

KILLING OF PLANT CELLS BY ERWINIA CAROTOVORA

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by

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ABSTRACT

Crude dialysed extracts from rots of cucumber fruit infected by Erwinia carotovora caused cell separation and death of protoplasts in normal tissues of different plants. By ammonium sulphate precipitation, gel filtration and isoelectric focusing a pectate trans-eliminase (PTE) was purified from rot extracts. When used at concentrations occurring in extracts it affected normal tissues in much the same way as rot extract. Cell separation by both preparations was inhibited by ions but inhibition could be removed by washing. In plasmolysed tissue rot extract caused cell separation and killed protoplasts, whereas PTE did not kill protoplasts although it did cause cell separation. Similar effects were found with single carrot cells.

Proteinase and phosphatidase purified from rot extracts separately ruptured isolated cucumber protoplasts and together were almost as active as rot extract when used at concentrations occurring in extracts. In contrast, PTE did not rupture isolated protoplasts. However, proteinase and phosphatidase even in combination with PTE and cellulase (CMCase) did not kill protoplasts of plasmolysed single carrot cells or potato tissue. This showed that some factor other than PTE, proteinase and phosphatidase in rot extracts caused death of plasmolysed protoplasts.

Release of electrolytes, protein and enzymes from potato tissue by rot extracts could be explained largely by their PTE content. Protein and enzymes but not electrolytes were released from walls of PTE treated plasmolysed tissue.

Changes in respiration rate caused by rot extract were not found with PTE and although respiration rate rapidly declined with rot extract treated plasmolysed tissue, PTE had little effect.

Electron microscopy of potato tissue showed changes in wall structure and cytoplasm. Carrot cells showed collapse of walls with apparent extrusion of contents.

Death of protoplasts in unplasmolysed tissue caused by FTE probably depends on alteration of cell wall structure so that protoplasts rupture under turgor pressure.

CONTENTS

	<u>Page</u>
ABSTRACT	2
INTRODUCTION	10
REVIEW OF THE LITERATURE	12
I. <u>Pectic enzymes and maceration</u>	12
II. <u>The cell wall</u>	17
III. <u>Permeability changes in disease</u>	22
IV. <u>The nature of the toxic factor in cell separation</u>	25
MATERIALS AND METHODS	32
1. Culture of pathogen	32
2. Living substrates	32
A. Tissue	32
B. Single cells	33
C. Isolated protoplasts	34
3. Chemical reagents and substrates	35
4. Pectic substrates	35
5. Buffers	35
6. Sterilization	36
7. pH measurements	36
8. Spectrophotometry	36
9. Preparation of cell-free cucumber rot extracts	36
A. Inoculation of tissue	36
B. Extraction of rotted tissue	37
10. Measurement of enzyme activities	37
A. Pectic enzymes	37
i) Pectin methyl esterase (PME)	37
ii) Chain-splitting enzymes	38

	a)	Viscosity reducing activity	38
	b)	Release of reducing groups	38
	c)	Thiobarbituric acid test	39
	d)	Cup-plate assay	40
B.		Toxicity	41
C.		Cell separation	42
D.		Permeability increasing activity	43
E.		Proteinase	43
	i)	Cup-plate assay	43
	ii)	Congocoll method	43
F.		Phosphatidase	44
G.		Cellulase (CMCase)	44
H.		Acid phosphatase	45
I.		Phenoloxidase	45
J.		Peroxidase	45
K.		Malic dehydrogenase	46
L.		Ribonuclease (RNase)	47
11.		Measurement of ion content	47
12.		Measurement of protein	47
	A.	Ultraviolet absorption method	47
	B.	Folin-Ciocalteu reagent method	48
13.		Ultrafiltration	48
14.		Purification of enzymes from crude cucumber rot extracts	49
	A.	Ammonium sulphate precipitation	49
	B.	Gel filtration	49
	C.	Ion-exchange chromatography	50
	D.	Isoelectric focusing	51
15.		Scanning electron microscopy	53
16.		Transmission electron microscopy	54

17.	Oxygen uptake by potato tuber disks	55
18.	Gel electrophoresis	56

EXPERIMENTAL WORK AND RESULTS	57
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I.	<u>Preliminary Experiments</u>	57
1.	Examination of the ability of <u>E. carotovora</u> to cause soft rots in different plant tissue	57
2.	Properties of extracts of rots from different tissues	58
A.	Changes of pH	58
B.	Pectic enzyme activity	59
	i) Viscosity reducing activity	59
	ii) Thiobarbituric acid test	60
C.	Cell separating activity and toxicity	61
II.	<u>Properties of crude dialysed cucumber rot extracts</u>	62
1.	Pectate <u>trans</u> -eliminase activity (PTE)	62
A.	pH optimum	62
B.	Effect of dilution	63
2.	Cell separating activity and toxicity	67
A.	pH optima	67
B.	Effect of dilution	68
3.	Permeability increasing activity	69
III.	<u>Purification of pectate <u>trans</u>-eliminase (PTE) from cucumber rot extracts</u>	73
1.	Ammonium sulphate fractional precipitation	73
2.	Gel filtration using Sephadex G75 Superfine	75
3.	Isoelectric focusing	77
4.	Activities of cucumber rot extract and purified PTE samples	81

5.	Gel electrophoresis of PTE	84
6.	Estimation of molecular weight of PTE	84
IV.	<u>Properties of PTE and cucumber rot extract</u>	86
1.	Cell separating activity, toxicity and effect of plasmolysis	86
A.	Effect of sucrose	86
B.	Effect of KNO_3	88
C.	Effect of different salts	90
D.	Effect of non-electrolytes at hypotonic concentration	93
E.	Effect of salts on NaPP degradation by cucumber rot extract and PTE	93
F.	Effect of washing on inhibition of cell separation by salts	95
2.	Permeability increasing activity	96
A.	Release of ions	96
B.	Permeability increasing activity of ethanol and Triton X-100	99
C.	Release of ions from cell walls	101
D.	Effect of plasmolysis	101
3.	Effect on different living substrates	108
A.	Tissues	108
B.	Single cells	111
C.	Isolated protoplasts	113
V.	<u>Purification of certain other enzymes in cucumber rot extracts</u>	117
1.	Phosphatidase	117
A.	Gel filtration on Sephadex G75 Superfine	117
B.	Isoelectric focusing	119
C.	pH optimum	119

2.	Proteinase	121
A.	Gel filtration on Sephadex G75 Superfine	121
B.	Isoelectric focusing	121
C.	Ion-exchange chromatography	122
D.	pH optimum	124
3.	Cellulase (CMCase)	125
A.	Ion-exchange chromatography	125
VI.	<u>Effect of purified enzymes on living substrates</u>	128
1.	Isolated protoplasts	128
2.	Single cells	128
3.	Tissues	131
4.	Nature of factor in cucumber rot extract toxic to plasmolysed protoplasts	138
VII.	<u>Toxicity of pectate <u>trans</u>-eliminase (PTE) to potato tuber tissue</u>	141
1.	Release of toxins	141
A.	Lower molecular weight toxins	141
B.	Higher molecular weight toxins	141
C.	Release of hydrogen peroxide	144
2.	Effect of deplasmolysis on PTE treated potato tissue	145
3.	Browning of reaction mixtures	146
4.	Release of protein	148
A.	Rate	148
B.	Relation to permeability changes	151
C.	Molecular weight of protein	152
5.	Release of enzymes	153
A.	Acid phosphatase	153
i)	Relation to permeability changes	155

B.	Other enzymes	155
6.	Release of RNA	164
A.	Gel electrophoresis	164
B.	Spectrophotometric estimation of RNA	165
C.	Orcinol method for estimation of RNA	166
7.	Gel electrophoresis of protein released from PTE treated disks	167
8.	Respiratory changes in enzyme treated potato tissue	167
VIII.	<u>Light and electron microscopy</u>	173
1.	Potato tuber tissue	173
2.	Carrot callus cells	179
DISCUSSION		183
REFERENCES		194
ACKNOWLEDGEMENTS		204

INTRODUCTION

This study investigates the killing of plant cells by soft rot pathogens.

The organism chosen for this work was Erwinia carotovora, an important pathogen of storage tissue in which it causes a rapidly spreading soft rot. A bacterial pathogen was preferred to a fungal pathogen because it rots tissue much more rapidly. Extracts of such rotted tissue were used throughout this work as it was considered that effects produced by substances present in diseased tissue would be better related to processes occurring in the diseased plant than effects produced by substances secreted by the pathogen in cultures on synthetic media.

A bacterium producing a soft rot of carrot was first described by Jones (1901) and called Bacillus carotovorus. The genus name Erwinia was adopted in the first edition of Bergey's Manual of Determinative Bacteriology in 1923, to include the petrichously-flagellated organisms within the plant pathogenic tribe Erwineae. Waldee in 1945, pointed out that the genus Erwinia contains two groups of organisms, those producing dry necroses and wilts and those, because of their ability to secrete pectolytic enzymes, producing soft rots. He suggested a separate genus for the pectolytic organisms which he named Pectobacterium. Graham (1964), in a review of the soft rot coliform bacteria considers that only one species, Pectobacterium carotovorum, should be recognised. This would then contain the closely related bacteria as varieties i.e. var. carotovorum, var. aroideae, var. atrosepticum and var. chrysanthemi.

This group is not recognised by most workers and thus in accordance with the reports of recent workers and the classification shown in the 7th edition of Bergey's Manual the name Erwinia carotovora will be used to describe the pathogen used in this study.

Soft rots, such as those caused by E. carotovora are important diseases of higher plants. They are typical of parenchyma, especially of

storage tissue, and are characterized by the formation of a rapidly spreading water-soaked lesion. The cells separate along the lines of the middle lamellae and the tissue rapidly loses its coherence. At about this time the protoplasts of the cells die and why they do so is the subject of this thesis.

The involvement of pectic enzymes in cell separation and the closely associated death of the cells was studied. The role of factors other than pectic enzymes was examined and attempts were made to separate the two processes of cell separation and death of protoplasts.

REVIEW OF THE LITERATURE

This review examines the factors causing cell separation especially the pectic enzymes. The structure of plant cell walls is described with particular reference to the middle lamella and primary wall. The involvement of changes in cell permeability in disease and the role of pectic enzymes in this context is considered together with the nature of the toxic factor associated with cell separation.

I. Pectic enzymes and maceration

The enzymatic nature of tissue degradation by plant pathogens has long been known. De Bary (1886), showed that juice from carrot roots and other tissues infected with Sclerotinia libertiana contained a thermolabile principle that dissolved cell walls. In the first major study of maceration, by the fungus Botrytis cinerea, Brown (1915), demonstrated that treatment of parenchyma with extracts of young hyphae led to loss of coherence between cells, solution and collapse of cell walls and death of protoplasts.

During maceration cells separate along the lines of the middle lamellae and thus the importance of enzymes that degrade pectic substances, generally regarded as the most important components of this zone, has been stressed (Wood, 1960).

It is known that most plant pathogens produce enzymes that degrade all components of primary cell walls of plants (Albersheim, Jones and English, 1969). In cell separation in soft rots the cell wall remains essentially intact. This indicates that cellulases do not play a major part in maceration as does the absence of cellulose from the middle lamella and the fact that it would probably not be exposed until the matrix around microfibrils had been removed by other enzymes (Wood, 1960). Also, it is known that cellulases and hemicellulases do not cause maceration and even when they occur with pectic enzymes it is considered that they are unlikely to contribute significantly to maceration (Bateman, 1963, 1968; Spalding,

1963; Cole and Bateman, 1969).

Cole and Wood (1970), have pointed out that galactanase might be important in maceration by filtrates of cultures of Penicillium digitatum. A galactanase from Phytophthora infestans caused maceration of potato disks, but only after 24h (Knee and Friend, 1970).

The presence of protein in the middle lamella was inferred by Ginzburg (1961), because after separation of cells with ethylene-diamine tetra-acetic acid (EDTA), treatment with divalent cations rejoined them. However, treatment with proteolytic enzymes prevented rejoining. Proteases have often been found in diseased tissue (Kuć, 1962; Keen, Williams and Walker, 1967; Reddy, Stuteville and Sorensen, 1971; Heath and Wood, 1971). A role for these enzymes in the degradation of the supposed protein-pectin-cation complex of the middle lamella has been postulated (Kuć and Williams, 1962).

Most studies on maceration have used a mixture of enzymes in which activity has usually been attributed to pectic enzymes (Bateman, 1963; McClendon, 1964). The macerating enzyme was referred to as pectinase or protopectinase by earlier workers.

Pectic enzymes have been classified into exo-enzymes which split off terminal galacturonic acid residues and endo-enzymes which cleave the α -1,4 glycosiduronic linkages randomly along the chain (Demain and Phaff, 1957). It was first thought that the hydrolytic polygalacturonases (PG) were the only type of chain splitting pectic enzyme. Albersheim, Neukom and Deuel (1960a), described an enzyme which ruptured the α -1,4 linkages by a trans-elimination reaction forming a product with an unsaturated bond between carbon atoms 4 and 5 which absorbed strongly at 235 nm. These enzymes are termed pectic trans-eliminases (TE) or lyases. The enzymic action is thought to occur similarly to that of the degradation of pectin in alkaline (Neukom and Deuel, 1958) or neutral solution (Albersheim, Neukom and Deuel, 1960b).

Lyases usually have pH optima at c. 9.0 and are activated by

calcium, usually PGs have pH optima c. 5.0 and are inhibited by calcium. Addition of EDTA inhibits TE but enhances PG activity (Starr and Moran, 1962; Ayers, Papavizas and Diem, 1966).

Pectic enzymes are further classified by preference for pectate or pectin substrates.

The activity of endo-enzymes which rapidly decrease the viscosity of solutions of pectic substrates has usually been associated with maceration. Exo-forms of pectolytic enzymes do not cause maceration (McClendon, 1964; Hancock and Millar, 1965).

'Macerating enzymes' have now been separated from pectic enzymes. Kaji (1958), separated a macerating enzyme from a PG of Clostridium felsineum var. sikokianum. Byrde and Fielding (1962), separated a macerating factor from endo-PG in filtrates from Sclerotinia fructigena and claimed that the macerating factor was an α -L-arabinofuranosidase (Byrde and Fielding, 1965). Later it was identified as a pectin TE (Byrde and Fielding, 1968).

Phytolysin, a macerating enzyme obtained from filtrates of cultures of Dothidea ribesia, has been separated from pectic enzymes (Kern and Naef-Roth, 1971).

The ability of a pathogen to produce pectic enzymes has been correlated with its virulence and the critical role of such enzymes in infection. Albersheim, Jones and English (1969), proposed that interactions between the pathogen and carbohydrate polymers of a host determine the production of wall-degrading enzymes and whether or not infection occurs. Friedman and Ceponis (1959), showed that ultraviolet light induced avirulent mutants of Pseudomonas marginalis did not produce pectolytic enzymes in culture. Friedman (1962), later showed that a wild-type strain of Erwinia carotovora was more virulent and produced more pectolytic enzymes than a UV-mutant strain.

Pectic enzymes are often found in diseased tissue and absent or at very low levels in healthy tissues. A correlation between macerating

activity of tissue extracts with that of particular pectic enzymes in such extracts is evidence that pectic enzymes are involved in pathogenesis. Activity of pectic enzymes is modified in vivo by various factors. The pH of bean tissue infected with Sclerotium rolfsii decreases to pH 4, the optimum for PG of this pathogen (Bateman and Beer, 1965). In alfalfa infected with Colletotrichum trifolii the pH rises to over 7, near the pH optimum of TE of this pathogen (Hancock and Millar, 1965). Friedman and Ceponis (1964), relate decreased virulence of a strain of E. carotovora to acid production in vivo and to a low pH unfavourable for the TE.

Calcium, inhibits the PG of Rhizoctonia solani and with pectic substances in bean cell walls gives insoluble compounds resistant to the PG produced in vivo (Bateman, 1964). In contrast, Hancock and Stanghellini (1968), suggest that the pectate trans-eliminase (PTE) in squash hypocotyls infected by Hypomyces solani f. sp. cucurbitae was enhanced by the localization of calcium in the infected tissue. Spalding (1969), has claimed that monovalent ions can also inhibit maceration by PG from Rhizopus stolonifer possibly by binding with and blocking sites of enzyme action in tissues.

Many other inhibitors of pectic enzymes have been found. Oxidized phenolic compounds inhibit pectic enzymes in vitro and have been linked to disease resistance (Cole, 1958; Deverall and Wood, 1961).

Albersheim and Anderson (1971), showed that proteins from the cell walls of Phaseolus vulgaris hypocotyls, tomato stems and sycamore cells inhibit the PGs of Colletotrichum lindemuthianum, Fusarium oxysporum and Sclerotium rolfsii. The inhibitors resembled phytohaemagglutinins and inhibition was specific for the enzyme of the particular pathogen, which suggested a role in resistance.

A further mechanism regulating pectic enzyme activity is the 'wall modifying enzyme' of a commercial preparation. Pretreatment of cell walls with enzyme was a prerequisite to degradation by other enzymes (Karr and Albersheim, 1970).

Recent work has implicated endo-PGs and endo-TEs in maceration in a number of diseases. The endo-PG of Sclerotium rolfsii causes tissue maceration (Bateman, 1968). Byrde and Fielding (1968), showed that maceration by culture filtrates of Sclerotinia fructigena depends on endo-pectin TE. A PTE from Erwinia aroideae caused maceration of potato tuber tissue (Dean and Wood, 1967), which is also macerated by endo-PG and endo-PTE present in extracts of rots of potato caused by Corticium praticola and Erwinia carotovora var. atroseptica respectively (Hall and Wood, 1970). Similar results have been obtained for the highly purified PTEs from culture filtrates of E. carotovora by Mount, Bateman and Basham (1970) and from E. chrysanthemi by Garibaldi and Bateman (1971).

Dean and Wood (1968), state that the use of potato tuber tissue in maceration tests with enzymes from different pathogens means that the enzymes are non-specific. This suggests that they are not important in specificity which is determined by other factors. However, a degree of substrate specificity is reported. Garibaldi and Bateman (1971), showed that of several PTE isoenzymes that degraded soluble pectic substrates, one did not cause maceration. Also, there were differences in susceptibility of different tissues to maceration by these isoenzymes. Although some readily macerated potato, carrot and cucumber tissues, one had little effect on carrot but readily macerated potato and cucumber. Turner and Bateman (1968), found that a strain of E. carotovora would cause a soft rot of carrot and cucumber but not of onion, but these tissues were equally susceptible to the enzymes from rots of carrot or cucumber. Spalding (1969), found that red beet tissue resists maceration by Rhizopus stolonifer which was found earlier for E. aroideae (Wood, 1955). Whether these differences reflect differences in pectic substances or some other factor is not known.

So far each macerating enzyme on purification has been found to be a chain splitting pectic enzyme.

Wood (1972), suggests 'cell separation' be used instead of maceration for loss of coherence in the early stages in some diseases especially soft rots because maceration is also used for the more drastic changes where cells lose their identity. The term cell separation will thus be used in this thesis.

II. The cell wall

There are many extensive reviews on the structure and composition of plant cell walls, especially those of Lamport (1965, 1971), Northcote (1958, 1972) and Roelofsen (1965).

The cell wall of higher plants is usually considered to have three regions, the middle lamella, the primary cell wall and the secondary cell wall. In parenchyma and similar tissues the secondary cell wall is often only poorly developed (Wood, 1967).

The primary and secondary cell wall consists of a matrix in which are embedded cellulose microfibrils. The matrix is continuous with the middle lamella which does not contain microfibrils. The microfibrils are composed of α -cellulose, consisting of chains of β -1,4 linked glucose residues (Northcote, 1958). These are aggregated to form the microfibril with a width of 100 - 200 Å. Approximately two-thirds of the microfibrils are crystalline, the remainder forming a paracrystalline sheath capable of hydrogen bonding to other microfibrils and to matrix polysaccharides (Roelofsen, 1965). The microfibrils are a relatively small proportion of the primary cell wall volume (c. 10 per cent) and thus in living hydrated cell walls they form a loose meshwork embedded in a matrix of much greater volume.

The polysaccharides of the cell wall are classified on a variety of extraction procedures. The matrix polysaccharides are the pectic substances, soluble in hot water or aqueous solutions of chelating agents and the hemicelluloses which are soluble in alkali. The concentration of the hemicelluloses relative to cellulose increases towards the middle

lamella where they are absent. The pectic substances are localized in the middle lamella and the primary cell wall. The secondary cell wall has more cellulose than the primary cell wall and a smaller proportion of hemicellulose. The pectic substances are essentially absent from the secondary cell wall (Northcote, 1958).

The pectic substances are polysaccharides in which the principle constituent is D-galacturonic acid joined by α -1,4 glycosiduronic linkages to form chains of polygalacturonic acid; high molecular weight forms are pectic acids. The carboxyl groups of pectic acid may be esterified with methanol to give pectinic acids. When the methoxyl content of the pectinic acids is c. 6% or higher these are called pectins. Pectic acids and their salts with polyvalent cations are insoluble. The solubility of pectinic acids and their salts increases with methoxyl content.

Much pectic material in the cell wall is insoluble and the term protopectin has been used to describe the native pectic substances in plant tissues insoluble in cold water (Joslyn, 1962; Wood, 1967). Joslyn (1962), suggests as explanations for the insolubility of protopectin, its large molecular size, ester formation with cellulose or hemicelluloses, linkage with the basic groups of proteins or with lignin or the formation of polyvalent ion bridges between the carboxyl groups of adjacent pectic chains.

The non-uronide of extracted pectic substances ranges from 15 - 50%. Usually, non-uronide sugars that appear to be components of a galacturonan molecule are α -D-galactose, β -L-arabinose and α -L-rhamnose. In addition, D-glucose and α -D-xylose may be present but usually in smaller amounts (McCready and Gee, 1960). The anhydrogalacturonic acid content of even highly degraded pectic acid is rarely over 90%.

The main galacturonan chain of pectic substances is interrupted at intervals by L-rhamnose radicals and at these points the chain is bent

or kinked (Northcote, 1972; Aspinall, 1973). This galacturonorhamnan is the main acidic component of pectic substances extracted from cell walls by water and occurs together with neutral polysaccharides. L-rhamnose occurs only in the main chains; whereas all other neutral sugars are found in the side chains which contain L-fucose, D-xylose, D-galactose and D-glucuronic acid. In more mature cell walls, blocks of large molecular weight, composed of L-arabinose and D-galactose are attached to the main galacturonorhamnan chain. L-arabinose forms a highly branched polysaccharide with α -1,3 and α -1,5 linkages, whereas galactose forms a linear chain of β -1,4 linked galactose units. In addition, highly branched arabinogalactans may form part of the pectic complex (Northcote, 1972).

It is now accepted that various gums e.g. gum arabic, gum tragacanth, closely resemble pectic substances. They have been much used in work on the nature and attachment of neutral sugar residues in side chains to galacturonorhamnans. Side chains of D-xylopyranose residues alone or with D-galactopyranose or L-fucopyranose were originally thought atypical but occur in pectic substances of rapidly enlarging or differentiating tissues (Aspinall, 1973). In the natural state, pectic chains may be packed in microcrystallites, similar to those of pectin and pectate gels. This would give cohesion, strength and water-holding and ion-exchange properties which may be the main functions of the matrix polysaccharides. Modification by branching and insertion decreases molecular cohesion in rapidly enlarging tissues (Rees and Wight, 1969). A similar role for pectic polysaccharides in water relationships and mechanical properties is also suggested by Northcote (1972).

The insoluble pectic substances in cell walls, despite their complexity, appear amorphous. However, the middle lamella of suspension-cultured cells have some structure such as electron-opaque fibres, electron transparent rods, vesicles and myelin-like figures but the latter were possible contaminants from ruptured cells. This complexity may depend on abnormal

conditions of growth but it is possible that the structures also occur in plant but are obscured by the strong compression between cells in a tissue (Leppard and Colvin, 1971).

The hemicelluloses are long chain, branched polysaccharides which on acid hydrolysis give a number of different monosaccharides, including D-galactose, D-xylose, D-mannose, L-arabinose and D-uronic acids and also acetic acid and methanol (Northcote, 1958). Pure glycans are relatively rare in higher plants; most glycans are heteroglycans containing up to five or six types of sugar residues (Whistler and Richards, 1970). Two main types of polymer are recognised, xylans and glucomannans. The D-xylans occur in almost all land plants and plant parts. Arabinogalactans are important constituents of some gymnosperm hemicelluloses. Polysaccharides of both groups contain interior chains differing only slightly in detailed structure but greatly in number, nature and mode of attachment of sugar residues and other substituents (Aspinall, 1973).

Protein as a structural component of cell walls was implied by the work of many workers including Ginzburg (1958). Lamport and Northcote (1960 a) demonstrated that a protein rich in the imino acid hydroxyproline was an integral part of walls of cells of sycamore and bean callus, and that only traces of this acid occurred in cytoplasm. This was also found for tobacco callus (Dougall and Shimbayashi, 1960) and confirmed for sycamore callus (Roberts and Northcote, 1972). This hydroxyproline-rich protein was called extensin by Lamport (1965), who proposed that cross-linking with other wall polymers controls extension growth.

In 1969, Lamport isolated five glycopeptides from cell walls from suspension cultures of tomato. The hydroxyproline was apparently linked through an O-glycosidic bond to arabinose of an oligosaccharide. A hydroxyproline-arabinose-galactose glycoprotein was later isolated from sycamore cell walls (Heath and Northcote, 1971). Cleland and Karlsnes (1967), proposed that in etiolated peas an increase in wall hydroxyproline with age may be a factor controlling the termination of growth.

Ridge and Osborne (1970), have suggested that the reduction of elongation in etiolated pea epicotyls by ethylene is related to the 'premature' increase in wall hydroxyproline and it has been shown that there is more hydroxyproline in walls of mature sycamore cells than in those of actively dividing cells (Roberts and Northcote, 1972).

There are a number of reports on enzymes firmly bound to cell walls. Lamport (1965), remarks on the ubiquity of wall-bound ascorbic acid oxidase, a glucose oxidase is present in the walls of cauliflower floret tissue (Lund and Mapson, 1970), and Ridge and Osborne (1971), found hydroxyproline-rich peroxidase in pea cell walls.

Chang and Bandurski (1964), found a range of hydrolytic enzymes bound to walls of corn root cells, some of which had previously been found in cell walls of corn coleoptiles (Kivilaan, Beaman and Bandurski, 1961). Many of these enzymes have been associated with the dynamic recycling functions of intracellular lysosomes (Matile, 1969). They can account for much of the total activity of the cell. Thus, at least 80% of acid phosphatase activity of suspension-cultured sycamore cells was found in wall preparations (Lamport and Northcote, 1960b).

Albersheim and Anderson (1971), isolated glycoproteins similar to phytohaemagglutinins from cell walls of Phaseolus vulgaris hypocotyls, tomato stems and suspension-cultured sycamore cells.

By use of purified hydrolytic enzymes and methylation analysis, a detailed study has been made of the pectic and hemicellulose polysaccharides of suspension-cultured sycamore cells (Talmadge, Keegstra, Bauer and Albersheim, 1973; Bauer, Talmadge, Keegstra and Albersheim, 1973). A model of the primary wall was proposed in which macromolecules are covalently cross-linked (Keegstra, Talmadge, Bauer and Albersheim, 1973). This contrasts with the suggestion that the cell walls of plants are held together by non-covalent forces (Rees and Wight, 1969). This model gives the first clear picture of the molecular structure of a cell wall. In it, the pectic rhamnogalacturonans are covalently attached by galactan side chains to the xyloglucan which in turn is bound

tightly to the cellulose by hydrogen bonds. Such connections between cellulose fibrils and different pectic polymers would cross-link the cellulose fibrils. It is also suggested that arabinogalactan side chains of the pectic rhamnogalacturonan are attached to seryl residues of wall protein which has arabinosyl tetrasaccharides glycosidically attached to hydroxyproline residues. Keegstra et al. (1973), consider that the primary cell wall of sycamore cells is a macromolecule with components interconnected by covalent bonds except for xyloglucan and cellulose. The macromolecules accounted for all structural components of primary cell wall which contained 34% pectin, 38% hemicellulose and 26% cellulose values which corresponded well with those previously obtained for sycamore viz. 36%, 34% and 27% respectively (Lamport, 1965), and the observation by Roelofsen (1959), that primary cell walls are typically one-third cellulose, one-third hemicellulose and one-third pectin plus protein.

In contrast to secondary cell walls, primary walls of a variety of plants have many features in common.

III. Permeability changes in disease

Altered permeability occurs with many plant diseases and may be characteristic of diseased plant tissue. Thatcher (1939, 1942, 1943), studied it in rusts, powdery mildews, soft rots, dry rots and wilts. He concluded that altered permeability of cells is a feature of disease caused by fungi. There is similar evidence for bacteria (Friedman and Jaffe, 1960; Cook and Stall, 1968), viruses (Ghabrial and Pirone, 1967), toxins (Wheeler and Black, 1963; Gardner, Mansour and Scheffer, 1972) and enzymes (Mount, Bateman and Basham, 1970; Hall and Wood, 1970).

Thatcher further proposed that an increase in permeability is characteristic of susceptibility which led to the hypothesis that this would release nutrients for the pathogen resulting in susceptibility whereas decreased permeability would restrict the nutrient supply resulting in resistance (Thatcher, 1943). Also, that the failure of cells from the

borders of necrotic flecks to deplasmolyse in hypertonic solution was due to a decreased permeability of the cell membranes or cytoplasm. It has been suggested that this failure to deplasmolyse might be due to a change in the cell wall permeability (Wheeler and Hanchey, 1968). Although primary cell walls are considered to be freely permeable to small molecules, they can be rendered impermeable even to water (Miller, 1938). Bennet-Clark (1959), found that cells adjacent to necrotic tissue when placed in hypertonic solution do not plasmolyse normally. The protoplast shrinks away from the wall only on the side away from the lesion and this was attributed to the impermeability of the walls adjacent to the lesion.

The plasmalemma is the chief permeation barrier in cells.

Samaddar and Scheffer (1968), considered that the rapid loss of electrolytes from tissues treated with the toxin from Helminthosporium victoriae and loss of ability to plasmolyse depended on the effect of the toxin on the plasmalemma. This toxin, victorin, causes leakage of electrolytes especially potassium from susceptible but not resistant oat tissues (Wheeler and Black, 1963). Loss of carbohydrate, inorganic phosphorus, nitrogenous compounds and ^{14}C -labelled metabolites also occurs (Black and Wheeler, 1966; Samaddar and Scheffer, 1971). Tissues of susceptible sorghum exposed to the toxin of Periconia circinata behaved similarly, much of this efflux was potassium, the most available cation in the cell. This suggested that efflux was non-specific (Gardner, Mansour and Scheffer, 1972).

In susceptible oat tissues treated with victorin there is an early change in the unit structure of the cell membrane; severe damage caused membrane disruption (Hanchey, Wheeler and Luke, 1968). This suggested that the early changes might explain increases in permeability. However, resistant tissues treated with victorin also showed marked increases in permeability but with no changes in membrane ultrastructure. This indicates that changes in permeability can occur with no detectable change in plasmalemma ultrastructure. It seemed that changes with susceptible tissue represented irreversible damage (Wheeler, 1971).

Change in permeability may be the initial response of plants to a variety of pathogens (Wheeler and Luke, 1963). They accompany water congestion, an early symptom of cucumber angular leaf spot (Williams and Keen, 1967). Changes in permeability also appear before visible symptoms (Cook and Stall, 1968), or in advance of the pathogen (Thatcher, 1939). Friedman and Jaffe (1960), found an increase in conductance of witloof chicory tissue treated with living cells or filtrates of cultures of Erwinia sp. and Pseudomonas sp. Conductance increased in advance of the margin of visible lesions. Increase in conductance was attributed to increased permeability, but it was also suggested that calcium and magnesium released from pectates by pectolytic enzymes contributed to the increase in conductance. A non-pectolytic mutant of Pseudomonas marginalis caused no soft rotting or conductance changes. Increases in membrane permeability were detectable before visible symptoms in mung bean hypocotyls infected with Rhizoctonia solani. Droplets from lesions induced leakage of electrolytes and amino acids when infiltrated into healthy hypocotyls, this ability was associated with an endo-PG (Lai, Weinhold and Hancock, 1968).

Hall and Wood (1970), established that the cell separating activity of rots caused by Erwinia carotovora var. atroseptica depended on a PTE and in rots caused by Corticium praticola on a PG. These extracts also had a striking effect on permeability of protoplasts in advance of cell separation. Disks in an extract causing cell separation in 60 min caused readily detectable leakage of electrolytes in 10 min. Increase in permeability was closely related to activity of the PTE or PG, strongly suggesting that these enzymes caused permeability changes.

A later publication reported that potato tuber disks killed by immersion in 30% ethanol and then leached in water did not increase conductivity of the ambient solution. When these disks were treated with C. praticola rot extracts containing high PG activity there was no efflux of electrolytes from the disks indicating that only a small proportion

of the electrolytes released when living cells are exposed to active rot extracts was due to ion release from cell walls (Hall and Wood, 1974).

Extracts containing cell-separating enzymes also cause cells to lose water rapidly; potato disks lose 25% fresh weight in c. 40 min, this being the time taken for cell separation (Webb, 1971).

Conclusive evidence for permeability increasing activity of pectic enzymes was provided when it was demonstrated that a highly purified endo-PTE from filtrates of cultures of Erwinia carotovora caused leakage of electrolytes from potato tissue disks, as measured by increased conductance of the bathing solution, and loss of accumulated ⁸⁶Rb. This loss of electrolytes preceded maceration (Mount, Bateman and Basham, 1970). Similar results were obtained by treatment of potato tuber tissue with a highly purified endo-PTE from Erwinia chrysanthemi (Garibaldi and Bateman, 1971). This and other recent work will be discussed more fully later in the thesis.

IV. The nature of the toxic factor in cell separation

Some of the earliest work on tissue degradation recognised that cells were killed, but no conclusion was reached on the nature of the toxic substance (de Bary, 1886). Brown (1915), recognised the close relationship between cell separation and death and tried to separate them by variously treating Botrytis cinerea extracts. Cell separating activity was decreased by heat treatment, mechanical shaking or adjustment of pH. Change in activity on cell walls paralleled decrease in death as measured by loss of ability to plasmolyse. Extracts with no cell separating activity did not kill cells. Brown suggested that both actions were due to the same substance or group of substances or, that the toxic factor did not reach the protoplast until permeability of the cell wall had been increased by the cell separating factor.

Brown found the first alternative more likely because if cell separation was stopped even at a late stage by alteration of the pH, then

the toxic action was strongly retarded.

The first separation of these two activities was achieved by Tribe (1955), working with filtrates from cultures of B. cinerea and Bacterium (=Erwinia) aroideae. Plasmolysing concentrations of salts and of non-electrolytes retarded killing by culture filtrates out of all proportion to effects on cell separation. This was interpreted as increased resistance of protoplasts to penetration by the toxin and death of protoplasts was explained by direct degradation of pectic substances only accessible in the unplasmolysed protoplasts. Isolated protoplasts were killed by thermostable and thermolabile toxins in culture filtrates. The former were probably metabolites, the latter enzymes. Protoplasts plasmolysed in situ were even more resistant to the toxins than isolated protoplasts.

Fushtey (1957), showed that cells recovered from plasmolysis regained their original sensitivity to the toxins and that toxicity of oxalic acid and mercuric chloride was retarded by plasmolysis. Partial cell separation gave increased sensitivity to oxalic acid, mercuric chloride or enzymes of Botrytis cinerea. This refuted the hypothesis that the toxin did not reach protoplasts until cell separation was complete and demonstrated the parallel effect of these two activities in hypotonic solution.

Heat treatment of Verticillium dahliae culture filtrates at 60 - 100°C for 5 min or longer decreased toxicity more than cell separating activity. It was considered that separate factors were in part responsible for the two activities (Kamal and Wood, 1956). It has, however, been suggested that this separation can be explained on the basis of different kinetics for two processes caused by a common factor (Mount et al., 1970).

