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BREAKDOWN OF PLANT TISSUES BY PECTIC ENZYMES OF FUNGAL
ORIGIN, WITH SPECIAL REFERENCE TO CITRUS FRUITS.

submitted by DAVID A. BUSH B.Sc.

for the degree of Ph.D.

of the Bath University of Technology.

1968.

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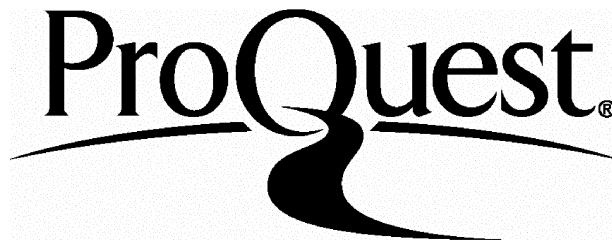
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CONTENTS.

SUMMARY.

The evidence for the participation of pectic enzymes in plant disease is reviewed, with particular reference to the part played by these enzymes in diseases of citrus fruits.

The pectic enzymes of Penicillium digitatum, a pathogen of citrus fruits were compared with those of Penicillium notatum a related species which is non pathogenic, giving evidence that pectin transeliminase and arabanase might be involved in pathogenicity. The pectic enzymes produced by P.digitatum and two other citrus pathogens, Penicillium italicum and Aspergillus fonsecaeus, under various growth conditions were investigated. An indication was thus given that , in the case of P.digitatum and P.italicum, pectin transeliminase was associated with maceration.

The pectin transeliminases of Penicillium digitatum and Penicillium italicum were purified till they were electrophoretically homogeneous. In such a state of purity, they were still found to be capable of macerating orange rind tissue. Evidence of another macerating system (possibly endo-polygalacturonase) was found in the case of P.italicum. Pectin transeliminase was not considered to play a significant part in maceration in the case of Aspergillus fonsecaeus, though the nature of the macerating factor produced by this organism is not known at present. Pectin transeliminase and macerating factor were shown to be present in orange tissue infected with P.digitatum or P.italicum.

The pectin transeliminases of Penicillium digitatum and Penicillium italicum were compared, and

were found to have the same electrophoretic mobilities. They were found to differ only in their susceptibility to temperature, and when injected into oranges they were found to cause areas of softening.

The significance of these results in the light of other work, is discussed; and suggestions are made for further research.

INTRODUCTION

The role of enzymes in host parasite relationships has long been established. Brown (1915) was one of the earliest workers to show this. He obtained an extract from germinated pathogen spores, and demonstrated that this extract was capable of destroying the coherence of any susceptible tissue placed in it. He noted that the action of the extract was firstly, to cause solution of the middle lamella so that the tissue lost coherence, and secondly, to cause the remaining layers of the cell wall to disintegrate, though complete solution of the cell was not observed. The extract was, he found, inactivated by heating thus suggesting its enzymic nature. In a later publication Brown (1936), suggested that although extracts from fungal pathogens were capable of causing disintegration of the host tissue, these extracts were not responsible for the primary invasion through the epidermis. Evidence for this view was obtained by injecting rose petals with an extract of Botrytis cinerea. After a quarter of an hour, the rose petals were destroyed, but the surface of the petals remained intact.

Plant Cellular Structure.

The nature of the enzymes produced by plant pathogens may be better understood by examining first the chemical structure of plant tissues. It will then be clear what substrates are available for such enzymes. Bailey (1938), has made a study of the physico-chemical composition of cell walls of higher plants. He distinguished three categories of cell structures as follows:-

1. Meristematic (embryonic) cells are capable of growth and have a wall consisting of cellulose and pectin.

2. Fibres and other differentiated cells, lose the potential for growth and retain their original primary wall in a modified form. They lay down a supplementary

wall with a mechanical function, consisting of cellulose
with or without hemicellulose. This is known as the
secondary wall and does not contain any appreciable
fraction of pectic compounds.

3. Adjacent cells are held together by a truly
isotropic substance of pectic nature. This intercellular
layer is known as the middle lamella.

All these structures may become lignified
with a corresponding reduction in pectin content.
On the other hand soft tissues generally give high yields
of pectic substances and low yields of hemicelluloses
and ligin . Fruits and roots usually have a high pectin
content, for example the peel of citrus fruits can
contain from 20-40% pectin on a dry weight basis (Kefford
1959). The cell wall pectic substances are different from
those in the intercellular layer, where they occur
frequently as a salt with calcium. There are also a
variety of other compounds associated with plant pectin
which will be considered later.

It is evident that in order to cause
disintegration of plant tissues, a pathogen must first
cause dissolution of the middle lamella. These enzymes
which break down pectic materials and associated compounds
are likely to be involved in pathogenicity.

The Chemistry of Pectic Materials and Associated Compounds

The majority of early work on the chemistry of pectic
substances was carried out by Hirst and Jones (1938, 1939,
1946). They isolated and analysed pectin and its associated
compounds from apples, citrus peel, peanuts and lupins.
Work on this subject has been reviewed extensively by
Kertesz and McColloch (1950), Kertesz (1951), Whistler and
Smart (1953), Deuel and Stutz (1958) and Joslyn (1962).

The pectin complex is not a definite structure,
but all pectins so far isolated have yielded on hydrolysis

mainly the sugars L-arabinose and D-galactose, the
uronide D-galacturonic acid and methyl alcochol. The
central molecule in this complex is a polygalacturonide
made up of D-galacturonic acid molecules, 1-4 α
glycosidically linked in a linear fashion. The carboxyl
groups of this polymer are either fully or partially
esterified with methyl alcochol, and those which are not
are sometimes involved in salt formation, in particular
with calcium and magnesium. The araban, a polymer of
L-arabinose and the galactan, a polymer of D-galactose,
are associated with the polygalacturonide, and all three
polymers are usually extracted together.

Whistler and Smart (1953) give a general class-
ification of terms pertaining to pectic substances. This
classification was laid down by a committee of the American
Chemical Society as follows:-

1. Pectic Substances. This is a group name for those
complex colloidal carbohydrate derivatives which occur in,
or are prepared from plants, and contain a large proportion
of anhydro-galacturonic acid units, which exist in a chain
like combination. The carboxyl groups of the polygalact-
uronic acids may be partly esterified by methyl groups and
partly or completely neutralized by one or more bases.

2. Pectic Acids. These are colloidal polygalact-
uronic acids, free from methyl alcohol, which form normal
or acid pectinates as salts.

3. Pectinic Acids. These are colloidal polygalact-
uronic acids containing more than a negligible proportion
of methyl ester groups. Under suitable conditions they are
capable of forming gels with sugar and acids, or if low
enough in methyl alcochol with certain metallic ions.
Salts are either normal or acid pectinates.

4. Pectins. These are water soluble pectinic acids
of varying methyl alcohol content and degree of neutraliza-
tion. They form gels with sugar and acids under certain
conditions.

5. Protopectin. This is the water insoluble
parent pectic substance, which occurs in plants, and
which ~on restricted hydrolysis yields pectin or pectinic
acids.

The term protopectin is now probably obsolete, as
the insolubility of native pectin is most likely to be due
to its association with other compounds. Before considering
this however, the more precise chemical structure of the
various parts of the pectin complex will be discussed.

The polygalacturonic acid is a linear polymer of
D-galacturonic acid monomers, which due to the resistance
of the polymer to hydrolysis, are likely to be in the
pyranose form (Whistler and Smart 1953). This, together
with the periodate oxidation technique has indicated that
the monomers are joined by 1-4 glycosidic links, and
enzyme hydrolysis experiments and the high optical rotation
of pectic acid have suggested the α type of linkage. The
polymer is a trans 1-4 polysaccharide, as the hydroxyl
groups at carbon atoms 1 and 4 are in the axial position.
Free rotation at the glycosidic linkages is therefore
hindered and the pectin macromolecule may be considered
a chain with restricted flexibility. Secondary hydroxyl
groups at carbon atoms 2 and 3, and the carboxyl group at
carbon atom 5 are in an equatorial position and are there-
fore easily accessible (Whistler and Smart 1953). X-ray
diffraction of sodium pectate chains showed a threefold
screw symmetry and chains are arranged in closest packing
around the axis (Deuel and Stutz 1958). Molecular weights
for pectic acid have been reported from 10,000 to 200,000.

The araban and galactan polymers are linked to the
galacturonan partly by covalent links and partly by
secondary binding forces. Thus pectin is part mixture
and part compound. Araban is a pure polymer of L-arabinose,
having a relatively low molecular weight. It is a chain of
α1-5 linked arabofuranose units, half of which bear an

OH
|
H————(4)
              (6)-OOH

                     (5)————————O
                    /
                  H

                          H
                          |
HO————(3)════════════(2)
        | |
        H   OH   (1)——H
                             OH
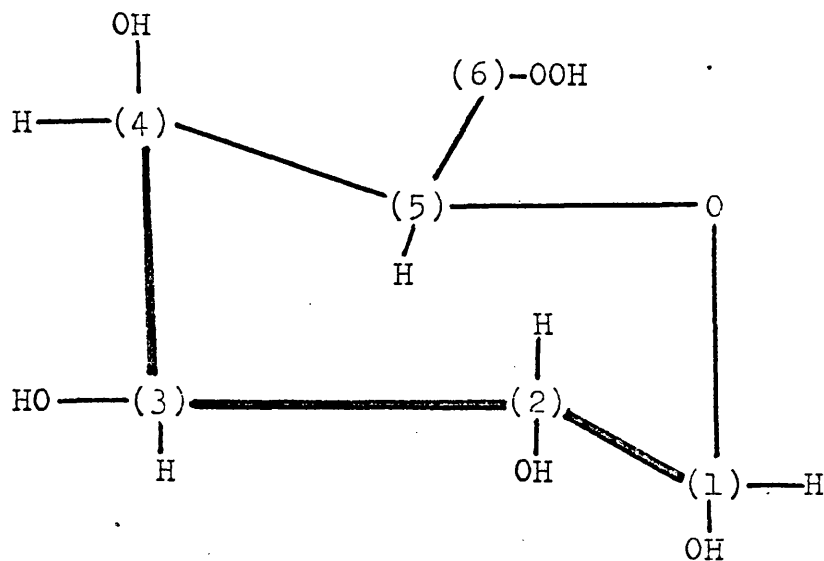
Form of Galacturonic Acid in Pectic Acid
Chain showing Threefold Screw Symmetry
(Deuel and Stutz 1958)

additional α 1-3 linked L-arabofuranose residue as a one
unit side chain. Araban is not found free from pectin in
nature, though L-arabinose is commonly met as an integral
portion of other polysaccharide molecules. The high negative
rotation and the rapid rate of acid hydrolysis suggests that
all arabinose units are in the furanose form and are
connected by α L-glycosidic linkages (Whistler and Smart
1953).

    Galactan is a linear molecule made up of β 1-4
linked D-galactopyranose units, shown in part by its
resistance to acid hydrolysis and its low optical rotation.
The usual chain length for the galactan associated with
pectin is about 100 D-galactose units (Whistler and Smart
1953)

Biosynthesis of Pectic Substances

The original theory that galactan was converted to pectic acid, which was then converted to araban is now discounted, due to the entirely different structures of the three polymers. Introduction of glucose-1-C 14, -2-C 14, -6-C 14 and galactose-1-C 14 into green strawberries through their stems, was carried out by Seegmillar, Jang and Mann (1956). After several days incubation the "pectin" fraction was isolated and hydrolysed to galacturonic acid and arabinose. The label in individual carbon atoms of the galacturonic acid and arabinose was determined by means of chemical degredation procedures. The results suggested that the intact carbon skeleton was preserved in the transformatiom of hexose to galacturonic acid. It also appeared that the intact chain of the first five carbon atoms of the hexose was preserved in the transformatiom of hexose to pentose (arabinose). U.D.P. appeared to be involved in this process (Deuel and Stutz 1958), though in fact UDP galacturonic acid has not been isolated from higher plants, only from Type I Pneumococcus which contains a polysaccharide rich im galacturonic acid. The methyl ester group is incorporated by trans-methylation using methionine, formate, or the α carbon of glycine or serine.

The Insolubility of "Native" Pectin

Isolated pectin is soluble to a greater or lesser extent depending on the degree of esterification, but native pectin is not. In considering the breakdown of plant material by enzymes from plant pathogens, it is of course native pectin that is of greatest interest. The term protopectin has been applied to insoluble plant pectin. This would now seem

to be a misnomer, as it suggests a substance different
in basic structure from the pectins that have been iso-
lated and analysed. It now appears more likely that it,
is the manner in which pectin and its asscociated mole-
cules are bound in plant material that accounts for their
insolubility. Josyln (1962) has reviewed the various
theories put forward to account for the insolubility
of plant pectin, and it would be of value to summarize
this review here.

The size of pectin molecules is known to have
an effect on their solubility, in that large molecules
are less soluble than smaller ones; and there has been
evidence to suggest that this is the most likely factor
involved in the anchorage of pectins in plant tissues.
If this was a significant factor, then the native pectin
would become noticably soluble on partial degradation,
offering an explanation for the maceration of plant
tissue by pectin depolymerases, which will be considered
later. An extension of this argument is that plant pec-
tin may be rendered insoluble due to the secondary agg-
regation of filamentous molecules of pectin with one
another, or with large polymers in the cell wall. Agg-
regation has been explained as simply intertwining of
the molecules,or possibly linking by bonds such as the
anhydride, lactone or ester linkage. There is, however,
much dispute over this last hypothesis, due to the diff-
erent interpretations of the results of viscosity exp-
eriments.

The existence of a pectin cellulose compound
has been suggested by several early workers in the pec-
tin field, to account for the insolubility of native
pectin. Ester formation between the free carboxyl groups
of pectin and the hydroxyl groups of cellulose, or com-
bination between the free carboxyl groups of pectin and
the carboxyl groups of cellulose was put forward to ex-
plain this. The formation of ester or ether linkages

between pectin and hemicelluloses has also been sug-
gested as an explanation for the insolubility of native
pectins, on the evidence that araban and galactan are
always found associated with pectin. Galactose and
arabinose have also been found in the main uronide
chain. Yet another theory is that salt linkages are
formed between the carboxyl groups of pectin and the
basic groups of proteins. Proteins have been identified
in the middle lamella of some plants,and apple tissue
proteins which are difficult to extract, are removed
best under alkaline conditions which also remove most
pectin. However, specific protein extracting agents
such as thioglycollic acid and some proteases, appear
to have no effect on the solubility of pectin in tissue
preparations from certain varieties of apples.

The fact that the relatively small amount of
pectin in lignified tissue is more difficult to extract
than the pectin in non lignified tissue, led to the
suggestion that an insoluble lignin pectin complex may
exist. Although chemical combination between lignin and
carbohydrates has been reported, the type of linkage
of pectin and lignin that would decrease solubility is
unknown and therefore such a complex is thought un-
likely. Calcium, magnesium and iron have been demon-
strated in the intercellular layer and in fact, highly
insoluble double salts of galacturonic acid have been
isolated. Also calcium pectinate and other polyvalent
cation salts are well known for their insolubility.
Thus the insolubility of "protopectin" could be ex-
plained entirely by pectin cation complexes. However
the cation complexing reagents used to test this view,
have been used under conditions that would in any case,
lead to extensive denaturation and depolymerization of
pectins. A great deal of further evidence is presented
by Joclyn both for and against this view, but he was

not able to draw any definite conclusions as to which,
of the several possible explanations for native pectin
insolubility, was the correct one. The most likely ex-
planation seemed to him to be that " Native pectin ex-
ists as a polygalacturonide, in which the hydroxyl
groups on carbon atoms 4 and 5 are masked by glycoside
and ring formation and the carboxyl on carbon atom 1 (sic 5)
is either free, esterified with methyl alcohol or es-
terified with araban, galactan or other polysaccharides.
The hydroxyl groups on carbon atoms 2 and 3 may be free,
esterified with acetyl groups, or linked by ether like
linkages to polysaccharides or lignins. It is likely
that non uronide sugars occur in the main chain as well
as in branched chains. Ester, hemiacetal and ether lin-
kage is suggested between chains of the polyuronide and
polysaccharide. The degree of acetylation may also be
a factor in determining the extractability of pectin."

Ginzburg, (1961) put forward evidence for a
protein gel structure, cross linked by metal cations
in the intercellular cement of pea root tips. Chelating
agents aided the separation of the cells, which could
then be recemented by treatment with divalent cations.
Proteolytic enzymes, or denaturing agents stop the cap-
acity to recement. Less importance was placed on the
role of cations by McClendon and Somers (1960). They
found that although chelating agents aided enzymic
maceration of potato tuber tissue, they had no action
alone, and calcium ions did not prevent maceration. Thus
they concluded that, although calcium ions were present
in this tissue, they did not contribute to its basic
structure.

Further evidence for the importance of the non-
uronide sugars in native pectin has been presented by
Barrett and Northcote (1965), who achieved the separ-
ation of pectic substances of the apple into a pure,

weakly acidic pectic compound and a neutral arabinan
(araban) galactan complex. The pectinic acid contained
galacturonic acid, arabinose, galactose, .rhamnose,
xylose and several trace sugars linked covalently to the
galacturonosyl residues. The suggestion was put forward
that the galacturonosyl-(1-2)-rhamnose link is a general
feature of pectinic acid structure. A similar situation
was found in sycamore, but in addition, there was a
strongly acidic polygalacturonic acid component. The
galacturonsyl-(1-2)-rhamnose link has also been found
in sycamore whole cambium.

An important point to be drawn out of the pre-
ceeding work, is that there is nothing which contradicts
the view that, pectic enzymes may be an important factor
in the breakdown of host tissue by plant pathogens. What
this work does suggest however, is that there may be other
enzymes working with, or as possible alternatives to,the
pectic enzymes in tissue decomposition.

Enzymes Attacking Pectic Materials

The literature dealing with pectic enzymes is
both prolific and confused. There have, however, been
several excellent reviews on the subject, namely, Phaff
and Joclyn (1947); Kertesz and McColloch (1950);Wood
(1955); Demain and Phaff (1957); Deuel and Stutz (1958);
Wood (1960); and Bateman and Millar (1966). Pectic enzymes
can be broadly classified into three groups. These are,
firstly, enzymes removing the methyl groups from pectin,
secondly, enzymes attacking the main uronide chain in one
of several ways, and lastly, enzymes attacking the non
uronide polymers associated with pectin.

The first group are known as the pectin methyl
esterases (P.M.E.'s), and are found in a wide range of
micro-organisms and plants. They have no effect on the
main uronide chain. In plants they tend to be bound

strongly to the cell wall and have to be extracted by
salt solutions (Jansen, Jang and Bonner, 1960). P.M.E.s
of microbial origin, particularly those from fungi,
generally show optimal activity at a lower pH than those
of higher plant origin, and are less affected by the
presence of salts in the reaction mixture (Bateman and
Millar 1966). Jansen, McDonnell and Jang (1945) reported
that polygalacturonase (P.G.), acting simultaneously
with P.M.E. on pectin at pH 4.0 had a favourable effect
on de-esterification and on the maintenance of the reac-
tion rate, as compared with the action of P.M.E. alone.
This suggests that P.M.E. is inhibited by its products.
In sufficiently high concentrations, P.M.E. enhanced
the action of P.G. on pectin to an extent that approached
the rate observed on pectic acid.

The most important group of pectic enzymes con-
sists of those enzymes attacking the main uronide chain
of pectin or pectic acid. Attempts to classify these
enzymes have been made by Demain and Phaff (1957) and
Deuel and Stutz (1958), but due to the lack of know-
ledge about the recently discovered transeliminases,
these classifications are now incomplete. Bateman and
Millar (1966), have put forward the most comprehensive
classification to date, and it is this that will be used
here.

All the chain splitting enzymes have the ability
to break the α1-4 glycosidic bonds between galacturonic
acid monomers of the pectin or pectic acid chain. The
main subdivisions in their classification depend on
whether they attack such bonds by a hydrolytic mechanism,
or by a transeliminative mechanism. Pectic enzymes of
the hydrolytic type have been known for a considerable
time, and split the glycosidic bond with the addition
of water across it. The transeliminases are of more

recent discovery.   : The earliest report of such
activity was by Albersheim, Neukom and Deuel (1960a).
Using a commercial pectinase preparation, they found
an enzyme which attacked only the methyl ester of
pectic acid, giving breakdown products which absorbed
strongly at 235nm., due to the presence of -4-5 un-
saturated galacturonic acid groups. The mechanism of
such action will be dealt with later. Hydrolases and
transeliminases can then be further classified on
whether they degrade methylated or demethylated pectin,
and whether they attack the substrate in a random or
terminal manner. Enzymes attacking the polymer chain
in a terminal manner, ie. by removing monomers from
the end, are known as exo-enzymes. Those attacking the
polymer randomly are known as endo-enzymes. Using such
criteria the classification of Bateman and Millar is
as follows :-

A. Enzymes causing hydrolytic cleavage of the α 1-4
glycosidic links of pectic substances.

  1. Random mechanism of hydrolysis.

      a. Pectin attacked in preference to pectic acid
      endo-polymethylgalacturonase (endo-P.M.G.).

      b. Pectic acid attacked in preference to pectin
      endo-polygalacturonase (endo-P.G.).

  2. Terminal mechanism of hydrolysis.

      a. Pectin attacked in preference to pectic acid
      exo-polymethylgalacturonase (exo-P.M.G.).

      b. Pectic acid attacked in preference to pectin
      exo- polygalacturonase (exo-P.G.).

B. Enzymes causing transeliminative cleavage of the α
1-4 glycosidic links of pectic substances.

  1. Random mechanism of transeliminative cleavage

      a. Pectin attacked in preference to pectic acid
      endo-pectin methyl transeliminase (endo-P.M.T.E.
                                        or P.T.E.)

      b. Pectic acid attacked in preference to pectin
      endo-polygalacturonate transeliminase (endo-P.G.T.E.)

2. Terminal mechanism of transeliminative attack
    a. Pectin attacked in preference to pectic acid
    exo-pectin methyl transeliminase (exo-P.M.T.E.
                                     or P.T.E.)
    b. Pectic acid attacked in preference to pectin
    exo-polygalacturonate transeliminase (exo-P.G.T.E.)
Demain and Phaff (1957) used pH optima and extent of bond
breakage to classify the enzymes further, but such
criteria were not used by Bateman and Millar (1966)
because they considered that there was too little
information on the degradation of substrates by purified
enzyme systems.

    Hydrolytic pectic enzymes were familiar to some
of the earliest workers in the field, but it is highly
probable that many of the enzymes considered as
hydrolases in the past were in fact transeliminases.
The hydrolase attacks the 1-4 α glycosidic bonds of
the uronic acid polymers in the following manner.
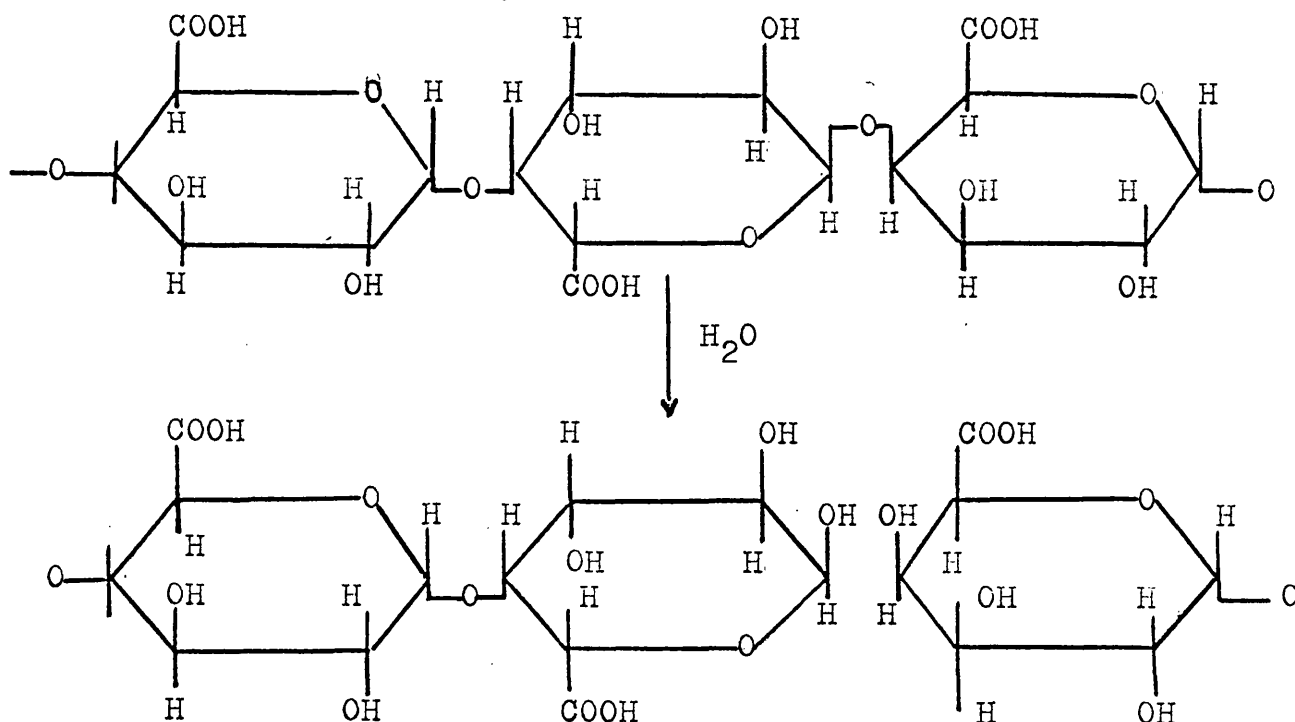
Hydrolysis of part of a Polygalacturonide Chain

Pectic hydrolases are found in fungi, bacteria and higher plants. Luh and Phaff (1951a), demonstrated a P.G. free of P.M.E. in yeast cultures. They later showed that this enzyme caused incomplete breakdown of pectic acid to a mixture of digalacturonic acid and galacturonic acid, (Luh and Phaff 1951b, Demain and Phaff 1956). Although this was a three stage process, they showed that only one enzyme, endo-P.G., was responsible for it. Mill and Tuttobello (1961) identified and purified an endo-P.G. from Aspergillus niger. They showed that it acted on pectic acid in the same way as yeast P.G.. Patel and Phaff (1960) isolated a P.G. from tomatoes. They considered that the purified system consisted of two components, as digalacturonic acid was attacked, in contrast to the yeast and aspergillus enzymes.

