water)	12	12	12

no. of infections out of 12
14 days after treatment

Good control was obtained in both experiments, irrespective of when Pimaricin was applied in relation to inoculation.

A white Pimaricin precipitate was visible on all treated fruit. There was also a slight 'sinking' of the fruit at the inoculation site.

Using the same technique as in the previous experiments, Pimaricin was applied in different concentrations immediately upon wounding and inoculating, and 24 hours after. The results after 7 and 14 days incubation can be seen in Table 3.

Table 3

Effect of Pimaricin concentration and time of treatment on infection

Pimaricin Concentration	Pimaricin application			
	Immediate		24h after	
	Days after treatment			
	7	14	7	14
2000 ppm	4	6	2	3
1000 ppm	5	6	4	5
500 ppm	5	5	6	7
100 ppm	3	7	6	8
10 ppm	7	9	12	12
Water Control	12	12	12	12

no. of infections out of 12

When Pimaricin was applied immediately, there was no significant difference between control given by 100 ppm Pimaricin and 2000 ppm Pimaricin. When applied 24 hours after wounding, the higher the concentration of Pimaricin the greater the control, but control was still not considered very satisfactory even at 2000 ppm Pimaricin.

Tests using Pimaricin at concentrations of 1000 ppm to control P. italicum infection of oranges conducted at R.N.F.I. Ltd. Research Laboratory in Delft, gave promising results when the Pimaricin was incorporated in a 1 or 2 per cent solution of lecithin.

Mr. T. Mulder: Oldenaller 19,
Amsterdam-Buitenveldert,
kindly supplied a quantity of lecithin (soya bean derivative) and Pimaricin was incorporated in it at various concentrations.

3. The effect of Pimaricin in controlling green mould infections when incorporated in 2 per cent lecithin

Spanish Navel oranges were wounded by the X technique to a depth of 1 mm and inoculated with a spore suspension of P. digitatum. After drying the fruit were dipped into the Pimaricin/lecithin solutions for 30 seconds
a) immediately and b) after 24 hours inoculation.

Pimaricin was incorporated into the lecithin solution at concentrations of:- 2000, 1000, 500, 250, 100 ppm. Controls dipped in water and lecithin only were also set up.

The results after 4, 7 and 14 days incubation are given in Table 4.

Table 4

Effect of Pimaricin in controlling green mould when incorporated in 2% lecithin

Pimaricin Concentration	Pimaricin application					
	Immediate			24 hr after		
	Days after treatment					
	4	7	14	4	7	14
2000 ppm	6	11	11	8	11	11
1000 ppm	8	12	12	4	12	12
500 ppm	5	11	11	7	12	12
250 ppm	5	11	11	6	12	12
100 ppm	12	12	12	9	12	12
Water Control	12	12	12	12	12	12
2% Lecithin Control	11	12	12	12	12	12

no. of infections out of 12

No control was obtained with Pimaricin incorporated into lecithin under the conditions employed.

4. The effect of immersion time in Pimaricin/lecithin solution in controlling green mould

Fruit were treated in the same way as in the previous experiment. They were dipped for different times, a) immediately b) after 24 hours after wounding and inoculating. Pimaricin was incorporated into the lecithin solution at a concentration of 2000 ppm. The results are shown in Table 5. Water and lecithin controls were set up as in the previous experiment.

Table 5

Effect of immersion time in Pimaricin/lecithin solution in controlling green mould

Time of immersion	Pimaricin application					
	Immediate			24 hr after		
	Days after treatment					
	4	7	14	4	7	14
30 sec.	2	2	2	4	4	7
1 min.	0	3	4	5	6	8
3 min.	1	3	3	2	8	8
5 min.	0	0	3	5	8	8
Water Control (30 sec)	12	12	12	12	12	12
(5 min)	10	10	10	12	12	12
Lecithin Control(5 min)	11	12	12	12	12	12

no. of infections out of 12

The time of immersion did not significantly affect the control achieved. Immersion for 30 seconds showed a much greater degree of control in this experiment than the previous one. The reason for this difference cannot be accounted for except perhaps the inherent difference between different consignments of oranges.

In none of these experiments was a good, consistent control of green mould obtained using Pimaricin alone or in combination with other materials.

B. DICHLORAN

DCNA has been successfully used as a fungistat on fruits, flowers, vegetables and root crops.