Toxicity has been estimated by plasmolytic methods based on retention of a vital dye or by efflux of electrolytes, both methods depending on the semipermeable nature of protoplast membranes. Decreases in cellular respiration have also been used (Spalding, 1969), but these are

probably less sensitive.

The plasmalemma is usually considered to be the chief permeation barrier in cells. Although the unit-membrane concept has been criticized, particularly by Korn (1966), it is accepted that the structural components of membranes are proteins and phospholipids. As pointed out by Brown (1965), it might be expected, therefore, that the enzymic toxin is proteolytic or lipolytic.

Proteinases are often found in diseased tissue (see section I for references). Tseng and Bateman (1968), showed that phosphatidase is produced by several phytopathogens including Sclerotium rolfsii, E. carotovora and B. cinerea. They suggested that phosphatidases contribute to changes in membrane permeability. Lumsden and Bateman (1968), found phosphatidase B in cultures of Thielaviopsis basicola and infected plant tissue and phosphatidase C occurs in cultures of E. carotovora (Mount and Bateman, 1969). Phosphatidases, however, play no part in permeability changes and cell death caused by extracts of potato rots caused by E. carotovora var. atroseptica and Corticium praticola (Hall and Wood, 1973). Similar results for phosphatidase and proteinase were obtained for E. carotovora by Mount et al. (1970) and for E. chrysanthemi by Garibaldi and Bateman (1971).

Ruesink and Thimann (1965), tested a range of enzymes on isolated oat coleoptile protoplasts. Proteinases and phosphatidases were relatively ineffective in disrupting protoplasts, but disruption was caused by ribonuclease indicating RNA as a possible important structural component of the plasmalemma. This enzyme has a high isoelectric point (9.6) and it was also suggested that its basic properties may disrupt anionic sites on the membrane critical to membrane integrity. Later work (Ruesink, 1971) showed that an enzymically inactive ribonuclease also disrupted protoplasts thus refuting the involvement of RNA in membrane structure. It was suggested that the action of RNA, as a basic molecule, may resemble that of some polyene antibiotics e.g. filipin, which disrupt sterol-containing

membranes (Mudd and Kleinschmidt, 1970). However, Garibaldi and Bateman (1971) pointed out that trypsin with an isoelectric point of 10.5 did not cause lysis of oat protoplasts, whereas the PG of S. rolfsii (pI 5.2) readily macerates and kills plant tissue.

Brown (1965), discussed the possibility that polysaccharide degrading enzymes act on a polysaccharide framework within the plasmalemma. Codner (1972), also suggests that cell separating enzymes act on substrates in cell membranes. In this hypothesis vesicles containing polyuronide and polysaccharide cell wall precursors are passed through the plasmalemma. While embedded in the cell membrane these substances may be degraded by pectic enzymes. The membrane would then be weakened leading to death of the protoplast. There is little evidence, however, to suggest that pectic substances are constituents of cell membranes or cytoplasm. Certain cytoplasmic particles of onion cells contain substances that stained with alkaline ferric hydroxylamine, a reagent developed for the detection of esterified pectic substances (Albersheim and Killias, 1963) and Branton (1969), has demonstrated, by freeze-etching, particles in the matrix of membranes which he interpreted as interpenetrations of protein or polysaccharide.

Wood (1967), suggested several possibilities concerning the relation between killing and cell separation.

- (a) the protoplasts are killed by toxins which are not the cell separating enzymes and which act independently. This is unlikely as the toxins and enzymes would need to have remarkably similar properties.
- (b) That toxins are present which cannot act until cells have been conditioned by the action of wall degrading enzymes.
- (c) That cell separation is associated with proteolytic enzymes

which themselves cause death of protoplasts. Phosphatidases could also be included in this category. Spalding (1969), has shown that a PG from Rhizopus stolonifer free of phosphatidase and with negligible proteinase activity, was able to cause cell separation and death of sweet potato tissue.

- (d) That the cell separating enzymes themselves cause the death of protoplasts. Recent work has demonstrated that purified endo-pectic enzymes cause cell separation and death of protoplasts as estimated by leakage of electrolytes and loss of ability to plasmolyse and retain neutral red (Mount et al., 1970; Garibaldi and Bateman, 1971; Hall and Wood, 1970; 1973; 1974).

It is now accepted that plant cells contain vesicles with hydrolytic enzymes that are the counterparts of the lysosomes of animal cells. Matile (1969), suggests that the central vacuole of plant cells is in fact one large lysosome. Pitt and Coombes (1968, 1969), have shown that infection of potato tissue by several fungal pathogens caused swelling and disruption of host cytoplasmic particles that contained a variety of enzymes including acid phosphatase and proteinase. Permeability alterations are often associated with disease and as Wilson (1973) points out, disruption of the host lysosomal system may release hydrolytic enzymes causing autolysis of the cell. High levels of hydrolytic enzymes have been found associated with cell walls (see section II for references). The action of cell wall degrading enzymes might release these enzymes which would then cause disruption of the protoplasts.

The action of pectin methylesterase on cell walls leads to the liberation of methanol which would be toxic to the protoplasts.

Mussell(1973), has demonstrated that a purified endo-PG from Verticillium albo-atrum was toxic to cotton leaves. He proposed that

toxicity was due to generation of hydrogen peroxide by oxidase enzymes and substrates released from walls by the pectic enzyme. Symptoms similar to those caused by endo-PG were observed after treatment of leaves with several substrate-oxidase combinations which generated hydrogen peroxide. The toxic action of PG could be blocked by pretreatment of leaf tissue with catalase.

Fushtey (1957), however, was unable to demonstrate any increase in toxicity of culture filtrates from B. cinerea or Bacterium (= Erwinia) aroideae after digestion of a large mass of turnip tissue. He interpreted this as indicating that no toxin was accumulating as a result of the action of pectic enzymes on the turnip cell walls.

Wood (1967, 1972), has suggested that once certain physical barriers in the cell wall have been removed by cell separating enzymes, the external membranes of the protoplast are ruptured by the rapid expansion of the protoplasts through absorption of water from the ambient solution. This is supported by the observation that death of protoplasts is delayed in hypertonic solution (Tribe, 1955). A suitable osmoticum is also a prerequisite for isolation of intact protoplasts by pectic enzymes (Cocking, 1972) and that once isolated the protoplasts will remain alive for some days and even regenerate a cell wall (Pojnar, Willison and Cocking, 1967). This suggests that under these conditions they are unaffected by the pectic enzymes.

Hall and Wood (1970, 1974), consider that possible points of weakness in the cell wall for rupture of the plasmalemma might be plasmodesmata. The structure of plasmodesmata is not clearly established but they are believed to be tubes, lined with plasmalemma continuous with that of the parent protoplasts. Numbers of these traversing cell walls has been placed as high as 6,000 - 24,000 by some workers (Krull, 1960; Strugger, 1957). It is envisaged that as the middle lamella is degraded by pectic enzymes the plasmalemma at plasmodesmata becomes exposed to the hypotonic ambient solution and ruptures under turgor pressure.

Mount et al. (1970) and Garibaldi and Bateman (1971), however, consider that their results indicate a direct toxic action by pectic enzymes due to their action on substrates within cell membrane or within the protoplast.

In conclusion, it is now recognised that pectic enzymes of the endo-type are able to cause cell separation, leakage of electrolytes and death of protoplasts. Work with purified preparations has shown that these effects can occur in the absence of other enzymes. Other cell wall degrading enzymes and phosphatidase and proteinase may play an important part in disease but at least in soft rots their role is apparently secondary.

Many workers have noted the parallel nature of cell separation and death first recognised by Brown in 1915. The only separation of these two activities was achieved by Tribe (1955).

Much is still unknown as to how the pectic enzymes are able to cause death of protoplasts. There are two main hypotheses, that of direct action against the protoplast or that of direct action on the cell wall which in some way indirectly causes protoplast death.

MATERIALS AND METHODS

1. Culture of pathogen

The organism used in this work was Erwinia carotovora strain 1747, isolated from onion in Japan by M. Goto. It was obtained from the National Collection of Plant Pathogenic Bacteria and maintained on slopes of nutrient agar prepared by dissolving 13g 'Oxoid' nutrient broth No. 2 (Oxoid Ltd., London) in 1l of water, adding 15g of agar and sterilizing by autoclaving for 15 min at 121°C. Slopes were prepared in McCartney bottles and inoculated by streaking a loopful of the bacteria over the surface of the agar. Slopes were subcultured about every 3 weeks and stored at 4°C.

Master cultures on nutrient agar slopes were stored at -20°C.

Suspension of the bacteria were also frozen in an acetone/dry ice mixture and then dried under a vacuum. The freeze-dried cells were stored in a sealed ampoule with a piece of self-indicating silica gel. Only those ampoules in which the silica gel remained unchanged in colour were retained.

2. Living substrates

Three types were used in enzyme assays; tissue, single cells and isolated protoplasts.

A. Tissue

Three types were used. Potato tubers (Solanum tuberosum cv. Majestic) were grown at Imperial College Field Station, Silwood Park, Ascot, Berks. and stored in the dark at 10°C. Carrot root (Daucus carota) and cucumber fruit (Cucumis sativus) were bought locally when required.

Disks were prepared from turgid potato tubers (soaked overnight in tap water) by cutting 0.5 mm thick slices from the medulla with a hand microtome. 7 mm disks were cut from these slices with a No. 4 cork borer and the disks were well washed in water before use. Disks were prepared in the same way from medulla of carrot root and mesocarp of cucumber fruit.

B. Single cells

A culture of carrot root callus was obtained from the Botany Department, University of Leicester. The culture was maintained at 25°C on modified White's medium (White, 1954) and subcultured every 4 - 6 weeks. The medium was prepared as follows. An inorganic stock solution contained the following in 800 ml. water :

$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	2.877 g
Na_2SO_4	2.00 g
KCl	0.80 g
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.2145 g
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	66.0 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	26.7 mg
H_3BO_3	15.0 mg
KI	7.5 mg

This was mixed slowly with 200 ml water containing 7.365g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and stored in the dark at 4°C. Vitamin stock solution contained the following in 100 ml water :

Glycine	300 mg
Nicotinic acid	50 mg
Thiamine	10 mg
Pyridoxine	10 mg

This was stored at 4°C. For 1l of medium, 20g of sucrose was dissolved in 500 ml water and to this was added 25 ml of a solution of $\text{Fe}_2(\text{SO}_4)_3 \cdot 7\text{H}_2\text{O}$ (18.3 mg/100 ml); 200 ml of inorganic stock solution, 20 ml of vitamin stock solution and 10 ml of a solution of 2,4-dichlorophenoxyacetic acid (2,4-D, 10 mg/100 ml) were added and the volume made to 1l with water. 50 ml aliquots of this medium were pipetted into 150 ml conical flasks containing 0.4g agar. The flasks were closed with a cotton wool bung, lightly covered with aluminium foil and sterilized by autoclaving for 10 min at 126°C.

The media was inoculated by placing a small piece (c. 5mm diam) of callus on the surface with a sterile spatula.

Liquid media was prepared as above but without agar and with 2,4-D at 0.1 mg/l. This was inoculated as before and incubated at 25°C on a 'Gallenkamp' orbital incubator at 150 r.p.m.

After 4 weeks' incubation suspension cultures of carrot callus mainly as single cells or as clumps of a few cells were obtained. The flask was removed from the incubator and the large clumps of cells allowed to settle. Some of the suspension from the top of the culture was decanted to a centrifuge tube and the cells sedimented by centrifugation at 1700 x g for 10 min. The cells were washed twice in water by centrifuging and the pellet was finally suspended in a small volume of water. This dense suspension of mainly single cells was used for enzyme assays.

C. Isolated protoplasts

Cylinders of tissue were cut from the mesocarp of cucumber fruit with a No. 4 cork borer. The cylinders were plasmolysed in 0.5M KNO_3 solution. After 45 min the cylinders were sectioned with a razor and the sections together with the contents of the ruptured cells transferred to a watchglass containing 0.5M KNO_3 solution. A sample of the bodies obtained were transferred to M KNO_3 solution and examined under the microscope. Intact protoplasts were recognised by an even covering of cytoplasm in which streaming was often observed. Tonoplasts were recognised by their hyaline appearance and absence of cytoplasmic covering or as hyaline spheres with lumps of disorganised cytoplasm adhering usually at one point. It was found that about 90% of bodies isolated in this way were tonoplasts.

In a second, better method, placenta of cucumber fruit was plasmolysed and sectioned in 0.5M KNO_3 solution. About 70% of the bodies released by this method were intact protoplasts.

Dense suspensions of these tonoplasts and protoplasts were used for enzyme assays.

3. Chemical reagents and substrates

All reagents were analytical grade and obtained from British Drug Houses Ltd., Poole, Dorset or Hopkin and Williams Ltd., Chadwell Heath, Essex.

Sources of substrates used in enzyme assays are indicated in the text. Davis standard agar was used throughout this work (Davis Gelatine Ltd., Leamington Spa).

Glass-distilled water was used wherever 'water' is referred to in the text.

Neutral red stain was obtained from Solmedia Ltd., London, Toluidine blue from Gurr Ltd., London and Coomassie brilliant blue from Bio-Rad Laboratories, Richmond, California, U.S.A.

4. Pectic substrates

Sodium polypectate (NaPP) and Pectin N.F. (P) from citrus (Sunkist Growers Inc., Ontario, California, U.S.A.) were washed before use in enzyme assays; 25g was added to 150 ml 70% ethanol containing 0.05N HCl and mixed for 1h on a magnetic stirrer. The substrate was collected on Whatman No. 41 filter paper and washed several times with 70% ethanol until no traces of chloride in the washings were detected with AgNO_3 solution. The residue was then washed once more with 95% ethanol, placed on aluminium foil and dried in an oven at 70°C to constant weight.

To prepare solutions of pectic substrates for enzyme assays, substrates were added slowly to water stirred vigorously on a magnetic stirrer. Solutions were made to twice the final concentration required and pH adjusted to an appropriate value. This solution was then mixed with an equal volume of buffer. Solutions were freshly prepared each day.

5. Buffers

These were prepared as described by Dawson, Elliott, Elliott and Jones (1959). Those used most frequently in extraction and assay of enzymes were :

Citric acid - sodium citrate (0.1M), pH 3.0 - 6.2

Sodium acetate - acetic acid (0.2M), pH 3.6 - 5.8

Na_2HPO_4 - NaH_2PO_4 (0.1M), pH 5.8 - 8.0

Tris - (hydroxymethyl)-aminomethane - HCl (0.05M), pH 7.2 - 9.1

Glycine - NaOH (0.05M), pH 8.6 - 10.6

6. Sterilization

Media were sterilized as indicated in the text. Pipettes were sterilized in metal canisters in an autoclave for 15 min at 121°C.

7. pH measurements

The pH of large volumes of liquid was measured with a 'Pye 290' pH meter (Pye Unicam, Cambridge). For smaller volumes BDH Narrow Range indicator papers were used.

8. Spectrophotometry

A 'Beckman' model D.B. spectrophotometer with a 'Beckman' potentiometric recorder was used for all absorption measurements in conjunction with fused silica cuvettes of 1 cm light path.

9. Preparation of cell-free cucumber rot extracts

A. Inoculation of tissue

Two day old cultures of E. carotovora on plates of nutrient agar were used for inocula. Bacteria were suspended in sterile 0.85% NaCl and the concentration adjusted to $\underline{c. 10^{10}}$ cells/ml by means of direct counting using a 'Thoma' counting slide and phase-contrast microscopy.

The surface of each cucumber fruit was washed and then swabbed in ethanol and allowed to air-dry. The fruits were then cut into 2 - 3 cm thick slices.

Plastic boxes were swabbed with ethanol and lined with tissue paper soaked with sterile distilled water. Glass supports, washed with ethanol, were placed on the wet tissue paper and the cucumber slices placed on top of these.

0.1 ml bacterial suspension was spread over the cut surface of each tissue slice. Boxes were closed and incubated at 25°C for 3d.

B. Extraction of rotted tissue

After three days rotted tissue was removed from slices, mixed with an equal volume of 0.85% NaCl and homogenized in a 'Kenwood' blender for 60 sec at full speed. The homogenate was strained through two layers of muslin and the filtrate made cell-free by centrifuging at 12000 x g for 20 min at 4°C. The supernatant was centrifuged for a further 20 min, then dialysed against water (10 ml rot/500 ml water) for 24h at 4°C using Visking tubing (H.M.C., England). The dialysed extract was bulked then divided into aliquots and stored at -20°C.

Rotted cucumber tissue (50 ml aliquots) was also placed in polythene bags and stored at -20°C for later use.

10. Measurement of enzyme activities

A. Pectic enzymes

i) Pectin methyl esterase (PME)

A cup-plate assay method was used (Mann, 1962); 2.5g of pectin was added to 245 ml water and the pH adjusted to 6.0. Methyl red solution (5.0 ml), salicylanilide (0.025g) and agar (5.0g) were added and the mixture sterilized by autoclaving for 15 min at 121°C. 25 ml aliquots were pipetted into 9.0 cm diam plastic petri-dishes and when set, 9 mm diam wells were cut in the agar with a No. 5 cork borer. 0.1 ml of enzyme sample was pipetted into each well and the plates incubated at 25°C for 18h. Acid liberated from pectin by the action of PME is detected as a red zone around the well. Activity is expressed as the area of the red zone.

ii) Chain-splitting enzymes

a) Viscosity reducing activity

'Cannon-Fenske' size 200 viscometers were suspended in a water bath at 25°C. Reaction mixtures contained :

8.0 ml 1% w/v pectic substrate in 0.1M Tris-HCl buffer pH 9.0

1.0 ml 0.01M CaCl_2 (0.001M final) or 1 ml H_2O

1.0 ml enzyme

Assays for polygalacturonase (PG) used pectic substrate in 0.1M sodium citrate buffer pH 5.0 with no CaCl_2 . For trans-eliminase (TE) assays CaCl_2 was mixed with enzyme before adding to the substrate. This prevented gelling of the substrate.

On addition of enzyme, the reaction mixture was mixed by blowing through the viscometer and then viscosity was measured at intervals. Enzyme activity is expressed as $100/t$ where t is the time (min) for 50% reduction in viscosity. All viscometers were calibrated against water, the flow time for 10 ml water representing 100% reduction in viscosity of solutions of pectic substrates.

b) Release of reducing groups

This method was used to estimate pectate trans-eliminase activity (PTE). Reaction mixtures were as described for viscometry and were incubated at 25°C. 0.2 ml samples were removed at intervals and reducing groups estimated by the Nelson-Somogyi method (Nelson, 1944).
Reagents used were as follows :

Copper reagent A

25g Na_2CO_3 (anhydrous)

25g potassium sodium tartrate

20g NaHCO_3

200g Na_2SO_4 (anhydrous)

dissolved in c. 800 ml water, then diluted to 1l and filtered and stored at room temperature.

Copper reagent B

15g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, one or two drops conc. H_2SO_4 in 100 ml water.

Arsenomolybdate reagent

Ammonium molybdate (25g) was dissolved in 450 ml water and 21 ml conc. H_2SO_4 added; 3g of $\text{Na}_2\text{AsO}_4 \cdot 7\text{H}_2\text{O}$ in 25 ml water was added and the solution incubated at 37°C for 48h. The reagent was stored in a brown glass bottle at room temperature.

Copper reagents were mixed on day of use, 25 parts A to 1 part B. The test sample (0.2 ml) was added to 0.8 ml water and 1.0 ml of copper reagent and heated for 30 min on a boiling water bath. After cooling 1 ml of arsenomolybdate reagent was added and thoroughly mixed on a 'Whirlimixer' until CO_2 evolution had ceased. The blue colour produced was estimated at 660 nm against a reagent blank.

A calibration curve was obtained with a series of glucose solutions containing between 5 and 50 $\mu\text{g}/\text{ml}$ and enzyme activity expressed as μg reducing groups (glucose equivalents) released.

c) Thiobarbituric acid test

The reaction mixtures were as described for viscometry with NaPP as substrate. They were incubated at 25°C . Samples (1.0 ml) were removed at intervals and added to 5.0 ml

thiobarbituric acid (0.01M) and 2.5 ml HCl (0.5N) in a test tube and mixed well. The tube was covered with a cap and placed on a boiling water bath for 30 min. After cooling, the red colour produced was read at 515 nm (PG) or 550 nm (TE), against samples prepared in the same way but with autoclaved enzyme.

In later tests this method was modified and unless otherwise stated used for all PTE estimations. Reaction mixtures contained :

1 ml enzyme sample

0.5 ml 0.008M CaCl_2 (0.001M final)

2.5 ml 1% w/v NaPP in 0.1M Tris - HCl buffer pH 9.0

This was incubated in a water bath at 25°C for 1h. 7.5 ml of a mixture of 5.0 ml thiobarbituric acid (0.04M) and 2.5 ml HCl (1N) were added and mixed. The procedure was then continued as above and the red colour produced read against a reagent blank prepared in the same way. One unit of PTE activity caused an increase in absorbance at 550 nm of 0.1 in 30 min.

d) Cup-plate assay

This method was used only for estimations of PTE activity. NaPP (2.5g) was dissolved in 125 ml water and the pH adjusted to 9.0. An equal volume of 0.2M Tris - HCl buffer (pH 9.0) was added to give a solution of 1% NaPP in 0.1M Tris-HCl buffer at pH 9.0. 2.5g agar(1% final concentration) was then added and the mixture autoclaved for 15 min at 121°C; 25 ml aliquots were pipetted into 9.0 cm plastic petri-dishes and when set, 7 mm diam wells were cut in the agar with a No. 4 cork borer. 0.02 ml CaCl_2 (0.002M) and 0.02 ml of enzyme sample was pipetted into each well. Plates were

incubated for 18h at 25°C, then 5N HCl was poured over the surface of each plate. Enzyme activity was revealed by the white halos that developed around the well after addition of the acid. Enzyme activity is expressed as the area of this zone around the well.

B. Toxicity

The plasmolytic method described by Tribe (1955) was used for tests with tissue. Disks prepared as described previously were well washed before use in water. When plasmolysed disks were to be used, disks were immersed in the plasmolyticum for 45 min prior to use. Reaction mixtures for the early experiments varied and details are given with individual experiments. For later experiments unless otherwise stated reaction mixtures were 3 ml total volume and contained; 0.001M CaCl_2 , 0.02M Tris - HCl buffer at pH 9.0, water or plasmolysing agent, 1.0 ml of a solution containing 0.4 ml untreated or autoclaved cucumber rot extract, or 1.0 ml solution untreated or autoclaved PTE ($\cong$ 0.4 ml cucumber rot extract) with activity 14.8 units/3 ml reaction mixture and 48 tissue disks.

At intervals 4 disks were removed, washed in water or plasmolyticum and placed in 0.85M KNO_3 containing 0.01% w/v neutral red and 0.005M sodium phosphate buffer pH 7.5. After 20 min disks were transferred to 0.9M KNO_3 in 0.01M sodium phosphate buffer pH 7.5. Live cells plasmolysed and retained neutral red which was rapidly leached from dead cells on washing. After a further 20 min disks were examined and graded on a scale 0 - 5 where 0 means that all cells are alive and 5 that all are dead. This scale is the neutral red index (NRI).

Death of isolated protoplasts was estimated by direct counting under low power of the microscope. Three Perspex rings (20 mm x 11 mm) were stuck to a microscope slide with 'Araldite'. Reaction mixtures were 1 ml total volume and contained, 0.9, glycerol, 0.001M CaCl_2 , 0.02M Tris - HCl buffer pH 9.0 and 0.3 ml crude cucumber rot extract or equivalent

concentrations of PTE, proteinase and phosphatidase. Glycerol replaced KNO_3 as plasmolyticum because it was found that the controls gave better survival. In another Perspex ring, autoclaved enzyme samples were used and in the third, water replaced the enzyme samples.

Into each reaction mixture was pipetted a small volume of the protoplast suspension and when counts were not being made, cover slips were placed over the reaction mixtures to prevent evaporation. Although both tonoplasts and protoplasts were present in reaction mixtures it was impractical to distinguish between these during counts, estimates being made at intervals of surviving 'protoplasts'.

Death of single carrot callus cells was estimated using similar slides and reaction mixtures. A small volume of the carrot callus cell suspension prepared as described previously was added to reaction mixtures. Death was estimated in one of two ways; small samples of the reaction mixture containing the carrot cells were removed at intervals and placed in cavity slides. An equal volume of neutral red solution (as used for tissue disks) was added and after 20 min the proportion of a sample of single cells (usually 100) that were plasmolysed, as visible by uptake of neutral red, were counted under the microscope. An alternative method was to remove the reaction mixture and replace it with the neutral red solution. After 20 min the solution was replaced by the wash solution and after a further 20 min plasmolysed single cells in a sample were counted. The first method allowed toxicity to be estimated with time, a single treatment time only being possible with the second procedure.

C. Cell separation

This was estimated with tissue disks by the needle test (Brown, 1915). Disks used for toxicity measurements were pulled apart with mounted needles and cell separation estimated using a scale 0 - 5 where 0 represents fully coherent tissue and 5 complete lack of coherence. It was not possible to grade cucumber tissue in this way with the same accuracy as carrot or potato, and so time for cell separation only was recorded.

D. Permeability increasing activity

Permeability changes in cells of potato disks were assessed by measuring increase in conductivity of bathing solutions with a 'Chandos' conductivity meter (Hall, 1971). Reaction mixtures were generally similar to those used for toxicity/cell separation estimations; differences are given with individual experiments. As reaction mixtures are highly conducting solutions, relatively small changes given by the disks would be masked if measurements were made directly. Thus, 5 disks were removed at intervals, washed in water or plasmolyticum, transferred to 15 ml water or plasmolyticum, shaken for 10 sec and the conductivity of the bathing solution taken; 15 min later disks were again shaken for 10 sec and the conductivity measured. Results are expressed as the increase in conductivity (μmhos) during the 15 min.

E. Proteinase

Two methods were used; a cup-plate assay and the congocoll method of Mount et al. (1970).

i) Cup-plate assay

3.0g gelatin (Gurr Ltd., London) and 6.0g agar were added to 300 ml 0.05M Tris - HCl buffer at pH 8.0. The mixture was autoclaved for 15 min at 121°C and 25 ml aliquots were pipetted into 9.0 cm diam plastic petri-dishes. When set, 10 mm diameter wells were cut in the agar with a No. 6 cork borer; 0.2 ml of enzyme sample was pipetted into each well and the plates incubated for 3h at 30°C . Activity was expressed as the area of the clear zone around the well.

ii) Congocoll method

5 mg finely-ground congocoll (Calbiochem, Los Angeles, California, U.S.A.) was added to 0.9 ml 0.05M Tris - HCl buffer at pH 8.0. 0.1 ml enzyme sample was added and the mixture

incubated in a water bath at 37°C. After 1h, 4 ml of water was added and the solid debris removed by filtration through Whatman No. 1 filter paper. The liberated congo red was then estimated at 495 nm against a water blank prepared in the same way. Activity is expressed as the increase in absorbance (OD units) at 495 nm.

F. Phosphatidase

A cup-plate assay method was used (Doery, Magnusson, Gulasekharan and Pearson, 1965). 1.5g soybean lecithin (Sigma Chemical Co. Ltd., St. Louis, Missouri, U.S.A.) was added to 135 ml water and homogenized in a 'Sorvall Omnimixer' for 1 min at full speed; 15 ml of a solution of thiomersal (0.2% w/v) was added.

Agar (6.0g) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1125g) were added to 150 ml 0.1M Tris - HCl buffer at pH 9.0 and the mixture autoclaved for 15 min at 121°C. The lecithin emulsion was added to molten agar and mixed; 25 ml aliquots were pipetted into 9.0 cm diam plastic petri-dishes and when set, 10 mm diameter wells cut in the agar with a No. 6 cork borer. 0.2 ml enzyme sample was pipetted into the wells and the plates incubated for 48h at 30°C. Enzyme activity caused a white zone around the well and activity was expressed as the area of this zone.

G. Cellulase (CMCase)

This was estimated by the release of reducing groups from carboxymethyl cellulose (CMC).

A 1 per cent w/v solution of CMC (Hercules Inc., Wilmington, Delaware, U.S.A.) was prepared, adjusted to pH 5.0 and mixed with an equal volume of 0.2M sodium citrate buffer at pH 5.0. 0.2 ml enzyme sample was added to 0.8 ml of CMC solution, mixed and incubated in a water bath at 30°C. After 1h release of reducing groups was estimated by the Nelson-Somogyi method described previously. Activity is expressed as m moles reducing groups (glucose equivalents) released.

H. Acid phosphatase

This was estimated by adding 0.2 ml enzyme sample to 0.3 ml water and 1.0 ml 2 mM p-nitrophenyl phosphate disodium salt (Koch-Light Ltd., Colnbrook, Bucks). After incubation for 30 min in a water bath at 37°C, 2.5 ml 0.2M NaOH was added and liberated p-nitrophenol measured at 400 nm against a water blank. Where enzyme samples were coloured, the sample was read against a control assayed immediately after adding the enzyme sample. Activity was expressed as µg p-nitrophenol liberated under the conditions of the assay.

I. Phenoloxidase

A 0.05M solution of catechol (BDH Chemicals Ltd.) and 0.2M sodium phosphate buffer at pH 7.0 were prepared. Reaction mixtures were prepared in spectrophotometer cuvettes as follows :

	Reference cuvette	Sample cuvette
catechol	2.0 ml	2.0 ml
water	0.5 ml	0.3 ml
buffer	0.5 ml	0.5 ml

After adjusting to zero absorbance at 495 nm, 0.2 ml enzyme sample was added to the sample cuvette and mixed. Absorbance readings were taken every 15 sec for 2 min. Corrections were made for colour in enzyme samples by measuring the absorbance at 495 nm of 0.2 ml enzyme sample in water and buffer against water and buffer. Phenoloxidase activity was expressed as the change in absorbance at 495 nm in 1 min.

Modifications of this method are given with individual experiments.

J. Peroxidase

Enzyme samples were diluted tenfold to decrease phenoloxidase activity. The oxidation of pyrogallol (Hopkin and Williams Ltd.) was measured using a spectrophotometric method similar to that previously described for phenoloxidase.

Solutions of 0.005M pyrogallol in 0.0082M sodium phosphate buffer at pH 6.0 and 1% v/v H_2O_2 were prepared. Reaction mixtures were prepared in spectrophotometer cuvettes as follows :

	Reference cuvette	Sample cuvette
pyrogallol solution	2.5 ml	2.5 ml
water	0.1 ml	-
H_2O_2	0.1 ml	-
enzyme sample	-	0.1 ml

The spectrophotometer was adjusted to zero absorbance at 420 nm and 0.1 ml. H_2O_2 was added to the sample cuvette and mixed. Absorbance measurements were made every 15 sec for 2 min and peroxidase activity expressed as the change in absorbance at 420 nm in 1 min.

F. Malic dehydrogenase

Enzyme activity was estimated by the oxidation of reduced nicotinamide adenine dinucleotide (NADH). Solutions prepared were ; 0.1M sodium phosphate buffer at pH 7.4, 5.0 ml water containing 2.66 mg NADH (BDH Chemicals Ltd.) and 0.06M cis-oxalacetic acid (Sigma Chem. Co. Ltd.). The oxalacetic acid was dissolved in a small volume of water, adjusted to pH 7.4 and made to the required volume with buffer. Reaction mixtures were prepared in spectrophotometer cuvettes as follows :

	Reference cuvette	Sample cuvette
NADH	0.2 ml	0.2 ml
buffer	2.7 ml	2.6 ml
enzyme sample	0.1 ml	0.1 ml

The spectrophotometer was adjusted to give maximum absorbance at 340 nm then 0.1 ml of oxalacetic acid solution was added to the sample cuvette, mixed, and malic dehydrogenase activity estimated from the decrease in absorbance at 340 nm in one min from readings taken every 15 sec for 2 min.

L. Ribonuclease (RNase)

The release of degradation products of ribonucleic acid (RNA) absorbing at 260 nm was estimated using the method of Pitt and Coombes (1969).

The enzyme sample (0.2 ml) was added to 0.3 ml water and 0.5 ml 0.1M sodium acetate buffer at pH 5.0. To this was added 0.05 ml 1% w/v highly polymerised yeast RNA (BDH Chemicals Ltd.) and after mixing incubated for 1h at 37°C. The reaction was stopped by the addition of 1.5 ml 0.25% w/v uranyl acetate in 4% perchloric acid. A duplicate set of reaction mixtures were prepared for each enzyme sample, the reaction being stopped immediately after addition of the substrate. The precipitated RNA in all reaction mixtures was removed by centrifugation (1000 x g for 10 min). To 1 ml of the supernatant fluid was added 3 ml water and RNase activity estimated from the increased absorbance at 260 nm against the corresponding unincubated control.

11. Measurement of ion content

Potassium, sodium and calcium ion estimations were made using an 'Eel' flame photometer (Evans Electroselenium Ltd., Essex). Using the appropriate filter the instrument was calibrated using dilutions of salt solutions of each cation i.e. KCl (0 - 10 p.p.m.), NaCl (0 - 4 p.p.m.) and CaCl₂ (0 - 100 p.p.m.). The upper and lower limits of the instrument scale were adjusted at regular intervals using the salt solutions and the instrument flushed through with water between each sample.

12. Measurement of protein

Two methods were used; an ultraviolet absorption method for partially purified preparations (Layne, 1957), or the more specific colour reaction with the phenol reagent method of Lowry, Rosebrough, Farr and Randall (1951).

A. Ultraviolet absorption method

The optical density of the sample was estimated at 260 nm and 280 nm

against water. Correction was made for the presence of nucleic acids absorbing strongly at 260 nm by the ratio :

$$\frac{\text{optical density at 280 nm}}{\text{optical density at 260 nm}} = F$$

F was then substituted in the following :

$$\text{protein concentration (mg/ml)} = F \times \frac{1}{d} \times \text{optical density at 280 nm}$$

where d = length of light path of cuvette in cm

B. Folin-Ciocalteu reagent method

Reagents prepared were :

- A - 2% w/v Na_2CO_3 in 0.1N NaOH
- B - 1% w/v $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution and 2% w/v potassium sodium tartrate solution, mixed in equal quantities on day of use
- C - alkaline copper reagent : 50 parts A to 1 part B
- D - Folin-Ciocalteu reagent diluted to 1N

1 ml protein sample and 5 ml reagent C were mixed and kept at room temperature for 30 min. 0.5 ml reagent D was then added and mixed. After a further 30 min the absorbance of the solution was estimated at 750 nm against a reagent blank. A calibration curve was prepared with solutions of crystalline bovine serum albumin (Koch-Light Ltd.) containing between 10 and 100 $\mu\text{g/ml}$.

13. Ultrafiltration

This method was used for concentration of enzyme solutions and separation of proteinaceous components by molecular weight size. For large volumes an 'Amicon' ultrafiltration cell model 52 was used and for smaller volumes model 10 - PA (Amicon Corp., Lexington, Massachusetts, U.S.A.). These cells were used with 'Diaflo' membranes which have cut-off points at different molecular weights. The membranes were washed in changes of water

for 1h on a magnetic stirrer before use and stored in 10% v/v ethanol at 4°C. Model 52 ultrafiltration cell is connected to a gas cylinder the solution being forced through the membrane by a pressure of 60 p.s.i. N₂ and is operated in conjunction with a magnetic stirrer. Cooling was achieved by standing the cell in a container of ice. Model 10 - PA cell is a self-contained unit used in conjunction with 'Amicon P - 10' pressurized propellant containers.

This method was used to concentrate crude cucumber rot extract prior to purification of its constituent enzymes and also the separation of proteinaceous material according to molecular weight.