Perombelen and Hadley (1964) identified an endo-P.G. and an endo-P.M.G. in certain strains of Rhizoctonia and a similar situation was found in Erwinia carotovora by Masao Goto and Norio Okabe (1963). Akira Endo (1964) isolated and purified three endo-P.G.s from Coniothryum diplodiella. These had different optimum pHs. Apart from tomatoes, exo-P.G.s have been identified in several Botrytis species by Hancock, Millar and Lorbeer (1964), and in Botrytis cinerea by Barash et al (1964). Garber Beraha and Shaeffer (1965) also identified an exo-P.G. and an endo-P.G. in culture filtrates of Penicillium digitatum and Penicillium italicum.

A study of the alkaline breakdown of pectin (Neukom and Deuel, 1958) and the breakdown of pectin in neutral solutions (Albersheim Neukom and Deuel 1960p), showed that under these conditions the glycosidic links were cleaved by a β-dealkoxylation mechanism. This meant that the glycosidic linkage in the β position to

the ester carbonyl group of pectin was cleaved , after
the removal of the activated hydrogen at carbon atom
5, and the formation of a double bond between carbon
atoms 4 and 5. The products of such a breakdown showed
an absorption maximum at 235 nm, and reacted with thio-
barbituric acid to give a product which absorbed at
547 nm. There then followed the discovery that pectin
and pectic acid could be broken down enzymically by
this mechanism (Albersheim, Neukom and Deuel, 1960a).
Investigations by Nagel and Vaughn (1961), on the deg-
radation of oligogalacturonides by the P.G. of Bacillus
polymyxa showed that this enzyme broke down trigalact-
uronic acid to give galacturonic acid and an altered
dimer. This dimer absorbed strongly at 235 nm, and it
was concluded that the enzyme was a transeliminase,
rather than a hydrolase. It appeared to be an endo-
P.G.T.E., as digalacturonic acid was not attacked.
Starr and Moran (1961) identified a P.G.T.E. in culture
filtrates of Erwinia carotovora and Bacillus polymyxa,
while Macmillan and Vaughn (1964) and Macmillan, Phaff
and Vaughn (1964), purified and studied the action of
an exo-P.G.T.E. from Clostridium multifermentans. This
enzyme was dependent on critical levels of certain di-
valent cations; calcium, strontium and manganese
showing the greatest stimulation. It showed no activity
on pectin, but removed units of $O-(4-deoxy-\beta-L-threo-$
hexopyranos-4-enyluronic acid$)-(1 \rightarrow 4)$-D-galacturonic
acid (orα,β-unsaturated digalacturonic acid) from the
reducing ends of polygalacturonic acid chains. Strangely
enough, although it was an exo- enzyme, it did not
attack digalacturonic acid.

Fuchs (1965) identified an inducible P.G.T.E.
from three strains of Pseudomonas fluorescens.This
enzyme was capable of converting sodium polygalact-
uronate into several reaction products, which strongly

absorbed light at 235 nm. Paper chromatography revealed
that these products constituted a homologous series of
oligouronides. The fastest moving compound appeared to
be an unsaturated monouronic acid, 4-deoxy-L-threo-5-
hexo-seulose uronic acid. This together with other in-
direct evidence, indicated that the enzyme was an endo-
P.G.T.E. cleaving the glycosidic linkages as shown.
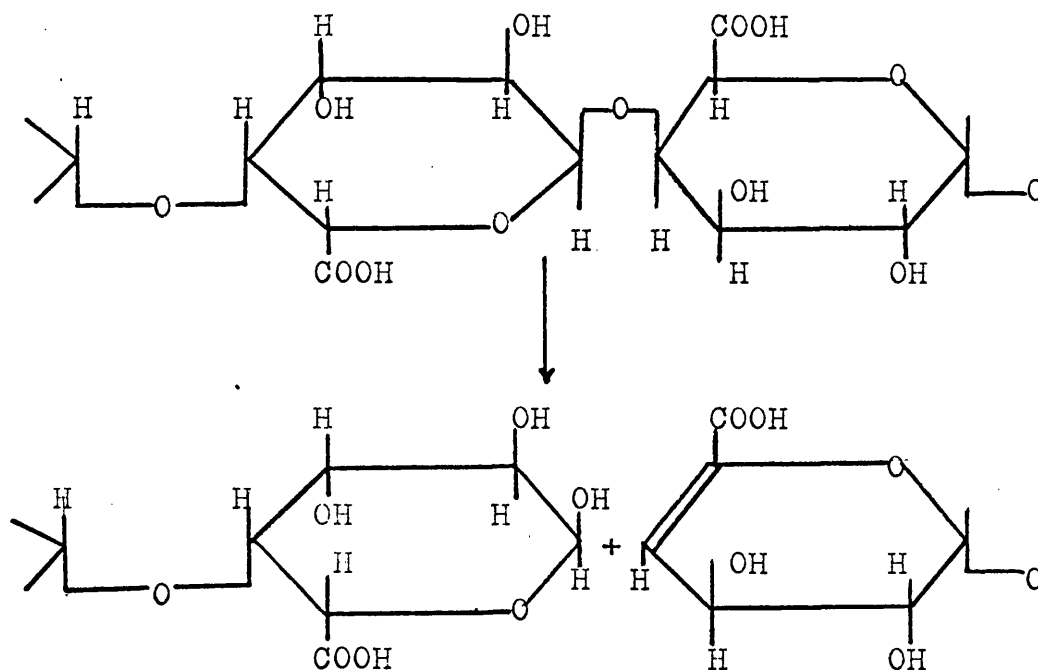
Transeliminative Cleavage of Part of a
Polygalacturonide Chain.

A P.T.E. from a fungal source was purified by
Albersheim and Killias (1962), taking advantage of the
low isoelectric point of the enzyme (pH 3•5). After
surveying several fungi, a study was made of the pro-
duction and purification of a P.T.E. from Aspergillus
fonsecaeus by Edstrom and Phaff (1964a and 1964b).
They found that the enzyme caused a random cleavage of

pectin, giving α, β unsaturated reaction products, and
that its action was enhanced by calcium ions. Another
P.T.E. was isolated,together with a hydrolase, from
cell extracts of an aerobic Bacillus (Hasegawa and
Nagel,1967). They separated the two enzymes, and found
that the transeliminase attacked higher uronides in a
random manner, but was unable to attack trigalacturonic
acid. Again the enzyme was stimulated by calcium ions
but unlike the transeliminase of Aspergillus fonsecaeus
it was able to attack pectic acid as well as pectin.

The final group of enzymes involved in the
breakdown of pectic substances, are those breaking
down the non-uronide polymers associated with pectic
acid and pectin. The most important of these are the
arabanases and the galactanases. Kaji et al have id-
entified an arabanase in a commercial enzyme prepar-
ation (1961) and also in the culture filtrates of
several Aspergilli (1963). On purification,these
enzymes appeared to attack the araban polymer in a
terminal manner, by removing arabinose units from the
end of the chain. In a later publication, Kaji et al
(1964), found that the enzyme preparations could hyd-
rolyse not only the 1:5 links of the main araban chain
but also the 1:3 links of the branch chains.

The Role of Pectic Enzymes in Pathogenèsis and Maceration

The enzyme responsible for breakdown of host
tissue by pathogens was, for a long time designated as
'protopectinase'. The work of Joclyn (1962), quoted
earlier, has indicated that protopectin is not a spec-
ific substancé thus the existance of a protopectinase
is unlikely.

Beavan and Brown (1949), attributed the macer-
ating power of Byssochlamys fulva to a 'pectinase',
which appeared to reduce the molecular size of pectin,

without the production of galacturonic acid. This en-
zyme was not found free of P.M.E., and it seems prob-
able in the light of modern knowledge, that it was an
endo P.G.. Similar P.G.s were isolated from Botrytis
cinerea, Penicillium expansum and Aspergillus aureas,
by Jermyn and Tom kins (1950). When a study of their
action was made, they were found to hydrolyse pectic
acid by a random scission, and to cause a rapid low-
ering of the viscosity of pectin solutions. It would
seem likely that these preparations contained a mixture
of P.M.E., P.G. and possibly P.M.G.. Such a situation
was in fact verified by Jarvis (1953), who found two
pectic enzymes in Botrytis cinerea, one acting on
pectin and the other acting on pectin and pectic acid.
Both enzymes caused viscosity reductions and the author
claimed that they resembled protopectinase (macerating
factor) more nearly than P.G.. It would seem that there
is a confusion of terminology here. The P.G. refered
to by Jarvis, is probably an exo-P.G. and the two
viscosity reducing enzymes, endo-P.M.G. and endo-P.G.

Botrytis cinerea causes rotting of apples as
do Sclerotinia fructigena and Sclerotinia laxa,but
when Cole J.S. (1956) examined rotted tissue for
pectic enzyme activity, he was unable to detect this.
He was however, able to show that pectin had been
broken down in rotted apple tissue, and he also found
that oxidised apple juice had a pronounced effect in
deactivating certain enzymes, particularly those prod-
uced by B. cinerea. This, he felt, was an adequate ex-
planation of the absence of pectolytic activity in
rotted apple tissue; though such enzymes were clearly
involved in pathogenisis. Apple pathogens, causing a
firm rot of apples, caused a reduction in the water
soluble pectic substances of about 10 - 20%, whereas

soft rot fungi caused larger changes, of about 70%_
(Cole M. and Wood 1961a). Galacturonic acid, absent
from sound tissue, was universally present in each
type of rotted tissue. In a subsequent publication
Cole M. and Wood (1961b), found that filtrates from
S. fructigena and Penicillium expansum liberated gal-
acturonic acid from apple fibre, which had been thor-
oughly extracted with cold water, leaving only the
insoluble pectic materials. Substances claimed by
these authors to be responsible for the inhibition of
P.G. in healthy apple, were leuco-anthocyanins, changed
to other compounds by the action of polyphenol oxidases.

Botrytis cinerea is pathogenic on a number of
hosts and investigations have not only centered around
its attack on apples. Leachates of Safflower blossoms
contained nutrients that stimulated the germination of
conidia of B. cinerea. Such leachates also contained
pectic substances that induced the production of P.G.
in germinating Botrytis conidia, according to Barash,
Klisiewicz and Kosuge (1964). The P.G. activity was
detected soon after the germ tubes appeared and in-
creased as they elongated. Extracts of flower heads,
infected with this organism, showed traces of galact-
uronic acid, not traceable in sound blooms.

In yet another host parasite relationship in-
volving Botrytis cinerea, the production of pectic
enzymes by this organism, together with Botrytis allii
and Botrytis squamosa, was investigated by Hancock,
Millar and Lorbeer, (1964a), using potato dextrose
broth, detached onion leaves and intact leaves. P.M.E.
and cellulase were found to be produced by all organ-
isms under all conditions. B. allii produced trace
amounts of P.G. in potato dextrose broth and both endo
and exo-P.G. in detached and intact leaves; whereas
B. cinerea and B. squamosa produced endo-P.G. under all

conditions, and exo-P.G. in detached leaves. When culture
filtrates were sprayed onto onion leaves, the leaf spots
induced were similar to those caused by the Botrytis
species (Hancock, Millar and Lorbeer 1964b). Culture fil-
trates with little P.G. failed to cause leaf spotting
even though they contained a moderate amount of cellulase.
Assays of extracts from diseased onion plants indicated
that endo-P.G. and P.M.E. were the principal pectic enzymes
associated with Botrytis leaf spot. The absence of exo-P.G.
from plants infected with B. cinerea and B. squamosa sug-
gested to these authors that this enzyme did not play an
important role in Botrytis leaf spot.

Sclerotinia sclerotiorum a pathogen of apples and
other fruits, was shown to produce P.M.E., P.G. and mac-
erating factor when grown on wheat bran (Echandi and Walker
1957). These activities were inhibited by various vegetable
storage organ extracts (c.f. Cole M. and Wood 1961 a and b).
Echandi and Walker claimed that Erwinia aroideae differed
from S. sclerotiorum in that it produced no P.G. or P.M.E.,
but only a pectin depolymerase. Here again is an apparent
confusion of terms, as the so called depolymerase was almost
certainly an endo-P.G. or endo-P.M.G. or endo-transeliminase.
E. aroideae was also investigated by Murant and Wood (1957),
with reference to the rotting of potato tubers. A filtrate
of this organism was also found to cause maceration of
potato tuber tissue tissue, but this activity could be red-
uced by potato sap. The mechanism of resistance to infection
by Erwinia, found in potato tuber tissue, has been inves-
tigated in detail by Lovrekovich, Lovrekovich and Stahmann
(1967). In contrast to the results of Echandi and Walker
(1957), Wood and Gupta (1958), detected both P.M.E.and P.G.
in E. aroideae but they were unable to detect these enzymes
in Pythium debaryanum, although this organism macerated
potato tissue. P. debaryanum produced an enzyme causing

a reduction in the viscosity of pectin at a high pH
which, it is now considered, was probably an endo-P.M.G.
or an endo-P.T.E..

P.G. and P.M.E. were found in culture filtrates
of Fusarium oxysporium and Fusarium lycopersici by
Waggoner and Dimmond (1955). They claimed that P.M.E.
plus P.G. caused maceration, and detected these enzymes
in the vascular stream of tomato plants infected with
Fusarium; thus indicating that these enzymes had a
role in pathogenicity. In Fusarium solani and Rhizoc-
tonia solani, Elasori (1958) correllated 'protopectin-
ase' activity with enzymes that reduced the viscosity
of pectin. He did this by comparing their pH optima.
He found that the enzymes from both fungi were more
active together than singly, and that only the Rhizoc-
tonia enzyme formed galacturonic acid as an end prod-
uct. The author concluded from these results that
'protopectinase' activity was probably due to a cum-
ulative effect of several enzymes.

Later work on Rhizoctonia solani (Bateman
1963a), revealed that water extracts of bean hypo-
cotyls infected with this organism, contained P.G.
and P.M.E.. The P.G. complex produced by R. solani in
bean medium, appeared to contain an exo-P.G. not prod-
uced in vivo, where only an endo-P.G. was present. The
optimum pH for maceration by culture filtrates of this
organism were found to vary, depending on the relative
proportions of P.G. and cellulase (Bateman 1963b).
From these experiments, he concluded that P.G. was
primarily responsible for maceration, whereas cellulase
appeared to play a secondary role. To confirm this he
extracted P.G. from Rhizoctonia infected bean tissue,
purified it and found that it macerated in the absence
of P.M.E. and cellulase.

When extracts were made of bean hypocotyls infected with Rhizoctonia solani, they appeared to contain a dialysable inhibitor to P.G. and maceration (Bateman 1964). Calcium ions seemed to be most effective in this respect, having been detected by radio tracers around lesions. The suggested mechanism for this inhibition was, that the calcium ions formed calcium pectate with demethylated pectin, which was then resistant to attack by P.G.. Though the preceeding evidence favours strongly the involvement of the pectic hydrolases in Rhizoctonia host parasite relationships, Perombelon and Hadley (1964) could detect no larger amounts of endo-P.G., endo-P.M.G. and macerating activity in pathogenic Rhizoctonia strains than they could in non pathogenic strains. This suggested that factors other than pectic enzyme production and the ability to macerate host tissue may be involved in pathogenicity. Further to this, no P.G. or macerating activity was detected in cultures of the pathogen Phytophthora infestans, though P.M.E. was constitutive (Clarke 1966). The absence of pectic enzyme activity in this organism may be accounted for by the fact that it does not cause a soft rot disease. Nevertheless, it indicates that pathogenicity may not necessarily be dependent on the presence of pectic enzymes.

Evidence for the participation of pectic enzymes in other host parasite relationships, was found by Leal and Villaneuva (1962), who examined forty strains of Verticillium species grown on a pectin medium. High pectic enzyme activities were found in pathogenic strains and no activity was found in the non pathogenic ones. Similarly Abo el-Dahab (1964), using Xanthomonas malvacearum, found that by adding pectin

to a medium, the pectic enzyme production was increased and the cells became more virulent. Cellulolytic activity was not detected. Further evidence along these lines was put forward by Singh and Akhitar (1964) who found that the more virulent strains of <u>Colletotrichum falcatatum</u> produced more P.G. than the less virulent ones. Cellulase and P.M.E. were produced equally by all organisms. Evidence of a rather different nature for the involvement of pectic enzymes in rotting, was put forward by Edney (1964). He found that if apples were stored in 5% CO_2 and 3% O_2, rotting by Gleosporium species was considerably reduced. If the pathogens were incubated under these conditions, then the pectic enzyme production was reduced.

Wood (1955), in a review on pectic enzymes, concluded that the secretion of pectic enzymes by plant pathogens had two purposes. These were, firstly the breakdown of plant tissue and secondly further degradation of primary breakdown products to give substances, which would pass into the cell and be used for growth. Although at this stage he was uncertain as to the exact nature of these enzymes, the truth of his statement still holds, in the light of modern knowledge. Deuel and Stutz (1958) concluded that pectic enzymes were involved in the maceration of plant material and the killing of plant cells, though they did not commit themselves very specifically on the exact identity of the enzymes,and the extent to which they were involved. In a later review Wood (1960), admitted that the macerating activity, shown by some plant pathogens, followed the activity of the chain chain splitting pectic enzymes, but pointed out that with other organisms, the maximum activity of P.G. and P.M.E. were obtained under different conditions from

the maximum activity of maceration. In the same review
Wood made the point, that to prove that an enzyme
plays a part in plant disease, it must be shown to
be present in diseased plant tissue. In conclusion he
suggested that other factors such. as proteases and
arabanases, might have a part to play in maceration.

The main evidence for the involvement of arab-
anases in maceration and pathogenicity was obtained
by Byrde and Fielding (1962 and 1965), These workers
separated an endo-P.G. and a macerating factor from
Sclerotinia fructigena and found that the latter lib-
erated arabinose from potato tissue and also from a
synthetic substrate, phenyl-α-L-arabinofuranoside. Further
evidence for this view came from the work of Fuchs
et al (1965), who detected highest arabanase activity
in fungi attacking legumes, which have a high araban
content. Extensive work by McClendon (1964), also pro-
duced a certain amount of evidence for the involvement
of arabanases in phytopathogenicity.

No mention has yet been made of the role of
the transeliminases in maceration and pathogenicity.
Their discovery has increased the evidence for the
general involvement of pectic enzymes in host pathogen
relationships. Starr and Moran (1961), have shown that
culture filtrates of Erwinia carotovora and Bacillus
polymyxa, grown in pectin, had transeliminase activity.
They later characterized the enzyme (Starr and Moran
1962), showing that it acted preferentially on pectic
acid rather than pectin. It was activated by calcium
ions to such an extent that E.D.T.A. produced complete
inhibition. Dean and Wood (1967), fractionated culture
fluids of Erwinia aroidea and found a close correlation
between P.G.T.E. activity and maceration. When they
incubated a sample of the enzyme with potato cell wall

material there was an increase in absorbance at 235 nm.
This explains the results of Echandi and Walker (1957),
mentioned earlier, who could find no P.G. or P.M.E. but
only a depolymerase in filtrates of E. aroidea.

The main conclusions to come out of the work
of McClendon (1964), apart from the possible importance
of arabanases in pathogenicity, were that maceration
could most likely be correlated with the endo forms of
both hydrolases and transeliminases; and that exo-
enzymes were unlikely to be involved in this process.

Calcium ions were found to stimulate the act-
ivity of a P.G.T.E. produced by Fusarium species grown
on autoclaved alfalfa roots (Millar 1965). Calcium
was also found to stimulate maceration of potato by
culture filtrates containing P.G.T.E. but no endo-P.G.
This contrasts directly with the results of Bateman
(1964), who found that calcium ions inhibited P.G. and
maceration in Rhizoctonia solani. Such a contrast is
interesting,as it gives strong evidence to the view
that maceration can be caused by both hydrolytic and
transeliminative activities. A P.G.T.E., similar to
that isolated from Fusarium, was detected in Colleto-
trichum trifollii , Ascochyta imperfecta and Stemphylium
botryosum , in the absence of endo-P.G. and endo- P.M.G.,
when these organisms were grown on autoclaved alfalfa
shoots (Hankock and Millar 1965a). However only
C. trifollii produced P.G.T.E. in vivo in any signifi-
cant amounts. This was associated with a greater
change in pectic materials in the disease of alfalfa
caused by this organism, than in the diseases caused
by the other two. Although all three organisms were
found to be capable of utilizing cellulose, only
C. trifollii produced this in vivo (Hankock and Millar
1965b). These authors also found certain evidence for

the involvement of proteolytic enzymes and xylanases
in C. trifollii infections. The P.G.T.E. production
was favoured by an increase in pH (Hankock 1965) and
it was therefore thought to be significant that such
pH increases took place during the course of patho-
genisis of alfalfa tissues infected with C. trifolli

Futher evidence of the participation of trans-
eliminases in pathogenesis, was obtained by Bateman
(1966), who showed that Fusarium solani produced three
pectic enzymes in vitro. These were, a hydrolase attack-
ing pectin at pH 5·0, a hydrolase attacking pectic acid
at pH 6·0 and a calcium dependent transeliminase, deg-
rading pectin or pectic acid at pH 8·6 or above. The
transeliminase only was produced on autoclaved bean
hypocotyls, and it was the predominant enzyme in vivo.
This work clarified the situation found by Elasori
(1958), quoted above, in F. solani, where he identified
pectin depolymerases distinct from P.G.s..P.G.T.E. has
also been isolated from phytopathogenic bacteria in
the genus Xanthomonas (Nasuno and Starr 1967).

Bateman and Millar (1966), have concluded, in
their review of the subject, that maceration is not
the property of a single enzyme, but that it could
most likely be correlated with the endo forms of the
pectic glycosidases (hydrolases) and lyases (trans-
eliminases), as suggested by McClendon (1964). Again
these authors found no evidence of maceration by exo-
enzymes. Although they found abundant circumstantial
evidence for the involvement of pectic enzymes in
pathogenicity, they felt that proof that the ability
of an organism to produce pectic enzymes accounted
for its pathogenicity was lacking.

The Pathogenicity of _Penicillium digitatum_, _Penicillium italicum_ and _Aspergillus fonsecaeus_ on Citrus Fruits.

Penicillium digitatum and Penicillium italicum
cause the green and blue rots of citrus fruit respec-
ively. The diseases are most prevalent after harvest,
in the packing houses and in transit. Both organisms
cause softening of the fruit and profuse sporulating
growth appears over the surface. The spores of P.dig
itatum are olive green, whereas those of P. italicum
are rather more of a blue green colour.

Klotz (1930), found that both Penicillium
digitatum and Penicillium italicum grew inter and intra-
cellularly in the albedo parenchyma, flavedo and epid-
ermis; finally invading the juice vesicles. He noticed
that the organisms produced a limited softening ahead
of the invading mycelium and suggested that these
macerating substances were for the most part intra-
cellular; being secreted only in a limited quantity
near the tips of the living hyphae. They were, he
thought, liberated en masse from old, dead, autolys-
ing mycelium in the regions of advanced decay. To test
this theory, Klotz made a mycelial extract of the
fungiin water and inserted this in cylindrical holes
made in the albedo of the orange. The result was a
distinct softening around the holes. This softening
was darker and more pronounced than that caused by ox-
alic acid.

Green (1932), working mainly on the epidemiology
of Penicillium digitatum and Penicillium italicum con-
cluded that there was a resistance to infection in the
rind of oranges, which could be broken down by acids
and ammonium oxalate. Penicillia grown in citrus juice
or rind, but not in synthetic media also appeared to
be able to break down the rind resistance. Once this

system was broken down, infection could occur through
the stomata in uninjured rind. Green admitted that
this so called resistance destroying system might be
enzymic. Further work on the epidemiology of these
diseases indicated that susceptibility to infection
wasto some extent, related to the mode of inoculation
(Nadel Schiffman et al 1953). For example, inoculations
pricked into the oil glands gave consistantly higher
rates of infection than those between the glands.
Kavanagh (1965) found that filtrates of Penicillium
digitatum grown in a pectin medium, did not promote
infection, whereas filtrates when glucose was the car-
bon source did. This worker demonstrated the production
of P.M.E. and P.G. by P. digitatum in vitro, although
he could find little cellulase. P.M.E. was demonstrated
in greater quantity in 0·2M NaCl extracts of infected
rind than in similar extracts of healthy rind. P.G.
and cellulase were found to a slight extent in infected
rind but not in healthy rind, though macerating activ-
ity, while produced in vitro, was not found in vivo.

Miyakawa (1962) found that both Penicillium
digitatum and Penicillium italicum excreted P.M.E. and
P.G. constitutively and also demonstrated P.G. activity
in infected orange peel tissue. The macerating activity
of the two moulds appeared to be lower than that of the
less pathogenic fungus Penicillium expansum, suggesting
to the author that pectic enzymes were not the sole
factor involved in pathogenicity. However he found
that both fungi could breakdown pectin and furthermore,
use the products of such a breakdown as carbon sources.
In a later publication Miyakawa (1963) suggested that
D-galacturonic acid, produced by both P. digitatum and
P.italicum on pectin media, might have an effect on
pathogenicity. He found that galacturonic acid caused

in vitro maceration of orange peel. This compound also
showed an activity in the promotion of infection by
the two Penicillia, suggesting that D-galacturonic acid
could cause a destruction of rind resistance. This ob-
servation contrasts directly with an observation made
by Kavanagh (1965), that filtrates of P.digitatum
grown in a pectin medium, did not promote infection.
It is assumed that such filtrates contained galact-
uronic acid.