Houck (1965) found that under certain conditions aqueous suspensions of DCNA substantially reduced decay by pre-treatment and post-treatment P. digitatum infections of lemons during storage.

DCNA was used in this investigation in an attempt to control P. digitatum infections of oranges.

Houck showed that pre-treatment infections were controlled by immersing lemons for 4 minutes in warm ($43^{\circ}C$ +) 0.5 per cent DCNA at pH 11.5 - 12.0. Less decay developed in fruit that was not rinsed than in fruit that was rinsed in cool water following treatment.

In the following experiments, Valencia oranges (count 100) were treated in one of the following ways:

1. The fruit were dipped in an agitated aqueous suspension of 0.5 per cent DCNA at 50°C and pH 11.7 (adjusted with N NaOH) for 5 minutes and then incubated at 25°C. and 100 per cent humidity for 24 hours. After 24 hours the fruit were wounded by the X technique and a 0.01 ml spore suspension (3 million/ml) applied to the wound. The fruit were then incubated as previously described.

2. The fruit were wounded, a spore suspension applied and incubated for 24 hours as described above.

3. The fruit were wounded, spore suspension applied and then dipped into DCNA (as above) immediately after drying. The fruit were then incubated as above.

The results of two identical experiments using 12 fruit/treatment are shown in Table 6.

Pre-treatment infections were not controlled by DCNA under these conditions. Post-treatment infections were successfully controlled when the spores and fungistat were applied at the same time, in one experiment control was successful, in the other was not. The cause of this variation was not known.

Table 6

The use of DCNA (0.5 per cent) to control pre- and post treatment green mould infections

Application	Experiment 1			Experiment 2			
	days after treatment						
	4	15	20	4	7	14	21
24 hr before	8	12	12	5	10	12	12
24 hr after	0	0	1	0	0	0	0
Immediate	0	1	1	0	7	10	11
Controls							
24 hr before	11	12	12	11	12	12	12
24 hr after	11	11	11	12	12	12	12
Immediate	12	12	12	9	12	12	12

no. of infections out of 12

In an attempt to obtain better control in all treatments, the concentration of DCNA used was increased to 1 per cent.

Spanish (Blancas) oranges were treated in one of the following ways.

1. The fruit were wounded by the X technique and dipped into a spore suspension containing 2 million spores/ml. After drying the fruit were dipped for 3 minutes into an agitated suspension of 1 per cent DCNA at

pH 11.5 and 45°C. The fruit were incubated in the normal manner.

2. The fruit were wounded and dipped into spore suspension as above, and then incubated for 24 hours before dipping in the DCNA suspension.

The results are shown in Table 7.

Table 7

The use of DCNA (1.0 per cent) to control pre- and post treatment green mould infections

DCNA Application	Days after treatment		
	7	14	21
Immediate	2	5	5
24 hr after	0	1	1
Control (Water)			
Immediate	11	11	11
24 hr after	10	10	10

no. of infections out of 12

Control was good in both treatments, but better control was obtained in the pre-treatment infections.

Houck showed for lemons that less decay developed in fruit that was not rinsed in cool water following treatment. In the following experiment, Spanish (Blancas)

oranges were treated as previously (see treatment 1 and 2 in previous experiment); after DCNA treatment the fruit were rinsed in running water for 1 minute. The results are shown in Table 8.

Table 8

The effect of rinsing on the control of green mould by DCNA

Application	Treatment	Days after treatment		
		7	14	21
Immediate	Unrinsed	2	5	5
	Rinsed	2	6	6
	Control (water)	11	11	11
24 hr after	Unrinsed	0	1	1
	Rinsed	0	0	0
	Control (water)	10	10	10

no. of infections out of 12

Rinsing after DCNA did not have a significant effect on the control of green mould. Rinsing had the advantage that a great amount of the yellow residue normally remaining on the fruit after DCNA treatment was largely removed.

Smoot and Melvin (1963) reported that Pineapple and Valencia oranges treated with hot water at 50°C. for 5 minutes was effective in reducing stem end rot caused by Phomopsis citri and decay due to P. digitatum. The control obtained by warm DCNA (45°C.) was, therefore, compared with the control obtained by warm water (45°C.) treatment at pH 11.5.