14. Purification of enzymes from crude cucumber rot extracts

A. Ammonium sulphate precipitation

The first step in all enzyme purification procedures was a fractional precipitation. Solid (NH₄)₂SO₄ was added to undialysed crude rot extract to give the desired percentage saturation i.e. 0 - 40, 41 - 60 and 61 - 80. The procedure was carried out at 23°C using a water bath. The precipitate was removed by centrifuging at 12000 x g for 20 min at 23°C in an 'MSE' high-speed centrifuge. The supernatant was decanted and further solid (NH₄)₂SO₄ added to increase the percentage saturation. At all stages the amount of solid (NH₄)₂SO₄ to be added was calculated from tables using the volume of fluid at hand. The precipitates at each stage were dissolved in a small volume of water and then dialysed against water at 4°C for 18h before storing at -20°C.

B. Gel filtration

Enzymes present in (NH₄)₂SO₄ precipitates were separated by gel filtration using Sephadex G75 Superfine (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden). This gel has a dry particle diameter of 10 - 40μ and separates proteins with molecular weights between 3,000 and 70,000.

40g of dry gel was added to a large volume of water and heated on a boiling water bath for 3h. The gel was stirred at intervals, but not

vigorously, to avoid damaging the gel particles. After cooling, excess water was decanted to give a concentrated slurry of gel.

The column used was a Sephadex K25/100 (Pharmacia Fine Chemicals Ltd.) 2.5 cm diam and 100 cm long. With the outlet tube closed the nylon net at the base was covered with water and air bubbles excluded. After replacing the lower end piece the gel slurry was carefully poured into the column down a glass rod. Care was taken not to trap any air bubbles. The column was filled and connected to a reservoir of 0.1M NaCl in 0.005M Tris - HCl buffer at pH 9.0. The column was flushed with this buffered salt solution at a flow rate of 12 ml/h using a 'Varioperpex' peristaltic pump (LKB-Produkter, Bromma, Sweden). This was continued until twice the column volume had flowed through, during which time the gel column had settled to a constant height.

A sample applicator was used to aid even layering of the sample. Buffer was removed from the top of the gel column until c. 1 cm remained. The sample applicator was pushed down the column until the net just made contact with the buffer surface and no air-bubbles were trapped under the net. The appropriate $(\text{NH}_4)_2\text{SO}_4$ precipitate was pipetted into the sample applicator and, with the outlet tube open, settled evenly on the surface of the gel. Buffer was carefully added to the top of the sample to avoid mixing. When the sample had completely entered the gel, the column was refilled with buffer and eluted at a flow rate of 12 ml/h at 4°C in a cold room. 5 ml fractions were collected with an 'Ultrorac' fraction collector (LKB - Produkter). The fractions were stored at -20°C until required.

C. Ion-exchange chromatography

Separation of certain enzymes present in cucumber rot extracts was most effectively achieved by this method.

A length of glass tubing (c. 25 x 1.8 cm diam) drawn to a fine capillary at one end served as a column. A small wad of glass wool was placed at the base and with the outlet closed, covered with water and air bubbles excluded.

The ion-exchange gel used was DEAE - Sephadex A.50 (Cl^- form) obtained from Pharmacia Fine Chemicals Ltd. 5g of dry gel was added to a large volume of 0.05M Tris - HCl buffer pH 8.0 and heated on a boiling water bath, with gentle stirring for 2h, to swell the gel. When cool, the slurry was carefully poured into the column ensuring that no air-bubbles were trapped. The column was then placed in a cold room at 4°C and flushed with twice the column volume of buffer.

Buffer was removed from the top of the gel column until c. 0.5 cm remained. The appropriate $(\text{NH}_4)_2\text{SO}_4$ precipitate was carefully pipetted on to the gel column, care being taken not to disturb the gel surface. With the outlet open, the sample was allowed to enter the gel and then followed by the addition of 25 ml buffer. The sample was eluted by the stepwise addition of 25 ml each of 0.05, 0.1, 0.2, 0.3 and 0.4M NaCl in 0.05M Tris - HCl buffer at pH 8.0. 5 ml fractions were collected using an 'Ultrorac' fraction collector and stored at -20°C .

D. Isoelectric focusing

This method was used as the final step in the purification procedure for both PTE and phosphatidase. The advantages of this method are that large samples can be used, the total volume of sample used is recovered, the individual protein constituents are concentrated rather than diluted and that the isoelectric point (pI) of each can be readily determined.

The principle of the method depends on the establishment of a pH gradient between two electrodes; the proteins in the sample then migrate to the points on this gradient corresponding to their individual isoelectric points. The pH gradient is produced by the use of low molecular weight 'Ampholine' carrier ampholytes (LKB - Produkter) giving gradients between pH 3 and 10 or fractions of this range. The LKB carrier ampholytes are mixtures of a large number of different aliphatic polyaminopolycarboxylic acids and were used at a final concentration of 1 per cent. When preparing the column a sucrose gradient is used to obtain a convection-free medium.

An LKB 8100 'Ampholine' electrofocusing column was used. Solutions were prepared as follows:

Cathode solution (light)

NaOH	0.1g
water	10 ml

Anode solution (dense)

phosphoric acid	0.2 ml
water	14 ml
sucrose	12g

Gradient solution (light)

0.7 ml carrier ampholytes
enzyme sample (fractions containing peak of activity from
either Sephadex or DEAE Sephadex columns)
water to 60 ml

Gradient solution (dense)

1.8 ml carrier ampholytes
water to 42 ml, then sucrose 28g

The column has an inner and outer water jacket through which externally-cooled water maintained at 4°C by a water-bath thermostat, is circulated.

To set up the column, the outlet was closed and the dense electrode solutions poured in carefully through a funnel in sufficient quantity to cover the lower electrode. The electrode solutions are included to prevent contact of the carrier ampholytes with the electrodes. The dense and light gradient solutions were then added by means of a gradient mixer. The flow was kept at a constant rate (4 ml/min) by gravity feed to ensure even mixing and thereby avoid discontinuities in the sucrose density gradient. Using this method the enzyme sample is evenly distributed throughout the gradient. Filling of the column was continued until the solution level was just below the upper electrode. The light electrode solution was then carefully placed on top of the gradient solution to cover the upper electrode. The

column was then connected to an LKB DC Power Supply and the voltage adjusted to give maximum power of not more than 2 - 3W. During the run the voltage is adjusted to higher values up to c. 1,000v until the current falls to a steady low level at which time the run is completed. Running time was usually 48 - 52h although in some experiments longer periods were used.

To empty the column, water was pumped through the column using a peristaltic pump and 5 ml fractions were collected. The tubes containing the fractions were capped to prevent exposure to CO₂ and subsequent change in pH. The pH values of the fractions were measured using a pH meter. These measurements were carried out at 4°C, this being the temperature used during electrofocusing.

Protein estimations were made on the fractions by the ultraviolet absorption method. The fractions were then dialysed against water at 4°C for 15h to remove the sucrose and carrier ampholytes and finally stored at -20°C.

15. Scanning electron microscopy

Samples from suspensions of carrot callus cells treated with PTE for 2h as described earlier, or untreated cells placed on small cover slips were covered with drops of 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.0 and placed in petri-dishes for 4h. The fixative was removed and the coverslips washed three times with water (10 - 15 min each).

The cells on the coverslips were then dehydrated in an alcohol series (10, 20, 30, 50, 70 and 80% - 10-15 min each, 90% - 30 min, 100% - three changes of 30 min each). Dehydration was followed by replacement of the alcohol by amyl acetate. The samples were placed in a mixture of 5 parts alcohol to 1 part amyl acetate for 10 - 15 min. This solution was replaced by one containing 4 parts alcohol to 2 parts amyl acetate (10 - 15 min). The samples were immersed in a series of solutions containing decreasing amounts of alcohol and increasing amounts of amyl acetate until the samples were in a solution containing 1 part alcohol and 5 parts amyl

acetate where they remained for 30 min. This solution was replaced by three changes of amyl acetate (30 min each). At each step in this procedure care was taken to ensure that the cells remained wet at all times to avoid distortion caused by drying.

The amyl acetate was removed from the cells by critical point drying. The amyl acetate was replaced by liquid CO₂ (2.5 - 3h) in an air-tight container and then the container heated to change the CO₂ from liquid to gas. The CO₂ gas was then run off to leave the samples dry.

The coverslips with the cell samples were then glued to stubs and coated with gold/palladium in a Micro - BA 3 high vacuum coating apparatus (Balzer, "Furstentum, Liechtenstein). Coated stubs were stored in a desiccator.

16. Transmission electron microscopy

Potato disks either untreated or treated with PTE for different times as described previously were transferred to 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.0. Small pieces (c. 1 mm²) from the disks were transferred to fresh glutaraldehyde in buffer and left for 3h. The fixative was removed and the material washed three times in buffer (15 min each).

The following steps were performed in the cold by placing the watch-glasses containing the material on ice. The material was treated with 1% osmium tetroxide in 0.1M sodium cacodylate buffer pH 7.0 (2h) then washed twice with buffer (15 min). The material was then dehydrated in an alcohol series (10, 20, 30, 40, 50, 60, 70 and 80% - 10 min each, 90% - 20 min), then left overnight in 100% alcohol. The alcohol was changed once (30 min) and the material was then ready for embedding. The alcohol was replaced by epoxypropane a substance soluble in alcohol and the embedding resin. The material was placed in a solution of 1 part alcohol to 1 part epoxypropane (30 min) and then into two changes of epoxypropane (30 min each). The epoxypropane was replaced by epoxy resin. The material was placed in a

mixture of 1 part epoxypropane to 1 part epoxyresin for 1h, covered, and then uncovered for a further 1h for the epoxypropane to evaporate. The material was then placed in epoxyresin (3 changes in 2d) and left open for the residual epoxypropane to evaporate. The resin containing the material was then placed in an oven at 60°C for 2d to harden.

The embedded material was then cut into blocks and sectioned on an ultra-microtome (Reichert, Wien, Austria), with a diamond knife. The sections were placed on grids and stained in 2% uranyl acetate in Reynolds lead citrate (Reynolds, 1963) for 30 min at room temperature.

17. Oxygen uptake by potato tuber disks

An oxygen electrode was used to measure uptake of oxygen by tuber disks. The water jacket of the electrode was connected to a circulating supply of water thermostatically controlled at 25°C.

The cathode was covered with saturated KCl and the electrode compartment separated from the reaction chamber above by a thin oxygen-permeable Teflon membrane. The reaction chamber is fitted with a plunger which has a capillary at its lower end which restricts oxygen diffusion to or from the atmosphere. The liquid in the reaction chamber is stirred rapidly using a magnetic stirrer.

When a voltage is applied to the electrode, the current flowing through the cell will be proportional to the oxygen concentration in the region of the cathode. This current after suitable amplification is used to operate a potentiometric chart recorder. The electrode was calibrated with air-saturated water for 100% oxygen concentration (26 μ moles O_2 /ml at 25°C). The zero level of oxygen concentration was obtained after the addition of sodium dithionite which removes oxygen from the solution.

The reaction mixture was 6.0 ml total volume and contained $CaCl_2$ (0.001M final), Tris - HCl buffer pH 9.0 (0.02M final) and crude cucumber rot extract (0.8 ml) or equivalent concentration of PTE (29.6 units) and 50 potato tuber disks (4 mm diam, 0.5 mm thick). In some experiments disks were plasmolysed in 0.5M sucrose.

On addition of the enzyme sample oxygen concentration in the reaction chamber was followed for a 2 - 3h period using the chart recorder.

18. Gel electrophoresis

This procedure was used for the resolution of protein or RNA. A 'Shandon' electrophoresis apparatus was used (Shandon Scientific Co. Ltd., London). Polyacrylamide gels of 7.5% were run at pH 2.3 and 5% gels at pH 9.5.

The sample (0.1 ml) made dense by the addition of sucrose was layered on the top of the gel and run at 6 mA per tube until the sample front had moved to a point c. three-quarters down the gel. The gels were removed from the running tubes and stained for protein with 0.1% w/v Coomassie brilliant blue in a solvent of 10% v/v glacial acetic acid and 10% v/v methanol in water. After c. 4h the gels were destained in changes of the solvent for several days.

Other gels were stained for RNA with 0.1% w/v Toluidine blue in 15% v/v ethanol for 1h then destained in solvent for several days.

Bands of protein or RNA resolved by this procedure became fixed by the stain which was leached from the gel on destaining in fresh solvent. Bands of protein were assessed visually or by measuring the absorbance of the Coomassie blue at 550 nm in a Gilford 240 Spectrophotometer with a Gilford linear attachment (Gilford Instrument Co., Oberlin, Ohio, U.S.A.).

Bands of RNA were assessed visually.

EXPERIMENTAL WORK AND RESULTS

I. Preliminary Experiments

These were performed to establish the virulence of the pathogen and the host tissue most easily rotted.

1. Examination of the ability of *E. carotovora* to cause soft rots in different plant tissue

Plant tissues used were; potato tuber, carrot root, onion bulb and cucumber fruit. The carrot and potato tissues were soaked overnight in tap water to ensure turgidity. The onion was cut into c. 2 - 3 cm segments along the vertical axis and the other tissues cut into 2 - 3 cm slices.

The tissues were inoculated with 0.1 ml of a suspension of *E. carotovora* (c. 10^{10} cells/ml) as described for cucumber tissue in 'Materials and Methods'. 0.1 ml sterile distilled water was spread over the cut surface of each carrot slice prior to inoculation with the bacteria as it was found that the cut surfaces dried very rapidly. Four slices of each tissue together with a control slice treated with 0.1 ml sterile distilled water were set up and incubated at 25°C.

The slices were examined after 3 and 5d. Rotting was estimated on a linear scale 0 - 4 where 0 means that no rotting was visible and 4 that the slices were almost or completely rotted.

Table 1. Soft rotting present in different tissues after 3d and 5d

Tissue	3d		5d	
	Inoculated	Control	Inoculated	Control
Potato	3.0	0	3.5	0
Carrot	1.0	0	2.0	0
Onion	3.3	0	3.7	0
Cucumber	3.3	0	4.0	0

Results represent mean grade for 4 slices

Rotting was visible in all tissues except carrot after 1d incubation. After 3d rotting was visible in carrot as a slightly darkened lesion. Rots were cream coloured in onion and cucumber and developed rapidly. In potato the rot was cream coloured but with a brown margin. All rots appeared water-soaked with a milky exudate as droplets on the surface. There was little apparent shrinkage in volume of rotted tissue.

E. carotovora was able to cause a soft rot in each of the tissues tested although less in carrot than in onion, potato and cucumber where there was considerable rotting within 3d (Table 1).

2. Properties of extracts of rots from different tissues

Potato, cucumber, onion and carrot tissues were inoculated with E. carotovora as before and incubated at 25°C for 3d. Extracts of the rots that developed were made cell free, dialysed against water for 24h at 4°C and stored at -20°C.

From the results of this experiment it was hoped to obtain a combination of pathogen and host which would provide large volumes of rotted tissue with high pectolytic, cell separating and toxic activities.

A. Changes of pH

About 10g fresh weight of rotted and uninoculated tissue was homogenized with 10 ml of water in a 'Sorvall Omnimixer' for 1 min at full speed. The pH of each was then measured (Table 2).

Table 2. pH values of tissues

Tissue	Healthy	Rotted
Potato	6.00	6.60
Carrot	6.15	6.15
Onion	5.45	5.95
Cucumber	6.05	7.05

The pH of rots in all tissues except carrot was higher than that of uninoculated tissue

B. Pectic enzyme activity

i) Viscosity reducing activity

Each rot extract was assayed by viscometry using NaPP and P substrates at pH 5.0 and pH 9.0 and with or without added CaCl_2 (0.001M final).

Reaction mixtures contained: 8.0 ml 1% w/v pectic substrate in 0.1M Tris-HCl buffer pH 9.0 or 0.1M sodium citrate buffer pH 5.0, 1 ml 0.01M CaCl_2 (0.001M final) and 1 ml of rot extract.

Table 3 summarizes the results.

Table 3. Viscosity reducing activity of rot extracts

Rot extract	NaPP				P			
	pH 9.0		pH 5.0		pH 9.0		pH 5.0	
	+Ca ²⁺	-Ca ²⁺	+Ca ²⁺	-Ca ²⁺	+Ca ²⁺	-Ca ²⁺	+Ca ²⁺	-Ca ²⁺
Potato	107	85	6	6	50	11	3	3
Carrot	67	46	<1	<1	77	15	0	<1
Onion	52	37	2	2	60	14	<1	<1
Cucumber	342	194	<1	<1	274	7	<1	<1

Activity expressed as $100/t$ where t is the time (min) for 50% reduction in relative viscosity

Viscosity reducing activity at pH 5.0 with both substrates was very low or negligible for each rot extract. Potato and cucumber rot extracts were most active on NaPP at pH 9.0 when calcium was present. Carrot and onion rot extracts were slightly more active on P substrate at pH 9.0 when calcium was present. All rot extracts were more active when 0.001M CaCl_2 was present in reaction mixtures. It appeared that NaPP was the preferred substrate for each rot extract. This was clearly seen at pH 9.0 in the absence of added calcium but was less clear when calcium was included. NaPP substrate

preference is shown even at pH 5.0 by potato and onion rot extracts.

It has been shown that the PTE in filtrates of cultures of E. carotovora has a calcium requirement with an optimum at 0.001M and that above this level there is a decrease in activity (Hall, 1971). As the viscosity reducing activity of these rot extracts is highest at pH 9.0 and enhanced by the addition of calcium (Table 3) it is likely that a trans-eliminase is involved. It is possible that some calcium remains in the rot extracts after dialysis and that further addition will be in excess of the optimum requirement leading to a decrease in activity.

The cucumber rot extract showed the highest viscosity reducing activity this being 3 x that of the next highest i.e. that shown by the potato rot extract.

ii) Thiobarbituric acid test

All rot extracts were assayed using NaPP and P substrates at pH 9.0 in the presence of 0.001M CaCl_2 . Reaction mixtures were the same as those used for viscometry.

Results shown in Table 4 are the absorbance values (OD units) at 550 nm shown by 1 ml samples removed from reaction mixtures after 1h. All samples showed a peak of absorbance at 550 nm indicating trans-eliminase activity in the rot extracts.

Table 4. Trans-eliminase activity of rot extracts

Rot extract	Absorbance 550 nm	
	NaPP substrate	P substrate
Potato	0.318	0.174
Carrot	0.288	0.271
Onion	0.248	0.220
Cucumber	0.465	0.157

All extracts showed pectate trans-eliminase (PTE) activity; the cucumber extract was the most active.

C. Cell separating activity and toxicity

These were estimated by the needle test and the plasmolytic method respectively, using potato tuber tissue disks. Reaction mixtures contained :

5 ml rot extract
1 ml 0.2M Tris-HCl buffer pH 9.0
1 ml 0.01M CaCl_2
3 ml water

Controls containing autoclaved rot extract were also set up. 50 disks were placed into each reaction mixture. At 10 min intervals 4 disks were removed and tested for toxicity. At 5 min intervals one or two disks were removed and cell separation estimated. This was considered to have occurred when the disks showed no resistance to being pulled apart by mounted needles. Results are shown in Table 5.

Table 5. Cell separating activity and toxicity of rot extracts

Rot extract	Cell separation	Toxicity
Potato	2.0	1.1
Carrot	1.8	1.7
Onion	2.0	2.0
Cucumber	4.0	2.0

Results for cell separation and cell death are expressed as $100/t$ where t (min) is the time required for cell separation or the time required for 4 disks to show a mean value of 4.0 on the neutral red index.

The cucumber rot extract showed the highest cell separating activity and toxicity. As this extract also showed the greatest viscosity reducing activity and PTE activity by the thiobarbituric acid test, it was selected for use for the rest of the work.

Large volumes of rotted tissue were prepared and extracted as described in 'Materials and Methods'. This extract was used wherever

cucumber rot extract is referred to in the text and contained 37.3 units/ml PTE activity.

II. Properties of crude dialysed cucumber rot extracts

1. Pectate trans-eliminase activity (PTE)

A. pH optimum

Pectolytic activity was estimated by the thiobarbituric acid test. Reaction mixtures contained 0.5 ml each of rot extract, water and CaCl_2 (0.008M) and 2.5 ml of 1% w/v NaPP in 0.1M buffer to give a range of pH values (4 - 10).

Table 6. Pectate trans-eliminase activity at different pH values

pH	Absorbance 550 nm
4	0
5	0
6	0
7	0.41
8	3.26
9	3.73
10	2.36

Results shown are mean values for 3 replicates and represent increased absorbance (OD units) compared with controls at the corresponding pH containing autoclaved rot extract.

The results in Table 6 show that PTE activity of cucumber rot extract was highest at pH 9.0 and was sharply reduced with decreasing pH and not detected at pH 6.

B. Effect of dilution

Activity was estimated by two methods, viscosity reduction and release of reducing groups. Reaction mixtures contained :

8 ml 1% w/v NaPP in 0.1M Tris-HCl buffer pH 9.0

1 ml 0.01M CaCl_2

1 ml diluted rot extract

Rot extract was diluted with autoclaved extract to give concentration of 0.1, 0.05, 0.02, 0.01 and 0.001. Viscosity reducing activity was expressed as $100/t$ where t is the time (min) for a 50% reduction in relative viscosity. The relation between activity and enzyme dilution is shown in Figure 1 (a).

Release of reducing groups was estimated against a reagent blank by the Nelson-Somogyi method using 0.2 ml samples taken from reaction mixtures at various time intervals. Results expressed as $\mu\text{g/ml}$ reducing groups released are shown in Table 7.

Table 7. Release of reducing groups ($\mu\text{g/ml}$) by different concentrations of cucumber rot extract

Time (min)	Extract dilution				
	0.1	0.05	0.02	0.01	0.001
1	0	0	0	0	0
5	26.0	15.5	0	0	0
10	46.5	-	0	0	0
30	178.5	56.0	27.5	17.5	0
60	460.0	129.0	-	-	0
90			56.0	-	0
120			73.5	23.5	0
150				30.5	0
180					10.0

The results obtained were considered as $\mu\text{g/ml}$ anhydrogalacturonic acid groups. These results were used to calculate the percentage hydrolysis of the NaPP by the relationship :

$$\% \text{ hydrolysis} = \frac{\text{mg. anhydrogalacturonic acid liberated}}{\text{mg. anhydrogalacturonic acid originally present in polymer form}} \times 100$$

Sunkist Growers Inc. the suppliers and producers of the NaPP substrate quote 78% as the purity of the product. Thus, 1% NaPP contains 7.8 mg/ml anhydrogalacturonic acid in polymer form.

The relation between enzyme dilution and % hydrolysis of NaPP in 30 min is shown in Figure 1 (b).

Figure 1 (a) and (b) shows that the relation between enzyme concentration and activity as estimated by both viscosity reduction and % hydrolysis of NaPP is linear.

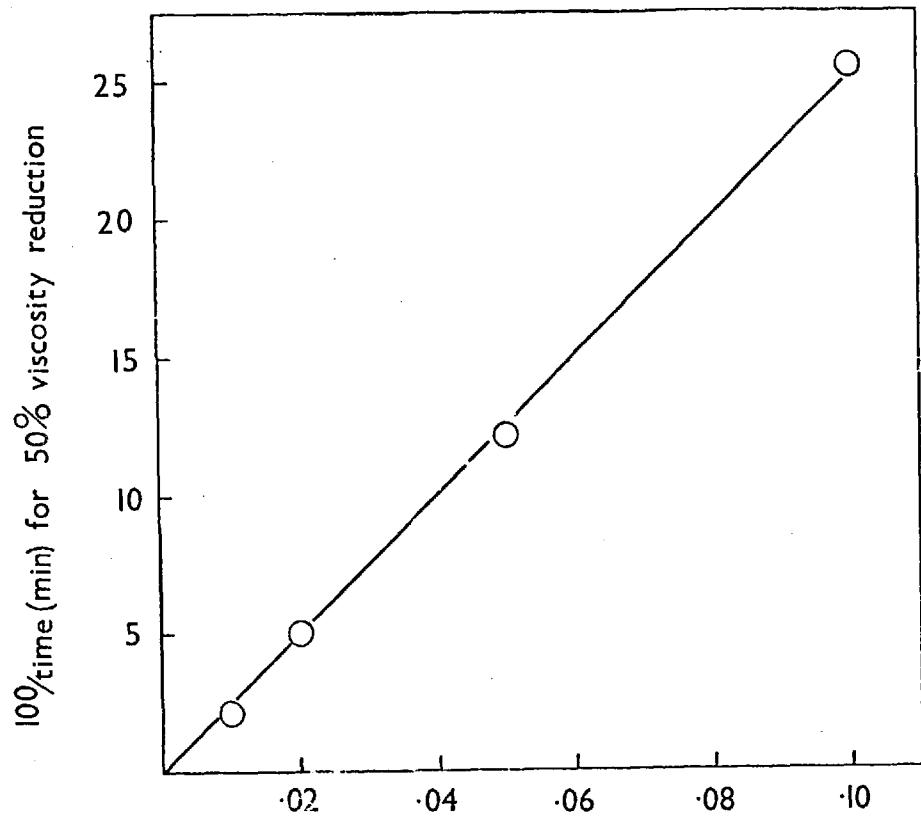
It has been shown that extracts contain pectate trans-eliminase. From the above results % hydrolysis of NaPP for 50% reduction in viscosity at each dilution was calculated. A 50% reduction in viscosity was equivalent to 0.3% hydrolysis of the NaPP. This showed that the pectic enzyme was strongly endo- in action.

In view of the report by Nasuno and Starr (1966), that PG is present in filtrates of cultures of E. carotovora, an attempt was made to detect PG in the cucumber rot extract. Nasuno and Starr state that their PG has a pH optimum 5.2 - 5.4.

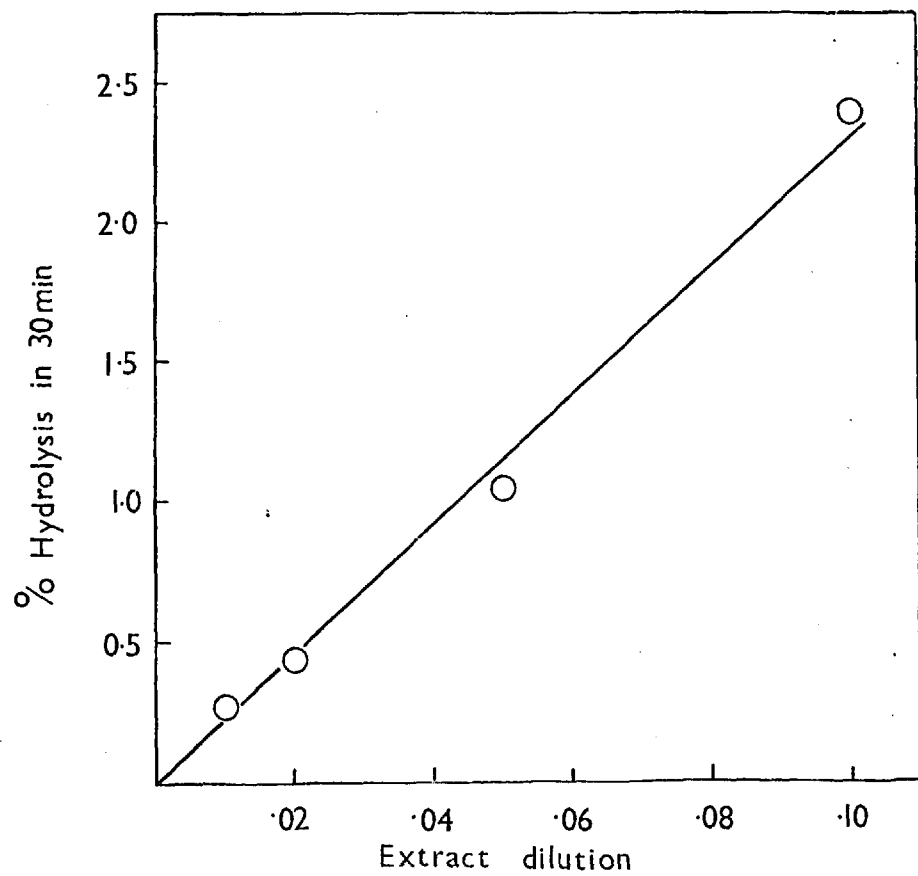
Only low activity was found at pH 5.0 using cucumber rot extract and this may have been due to PTE. It has been reported that PTE activity is inhibited when ethylenediamine tetra-acetic acid (EDTA) is included in reaction mixtures at $3 \times 10^{-5} \text{M}$ final concentration (Starr and Moran, 1962). In the present work although only very low activities were detected by viscometry with NaPP at pH 5.0 in the absence of calcium, PG if present may have been inhibited by calcium present in the cucumber rot extract. The viscometry assay was repeated at pH 5.0 as before but 1 ml EDTA ($3 \times 10^{-4} \text{M}$)

Figure 1. Dilution of cucumber rot extract and pectolytic activity

(a) Viscosity reducing activity



(b) %Hydrolysis



replaced CaCl_2 in the reaction mixture.

Viscosity reducing activity expressed as $^{100}/t$ min for 50% reduction in relative viscosity was ≤ 1 . Again, this slight activity may have been due to PTE with a much reduced activity at this low pH.

Using the thiocarbituric acid test a range of concentrations of EDTA were used to inhibit PTE activity.. Reaction mixtures contained:

1 ml rot extract

0.5 ml EDTA at appropriate concentration

2.5 ml 1% w/v NaPP in 0.1M sodium citrate buffer at pH 5.0

EDTA was added to give final concentrations of 0, 0.1, 0.01, 0.001 and 0.0001M. After 1h at 25°C the reaction mixtures were treated as described in 'Materials and Methods'. When estimated against controls prepared in the same way but with rot extract replaced by autoclaved rot extract, there was no increase in absorbance at 510 nm or 550 nm indicating the absence of PG or PTE activity.

To confirm that PTE activity was inhibited by EDTA further reaction mixtures were set up containing:

1 ml rot extract

0.5 ml EDTA at appropriate concentration

2.5 ml 1% w/v NaPP in 0.1M Tris-HCl buffer at pH 9.0

EDTA was included to give the same final concentrations as the previous experiment.

Table 8. Effect of EDTA on PTE activity at pH 9.0

EDTA final conc.(M)	Absorbance 550 nm
0	3.0
0.1	0.015
0.01	0.029
0.001	2.67
0.0001	3.30

Results expressed as increase in absorbance (OD units) compared with controls containing autoclaved rot extract.

The results (Table 8) show that a reduction in PTE activity was found with increasing EDTA concentration. Concentrations of EDTA above 0.01M almost completely inhibited PTE activity of cucumber rot extract. These concentrations are much higher than $3 \times 10^{-5}M$ quoted by Starr and Moran (1962).

2. Cell separating activity and toxicity

A. pH optima

These were estimated by the needle test and the plasmolytic method respectively using potato tuber tissue disks. Reaction mixtures contained :

- 2 ml rot extract
- 2 ml water
- 2 ml 0.004M $CaCl_2$
- 2 ml 0.08M buffer

Buffers were added to give a range of pH values (4 - 10). Thirty disks were placed in each reaction mixture and samples of 5 disks removed at intervals and tested for viability. After estimating toxicity the disks

were pulled apart with mounted needles and estimates made of cell separation.

Table 9. Cell separating activity and toxicity at different pH values

pH	Time (min)							
	20		40		60		80	
	CS	CD	CS	CD	CS	CD	CS	CD
4	0	0	0	0	0	0	0	1.4
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	1.2	0.4	1.7	1.0
8	0	0	1.2	1.2	2.5	2.9	4.5	4.6
9	0.2	0.4	1.5	2.0	3.2	3.1	4.7	4.7
10	0.5	0.6	1.2	1.6	3.2	2.9	4.7	4.7

CS - cell separation, CD - cell death, both expressed as mean grade per 5 disks.

The results (Table 9) show that there is a close relation between cell separation and death at each pH value used. The two activities were highest at pH 9 - 10 which is only slightly higher than the optimum recorded for degradation of NaPP. Below pH 7 no cell separating activity was detected. The toxicity found at pH 4 after 80 min was probably due to the low pH.

B. Effect of dilution

Cucumber rot extract was diluted with autoclaved rot extract to give concentrations of 1.0, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05. Reaction mixtures contained :

5 ml diluted rot extract
2 ml 0.005M CaCl_2
2 ml 0.2M Tris-HCl buffer pH 9.0
1 ml water

70 disks were added to each reaction mixture and at intervals 5 disks were removed for estimations of cell separation and toxicity as described earlier.

The relation between cell separation and rot extract concentration is shown in Figure 2(a) and that between cell death and rot extract concentration in Figure 2(b).

Figure 2(a) and (b) demonstrates that cell separation and cell death were not linear with extract concentration. The two curves are very similar emphasizing the relation between these two activities. At low concentrations of rot extract, cell death and cell separation increased rapidly with concentration but at higher concentrations the curves levelled off indicating that some factor other than extract concentration was limiting activity. This factor was probably the time taken for the extract to diffuse into the tissue.

When cell separating activity and toxicity ($^{100}/t$) were plotted against the log extract concentration (Figure 3 (a) and (b)) both gave a linear relation. This is in contrast with the relation between degradation of NaPP and extract concentration showing that cell separation or toxicity measured in this way is dependent on availability of substrate not encountered with substrates in solution. Similar results have been obtained by Dean and Wood (1967) and Hall (1971).