Cole A.L.J. (1967), in a very extensive survey
of enzyme production by Penicillium digitatum in rel-
ation to the attack of oranges by this organism, found
that it produced constitutively P.M.E., enzymes that
reduced the viscosity of both pectin and pectic acid,
P.T.E., cellulase,α-L-arabinofuranosidase,β-D-gal-
actopyranosidase and macerating factor. On passing
culture filtrates through Sephadex G75 he found that
the peak of macerating activity coincided with the
peak of the viscosity reducing enzymes and P.T.E.. The
peak of theα-L-arabinofuranosidase was slightly dif-
ferent. A similar situation was encountered with cul-
ture filtrates of the organism, grown on pectin and
sodium polypectate media, although less growth was
obtained than in the medium without pectin. On a syn-
thetic medium containing pectin, P.digitatum produced
less viscosity reducing activity than before, though
macerating activity was high. P.T.E. was also present
on this medium and appeared to have the same optimum
pH as macerating activity. No difference was found
between pectic hydrolase, pectic lyase and macerating
activity, by thermal inactivation experiments. Growth
on a slightly altered synthetic medium led to a sit-
uation where P.T.E. was absent, but viscosity reduc-
ing activity and macerating activity were high. The

enzyme reducing the viscosity of pectin was found to
lose some of its activity on freeze drying, whereas
neither the enzyme reducing the viscosity of pectate
nor macerating activity were affected by such a treat-
ment. Dialysis appeared to reduce the activity of the
viscosity reducing enzymes and maceration, none of
which recovered after dialysis against salts.

Cole found that Penicillium digitatum, when
grown on an orange albedo medium, produced P.T.E.,
pectin viscosity reducing activity and macerating
activity, but no P.M.E... There was a correlation bet-
ween the loss of weight of an orange tissue preparation
during degradation and the release of reducing groups
from it. Galacturonic acid, galactose and arabinose
were identified as end products of this degradation
which occured in the absence of P.T.E.. Macerating
activity and P.M.E. were detected in infected rind but
viscosity reducing enzymes, P.T.E., cellulase and pro-
teolase were absent. Cole found that macerating activity
and pectin viscosity reducing activity were present in
the juice of infected fruits, but P.M.E. and P.T.E.
were not. Orange juice appeared to have a thermolabile
inhibitor to macerating activity and viscosity reduc-
ing activity.

A comparison of pectolytic and cellulolytic
enzyme production by Penicillium digitatum, Penicillium
italicum and Penicillium expansum with enzyme production
by avirulent mutants of these organisms, was made by
Garber, Beraha and Schaeffer, (1965). Pectolytic and
cellulolytic activity appeared to depend on the carbon
source in the growth medium, even with the mutants.
The avirulent forms of P.digitatum and P.italicum had
pectolytic activity when they were grown on a pectin
containing medium. Orange tissue rotted by P.digitatum
and P.italicum had both pectolytic and cellulolytic

activity, though necrotic tissue, produced by aviru-
lent mutants had only cellulolytic activity. In a
later publication Garber and Beraha (1966), showed
that endo- and exo-P.G. were produced by P.digitatum
and P.italicum grown on a variety of media, whereas
P.T.E. was not. These authors also found exo- and endo-
P.G. in the tissue of oranges infected with these org-
anisms.

A wide range of pectic enzymes have been shown
to be produced by Penicillium digitatum and Penicillium
italicum both in vitro and in infected fruits, thus
indicating their role in the pathogenicity of these
organisms. The abundant evidence quoted earlier, for
the involvement of pectic enzymes generally in host
parasite relationships, supports such an indication.
However what still needs to be shown, is the exact
nature of the enzymes responsible for the pathogen-
icity and more particularly, the macerating activity
of P.digitatum and P.italicum. Further studies on the
enzymes produced both in vitro and in vivo by these
organisms; and fractionation of culture filtrates of
them,may help to discover the identity of the factor
causing maceration. Such studies may also reveal to
what extent pectic enzymes are involved in the rotting
of citrus fruits by Penicillia.

A fungal disease of citrus fruits known as the
'black rot' has been reported by Fawcett, (1926). This
is not as prevalent as the Penicillium rots and is
normally only found in countries with a very hot cli-
mate. The organism causing such a disease is Asper-
gillus niger. A wide range of pectic enzymes have been
shown to be produced by this mould. Tuttobello and
Mill (1961), identified an endo-P.G., Kaji et al (1963)
an arabanase and Edstrom and Phaff (1964), purified a
P.T.E. from Aspergillus fonsecaeus, a species of the

A.niger group. It was decided therefore, to investig-
ate the pectic enzymes produced by A.fonsecaeus and
to compare them with the enzymes produced by the Pen-
icillia mentioned above,in order to determine whether
the same factors were responsible for pathogenicity
and maceration in each organism.

EXPERIMENTAL PART 1. Materials and Methods.

     All chemical used, were supplied by British
Drug Houses Ltd., unless otherwise stated. ANALAR re-
agents were used wherever possible, especially in the
analytical techniques.

a. Growth and Maintenance of Cultures

     Penicillium digitatum Saccardo was obtained
from a mouldy orange and isolated on malt agar. Peni-
cillium notatum Wehmer, Penicillium italicum and
Aspergillus fonsecaeus (Aspergillus niger 41875) were
obtained from the Bath University of Technology fungal
culture collection. P.digitatum and P.notatum were
maintained on malt extract agar (3% Oxoid malt extract
+ 0·5% Oxoid mycological peptone + 1·5% Oxoid agar in
water), whereas P.italicum and A.fonsecaeus were main-
tained both on this medium and on Czapek Dox agar in
Universal bottles.

     In order to obtain large spore crops for inoc-
ulation of liquid or bran cultures, 5ml. of a sterile
0·05% Tween 80 solution were added to a Universal
bottle culture. After gentle shaking by hand, 1ml. of
the spore suspension so formed, was pipetted aseptic-
ally into a 20oz. medical flat, containing a 5mm.
layer of malt extract agar. The bottle was manipul-
ated so that the spore suspension covered the entire
surface of the agar; and was incubated at 25°C till
profuse sporulating growth was apparent. In order to
harvest the spores, about 20 sterile glass beads size
7 - 8mm., and 50ml of a 0·01% sterile Tween 80 solu-
tion were poured aseptically into the medical flat,
which was then agitated vigorously to suspend the
spores. The number of spores per ml in the suspension

was determined by means of a Thoma haemocytometer cell
and an appropriate amount of suspension could then be
used to seed either liquid or bran cultures. Liquid
shaken cultures were grown in 250ml flasks containing
75ml of medium, on either a New Brunswick gyrotatory
shaker or a Gallenkamp orbital incubator at $25^{\circ}C$, using
a 1" throw at 330 rev/min. Liquid surface cultures
were also seeded in 250ml flasks, containing 75ml of
medium and were incubated at $25^{\circ}C$ in a constant temp-
erature room. Bran cultures were likewise grown at
$25^{\circ}C$ in a constant temperature room, in either 250ml
or 1 litre conical flasks, depending on the amount of
bran used (10g or 40g respectively, moistened with an
equal amount of water).

The filtrates of liquid cultures were harvested
by filtering through Green's no. 904 paper, in a Buch-
ner funnel. The bran 'filtrates' were obtained by add-
ing water to the bran (100ml of water to 10g of dry
bran), and allowing this to stand for an hour. At the
end of this time, the bran was removed from the liquid
by filtering through muslin and the filtrate was cen-
trifuged at 2500 rev/min (2075 g) for 1 hour in a
M.S.E. Mistral 6 litre centrifuge. After harvesting,
filtrates were either frozen immediately, or freeze
dried in an Edwards Model 30 centrifugal freeze drier.

b. Ammonium Sulphate Fractionations

Partial separation of the enzymes in culture
filtrates was effected by fractional precipitation
with ammonium sulphate. The precipitate formed after
each addition of ammonium sulphate was removed and re-
dissolved. The amounts of ammonium sulphate to be added
to a solution to give the desired degree of saturation,
were obtained from a nomogram by Dixon (1953). Additions

were always made to solutions at refrigeration temp-
eratures (4°C or less). After the requisite amount of
ammonium sulphate had been added to a culture filtrate,
this was left to stand at 4°C for 15 minutes to allow
all the protein be precipitated. The solution was then
centrifuged at 12,000 rev/min (23,000 g) for 20 min-
utes in an M.S.E. 18 high speed centrifuge, and the
supernatant was removed. The precipitated protein was
then dissolved in cold water and either stored in a
deep freeze at - 25°C, or used immediately.

c. Column Chromatography

1. Sephadex.   Columns of Sephadex G25 were
used for desalting enzyme solutions, prior to their
passage through ion exchange cellulose. The Sephadex
was prepared by first immersing it in distilled water
(wt./vol. ratio 1/10) for three days to allow the gel
particles to swell. A column 30cms by 1·8cms was then
poured from this slurry, containing 10g dry weight of
Sephadex, in the following way. The column was filled
to one third of its height with distilled water, the
drain tap having been closed. The suspension of Seph-
adex was then poured into a separating funnel which
fitted, via a ground glass joint, into the top of the
column. The funnel tap was then opened, followed by
the drain tap at the bottom of the column. In this way
Sephadex flowed into the column as water flowed out
and packing of the gel was completely even. The top
of the gel was protected from disturbance by placing
on top of it, a piece of filter paper, with the same
diameter as the inside of the column. Samples were
applied to the top of the column and were eluted with
distilled water.

2. Ion Exchange Cellulose.   The cellulose
used in this work was Whatman's ET 11 ion exchange
cellulose for column chromatography, often known as
ECTEOLA cellulose. It was prepared for use according
to the manufacturer's instructions and the method of
Byrde and Fielding (1962). 25g of cellulose were
stirred into 500ml of water and allowed to stand for
30 minutes. At the end of this time the supernatant
was decanted off and the process was repeated, result-
ing in the removal of very fine particals which would
hinder column flow. The cellulose was then suspended
in 500ml of 0·5 N HCl for 1 hour, after which it was
filtered under suction. Washing with water then
followed until the pH of the washings collected in the
filter flask was between 4 and 5. The cellulose was
then suspended in 500ml of 0·5 N NaOH for 1 hour, fol-
lowed by washing as before, until the pH of the wash-
ings was between 6 and 7. The purpose of the acid/alk-
ali treatment was to impart sufficient hydrodynamic
force to the ion exchanger, in order to break the hyd-
rogen bonding in the vicinity of the ionic groups.
Such hydrogen bonding is induced by drying. The cell-
ulose was then suspended in 500ml of 0·5 M KH_2PO_4 to
remove U.V. absorbing impurities, washed again in
water and finally in 0·01 M phosphate buffer pH 7·5.
It was then suspended in distilled water prior to
column preparation. From 25g of cellulose, a column
45cms by 2·5cms was prepared. The cellulose suspension
was poured into the column which was half filled with
water, and at the same time the drain tap at the bot-
tom of the column was opened to allow the water to
flow through, thus ensuring even packing of the cell-
ulose. The top of the column was protected from dis-
turbance by a filter paper disc, in the same way as

the Sephadex. Desalted samples were applied to the top
of the column and elution was carried out, using water
and a stepwise salt gradient. Fractions from column
chromatography were collected on a Central fraction
collector and frozen or used immediately the run was
completed.

d. Dialysis

Salts were sometimes removed from enzyme
samples by dialysis, using a 1" Visking dialysis tub-
ing supplied by the Scientific Instrument Centre Ltd..
100ml of sample were dialysed against two changes of
5 litres of distilled water at $4^{o}C$.

e. Conductivity Measurements

Salt concentration was measured in enzyme sam-
ples by means of a Mullard conductivity bridge, made
by the M.E.L. company. Samples with conductivities of
less than 5×10^{2} μ mhos were subjected to ion exchange
chromatography.

f. Thin Layer Chromatography

The apparatus of Shandon and the media of
Merck were used in all the thin layer chromatography
carried out in this work.

1. Chromatography of Sugars.   The thin layer
method of Stahl and Kaltenbach (1961), was used to sep-
arate sugar monomers found as a result of the action
of culture filtrates on orange tissue. Chromatoplates
were prepared by spreading a mixture of 30g of Kiesel-
guhr G. and 60ml of 0·02 M sodium acetate (shaken for
1 minute) in a layer 0·3mm thick. The plates were dried
for 1 hour at $100^{o}C$ and stored in a container with
silica gel to protect them from moisture. Chromatograms
were run in an ethyl acetate:isopropanol:water (64:24:12)

solvent system and when dry, they were developed by
spraying with aniline hydrogen phthalate and heating
for 5 minutes at 105°C. The aniline hydrogen phthalate
was made up by dissolving 0'96g of aniline and 1·62g
of phthalic acid in 100ml of water saturated butanol.
This spray distinguished pentoses by a pink colour
and hexoses by a brown yellow colour.

2. Chromatography of the Breakdown Products
of P.T.E. Acting on Pectin.   The thin layer method
of Ovodov et al (1967), was used to separate the prod-
ucts of the action of P.T.E. on pectin. Chromatoplates
were prepared by spreading a mixture of 30g of Silica
Gel G. and 60ml of 0·07 M NaH_2PO_4 in a layer 0·3mm
thick. Plates were dried and stored as before. The
chromatograms were run in an ethanol:butanol:0·1 M
phosphoric acid (10:1:5) solvent system and when dry
they were developed by spraying with conc. H_2SO_4 in
ethanol (1:1 v/v), followed by heating for 5 minutes
at 100°C. This reagent detected organic material by a
charring effect, therefore galacturonic acid and all
of its oligomers right up to pectin would be detected.

g. Arabanase Assay
        Arabanase was assayed in culture filtrates by
measuring the rate of release of arabinose from an
araban substrate under defined conditions

        1. Preparation of Araban.   Preliminary ex-
periments indicated that araban could be obtained in
greatest yield from sugar beet. This was therefore
used as a source and extracted by the method of Jones
and Tanaka (1965). 3·3kg of molassed beet pulp nuts,
kindly supplied by the British Sugar Beet Corporation,
were soaked in running water for 24 hours to remove
the molasses. The remaining beet pulp was then suspended

in 15 litres of distilled water, to which had been
added 100g of calcium hydroxide and steam was blown
through this mixture for 24 hours. At the end of this
time the material was filtered through muslin and cen-
trifuged at 2,000 rev/min (1320 g) for 30 minutes in
a M.S.E. Mistral 6 litre centrifuge. The resultant
supernatant was concentrated to 3 litres by means of
a Quickfit climbing film evaporator and freed from
calcium salts by precipitation with oxalic acid. This
solution was then freeze dried in an Edwards Model 30
centrifugal freeze drier, giving a cream coloured
powder which, on analysis was found to contain about
50% araban. This material proved to be a satisfactory
substrate for the arabanase assay and was therefore
used without further purification.

2. Measurement of Arabinose.   1ml of enzyme
was added to 3ml of a 1% solution of the araban in
citrate phosphate buffer pH 3·9 and this was incubated
for one hour at $36^{\circ}C$ in Universal bottles. These con-
ditions were determined by experiments to be recorded
later. The reaction was stopped by boiling for 15 min-
utes. The reaction mixture was diluted 10 times to
measure its arabinose content, which was done by the
method of Tracey (1950). In this method, 6ml of reagent
made up by mixing 16ml of colourless aniline with 100
ml of glacial acetic acid followed by the addition of
24ml of distilled water and 3ml of 5% oxalic acid, was
added to 2ml of diluted reaction mixture and left for
24 hours. The resultant colour was measured at 470 nm
on a Unicam SP 600 spectrophotometer and was converted
to μg/ml arabinose by reference to a standard line. A
unit of arabanase activity was defined as that amount
of enzyme causing the release of 1μg of arabinose per
minute under the conditions stated.

h. Pectin transeliminase Assay.

Pectin transeliminase was measured by the method of Albersheim and Killias (1962), with certain modifications. $0 \cdot 1ml$ of enzyme was allowed to act on 3ml of $0 \cdot 5\%$ Kodak citrus pectin solution in $0 \cdot 1$ M citrate phosphate buffer at pH $5 \cdot 2$. The reaction was carried out in a 1cm silica cuvette at room temperature, and the increase in U.V. absorption at 235 nm was recorded. In the published method, the substrate was clarified by means of an enzyme preparation, but in the present work it was found to be more convenient to clear the substrate by centrifuging at 18,000 rev/min (40,000 g) for one hour at $4^{\circ}C$. One unit of transeliminase activity was that amount of enzyme which caused an increase in optical density at 235 nm of $0 \cdot 01$ optical density units, in one minute, under the conditions described above. Samples were assayed for pectic acid transeliminase activity by this method, using pectic acid $(0 \cdot 5\%)$ in buffer at pH $5 \cdot 2$, instead of pectin.

i. Assay of Reducing Group Liberating Enzymes.

P.G. activity was measured by determining the amount of reducing groups liberated from pectic acid, according to the method of Jansen and McDonnell(1945). Pectic acid was washed in 70% ethanol to remove any traces of reducing sugar impurities and was then dissolved in $0 \cdot 1$ M acetate buffer pH $5 \cdot 0$ to give a 0 5% solution. The pH of the solution was adjusted to $5 \cdot 0$ with 1 N NaOH. To 15ml of this substrate was added $0 \cdot 5ml$ of enzyme, and after mixing, 5ml was immediately withdrawn and pipetted into $0 \cdot 9ml$ of M Na_2CO_3 in a glass stoppered bottle. The enzyme substrate mixture was incubated for 8 minutes at $30^{\circ}C$ and a further 5ml

was withdrawn and pipetted into 0·9ml of M Na_2CO_3 in another glass stoppered bottle. 5ml of 0·1 N iodine were then added to each bottle and left for 20 minutes at the end of which time 2·0ml of 2 N H_2SO_4 were added and the mixture was shaken. The contents of each bottle were then titrated against 0·05 N sodium thio-sulphate. From the difference in titration values, the number of milli equivalents of iodine used, and consequently the number of milli moles of reducing group liberated could be calculated, having ascertained that one milli equivalent of iodine = 0·530 milli moles of reducing group. The P.G. unit of activity is then defined as the amount of enzyme which causes a liberation of 1 μ mole of reducing group per minute under the conditions described above.

j. Assay of Viscosity Reducing Enzymes

Enzymes reducing the viscosity of pectin were assayed by the method of Roboz, Barratt and Tatum (1952), using 0·5% pectin in citrate phosphate buffer pH 5·2 as a substrate. Those reducing the viscosity of pectic acid were assayed using 2·0% pectic acid in acetate buffer pH 5·0 to correspond with the reducing group assay. 0·25ml of enzyme was added to 7·5ml of substrate and the time noted. 4ml of this enzyme substrate mixture were then pipetted into a viscometer immersed in a water bath at $25^{\circ}C$, and at recorded time intervals the time for the liquid to fall between two points was determined, using a 0·1 second stop watch. The viscosity of the substrate + boiled enzyme and the buffer + boiled enzyme was also measured as controls. The percentage decrease in viscosity was calculated by the following formula:- $A = \dfrac{Vo - Vt}{Vo - Vs} \times 100$

where, A = percentage decrease in viscosity.

Vo = flow time in seconds of the substrate +
the heat inactivated enzyme.

Vt = flow time in seconds of the substrate +
the enzyme, at recorded time intervals.

Vs = flow time of the buffer + the heat in-
activated enzyme.

A unit of viscosity reducing activity can then
be defined as that amount of enzyme, which causes a
decrease in viscosity of 1% under the conditions
described.

k. Plate Assays for P.M.E. and P.G.

The methods used were those developed by
Dingle, Reid and Solomons (1953).

1. P.M.E. The agar contained Kodak citrus
pectin (1%), salicylanilide (0·01%) and agar (2%)
dissolved in water to 100%. The pH was adjusted to 6·0
with NaOH. After autoclaving, sufficient methyl red in
ethanol was added to the solution to give a final
concentration of 0·01%. The pH was then readjusted
with NaOH if necessary to discharge any trace of red
colour of the indicator. The molten agar was poured
into Petri dishes in layers 3mm thick and when it had
set, holes were cut in it with an 8mm cork-borer.
0·01ml of enzyme solution was added to each well and
after 20 hours incubation P.M.E. activity was detected
by the presence of a red zone around the well.

2. P.G. the agar contained sodium poly-
pectate (1%), prepared as described below, salicyl-
anilide (0·01%), ammonium oxalate (0·7%) and agar (2%)
dissolved in 0·2 M acetate buffer pH 5·0 to 100%. The
medium was autoclaved at 15 lbs/in^2 pressure for 15
minutes and poured into Petri dishes in layers 3mm

deep. Holes were cut in the agar with an 8mm cork--
borer and 0·1ml of enzyme solution was added to the
well. After 20 hours incubation at $25^{\circ}C$, P.G. activity
was detected by the presence of a clear area surrounded
by a white halo around the well, after developement
with 5 N HCl.

Though it is claimed that the zone diameter
varies linearly with the log. of the enzyme concen-
tration, these tests were only used qualitatively in
the present work.

The sodium polypectate used in the P.G. assay
was prepared from Kodak citrus pectin by the method
of Jermyn and Tom kins (1950). 100g of pectin was
stirred with a mixture of 375ml of 95% (v/v) aqueous
ethanol and 125ml of N NaOH twice for 20 minutes. It
was then washed with a mixture of 200ml of 95% ethanol
20ml of water and 10ml of N HCl. Finally it was dried
in an oven at $37^{\circ}C$. The product was the sodium salt
of pectic acid.

1. Assay of Macerating Activity

Macerating activity was assayed by placing
discs of orange rind cut with a 9mm cork-borer into
solutions of enzyme for 1 hour at room temperature.
Maceration was scored at the highest possible dilution
on the following arbitrary scale.

$\pm$ very slight softening compared with the
control.

+   softening

++   extreme softening

+++   partial disintegration

++++   almost complete disintegration

m. Protein Estimations

Protein was estimated by the method of Lowry et al (1951). This involved the reaction of the phenol groups in tyrosine and tryptophan with the Lowry reagents to give a blue colour that could be measured spectrophotometrically. The reagents used were made up as follows:-

Reagent 1. 2% sodium carbonate in 0·1 N NaOH.

Reagent 2. 0·5% copper sulphate ($CuSO_4.5H_2O$) in 1% sodium tartrate $(CH(OH).COONa)_22H_2O$

Reagent a. was made up by adding 50ml of 1. to 1ml. of 2.

Reagent b. was standard B.D.H. Folin and Ciocalteau's reagent diluted in the proportion of 10ml of reagent to 18ml of water.

To 1ml of protein sample in a boiling tube was added 4ml of reagent a., and the mixture was immediately shaken. After 10 minutes 0·4ml of reagent b. was added to the mixture and the tube was shaken and left for at least 30 minutes. The blue colour which developed could then be read on a Unicam SP 600 spectrophotometer against a reagent blank at 750 nm. Absorption values were converted to μg of protein by reference to a standard line, using bovine serum albumin in concentrations of 10 to 280 μg/ml.

n. Electrophoresis

Fractions were examined for electrophoretic homogeneity by disc electrophoresis on polyacrylamide gels at pH 9·5, according to the technique of Davis . and Ornstein (1964), using the apparatus of Shandon. Gels were loaded with about 250ug of protein and run at 5 m.a. per tube. They were stained with Naphthalene Black 12 B to locate the protein bands and the gel was destained in 7·5% acetic acid.

o. Estimation of the Methoxyl Content of Pectic Substances

Methoxyl estimations were done by the method
of Nanji and Norman (1926), which involved first a
calibration of the method with methanol and then the
measurement of the methanol released from a sample of
pectin or pectic acid. 100ml of potassium permanganate
solution (9·75g/l) and 40ml of NaOH (150g/l) were in-
troduced into a 500ml Quickfit conical flask. To this
mixture was added the dilute methanol solution con-
taining not more than 10mg. The mixture was well shaken
and boiled under a reflux condenser for three minutes.
100ml of oxalic acid (20g/l) were then added to this
without cooling, followed by 40ml of H_2SO_4 (conc. acid
to water, 2:5). After shaking, the excess oxalic acid
was titrated against 0·05 N K_2MnO_4 (1·58g/l). A con-
trol experiment was carried out without the addition
of methanol. By using at least 5 methanol solutions of
known concentration, it was possible to obtain a stan-
dard curve of methanol against the volume of perman-
ganate used.

Methanol was released from pectin by treat-
ment with sodium hydroxide. To a 0·5% solution of the
pectin or pectic acid, containing not more than 0·05g
of the substance, was added 100ml of 0·1 N NaOH in a
glass stoppered flask. This was left overnight, after
which it was neutralized to pH 4·5 with 0·5 N H_2SO_4,
transferred to a distillation flask and distilled
until approximately nine tenths had distilled over.
The methanol estimation was then applied to the whole
distillate as described before.

EXPERIMENTAL PART 2. Results.

a. Determination and Verification of Reaction Conditions
for Enzyme Assays.

      1. Arabanase.   The method used for the assay
of arabanase depended upon the measurement of arabinose
released from an araban substrate. Since this method
has not been published, it was necessary to determine
the conditions for the assay, as described in 'mat-
erials and methods'. The pH and temperature optima
were estimated approximately at first; then using
these conditions an assay of enzyme activity over a
period of time, at two substrate levels was carried
out. The results are shown in Fig 1. and from them it
was decided to use a substrate level of 1% araban and
an incubation time of 1 hour for the assay.

      Using these conditions and the approximate pH
optimum, the reaction was studied over a range of temp-
eratures. The results of this experiment can be seen
in Fig 2.. The enzyme appeared to be almost inactive
below about $5^{\circ}C$ and above $50^{\circ}C$. The optimum temperature
for enzyme activity was between 34 and $38^{\circ}C$. Results
were not consistant enough to obtain a more accurate
value than this, so it was decided to carry out the
assay at $36^{\circ}C$. The effect of pH on enzyme activity was
then examined at a reaction temperature of $36^{\circ}C$ and
the results are shown in Fig 3. The enzyme appeared to
be active in the range pH 2.5 to 6.5 with a fairly
sharp optimum at about pH 3.9.