The treatments previously described were used immediately and 24 hours after inoculation. The results are shown in Table 9.

Table 9

Comparison of warm DCNA and warm tap water on development of green mould

Application	Treatment	Days after treatment		
		7	14	21
Immediate	Warm DCNA	2	5	5
	Warm water	4	5	7
	Control (cool water)	11	11	11
24 hr after	Warm DCNA	0	1	1
	Warm water	4	5	6
	Control (cool water)	10	10	10

no. of infections out of 12

The incidence of infection was slightly decreased with warm water at pH 11.5. Using DCNA the control was substantially better, especially in treatment 2.

C. DECIQUAM AND PANACIDE

Deciquam and Panacide are reported to be highly active against a wide range of microorganisms, including many fungi, and are being used for sterilising purposes in the food, beverage, catering and allied industries. It was thought they might possibly be of use in controlling green mould infections of citrus fruit.

1. Deciquam

Spanish Navel oranges (count 80) were wounded by X technique and inoculated with P. digitatum spores.

The fruit were then dipped in the following:

a) water (control) 30 secs dip

b) 0.05% Deciquam 30 secs dip

c) 0.1% Deciquam 30 secs dip

d) 0.1% Deciquam 5 mins dip

The pH of 0.1% solution Deciquam = 5.6.

After drying the fruit were incubated as normal. The results are shown in Table 10.

Table 10

Effect of immersion time in two concentrations of Deciquam on the control of green mould

Treatment	Days after treatment			
	2	3	4	7
0.05% Deciquam/30 secs.	8	12	12	12
0.1% Deciquam/30 secs.	3	9	10	10
0.1% Deciquam/5 mins.	1	6	8	9
Water control/30 secs.	10	11	12	12
	no. of infections out of 12			

Under these conditions, Deciquam did not satisfactorily control green mould infection.

2. Deciquam and Panacide

Spanish (Blancas) oranges were treated in one of the following ways:

a) No wounding, immersion in 2 million/ml spore suspension for 1 minute.

b) X wounded, immersion in the above spore suspension for 1 minute.

After drying the fruit were treated with:

i) 1% Panacide solution (pH 10.5) 3 minutes

ii) 0.1% Deciquam solution (pH 11.0) 3 minutes

iii) 0.2% Deciquam solution (pH 11.0) 3 minutes

iv) Water - Control.

The fruit were then incubated in the normal manner.

The results are shown in Table 11.

Table 11

Effect of Deciquam and Panacide in controlling green mould

Treatment	Days after treatment		
Unwounded	7	14	21
1% Panacide	1	2	2
0.1% Deciquam	2	5	5
0.2% Deciquam	0	2	5
Water Control	9	9	11
X Wounded			
1% Panacide	6	7	7
0.1% Deciquam	8	10	10
0.2% Deciquam	1	3	4
Water Control	8	11	12

no. of infections out of 12

Quite good control by all treatments was obtained when the oranges were not artificially wounded. Of those that were artificially wounded, only Deciquam at 0.2 per cent concentration was satisfactory.

In all fruit treated with either Panacide or Deciquam there was a marked burning of the fruit and sections of the rind became sunken, so no further tests were made with these chemicals.

D. THIABENDAZOLE

Thiabendazole (TBZ) is an antihelminthic agent that has recently been found to be fungicidal. Grivelli (1966) demonstrated that it had a high fungistatic activity on P. italicum and P. digitatum in vitro and in vivo. He showed that the decay produced by these two fungi could be inhibited by immersion of the fruit in solutions containing 0.075 per cent or more TBZ and that the most convenient formulation is in a solution in lactic acid (thiabendazole lactate - TBZL).

Two experiments were done in an attempt to control P. digitatum decay.

Experiment 1

Spanish (Blancas) oranges (count 100) were treated in one of the following ways:

1) Fruit were dipped for 1 minute in a spore suspension containing 2 million spores/ml. The fruit were not wounded.

2) Fruit were wounded by the X technique and dipped into the spore suspension for 1 minute.

3) Fruit were neither wounded nor dipped into spore suspension.

After treating the fruit in one of these ways, the fruit were treated with TBZL in the following concentrations for 3 minutes:

a) 0.1% TBZL

b) 0.2% TBZL

c) water control.

After drying the fruit at laboratory temperature, they were incubated in the normal way. Twelve fruit were used for each treatment and the results are shown in Table 12.