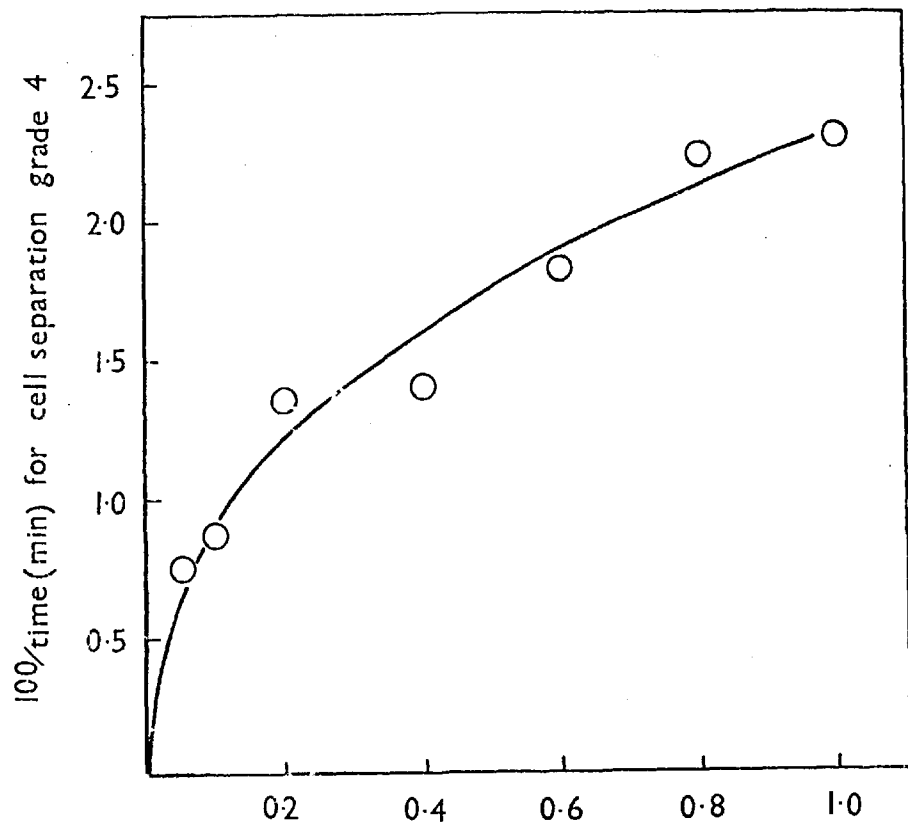
3. Permeability increasing activity

Reaction mixtures contained :

2 ml rot extract
2 ml 0.004M CaCl_2
2 ml 0.08M Tris-HCl buffer pH 9.0
2 ml water

Figure 2. Dilution of cucumber rot extract and cell separating activity and toxicity

(a) Cell separating activity



(b) Toxicity

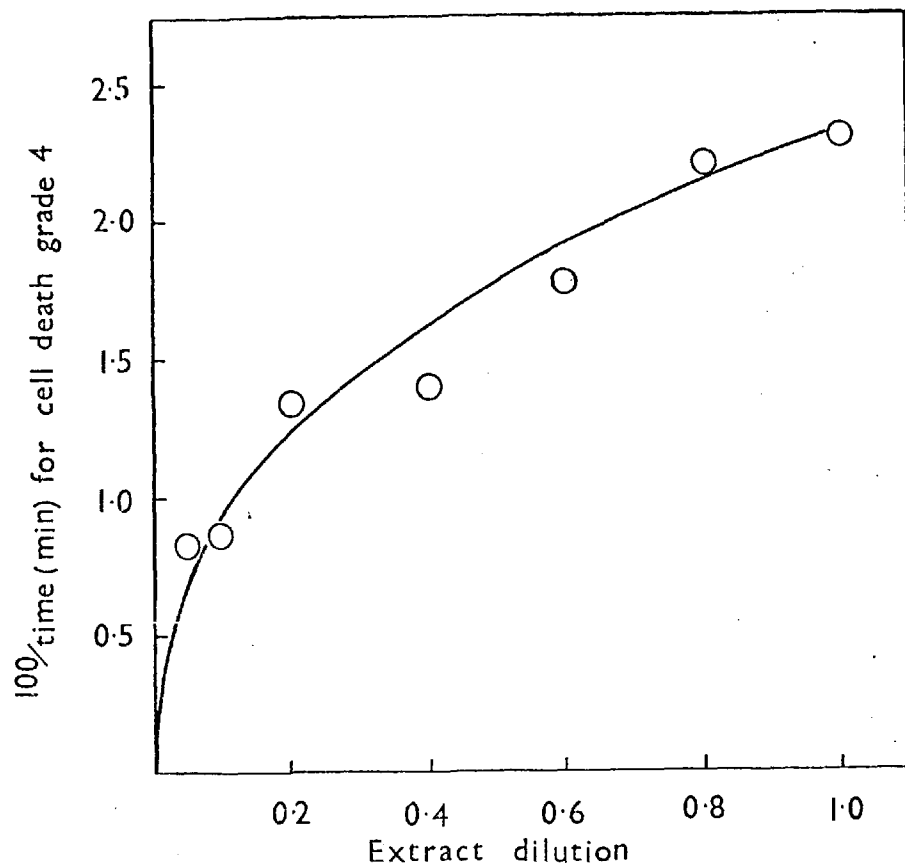
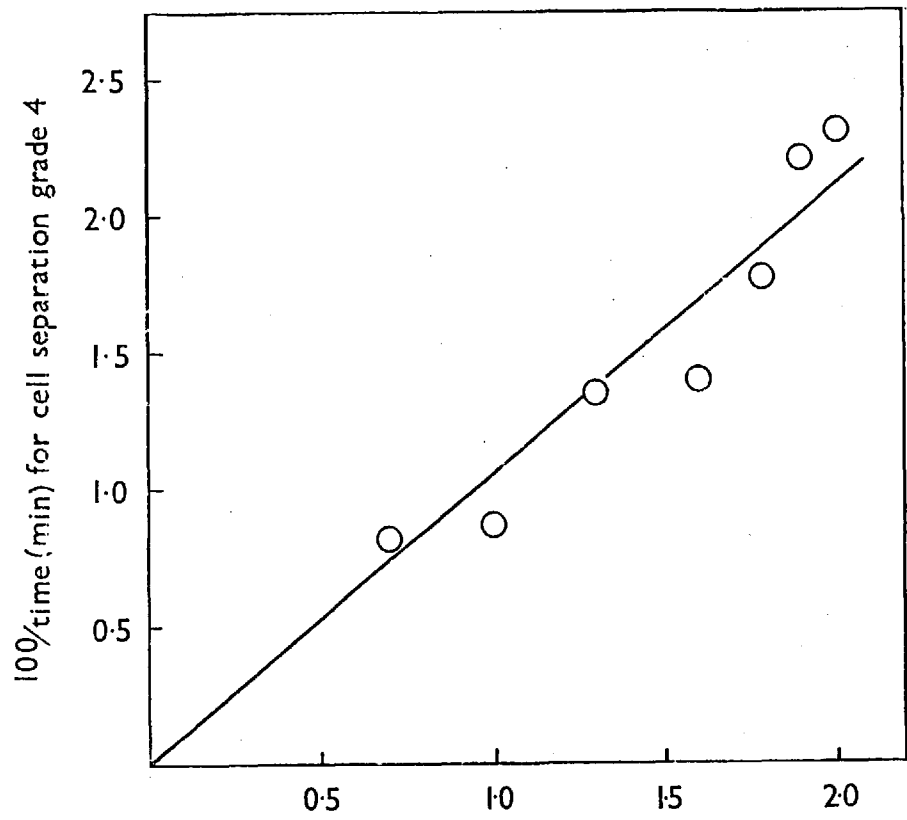
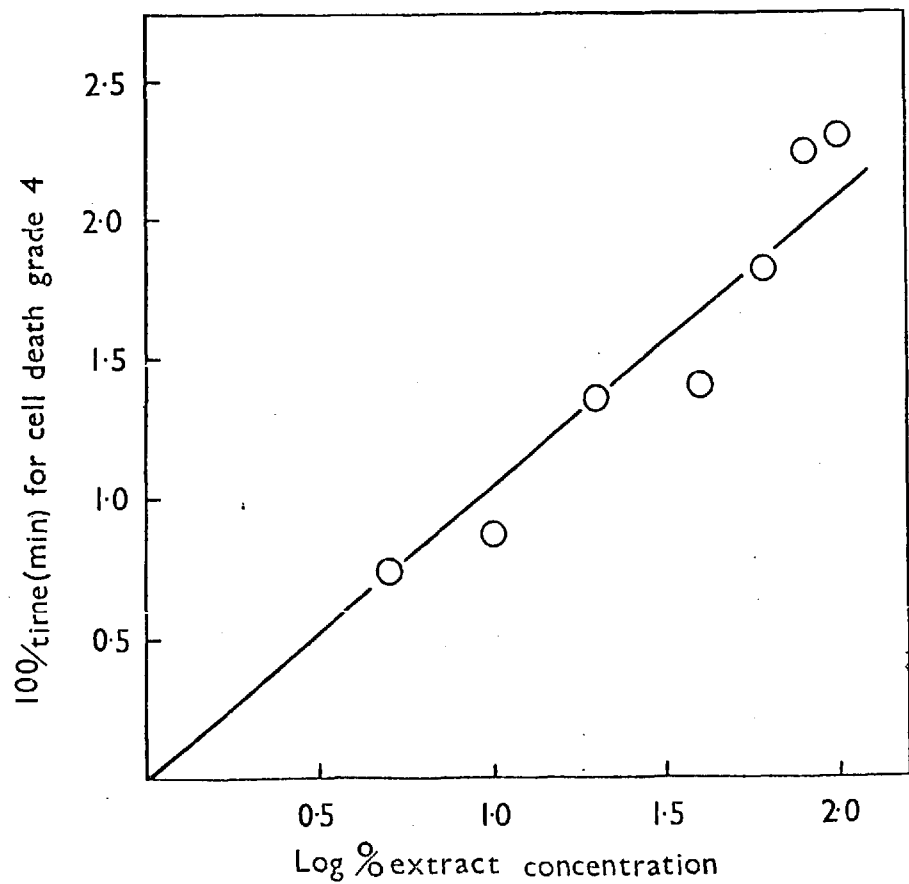


Figure 3. Log concentration of extract and cell separating activity and toxicity

(a) Cell separating activity



(b) Toxicity



Control reaction mixtures were also set up containing autoclaved rot extract or water. 50 disks were placed in reaction mixtures and at intervals 5 disks removed for conductivity measurements. Results are shown in Table 10.

Table 10. Permeability increases shown by potato tuber disks

Δ Conductivity μ hos						
Time (min)	Water	Extract (A)	Extract *	Extract	Extract *	Extract **
1	1.8(16)	2.1(10)	0.3(6)	5.3(3)	3.5(4)	3.2(2)
5	1.8(16)	3.6(16)	1.8(37)	18.5(12)	16.7(19)	14.9(11)
10	1.8(16)	2.4(11)	0.6(12)	20.4(13)	18.6(21)	18.0(14)
15	-	2.2(10)	-	24.6(16)	-	22.4(17)
20	1.7(15)	2.5(11)	0.8(16)	21.3(14)	19.6(23)	18.8(14)
25	-	2.0(9)	-	18.6(12)	-	16.6(13)
30	1.4(13)	1.6(7)	0.2(4)	14.3(9)	12.9(15)	12.7(10)
45	-	1.9(9)	-	13.4(9)	-	11.5(9)
60	1.3(12)	1.9(9)	0.6(12)	11.5(7)	10.2(12)	9.6(7)
90	1.2(11)	1.8(8)	0.6(12)	6.5(4)	5.3(6)	4.7(4)
Total	11.0(100)	22.0(100)	4.9(100)	154.4(100)	86.8(100)	132.4(100)

Extract - cucumber rot extract

(A) -- autoclaved

Results are expressed as increase in conductivity in μ hos/15 min/5 disks/15 ml and represent means for two replicates

* - increase compared with water control

** - increase compared with autoclaved extract control

Numbers in parentheses indicate percentages of total

Crude cucumber rot extract rapidly caused an increase in the permeability of potato tuber disks. Maximum increases occurred after about 15 minutes which was long before cell separation (almost complete after 80 minutes). Autoclaved rot extract showed little more permeability increasing activity than water indicating that activity may be enzymic in nature.

III. Purification of pectate trans-eliminase (PTE) from cucumber rot extracts

1. Ammonium sulphate fractional precipitation

100 ml of undialysed cucumber rot extract was brought to different saturations by the addition of solid $(\text{NH}_4)_2\text{SO}_4$. Precipitates obtained between 0 - 40%, 41 - 60% and 61 - 80% saturation were taken up in a small volume of water, dialysed for 24h at 4°C against water and finally made up to 10 ml with water. These samples were stored at -20°C and used for estimations of different activities present in each fraction of cucumber rot extract.

PTE activity was estimated by the thiobarbituric acid test and expressed as units/ml PTE.

Cell separation and toxicity were estimated using samples of 5 disks removed at intervals from reaction mixtures containing :

- 1 ml enzyme sample
- 1 ml 0.04M CaCl_2
- 1 ml 0.08M Tris-HCl buffer pH 9.0
- 1 ml water
- 70 potato tuber disks

Permeability increasing activity was estimated after placing 50 potato disks in reaction mixtures containing :

- 1 ml enzyme sample
- 2 ml 0.008M CaCl_2
- 2 ml 0.16M Tris-HCl buffer pH 9.0
- 3 ml water

5 disks were removed at intervals for conductivity measurements.

Proteinase and phosphatidase activities were estimated by the congocoll and cup plate methods.

Cellulase (CMCase) was estimated by the Nelson-Somogyi method using 0.2 ml enzyme sample and 0.8 ml 0.5% w/v CMC in 0.1M sodium citrate buffer at pH 5.0.

The results of all these estimations are summarized in Table 11.

Table 11. Activities of $(\text{NH}_4)_2\text{SO}_4$ fraction precipitates of cucumber rot extract

Activity*	Fraction precipitate		
	0 - 40%	41 - 60%	61 - 80%
PTE	16.5	23.0	27.5
Cell separation (30 min)	0	1.2	4.2
(60 min)	0.3	3.7	5.0
Toxicity (30 min)	0	1.2	3.4
(60 min)	0	3.0	5.0
Permeability increasing activity	3.1	10.2	62.5
Proteinase	0.92	1.08	0.70
Phosphatidase	0.98	4.94	2.36
Cellulase (CMCase)	0.26	0.94	1.19

* - PTE - units/ml; cell separation and toxicity expressed as mean grade for 5 disks; permeability increasing activity expressed as $100/t$ where t is the time (min) for 10 μmhos increase in conductivity of 15 ml water bathing

5 disks in 15 min; proteinase expressed as absorbance (OD units) at 495 nm; phosphatidase expressed as area (cm^2) of zone around well; CMCase expressed as m moles reducing groups released/0.2 ml enzyme/60 min.

PTE, cell separating, toxic and permeability increasing activities were highest in the 61 - 80% fraction. Cell separating activity and toxicity in each of the fractions used were very similar demonstrating the close association of these activities. The highest cellulase activity was also found in the 61 - 80% fraction.

Proteinase and phosphatidase showed the highest activities in the 41 - 60% fraction.

These results showed some considerable separation of activities by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$. This procedure was used as the first step in all subsequent purification used. Before precipitation with $(\text{NH}_4)_2\text{SO}_4$ the rot extract was concentrated two fold by ultrafiltration.

2. Gel filtration using Serphadex G75 Superfine

The 61 - 80% $(\text{NH}_4)_2\text{SO}_4$ fraction precipitate with highest PTE activity was separated on Sephadex G75 gel. The column height was 79 cm and the sample volume applied was 5 ml. Fractions of 5 ml were collected, their protein content measured by the ultraviolet absorption method and then divided into small aliquots and stored at -20°C .

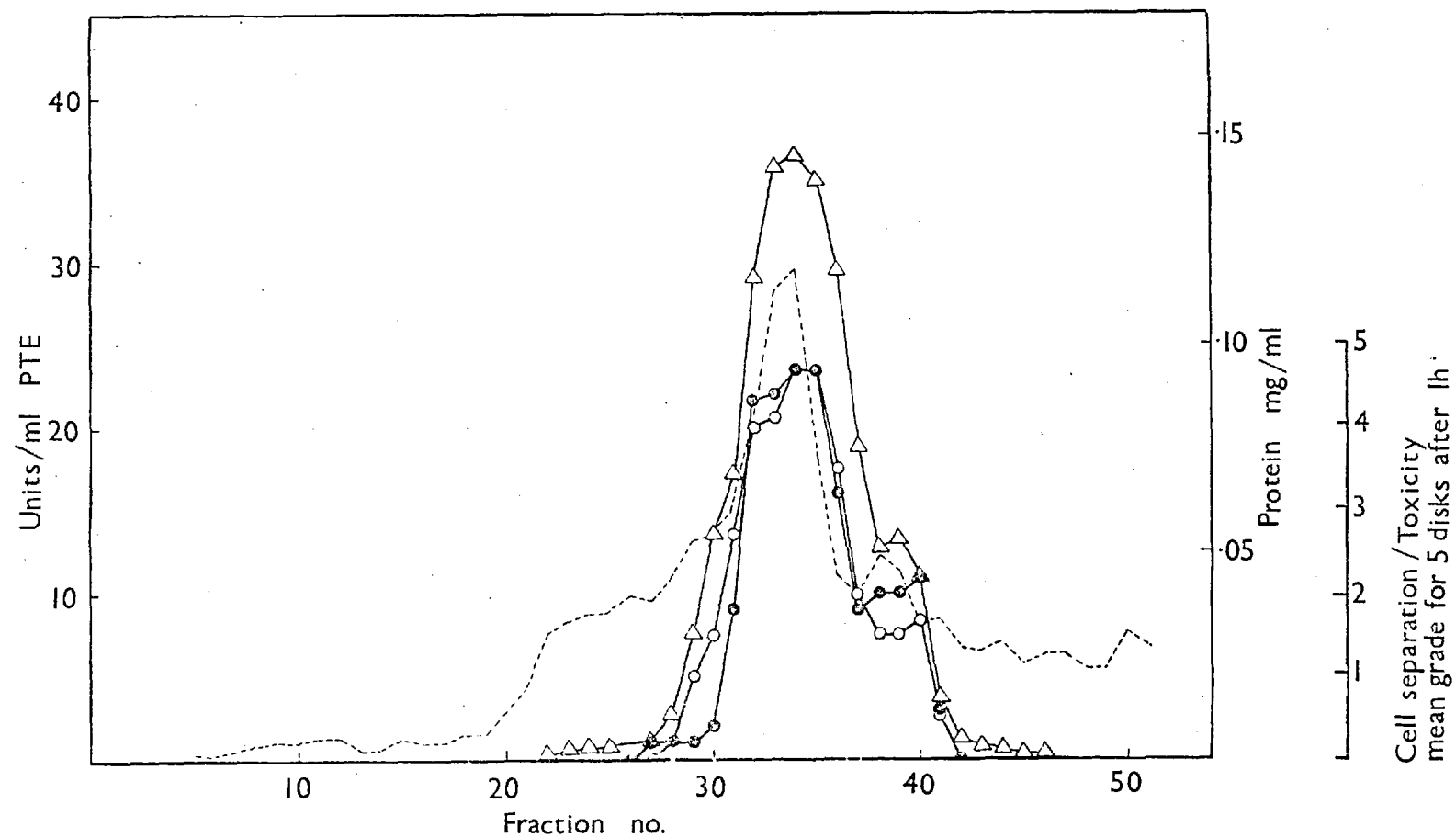
PTE activity was estimated by the thiobarbituric acid test and expressed as units/ml PTE.

Cell separation and toxicity were estimated using reaction mixtures containing 1 ml of the appropriate fraction, 0.001M CaCl_2 , 0.02M Tris-HCl buffer at pH 9.0 and 15 potato tuber disks. 5 disks were removed at 20 min intervals and cell separation and toxicity estimated by the needle test and the plasmolytic method respectively.

The results for these estimations are shown in Figure 4.

Gel filtration on Sephadex G75 Superfine gave a large protein peak

Figure 4. Separation of the 61-80% $(\text{NH}_4)_2\text{SO}_4$ fraction precipitate of cucumber rot extract on Sephadex G75 Superfine



△-△ PTE ○-○ Cell separation ●-● Toxicity ---- Protein

associated with maximum PTE activity in fractions 32 - 40. Some purification of the PTE was achieved as much protein was eluted before the PTE. Cell separation, toxicity and PTE showed maximum activities in the same fractions demonstrating again the close association of these activities.

3. Isoelectric focusing

Fractions from Sephadex gel filtration containing the bulk of the PTE activity were bulked and further purified by isoelectric focusing. 31 ml of sample containing the PTE was subjected to isoelectric focusing between pH 7 and 10 for 52h at 4°C.

It has been stated that as sucrose reduces the dielectric constant of aqueous media then the higher the sucrose concentration at the focused protein zone, the lower the pI value obtained for that protein (Flatmark and Vesterbeg, 1966). In preliminary runs in this work, it was found that the PTE in cucumber rot extract became focused near the cathode and thus showed a high pI. In order to minimize distortion of the measured pI of the PTE by the concentration of sucrose, the top electrode of the column was used as the cathode.

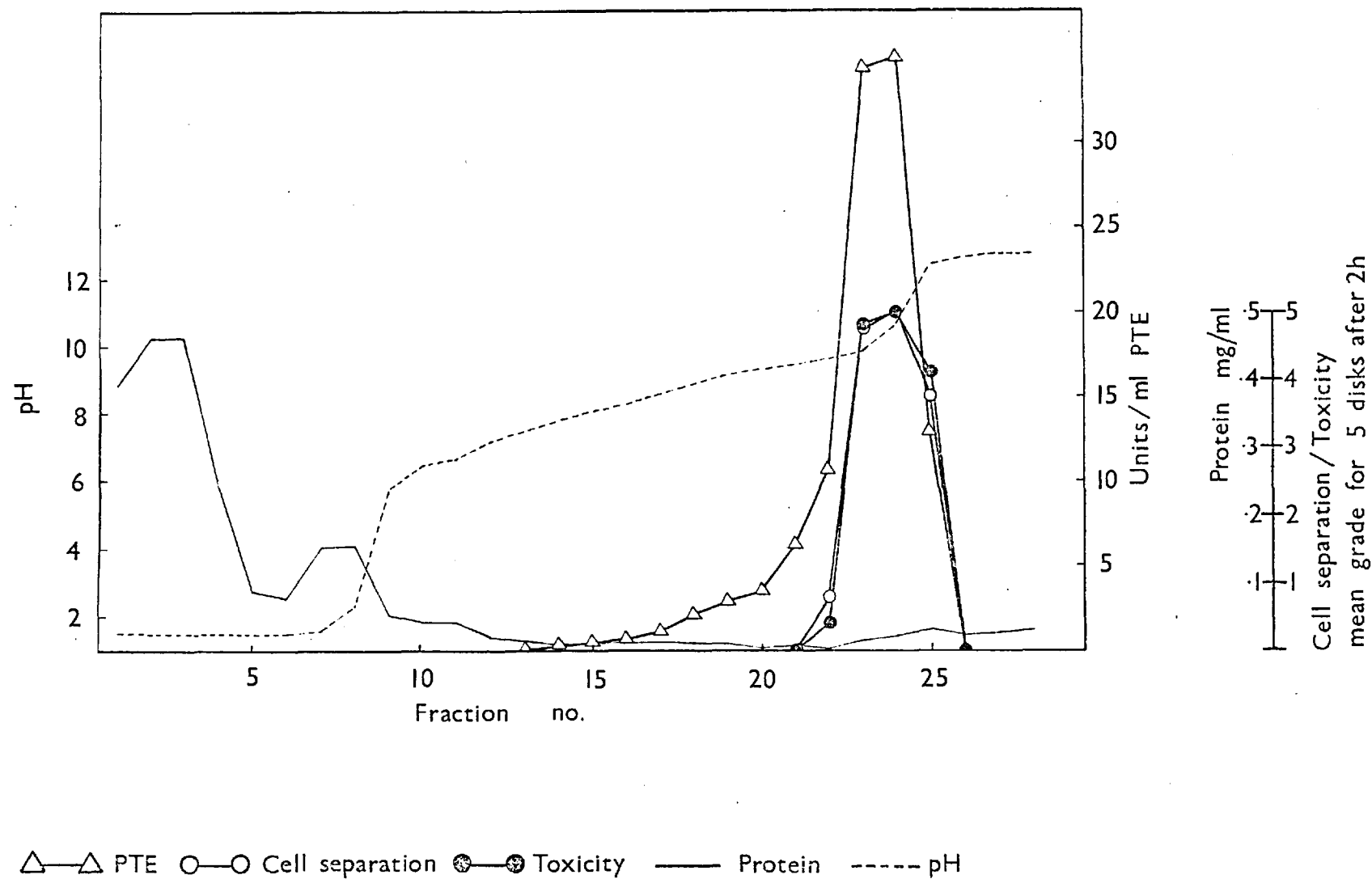
5 ml fractions were collected from the column and after determinations of pH and protein (by the ultraviolet absorption method) had been made, dialysed for 18h against water at 4°C to remove sucrose and the ampholine carriers. After dialysis all fractions had a pH about neutrality. The volume of each fraction after dialysis was measured before dividing into small aliquots and storing at -20°C.

PTE activity was estimated by the thiobarbituric acid test and expressed as units/ml PTE before dialysis.

Cell separation and toxicity were estimated using reactions mixtures containing 0.5 ml of the appropriate fraction, 0.5 ml water, 0.001M CaCl_2 , 0.02M Tris-HCl buffer at pH 9.0 and 15 potato tuber disks. 5 disks were removed after 60, 90 and 120 min and cell separation and toxicity were estimated.

Results are shown in Figure 5.

Figure 5. Isoelectric focusing of PTE after partial purification by gel filtration



A single peak of PTE activity became focused in fractions 22 - 25. The pH at this point and thus the pI of the PTE was 10.3. Good separation of protein and PTE was achieved by this method, the bulk of the protein being present in first few fractions.

Cell separating and toxic activities were highest in those fractions containing maximum PTE activity, and absent from fractions showing no or little PTE activity.

The work of Mount et al. (1970) and Garibaldi and Bateman (1971), has shown that isoelectric focusing can separate isoenzymes of PTE. From the results obtained in the present work it appeared that the PTE from cucumber rot extract was a single component of high pI, focusing at about the limit of the pH gradient obtained with the carrier ampholytes available. It was possible that isoenzymes of this PTE with similar isoelectric points occurred within a narrow pH range around 10.3. Because no carrier ampholytes were available for pH gradients with an upper limit in excess of pH 10, attempts were made to extend the gradient with basic amino acids because Haská^O (1972), found that arginine and lysine acted this way in combination with LKB carrier ampholytes (pH 7 - 10).

Two of the three methods described by Haská^O (1972) were used. In the first, 400 mg arginine, 100 mg lysine and 600 mg pH 7 - 10 ampholine were used to give an expected pH gradient of 7 - 11 with dialysed fractions containing PTE obtained in a previous isoelectric focusing run. Focusing was carried out for 43h at 4°C. The pH of each fraction was determined and after dialysis PTE activity estimated by the thiobarbituric acid test.

No PTE activity was recovered from the column. Haská^O (1972), states that some loss of activity of his enzyme samples occurred during focusing. The pH gradient obtained is shown in Table 12.

In the second method 800 mg arginine, 200 mg lysine and 200 mg pH 7 - 10 ampholine were used to give an expected pH gradient of 7 - 12. Five ml dialysed cucumber rot extract was used and 5 ml fractions were collected after focusing for 46h at 4°C. After estimation of pH and protein content

(by the ultraviolet absorption method) all fractions were dialysed against water. PTE activity was estimated by the thiobarbituric acid method and expressed as units/ml before dialysis. The results are shown in Table 12.

Table 12. Isoelectric focusing using extended pH gradients

Fraction No.	Method 1 (pH 7 - 11)	Method 2 (pH 7 - 12)		
	pH	pH	Protein mg/ml	Units/ml PTE
1	1.60	1.70	0.277	0.15
2	1.60	1.70	0.387	0.42
3	1.60	1.70	0.487	0.24
4	3.10	4.81	0.310	0.67
5	6.40	6.71	0.218	1.93
6	6.73	7.23	0.169	0.92
7	7.57	8.92	0.168	4.40
8	8.17	9.85	0.101	5.40
9	8.74	10.20	0.098	2.80
10	9.22	10.27	0.127	2.60
11	9.57	10.31	0.105	1.80
12	9.99	10.33	0.073	1.96
13	10.23	10.37	0.050	1.80
14	10.30	10.40	0.030	1.10
15	10.32	10.37	0.017	0.72
16	10.32	10.43	0.010	1.24
17	10.35	10.46	0.008	1.00
18	10.37	10.47	0.005	0.76
19	10.37	10.48	0.005	1.60
20	10.39	10.48	0.003	0.22
21	10.39	10.58	0.001	0.18
22	10.43	10.83	0.002	0.12
23	10.58	11.56	0.004	0.16
24	11.80	12.50	0.015	0
25	12.50	12.80	0.017	0

With both methods a large plateau in the pH gradient was obtained. The first method gave 12 fractions (12 - 23) with a pH difference of only 0.59. The second method gave a slightly extended pH range compared with the first method, but again 14 fractions were obtained giving a pH

difference of 0.63. In the second method low PTE activity was detected in most fractions and was highest in fraction 8 (pH 9.85). Satisfactory focusing of the PTE was not obtained by these methods and thus purification of PTE in subsequent runs was achieved by use of standard LKB carrier ampholytes (pH 7 - 10).

Samples from Sephadex gel, or in later experiments ion-exchange gel, columns were subjected to isoelectric focusing as described in 'Materials and Methods'. Five ml fractions were collected and the pH of each measured. As the protein concentration of the majority of the fractions was very low estimates were not made in subsequent runs.

PTE activity of the fractions was estimated initially by either the thiobarbituric acid test or the cup plate method. The latter had the advantage that large numbers of samples could be used relatively easily and that estimates of activity could be made before dialysis of the fractions. The plates were incubated at 25°C for 18h and during this time the fractions were dialysed against water at 4°C. When using the thiobarbituric acid method the fractions were assayed directly after dialysis.

Fractions containing high PTE activity were bulked, then divided into 1 ml aliquots and stored at -20°C. The PTE activity of these samples was finally estimated by the thiobarbituric acid test. The activity of the first batch of PTE was 14.8 units/ml and for subsequent batches fractions were selected to give a final PTE activity in excess of this amount.

In several isoelectric focusing runs the estimated pI of the PTE was 10.1, 10.4, 10.3 and 10.7 giving a mean pI of 10.4.

4. Activities of cucumber rot extract and purified PTE samples

Fractions from the isoelectric focusing run shown in Figure 5 containing the bulk of the PTE activity were tested for a range of enzyme activities. Cucumber rot extract was also tested to detect activities present before and after purification of the PTE in order to determine how successful the purification had been.

The activities of the isoelectric focusing fractions were estimated against a water control and cucumber rot extract estimated against an autoclaved rot extract control.

Pectin methylesterase (PME) and phosphatidase activities were estimated by cup-plate methods.

Proteinase activity was estimated by the congocoll method.

α -D-galactopyranosidase and β -D-mannosidase activities were estimated at pH 5 and pH 8 using p-nitrophenyl substrates (Koch-Light Ltd., Colnbrook, Bucks). Reaction mixtures contained 1.8 ml 0.1M sodium citrate buffer at pH 5 or Tris-HCl buffer at pH 8 containing 0.8 mg of p-nitrophenyl substrate and 0.2 ml enzyme sample. After 2h at 30°C, 1 ml of N NH₄OH containing 2 mM disodium EDTA was added and the yellow colour estimated at 400 nm.

Cellulase (CMCase), xylanase and arabinanase activities were estimated by the Nelson-Somogyi method. Solutions of CMC (1% w/v) adjusted to pH 5 and 8, were mixed with an equal volume of 0.2M sodium citrate buffer at pH 5 or 0.2M Tris-HCl buffer at pH 8. Reaction mixtures contained 0.8 ml 0.5% w/v CMC in 0.1M buffer and 0.2 ml enzyme sample.

Xylanase activity was estimated in a similar way using 0.25% w/v Larchwood xylan (Sigma Chem. Co. Ltd.) in 0.1M buffer at pH 5 or 8.

Arabinanase activity was estimated using 0.25% w/v arabinan (Koch-Light Ltd.) in 0.1M sodium citrate buffer at pH 5.

The results in Table 13 show that some colour was present in the isoelectric focusing fractions after incubation with congocoll. This absorbance was low and probably due to uneven leaching of congo red in the different samples rather than to release by enzyme action. Subsequent samples of PTE purified in this way showed no release of congo red when the substrate was finely ground in a pestle and mortar.

Cucumber rot extract contains proteinase, phosphatidase, α -D-galactopyranosidase, xylanase, arabinanase (although low) and cellulase but not PME or β -D-mannosidase activities. All these activities were absent from the isoelectric focusing fractions high in PTE activity, demonstrating that purification had been successful.

Table 13.

Activities of cucumber rot extract and purified PTE samples

Sample		Activity*											
		PME	Proteinase	Phosphatidase	α -D-galactopyranosidase		β -D-mannosidase		Xylanase		Arabinanase	CMCase	
					pH5	pH8	pH5	pH8	pH5	pH8		pH5	pH8
Isoelectric focusing fraction	20	0	0.009	0	0	0	0	0	0	0	0	0	0
	21	0	0	0	0	0	0	0	0	0	0	0	0
	22	0	0.015	0	0	0	0	0	0	0	0	0	0
	23	0	0.012	0	0	0	0	0	0	0	0	0	0
	24	0	0.010	0	0	0	0	0	0	0	0	0	0
	25	0	0.012	0	0	0	0	0	0	0	0	0	0
Cucumber rot extract		0	0.90	1.8	1.67	0.16	0	0	0.19	0.23	0.02	0.65	0.38

* - PME and phosphatidase expressed as area (cm²) of zone around the well; proteinase expressed as increase in absorbance (OD units) at 495 nm; α -D-galactopyranosidase and β -D-mannosidase expressed as μ g p-nitrophenol liberated/0.2 ml enzyme/2h; CMCase, xylanase and arabinanase expressed as μ moles reducing groups released/0.2 ml enzyme/2h.

5. Gel electrophoresis of PTE

A sample of PTE prepared by Sephadex gel filtration followed by isoelectric focusing was run on 7.5% polyacrylamide gel at pH 2.3. The gel was stained for 4h in Coomassie brilliant blue and then destained for several days in changes of solvent. Only one band of protein was found indicating that the PTE was composed of a single protein component.

This was repeated using a sample of PTE prepared by ion-exchange chromatography followed by isoelectric focusing. Plate 1 shows that a single band of protein resulted after staining.

This demonstrates that PTE is composed of a single protein component which is enzymically active. There are apparently no isoenzyme forms of this enzyme when prepared in this way from cucumber rot extract.

6. Estimation of molecular weight of PTE

A procedure of gel filtration on Sephadex G75 was used as described by Andrews (1964). The column was equilibrated with 0.005M Tris-HCl buffer at pH 9.0 containing 0.1M NaCl which was used as the eluant. Blue dextran (9 mg), yellow dextran (9 mg), bovine serum albumin (9 mg), cytochrome c (5 mg) and vitamin B₁₂ (0.27 mg) were used as reference standards. These standards were taken up in 4 ml of purified PTE solution obtained by isoelectric focusing. 1 ml fractions were collected.

Blue dextran was estimated by measuring absorbance at 630 nm, yellow dextran at 400 nm, cytochrome c at 410 nm and vitamin B₁₂ at 550 nm. Bovine serum albumin was estimated with the Folin-Ciocalteu reagent and PTE by the thiobarbituric acid test.

Blue dextran (molecular weight 2×10^6) was used to determine the void volume of the column ($V_e/V_o = 1.0$). The V_e/V_o values for bovine serum albumin (molecular weight 67,000), cytochrome c (molecular weight 12,400) and vitamin B₁₂ (molecular weight 1,357) were 1.13, 2.32 and 3.49 respectively. No estimate could be obtained for the elution volume of the yellow dextran. The V_e/V_o value for PTE was 1.66 in this system



Plate 1. x 1. Electrophoresis of PTE on 7.5% gel at pH 2.3

which gave an estimated molecular weight of c. 31,000.

This result is in agreement with the findings of Mount et al. (1970) who estimated the molecular weight of the PTE of E. carotovora to be 31,000. Garibaldi and Bateman (1971) estimate the molecular weight of the PTE of E. chrysanthemi to be c. 30,000. Both of these values were estimated by gel filtration.

IV. Properties of PTE and cucumber rot extract

1. Cell separating activity, toxicity and effect of plasmolysis

It has already been shown that cucumber rot extract and PTE can cause cell separation and death of potato tuber tissue and that these activities are closely related. A separation of these two activities has been achieved when tissue is treated by enzyme preparations in hypertonic solutions of salts or non-electrolytes (Tribe, 1955). Resolution of cell separating activity and toxicity by this method was attempted for cucumber rot extract and also for PTE.

A. Effect of sucrose

Potato tuber disks were treated with rot extract or equivalent activity of PTE (14.8 units PTE/3 ml reaction mixture). Sucrose was included in the reaction mixture to give final concentrations of 0, 0.1, 0.2, 0.3 and 0.5M. Disks were kept in each concentration of sucrose for 45 min before adding to reaction mixtures.

The results (Table 14) show that in the absence of sucrose both rot extract and PTE show very similar cell separating and toxic activities. The cucumber rot extract caused complete loss of coherence of disks and total killing of protoplasts in slightly less time than did PTE. This may have been due to the influence of other enzymes present in the crude extract.

Table 14.