      In order to check whether, under these con-
ditions, an increase in activity really represented a
proportional increase in enzyme concentration, a sam-
ple of culture filtrate, containing arabanase was

Fig 1. Activity Time Curves for Arabanase at Substrate
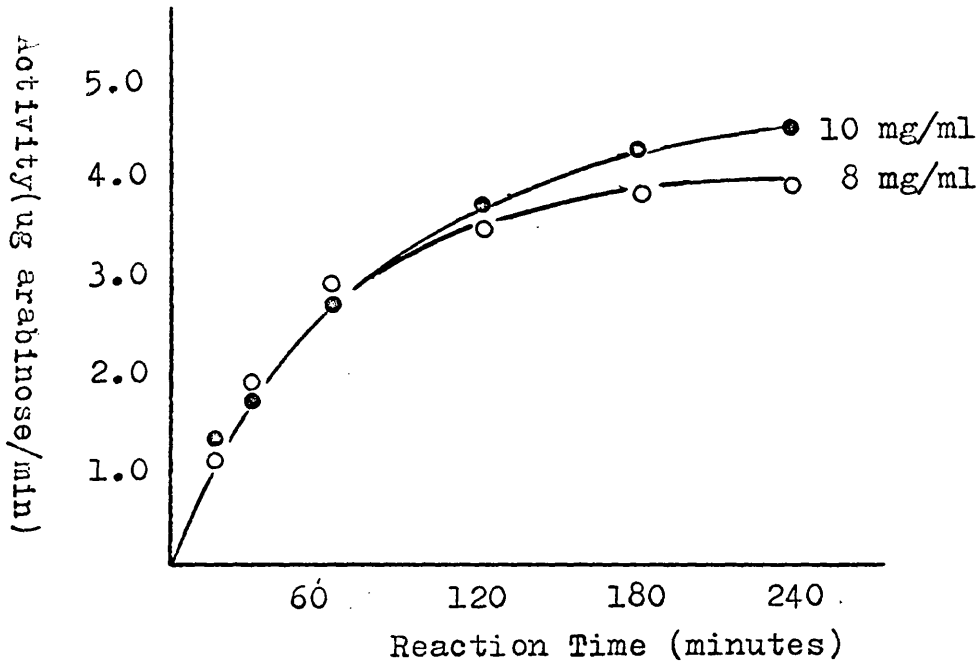Levels of 8 and 10 mg/ml.

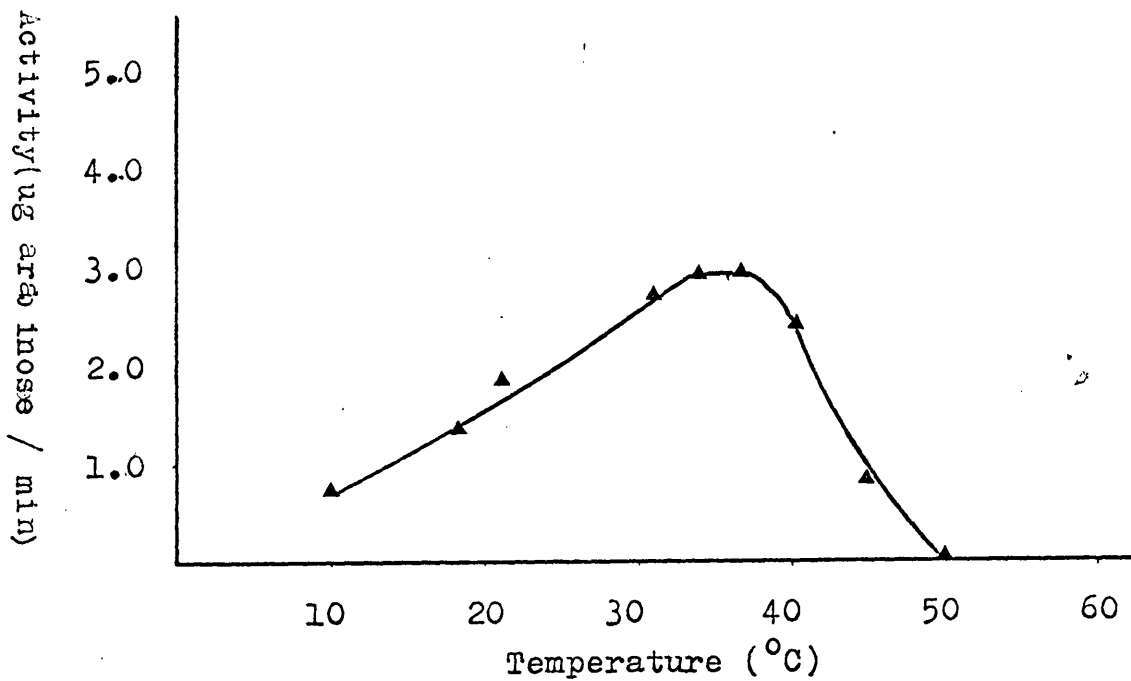Fig 2. Activity Temperature Curve for Arabanase.
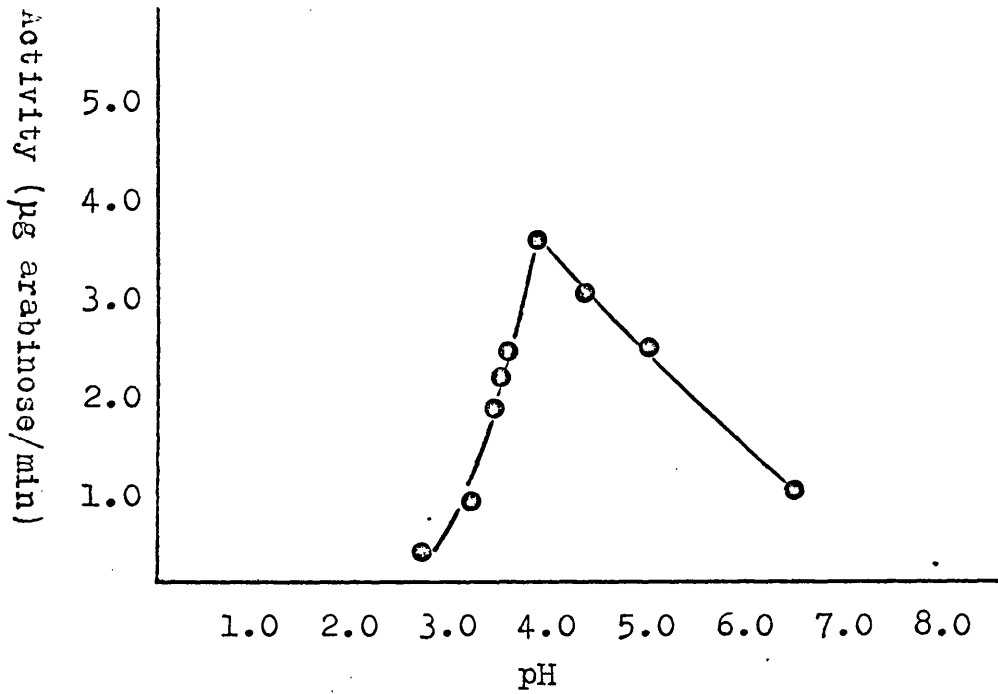
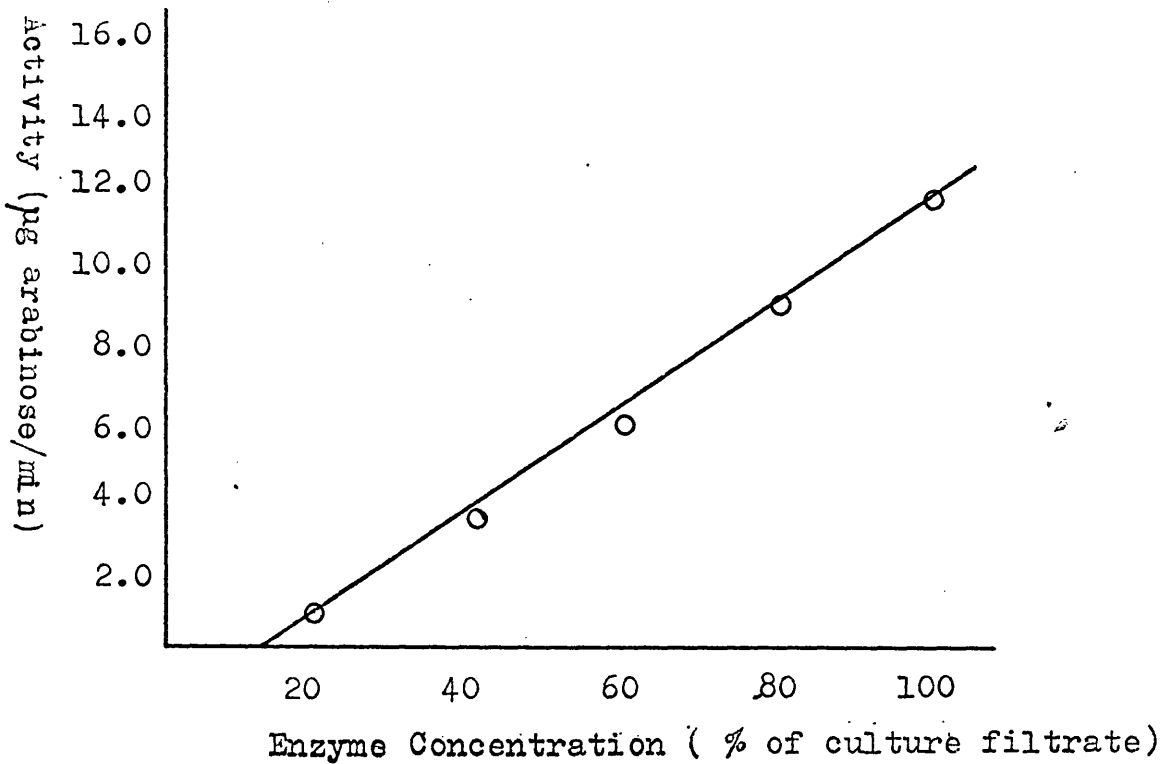Fig 3. Activity pH Relationship of Arabanase.

Fig 4. Enzyme Concentration : Activity Relationship
of Arabanase.

diluted to give the following concentrations:- 80%, 60%, 40%, and 20% . A sample of each concentration was then assayed for arabanase activity. The results of this experiment are shown in Fig 4. and verify that over the range tested (0 - 12μg arabinose/min.), activity was proportional to enzyme concentration.

2. Pectin transeliminase. This enzyme was assayed by the method of Albersheim and Killias (1962), with only minor modifications. In order to determine a suitable reaction time for the assay, the reaction was followed spectrophotometrically as described in 'materials and methods', over a period of time. Dilutions of a sample of culture filtrate containing this enzyme were made, in order to demonstrate a straight line relationship between enzyme concentration and activity. The results of these experiments are shown in Figs 5 and 6 respectively. At 10 and 15 minutes reaction time, chosen from Fig 5. it was found that enzyme concentration was proportional to activity over the range tested.

3. Reducing Group Releasing Enzymes. Enzymes causing the liberation of reducing groups from pectic acid were measured by the method of Jansen and Mac-Donnell (1945). This was a modified Willstatter – Schudel hypoiodate method, depending on the reduction of iodine by the reducing groups liberated from pectic acid during the course of its breakdown. The method was first calibrated by obtaining a standard curve with galacturonic acid (Fig 7.), making use of the facts that 1 milli mole of galacturonic acid produces 1 milli mole of reducing group; and that 1 milli equivalent of iodine = 10ml of 0.1 N iodine. The values of milli moles of reducing group to milli equivalents of iodine , that were obtained using galacturonic acid concentrations from 0.5 to 10.0 mg/ml

are shown in table 1.

Table 1. Relationship between Milli Moles
of Reducing Group from Galacturonic Acid
and Milli Equivalents of Iodine.

Galacturonic Acid Concentration (mg/ml)	Milli Moles of Reducing Group	Milli Equivalents of Iodine
10	0.2577	0.410
8	0.2062	0.367
6	0.1546	0.287
5	0.1288	0.246
4	0.1030	0.195
2	0.0516	0.097
1	0.0258	0.049
0.5	0.0128	0.023

Taking values which fell on the linear portion of the curve (Fig 7.), it was found that 1 milli equivalent of iodine was reduced by 0.530 milli moles of reducing group.

A suitable reaction time for the assay was determined using a fungal enzyme preparation,known as Carterzyme. This preparation, kindly supplied by Beechams Food and Drink Division of Coleford, contained a P.G. which had previously been shown to release reducing groups from low methoxy pectin. This enzyme was incubated with buffered pectic acid under the conditions stated previously, in order to determine a suitable reaction time for the assay. The reducing group liberation was then plotted against reaction time as shown in Fig 8. From this experiment it was

decided to use a reaction time of 8 minutes for the
reducing group assay. In order to check that activity
was proportional to enzyme concentration, a series of
dilutions was prepared from a sample of Carterzyme.
The activity of each dilution was then assayed and act-
ivity was plotted against concentration. The concen-
trations used were, 100, 80, 60, 40, and 20% solutions
of the Carterzyme. The results of this experiment are
shown in Fig 9., indicating that concentration was
proportional to activity over the activity range 0 - 2
μ moles reducing group/minute.

      4. Viscosity Reducing Enzymes.   The method
for estimating viscosity reducing enzymes used in the
present work,was that of Roboz, Barratt and Tatum (1952),
with certain modifications as indicated in section 2.
(materials and methods). The assay was carried out on
0.5% pectin at pH 5.2 and on pectic acid(2.0%) at pH
5.0, to correspond with the transeliminase assay and
the reducing group assay respectively. The reaction
with both substrates was followed over a period of
time in order to determine a suitable reaction time.
The results of one such experiment are shown in Figs
10 and 11. From these results it was clear that the
viscosity reducing activity fell off rapidly after a
period of only one or two minutes. It was therefore
decided to run the assay routinely over 4 minutes,
taking readings every minute. In order to check
whether reduction in viscosity over this period could
be used to measure the amount of enzyme present, sam-
ples of enzyme were diluted to 80%, 40%, 20% and 10%
of their original concentrations and these diluted
samples were assayed for enzyme activity, by measuring
the reduction in viscosity produced in 1 minute. The
percentage decrease in viscosity was then plotted

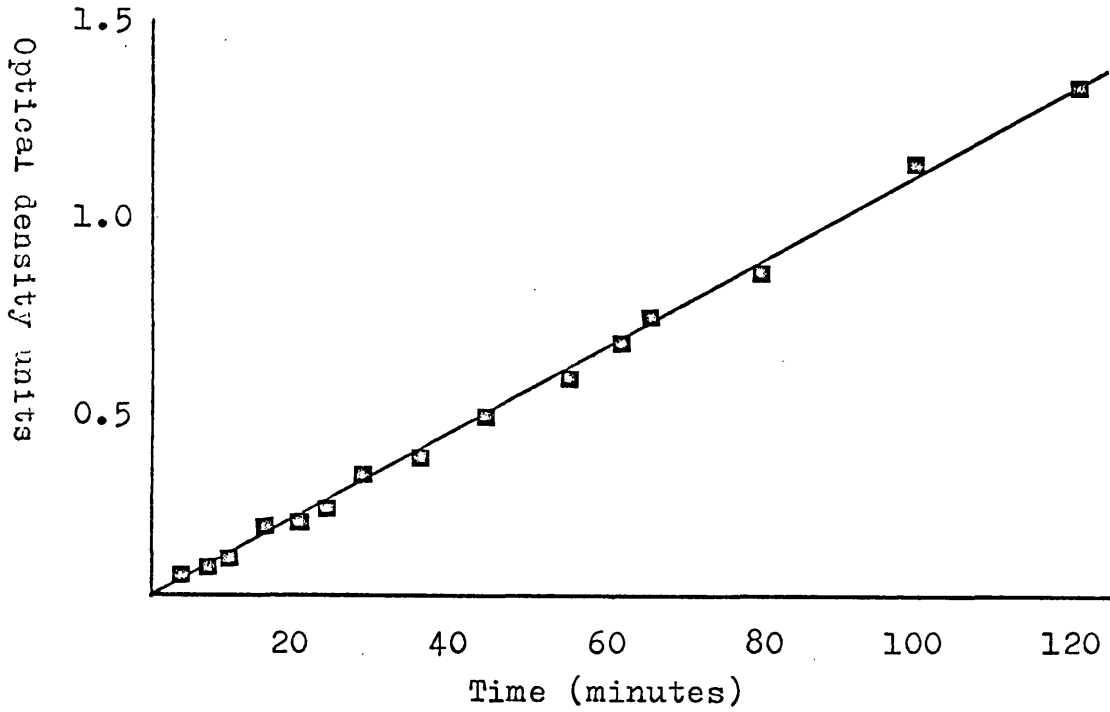Fig 5. Increase in Absorption at 235 nm of a Pectin
Solution due to P.T.E.

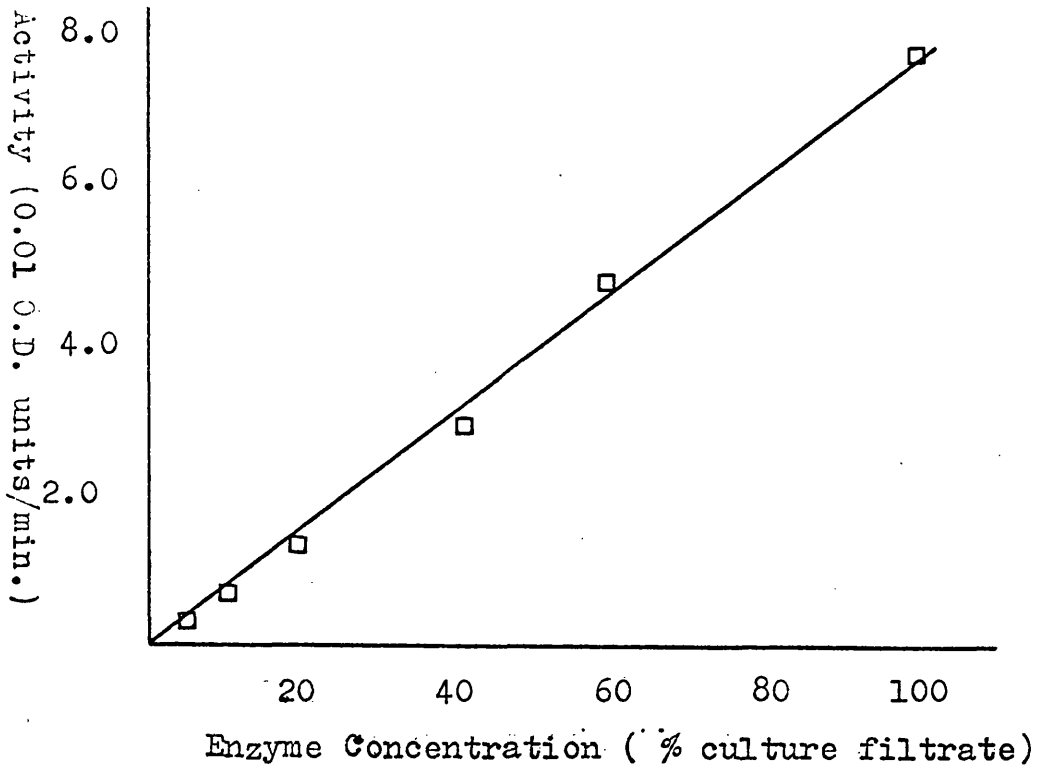Fig 6. Relationship of P.T.E. Concentration with Activity

Fig 7. Relationship between Galacturonic Acid Concentration (Reducing Group) and Milli Equivalents of Iodine Reduced.
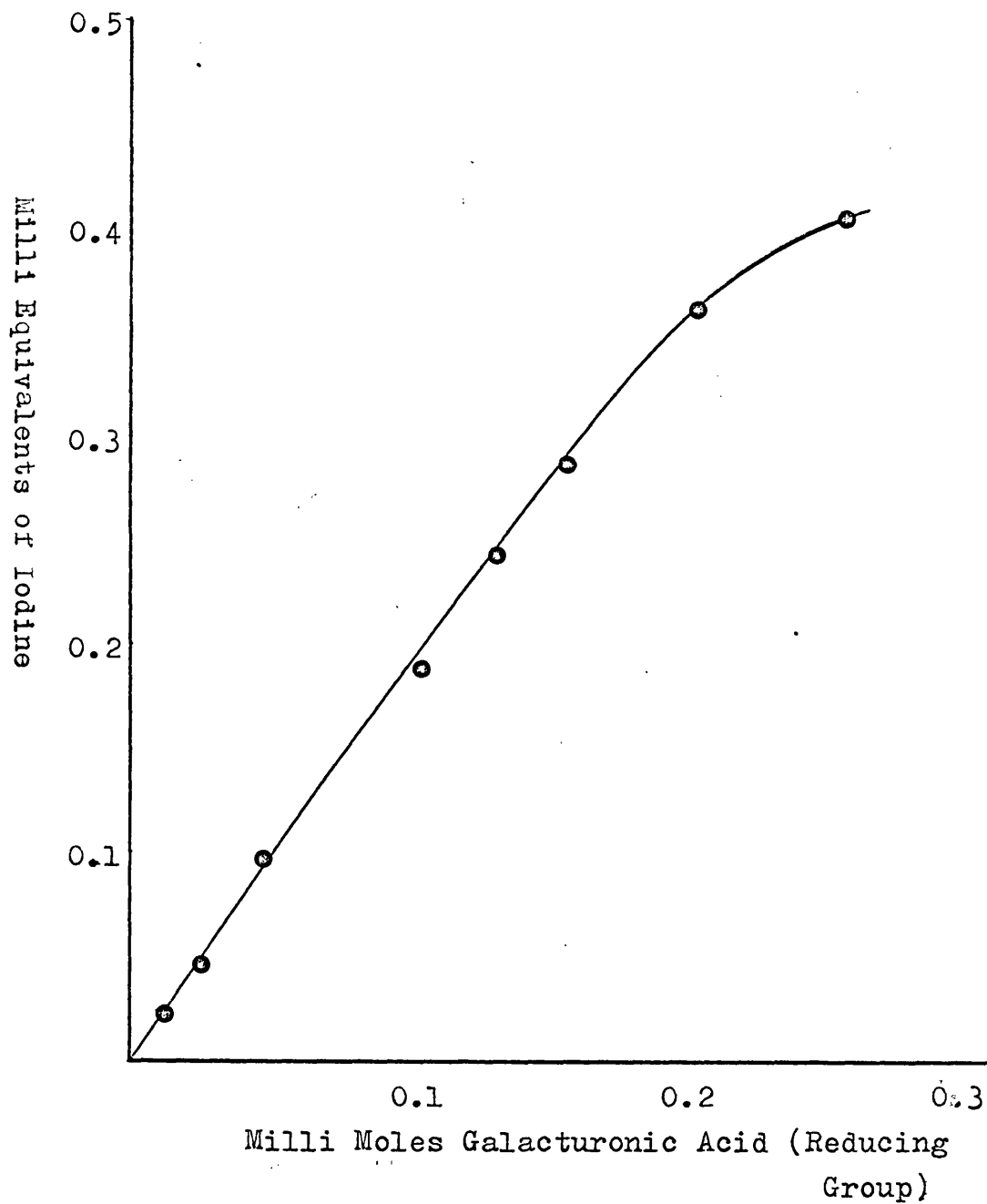
Fig 8. Release of Reducing Groups From Pectic Acid
by Carterzyme.
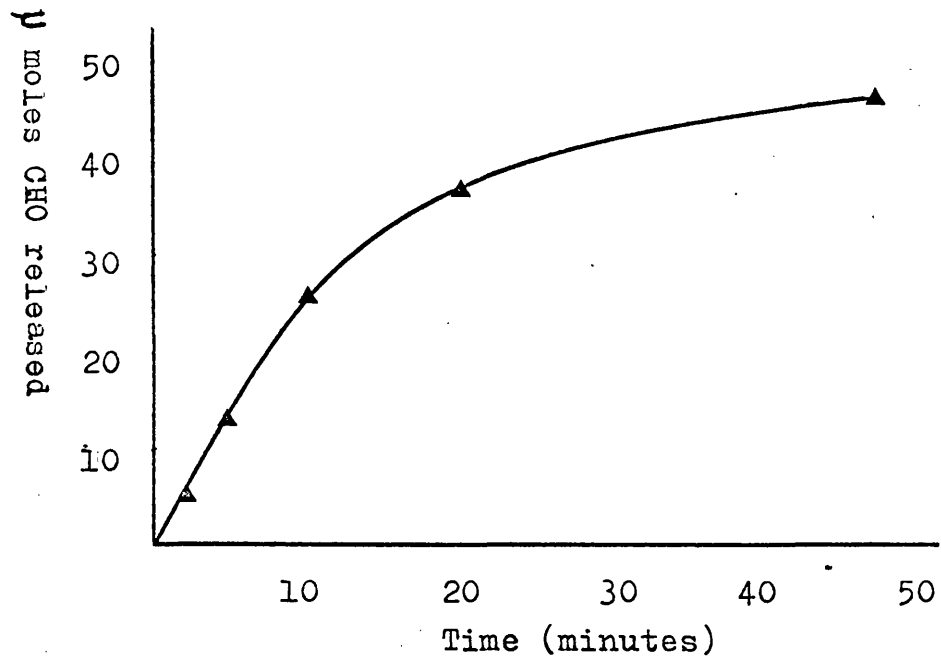
Fig 9. Relationship between Concentration and Reducing
Group Liberating Activity of Carterzyme.
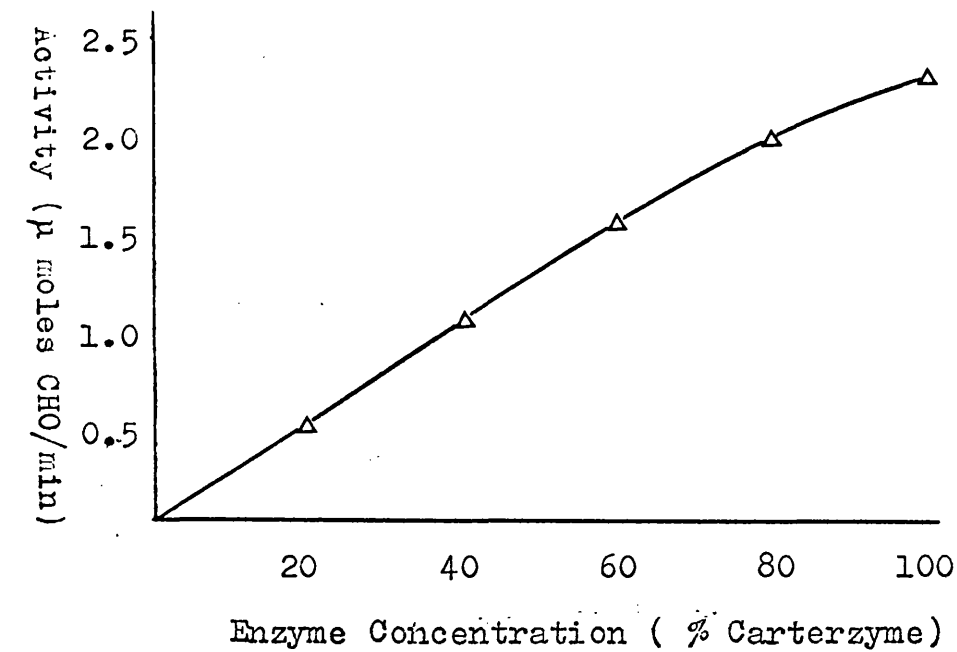
against the enzyme concentration as shown in Figs 12
and 13. Percentage decrease in viscosity was thus
shown to be directly proportional to enzyme concen-
tration, over the range 0 - 20% decrease in viscosity
for pectin and 0 - 10% decrease in viscosity for
pectic acid. In this experiment Penicillium digitatum
culture filtrate was used to reduce the viscosity of
pectin and Carterzyme was used with pectic acid. The
reason for such a choice of enzyme-substrate systems
was that, while P.digitatum culture filtrate contained
an enzyme directly effecting the viscosity of pectin
(P.T.E.), Carterzyme had an enzyme acting directly
on pectic acid (endo-P.G.). Neither enzyme preparation
affected the viscosity of the other substrate.

b. Determination of Methoxyl Content of Pectic
Substances used as Substrates.