From this table it can be seen that treating fruit with 0.1 and 0.2 per cent TBZL substantially reduced decay. Wounded fruit treated with 0.2 per cent TBZL showed no infection after 21 days incubation in conditions ideal for supporting fungal growth, whereas untreated fruit showed 100 per cent infection.

No deleterious effects to the fruit could be seen.

Table 12

Use of TBZL to control green mould

	Days after treatment		
	7	14	21
Treatment 1			
0.1% TBZL	1	1	1
0.2% TBZL	0	0	0
Water Control	9	9	11
Treatment 2			
0.1% TBZL	0	0	1
0.2% TBZL	0	0	0
Water Control	10	11	12
Treatment 3			
0.1% TBZL	0	0	0
0.2% TBZL	0	0	0
Water Control	1	1	1

no. of infections out of 12

Experiment 2

Spanish (Blancas) oranges (count 100) were treated in one of the following ways:

1) Fruit were wounded as in Experiment 1, dipped in a spore suspension and left to dry.

2) Fruit were wounded (X technique), dipped into spore suspension, dried and then incubated at 25°C. and 100 per cent humidity for 24 hours.

After treatments 1 or 2, the fruit were dipped in the following solutions for 3 minutes:

a) 0.01% TBZL

b) 0.05% TBZL

c) 0.1% TBZL

d) water control.

After drying, the fruit were incubated in the usual way. The results are shown in Table 13.

Table 13

Use of TBZL to control green mould

	Days after treatment		
Treatment 1	7	14	21
0.01% TBZL	0	0	0
0.05% TBZL	0	0	0
0.1% TBZL	0	0	0
Water Control	11	11	11
Treatment 2			
0.01% TBZL	4	6	6
0.05% TBZL	0	0	0
0.1% TBZL	0	0	0
Water Control	11	11	11

no. of infections out of 12

Decay was effectively controlled in fruit which had been inoculated and treated immediately by all the concentrations of TBZL down to 0.01 per cent. In fruit which had been inoculated 24 hours before TBZL was applied, complete control was obtained with solutions of TBZL of 0.05 per cent and 0.1 per cent concentration. No deleterious effects to the fruit were observed.

The very good control obtained by TBZL, without any accompanying rind damage, would indicate the possibility of commercial application.

E. ORTHOPHENYL PHENOL ACETATE

Transport Experiments EKI.65 EKM.65

The effect of OPP-acetate, diphenyl wrappers and interleaves on the control of wastage

Variety of fruit	Navels
Examination	London. 14 days after arrived at Southampton.
Replicates	16 containers - count 72
Wrappers	Plain
	Diphenyl
	OPP-acetate

Table 14

Effect of OPP-acetate on the control of wastage and OPP residues on the fruit

Treatment	EKI		EKM	
	% wastage	Residues in ppm	% wastage	Residues in ppm
Plain wraps	3.0	-	1.5	-
Diphenyl pads	4.3	-	-	-
Diphenyl wraps	-	-	0.1	-
Diphenyl interleaves	3.7	-	1.4	-
OPP acetate wraps (25 mg OPP acetate)	1.3	12.8 after 4 days 29.2 after 10 days	0	21.3 after 10 days
OPP acetate wraps (50 mg OPP acetate)	1.6	36.3 after 4 days 45.5 after 10 days	0.2	41.2 after 10 days
No. of fruit/ treatment	1148		1056	

Wastage in the controls was not high. The chemical reduced green mould incidence, although no significant differences were found. Residue analyses of the fruit using OPP-acetate wraps were high (in excess of E.E.C. countries residue tolerance of 10 ppm).

DISCUSSION

Green mould is responsible for 80-90 per cent of wastage found in South African fruit. Other diseases, of secondary importance, include Trichoderma brown rot (Trichoderma viride), Diplodia stem end rot (Diplodia natalensis), sour rot (Geotrichum candidum) and Anthracnose (Colletotrichum gleosporoides). These are largely kept under control by the low shipping temperature (40°F.). Only two chemicals are at present permitted by the import regulations of customer countries for post harvest decay control - sodium orthophenyl phenate (SOPP), and diphenyl. SOPP is used as a dipping treatment and is effective only against germinated spores of P. digitatum. Germination rarely occurs before the fruit is packed (Christ, 1965). After the fruit is dipped into SOPP, it is then washed, so the SOPP can only be effective during the time the fruit is immersed in the solution. Its effectiveness as a fungicide has been recently questioned. This, and the difficulty encountered in its usage has greatly reduced its use in South Africa.