Effect of sucrose on cell separating activity and toxicity of PTE and cucumber rot extract

Time (h)	Sucrose concentration																			
	OM				0.1M				0.2M				0.3M*				0.5M*			
	PTE		EXTRACT		PTE		EXTRACT		PTE		EXTRACT		PTE		EXTRACT		PTE		EXTRACT	
	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD
0.5	0.6	1.0	1.5	2.5	0.6	0.2	1.2	1.0	0.6	0	0.6	1.5	0.9	0	0.6	0.2	0.6	0	0.6	0.2
1	2.7	3.2	3.4	3.7	3.1	3.0	3.4	3.2	2.7	0	2.7	2.5	1.2	0	2.7	1.7	1.9	0	2.5	1.0
1.5	4.4	4.2	4.6	4.6	4.0	3.5	4.6	4.4	3.4	0	3.4	3.0	3.1	0	3.1	2.5	2.5	0	3.4	2.0
2	4.6	4.7	5.0	5.0	4.6	4.4	5.0	4.7	4.0	0	4.4	3.5	3.4	0	4.4	3.0	3.1	0	4.0	3.0
2.5	5.0	5.0			5.0	4.5	5.0	5.0	4.0	0	5.0	4.0	4.0	0	5.0	3.6	4.0	0	5.0	3.5
3					5.0	4.7			4.6	0	5.0	4.2	4.6	0	5.0	4.4	4.0	0	5.0	4.2
3.5					5.0	4.9			5.0	0.5	5.0	4.5	5.0	0	5.0	4.5	5.0	0	5.0	4.4
4					5.0	5.0			5.0	0.5	5.0	4.9	5.0	0	5.0	4.7	5.0	0	5.0	4.6
5									5.0	0.7	5.0	5.0	5.0	0	5.0	5.0	5.0	0	5.0	4.7
6									5.0	1.5			5.0	0			5.0	0	5.0	5.0
24									5.0	2.2			5.0	0			5.0	0		

Extract - cucumber rot extract

CS - cell separation

CD - cell death

Results denote mean grade for 4 disks

* - concentration hypertonic for potato tissue

It could be seen that much of the cell separating activity and toxicity of crude cucumber rot extract was due to its PTE content. In the absence of sucrose cell death paralleled cell separation with both enzyme preparations.

As the concentration of sucrose was increased the action of rot extract in killing protoplasts was considerably delayed. This was in contrast to the slight effect on cell separation which may have been due to a dehydration of the cell walls by the plasmolysing agent.

Sucrose caused a similar effect on the cell separating activity of PTE. The toxic action of PTE was delayed for much longer periods at 0.2M and abolished at higher concentrations which cause strong plasmolysis of the protoplasts of potato tuber tissue. In subsequent experiments all protoplasts were alive after 24h in PTE in 0.25M sucrose but only protoplasts that were plasmolysed remained alive in 0.2M sucrose.

Autoclaved PTE or rot extract caused no cell separation or toxicity and are thus omitted from Table 14. Similarly 0.5M sucrose in the absence of the enzyme preparations showed no effect on potato disks within 24h.

These results suggest that crude cucumber rot extracts contain a heat labile factor or factors other than PTE able to cause death of plasmolysed protoplasts of potato tuber tissue.

B. Effect of KNO_3

The previous experiment was repeated with an ionic plasmolysing agent KNO_3 in reaction mixtures at final concentrations of 0, 0.05, 0.1 and 0.2M. Protoplasts of potato tuber tissue were plasmolysed at 0.2M.

The results (Table 15) show that the cell separating activity and toxicity of cucumber rot extract are affected by KNO_3 in a similar way to that found with sucrose. KNO_3 below 0.2M had little effect on cell separation or toxicity but both activities were greatly decreased at 0.2M.

With PTE different effects were found. Whereas sucrose had little effect on cell separation even at plasmolysing concentrations, KNO_3 even at

Table 15. Effect of KNO₃ on cell separating activity and toxicity of PTE and cucumber rot extract

Time	KNO ₃ concentration															
	OM				0.05M				0.1M				0.2M*			
	PTE		EXTRACT		PTE		EXTRACT		PTE		EXTRACT		PTE		EXTRACT	
	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD
20 (min)	0.9	1.2	0.9	1.2	-		0.6	0.5	-		0.2	0.2	-		0	0
40	1.2	1.7	2.7	3.0	-		2.7	2.5	-		2.1	1.0	-		2.1	0.2
60	2.5	3.0	4.4	4.6	0	0	4.0	3.5	0	0	3.7	3.4	0	0	2.7	1.0
80	4.0	4.1	5.0	4.6	-		5.0	4.6	-		4.4	3.9	-		3.7	1.0
120	5.0	4.6	5.0	5.0	2.1	2.0	5.0	5.0	0.6	0.2	5.0	4.5	0	0	4.0	1.7
2.5(h)	5.0	5.0			2.5	2.0			1.2	1.0	5.0	5.0	0.6	0	4.4	2.0
3					2.5	2.6			1.9	2.2			1.5	0	5.0	2.7
3.5					3.1	3.6			-				1.5	0	-	
4					4.4	4.2			2.1	3.0			1.5	0	5.0	3.0
5					5.0	4.6			3.1	3.4			1.9	0	5.0	4.2
6					5.0	4.6			3.7	4.4			2.1	0	5.0	4.6
20					5.0	5.0			5.0	5.0			2.5	0		

Extract - cucumber rot extract CS - cell separation CD - cell death Results denote mean grade for 4 disks

* - concentration hypertonic for potato tissue

0.05M greatly delayed cell separation and also death. At 0.2M which is hypertonic for potato the toxic action was abolished as found previously with sucrose. In striking contrast, even after 20h, cell separation showed only a grade 2.5 i.e. 50% loss of coherence of the tissue.

From these results it is clear that the cell separating action of PTE is strongly inhibited by the KNO_3 . This effect was virtually absent from disks treated with cucumber rot extract. As both cell separation and death with PTE are delayed even with hypotonic concentrations of KNO_3 it appears that the toxic action is somehow dependent on cell separation.

C. Effect of different salts

Effects similar to those obtained in the last experiment were reported by Spalding (1969). He found that many ions decreased cell separating action of PG in extracts from sweet potatoes rotted by Rhizopus stolonifer. Anions were more effective than cations and the more reactive the anion the greater its inhibitory effect. Thus $\text{NO}_3^- < \text{NO}_2^- < \text{N}_3^-$.

The apparent inhibition of the cell separating action of PTE by subplasmolytic concentrations of KNO_3 was further investigated using a range of salts. These were selected to provide a variety of cations and anions and all were tested at 0.1M which is hypotonic for potato disks.

Reaction mixtures were as in the previous experiment and disks were kept in the appropriate salt solution for 45 min before adding to reaction mixtures. The results are summarized in Table 16 (a) and (b).

Each of the salts showed some inhibition of cell separation caused by both enzyme preparations. The effect was more pronounced with PTE. Nitrite ions were no more inhibitory than nitrate ions and cations were more effective than anions. When cell separation was delayed there was a corresponding delay in toxicity. The salts used showed little toxicity to the potato disks even after 20h.

It appeared that inhibition of cell separation was related to the valency of the cation used. Thus $\text{Mg}(\text{NO}_3)_2$ was more effective than KNO_3 and

Table 16(a) Effect of different salts on cell separating activity and toxicity of cucumber rot extract

Time (min)	Salt (0.1M)																	
	No salt		KNO ₃		KNO ₂		KCl		NaNO ₃		NaCl		Na ₂ SO ₄		Mg(NO ₃) ₂		MgSO ₄	
	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD
20	0.2	0.2	0	0	0	0	0	0	0	0	0	0.5	0	0.7	0	0.5	0	0.5
40	1.5	1.5	0.9	0.2	1.5	1.0	0.2	0.2	0.6	0.2	0	0.7	0.9	1.2	0	0.5	0	0.5
60	3.1	2.7	2.5	1.7	1.9	1.2	1.9	1.7	0.9	0.5	1.5	2.0	0.9	1.2	0.6	0.5	0.9	2.0
80	4.4	4.2	3.7	2.2	2.7	2.1	2.7	3.7	3.1	2.0	2.7	4.0	1.9	1.2	0.2	0.2	1.2	2.2
100	5.0	4.9	4.4	3.0	4.4	4.4	4.4	3.7	3.1	2.0	4.4	4.1	1.5	1.5	0.9	0.7	1.5	1.5
120	5.0	5.0	4.6	4.0	5.0	4.4	5.0	3.9	3.1	2.7	4.4	3.9	1.5	0.7	0.9	0.7	2.1	1.7
150			5.0	4.0	5.0	4.4	5.0	4.1	4.6	4.0	5.0	4.7	2.1	2.3	1.2	1.0	2.5	1.6
180			5.0	4.0	5.0	4.6	5.0	4.7	5.0	4.4	5.0	4.9	3.7	3.4	1.2	0.5	2.5	1.6
210			5.0	4.5	5.0	5.0	5.0	4.7	5.0	4.7			-		-		-	
240			5.0	4.7									3.7	3.2	1.2	0.5	3.1	3.0
270			5.0	4.7									-		-		-	
300													4.4*	4.0*	2.5*	3.5*	3.7*	3.9*
360													5.0*	4.2*	3.4*	3.2*	3.7*	3.0*

CS - cell separation

CD - cell death

Results denote mean grade for 4 disks (* - mean for 2 disks)

Table 16(b)

Effect of different salts on cell separating activity and toxicity of PTE

Time (min)	Salt (0.1M)																	
	No salt		KNO ₃		KNO ₂		KCl		NaNO ₃		NaCl		Na ₂ SO ₄		Mg(NO ₃) ₂		MgSO ₄	
	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD
20	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40	0.6	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	1.5	2.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
80	2.1	2.6	0	0	0	0.2	0.2	0.2	0	0	0.2	1.2	0	0	0	0.2	0	0.5
100	4.0	3.7	0	0	0.6	1.7	0.2	0.5	0	0	0.9	1.5	0	0.7	0	0.2	0	0.7
120	5.0	4.9	0	0.2	0.9	1.7	0.6	0.5	0	0.2	0.9	1.0	0.2	1.5	0	0.5	0	1.7
150	5.0	5.0	0.6	0.5	1.9	2.5	0.9	1.7	0.6	1.2	1.2	2.0	0.2	0.7	0	0.5	0	2.0
180			0.9	0.7	2.5	2.5	3.1	3.5	1.5	3.0	2.5	3.5	1.5	1.0	0	0.5	0	2.0
210			1.2	1.2	2.5	2.2	3.4	3.9	1.5	3.0	2.1	2.5	1.2	0.2	0	0.5	0	1.2
240			1.9	2.5	3.1	3.1	4.0	3.9	1.9	3.2	1.9	2.0	1.2	1.2	0	0.5	0	2.2
270			2.7	3.2	3.1	3.7	4.4	4.2	2.5*	3.0*	2.1	2.9	1.2	0.8	0	0.2	0	3.2
300			3.1	3.4	3.7*	4.2*	4.4*	4.2*	2.7	3.2	3.1	3.0	1.2	1.5	0	0.5	0	2.2
360			2.7	3.5	4.4	4.5	5.0	4.7	3.4	4.1	3.7	3.6	1.2	1.5	0	1.0	0	1.8
20(h)											5.0*	4.7*	2.5*	3.0*	0.2	2.0*	1.2*	2.0*

CS - cell separation

CD - cell death

Results denote mean grade for 4 disks (* - mean for 2 disks)

MgSO₄ more effective than Na₂SO₄.

D. Effect of non-electrolytes at hypotonic concentrations

Sucrose at 0.1M final concentration had little effect on cell separation by rot extract or PTE. 0.1M mannitol was also tested for comparison. Reaction mixtures were as above and results shown are in Table 17.

Mannitol at 0.1M had little effect on cell separation and toxicity with both enzyme preparations as, again, did 0.1M sucrose. The fact that cell separating and toxic activities of PTE especially were greatly delayed by KNO₃ shows that inhibition is an ionic effect.

Table 17. Effect of non-electrolytes at hypotonic concentrations on cell separating activity and toxicity of cucumber rot extract and PTE

Time (min)	0.1M Sucrose				0.1M Mannitol			
	PTE		EXTRACT		PTE		EXTRACT	
	CS	CD	CS	CD	CS	CD	CS	CD
20	0	0	0	0	0	0	0	0
30	0	0	0.6	0.2	0	0	0.2	0.2
40	0.9	0.7	2.5	1.2	1.5	0	1.5	1.2
50	1.5	0.7	2.7	1.7	1.5	1.0	1.9	1.2
60	2.1	2.5	2.7	2.0	1.9	1.7	2.7	2.0
70	2.1	2.2	3.1	2.2	1.2	1.5	3.4	3.0
80	2.1	2.5	4.4	3.4	2.7	2.5	4.0	3.2
90	3.1	3.0	4.6	4.0	3.4	3.0	4.4	3.9
100	4.0	3.7	4.6	4.6	4.4	3.7	4.4	4.2
120	4.6	4.2	5.0	4.7	4.4	4.0	4.6	4.5

Extract - cucumber rot extract CS - cell separation

CD - cell death Results denote mean grade for 4 disks

E. Effect of salts on NaPP degradation by cucumber rot extract and PTE

Divalent cations such as Mg²⁺ or Ca²⁺ inhibit pectic enzymes by

binding with pectic substrates to form compounds resistant to degradation (Bateman, 1964).

In an attempt to explain inhibition of cell separation by different salts a cup plate assay was used. 7 mm diam wells were cut in 1% w/v NaPP buffered at pH 9.0 in 1% w/v agar and into these were pipetted 0.01 ml 0.004M CaCl_2 , 0.01 ml of each salt solution at 0.4M and 0.02 ml of cucumber rot extract or PTE at equivalent activity. After 18h at 25°C activity was estimated as the area (cm^2) of the zone around each well.

Table 18. Effect of salts on degradation of NaPP by cucumber rot extract and PTE

Salt (0.1M)	Area of zone (cm^2)	
	PTE	EXTRACT
No salt	2.92	2.92
KNO_3	2.92	2.92
KNO_2	3.00	3.00
KCl	3.00	3.00
NaNO_3	2.92	3.00
NaCl	3.00	2.92
Na_2SO_4	3.08	3.08
$\text{Mg}(\text{NO}_3)_2$	1.95	2.38
MgSO_4	2.04	2.45
Extract - cucumber rot extract		

Results denote mean area for 2 replicates

The results (Table 18) show that the action on NaPP by both enzyme preparations was similar in the absence of salt or with the monovalent cation salts. Divalent cation salts reduced degradation of NaPP.

Spalding (1969) regards inhibition of cell separation by ions to be

due to blocking of enzyme sites in the walls. From these results it appears that there is a two-fold effect (a) ionic binding and blocking of enzyme sites in walls of potato disks which to a certain extent can be overcome by factors in cucumber rot extract other than PTE, and (b) chemical binding by multivalent cations to substrate in walls producing a compound resistant to attack by the enzymes.

F. Effect of washing on inhibition of cell separation by salts

Spalding (1969) has shown that the inhibitory effect of ions on cell separation can be removed by washing the tissue in water. He considered this as evidence against any chemical binding with substrates in walls.

This effect was investigated with PTE. Fifty six potato disks after equilibration with salt solutions for 45 min were placed in reaction mixtures as described previously. Disks were sampled at intervals. After 2h 16 disks were removed washed well in water and placed in fresh reaction mixtures. These reaction mixtures were of the same composition and prepared at the same time as the previous set but contained no salt. Total volume of the second set was 0.75 ml. Disks were sampled at intervals from both sets of reaction mixtures and cell separation and toxicity estimated. Results are shown in Table 19.

No cell separation occurred with disks in solutions of divalent cation salts within 6h. After washing, disks from divalent salt solutions showed complete loss of coherence within 2h. This demonstrates that most of the inhibitory effect of the salts tested could be removed by washing in water. This suggests that ionic binding to sites and thereby blocking enzyme action is the more likely explanation for this inhibitory effect.

This experiment also demonstrated that when cell separating activity was restored by washing free of the inhibitors toxicity was also restored. This provides further evidence for the interrelation of these two activities.

Table 19. Effect of washing on inhibition of cell separation by salts

Time (min)	0.1M KNO_3				0.1M $\text{Mg}(\text{NO}_3)_2$			
	CS	CD	CS	CD	CS	CD	CS	CD
30	0	0			0	0		
60	0	0			0	0		
90	0	0.2			0	0		
120	0	0.5 →			0	0 →		
150	0.2	0.2	2.7	2.7	0	0	1.5	2.0
180	0.2	0.5	4.6	4.6	0	0	3.7	3.9
210	1.5	2.2	5.0	5.0	0	0.2	4.6	4.4
240	1.5	2.0	5.0	5.0	0	0.2	5.0	5.0
300	2.7	2.2			0	0.2		
360	3.1	3.2			0	0.2		

CS - cell separation

CD - cell death

→ - Transfer of washed disks to fresh reaction mixture

Results indicate mean grade for 4 disks

2. Permeability increasing activity

A. Release of ions

Rot extract and PTE were tested for permeability increasing activity. After conductivity measurements had been made the bathing solution was decanted and filtered through Whatman No. 40 ashless filter paper. Ion determinations were made on these samples using a flame photometer. These estimates are shown in Table 20 (a) and (b).

Rot extract and PTE rapidly increased the permeability to electrolytes of the potato tuber disks increases being detectable in the first 15 minutes. Conductivity of the bathing solutions reached a

Table 20 (a). Permeability increases and release of ions by potato disks treated with cucumber rot extract

Time (min)	EXTRACT (A)				EXTRACT *			
	Δ Condu- ctivity μ mos	K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm	Δ Condu- ctivity μ mos	K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm
5	2.4(16)	0.7(13)	0.4(13)	0.7(13)	12.2(11)	3.8(9)	1.8(9)	0.3(17)
15	1.9(13)	0.6(11)	0.4(13)	0.7(13)	21.9(20)	7.0(16)	3.2(16)	0.1(6)
30	1.6(11)	0.6(11)	0.3(10)	0.6(11)	23.8(21)	8.3(19)	3.8(19)	0.5(28)
45	2.2(15)	0.7(13)	0.4(13)	0.7(13)	16.9(15)	6.2(14)	2.8(14)	0 (0)
60	1.6(11)	0.7(13)	0.3(10)	0.7(13)	12.3(11)	5.1(12)	2.4(12)	0.4(22)
75	1.7(11)	0.6(11)	0.3(10)	0.6(11)	10.9(10)	5.7(13)	2.5(12)	0.2(11)
90	1.8(12)	0.7(13)	0.4(13)	0.8(14)	8.7(8)	4.7(11)	2.0(10)	0.1(6)
120	1.8(12)	0.8(15)	0.5(17)	0.8(14)	4.5(4)	3.5(8)	1.6(8)	0.2(11)
Total	15.0(100)	5.4(100)	3.0(100)	5.6(100)	111.2(100)	44.3(100)	20.1(100)	1.8(100)

Extract - cucumber rot extract (A) - autoclaved

Results are expressed as increase in conductivity μ mos/15 min/5 disks/15 ml together with content (ppm) of K⁺, Ca²⁺ and Na⁺ of 15 ml bathing solution and represent means for two replicates

* - increase compared with autoclaved enzyme control

Numbers in parentheses indicate percentages of total

Table 20 (b). Permeability increases and release of ions by potato disks treated with PTE

Time (min)	Δ Condu- ctivity μ hos	PTE (A)			Δ Condu- ctivity μ hos	PTE *		
		K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm		K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm
5	2.5(19)	0.7(12)	0.6(10)	0.6(9)	1.9(0.2)	0.8(22)	0.5(3)	0.4(13)
15	1.8(14)	0.6(10)	0.9(15)	0.7(10)	12.0(10)	3.6(10)	1.4(8)	0.2(7)
30	1.1(8)	0.6(10)	0.5(8)	0.6(9)	21.0(17)	5.7(15)	2.8(17)	0.5(17)
45	1.2(9)	0.8(14)	0.9(15)	0.9(13)	27.0(22)	7.1(19)	3.0(18)	0.2(7)
60	1.6(12)	0.9(16)	1.0(16)	1.3(19)	20.1(16)	5.4(15)	2.5(15)	0 (0)
75	1.6(12)	0.6(10)	0.6(10)	0.9(13)	18.1(14)	5.9(16)	2.7(16)	0.1(3)
90	1.7(13)	0.8(14)	0.8(13)	1.0(15)	13.6(1)	4.3(12)	1.9(11)	0.5(17)
120	1.8(14)	0.8(14)	0.8(13)	0.8(12)	11.6(1)	4.1(11)	1.9(11)	1.1(37)
Total	13.2(100)	5.8(100)	6.1(100)	6.8(100)	125.3(100)	36.9(100)	16.7(100)	3.0(100)

(A) - autoclaved

Results are expressed as increase in conductivity μ hos/15 min/5 disks/15 ml together with content (ppm) of K⁺, Ca²⁺ and Na⁺ of 15 ml bathing solution and represent means for two replicates

* - increase compared with autoclaved enzyme control

Numbers in parentheses indicate percentages of total

maximum after 30 and 45 min respectively and then declined probably because of depletion of ions within the disks. Autoclaved enzyme preparations caused no such increases.

Leakage from the disks of K^+ and to a lesser extent Ca^{2+} closely followed the conductivity changes with both enzyme preparations. The levels of Na^+ were consistently low and did not follow the same pattern of leakage. Levels of all three ions remained low with the autoclaved enzyme preparations.

This demonstrates that much of the permeability increasing activity of rot extracts is due to their PTE content although effects with extracts were somewhat more rapid. Leakage of electrolytes from potato disks especially K^+ occur very rapidly reaching a maximum long before cell separation is complete. Thus for PTE, cell separation is complete within 120 min but much of the K^+ of the cell is lost in the first 45 min.

B. Permeability increasing activity of ethanol and Triton X-100

As the plasmalemma is composed of protein and phospholipid it is difficult to understand how a purified pectic enzyme can cause such drastic alterations in the properties of this membrane. Ethanol and Triton X-100, a non-ionic detergent, were used to determine whether similar alterations in permeability and consequent leakage of ions from potato tuber disks could be induced. Both substances disrupt membranes.

Fifty potato tuber disks were placed in 8 ml 30% v/v ethanol, 2% v/v Triton X-100 or water containing 0.001M $CaCl_2$ and 0.02M Tris-HCl buffer at pH 9.0. Conductivity measurements were made on samples of 5 disks as before and after filtration ions in bathing solutions were determined. Results are shown in Table 21.

Ethanol and Triton X-100 both rapidly increased the permeability of potato disks. Maximum conductivity changes occurred within 5 and 10 min respectively. Leakage of ions followed the same pattern as changes in conductivity especially leakage of K^+ caused by both treatments. There were no conductivity changes or increased leakage of ions in water controls.

Table 21. Permeability increases and release of ions by potato disks in ethanol, Triton X-100 or water controls

Time (min)	Water control				30% Ethanol*				2% Triton X-100*			
	Δ Conduc- tivity μ hos	K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm	Δ Conduc- tivity μ hos	K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm	Δ Conduc- tivity μ hos	K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm
1	1.8(14)	0.8(22)	0.7(19)	0.4(22)	6.2(7)	2.3(6)	0 (0)	0 (0)	3.5(5)	1.4(5)	0 (0)	0 (0)
5	1.8(14)	0.6(17)	0.5(14)	0.2(11)	29.7(32)	9.0(24)	1.4(33)	0.1(25)	16.4(25)	6.8(23)	0.9(24)	0 (0)
10	1.8(14)	0.5(14)	0.5(14)	0.2(11)	21.2(23)	7.8(21)	1.0(24)	0.1(25)	28.8(44)	10.2(34)	1.4(38)	0 (0)
20	1.7(14)	0.4(11)	0.4(11)	0.2(11)	15.2(17)	7.7(20)	1.1(26)	0.1(25)	10.0(15)	5.4(18)	1.0(27)	0.1(100)
30	1.4(11)	0.3(8)	0.4(11)	0.2(11)	11.3(12)	5.8(15)	0.6(14)	0.1(25)	4.3(7)	3.3(11)	0.3(8)	0 (0)
40	1.4(11)	0.4(11)	0.5(14)	0.2(11)	5.8(6)	3.0(8)	0 (0)	0 (0)	2.0(3)	1.7(6)	0 (0)	0 (0)
50	1.3(10)	0.3(8)	0.4(11)	0.2(11)	-	-	-	-	-	-	-	-
60	1.3(10)	0.3(8)	0.3(8)	0.2(11)	2.6(3)	2.1(6)	0.1(2)	0 (0)	0.5(1)	1.1(4)	0.1(3)	0 (0)
Total	12.5(100)	3.6(100)	3.7(100)	1.8(100)	92.0(100)	37.7(100)	4.2(100)	0.4(100)	65.5(100)	29.9(100)	3.7(100)	0.1(100)

Results are expressed as increase in conductivity μ hos/15 min/5 disks/15 ml together with content (ppm) of K⁺, Ca²⁺ and Na⁺ of 15 ml bathing solution and represent means for two replicates

* - increase compared with water control

Numbers in parentheses indicate percentages of total

The effects of both ethanol and Triton X-100 although somewhat more rapid were similar to those caused by rot extract and PTE.

C. Release of ions from cell walls

It has been suggested that ions liberated from cell walls by action of pectic enzymes could contribute to conductivity changes (Friedman and Jaffe, 1960). This was investigated with potato tuber disks killed by immersion in 30% v/v ethanol for 15 min. The disks were then leached for 3h in several changes of water stirred on a magnetic stirrer. After this time a sample of 5 disks gave no increase in the conductivity of 15 ml water in 1h and gave a grade 5 on the neutral red index indicating that all protoplasts were killed.

Fifty dead, leached disks were placed in 8 ml reaction mixtures containing 2 ml cucumber rot extract, 0.001M CaCl_2 , 0.02M Tris-HCl buffer at pH 9.0 and water. Control reaction mixtures contained autoclaved rot extract. Conductivity measurements and ion determinations carried out as before are shown in Table 22.

Disks treated with rot extract showed negligible increases in conductivity compared with autoclaved rot extract controls. With the exception of Na^+ which has previously been shown not to contribute to the conductivity changes caused by cucumber rot extract, there were no increases in ion release shown by either rot extract or autoclaved rot extract treated disks. This demonstrates that increases in conductivity measured in previous experiments are due to ions released from the protoplast and not the walls of cells of potato tuber disks.

D. Effect of plasmolysis

Plasmolysing concentrations of sucrose have previously been shown to retard toxicity but not cell separating activity of cucumber rot extract. Sucrose was used to determine whether permeability increasing activity of rot extract would be affected in the same way as toxicity.

Fifty potato tuber disks were placed in 8 ml reaction mixtures

Table 22. Permeability increases and release of ions from dead, leached potato disks

Time (min)	Δ Condu- ctivity μ mos	EXTRACT (A)			Δ Condu- ctivity μ mos	EXTRACT *		
		K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm		K ⁺ ppm	Ca ²⁺ ppm	Na ⁺ ppm
1	0.5(3)	0.1(9)	0.1(17)	0.2(10)	0.2(9)	0.1(6)	0 (0)	0.1(4)
20	3.6(21)	0.3(27)	0.1(17)	0.3(15)	0 (0)	0 (0)	0 (0)	0 (0)
40	2.8(16)	0.1(9)	0.1(17)	0.3(15)	0.2(9)	0.3(19)	0.1(17)	0.6(22)
60	2.8(16)	0.1(9)	0 (0)	0.3(15)	0.6(27)	0.4(25)	0.2(33)	0.6(22)
80	2.9(17)	0.3(27)	0.2(33)	0.5(25)	0.7(32)	0.1(6)	0 (0)	0.3(11)
100	2.5(14)	0.1(9)	0.1(17)	0.2(10)	0.4(18)	0.4(25)	0.1(17)	0.6(22)
120	2.3(13)	0.1(9)	0 (0)	0.2(10)	0.1(5)	0.3(19)	0.2(33)	0.5(19)
Total	17.4(100)	1.1(100)	0.6(100)	2.0(100)	2.2(100)	1.6(100)	0.6(100)	2.7(100)

Extract - cucumber rot extract

(A) - autoclaved

Results are expressed as increase in conductivity μ mos/15 min/5 disks/15 ml together with content (ppm) of K⁺, Ca²⁺ and Na⁺ of 15 ml bathing solution and represent means for two replicates

* - increase compared with autoclaved enzyme control

Numbers in parentheses indicate percentages of total

containing 2 ml rot extract (autoclaved for control), 0.001M CaCl_2 , 0.02M Tris-HCl buffer at pH 9.0 and sucrose at 0, 0.1, 0.25 and 0.5M. Conductivity measurements were made as before on samples of 5 disks removed at intervals from the reaction mixtures.

Results (Table 23) show that maximum increases in conductivity were delayed with both 0.25M and 0.5M sucrose and were found to be lower than those in water. 0.1M sucrose which is hypotonic for potato tissue was ineffective in delaying conductivity changes. These results are similar to the effects caused by sucrose in delaying toxicity of cucumber rot extract.

Table 23. Effect of sucrose on permeability increasing activity of cucumber rot extract

Time (min)	Δ Conductivity μmhos			
	0M sucrose	0.1M sucrose	0.25M sucrose	0.5M sucrose
1	5.3(3)	1.8(2)	-	-
5	18.5(12)	8.2(10)	2.4(6)	3.4(6)
10	20.4(13)	14.1(17)	-	-
15	24.6(16)	-	3.4(8)	4.6(8)
20	21.3(14)	18.2(22)	-	-
25	18.5(12)	-	-	-
30	14.2(9)	14.1(17)	4.0(10)	8.7(14)
40	-	11.5(14)	-	-
45	13.4(9)	-	4.9(12)	8.8(15)
50	-	8.4(10)	-	-
60	11.5(8)	5.7(7)	6.3(15)	11.2(19)
70	-		6.7(16)	8.4(14)
80	-		7.9(19)	7.8(13)
90	6.5(4)		6.7(16)	7.5(12)
Total	154.2(100)	82.0(100)	42.3(100)	60.4(100)

Results are expressed as increase in conductivity $\mu\text{mhos}/15 \text{ min}/5 \text{ disks}/15 \text{ ml}$ and represent means for two replicates. Numbers in parentheses indicate percentages of total

To ensure that sucrose itself did not increase the permeability of potato disks to electrolytes, 50 disks were placed in control reaction mixtures without cucumber rot extract. Increases in conductivity are shown in Table 24.

Disks in 0.1M and 0.25M sucrose showed little increase in permeability. Permeability was slightly increased in 0.5M sucrose which might explain why in the previous experiment maximum increases shown by disks treated with cucumber rot extract occurred more rapidly in 0.5M sucrose than in 0.25M sucrose.

Table 24. Effect of sucrose on permeability of potato disks

Time (min)	Δ Conductivity μmhos		
	0.1M sucrose	0.25M sucrose	0.5M sucrose
5	1.0(11)	1.1(12)	4.1(21)
15	1.1(13)	0.8(9)	1.8(9)
30	1.1(13)	1.0(11)	2.7(14)
40	0.9(10)	1.1(12)	1.5(8)
50	0.9(10)	1.0(11)	2.0(10)
60	0.7(8)	1.1(12)	1.7(9)
70	1.3(15)	1.0(11)	2.2(12)
80	1.0(11)	1.1(12)	1.4(7)
90	0.8(9)	1.1(12)	1.8(9)
Total	8.8(100)	9.3(100)	19.2(100)

Results are expressed as increase in conductivity $\mu\text{mhos}/15 \text{ min}/5 \text{ disks}/15 \text{ ml}$

Numbers in parentheses indicate percentages of total

Potato disks were treated with cucumber rot extract in the same way as before but sucrose was replaced by mannitol as the plasmolysing agent.

Table 25. Effect of mannitol on permeability increasing activity of cucumber rot extract

Time (min)	Δ Conductivity μ mos		
	0M Mannitol [*]	0.25M Mannitol	0.5M Mannitol
5	19.3(28)	3.1(6)	2.8(4)
10	22.2(32)	3.8(8)	3.1(4)
20	10.9(16)	5.9(12)	6.0(8)
30	9.3(13)	6.9(14)	11.8(16)
40	4.3(6)	7.1(14)	12.1(16)
50	3.8(5)	9.1(18)	14.8(19)
60		7.3(15)	14.5(19)
75		6.9(14)	11.0(14)
Total	69.8(100)	50.1(100)	76.1(100)

Results are expressed as increase in conductivity μ mos/15 min/5 disks/15 ml and represent means for two replicates (* - 1 replicate only)
Numbers in parentheses indicate percentages of total

Results shown in Table 25 demonstrate that mannitol in plasmolysing concentrations delays increases in permeability of potato disks treated with cucumber rot extract in a similar way to sucrose.

Plasmolysis of potato disks with 0.5M sucrose has been shown to nullify completely toxicity of PTE as estimated by the plasmolytic method whereas the toxicity of cucumber rot extract is delayed but not prevented. The following experiment investigated these effects. Ninety six potato disks after plasmolysis for 45 min in 0.5M sucrose were placed in reaction mixtures containing :

0.8 ml cucumber rot extract or equivalent activity of PTE

(autoclaved for controls)

0.5 ml 0.012M CaCl_2

0.5 ml 0.24M Tris-HCl buffer pH 9.0

3.0 ml M sucrose

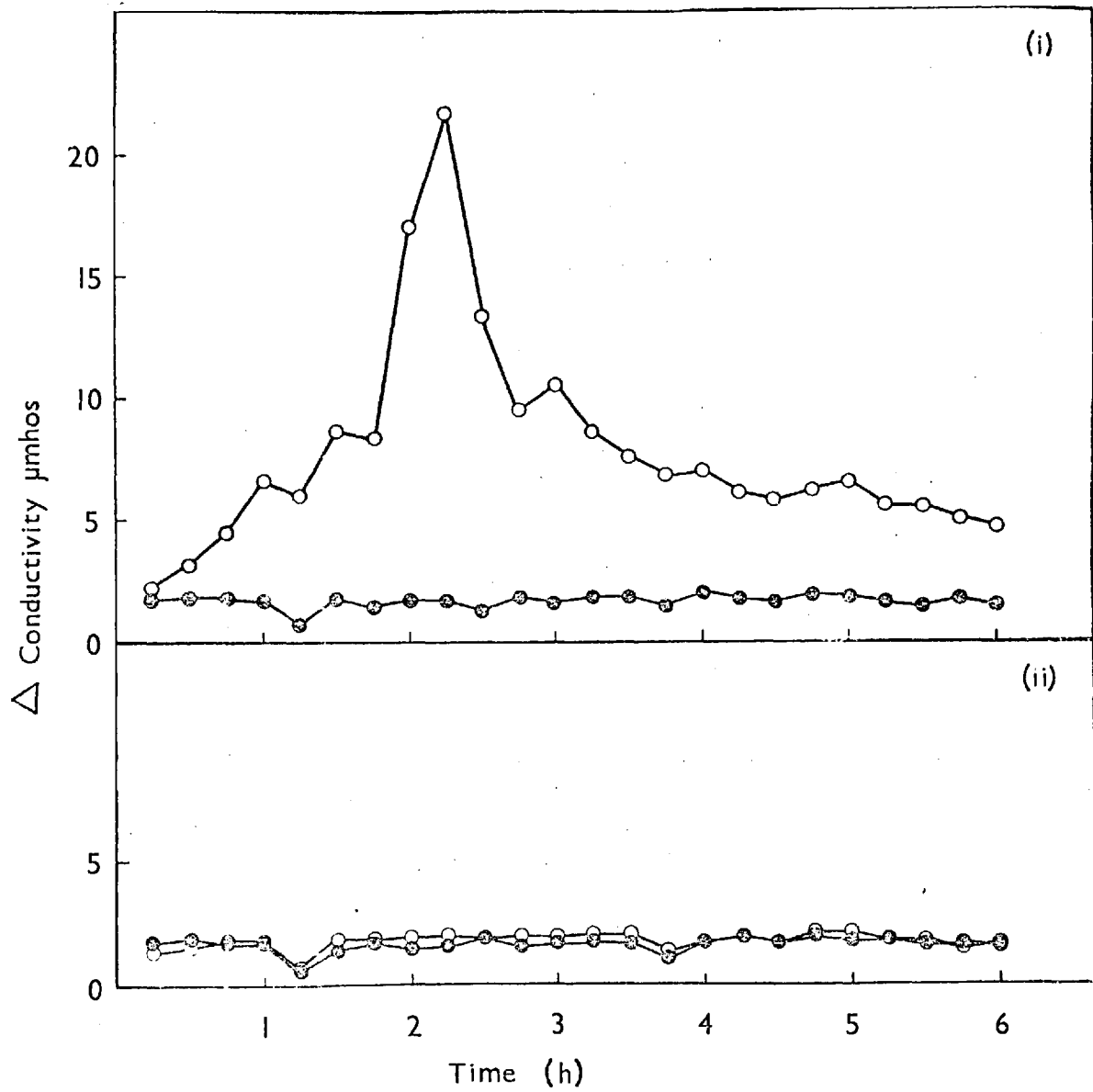
water to 6 ml

At intervals, 4 disks were removed, washed in 0.5M sucrose and placed in 10 ml 0.5M sucrose. Conductivity measurements were then made as before. Results (Figure 6) show that plasmolysed disks treated with cucumber rot extract gave an increase in permeability, conductivity changes reaching a maximum after about 135 min and then declining as the pool of ions in the disks became depleted. In hypotonic solution disks treated with similar concentrations of cucumber rot extract showed maximum conductivity changes within the first 15 min. Autoclaved cucumber rot extract or PTE did not cause these changes.