As has been mentioned earlier, one of the
criteria used in classifying pectic enzymes is the
nature of the substrate attacked, with respect to its
degree of methylation. As no information was available
on the methoxyl content of the commercial preparations
used as substrates in the present work, it was decided
to attempt to determine these values, in order to
define more specifically the enzymes investigated. The
method used to determine such methylation values was
developed by Nanji and Norman (1926) and has been des-
cribed earlier.

The substrates examined by this method were
the following:-

1. Kodak Citrus Pectin (Prac.) - Eastman
Organic Chemicals P2569

2. Sodium Polypectate, prepared from Citrus
Pectin by the Method of Jermyn and Tom kins (1950).

Fig 10. The Reduction in Viscosity of a 0.5% Pectin
Solution by Penicillium digitatum Culture Filtrate
over 1 Hour.
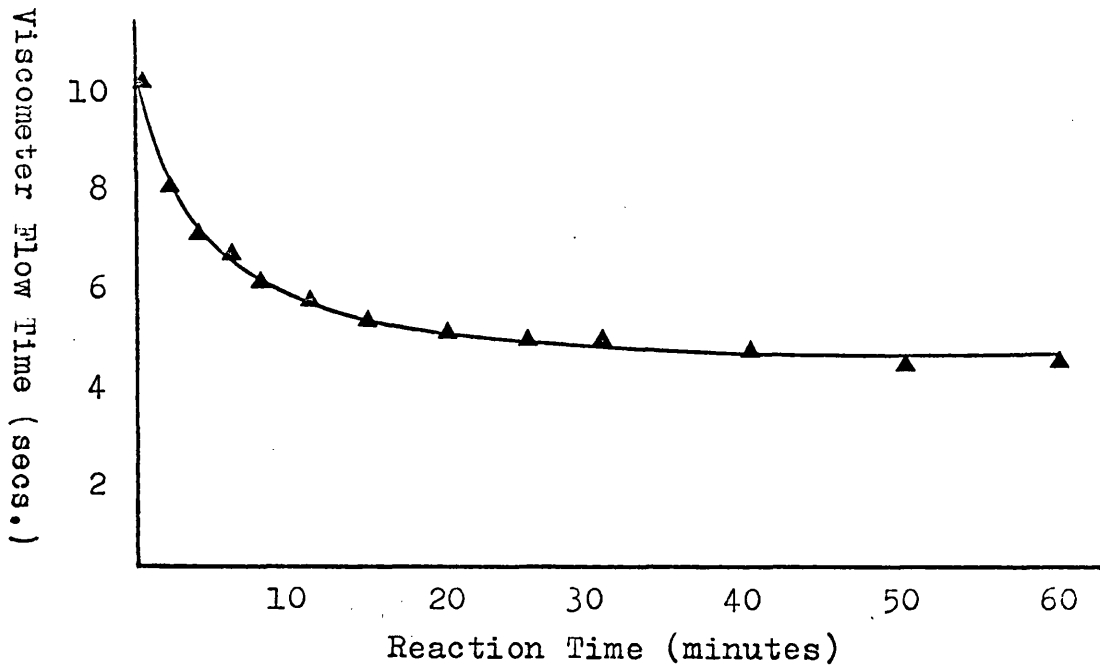
Fig 11. The Reduction in Viscosity of a 2.0% Pectic
Acid Solution by Carterzyme over 1 Hour.
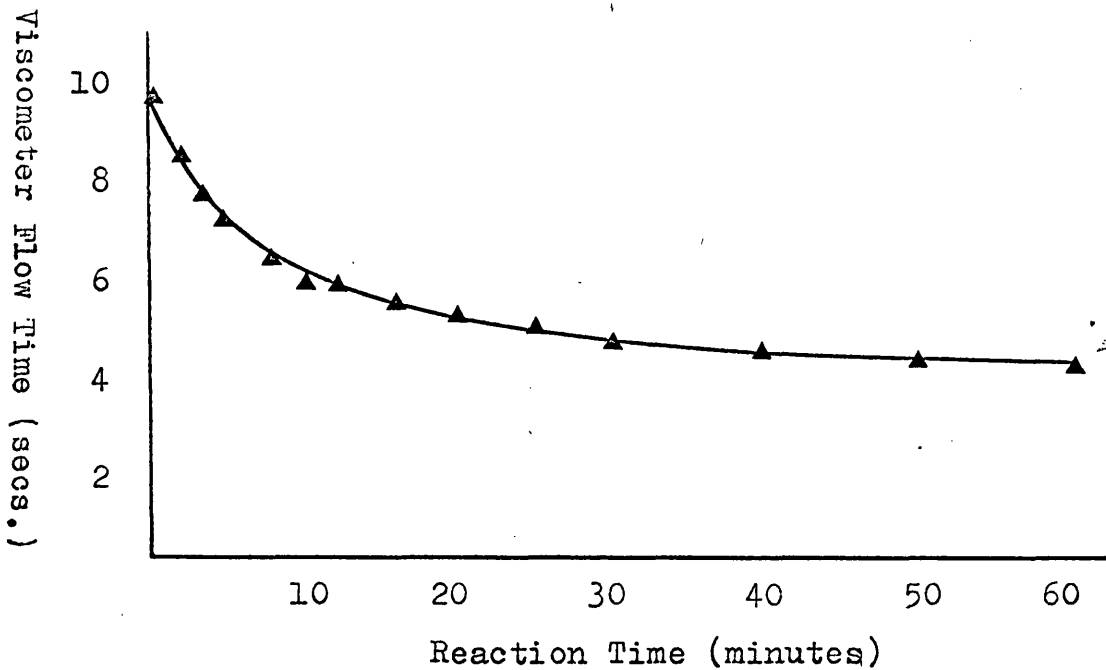
Fig 12. Relationship between Percentage Decrease in
Viscosity of a 0.5% Pectin Solution and Enzyme
Concentration.

Concentration of P.digitatum culture
filtrate (% culture filtrate)
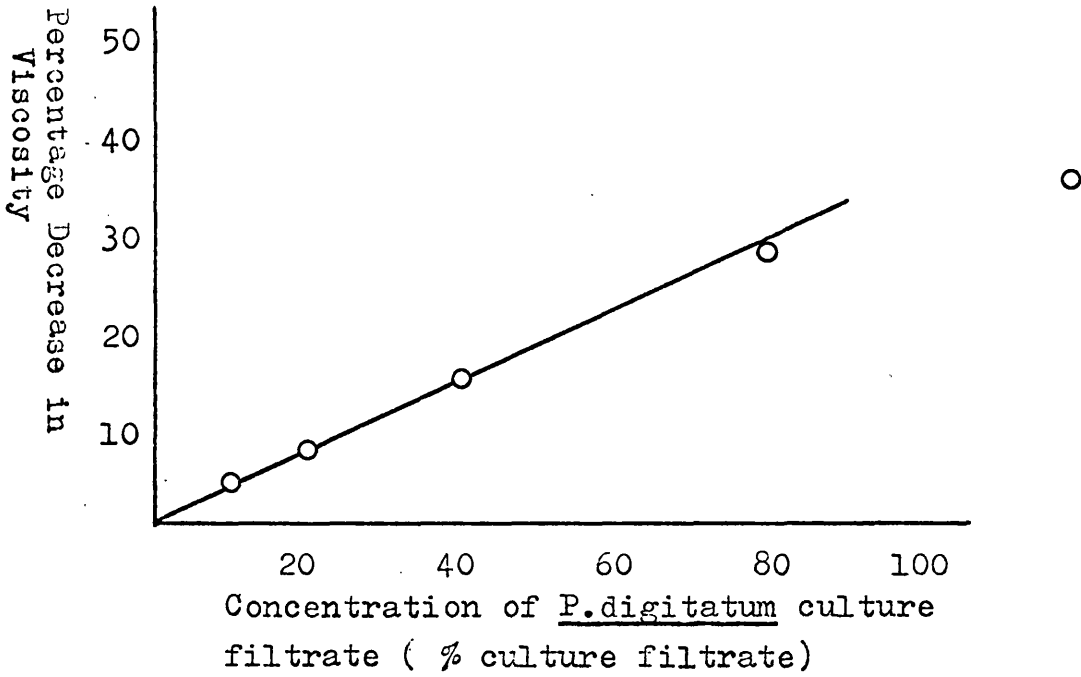
Fig 13. Relationship between Percentage Decrease in
Viscosity of a 2.0% Pectic Acid Solution and Enzyme
Concentration.

Concentration of Carterzyme (% Carterzyme)
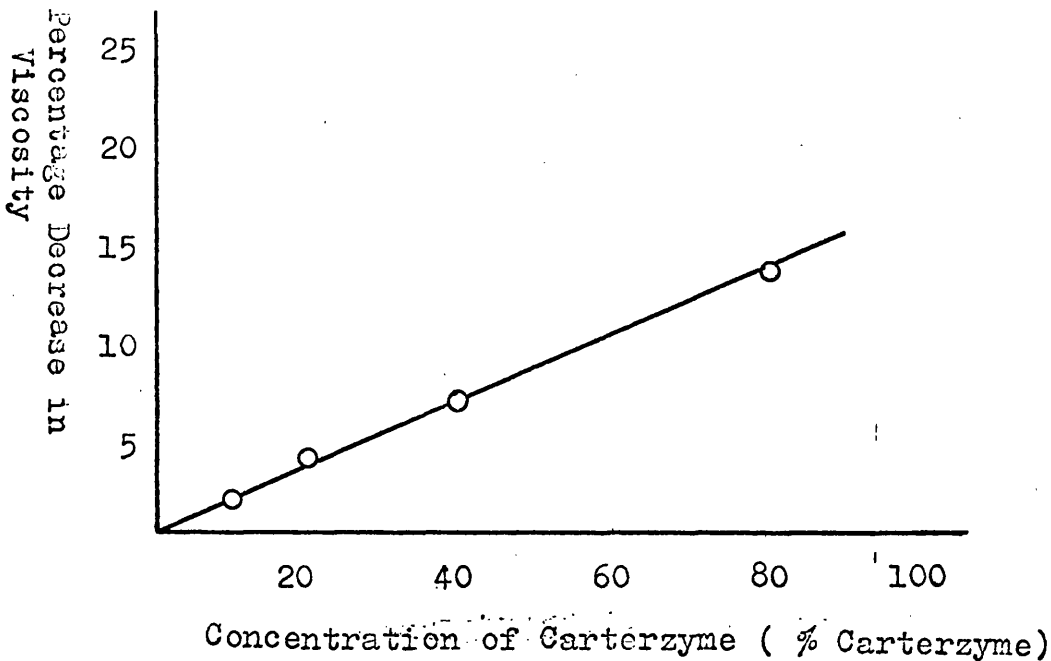
3. Kodak Polygalacturonic Acid - Eastman
Organic Chemicals P4429.
    4. Pectic Acid - K.and K. Labs. Inc. 17697

After calibration with methanol, the estimation
was applied to distillates of each of these substances.
The results of such an analysis are shown in table II,
percentage methoxyl being calculated as:-

the wt. of methanol x 100
the wt. of substance

Table II. Methoxyl Content of Pectic Substances

Substance Analysed.	Percentage Methoxyl.	Fraction of COOH groups Methylated.
1.Pectin	6.5	0.42
2.Sodium Polypectate	2.4	0.16
3.Polygalact-uronic acid	0.7	0.05
4.Pectic acid	1.2	0.08

The fraction of carboxyl groups in a molecule
that are methylated, is calculated on the basis that
fully methylated pectin would contain 15.4% methoxyl
groups. This figure is reached theoretically from the
molecular weights of galacturonic acid and methanol as
follows:-

M.W. Galacturonic acid = 194.6
M.W. Methanol = 32.0
M.W. Methylated galacturonic
                    acid = 208.6

It can be seen therefore that fully methylated pectin
would have a methanol : pectin ratio of 32 : 208 or
0.154, ie fully methylated pectin would contain 15.4%
methanol. Thus a sample of pectin containing x%
methoxyl would have x/15.4 of its carboxyl groups
methylated.

c. Preliminary Comparison of Enzyme Production by Penicillium digitatum and Penicillium notatum

A preliminary investigation of physiological
aspects of Penicillium notatum, which will not in-
vade citrus fruits was made and compared with certain
aspects of the physiology of Penicillium digitatum.
It was found that if an orange was sliced in half and
seeded with P.notatum spores, this organism grew
readily over the cut surface of the fruit. However it
did not penetrate the tissue, nor did it cause any
softening of the fruit as is apparent when an orange
is infected with P.digitatum. From this experiment a
number of conclusions could be drawn regarding the
differential abilities of P.digitatum and P.notatum
to parasitise oranges. The inability of P.notatum
to attack oranges was not therefore, as had been sug-
gested by Kirby (1964, unpublished), due to its in-
hibition at the pH of citrus tissue, its inhibition
by substances present in the citrus tissue, or its
inability to utilize citrus tissue as a suitable nut-
rient source. It appeared that the most significant
difference between these two moulds, was the inability
of Pnotatum , even when it had grown on cut fruit, to
penetrate beyond the outer layer of damaged cells. This
was coupled with the fact that it produced no softening
of the fruit. It was therefore concluded that P.digit-
atum produced on oranges, a factor capable of causing

the tissue to soften and eventually to disintegrate; and it seemed likely that this factor enabled the organism to spread throughout the orange rind tissue.

When liquid cultures of both organisms were grown in malt extract medium, it was found that the culture filtrate of Penicillium digitatum caused softening and eventually disintegration (maceration) of orange rind discs placed in it, whereas Penicillium notatum filtrate had no such action. If the P.digitatum filtrate was boiled, then its ability to macerate orange tissue was lost, suggesting that the factor responsible for this activity was enzymic in nature.

Filtrates of both organisms were allowed to act on finely cut orange rind and the sugars in the resulting digests were separated by thin layer chromatography. The Penicillium digitatum digest was found to contain arabinose and a trace of galacturonic acid, but the Penicillium notatum digest contained only a trace of galacturonic acid. Both digests contained glucose. These results suggested that P.digitatum produced an arabanase not produced by P.notatum, but that both organisms synthesised an enzyme capable of producing galacturonic acid from orange rind. It was thought likely that this was an exo-P.G., which removes galacturonic acid units from the end of pectin chains, after this has been demethylated by P.M.E.. Confirmation that both organisms produced a P.M.E. and a P.G. was obtained using the cup plate assay techniques. Culture filtrates were examined for enzymes of the transeliminase type, by observing the change in U.V. absorption at 235 nm of a clarified citrus pectin solution. P.T.E. was detected in P.digitatum culture filtrate, but not in that of P.notatum. These results indicated that P.T.E. and arabanase might be

more important in maceration and pathogenicity than
exo-P.G. and P.M.E. Such results are in agreement with
the work of McClendon (1964) and Bateman and Millar
(1966).

d. Investigation of Pectic Enzyme Production by Penicillium
   digitatum, Penicillium italicum and Aspergillus fon-
   secaeus on a Variety of Media.

   Preliminary work has indicated that arabanase
and P.T.E. might be involved in the pathogenicity of
Penicillium digitatum on citrus fruits. It was therefore
decided to examine in greater detail, the array of
pectic enzymes produced by this organism on different
media and to compare these with the enzymes produced
by Penicillium italicum and Aspergillus fonsecaeus on
the same media. The object of such an investigation
was twofold. Firstly it was hoped to gain some addit-
ional information on the association of particular enzymes with
macerating activity and secondly the investigation
was designed to show to what extent the three citrus
pathogens showed any similarity in the production of
pectic enzymes.

   The three media used in this investigation
were:-

   1. 3% malt extract + 0.5% mycological peptone
   2. 3% malt extract + 0.5% mycological peptone
+ 0.5% pectin.
   3. Bran.

   Both surface and submerged cultures were grown
in the liquid media, in 250ml conical flasks at 25°C.
The bran cultures were also grown in 250ml flasks at
25°C. When the cultures were harvested, the mycelium
of the liquid cultures was dried and weighed and the
amount of extracellular protein in each filtrate was

measured. The enzyme assays carried out were those
for P.T.E., arabanase, enzymes reducing the viscosity
of pectin and pectic acid, enzymes liberating reducing
groups from pectic acid, P.M.E. and macerating activity.

Penicillium digitatum cultures were inoculated
with 1.25×10^8 spores per flask. Submerged cultures
were grown for 48 and 72 hours, surface cultures for
144 and 168 hours and bran cultures were grown for
120 and 144 hours. Frequently difficulty was encoun-
tered in obtaining a continuous mycelial mat in the
case of surface cultures, although this occurred more
easily on the medium containing pectin than on the
malt extract alone. The results of such an investig-
ation are shown in table III.

Cultures of Penicillium italicum were inoc-
ulated with 1.5×10^8 spores per flask. Submerged cul-
tures were harvested after 48 and 72 hours growth,
surface cultures after 120 and 148 hours and bran cul-
tures after 72 and 96 hours. This organism grew con-
siderably faster on bran than did Penicillium digitatum
but the same difficulty in obtaining a continuous
mycelial mat in surface cultures was encountered. The
results of this experiment are shown in table IV.

Cultures of Aspergillus fonsecaeus were inoc-
ulated with 1.25×10^8 spores per flask. This organism
grew both quickly and profusely, especially in sub-
merged cultures and these were therefore harvested
after 24 and 48 hours growth. Surface cultures were
harvested after 120 and 144 hours and bran cultures
after 72 and 96 hours. The effect of growth conditions
on enzyme production by A.fonsecaeus are shown in
table V. Although growth, as measured by dry weight
of mycelium, was from two to three times greater than
with the two Penicillia, protein production by

Table III. Enzymes Produced by Penicillium digitatum under a Variety of Growth Conditions.

Growth Conditions.	Dry Wt. of Mycelium(g).	Protein (mg/ml)	P.G. Activity (μ moles reducing group/min)	P.M.E. (presence in plate assay)	P.T.E. Activity. (0.01 OD. units/min.)	Viscosity Reducing Activity (% reduction in viscosity/min.)		Arabanase Activity (ug arabinose/min)	Maceration (degree of softening)	
						Pectin	Pectic Acid		2hr.	24hr.
1. Sb M 48.	0.224	2.8	0	-	4.07	17.1	0	5.7	++	+++
2. Sb M 72.	0.197	2.7	0	-	4.17	17.1	0	10.0	++	++++
3. Sb MP 48.	0.274	2.1	0	-	2.04	13.1	0	22.4	++	+++
4. Sb MP 72.	0.252	2.0	0	-	1.43	11.2	0	11.6	+	+++
5. Sr M 144.	0.432	2.6	0	+	2.29	13.1	0	10.5	+	+++
6. Sr M 168.	0.443	2.3	0	+	3.00	15.0	0	14.3	+	+++
7. Sr MP 144.	0.339	2.2	0	+	1.45	9.40	0	16.4	+	+++
8. Sr MP 168.	0.415	2.1	0	+	1.59	10.2	0	25.4	+	+++
9. B 120.		2.1	0.2	+	4.93	24.2	2.2	45.7	++	++++
10. B 144.		1.8	0.13	+	3.02	12.9	2.9	44.3	++	+++

Key. Sb. = Submerged culture. Sr. = Surface culture.
M = Malt medium. MP = Malt + Pectin medium.
B = Bran. Figure = growth time in hours.

Table IV. Enzymes Produced by Penicillium italicum under a Variety of Conditions.

Growth Conditions.	Dry Wt. of Myc-elium(g)	Protein (mg/ml)	P.G. Activity (u moles reducing group/min)	P.M.E. (presence in plate assay)	P.T.E. Activity (0.01 OD. units/min.)	Viscosity Reducing Activity (% reduction in viscosity/min.)		Arabanase Activity (ug arab-inose/min)	Maceration (degree of softening)	
						Pectin	Pectic Acid		2hr.	24hr.
1. Sb M 48	0.307	2.4	0	+	0.11	0	0	2.0	±	±
2. Sb M 72	0.296	2.6	0	+	0.08	0	0	2.0	±	±
3. Sb MP 48	0.412	2.0	0	+	0.14	0	0	4.5	±	±
4. Sb MP 72	0.398	2.0	0	+	0.27	0	0	3.8	±	+
5. Sr M 120	0.142	1.8	0	+	0.71	10.0	0	9.4	+	++
6. Sr M 148	0.193	1.7	0	+	0.83	8.0	0	8.6	+	++
7. Sr MP 120	0.158	1.6	0	+	0.11	0	0	13.9	±	-
8. Sr MP 148	0.148	1.6	0	+	0.16	0	0	14.2	±	-
9. B 72		1.9	0.10	+	3.11	17.0	0	9.5	++	+++
10. B 96		1.9	0.13	+	3.06	17.0	0	9.7	++	+++

Key. Sb = Submerged culture. Sr = Surface culture
M = Malt medium MP = Malt + Pectin medium
B = Bran Figure = growth time in hours

Table V. Enzymes Produced by Aspergillus fonsecaeus
under a Variety of Conditions.

Growth Conditions	Dry Wt. of Mycelium(g)	Protein (mg/ml)	P.G. Activity (u moles reducing group/min)	P.M.E. (Presence in plate assay)	P.T.E. Activity (0.01 O.D. units/min)	Viscosity Reducing Activity (% reduction in viscosity/min.)		Arabanase Activity (ug arabinose/min.)	Maceration (degree of softening)	
						Pectin	Pectic Acid		2hr.	24hr.
1. Sb M 24	0.67	1.77	0	+	0.17	1.4	0	2.0	−	±
2. Sb M 48	1.01	1.10	0	+	0	0	0	2.9	−	±
3. Sb MP 24	0.58	1.38	0	+	0	0	0	4.0	−	±
4. Sb MP 48	0.82	0.93	0	+	0	0	0	6.1	−	±
5. Sr M 120	0.89	1.80	0	+	0	0	0	4.7	±	++
6. Sr M 144	0.89	1.68	0	+	0	0	0	4.5	+	++
7. Sr MP120	0.65	1.23	0	+	0	0	0	4.7	+	+++
8. Sr MP148	0.73	1.24	0	+	0	0	0	5.1	+	++
9. B 72		3.40	0.15	+	0	2.8	2.0	40.5	±	+++
10. B 96		3.29	0.10	+	0	3.2	2.3	33.8	±	+++

Key. Sb = Submerged culture. Sr = Surface culture.
M = Malt medium MP = Malt + Pectin medium.
B = Bran Figure = growth time in hours

A.fonsecaeus was slightly less than with these fungi
in the liquid media. Protein production by A.fonsecaeus
on bran was however significantly higher than that of
Penicillium digitatum and Penicillium italicum. Enzyme
production was generally less by A.fonsecaeus than by
P.digitatum and P.italicum. P.M.E. was produced by all
three fungi under all growth conditions except the sub-
merged cultures of P.digitatum. Exo-P.G. as measured
by the liberation of reducing groups from pectic acid
was produced only in bran cultures of each organism.
Arabanase was produced by all three organisms, though
in greatest quantity by P.digitatum. Bran appeared to
be the most satisfactory substrate for the production
of this enzyme, except in the case of P.italicum,
when liquid surface cultures were equally satisfactory.

P.T.E. was produced in all media by Penicillium
digitatum and Penicillium italicum , not to any great
extent by the latter, except in bran. P.T.E. was not
detected in cultures of Aspergillus fonsecaeus, apart
from a trace in young submerged cultures, without
pectin. Pectin appeared to have an inhibitory effect
on the production of P.T.E. (c.f. tables III.and IV.),
a phenomenon that does not seem to have been reported
previously. The reason for this is at present unknown,
although it is considered that it might be explained
in terms of either enzyme substrate absorption, or
product inhibition. In either case P.T.E. would be
produced by the organism in question, but would then
start to break down the pectin in the medium. In doing
this,it is possible that some of the enzyme might be-
come bound to the pectin, so that it would not show
any activity in the assay. Alternatively the enzyme
might be partially inactivated by the products of the
pectin breakdown, and again the organism would appear

to have produced less P.T.E. in the pectin medium.
Product inhibition is by no means an unknown phenom-
enon, although it must be admitted that no evidence
has yet been found to verify either of these explan-
ations.