Diphenyl has proved to be the only effective preservative to be used on a commercial scale for any

length of time. There have been indications that lower residue tolerances will be tolerated in certain consumer countries in the future. This would mean a reduction in the amount of diphenyl which is incorporated into protective wrappers or packs to levels which would make it ineffective. It has, therefore, become necessary to find an effective alternative to diphenyl and this investigation was conducted with this aim in view.

Any new material to replace diphenyl must satisfy certain requirements. It must be effective and not toxic; it must not impart a pronounced odour to the fruit; it must not burn or disfigure the fruit in any way; it must be effective over a long period of time, and it must also be cheap and easy to use. It is very difficult to satisfy all these requirements.

Pimaricin, under some circumstances, was shown to be effective as a fungistat. The main problem was to find a method of application where Pimaricin was not inactivated and therefore an additional difficulty was to find a suitable "carrier". Pimaricin was only effective at relatively high concentrations and its cost would, therefore, probably be prohibitive. There was also some indication that rind injury could be caused by using Pimaricin.

Fruit with any rind disfugurement becomes unacceptable to the trade. This would certainly eliminate Deciquam and Panacide in their present form, even if the control obtained by their use was good, which it was not.

Pimaricin, Deciquam and Panacide are used in food industries and the toxicological hazard with these materials as far as has been determined is small. In this respect, the residues figures obtained in using OPP-acetate were not encouraging, all the figures obtained being in excess of the "European Economic Community's" residue tolerance of 10 ppm. Furthermore, the residues appear to increase during storage. As far as waste protection was concerned, it was difficult to estimate its effect because of the low wastage found in the controls. Further experiments would be justified.

DCNA proved the second most successful material tested and it is reported to be of very low toxicity. Difficulty was encountered in its use because it is practically insoluble in water and only sparingly soluble in most organic solvents which necessitates a method of agitating the suspension to prevent it from settling out. Even after washing the fruit, a yellow residue could be seen on the fruit. Some rind burning and sinking was observed using DCNA, especially at the higher concentration (1 per cent).

Thiabendazole was the most successful material investigated. With the lactic acid formulation, it was easy to use and 100 per cent control was obtained in fruit which had been inoculated 24 hours before treatment with 0.05 per cent TBZL. No rind injuries or disfigurement were observed at any of the concentrations of TBZL used.

TBZ is reported as practically devoid of toxicity and it is odourless and colourless. Results with TBZ would appear promising and tests on a commercial scale would be most interesting to ascertain its use for commercial application.

SUMMARY

PART II

CONTROL OF GREEN MOULD OF ORANGES

A. PIMARICIN

1) Pimaricin alone, or dissolved in dimethyl sulphoxide, did not give good, consistent disease control, with Pimaricin concentrations of up to 2000 ppm.

2) No control was obtained with Pimaricin incorporated into a 2 per cent lecithin solution for Pimaricin concentrations up to 2000 ppm.

B. DICHLORAN

1) Immediate and post-treatment infections were successfully controlled using 0.5 per cent DCNA suspension at pH 11.5 and at a temperature of 45-50°C.

2) Rinsing fruit after DCNA application did not have a significant effect on the control of green mould.

3) Warm water gave some control of green mould. However, control was substantially better with DCNA added.

C. DECIQUAM AND PANACIDE

1) 0.05 and 0.1 per cent solutions of Deciquam did not give any control of green mould.

2) A one per cent Panacide solution did not give satisfactory control.

3) 0.2 per cent Deciquam gave a reasonable control of infection. However, there was a marked 'burning' of the fruit and treated fruit developed sunken areas on the rind.

D. THIABENDAZOLE

Decay was effectively controlled in fruit which have been inoculated and treated immediately by all concentrations of TBZL used. In fruit inoculated 24 hr before TBZL was applied, complete control was obtained with a 0.05 per cent TBZL solution.

E. ORTHOPHENYL PHENOL ACETATE

This chemical reduced disease incidence in experimental trials but the residue levels on the fruit were high, which would prohibit its use.

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