In marked contrast, disks treated with PTE showed changes similar to autoclaved PTE treated disks. This demonstrates that plasmolysis abolishes both the permeability increasing activity and toxicity of PTE which suggests that the two activities are similar in action. Although delayed, cucumber rot extract increased the permeability of the plasmolysed disks demonstrating that this activity is due to some heat labile factor present in the extract which is not the PTE. This was found earlier for toxicity of cucumber rot extract as estimated by the plasmolytic method.

Ethanol and Triton X-100 both rapidly increased the permeability of potato disks to ions in hypotonic solution in a similar way as did rot extract and PTE. With both enzyme preparations activity is decreased sharply by plasmolysing concentrations of sucrose. Ethanol and Triton X-100 were used with different sucrose concentrations to determine if their permeability increasing activity would be similarly affected. Forty potato tuber disks, equilibrated with the appropriate sucrose concentration were

Figure 6. Conductivity changes shown by plasmolysed potato disks treated with
cucumber rot extract and PTE



○—○ Cucumber rot extract

●—● PTE

(i) Untreated enzyme

(ii) Autoclaved enzyme

placed in 8 ml 30% v/v ethanol or 2% v/v Triton X-100^{with}/sucrose at 0, 0.1, 0.25 and 0.5M final concentration. Conductivity changes are expressed as before.

Results (Table 26) show that permeability of disks was increased by ethanol and Triton X-100 at each sucrose concentration compared with sucrose alone (Table 24).

None of the sucrose concentrations used much delayed the permeability increases caused by ethanol and Triton X-100. With increasing sucrose concentration this effect decreased as noted for plasmolysed disks treated with rot extract. But conductivity increases remained considerably higher than in water controls.

This experiment showed that plasmolysing concentrations of sucrose did not delay or abolish the permeability increasing activity of ethanol or Triton X-100. Indeed, 0.25M sucrose which is hypertonic for potato tissue enhanced the permeability increasing activity of Triton X-100.

3. Effect on different living substrates

A. Tissues

Cell separation and toxicity of cucumber rot extract and PTE was estimated with disks of potato tubers, carrot roots and mesocarp of cucumber fruits. Forty eight disks were placed in reaction mixtures with or without sucrose (0.5M final concentration). For cucumber disks where cell separation is more rapid, amounts of enzyme were a tenth of those for the potato and carrot disks. In plasmolysis experiments, disks were placed in 0.5M sucrose solutions for 45 min before placing in reaction mixtures. At intervals, samples of 4 disks were removed for estimations of cell separation and toxicity. In the results (Table 27) those for controls are omitted because autoclaved preparations did not cause cell separation or death of protoplasts.

Normal potato and carrot disks were affected similarly by rot extract and by PTE. Cell separation was closely associated with death of

Table 26. Effect of sucrose on the permeability increasing activity of ethanol and Triton X-100

Time (min)	Δ Conductivity μ hos							
	30% v/v Ethanol				2% v/v Triton X-100			
	OM sucrose *	0.1M sucrose	0.25M sucrose	0.5M sucrose	OM sucrose	0.1M sucrose	0.25M sucrose	0.5M sucrose
1	12.2(11)	2.6(3)	2.4(3)	6.6(9)	4.1(4)	6.7(8)	9.6(17)	6.7(12)
5	31.8(28)	15.9(19)	15.7(22)	19.4(25)	9.2(9)	23.7(29)	11.9(21)	9.2(17)
10	30.8(27)	22.6(28)	14.7(21)	17.2(22)	29.4(27)	20.1(24)	10.7(19)	8.4(15)
15	20.7(18)	14.7(18)	14.2(20)	13.5(17)	27.4(25)	13.6(16)	8.7(16)	9.3(17)
20	8.0(7)	13.8(17)	12.1(17)	8.4(11)	20.3(19)	9.6(12)	6.3(11)	8.3(15)
25	5.6(5)	8.1(10)	6.7(10)	6.8(9)	11.9(11)	5.6(7)	5.1(9)	6.7(12)
30	3.5(3)	4.3(5)	4.7(7)	5.4(7)	5.9(5)	3.7(5)	3.8(7)	6.0(11)
Total	112.6(100)	82.0(100)	70.5(100)	77.3(100)	108.2(100)	83.0(100)	56.1(100)	54.6(100)

Results are expressed as increase in conductivity μ hos/15 min/5 disks/15 ml and represent for ethanol means of five replicates (* - 2 replicates only) and for Triton X-100 three replicates

Numbers in parentheses indicate percentages of total

Table 27. Effects of cucumber rot extract and PTE on normal and plasmolysed disks of tissue

Time	Cucumber rot extract				PTE			
	Normal		Plasmolysed		Normal		Plasmolysed	
	CS	CD	CS	CD	CS	CD	CS	CD
<u>Potato</u>								
20(min)	0.2	0.2	-	-	0	0.2	-	-
40	1.5	1.5	-	-	0.6	0.5	-	-
60	3.1	2.7	1.5	2.0	1.5	2.0	1.2	0
80	4.4	4.2	-	-	2.1	2.6	-	-
100	5.0	4.9	-	-	4.0	3.7	-	-
2(h)	5.0	5.0	4.4	3.2	5.0	4.9	2.5	0
3			5.0	4.1	5.0	5.0	4.0	0
4			5.0	4.2			5.0	0
5			5.0	4.4			5.0	0
6			5.0	4.6			5.0	0
24			5.0	5.0			5.0	0
<u>Carrot</u>								
20(min)	0.6	0.5	-	-	0	0	-	-
40	2.5	2.4	-	-	0.9	1.2	-	-
60	4.6	4.0	3.7	0.5	3.7	2.7	2.5	0
80	5.0	4.2	-	-	4.4	3.4	-	-
100	5.0	4.5	-	-	5.0	4.4	-	-
2(h)	5.0	4.5	5.0	1.5	5.0	4.5	4.4	0
3			5.0	3.5			4.6	0
4			5.0	4.1			5.0	0
5			5.0	4.5			5.0	0
6			5.0	4.5			5.0	0
20			5.0	4.5			5.0	0
<u>Cucumber</u>								
20(min)	-	0.5	-	-	-	0.5	-	-
40	-	2.5	-	-	-	1.7	-	-
60	5.0	4.2	5.0	0	5.0	2.7	-	0
80	5.0	4.5	-	-	5.0	4.4	-	-
100	5.0	4.5	-	-	5.0	4.5	-	-
2(h)	5.0	4.6	5.0	0	5.0	4.5	5.0	0
3			5.0	0			5.0	0
4			5.0	0			5.0	0
5			5.0	0			5.0	0
6			5.0	0			5.0	0
24			5.0	4.2			5.0	0

CS - cell separation

CD - cell death

Results denote mean grade for 4 disks

protoplasts. With plasmolysed disks although toxicity of rot extract was delayed it was still rapid and most of the protoplasts were dead after 5h. In contrast, although PTE had caused complete cell separation after 5h very few protoplasts were killed even after 24h.

This separation of effects was obtained with plasmolysed cucumber disks only after 24h exposure to diluted preparations. Substantial differences were obtained with enzyme at the concentration used for the other tissues. Toxicity of cucumber rot extract was detected after 2.5h and after 6h was quite high (cell separation and toxicity 5.0 and 1.7 for cucumber rot extract and 5.0 and 0 for PTE treated disks).

B. Single cells

The toxicity of cucumber rot extract and PTE was estimated using single carrot callus cells, washed and suspended in water. They were added to 1.0 ml reaction mixtures in Perspex rings fixed to glass slides. Sucrose (0.5M final concentration) was included in some of the reaction mixtures and cells used with these were plasmolysed for 45 min in 0.5M sucrose before adding to reaction mixtures. After 2 or 3h the ambient solution was replaced by neutral red solution. After 20 min this was replaced by the wash solution and after a further 20 min cells coloured with neutral red and assumed to be alive were counted.

The results (Table 28) show that after 2h almost all unplasmolysed carrot cells were dead when treated with rot extract or PTE. With plasmolysed cells although rot extract killed cells, PTE did not. This means that protoplasts in isolated cells are affected by cucumber rot extract and PTE in much the same way as protoplasts in cells of tissues.

With tissues the toxicity of rot extract and PTE has been shown to parallel cell separating activity in hypotonic solution. Cell separation in potato tuber tissue is inhibited with both enzyme preparations by ions.

The toxicity of both preparations on single carrot cells was tested in the presence of added ions. As the ions would probably inhibit action

Table 28. Effects of cucumber rot extract and PTE on normal and plasmolysed single carrot cells

Treatment	% Cells alive			
	Normal		Plasmolysed	
	2h	3h	2h	3h
Extract	9(4)	-	15(1)	8(2)
Extract (A)	72(4)	-	69(1)	73(2)
Water control	71(4)	-	73(1)	71(2)
PTE	11(1)	12(3)	71(3)	72(4)
PTE (A)	67(1)	69(3)	71(3)	71(4)
Water control	64(1)	72(3)	71(3)	74(4)

Extract - cucumber rot extract

(A) -- autoclaved

Numbers in parentheses indicate number of replicates from which mean percentages were calculated.

of the enzymes on cell walls it was considered that there would be some effect on death of cells in hypotonic solution.

Single carrot cells were treated with rot extract and PTE using similar reaction mixtures as before but with added 0.1M NaCl or $Mg(NO_3)_2$. At intervals, samples of cells were transferred to cavity slides and toxicity estimated by the plasmolytic method. Results are shown in Table 29.

Numbers of live cells in control reaction mixtures containing no salt and autoclaved enzyme declined throughout the experiment. When treated with active rot extract or PTE in the absence of salts cells were rapidly killed. Both of the salts decreased the rate of killing which in turn was related to the valency of the ion used. Numbers of surviving cells

Table 29. The effect of ions on the toxicity of cucumber rot extract and PTE to single carrot cells

Time (h)	% Cells alive							
	Cucumber rot extract				PTE			
	No Salt	No [*] Salt	0.1M NaCl	0.1M Mg(NO ₃) ₂	No Salt	No [*] Salt	0.1M NaCl	0.1M Mg(NO ₃) ₂
0.5	20	58	58	68	61	74	69	77
1	16	70	52	74	48	76	60	68
1.5	4	68	44	76	26	72	58	68
2	0	74	40	76	16	72	52	64
2.5		66	30	70	12	74	44	64
3		68	32	68	10	72	50	66
4		74	28	66	2	66	46	64
5		72	22	60	0	76	46	58
24		28	0	18		26	22	26

* - autoclaved enzyme control

comparable with controls were obtained after 24h with both salts and PTE and with Mg(NO₃)₂ and rot extract.

This experiment demonstrates that toxicity of both enzyme preparations to single carrot cells in hypotonic solution is closely related to action on the cell wall. When this is prevented by ions, killing of cells also stops suggesting that death of protoplasts of single cells like those of tissues results from the action of the enzymes on the cell wall.

C. Isolated protoplasts

In this first series of experiments protoplasts obtained by sectioning mesocarp of cucumber fruits plasmolysed in 0.5M KNO₃ were treated

with rot extract and PTE. A large proportion (c. 90%) of these "protoplasts" were in fact tonoplasts.

Table 30. Effects of cucumber rot extract and PTE on isolated cucumber protoplasts

Time (min)	% Protoplasts ruptured		
	Untreated Enzyme	Autoclaved Enzyme	Water Control
<u>Cucumber rot extract</u>			
0	0	0	0
20	37.8	17.1	2.5
40	55.1	34.1	21.4
60	75.2	45.6	35.2
80	90.9	55.0	49.7
100	94.6	60.8	67.5
120	98.5	69.3	71.4
<u>PTE</u>			
0	0	0	0
20	14.3	16.1	18.5
40	35.6	30.2	34.0
60	47.6	43.3	42.1
80	54.4	50.4	51.5
100	63.9	58.4	57.5
120	68.4	66.6	64.5

Results are means for 2 replicates

The results (Table 30) show that protoplasts did rupture in the water controls; only about 30 - 35% remained intact after 2h. Survival of protoplasts in autoclaved rot extract and autoclaved PTE was similar to that in water controls. Untreated rot extract ruptured protoplasts more rapidly. Very few (1.5%), survived after 2h. In contrast untreated PTE was no more damaging than the controls.

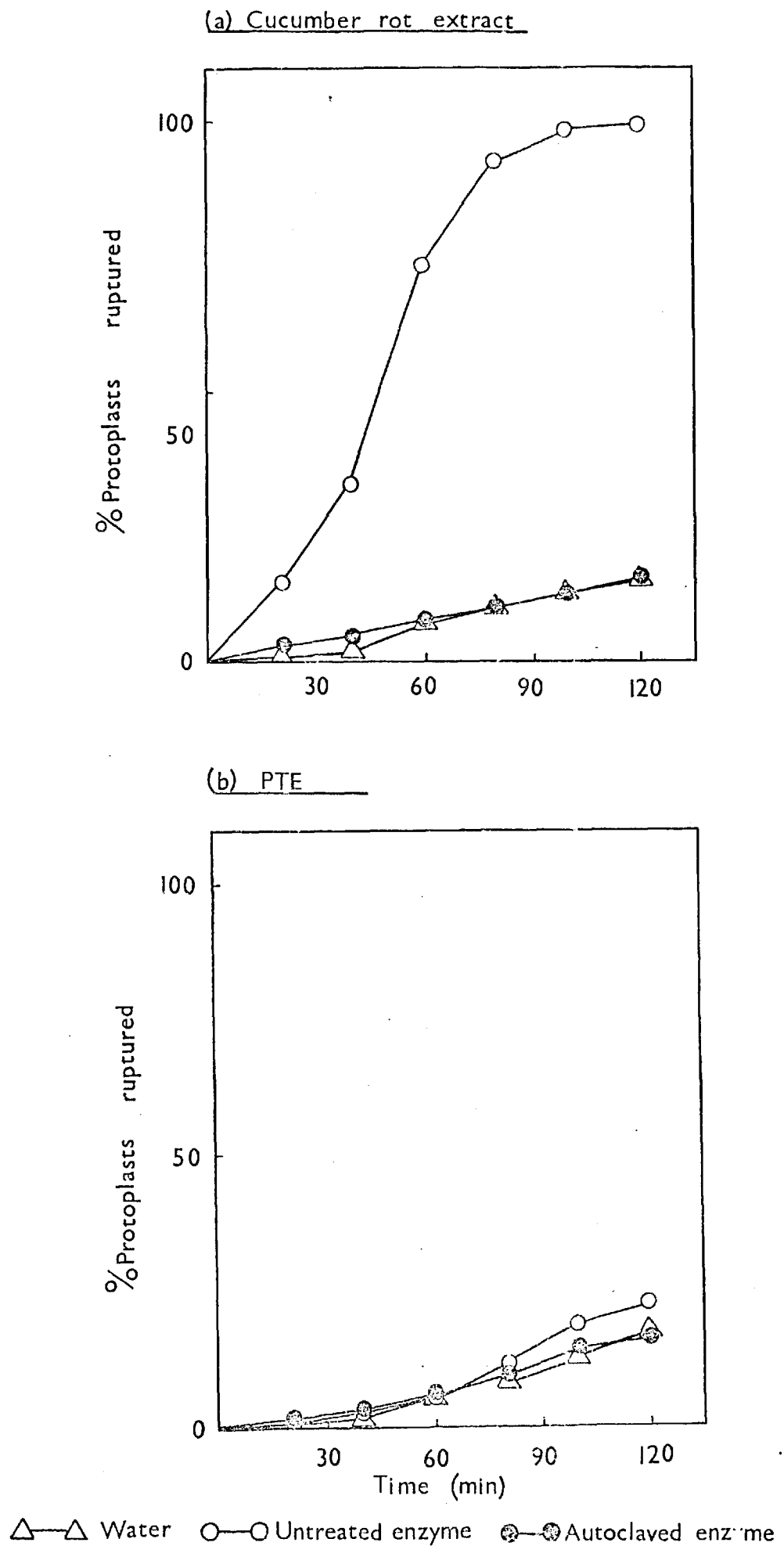
This shows that even when removed from cell walls, plasmolysed protoplasts are protected from toxicity of PTE but not from that of another heat labile factor present in rot extract.

This experiment was repeated with "protoplasts" from placenta tissue of cucumber fruit which were c. 70% intact protoplasts and c. 30% tonoplasts. These were treated with rot extract and PTE as before. Results in Figure 7 (a) and (b) represent mean for two replicates. Figure 7 (a) shows that untreated rot extract rapidly ruptured protoplasts compared with autoclaved rot extract or water controls. Untreated PTE in striking contrast was little more damaging than controls (Figure 7 (b)). This confirms for protoplasts the results obtained earlier with suspensions containing c. 90% tonoplasts.

In summary, both rot extract and PTE caused cell separation and death of potato, carrot and cucumber tissue and also killed protoplasts of single carrot cells in hypotonic solution. In hypertonic solution, cell separation by rot extract and PTE was little delayed but killing by rot extract was delayed and did not occur with PTE. Killing of protoplasts of single carrot cells by rot extract was delayed in hypertonic solution and did not occur with PTE. Likewise, isolated cucumber protoplasts were rapidly ruptured by rot extract but PTE was no more damaging than the controls.

Rot extract and PTE rapidly increased the permeability of potato tissues to ions especially K^+ . In hypertonic solutions PTE did not increase permeability and rot extract was much less active than in hypotonic solutions. In contrast, ethanol and Triton X-100 rapidly increased permeability of potato tissue in hypertonic solutions.

Figure 7. Effects of cucumber rot extract and PTE on isolated cucumber protoplasts.



The inclusion of salts at subplasmolytic concentrations in reaction mixtures inhibited cell separation by both enzyme preparations, especially PTE with a corresponding decrease in rate of killing of protoplasts. Inhibition depended on valency of the cation. This effect was abolished by washing (treated) tissues and may have depended on blocking of enzyme sites in cell walls.

V. Purification of certain other enzymes in cucumber rot extracts

In hypotonic solution the toxicity of rot extract and PTE paralleled cell separation. In hypertonic solution PTE is not toxic. Rot extract is toxic but much less so. Toxicity of rot extract to plasmolysed protoplasts may have depended on an enzyme other than PTE. Rot extract was known to contain a variety of enzymes including phosphatidase and proteinase both of which have been suggested as possible toxic agents (Brown, 1965; Tseng and Bateman, 1968).

These two enzymes and cellulase (CMCase) in cucumber rot extracts were purified to assess their roles in the toxicity of rot extract to plasmolysed protoplasts.

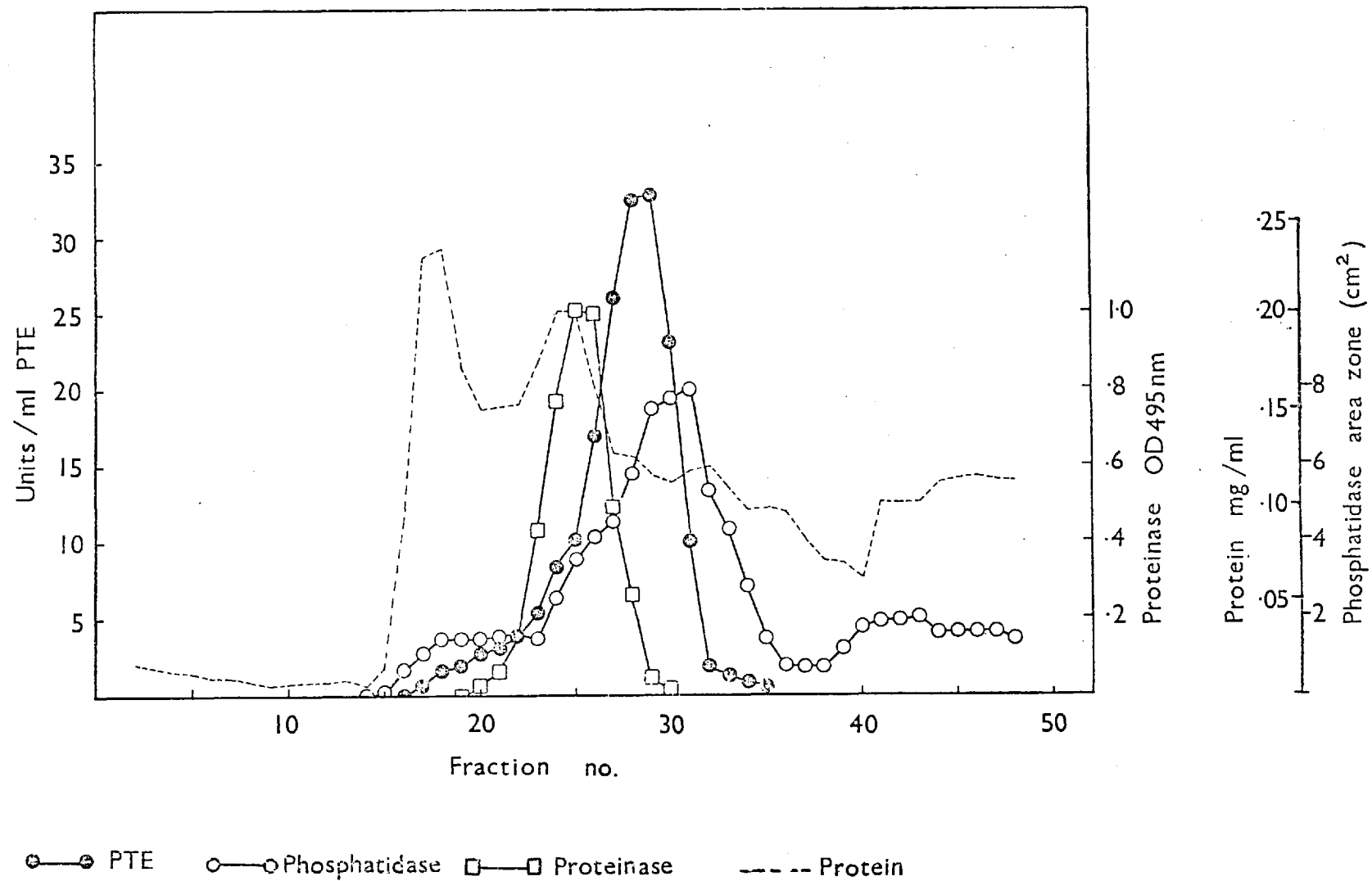
1. Phosphatidase

A. Gel filtration on Sephadex G75 Superfine

The 41 - 60% $(\text{NH}_4)_2\text{SO}_4$ fraction precipitate of rot extract, after dialysis was separated on Sephadex G75 Superfine gel. This fraction contains most of the phosphatidase of rot extracts. The column height was 68 cm and the sample volume was 7 ml. Fractions of 5 ml were collected, their protein content measured by the ultraviolet absorption method and they were then divided into small aliquots and stored at -20°C .

Phosphatidase activity was estimated by the cup plate assay method. PTE activity was estimated by the thiobarbituric acid test and proteinase by the congocoll method.

Figure 8. Separation of the 41-60% $(\text{NH}_4)_2\text{SO}_4$ fraction precipitate of cucumber rot extract on Sephadex G75 Superfine



The results (Figure 8) show that sharply defined peaks were obtained for each of the three activities. A large peak for protein was eluted in advance of the enzymes showing that some degree of purification had been achieved.

Phosphatidase activity closely followed that of PTE indicating that the molecular weight of phosphatidase was slightly lower (PTE known to be c. 31,000). Proteinase was eluted slightly in advance of the PTE indicating a slightly higher molecular weight. The three enzymes were not adequately separated by this method.

B. Isoelectric focusing

Fractions from Sephadex gel filtration containing most of the phosphatidase but also some PTE were subjected to isoelectric focusing between pH 3 and 10 for 50h at 4°C. Fractions of 5 ml were collected and their pH and protein content (by the ultraviolet absorption method) measured. They were then dialysed and divided into small aliquots which were stored at -20°C. Phosphatidase and PTE activities were estimated and the results are shown in Figure 9.

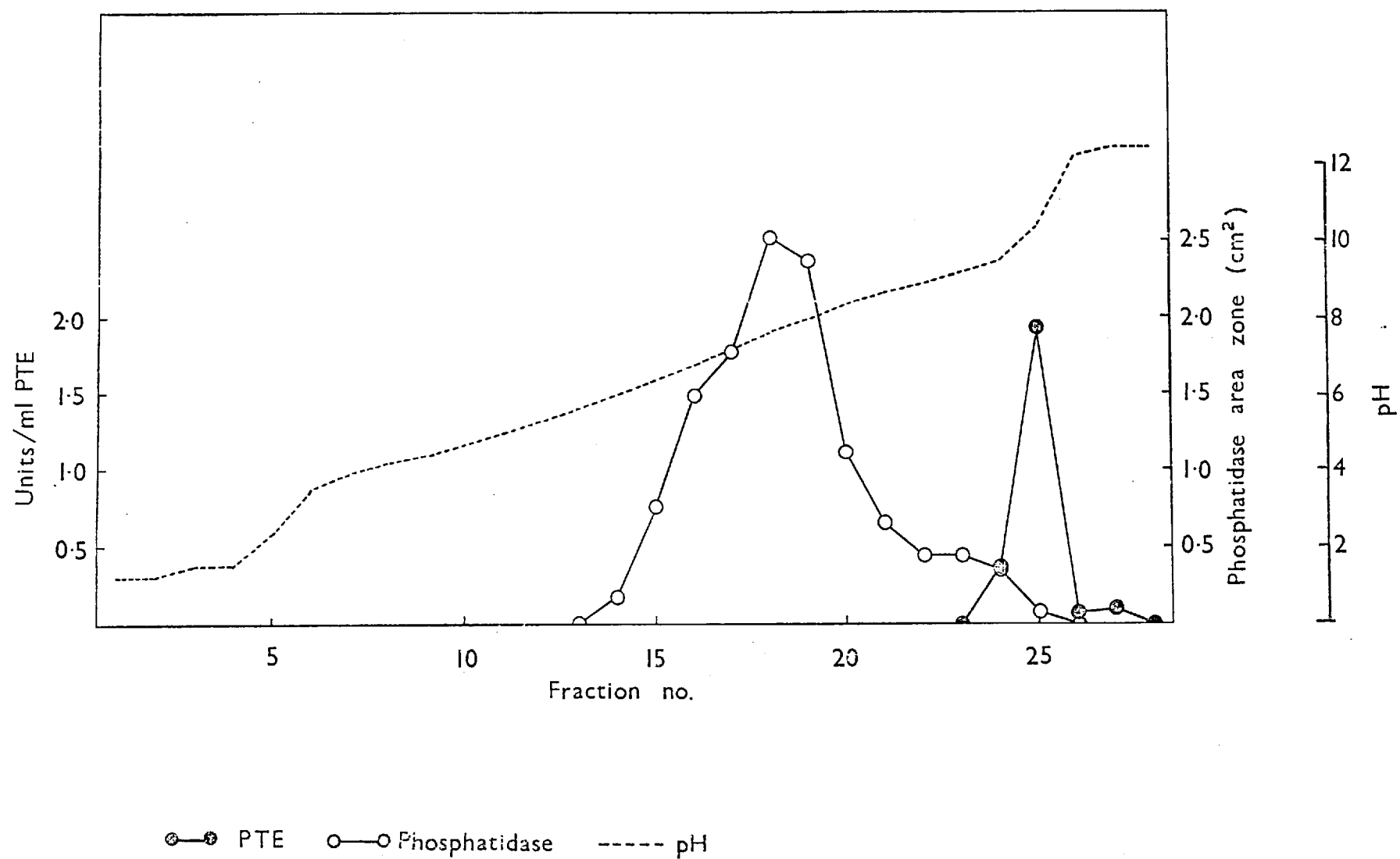
Phosphatidase and PTE were now well separated. The PTE focused at about pH 10.4 whereas the phosphatidase focused at about pH 7.7 thus giving the estimated isoelectric point(pI) of this enzyme.

Fractions containing most of the phosphatidase were bulked and divided into small aliquots which were stored at -20°C. The activity of these samples was estimated by the cup plate assay method to be the same as the rot extract. 0.2 ml cucumber rot extract causes a zone 1.8 cm² in lecithin agar and three replicates of phosphatidase (0.2 ml) showed an equal activity.

C. pH optimum

Lecithin agar media were made up with a range of buffers at 0.1M to give pH values between 4 and 10; 0.2 ml enzyme sample was added to each

Figure 9. Isoelectric focusing of phosphatidase and PTE after partial purification by gel filtration



well and plates were incubated at 30°C for 48h. Activity was expressed as area (cm²) of the white zone (Table 31).

Table 31. Phosphatidase activity at different pH values

pH	Area of zone (cm ²)
4	-
5	0.21
6	0.65
7	0.87
8	1.83
9	1.83
10	1.23

Results denote means for two replicates

Plates at pH 4 could not be used because the medium did not set properly. Phosphatidase activity on the remaining plates was highest at pH 8 and pH 9. The pH optimum for phosphatidase is thus between pH 8 and 9.

2. Proteinase

A. Gel filtration on Sephadex G75 Superfine

A certain degree of separation of proteinase from PTE and phosphatidase was achieved by gel filtration on Sephadex G75 Superfine (Figure 8). 41 - 60% (NH₄)₂SO₄ fraction precipitates of rot extracts were used as they contained the most proteinase.

B. Isoelectric focusing

These fractions with most of the proteinase but also with some PTE

and phosphatidase were subjected to isoelectric focusing between pH 3 and 10 for 50h at 4°C. On this and several subsequent occasions no proteinase was recovered. This is contrast to the findings of Tseng and Mount (1974) who used a similar procedure to obtain proteinase from filtrates of cultures of E. carotovora. Ries and Albersheim (1973) purified a proteinase from filtrates of cultures of Colletotrichum lindemuthianum by ion-exchange chromatography and also found that further attempts at purification resulted in sharply reduced yields.

C. Ion-exchange chromatography

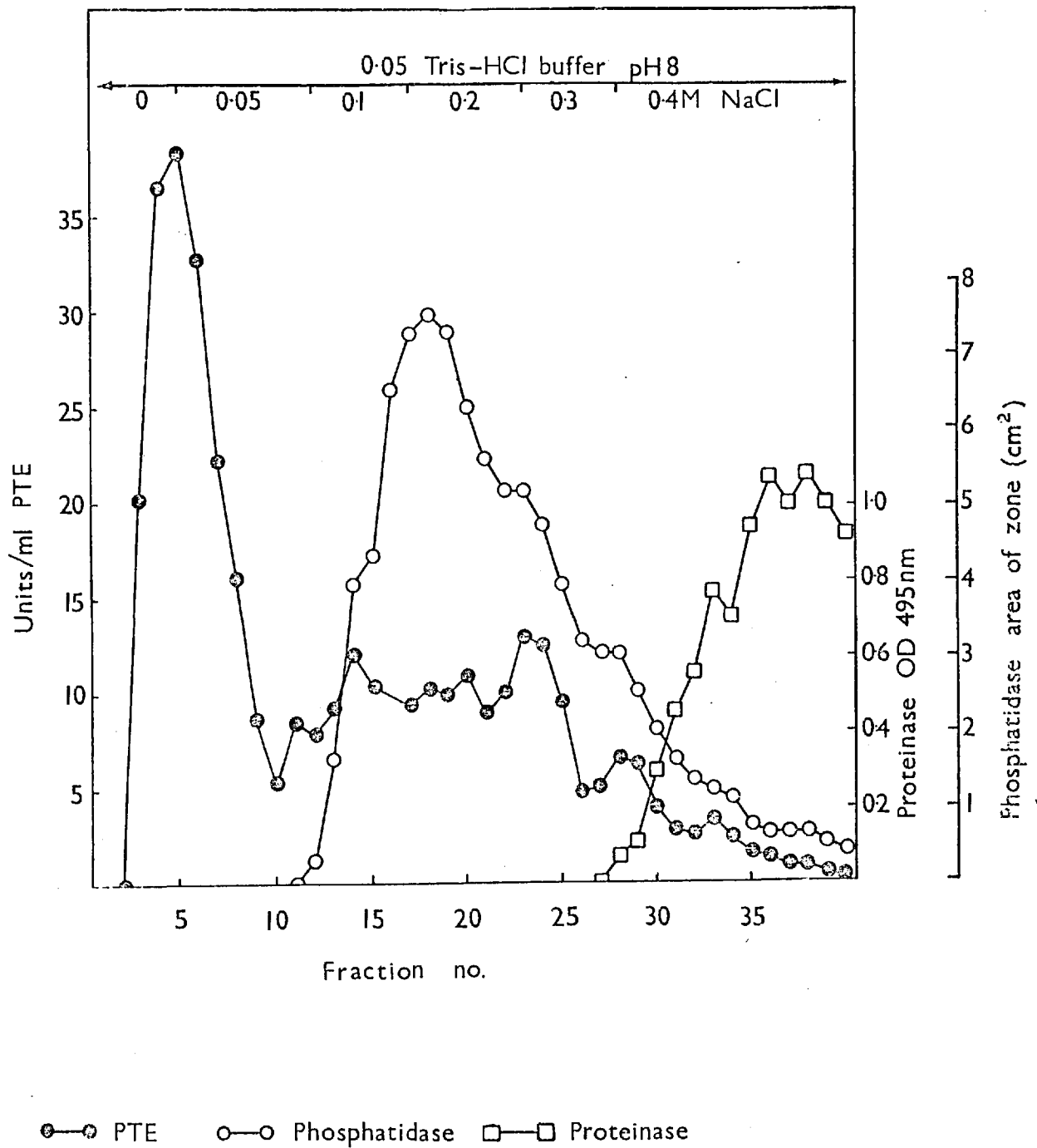
A dialysed solution of the 41 - 60% $(\text{NH}_4)_2\text{SO}_4$ fraction precipitate of rot extract was separated on a column (15 x 1.8 cm diam) of DEAE - Sephadex A-50 (Cl^- form) equilibrated with 0.05M Tris-HCl buffer at pH 8.0. The sample (8 ml) was eluted by stepwise addition of NaCl (up to 0.4M) in buffer. Fractions of 5 ml were collected and assayed for proteinase, phosphatidase and PTE as before.

Figure 10 shows that much of the PTE passed through with the void volume of the column though a little was adsorbed by the gel and then released as the concentration of NaCl was increased. Phosphatidase was eluted by NaCl at c. 0.1M whereas proteinase came off at c. 0.4M NaCl so that these two enzymes could be collected separately. Later, proteinase free from phosphatidase and all but traces of PTE was obtained by first eluting the column with large volumes of NaCl, up to 0.3M in 0.05M Tris - HCl buffer at pH 8.0; increasing the concentration of NaCl to 0.4M then gave purified proteinase which was dialysed before use.

Initial estimations of proteinase were usually made by the cup-plate assay which allowed rapid estimations to be made on large numbers of samples. Later estimations were made by the congocoll method.

For each of the two batches of proteinase prepared in this way activity by the congocoll method was slightly less than that of cucumber rot extract (1 ml cucumber rot extract $\cong$ 1.2 ml proteinase). 0.1 ml

Figure 10. Ion-exchange chromatography of the 41-60% $(\text{NH}_4)_2\text{SO}_4$ fraction
precipitate of cucumber rot extract



cucumber rot extract caused an increase in absorbance of 0.90 at 495 nm. PTE activity of these preparations was 0.10 - 0.13 units/ml compared with 37.3 units/ml for rot extract.