The activity of the enzyme(s) causing a red-
uction in viscosity of pectin, followed the activity
of P.T.E. in most cases, although where P.T.E. act-
ivity was very low, as in the liquid cultures of
P.italicum, no viscosity reducing activity on pectin
was recorded. Enzymes reducing the viscosity of pectic
acid were only produced on cultures grown on bran, as
were enzymes causing the liberation of reducing groups
from pectic acid. It may be therefore that there is an
association between these activities.

Macerating activity was produced under the same
conditions as P.T.E. by Penicillium digitatum and
Penicillium italicum. This was not the case however in
Aspergillus fonsecaeus, where macerating activity was
present in the surface and bran cultures, but P.T.E.
activity was not detected. The indication is there-
fore, that P.T.E. may be responsible for the tissue
maceration caused by P.digitatum and P.italicum, but
that this is most unlikely to be the case with
A.fonsecaeus. This view was investigated further by
applying enzyme separation proceedures to filtrates
of the three organisms, in an attempt to purify the
enzyme(s) responsible for the maceration process.
Growth conditions chosen for the production of filtrates
for separation, were those which stimulated the great-
est production of macerating factor and P.T.E.

e. Separation and Purification of Enzymes from Culture
Filtrates of Penicillium digitatum, Penicillium ital-
icum and Aspergillus fonsecaeus.

1. Penicillium digitatum.   Submerged cultures
of Penicillium digitatum were grown in a malt extract
medium and harvested after 48 hours. Four litres of
culture filtrate was freeze dried and re-dissolved in
water to 1 litre. This concentrated filtrate was frac-
tionated by an ammonium sulphate precipitation, the
results of which are shown in table VI. Solutions of
precipitates from this fractionation were made in
100ml, and that solution prepared from the precipitate
formed at 0.5 $(NH_4)_2SO_4$ saturation, which contained
the greatest P.T.E. activity, was subjected to a second
fractionation with ammonium sulphate. Solutions of the
precipitates from the second fractionation were made
in 15ml and were assayed for enzyme activity, as shown
in table VII. The solutions of precipitates formed at
0.4 and 0.5 $(NH_4)_2SO_4$ saturation in the second frac-
tionation, were passed through a column of Sephadex G25
to desalt them, and then through a column of ECTEOLA
cellulose to effect a separation of P.T.E. and arabanase.
The enzymes were eluted by water and stepwise additions
of increasing concentrations of sodium chloride, as
shown in Fig 14.

The specific activity of P.T.E. was increased
from 18.8 units/mg protein in the culture filtrate, to
4852.0 units/mg protein in the purest fraction (fract-
ion 5) from the ECTEOLA cellulose column, giving an
overall 258 fold increase in specific activity for the
purification process. Similarly the specific activity
of the arabanase was increased from 8.1 units/mg prot-
ein in the culture filtrate, to 495 units/mg protein
in fraction 21 from the ECTEOLA cellulose, giving an
overall 61 fold increase for the purification process.

Table VI. First Ammonium Sulphate Fractionation
of Penicillium digitatum Culture Filtrate

Fraction	P.T.E.		Arabanase		Maceration
	Activity (0.01 O.D. units/ min.)	Specific Activity (units activity per mg protein)	Activity (ug arab- inose/min)	Specific Activity (units activity per mg protein)	(Degree of Soften- ing)
Culture Filtrate	6.2	18.8	26.7	8.1	+
Conc. Culture Filtrate	27.4	20.8	136.7	10.4	+++
$(NH_4)_2SO_4$ Sat.					
0.2	0	—	5.2	2.3	—
0.4	30.0	97.4	346.7	112.6	+++
0.5	206.0	647.8	640.0	201.6	+++ *
0.6	30.2	95.0	110.7	34.8	+++
0.8	2.4	4.3	43.3	7.8	+
1.0	0	—	10.0	2.8	—
Residue (mater- ial not ppted. by sat. $(NH_4)_2SO_4$	0	—	13.0		—

(* = 1/10th. dilution, otherwise
undiluted fractions used)

Table VII. Second Ammonium Sulphate Fractionation using the Precipitate Formed at 0.5 $(NH_4)_2SO_4$ Saturation in Table VI.

Fraction	P.T.E.		Arabanase		Maceration
	Activity 0.01 OD. units/ min.)	Specific Activity (units activity per mg protein)	Activity (μg arabinose/ min.)	Specific Activity (units activity per mg protein)	(Degree of Softening)
0.5 sat. $(NH_4)_2SO_4$ ppt. from table VI. Saturation.	2265.0	647.8	640.0	201.6	+++ *
0.2	54.0	135.0	5.2		+
0.4	4120.0	710.0	1174.0	202.4	+++ **
0.5	2618.0	808.0	55.0	17.1	+++ *
0.6	82.0	49.4	0	-	+
0.8	0	-	0	-	-
1.0	0	-	0	-	-
Residue (material not ppted. by sat. $(NH_4)_2SO_4$	0	-	0	-	-

(* = 1/10th dilution, ** = 1/20th dilution, otherwise undiluted fractions used)

Fig. 14. The Separation of P.T.E. and Arabanase from Penicillium digitatum
by Column Chromatography on ECTEOLA Cellulose.
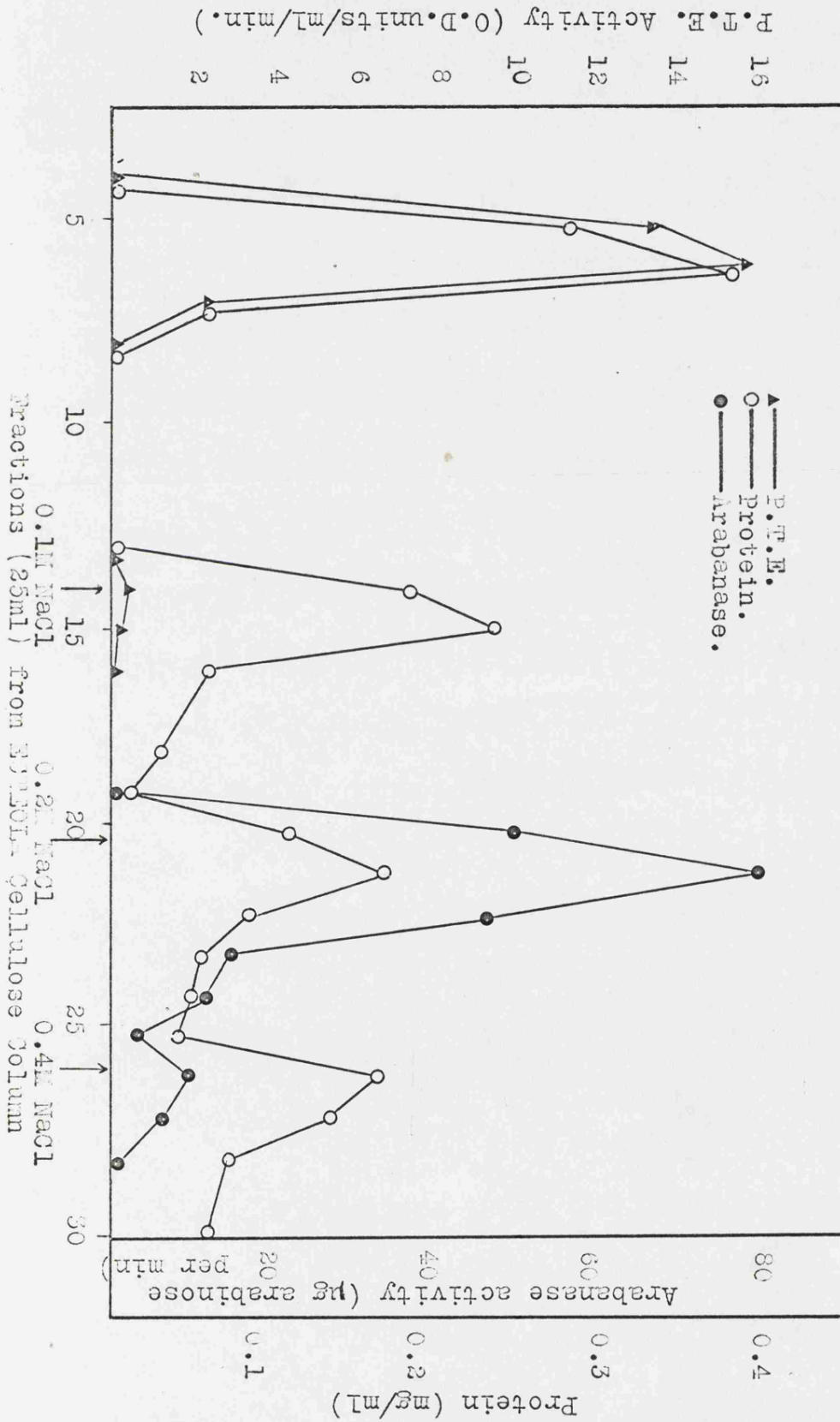
Arabanase activity was separated from P.T.E. by this
process, and fractions containing only arabanase did
not produce maceration. It was not found possible
however, to separate P.T.E. and macerating activity.
This was the first direct evidence obtained in the
present work, indicating that P.T.E. rather than arab-
anase was responsible for maceration.

2. Penicillium italicum.   Penicillium italicum
 bran cultures were set up in 1 litre conical flasks,
using 50g of bran per flask. The cultures were harv-
ested by extraction with water, after 72 hours, as
described previously, and the 4 litres of 'filtrate'
so obtained were freeze dried and reconstituted to
1 litre. To this concentrated bran filtrate was added
ammonium sulphate as with the Penicillium digitatum
culture filtrate, and the precipitate formed at each
saturation was dissolved in water and assayed for
enzyme activity. The results of such a fractionation
are shown in table VIII.

The solution of the precipitate formed at
0.5 $(NH_4)_2SO_4$ saturation was subjected to a further
ammonium sulphate fractionation. The precipitates so
formed were then dissolved in 15ml of water and
assayed for enzyme activity as shown in table IX. The
solution of that precipitate formed at 0.5 $(NH_4)_2SO_4$
saturation in table IX was passed through a column of
Sephadex G25 to remove salts and was then subjected
to column chromatography on ECTEOLA cellulose. Separa-
tion of P.T.E. and arabanase was effected using a step-
wise elution with sodium chloride, as shown in Fig 15.
The P.T.E. from Penicillium italicum was denatured to
a greater extent than that from penicillium digitatum
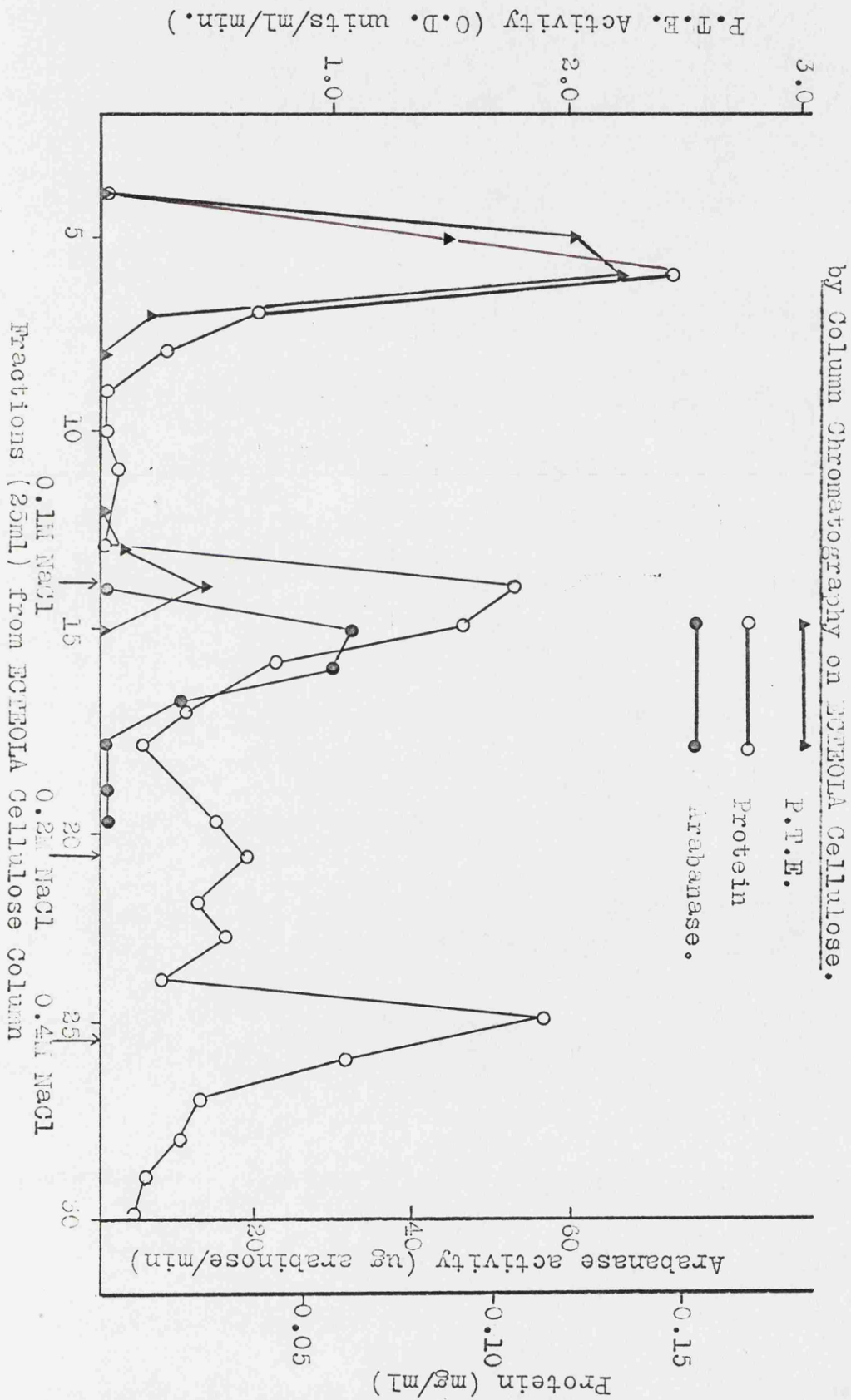during the purification process.

Table VIII. First Ammonium Sulphate Fractionation of Penicillium italicum Bran Filtrate.

Fraction	P.T.E. Activity (0.01 OD units/min)	P.T.E. Specific Activity (units activity/mg protein)	Arabanase Activity (ug arabinose/min)	Arabanase Specific Activity (units activity/mg protein)	P.G. Activity (u moles reducing group/min)	P.G. Specific Activity (units activity/mg protein)	P.M.E. Presence in plate Assay	Maceration Degree of Softening
Bran Filtrate	7.1	15.0	26.7	6.3	0.32	0.15	+	+
Conc. Filtrate	15.4	16.9	61.7	7.6	0.64	0.16	+	++
(NH$_4$)$_2$SO$_4$ Saturation								
0.2	12.0	6.6	0	-	0	-	-	++
0.4	24.5	49.7	126.7	25.7	0	-	-	++++
0.5	76.0	253.3	126.7	42.2	0	-	-	++*
0.6	23.9	52.5	20.0	4.0	0	-	-	+++
0.8	2.2	1.7	90.0	6.9	1.96	0.30	+	+
1.0	0.2	0.3	86.7	14.4	1.09	0.36	+	+₋
Residue (material not precipitated by sat. (NH$_4$)$_2$SO$_4$)	0	-	16.7	4.9	0	-	+	-

* = diluted 1/4 in the maceration assay, otherwise undiluted fractions used.

Table IX. Second Ammonium Sulphate Fractionation using the Precipitate Formed at 0.5 $(NH_4)_2SO_4$ Saturation in Table VIII. (Penicillium italicum)

Fraction	P.T.E.		Arabanase		Maceration
	Activity (0.01 OD units per min)	Specific Activity (units per mg protein)	Activity (ug arabinose per min)	Specific Activity (units activity per mg protein)	(degree of softening)
0.5 sat. $(NH_4)_2SO_4$ ppt. from table VIII.	71.2	250.0	113.0	40.0	++++
Saturation. 0.2	3.1	67.4	0	-	±
0.4	77.0	315.6	477.0	195.5	++++
0.5	218.6	888.6	247.0	100.4	++ *
0.6	35.8	303.4	8.0	6.8	+++
0.8	4.0	26.1	8.0	5.4	+
1.0	1.8	24.0	4.0	4.8	±
Residue (material not ppted. by sat. $(NH_4)_2SO_4$	0	-	10.0	-	-

(* = 1/10th. dilution otherwise undiluted fractions used.)

Fig. 15. The Separation of P.T.E. and Arabanase from Penicillium italicum by Column Chromatography on ECTEOLA Cellulose.

The specific activity of the P.T.E. was increased from 15.0 units/mg protein in the Penicillium italicum bran filtrate to 2174.0 units/mg protein in the purest fraction (fraction 5) from the ECTEOLA cellulose column, giving an overall increase in specific activity of 145 times. Similarly the specific activity of the arabanase was increased from 6.3 in the bran filtrate to 697.0 in fraction 16 from the ECTEOLA cellulose, giving an overall increase in specific activity of 110 times. As with Penicillium digitatum this procedure did not effect any separation of P.T.E. from macerating activity.

The precipitate formed at 0.8 $(NH_4)_2SO_4$ saturation in table VIII, appeared to have a higher macerating activity than could be accounted for by its P.T.E. content. It was therefore decided to attempt a further purification of this fraction, in order to determine the nature of other possible macerating enzymes. An ammonium sulphate fractionation was thus applied to this fraction and the solutions of the precipitates formed were assayed for enzyme activity. The results are shown in table X. From them it was clear that maceration could be produced by factors other than P.T.E. in Penicillium italicum bran filtrate. The indications were that this might be accounted for by a P.G. probably in conjunction with P.M.E. However attempts to further purify the P.G. fraction (fraction 0.8., table VIII) both by column chromatography and absorption onto pectic acid gel proved unsuccessful. The column chromatography did however, give further indication that a component other than P.T.E. and arabanase had a macerating activity, and that this component might be a P.G.

Table X. Ammonium Sulphate Fractionation of the
Precipitate Formed at 0.8 $(NH_4)_2SO_4$ Saturation
in Table VIII.   (Penicillium italicum)

Fraction	P.T.E. (0.01 OD. units per min.)	Reducing Group Liberation (μ moles CHO per min.)	Arabanase (μg arabinose per min.)	P.M.E. (presence in plate assay)	Maceration (degree of softening)
0.8sat. $(NH_4)_2SO_4$ ppt. from table VIII.	2.2	1.96	9.0	+	++
Saturation. 0.2	0	0.26	4.13	−	±
0.4	0	0.19	3.33	±	±
0.5	0.9	0.19	2.33	±	±
0.6	7.6	0.96	4.50	+	+
0.8	2.3	3.40	80.00	+	+
1.0	0	2.38	48.30	+	+
Residue (material not ppt. by sat. $(NH_4)_2SO_4$	0	0	10.00	−	−

3. <u>Aspergillus fonsecaeus</u>.   The organism was
grown on bran, as this medium induced the best product-
ion of macerating activity. The filtrate, prepared as
before, was freeze dried and made up to 1 litre in
distilled water(i.e concentrating it 4 times). This conc-
entrated filtrate was fractionated by an ammonium sul-
phate precipitation, the results of which are shown in
table XI.

Contrary to the results of the experiments on
enzyme production in various media, a small amount of
P.T.E. was detected in the bran filtrate of this org-
anism. Such activity appeared in the same fractions,
during the ammonium sulphate precipitation, as P.T.E.
activity produced by the two Penicillia. The P.T.E.
in these fractions appeared to be associated with part
of the macerating activity produced by <u>Aspergillus fon-</u>
<u>secaeus</u>. The activity of enzymes reducing the viscosity
of pectin and pectic acid was also assayed in this
fractionation. Viscosity reducing activity on pectin
appeared in the same fractions as P.T.E., whereas
activity on pectic acid was found in the same fractions
as enzymes liberating reducing groups from this sub-
strate. Arabanase activity also corresponded with
maceration to some extent. An attempt to purify the
P.T.E. in the 0.5 $(NH_4)_2SO_4$ saturated fraction from
table XI, by cellulose ion exchange chromatography,
was unsuccessful. Only a very small fraction of the
P.T.E. was eluted by water in the initial fractions
from the column, the majority being eluted by 0.2 M
NaCl in later fractions. This suggested that the P.T.E.
produced by <u>A.fonsecaeus</u> differed, at least in its
ionic properties, from those of <u>Penicillium digitatum</u>
and <u>Penicillium italicum</u>. It was clear however from
table XI, that by comparison with <u>P.digitatum</u> and

Table XI. Ammonium Sulphate Fractionation of Aspergillus fonsecaeus Bran Filtrate.

Fraction	P.T.E. Activity (0.01 OD. units/min)	Viscosity Reducing Activity (% reduction in viscosity per minute)		P.G. Activity (u moles reducing group/min)	Arabanase Activity (ug arabinose/min)	P.M.E. Presence in Plate Assay	Maceration Degree of Softening
		Pectin	Pectic Acid				
Bran Filtrate	1.0	5.50	3.00	0.76	62.0	+	+
Conc. Filtrate	2.6	15.50	13.00	1.50	183.0	+	++
$(NH_4)_2SO_4$ Saturation							
0.2	0.2	0	0	0.20	0	-	-
0.4	1.5	7.30	1.00	0	22.0	-	+
0.5	11.1	31.60	1.00	1.10	270.0	+	++
0.6	6.0	23.70	3.00	1.50	587.0	+	++
0.8	7.0	25.50	28.00	2.10	613.0	+	++
1.0	0.5	12.30	25.00	2.30	53.0	+	++
Residue (material not precipitated by sat. $(NH_4)_2SO_4$)	0	1.30	3.00	0	23.0	+	+

P.italicum the P.T.E. produced by A.fonsecaeus under
the conditions used, was insufficient to account for
the maceration produced by this organism.

The viscosity reducing activity on pectic acid
was found in fractions causing high maceration, point-
ing to a possible association of these two activities.
The enzyme responsible for reducing the viscosity of
pectic acid is most likely to be an endo-P.G. Macer-
ation of plant tissues by endo-P.G.s usually involves
P.M.E. (Bateman and Millar 1966), an enzyme which does
in fact occur in fractions from Aspergillus fonsecaeus
having endo-P.G. and macerating activity.

Although arabanase was also found in the same
fractions as macerating activity, it was not considered
that this played a part in maceration, as arabanase
fractions have been found free of macerating activity
during the fractionation of culture filtrates of
Penicillium digitatum and Penicillium italicum. It is
realized however, that Aspergillus fonsecaeus arabanase
may differ from the arabanase produced by the Penicillia
although no evidence for this has been found.

f. Comparison of the Properties of the P.T.E.s from
Penicillium digitatum and Penicillium italicum

Samples of P.T.E. from Penicillium digitatum
and Penicillium italicum were prepared by the fraction-
ation procedures previously described. The protein con-
tent was measured by the Lowry method and samples of
known protein content were then freeze dried. The dry
weight of these samples corresponded to the protein
values, previously calculated.

1. Electrophoretic Homogeneity.   Samples of
both transeliminases were examined for electrophoretic
homogeneity by disc electrophoresis, as previously

described. 0.1ml of solution, containing about 250µg
of protein were applied to the tubes, and run at 5m.a.
per tube. The P.T.E.s from Penicillium digitatum and
Penicillium italicum were run singly and in mixtures
of equal parts of each. In every case however, only
one protein band was detected on the gel, as shown in
plate 1. This suggested firstly, that each trans-

Plate 1. Electrophoresis of P.T.E.s from Penicillium
digitatum and Penicillium italicum.

eliminase was electrophoretically homogeneous and sec-
ondly that both enzymes had the same electrophoretic
mobility. Such evidence points strongly to the fact
that the enzymes from both organisms may be identical
in structure. To obtain further evidence for or against

this view, it was decided to examine the effect of-
reaction conditions on both P.T.E.s. The. effect of
reaction conditions on the macerating activity and the
pectin viscosity reducing activity, still found in the
purified P.T.E. preparations, was also investigated.

     2. Scan of Reaction Products.   The P.T.E.s
from Penicillium digitatum and Penicillium italicum
were allowed to react with buffered pectin at pH 5.2
for 15 minutes. After this time wavelength scans of
the reaction products were carried out on a Unicam
SP 800 recording spectrophotometer, using a buffered
pectin solution as a blank, in order to determine the
absorption peaks of these mixtures. The results are
shown in Figs 16 and 17, and the peaks for both reaction
mixtures appeared to be about 239 nm., rather than the
reported value of 235 nm. However the latter value was
so little removed from the peak obtained in the scans,
that it was decided to continue to measure P.T.E.
activity at this value for the sake of consistancy.