D. pH optimum

Activity of proteinase at different pH values was estimated by the congocoll method using a range of buffers at 0.1M to give pH values between 4 and 10. A control reaction mixture with water replacing enzyme was prepared for each pH value used.

Activity was expressed as the increase in absorbance at 495 nm (Table 32).

Table 32. Proteinase activity at different pH values

pH	Absorbance 495 nm
4	0
5	0.122
6	0.300
7	0.413
8	0.583
9	0.467
10	0.194

Results denote mean values for three replicates

Activity was highest at pH 8 presumably close to the pH optimum of proteinase.

3. Cellulase (CMCase)

A. Ion-exchange chromatography

A dialysed solution of the 61 - 80% $(\text{NH}_4)_2\text{SO}_4$ fraction precipitate of rot extract which contained most of the CMCase was separated on a column (16 x 1.8 cm diam) of DEAE-Sephadex A-50 as described previously. CMCase together with some PTE and phosphatidase was eluted with 0.2M NaCl in 0.05M Tris-HCl buffer at pH 8.0 (Figure 11).

Fractions containing most of the CMCase, after dialysis, were bulked and re-run on an ion-exchange column as before. It was hoped that PTE activity associated with CMCase would be separated in this way. No separation was achieved, the CMCase recovered being associated with a high activity of PTE as before.

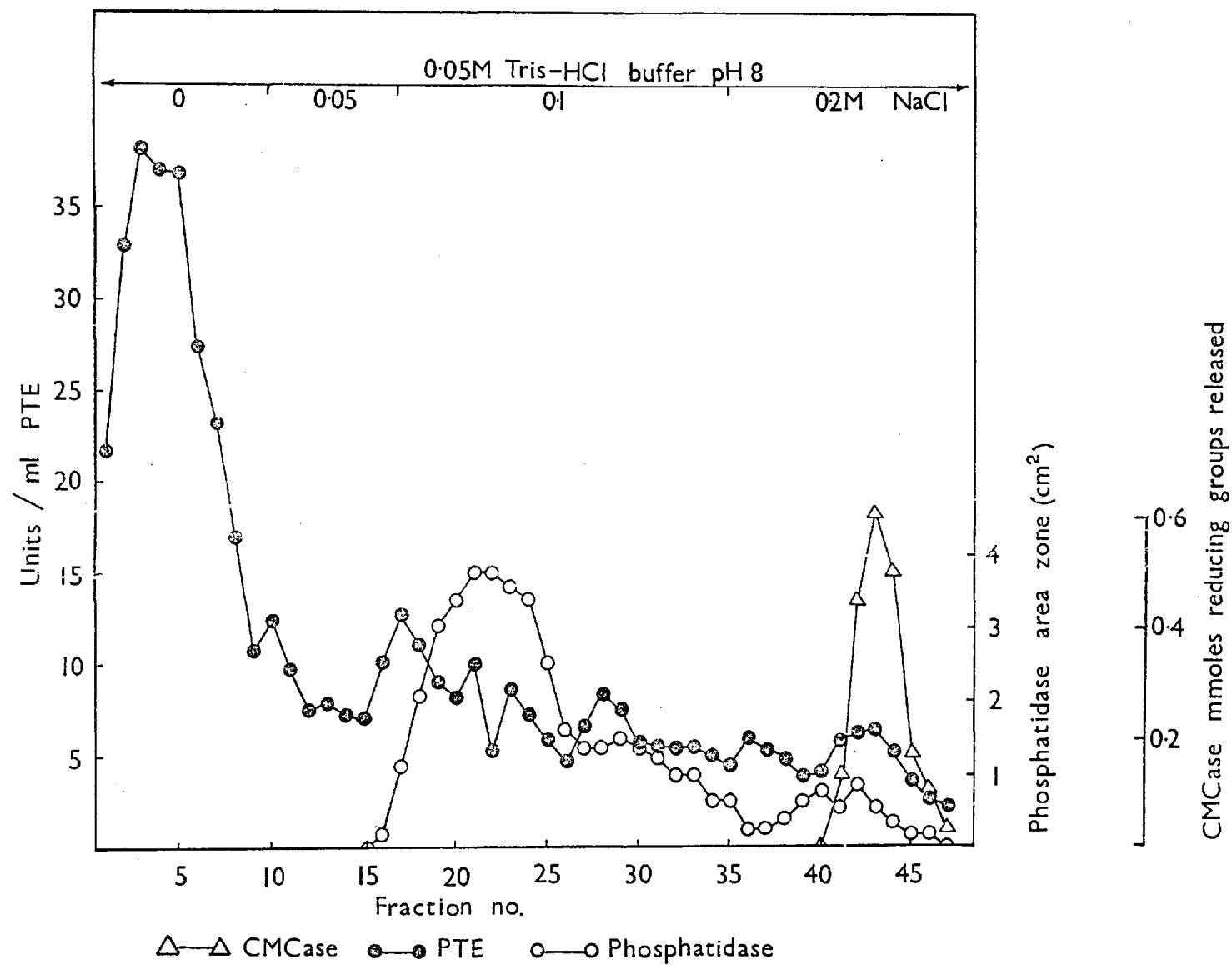
Separation of these activities was attempted by isoelectric focusing between pH 3 and 10 for 50h at 4°C. No CMCase was recovered from the column on this or on a subsequent occasion when focusing was carried out between pH 3 and 10 for 70h at 4°C.

Samples of CMCase free from proteinase and phosphatidase were obtained by ion-exchange chromatography by eluting the column with large volumes of NaCl in buffer up to 0.1M NaCl. CMCase was recovered on elution with 0.2M NaCl and samples were dialysed before use.*

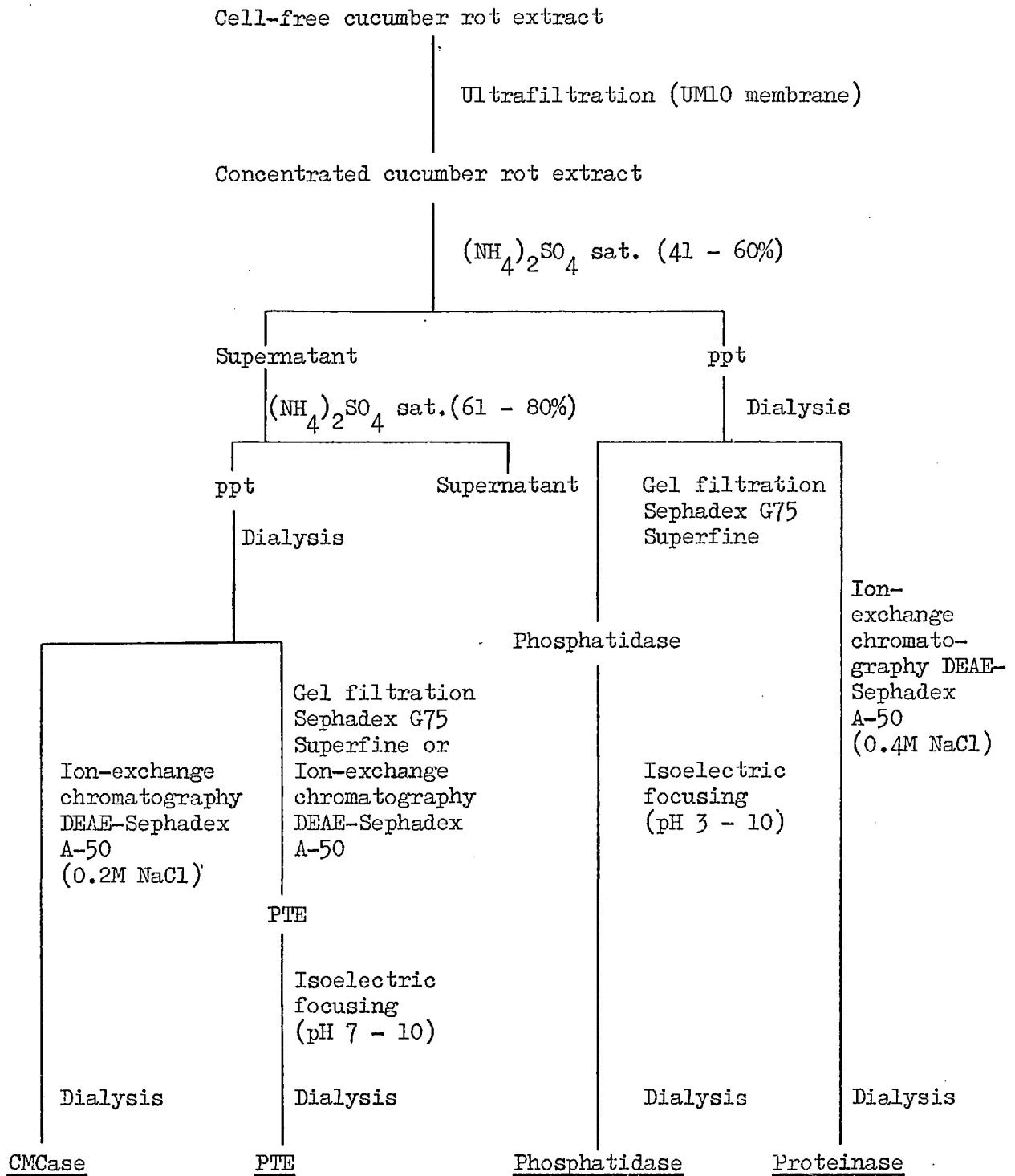
Activity of CMCase was greater than cucumber rot extract as estimated by the Nelson-Somogyi method (1 ml cucumber rot extract $\approx$ 0.67 ml CMCase). 0.2 ml cucumber rot extract released 0.62 m moles reducing groups under the conditions of the assay. PTE activity of this preparation was 18.1 units/ml which was allowed for in certain experiments.

* Elution of CMCase activity from ion-exchange columns in this work at 0.2M NaCl is in contrast to the findings of Garibaldi and Bateman (1970), that the CMCase of E. chrysanthemi was eluted from similar columns by 0.1M NaCl.

Figure 11. Ion-exchange chromatography of the 61-80% $(\text{NH}_4)_2\text{SO}_4$ fraction precipitate of cucumber rot extract



Summary of preparation of enzymes from cucumber rot extracts



VI. Effect of purified enzymes on living substrates

1. Isolated protoplasts

Isolated protoplasts from placenta of cucumber fruit were treated with proteinase and phosphatidase separately and in combination, using amounts equivalent to 0.3 ml rot extract as used previously. The results shown in Figure 12 (a), (b) and (c) represent means for two replicates.

Survival of protoplasts after 120 min in controls containing water or autoclaved proteinase or phosphatidase was between 67 - 81%. With untreated proteinase only 43% of the protoplasts were alive and with untreated phosphatidase only 31% after 2h.

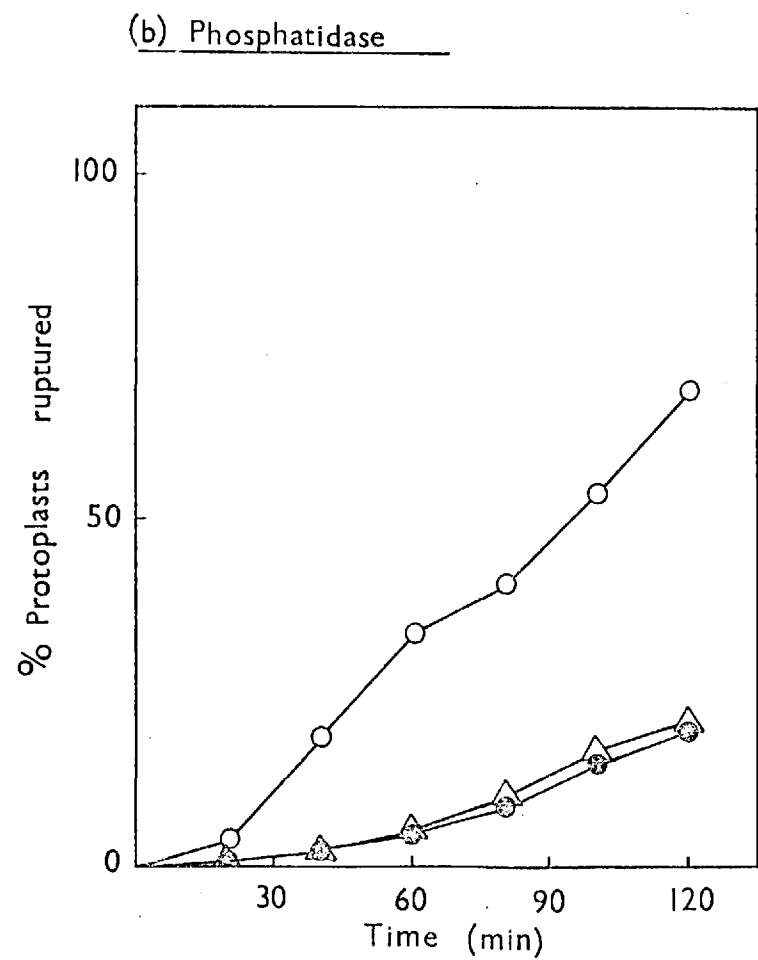
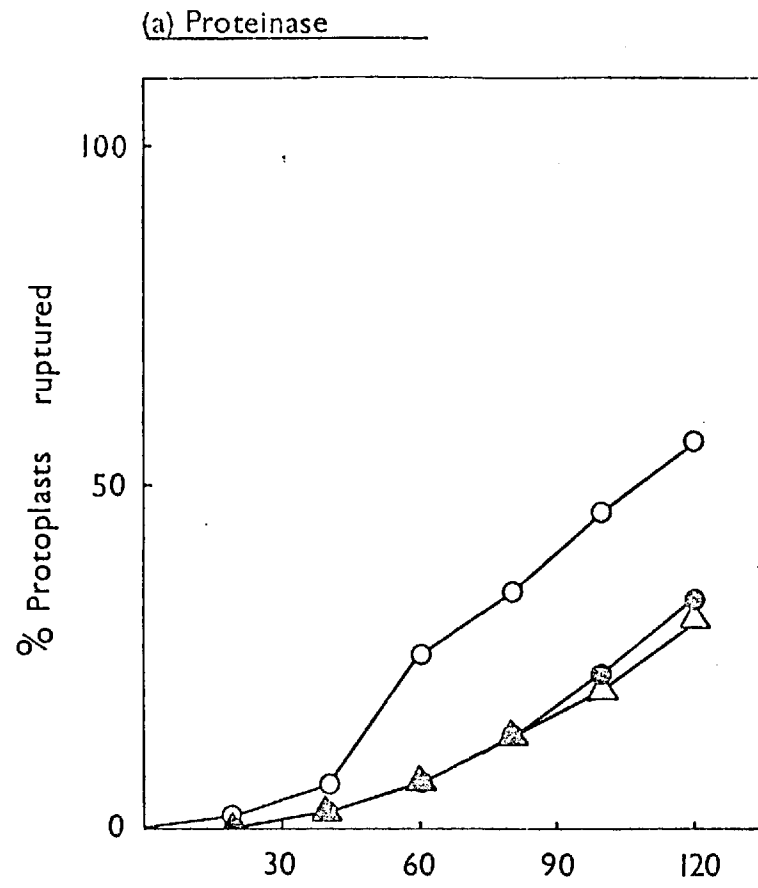
When tested in combination very few (c. 4%) protoplasts were alive after 2h. Thus proteinase and phosphatidase together were almost as active as cucumber rot extract in causing rupture of isolated cucumber protoplasts (see Figure 7 (a)).

2. Single cells

Plasmolysed carrot callus cells (0.5M sucrose) were treated with cucumber rot extract or purified enzyme preparations at equivalent concentrations. Reaction mixtures and procedure were as used for rot extract and PTE. Samples of cells were removed from the reaction mixtures at intervals and toxicity estimated by the plasmolytic method. The results are shown in Table 33.

Rot extract did not kill cells as rapidly as found earlier. PTE and PTE with CMCase did not kill cells any more rapidly than did water controls. Proteinase and phosphatidase together did kill cells, an effect probably enhanced by adding PTE. Rot extract was the most effective in killing plasmolysed cells. Proteinase and phosphatidase even in combination with PTE and CMCase did not kill plasmolysed cells as quickly as did rot extract. In view of the results obtained with isolated protoplasts it appears that the cell wall nullifies the toxicity to protoplasts of proteinase and

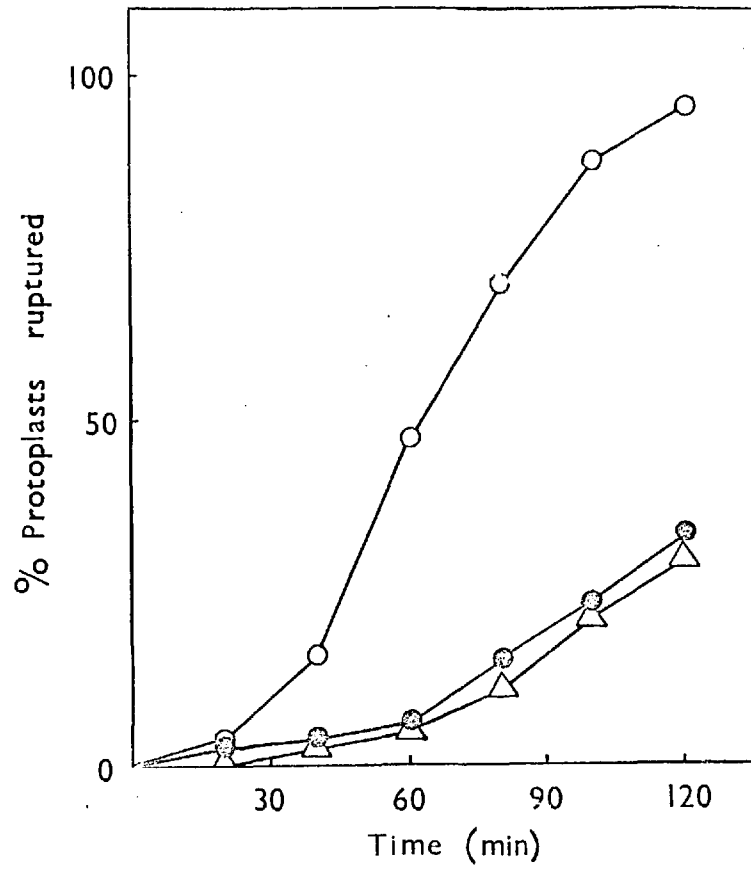
Figure 12. Effects of proteinase and phosphatidase on isolated cucumber protoplasts



△—△ Water ○—○ Untreated enzyme ●—● Autoclaved enzyme

Figure 12. Effects of proteinase and phosphatidase on isolated cucumber protoplasts

(c) Proteinase and phosphatidase



△—△ Water ○—○ Untreated enzyme ●—● Autoclaved enzyme

Table 33. Effects of cucumber rot extract and purified enzymes on plasmolysed single carrot cells

Time (h)	Cells alive (% of water control)						Water Control
	PTE	PTE + CMCase	Pr+Ph	PTE + Pr+Ph	PTE + CMCase + Pr+Ph	Cucumber rot extract	
1	95	101	89	66	63	53	100
2	93	104	82	57	62	48	100
3	98	102	73	54	56	46	100
4	90	100	63	47	48	42	100
5	93	95	67	47	52	43	100
6	93	95	62	46	47	36	100
10	100	98	61	58	58	30	100
20	96	94	60	54	44	24	100
24	100	102	74	68	53	20	100

Pr - proteinase

CMCase - cellulase assayed on carboxymethyl cellulose

Ph - phosphatidase

(CMC).

phosphatidase. This effect is little modified by PTE and CMCase.

3. Tissues

Disks of potato tuber tissue plasmolysed with 0.5M sucrose were treated with rot extract or purified enzyme preparations at equivalent concentrations. Samples of 4 disks were removed at intervals and cell separation and toxicity estimated. Results are shown in Table 34.

Killing of plasmolysed protoplasts of potato disks by rot extract which is well advanced after 1h is complete after 6h. Proteinase and phosphatidase, acting separately or together, even in the presence of PTE

Table 34.

Effects of cucumber rot extract and purified enzymes on plasmolysed potato disks

Time (h)	PTE		Extract		Ph		Pr		Ph+Pr		PTE + Ph		PTE + Pr		PTE + Ph+Pr		PTE + Ph(A)+ Pr(A)		PTE + Extract(A)	
	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD
0.5	0.2	0	0.6	1.5	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	1.2	0
1	1.2	0	1.5	2.0	0	0	0	0	0	0	0.6	0	0.9	0	0.9	0	0.9	0	1.5	0
1.5	1.9	0	3.1	2.5	0	0	0	0	0	0	1.5	0	2.1	0	1.5	0	1.5	0	2.5	0
2	2.5	0	4.4	3.2	0	0	0	0	0	0	3.1	0	2.7	0	2.7	0	3.1	0	2.7	0
2.5	3.7	0	5.0	4.0	0	0	0	0	0	0	4.0	0	4.0	0	3.4	0	3.4	0	4.0	0
3	4.0	0	5.0	4.1	0	0	0	0	0	0	4.4	0	4.4	0	4.4	0	4.0	0	4.6	0
3.5	4.6	0	5.0	4.1	0	0	0	0	0	0	5.0	0	4.6	0	4.6	0	5.0	0	5.0	0
4	5.0	0	5.0	4.2	0	0	0	0	0	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0
5	5.0	0	5.0	4.4	0	0	0	0	0	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0
6	5.0	0	5.0	4.6	0	0	0	0	0	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0
20	5.0	0	5.0	5.0	0	3.0	0	1.7	0	3.7	5.0	4.2	5.0	0.2	5.0	4.9	5.0	0	5.0	0
24	5.0	0	5.0	5.0	0	3.5	0	1.7	0	4.2	5.0	4.5	5.0	0.8	5.0	5.0	5.0	0	5.0	0

CS - cell separation

CD - cell death

Extract - cucumber rot extract

Ph - phosphatidase

Pr - proteinase

(A)- autoclaved

Results denote mean grades for 4 disks

which causes complete cell separation, did not kill protoplasts even after 6h. Some killing by proteinase and phosphatidase was observed after 20h but was apparently little enhanced by PTE. Protoplasts were not killed by autoclaved rot extract in combination with PTE which alone causes no killing within 24h.

These results indicate that the thermolabile factor present in rot extract and responsible for the killing of plasmolysed protoplasts of tissues and also single cells was not proteinase or phosphatidase.

A similar result was obtained when unplasmolysed potato disks were treated with proteinase plus phosphatidase; there was no cell separation or toxicity even after 24h.

These results were somewhat unexpected in view of the toxicity of proteinase and phosphatidase for isolated protoplasts. It seemed that the cell wall prevented these enzymes reaching protoplasts. Although cell separation was complete with PTE much of the structure of the cell wall would have been retained. However, when CMCase was used in combination with PTE there was little increased killing of plasmolysed single carrot cells by proteinase and phosphatidase.

This experiment was repeated with plasmolysed potato disks and the commercial cellulases Meicelase P (Meiji Seika Kaisha Ltd., Tokyo, Japan) and Onozuka SS (All Japan Biochemicals Co. Ltd., Nishinomiya, Japan) used after passing through Sephadex G50 Medium. Fractions from both preparations with highest activity on CMC were retained.

The three cellulase preparations were included in standard reaction mixtures at activities equivalent to 0.4 ml rot extract the amount used in standard tests. In this way it was hoped that the importance of cellulase in rot extracts in the killing of plasmolysed protoplasts could be assessed in the light of the possibility that cellulase in combination with PTE might increase wall degradation and, therefore, access to the protoplasts of proteinase and phosphatidase.

Results in Table 35 show that cell separating activity of PTE was

Table 35. Effect of cellulase preparations on plasmolysed potato tuber disks

Time (h)	PTE + CMCase		PTE + Ono.		PTE + Meic.		PTE + CMCase +Pr + Ph		PTE + Ono. +Pr + Ph		PTE + Meic. +Pr + Ph		PTE(A) + Ono.(A) + Meic.(A)	
	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD
1	1.9	0	1.9	0	2.1	0	1.9	0	1.9	0	2.1	0	0	0
2	3.1	0	3.4	0	3.4	0	3.1	0	3.7	0	3.4	0	0	0
3	5.0	0	5.0	0	4.6	0	5.0	0	4.6	0	4.4	0	0	0
4	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0	0	0
5	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0	0	0
6	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0	0	0
8	-		5.0	0	5.0	0	-		5.0	0	5.0	0	0	0
10	-		5.0	0	5.0	0	-		5.0	0	5.0	0	0	0
24	5.0	3.5	5.0	0	5.0	0	5.0	4.1	5.0	1.7	5.0	2.0	0	0

CS - cell separation

CD - cell death

Ono. - Onozuka SS

Meic. - Meicelase P

(A) - autoclaved

Results denote mean grades for 4 disks

little increased by any of the cellulases. Onozuka SS and Meicelase P were not toxic to plasmolysed protoplasts but some toxicity was shown by CMCCase from rot extract after 24h. None of the cellulases in combination with PTE and proteinase and phosphatidase caused death of protoplasts in 6h.

It was considered possible that degradation of cell walls sufficient to allow access of proteinase and phosphatidase to plasmolysed protoplasts did not occur for several hours and that during this time toxic enzymes may have lost some activity (possible autodegradation for proteinase). Plasmolysed potato disks were thus pretreated with PTE or PTE and Onozuka SS before adding proteinase and phosphatidase. Forty eight such disks were added to a reaction mixture containing CaCl_2 , Tris-HCl buffer at pH 9.0, PTE with or without Onozuka SS and sucrose to 0.5M final concentration. After 3h, proteinase and phosphatidase in plasmolyticum were added to give 3 ml final volume of reaction mixture.

Plasmolysed potato disks were also treated similarly but with the concentration of Onozuka SS increased threefold or that of proteinase and phosphatidase increased by a half. Results for these treatments are shown in Table 36.

Increasing the concentration of Onozuka SS, proteinase or phosphatidase did not cause death of plasmolysed protoplasts in 8h. Neither did pretreatment of disks with PTE or PTE and Onozuka SS. This contrasts with the action of rot extract which killed almost all plasmolysed protoplasts of potato tissue within 6h.

The purified enzymes were tested against cucumber disks in the same way as potato disks; 48 were placed in standard reaction mixtures containing phosphatidase and proteinase at the concentration used for potato disks. Disks plasmolysed in 0.5M sucrose were also treated in the same way and also with the addition of PTE or PTE and CMCCase. Results are shown in Table 37.

Table 36.

Effect of purified enzymes on plasmolysed potato tuber disks

Time (h)	PTE + Ono +Pr* + Ph*		PTE + Ono**		PTE***		PTE*** + Ono***	
	CS	CD	CS	CD	CS	CD	CS	CD
1	2.1	0	1.9	0	5.0	0	5.0	0
2	3.4	0	3.4	0	5.0	0	5.0	0
3	4.6	0	5.0	0	5.0	0	5.0	0
4	5.0	0	5.0	0	5.0	0	5.0	0
5	5.0	0	5.0	0	5.0	0	5.0	0
8	5.0	0	5.0	0	-	-	-	-
21	-	-	-	-	5.0	0.7	5.0	0.5
24	5.0	4.1	5.0	2.0				

CS - cell separation

CD - cell death

Pr - proteinase

Ph - phosphatidase

Ono - Onozuka SS

* - enzyme concentration increased by a half

** - enzyme concentration
increased threefold

*** - Pretreatment for 3h

Results denote mean grades for 4 disks

Table 37.

Effect of purified enzymes on cucumber disks

Time (h)	Normal						Plasmolysed									
	Pr		Ph		Pr+Ph		Pr		Ph		Pr+Ph		Pr+Ph+PTE		Pr+Ph+ PTE+CMCase	
	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD	CS	CD
1		0		0		0		0		0		0	5.0	0	5.0	0
2		0		0		0		0		0		0	5.0	0	5.0	0
3		0.5		0.2		1.7		0		0		0	5.0	0	5.0	0
4		1.2		1.0		1.7		0		0		0	5.0	0	5.0	0
5		1.2		1.0		1.7		0		0		0	5.0	0	5.0	0
6		2.0		1.0		2.2		0		0		0	5.0	0	5.0	0
24	4.0	3.2	-	2.7	4.0	3.2	4.0	1.0	-	1.0	4.0	1.0	5.0	1.5	5.0	1.7

CS - cell separation

CD - cell death

Pr - proteinase

Ph - phosphatidase

Results denote mean grades for 4 disks

Disks treated with proteinase had softened after 24h. Proteinase did contain small amounts of PTE which could account for the limited cell separation because cucumber tissue is very susceptible to PTE. Some protoplasts in unplasmolysed disks treated with proteinase were killed within 6h but this did not occur in plasmolysed disks. There was no cell separation in phosphatidase treated disks. Residual PTE in the proteinase preparation did not cause these effects in potato disks. Plasmolysed cucumber disks behaved similarly to plasmolysed potato disks with rot extract which caused substantial killing within 6h (Table 27). Phosphatidase and proteinase together had little effect except after 24h even in the presence of PTE and CMCase.

From these experiments it was clear that neither proteinase nor phosphatidase were the thermolabile factors toxic to plasmolysed protoplasts of potato tissue present in rot extract and, that cell walls of tissue disks were not degraded sufficiently by PTE and cellulase to allow access of the toxic enzymes to protoplasts.

4. Nature of factor in cucumber rot extract toxic to plasmolysed protoplasts

Cucumber rot extract was fractionated in an attempt to relate toxicity to plasmolysed protoplasts of potato tissue to specific enzymes in the extract.

100 ml of rot extract was reduced to 5 ml by ultrafiltration using a UM10 membrane. This sample was separated on a column (73 x 2.5 cm diam) of Sephadex G75 Superfine. Five ml fractions were collected and stored at -20°C.

Cell separation and toxicity were estimated with 1 ml of each fraction in standard reaction mixtures containing 32 plasmolysed potato disks and 0.5M sucrose as plasmolyticum. Estimates were made with samples of 4 disks.

PTE, proteinase and phosphatidase estimations were made as before.

It is possible that the factor in rot extract toxic to plasmolysed protoplasts might be of host origin. Lysosomal enzymes are possibly released on disruption of host lysosomes during rotting. Such enzymes have been cited as possible toxic agents in disease (Wilson, 1973) and RNase has been shown to disrupt isolated protoplasts (Ruesink and Thimann, 1965).

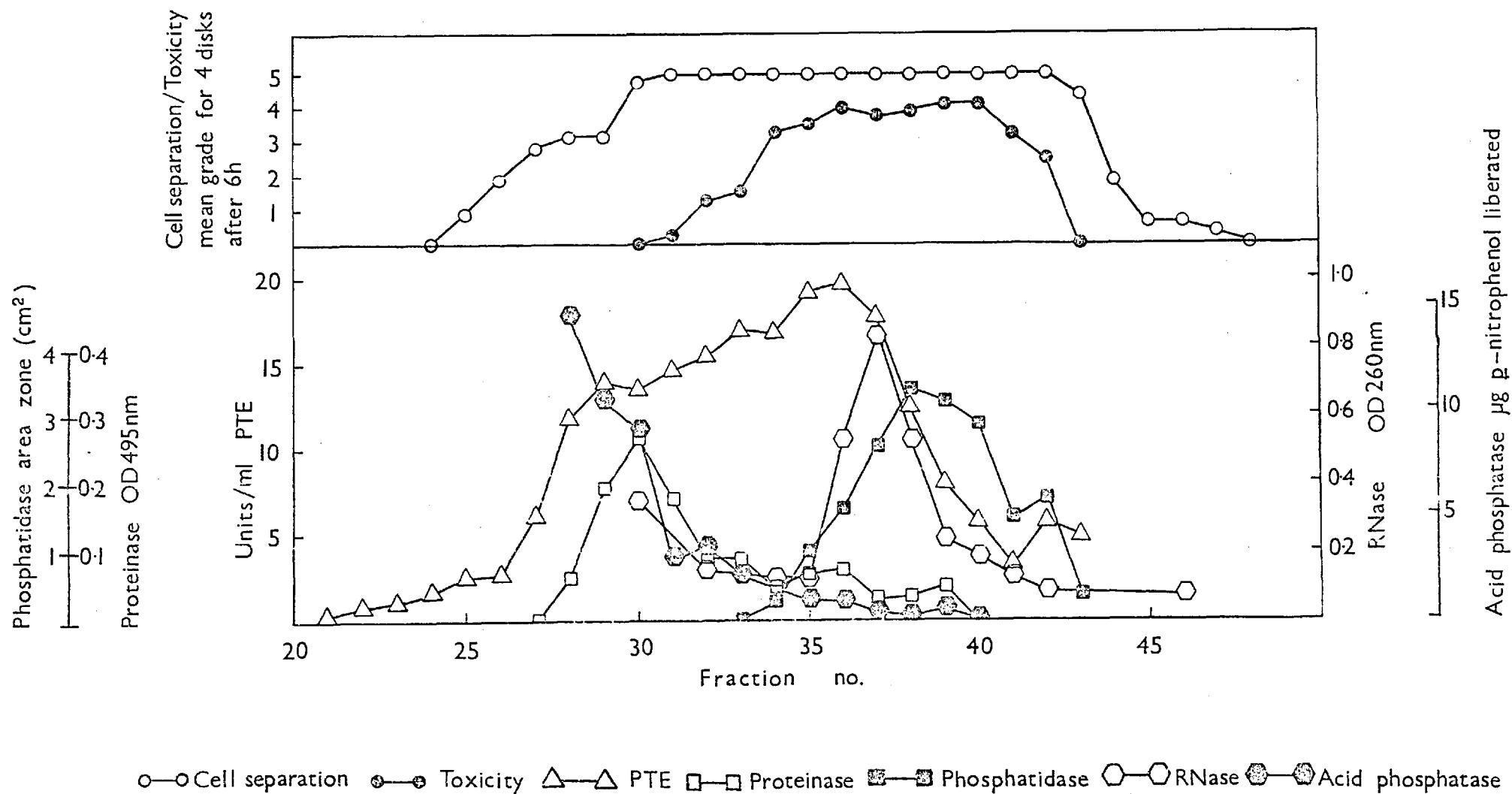
Acid phosphatase and RNase together with proteinase and phosphatidase in rot extracts were therefore estimated.

Figure 13 shows that cell separation in potato tissue after 6h was complete with fractions 31 - 42 from the Sephadex column. This activity was fairly well correlated with PTE activity which was greatest in fraction 36. Death of plasmolysed protoplasts of potato tissue did not occur in the absence of cell separation but death was not so well correlated with PTE activity; toxicity $>$ grade 3 after 6h was found with fractions 34 - 40. Most proteinase and acid phosphatase was eluted before toxic factors and thus did not contribute significantly to killing of plasmolysed protoplasts of potato tissue.

Most of the phosphatidase and RNase was eluted with most of the toxic factors, but it was clear that these two enzymes alone or together could not wholly account for the toxicity observed. Also, fractions containing only low activities of these enzymes were highly toxic.

PTE, phosphatidase and RNase (from bovine pancreas - Sigma Chem. Co. Ltd.) were used at concentrations equivalent to those present in fraction 36 (Figure 13) to determine whether RNase in combination with the other enzymes could kill plasmolysed protoplasts of potato tissue within 6h. Although cell separation was complete there was no killing.

Figure 13. Separation on Sephadex G75 Superfine of factors in cucumber rot extract toxic to plasmolysed protoplasts of potato tissue



VII. Toxicity of pectate trans-eliminase (PTE) to potato tuber tissue

There are a number of possible ways in which the PTE of cucumber rot extract could cause death of protoplasts of normal (unplasmolysed) tissues. First, there is the action of the enzyme on cell walls of tissue which might release products that are toxic to the protoplasts. The following experiments dealt with this possibility.