     3. The Effect of Substrate Concentration on
the Activity of the P.T.E.s.   The effect of substrate
concentration on enzyme activity varies with different
enzyme substrate systems. The Michaelis constant, or
the substrate concentration when half the maximum act-
ivity is reached, is a measure of the affinity of an
enzyme for its substrate, and can be used to compare
different enzyme substrate systems. The most usual
method of obtaining the Michaelis constant (Km), is to
plot $1/v$ against $1/S$, where v = the initial velocity
or activity and S = the substrate concentration. Km is
then taken as the reciprocal of the value where the plot
bisects the negative part of the abscicca. The substrate
concentration is measured in molarities for the purpose
of obtaining Km. Therefore in the case of P.T.E. acting

Fig 16. Absorption of the Reaction Products of P.T.E. from Penicillium digitatum and Pectin at pH 5.2. (traced from original scan).
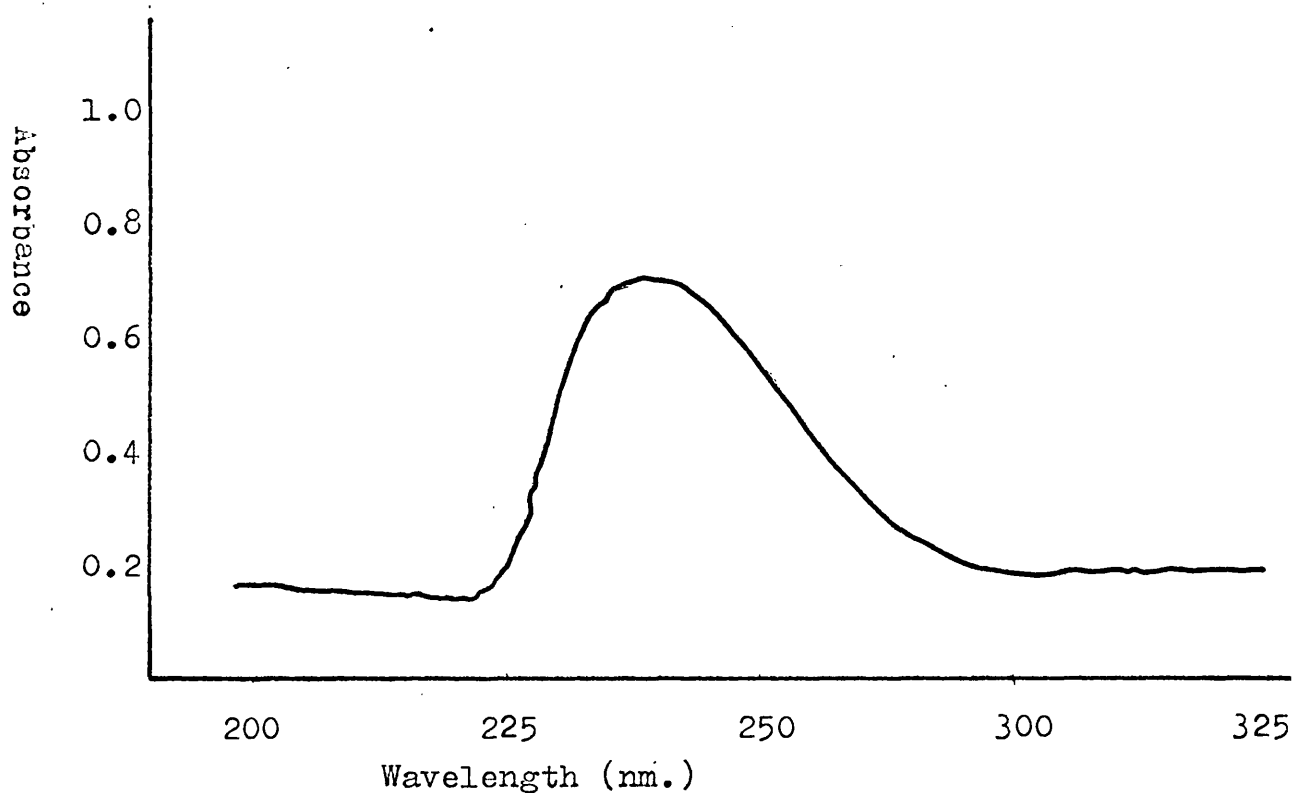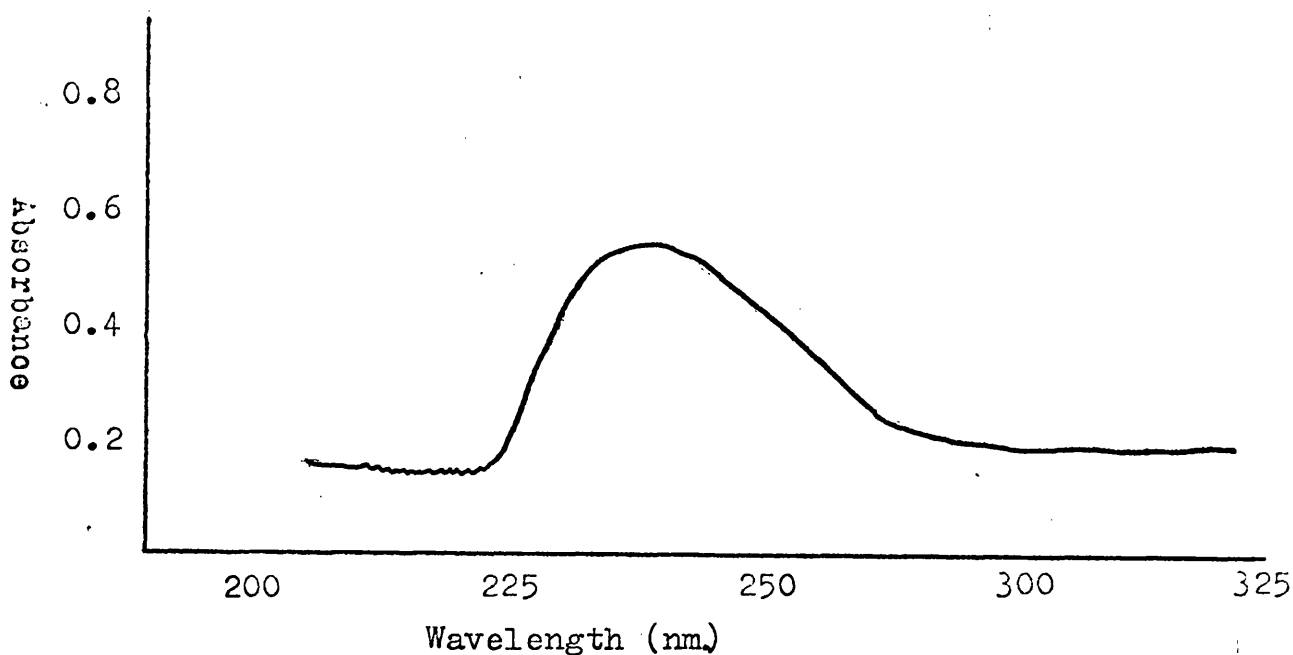

Fig 17. Absorption of the Reaction Products of P.T.E. from Penicillium italicum and Pectin at pH 5.2. (traced from original scan).

on pectin of unknown molecular weight, it is necessary
to take an approximate value for this. Molecular weights
for pectin have been reported to vary from 20,000 to
200,000. Thus as the pectin used in the present work
was an extracted material, and was likely to have been
considerably deploymerised during extraction, it was
decided to take the lower value as the approximate
molecular weight for the calculation of Km. The Michae-
lis constants obtained by this method are not therefore
absolute values, but they can be used to compare the
P.T.E.s from each organism. The activity of each P.T.E.
was thus measured at a variety of substrate concentra-
tions and the results obtained are shown in table XII
and Figs 18 and 19. From these the apparent Km value
for both P.T.E.s was 0.003 milli molar.

Table XII. The Effect of Substrate Concentration
on P.T.E. Activity

Substrate Conc.(%)	Substrate Conc.(milli molar)	Reciprocal of Sub- strate Conc.	Reciprocal of P.T.E. Activity.			
			P.digitatum		P.italicum	
			a.	b.	a.	b.
0.5	0.025	40	0.146	0.173	0.194	0.199
0.4	0.020	50	0.146	0.176	0.216	0.211
0.3	0.015	67	0.186	0.207	0.272	0.260
0.2	0.010	100	0.270	0.289	0.379	0.356
0.1	0.005	200	0.457	0.490	0.662	0.667
0.05	0.0025	400	0.719	0.775	1.042	1.020
0.01	0.0005	2000	4.167	3.704	4.546	5.000

Fig 18. 1/v : 1/S Plot for the P.T.E. from <u>Penicillium</u> <u>digitatum</u>.
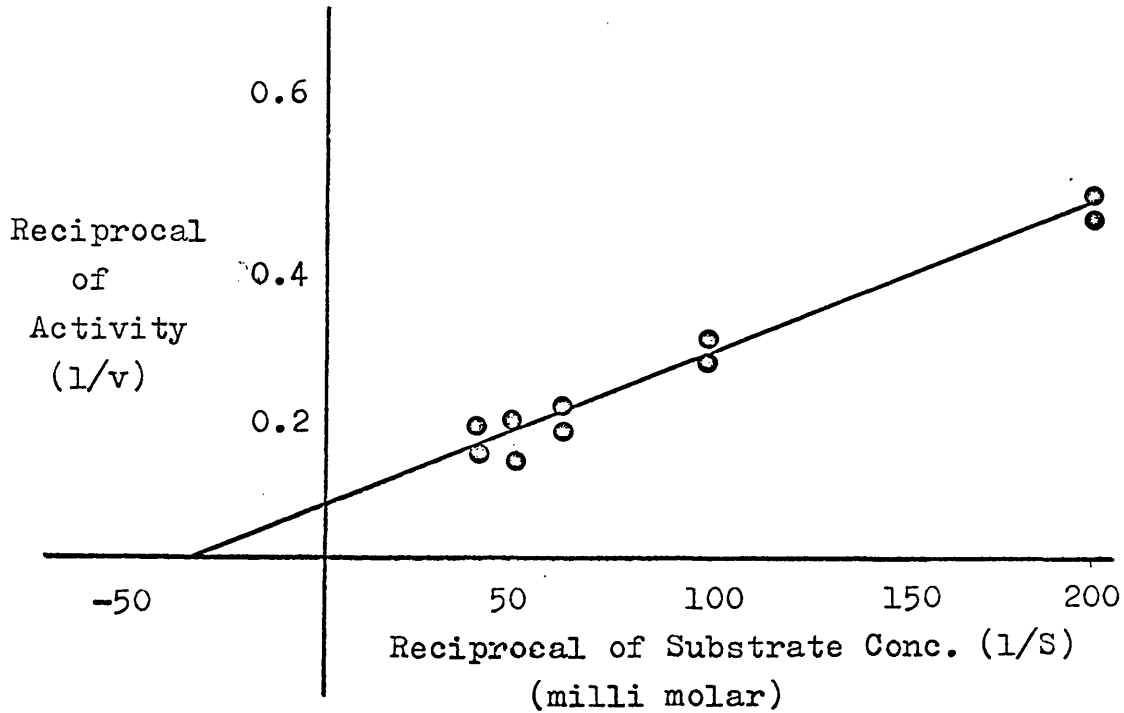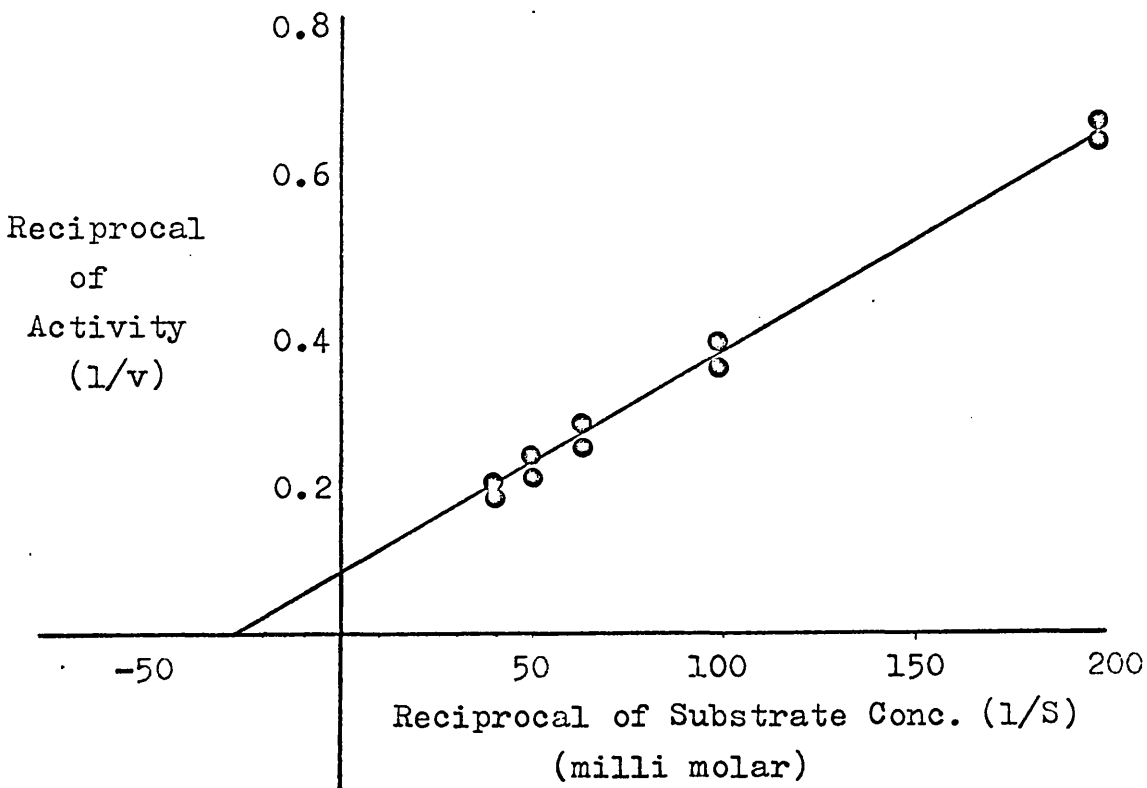

Fig 19. 1/v : 1/S Plot for the P.T.E. from <u>Penicillium</u> <u>italicum</u>.

4. **Effect of pH on P.T.E. Activity.** Activity of the two P.T.E.s was measured over a range of pH. The optimum pH for both enzymes was about 5.5. This is higher by 0.3 pH units than the reported value for P.T.E. of 5.2. (Albersheim and Killias 1962). The effects of pH on enzyme activity are shown in Figs 20 and 21.

5. **Thermal Inactivation of P.T.E.** 0.5ml samples of undiluted, purified P.T.E. were held at temperatures ranging from 50 to 75°C, for periods of 5 minutes. Preliminary experiments had indicated that inactivation occured in this temperature range. At the end of the immersion period, the 0.5ml samples were removed from the water bath and diluted to 5ml with distilled water at room temperature. This ensured that the samples were maintained at the inactivation temperature for exactly 5 minutes. Controls were treated in the same way, except that they were kept at room temperature continuously. The results are shown in table XIII. Percentage reduction in activity was calculated by the formula:- $R = \dfrac{C - A}{C} \times 100$ where:-

R = % age reduction in activity

C = activity of control

A = activity of sample after immersion

There is as can be seen from the results, a distinct difference between the thermal stabilities of the two P.T.E.s. <u>Penicillium italicum</u> P.T.E. is inactivated at a lower temperature than the P.T.E. produced by <u>Penicillium digitatum</u>. The <u>P.italicum</u> P.T.E. was also inactivated more than the <u>P.digitatum</u> P.T.E. during the purification process. It is therefore possible that there is a structural difference between the enzymes, although the variations in thermal stability might be explained by the presence of traces of impurities

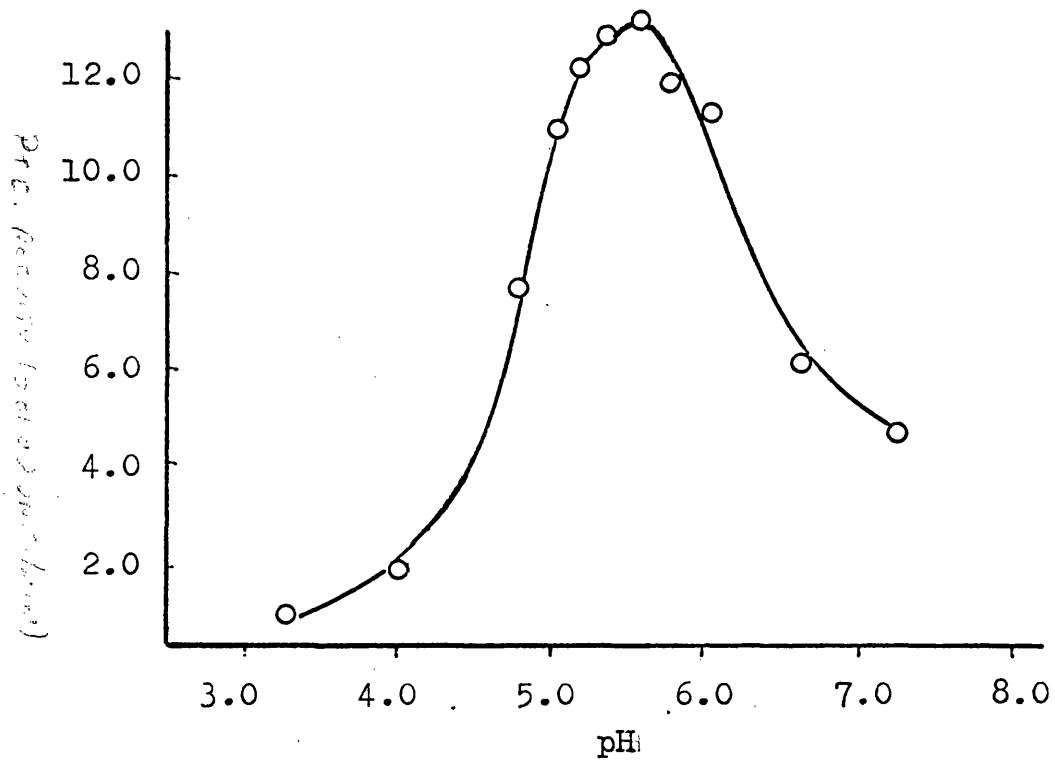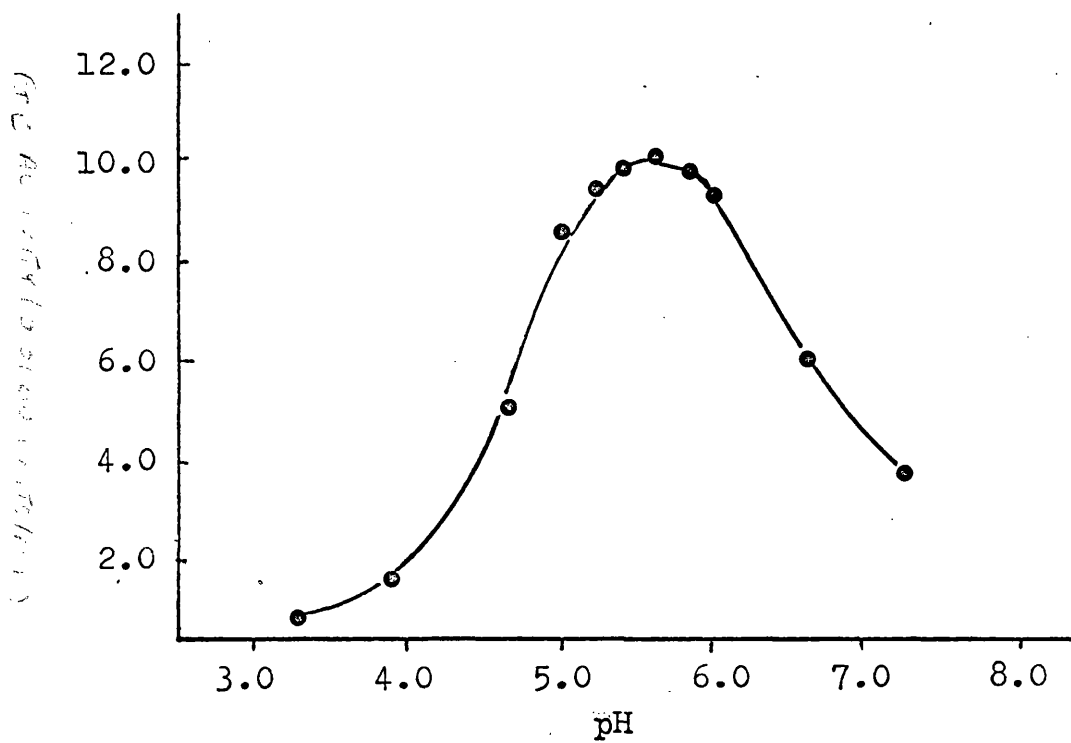Fig 20. Relationship between pH and Activity of P.T.E. from Penicillium digitatum.

Fig. 21. Relationship between pH and Activity of P.T.E. from Penicillium italicum.

other than proteins, in the electrophoretically homo-
geneous preparations.

Table XIII. Thermal Inactivation of P.T.E. from
Penicillium digitatum and Penicillium italicum

Temp. ^{o}C Samples held at this for 5 mins.	Penicillium digitatum P.T.E.		Penicillium italicum P.T.E.	
	Activity	%Reduction in Activity	Activity	%Reduction in Activity
50	3.17	2.2	2.77	11.8
55	3.14	3.1	1.81	42.4
60	1.98	38.9	0.19	93.9
65	0.11	96.6	0.02	99.4
70	0.0	100.0	0.0	100.0
75	0.0	100,0	0.0	100.0
Control	3.24	'	3.14	

6. Effect of pH on the Pectin Viscosity Reducing
Activity of the P.T.E. Preparations.   Previous experi-
ments had indicated that P.T.E. was responsible for the
reduction in viscosity of pectin solutions, caused by
filtrates of Penicillium digitatum and Penicillium
italicum. It was also found that the electrophoretically
homogeneous P.T.E. preparations from both organisms
were able to cause a decrease in viscosity of pectin

solutions. To obtain further confirmation that P.T.E.
was responsible for viscosity reducing activity, the
action of the purified P.T.E. preparations on the
viscosity of pectin, was examined over a range of pH.
The activity pH curves, obtained in this experiment
(Figs 22 and 23.) are of the same form as the activity
pH curves found when P.T.E. activity was measured
spectrophotometrically. The optimum pH for both types
of activity differed by less than half a pH unit. The
apparent ability of the P.T.E.s from both organisms to
reduce the viscosity of pectin, points very strongly
to their being endo enzymes. An exo enzyme would be
unlikely to have enough effect on the molecular size
of pectin, to cause a reduction in viscosity. This in
turn substantiates evidence so far put forward, that
P.T.E. can be responsible for maceration of orange
tissue, as it is recognised (Bateman and Millar 1966)
that endo enzymes are more likely to be responsible
for the maceration of plant tissues than exo enzymes.

7. Effect of pH on the Maceration of Orange
Tissue by the Purified P.T.E. Preparations.   Although
it is realised that the macerating activity assay used
in the present work is only partially quantitative, an
attempt was made to determine the effect of pH on mac-
eration, within the limits of the maceration assay.
Four ml of 0.1 M McIlvaines citrate phosphate buffer
were added to 1ml of undiluted, purified P.T.E. prep-
aration, and two orange discs were placed in this.
Softening of the discs was estimated after 1 and 24
hours at the following pH values:- 3.0, 4.0, 5.0, 5.5,
6.0, 7.0, and 8.0. Orange discs were also placed in
buffer at the various pH values in order to determine
how much softening was caused by the buffer alone. The
results are shown in table XIV.

Fig 22. Relationship of pH with Viscosity Reducing
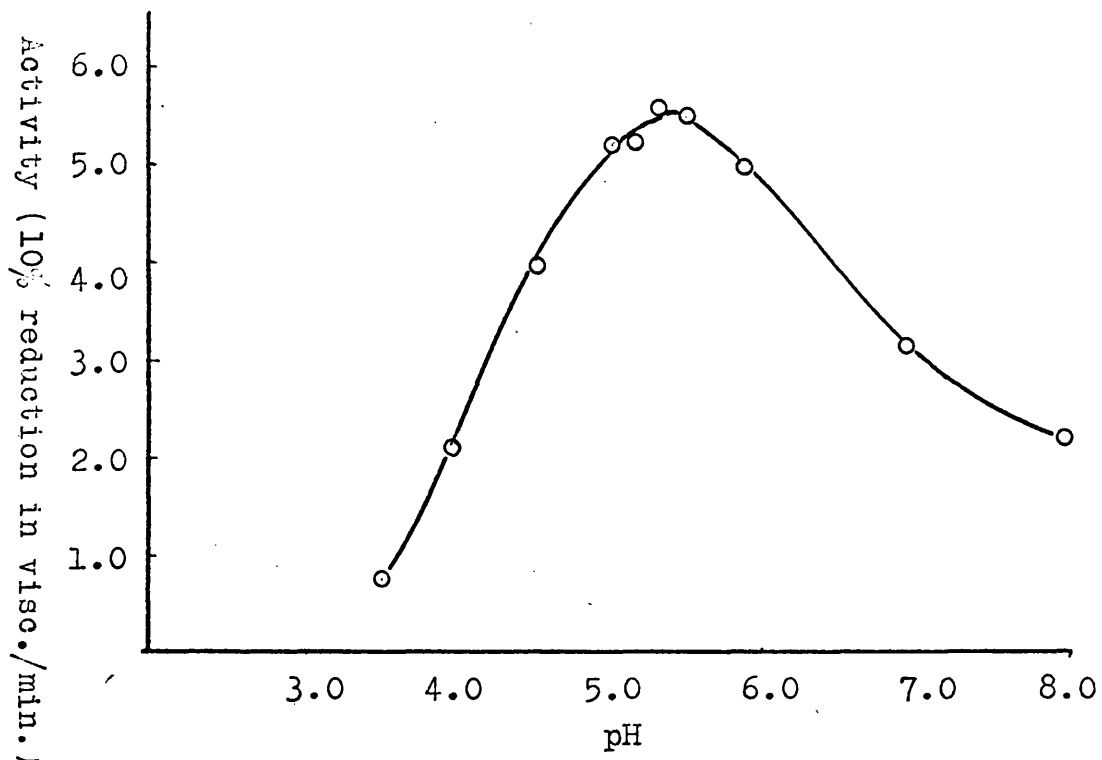Activity on Pectin of P.T.E. from Penicillium digitatum.

Fig 23. Relationship of pH with Viscosity Reducing
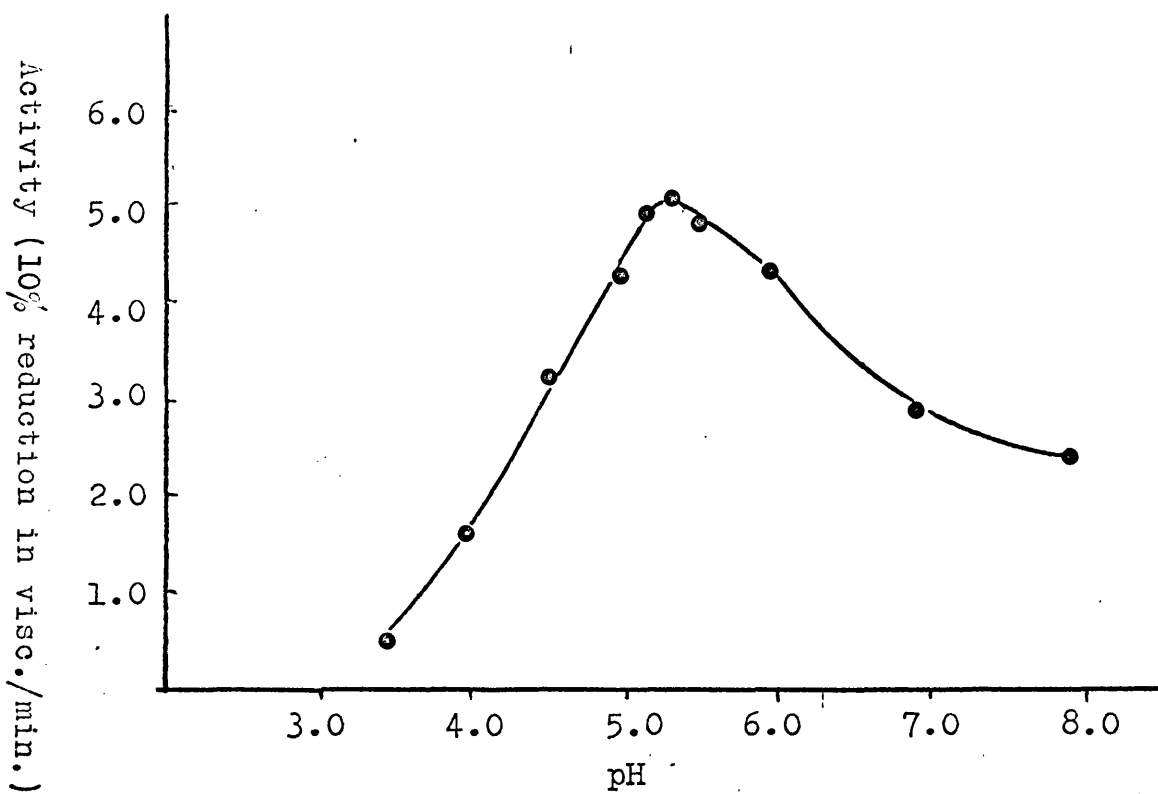Activity on Pectin of P.T.E. from Penicillium italicum.

Table XIV. The Effect of pH on Macerating Activity of
the Purified P.T.E. Preparations.

pH	Penicillium digitatum P.T.E.		Penicillium italicum P.T.E.		Controls (buffer without enzyme)	
	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.
3.0	±	++	−	++	±	++
4.0	+	+++	±	+++	±	+
5.0	+++	+++++	++	++++	−	±
5.5	+++	+++++	++	++++	−	±
6.0	+++	+++++	++	++++	−	−
7.0	±	++	+	++	−	−
8.0	±	+	±	+	−	−

In view of the softening which occured in the
controls, at pH 3.0 and 4.0, it is questionable how much
softening is due to enzyme activity and how much to pH,
in the course of the rotting of fruits by Penicillium
digitatum and Penicillium italicum. The pH of orange
rind, macerated in water (wt./vol. 1:1), is between
4.0 and 4.5, thus both enzymic and pH maceration could
theoretically occur.

8. Action of P.T.E. on Pectic Acid.   Pectic
acid transeliminase was not detected in culture filtrates
of the 3 organisms examined, in the course of the enzyme
production experiments. However, during the fractionation
of the culture filtrates, a small amount of pectic acid
transeliminase (P.G.T.E.) activity was detected in frac-
tions containing P.T.E. It therefore seemed likely that
P.T.E. had a very limited action on pectic acid. In
order to substantiate this theory, the action of the
purified P.T.E. samples on pectic acid was investigated
over a range of pH values. The level of activity found
was so small that it was not practicable to represent
this graphically; the results are therefore shown in
table XV. P.G.T.E. activity was only found at pH values
where P.T.E. was most active (between pH 4.5 and 6.0).
It is therefore considered most likely that none of
the organisms investigated produced a P.G.T.E.,and
that any such action recorded, was entirely due to the
action of P.T.E. (Aspergillus fonsecaeus produced so
little P.T.E. in any case, that transeliminative act-
ivity on pectic acid was never recorded). From the
results it appeared that P.T.E. attacked methylated
pectin at about 500 times the rate which it attacked
pectic acid.

9. Chromatography of Reaction Products of P.T.E.
Acting on Pectin.   Samples of P.T.E. from Penicillium
digitatum and Penicillium italicum were mixed with 0.5%
pectin in 0.1 M citrate phosphate buffer at pH 5.2.
and after 1, 12 and 24 hours part of the reaction mix-
ture was applied to a thin layer chromatogram. Galactur-
onic acid and pectin were used as markers and the enzyme
and buffer alone was applied as a control. No galactur-
onic acid was detected in the reaction mixtures, though
a trace of material between the markers of pectin and

galacturonic acid was observed. This was probably one
of the smaller oligomers of unsaturated galacturonic
acid. The results give further indication that both
P.T.E.s are endo enzymes.

Table XV. Action of the Purified P.T.E. Preparations
on Pectic Acid, over a Range of pH Values.

| pH | Pectic Acid Transeliminase Activity (0.01 O.D. units/min) | |
	Penicillium digitatum P.T.E.	Penicillium italicum P.T.E.
3.5	0	0
4.0	0	0
4.5	0.02	0.03
5.0	0.05	0.04
5.2	0.05	0.03
5.4	0.04	0.04
5.6	0.04	0.03
5.8	0.04	0.04
6.0	0.07	0.02
7.0	0	0
8.0	0	0

10. Softening of an Intact Orange with Purified
P.T.E.   Oranges were injected with 1ml of purified
P.T.E. (about 400 units of activity), from both Penicillium
digitatum and Penicillium italicum. One ml portions of
boiled enzyme were injected as controls. After 1 hour,
slight softening was noticed around the sites of in-
jection of the active enzymes, but not around the sites

of injection of the boiled enzymes. After 12 hours the
the softening became more apparent, with the softened
area turning brown and becoming sunken. The fruits
with these softened areas dried out more' quickly than
the control fruits. The area of softening remained dis-
crete, and the diameter varied from about 2 to 3 cms.
This suggested that the enzyme was absorbed onto the
orange tissue fairly quickly and was therefore pre-
vented from diffusing over a wide area. Alternatively
the discrete areas of softening may be due to the
enzyme being inactivated by substances in the rind
tissue. The effects of the purified enzymes injected
into oranges are shown in plate 2.

Plate 2. Oranges Injected with Purified P.T.E.

P.italicum   P.digitatum.

P.T.E.
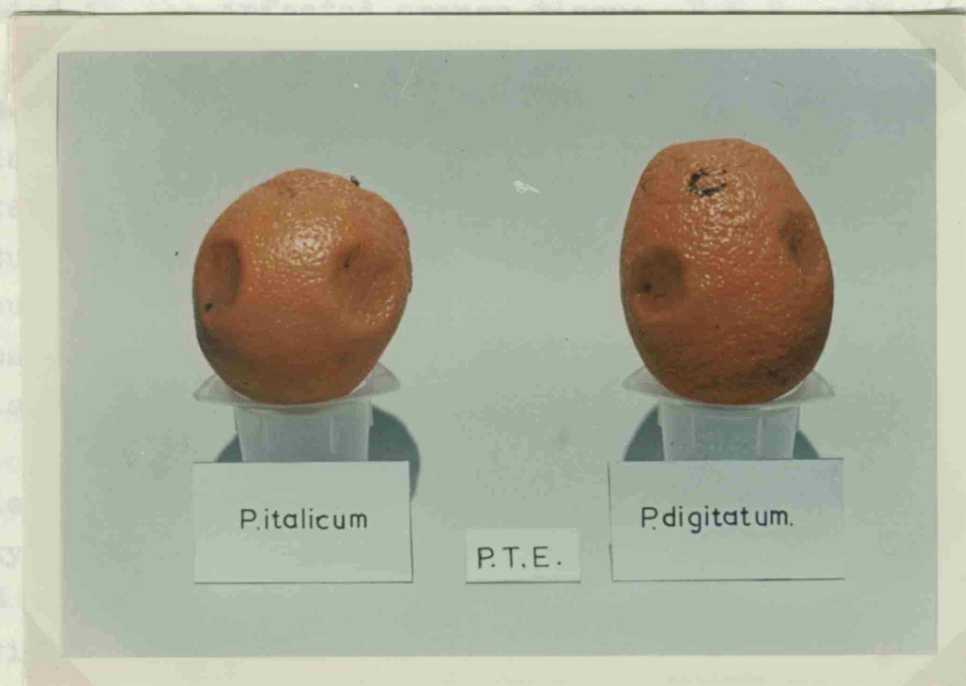
g. Production of Enzymes in Vivo by Penicillium digitatum and Penicillium italicum.

      Oranges infected with Penicillium digitatum and Penicillium italicum, and non infected oranges were incubated for 5 days at $25^{\circ}C$. At the end of this time the peel from all the oranges was removed, and 50g of peel from each treatment was macerated with 100ml of 0.5 M $Na_2H.PO_4$ at about pH 8.0, and stirred for an hour. The macerates were then centrifuged and the supernatants were made up to 0.8 saturation with ammonium sulphate. The precipitates so formed, were centrifuged at 12,000 rev/min. (23,000 g) for 20 minutes, and were then made up to 50ml with distilled water. Enzyme assays, were then carried out on these solutions as shown in table XVI.

      P.T.E. activity, pectin viscosity reducing activity, P.M.E. and macerating activity were demonstrated in the infected orange tissue. P.M.E. activity was also demonstrated in the healthy orange tissue. Although arabanase activity was not demonstrated, evidence that such activity had been present in infected tissue was obtained, as extracts from this tissue had a higher pentose content than the healthy tissue extract. It is considered likely that the arabanase was inactivated by the high pH of the extracting agent ($Na_2H\ PO_4$)

      Considerable difficulty was experienced at first in demonstrating any activity in vivo. Initially activity was shown by dispersing infected peel in a 0.5% pectin solution at pH 5.2 and observing the drop in viscosity of this mixture after centrifugation. With such evidence that infected tissues contained enzyme activity, various extraction methods were attempted. It is considered that with more investigation into

extraction proceedures, greater in vivo activity could be demonstrated. It is also probable that enzymes not demonstrated in this work, could be shown to be present by using different and improved methods of extraction.

Table XVI. Enzymes Present in Extracts of Healthy and Infected Oranges.

Enzyme Assay. (activity units as described previously)	Extract of Healthy Fruit	Extract of Fruit infected with P.digitatum	Extract of Fruit infected with P.italicum
P.T.E.	0	0.27	0.31
Viscosity Reducing Activity on Pectin	0	0.18	0.16
Arabanase	0	0	0
Liberation of Reducing Groups from Pectic Acid	0	0	0
P.M.E.	+	+	+
Maceration 1 hr. 24hrs.	− −	± ++	± ++

DISCUSSION

Penicillium digitatum and Penicillium italicum
have been shown to produce P.T.E., macerating factor,and
the ability to reduce the viscosity of pectin, under a
variety of conditions, including the in vivo state.
Arabanase was also produced in culture media, but it
was not shown in extracts of infected orange peel.
Evidence that arabanase had been active in infected
fruits was obtained. P.M.E. was produced by both org-
anisms under all conditions, but it was also present
in healthy orange tissue. P.G.,as measured by the lib-
eration of reducing groups from pectic acid, was detected
under certain culture conditions, but was not shown to
be present in extracts of infected orange rind tissue.
The fact that Penicillium notatum, an organism not
pathogenic on citrus fruits, produced P.M.E. and P.G.
as detected by plate assay, was taken as evidence for
the view that these enzymes were unlikely to be con-
cerned in the initial invasion of citrus fruits by the
pathogenic moulds. Macerating activity was produced
under the same conditions as P.T.E., throughout the ex-
periments on growth conditions, and when purified, P.T.E.
from both P.digitatum and P.italicum, was found to cause
maceration of orange rind tissue. The two P.T.E.s were
compared electrophoretically and were found to have
the same mobilities, although at present electrophor-
esis has only been carried out at one pH value. Apart
from causing maceration, both purified P.T.E.s were
able to reduce the viscosity of pectin. Their pH :
activity curves when measured by,a. the increase in
optical density at 235 nm of a pectin solution, b. the
reduction in viscosity of a pectin solution and c. the
maceration of orange tissue, were all similar; the pH

optima recorded with a., b., and c., were all of the
same order.

The P.T.E.s from <u>Penicillium digitatum</u> and
<u>Penicillium italicum</u>, were shown by thin layer chrom-
atography to be endo enzymes. They were found to be
similar in all respects, apart from their thermal
stability. <u>P.italicum</u> P.T.E. was inactivated at a
lower temperature than the P.T.E. of <u>P.digitatum</u>. At
present no explanation can be put forward for this
discrepancy, although conclusive evidence that it was
a discrepancy rather than a real difference between
the enzymes, could perhaps be obtained, by carrying out
electrophoresis of the purified samples at several pH
values, or by further purifying the samples before
carrying out the thermal inactivation tests on them.
Finally, when samples of the purified enzymes were
injected into whole oranges, they caused areas of soft-
ening in the rind, whereas boiled enzyme did not. The
results obtained are significant on three accounts.
Firstly they shed further light on the attack of citrus
fruits by <u>P.digitatum</u> and <u>P.italicum</u>. Secondly they
support certain evidence on the structure of plant cell
walls, and thirdly they add to the general knowledge
of the role of pectic enzymes in host parasite relation-
ships.

P.G. and P.M.E. were shown to be constitutively
produced by both <u>Penicillium digitatum</u> and <u>Penicillium
italicum</u> (Miyakawa 1962, Kavanagh 1965.), whereas only
P.M.E. was found to be produced under all conditions
in the present work. However, as these workers were
using different media, it was difficult to make a com-
parison. It did appear however from the present results
that the production of P.G. was dependent on the nature
of the medium (bran strongly favoured its production)

and aeration conditions, rather than its pectin content.
Miyakawa (1963), also suggested that D-galacturonic acid,
identified by him in infected citrus peel, might have
a role in the maceration of orange tissue. Although no
direct evidence for this has been found in the present
work, it has been observed that maceration of orange
tissue can occur in enzyme free buffer below about pH
4.5.

Much of the work carried out on enzyme produc-
tion by Penicillium digitatum, has been done by Cole
(1967). Although his results are not in complete
agreement with the present work, no serious problem is
encountered in reconciling the two groups of observations.
Enzyme production by P.digitatum was examined under a
wide variety of conditions by Cole, whereas in the pre-
sent work, only three media, all different from those
used by Cole, were employed. Comparison from this point
of view is therefore difficult, but evidence from both
sets of results has suggested that arabanase is not
principally concerned in maceration. The present work
differs mainly from that of Cole, in that he casts
doubt on the suggestion that, the P.T.E. component of
the pectic enzyme complex produced by P.digitatum is
responsible for maceration. The evidence presented by
Cole in support of this view, is that maceration has
been shown by him, to be produced under conditions of
growth, where he was unable to detect P.T.E. In no case
however, did he report that macerating activity was
absent, when P.T.E. was present. The results of the
fractionation of P.digitatum culture filtrate (tables
VI and VII), clearly show that macerating activity was
associated with P.T.E., and this view is borne out by
the fact that the electrophoretically homogeneous P.T.E.
preparation from P.digitatum, was able to macerate

orange tissue. Further to this, no other enzyme activity
was detected in such pure preparations. However it is
not denied that more than one macerating system may
operate in P.digitatum culture filtrates, produced
under varying medium and culture conditions. Strong
evidence of an alternative macerating system, has been
found using fractions of culture filtrates of P.italicum
low in, or free from P.T.E.; and in the case of Asper-
gillus fonsecaeus it seems probable that the amount of
maceration caused by P.T.E. is of limited significance.
However the results given above, strongly indicate that
in culture conditions where P.T.E. is produced, the pre-
sence of this enzyme can account for all or part of the
macerating activity of the filtrate.

A further contrast between the results found
by Cole (1967) and those presented in this work, occured
in the detection of enzymes in infected orange tissue.
P.T.E., the ability to reduce the viscosity of pectin,
macerating activity and P.M.E. have been detected in
vivo in the present work, whereas P.G. and arabanase
have not. Cole was unable to detect P.T.E. in vivo,
although he detected a small amount of viscosity red-
ucing activity. The detection of enzyme activity in
infected tissue is difficult for two reasons. Firstly
the enzymes may be inactivated by substances in the
tissue. For example, Cole himself found that orange
juice contained a thermolabile inhibitor to macerating
activity and viscosity reducing enzymes, and Cole J.S.
(1956), found that oxidised apple juice was
inhibitory to the pectic enzymes of Botrytis cinerea.
Secondly enzymes can become absorbed strongly to the
host tissue, when vigorous methods have to be used to
remove them. This in itself may result in inactivation
of the enzymes. A further difficulty is experienced in

the demonstration of P.T.E. in infected orange peel,
due to the high U.V. absorption of the extract. The
sensitivity of the spectrophotometer is then inadequate
to detect small increases in absorption. This difficulty
was overcome by reading the optical density of reaction
mixtures against a blank with a high U.V. absorbance
and by using an ammonium sulphate precipitate of the
extract, rather than the extract itself, to detect
activity. When these difficulties are taken into con-
sideration it is suggested that, the inability to
demonstrate the presence of an enzyme in vivo, is not
conclusive evidence that this enzyme is not produced
under such conditions. This suggestion is emphasized
by the work of Garber and Beraha (1966), who demon-
strated both an exo and an endo-P.G. in infected orange
tissue, in contrast to the results presented here. On
the evidence therefore, that both <u>Penicillium digitatum</u>
and <u>Penicillium italicum</u> produce P.T.E. in vitro and
in vivo; and that when purified, this enzyme will mac-
erate orange tissue and cause softening of the rind of
intact oranges, if injected into them, it is suggested
that P.T.E. has a very significant role to play in the
host parasite relationships of these fungi and citrus
fruits.

That other pectic enzymes have an important
role in this host parasite relationship, either spec-
ifically in maceration or in pathogenicity in general
is clear from the work of others (Cole, 1967; Garber,
Beraha and Shaeffer, 1965; Kavanagh, 1965.). It is
considered likely that endo-P.G. may have a role in
maceration together with P.M.E.; but that other
enzymes, eg. arabanases and exo-P.G. may act on the
breakdown products of the chain splitting enzymes,
to produce monomeric forms that can be absorbed by

pathogens. These substances will then presumably take part in the metabolism of the fungus. From the results presented here, there appears no need to invoke a macerating system in either <u>Penicillium digitatum</u> or <u>Penicillium italicum</u>, which does not involve either endo-hydrolases or endo- transeliminases breaking down pectin or pectic acid, as postulated by Cole (1967). Evidence of such an unknown system has however, been found in the case of <u>Aspergillus fonsecaeus</u>, where maceration has been detected in filtrates containing no pectic enzymes.

In more general terms of host parasite relationships, the results lend further weight to the argument of McClendon (1964), that pectic materials are of fundamental importance in the structure of the middle lamella of plants. The P.T.E. of <u>Penicillium digitatum</u>. was found to be capable of causing maceration of not only orange tissue, but also rose leaves. The rose leaves presented a more suitable substrate for microscopic examination, than the rather heterogeneous tissue of orange peel. When a rose leaf was placed into a solution of enzyme, it gradually disintegrated. If then the suspension of macerated leaf tissue was examined microscopically, it was found that the enzyme had caused a complete separation of the cells, although the cells themselves, remained intact for several days. It is thus significant that P.T.E., an enzyme having a specific action on extracted pectin, was able to cause a disintegration of plant material, by breaking down plant pectin. This gives support to the view of Joclyn (1962), who considered that protopectin (plant pectin) was not a substance basically different in structure from extracted pectin The results of the present work can be reconciled either with the view

that it is the size of the pectin molecule, which
renders it insoluble in plant tissue, or that it is
the way in which the molecule is bound with other sub-
stances in the tissue that is the cause of its insol-
ubility.

The results of the present work satisfy the
conditions laid down by Wood (1960), to prove that
pectic enzymes play a part in plant disease. These are
that such enzymes are,or have been present and active
in infected plant tissue, as is the case with P.T.E.
in the present investigation. The results are also in
agreement with the main conclusions of Bateman and
Millar (1966), which were that maceration was most likely
to be correlated with the endo forms of both hydrolases
and transeliminases, although it was admitted that
other enzymes might play a part in pathogenicity. As
has been mentioned, a certain amount of evidence was
obtained in the present work, for the involvement of
endo-P.G. in maceration in the case of Penicillium
italicum. What has not been obtained in work up to the
present time, in Bateman and Millar's view, is proof
that the ability of an organism to produce pectic
enzymes, is sufficient to account for its pathogenicity.
Although it is not claimed that such proof has now been
obtained, it is felt that strong evidence for this view
has been put forward. This evidence is based on the
observation that Penicillium digitatum and P.italicum
will both invade oranges wounded with pin pricks,
whereas Penicillium notatum will not. P.notatum will
however, grow on the surface of a cut orange, but will not
soften the fruit or grow throughout the tissue. It thus
appears that the ability of the pathogens to spread
through the orange tissue, is connected with their
ability to cause softening of the fruit. In addition

to this, strong evidence that the softening is caused
by pectic enzymes (in particular P.T.E.), has been
presented in these results. Further proof that the path-
ogenicity of P.digitatum and P.italicum on citrus fruits
depends on their ability to produce P.T.E., might be
obtained by using a specific inhibitor to P.T.E., if
such a substance could be obtained. If an orange
treated with this inhibitor was found to be immune to
attack by the two pathogens, the importance of P.T.E.
would have been shown.

The P.T.E.s produced by Penicillium digitatum
and Penicillium italicum appear to be identical in all
respects, apart from their thermal inactivation temperatures.
They resemble the transeliminase isolated by Albersheim,
Neukom and Deuel (1960), although the absorption peak
of the reaction mixture using this enzyme was reported
to be 235 nm., whereas a slightly higher value (239 nm.)
has been found in the present work. The P.T.E. isolated
by Edstrom and Phaff (1964a.), from Aspergillus fonse-
caeus, appeared to resemble the P.T.E.s of P.digitatum
and P.italicum. However when the A.fonsecaeus P.T.E.
was examined in the present work, it was found to behave
differently on ion exchange cellulose, from the P.T.E.s
produced by the two Penicillia. In general fungi have
been shown to produce transeliminases attacking pectin
(Albersheim and Killias, 1962; Edstrom and Phaff, 1964),
and bacteria have been shown to produce transeliminases
attacking pectic acid (Nagel and Vaughn, 1961; Fuchs,
1965.). An exception to this situation, is the trans-
eliminase isolated by Hasegawa and Nagel (1967), from
an aerobic Bacillus, which was able to attack both
pectin and pectic acid. The transeliminases from
P.digitatum and P.italicum,however, appeared to follow
the general rule, in that they attacked only pectin

significantly. No pectic acid transeliminase was det-
ected in either of these fungi, nor in A.fonsecaeus.

Apart from providing evidence for the view that
P.T.E. and macerating factor in Penicillium digitatum
and Penicillium italicum, are synonymous, and that
these enzymes play a significant role in pathogenisis,
the present work has also suggested several possible
lines for further research. Such research could follow
either an applied course or an academic one. Applied
research could be carried out with the aim of obtain-
ing a specific inhibitory agent to P.T.E., which, if
non-toxic, might be used to control the green and blue
rots of citrus fruits. At the moment, di-phenyl impreg-
nated wrapping paper is used in an attempt to control
these diseases. This is unsatisfactory both from the
toxicity point of view, and in its efficiency in
controlling the diseases. Thus the develop ment of an
improved antifungal agent, for use in controlling the
Penicillia rots of citrus fruits, would be a worth-
while research aim.

The method used in this work for the puri-
fication of P.T.E. is both quick and simple. It can
be envisaged therefore, that pure P.T.E. might have a
use in the fruit juice industry. At the present time
a mixture of P.M.E. and P.G.,in crude extracts, is
used to reduce the viscosity of fruit pectins in the
manufacture of fruit juices. The disadvantages of this
system are twofold; firstly the rate of the viscosity
reduction is limited by the rate of P.M.E. activity,
and secondly, the crude extract has to be used in rel-
atively large quantities, risking the addition of un-
desirable impurities to the juice. Both disadvantages
might be overcome by using a purified P.T.E. preparation
of the kind obtained in this work.

Work of a more academic nature might involve
the study of the products of transeliminase action.
The double bond between carbon atoms 4 and 5 of the
galacturonic acid residues should theoretically
render these structures unstable. It would be of
interest therefore, to examine their fate in the met-
abolism of the fungi, if indeed they are absorbed
without further degradation. It is possible to en-
visage the conversion of these unsaturated structures
to galacturonic acid by a separate enzyme system(s),
although no evidence has been found for such a system
at present.

As comparatively little is known about P.T.E.
at the present time, further studies on its substrate
specificity would be of interest. This would involve
the preparation of a series of oligomers of saturated
and unsaturated galacturonic acid, which could un-
doubtedly be carried out, using a range of Sephadex
gels. A great deal could then be learnt about the
mode of action of P.T.E., using these defined sub-
strates and a purified enzyme preparation

Finally the nature of other enzymes systems
responsible for maceration, might be investigated
further. Cole (1967), found evidence of such systems
in Penicillium digitatum, and the results shown above
have presented evidence of macerating systems other
than P.T.E. in Penicillium italicum and Aspergillus
fonsecaeus. Geotrichum citri-auranti has been reported
to invade citrus fruits (Fawcett, 1926). However,
apart from ascertaining that the organism will do
this, no work has been carried out on its path-
ogenicity of citrus fruits. Thus an investigation of
the pectic enzymes produced by this organism, and a
comparison of these with the enzymes produced by

P.digitatum, P.italicum and A. fonsecaeus, might prove
very fruitful .

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