1. Release of toxins

A. Lower molecular weight toxins

About 200 potato tuber disks were placed in a reaction mixture of 8 ml containing 2 ml rot extract, 0.001M CaCl_2 and 0.02M Tris-HCl buffer at pH 9.0. After 1h, by which time cell separation and killing of protoplasts were well advanced, the reaction mixture was filtered through Whatman No. 40 ashless filter paper to remove the disks and debris. The filtrate was then passed through a UML0 ultrafiltration membrane to remove material with a molecular weight in excess of 10,000. This filtrate was then tested against normal and plasmolysed potato disks using 1 ml filtrate/3 ml reaction mixture containing 0.001M CaCl_2 , 0.02M Tris-HCl buffer at pH 9.0 and water or 0.5M sucrose. Samples of 4 disks were removed at intervals and toxicity estimated by the plasmolytic method.

No toxicity was detected with normal or plasmolysed potato disks within 5h. Thus it appears that substances of molecular weight $< 10,000$ released from potato tissue by rot extract are not toxic to cells of potato tuber tissue.

B. Higher molecular weight toxins

About 500 potato tuber disks were placed in a 30 ml reaction mixture containing 5 ml cucumber rot extract, 0.001M CaCl_2 and 0.02M Tris-HCl buffer at pH 9.0. After 40 min, the reaction mixture was filtered

through Whatman No. 40 ashless filter paper and subjected to isoelectric focusing between pH 3 and 10 for 66h at 4°C. Fractions of 5 ml were collected, pH and protein content (by the ultraviolet absorption method) determined and then dialysed and stored at -20°C. Low molecular weight materials released from the disks would be removed by the dialysis.

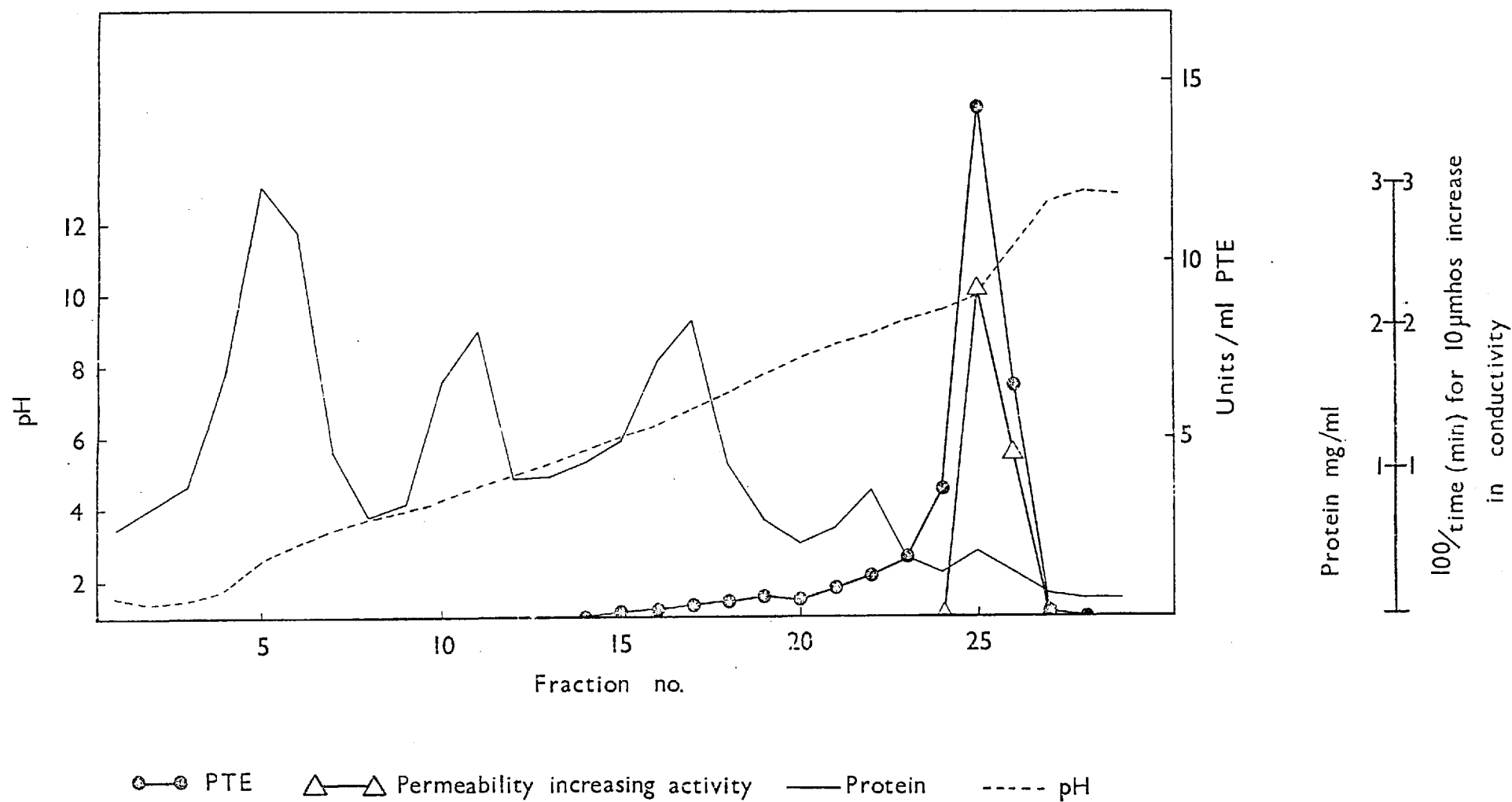
PTE activity was estimated by the thiobarbituric acid test and expressed as units/ml.

Toxicity was estimated as permeability increasing activity because it was considered that this would be more sensitive than the plasmolytic method. Samples of 15 disks were placed in 3 ml reaction mixtures containing 1 ml of fraction, 0.001M CaCl_2 and 0.02M Tris-HCl buffer at pH 9.0. Five disks were sampled after 30, 60 and 90 min and permeability increasing activity estimated as before. Activity was expressed as $100/t$ where t was the time in minutes for a 10 μmhos increase in conductivity of 15 ml water bathing 5 disks in 15 min.

Figure 14 shows that several well defined protein peaks were resolved by this method. A single peak of PTE activity was focused at c. pH 10. Only two fractions increased permeability these being the fractions with highest PTE activity. This shows that no high molecular weight toxins, other than PTE, are present in reaction mixtures after treatment of potato tissue with rot extract. PTE was present in the rot extract of the reaction mixture but many of the protein peaks resolved probably originated from the potato disks as similar patterns for protein are not obtained when rot extract alone is subjected to isoelectric focusing.

The results of this and the previous experiment agree with the findings of Fushtey (1957), who was unable to show an increased toxicity of cell separating preparations after digestion of a large mass of tissue. Thus, apparently no toxins are released by the action of PTE on the cell walls of potato tuber tissue.

Figure 14. Separation by isoelectric focusing of material released from potato disks by cucumber rot extract



C. Release of hydrogen peroxide

It has been suggested that the toxicity observed in cotton leaf tissue treated with PG from Verticillium albo-atrum is due to hydrogen peroxide. It is thought that PG releases oxidases and their substrates by its action on the cell walls which together produce H_2O_2 . Toxicity by PG could be prevented by treatment of tissues with 1,000 units of catalase (Mussell, 1973). Release of H_2O_2 from potato tuber tissue by the action of the PTE of cucumber rot extract was investigated.

Reaction mixtures of 8 ml contained 2 ml untreated or autoclaved rot extract, 0.001M $CaCl_2$, 0.02M Tris-HCl buffer at pH 9.0 and 5 potato disks (15 mm diameter, 1 mm thick). There were two replicates of these treatments and also one replicate of autoclaved rot extract and 1 mM H_2O_2 .

At intervals 1 ml was removed from each reaction mixture and added to 9 ml 6 mM o-dianisidine-diHCl (Sigma Chem. Co. Ltd.) in 0.1M sodium phosphate buffer at pH 7.0 containing 2,000 units horseradish peroxidase (BDH Ltd.). After 10 min at 23°C, 0.2 ml 4N HCl was added and mixed. After a further 5 min the absorbance of the solution was estimated at 460 nm against water. Results are shown in Table 38.

Table 38. Release of H_2O_2 from potato tuber disks

Time (min)	cucumber rot extract	cucumber rot extract (A)	cucumber rot extract (A) + 1 mM H_2O_2 [*]
30	0.111	0.096	0.272
60	0.114	0.104	0.181
90	0.129	0.107	-
120	0.134	0.121	0.159
150	0.139	0.125	-
180	0.150	0.129	0.157

(A) - autoclaved

Results are means for 2 replicates (*- 1 replicate only)

Increases in absorbance with both untreated and autoclaved rot extract were in part due to oxidation of the o-dianisidine reagent. The reaction mixture containing 1 mM H_2O_2 showed a depletion of the H_2O_2 over 3h.

After 3h there was a slight increase in absorbance for samples from rot extract treated disks compared with the controls. This may have been due to low H_2O_2 evolution.

In a further test to determine toxicity of H_2O_2 , 40 potato disks (7 mm diameter, 0.5 mm thick) were added to 4 ml of 1 mM H_2O_2 . Five disks were removed at intervals and toxicity estimated by the plasmolytic method. After 3h the disks showed only a grade 1.0 on the neutral red index although this had risen to 4.6 after 18h. Thus even 1 mM H_2O_2 did not cause the rapid killing of protoplasts observed when potato tissue is treated with rot extract. Mussell (1973) has shown evolution of 3 μ M H_2O_2 in 2.5h by cotton leaf tissue treated with PG. In the present work 3,000 units of catalase did not decrease the ability of rot extract to increase permeability of potato tuber tissue. This is three times the concentration that completely nullified toxicity of PG to cotton leaf tissue (Mussell, 1973).

From the results obtained in these experiments it was clear that even if a low level of H_2O_2 was produced from potato tissue by the action of rot extract it would not be able to cause the rapid killing of protoplasts observed. Furthermore, toxicity is not prevented by catalase which alone is evidence that H_2O_2 is not involved.

2. Effect of deplasmolysis on PTE treated potato tissue

It has been proposed that the action of pectic enzymes on tissues in hypotonic solution so alters the cell walls that the protoplasts are no longer restrained from bursting under turgor pressure. Protoplasts in plasmolysed tissue treated with PTE remain alive long after cells have separated.

To study effects of deplasmolysis, 48 potato tuber disks were

plasmolysed in 0.3M sucrose and treated with PTE for 2h. Disks remained intact after this treatment but cells separated readily under slight tension. Intact disks were washed with 0.3M sucrose to remove PTE other than that adsorbed by cells, and left in 20 ml 0.3M sucrose. Water was added, 0.5 ml at a time and at 5 min after each addition conductivity was measured. This was continued until the molarity of the solution was 0.16M, hypotonic for potato disks.

The results (Figure 15) show that with autoclaved PTE in the control there was little or no change in conductivity as molarity of sucrose decreased indicating that the semipermeability of the protoplasts had not been impaired by plasmolysis. But loss of ions from PTE treated disks started to increase as molarity decreased to 0.22M and rapidly increased to a maximum as molarity declined to 0.20M. As molarity decreased to 0.19M increases in conductivity became smaller presumably because of cumulative losses of ions from protoplasts.

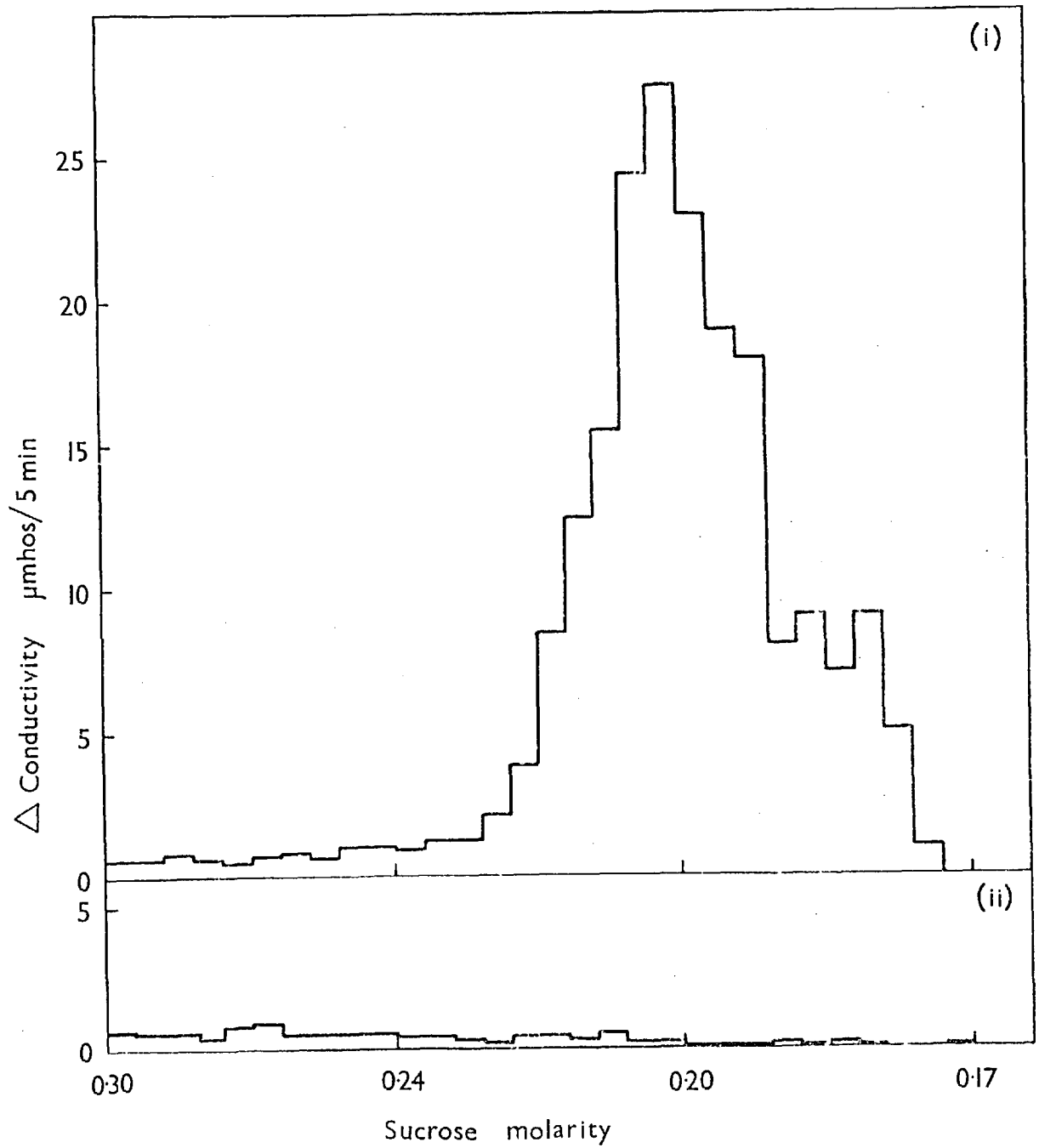
These data show that losses of ions increase sharply when the ambient solution is just about hypotonic for protoplasts. Similar results were obtained on two subsequent occasions.

It seems that PTE alters the composition of the cell wall so that it no longer can restrain expansion of the protoplasts as they accumulate water by osmotic forces. Disks were tested for viability by the plasmolytic method at the end of this experiment. Disks treated with PTE showed a mean grade of 4.5 on the neutral red index indicating that most of the protoplasts were dead. In contrast the controls showed a mean grade of 0.

3. Browning of reaction mixtures

It had been noticed that reaction mixtures containing potato disks and rot extract or PTE often became brown in colour according to the following pattern :

Figure 15. Conductivity changes given by plasmolysed potato disks on deplasmolysis



(i) PTE treated disks

(ii) Autoclaved PTE treated disks

	+, browning	-, no browning
	rot extract	PTE
Normal disks	+	+
Plasmolysed disks	+	-

Cells separate and protoplasts are killed except for plasmolysed disks and PTE, the only treatment in which browning did not occur.

The ambient solutions in reaction mixtures containing rot extract or PTE, untreated or autoclaved, and normal or plasmolysed potato disks were assayed for capacity to oxidize catechol. At intervals, 0.2 ml was removed and added to 2 ml 0.05M catechol, 0.5 ml 0.2M sodium phosphate buffer at pH 7.0 and 0.3 ml water; 1 min later absorbance at 495 nm was measured against a reagent blank as an estimate of oxidase activity. Results (Table 39) for samples from rot extract and PTE treated normal disks were corrected for any substrate released from protoplasts and subsequently carried over to the catechol solution.

Autoclaved enzymes caused no significant changes and results are not shown. Rot extract and PTE released oxidase from normal and plasmolysed disks. This suggests that ambient solutions do not become brown when plasmolysed tissue is treated with PTE because substrate remains within the plasmolysed protoplasts. Also, from the results for plasmolysed protoplasts, that the oxidase is released from the host cell walls.

4. Release of protein

A. Rate

If PTE kills protoplasts of tissues in hypotonic solution indirectly by its action on cell walls, then on rupture of the protoplasts under turgor pressure cytoplasmic protein might be released into the ambient solution. This possibility was investigated.

Table 39.

Release of phenoloxidase (catecholase) from enzyme treated disks

Time (min)	Absorbance 495 nm after 1 min			
	Normal		Plasmolysed	
	Extract	PTE	Extract	PTE
5	0.046	0.032	-	-
15	0.055	0.034	-	-
30	0.088	0.062	0.069	0.049
45	0.138	0.079	-	-
60	0.165	0.115	0.109	0.072
75	0.171	0.138	-	-
90	0.193	0.149	0.153	0.107
120	0.196	0.176	0.188	0.132
150			0.208	0.165
180			0.246	0.174
240			0.252	0.209

Extract - cucumber rot extract

Potato disks were treated as before with rot extract or PTE. Disks plasmolysed in 0.5M sucrose were also treated with PTE. At intervals, 0.2 ml was removed from the reaction mixtures, added to 0.8 ml water and protein content estimated by the Folin-Ciocalteu method. Although some samples were coloured, after dilution with the reagents they gave negligible absorbance at 750 nm.

Fifty disks were also placed in 30% v/v ethanol or 2% v/v Triton X-100 and the ambient solutions treated as above. These treatments were included because both cause rapid changes in permeability with consequent loss of ions from potato disks through disruption of membranes. In this respect their action was considered to be comparable to the presumed rupture of membranes caused by PTE and would therefore, also be expected to release cytoplasmic protein to the ambient solution.

Table 40. Release of protein from potato disks

Time (min)	Protein $\mu\text{g/ml}$					Ethanol	Triton X-100
	Extract	Extract (A)	PTE	PTE (A)	PTE [*]		
5	38	29	37	30	42	64	51
15	105	39	54	43	51	220	243
30	186	40	88	46	55	428	434
45	240	43	145	48	66	-	-
60	282	45	188	56	74	470	490
75	302	52	226	51	81	-	-
90	320	47	256	68	84	-	-
120	354	51	295	58	90	-	-

Extract - cucumber rot extract

* - disks plasmolysed in 0.5M sucrose

(A) - autoclaved

Results (Table 40) show that protein was readily detectable after 15 min and increased rapidly over the next 105 min in reaction mixtures containing untreated rot extract or PTE and normal potato disks. PTE treated plasmolysed disks released slightly more than controls but much less than normal PTE treated disks.

High levels of protein were also released from disks treated with ethanol or Triton X-100.

B. Relation to permeability changes

This was studied using the technique described earlier for conductivity changes and release of ions, except that disks were transferred to 5.0 and not 15.0 ml of water. Protein content of the water samples was estimated by the Folin-Ciocalteu method after measuring conductivity.

Results for normal potato disks treated with rot extract and PTE are shown in Table 41. Autoclaved enzyme caused no significant changes so results for these controls are omitted.

Table 41. Release of protein from and conductivity changes caused by enzyme treated disks

Time (min)	Cucumber rot extract		PTE	
	Δ Conductivity μmhos^*	Protein $\mu\text{g/ml}$	Δ Conductivity μmhos^*	Protein $\mu\text{g/ml}$
5	35.1	27	16.1	22
15	43.5	42	35.5	35
30	32.0	49	45.9	51
45	23.5	39	31.0	48
60	13.0	32	28.8	48
75	11.0	28	23.5	52
90	10.0	32	20.0	46

* - change in conductivity of 5 ml water bathing 5 disks in 15 min

Results are means for 2 replicates

Increases in conductivity caused by rot extract reached a maximum after 15 min, then declined sharply; release of protein from disks was maximal 15 min later and then declined less rapidly. With PTE maximum increases in conductivity changes and in protein release occurred at 30 min; conductivity increases then declined rapidly whereas release of protein continued at the same rate for at least another 60 min.

A possible explanation of these differences is that the permeability of the wall to macromolecules after PTE treatment is less than that after treatment with cucumber rot extract so that protein leaks more slowly to the ambient solution as shown by the slower response to the PTE treatment.

C. Molecular weight of protein

Molecular weight of protein released from potato disks was estimated by placing 48 disks for 2h in 3 ml standard reaction mixture containing cucumber rot extract or PTE. The mixture was filtered using Whatman No. 40 ashless paper and, after dilution, filtering in an Amicon Ultrafiltration cell through membranes with cut-off points of MW 1,000, 10,000 and 15 - 25,000 under pressure of 60 p.s.i. N_2 . Protein content of original and filtrate was estimated using the Folin-Ciocalteu method.

The results (Table 42) show that c. 70 per cent of the total measurable protein leached from rot extract and PTE treated potato disks had a molecular weight $>15 - 25,000$.

Table 42. Ultrafiltration of protein released from enzyme treated potato disks

Cut off MW of membrane	Protein in filtrate as % of original [*]	
	Cucumber rot extract	PTE
1,000	26.0	29.5
10,000	28.0	27.0
15 - 25,000	34.0	27.0

* - Protein passing through membrane as % of total leached from disks treated with cucumber rot extract or PTE

5. Release of enzymes

A. Acid phosphatase

The previous experiments showed that protein as phenoloxidase was released into the ambient solution from potato tissue treated with cucumber rot extract or PTE. Samples from reaction mixtures were next assayed for acid phosphatase. This enzyme has been used as a marker for lysosomal enzymes so that, should lysosomes of cells be disrupted by rot extract or PTE then acid phosphatase and possibly other enzymes should pass into the ambient solution with the cytoplasmic protein.

Samples of the ambient solution from potato tissue treated with rot extract or PTE were assayed for acid phosphatase. Activity was expressed as μg p-nitrophenol liberated under the conditions of the assay.

Samples from the ambient solution from disks treated with 30% v/v ethanol or 2% v/v Triton X-100 (50 disks/8 ml) were also assayed.

Results (Table 43) show that acid phosphatase is rapidly released from unplasmolysed potato tissue treated with rot extract or PTE in a manner similar to that for protein (see Table 40). Release from disks treated with autoclaved PTE was similar to that from untreated disks or ones treated with autoclaved rot extract. However, acid phosphatase was also released from plasmolysed disks treated with PTE; the rate was c. 40 per cent of that from normal disks. Because protoplasts remain alive in plasmolysed tissue treated with PTE and do not release ions, this result suggests that about 40 per cent of acid phosphatase released from enzyme treated normal tissues comes from cell walls and the balance from the protoplast.

Ethanol and Triton X-100 although causing release of large amounts of protein apparently released little acid phosphatase. But absence of activity in the ambient solution may also reflect inactivation after release.

Table 43.

Release of acid phosphatase from potato disks

$\mu\text{g p-nitrophenol liberated/0.2 ml sample}$										
Time (min)	Extract	Extract (A)	PTE	PTE (A)	Water	PTE*	PTE* (A)	Water	Ethanol	Triton X-100
5	8	2	5	2	1	3	1	1	1	0
15	40	3	21	3	2	10	2	2	3	2
30	81	3	72	4	3	24	3	3	4	4
45	106	3	86	5	4	42	4	3	-	-
60	119	4	103	6	4	51	5	4	5	4
75	131	5	117	6	5	60	5	4		
90	134	5	128	7	5	69	6	5		
120	140	5	137	8	5	73	7	5		

Extract - cucumber rot extract

(A) - autoclaved

* - disks plasmolysed in 0.5M sucrose

i) Relation to permeability changes

In a previous experiment an attempt was made to relate conductivity changes with release of protein. This was extended by estimating acid phosphatase activity in the same water samples. Results for conductivity changes and release of acid phosphatase (expressed as μg p-nitrophenol released) by normal disks treated with rot extract or PTE are shown in Table 44. Controls are omitted as they caused no significant changes.

Release of acid phosphatase from disks treated with rot extract was maximal after 30 min which was soon after maximum changes of conductivity and at the same time as release of protein (see Table 41). Release from PTE treated disks was maximal after 60 min which was 30 min after maximum conductivity changes but at a time when there was still a high rate of protein release. Thus acid phosphatase release occurs soon after maximum release of protein from disks treated with rot extract or PTE.

B. Other enzymes

The activity of a number of lysosomal enzymes associated with the supernatant fraction of tissue homogenates has been demonstrated. Among these are acid RNase, acid phosphatase and acid phosphodiesterase (Pitt and Galpin, 1971) and β -glucosidase (Matile and Winkenbach, 1971).

As acid phosphatase is released from potato tissue treated with cucumber rot extract or PTE attempts were made to detect other enzymes considered to be of lysosomal origin. Plasmolysed disks were also treated with PTE to determine whether other enzyme activities were associated with cell walls in the same way as acid phosphatase.

Samples from the ambient solution from potato disks treated with rot extract or PTE were assayed for alkaline phosphatase, acid phosphodiesterase or alkaline phosphodiesterase.

The procedure for estimations was similar to that described for

Table 44. Release of acid phosphatase from and conductivity changes caused by enzyme treated disks

Time (min)	Cucumber rot extract		PTE	
	Δ Conductivity μmhos^*	Acid phosphatase ^{**}	Δ Conductivity μmhos^*	Acid phosphatase ^{**}
5	35.1	111	16.1	55
15	43.5	186	35.5	103
30	32.0	198	45.9	130
45	23.5	150	31.0	178
60	13.0	136	28.8	225
75	11.0	133	23.5	220
90	10.0	147	20.0	211

* - change in conductivity of 5 ml water bathing 5 disks in 15 min

** - μg p-nitrophenol liberated/0.2 ml

Results represent means for 2 replicates

acid phosphatase. Alkaline phosphatase was estimated using 2 mM p-nitrophenyl phosphate disodium salt in 0.1M Tris-HCl buffer at pH 8.8. Acid and alkaline phosphodiesterase was estimated similarly using 2 mM bis p-nitrophenyl phosphate (Sigma Chem. Co. Ltd.) in 0.1M sodium acetate buffer at pH 5.0 or in 0.1M Tris-HCl buffer at pH 8.8 respectively. Results are shown in Table 45. Water controls or those containing autoclaved enzyme showed negligible increases in activity and are omitted.

Acid phosphodiesterase was released from normal disks treated with rot extract or PTE. Release also occurred with plasmolysed PTE treated disks, the rate being c. 30 per cent of that from normal disks. These results are similar to those obtained for acid phosphatase. In contrast, levels of alkaline phosphatase and phosphodiesterase were much lower but release from disks was detected in a manner similar to that of the other enzymes.

Attempts were made to detect other enzymes associated with lysosomes to see if these also were released from cell walls. It was hoped that an enzyme typically associated only with the protoplast could be detected; release of such an enzyme from normal PTE treated disks would provide evidence for leakage of cytoplasmic macromolecular material consistent with a hypothesis of membrane rupture caused by PTE treatment of normal but not plasmolysed disks. Estimates were made of RNase, DNase and β -glucosidase activities in the ambient solution from PTE treated normal or plasmolysed disks. In addition, estimates were made of K^+ and malic dehydrogenase, an enzyme believed to be associated exclusively with cytoplasmic fractions of tissue homogenates.

Assay of DNase was similar to that of RNase but 0.05 ml 1% w/v RNA was replaced by 0.1 ml 0.5% w/v calf thymus DNA (BDH Ltd.). Activity was expressed as increased absorbance (OD) at 260 nm.

Assay of β -glucosidase was similar to that of acid phosphatase; the substrate used was 2 mM p-nitrophenyl- β -D-glucopyranoside (Koch-Light Ltd.) in 0.1M sodium acetate buffer at pH 5.0. Activity was

Table 45. Release of acid phosphodiesterase, alkaline phosphodiesterase and alkaline phosphatase from disks

µg p-nitrophenol liberated/0.2 ml sample									
	Acid phosphodiesterase			Alkaline phosphodiesterase			Alkaline phosphatase		
	Extract	PTE	PTE [*]	Extract	PTE	PTE [*]	Extract	PTE	PTE [*]
5	11	9	2	1	1	1	0	0	0
15	42	25	8	3	2	1	1	0	0
30	92	80	11	7	6	2	2	1	0
45	113	90	17	9	8	4	4	3	1
60	130	112	30	11	11	4	4	3	1
75	142	124	35	12	11	5	4	4	1
90	150	128	41	13	12	6	4	4	1
120	154	139	43	14	13	7	5	5	1

Extract - cucumber rot extract

* - disks plasmolysed in 0.5M sucrose

expressed as μg p-nitrophenol liberated/0.2 ml enzyme sample.

Malic dehydrogenase activity was estimated as described in 'Materials and Methods' and expressed as change in absorbance at 420 nm per minute under the conditions of the assay. Mean activity for two replicates was estimated for each sample time.

No DNase activity was detected with PTE treated disks. Only low β -glucosidase activity was detected; increase in liberation of p-nitrophenol after 2h was only 2 μg for PTE treated normal disks and 1 μg for plasmolysed disks when compared with controls.

Results for K^+ , RNase and malic dehydrogenase are shown in Table 46. High levels of K^+ were released from PTE treated normal disks and much lower levels were released from autoclaved enzyme controls or PTE treated plasmolysed disks. This indicates that much of the K^+ efflux is from the protoplasts; release from cell walls as shown by PTE treated plasmolysed disks was no greater than that from controls and was probably due to the rupturing of a few protoplasts.

RNase release was high from PTE treated normal disks but was also high from autoclaved PTE treated disks being c. 50 per cent of the former. No explanation could be found for this high background level of activity shown by the control. Release of RNase from plasmolysed disks was c. 75 per cent of that from normal disks indicating that a large proportion of the total RNase activity of the cell is associated with the cell wall.

Malic dehydrogenase was released from PTE treated normal disks and also from plasmolysed disks the rate of release being c. 80 per cent of the former after 2h. Release from controls, in contrast, was very low. These results were unexpected as they demonstrate the apparent release of malic dehydrogenase from cell walls, the amount released being a large proportion of that released from PTE treated normal disks which might be considered as an estimate of wall plus protoplast activities.

Attempts were made to detect glucose-6-phosphate dehydrogenase in the ambient solution from PTE treated disks in the same way as before.

Table 46.

Release of K^+ , RNase and malic dehydrogenase from disks

Time (min)	K^+ (ppm)				RNase (OD 260 nm)			Malic dehydrogenase Δ OD 340 nm/min/0.1 ml sample			
	PTE	PTE (A)	PTE*	PTE* (A)	PTE	PTE (A)	PTE*	PTE	PTE (A)	PTE*	PTE* (A)
15	64.5	42.4	40.6	42.5	0.078	0.056	0.050	0.030	0.011	0.010	0.012
30	184	50	47.5	46.6	0.085	-	-	0.062	0.012	0.029	0.013
60	448	57.5	60	52.5	0.088	0.060	0.062	0.137	0.013	0.060	0.017
90	680	66	67	56.4	0.113	-	0.078	0.149	0.019	0.095	0.018
120	795	72.5	70.2	62	0.121	0.065	0.092	0.169	0.017	0.136	0.021

(A) - autoclaved

* - disks plasmolysed in 0.5M sucrose

A photometric method measuring the reduction of NADP in the presence of glucose-6-phosphate was used. No activity was detected in samples taken from ambient solutions of disks treated with PTE for 2h.

The apparent release of enzymes but not K^+ from cell walls of potato by the action of PTE was further investigated. Samples of 48 disks were treated in one of the following ways.

- a) 3 ml water containing 14.8 units PTE, 0.001M $CaCl_2$, 0.02M Tris-HCl buffer (pH 9.0) for 3h
- b) as (a) but in 0.5M sucrose as plasmolyticum
- c) as (b), then liquid removed, disks washed in 0.5M sucrose, added 3 ml water for 10 min
- d) 3 ml water, frozen at $-20^{\circ}C$, thawed and kept for 10 min
- e) as (d), liquid removed, disks then treated as (a)

At the end of each treatment, the ambient liquid was collected and assayed for protein, K^+ , acid phosphatase, phenoloxidase, peroxidase and malic dehydrogenase.

Table 47 summarizes the effects of the different treatments and also shows in column (d) + (e) the totals of protein, K^+ and enzymes liberated from frozen tissue after thawing and released from residual tissue after treatment with PTE for 3h. Other results are presented with percentages of these totals in parentheses.

Comparison of columns (d) and (e) show that K^+ liberated by PTE treatment of residual tissue was only about 10 per cent of that liberated by freezing and thawing and for protein liberated was 38 per cent. The corresponding figures for the enzymes were 72 - 91 per cent.

Similarly, whereas very little K^+ was released from tissues treated with PTE in a plasmolyticum, large amounts of peroxidase, acid phosphatase and malic dehydrogenase and smaller though substantial amounts of phenoloxidase were liberated under these conditions. There were

Table 47. Effect of PTE on release of protein, K⁺ and enzymes from potato tuber tissue

Activity [*]	Treatment ^{**}					
	(a)	(b)	(c)	(d)	(e)	(d) + (e)
Protein	335(73)	85(18)	250(54)	287(62)	173(38)	460(100)
K ⁺	625(81)	17(2)	685(88)	715(92)	60(8)	775(100)
Acid phosphatase	0.132(82)	0.059(37)	0.056(34)	0.040(25)	0.121(75)	0.161(100)
Phenoloxidase	0.13 (78)	0.032(19)	0.059(35)	0.046(28)	0.12 (72)	0.166(100)
Peroxidase	0.10 (110)	0.060(66)	0.023(25)	0.008(9)	0.083(91)	0.091(100)
Malic dehydrogenase	0.132(49)	0.082(30)	0.115(42)	0.052(19)	0.22(81)	0.272(100)

* Protein - µg/ml; K⁺ - ppm; acid phosphatase - mg p-nitrophenol liberated/0.2 ml enzyme solution; phenoloxidase - Δ OD 495 nm/min/0.2 ml enzyme solution; peroxidase - Δ OD 420 nm/min/0.01 ml enzyme solution; malic dehydrogenase - Δ OD 340 nm/min/0.1 ml enzyme solution

** Treatment see text for details

corresponding differences when plasmolysed protoplasts in PTE treated tissue were rapidly deplasmolysed.

Release of protein was high in treatments (a), (c) and (d); that is in those treatments where there is presumably rupture of membranes. Release in treatment (b) which is presumably from cell walls was comparatively low but this protein is associated with high enzyme activities. It must be assumed that this protein consists largely of enzyme protein unlike that released in treatments (a), (c) and (d) which contains a relatively smaller proportion of the enzymes measured.

The results of this and the previous experiments suggest that large proportions of the four enzymes in cells are bound to cell walls and are liberated when these are exposed to PTE.

The possibility that these results could be explained, at least in part, by adsorption by cell walls of cytoplasmic enzymes released when cells are broken during preparation of the potato tuber disks or by residual cytoplasm on cut surfaces was investigated.

Samples of 48 potato disks were placed in 0, 0.05, 0.1, 0.15 or 0.2M NaCl for 30 min. These solutions were changed twice during this time. All disks were then washed well in water before plasmolysing in 0.5M sucrose and treating with PTE as before. After 3h the ambient liquid from each sample was collected and assayed for acid phosphatase and malic dehydrogenase.

Table 48. Effect of NaCl on the release of enzymes from PTE treated plasmolysed disks in 3h.

Activity*	NaCl molarity				
	0	0.05	0.1	0.15	0.2
Acid phosphatase	0.065	0.060	0.078	0.068	0.080
Malic dehydrogenase	0.094	0.107	0.112	0.129	0.119

* Acid phosphatase - mg p-nitrophenol liberated/0.2 ml enzyme solution;
malic dehydrogenase - Δ OD 340 nm/min/0.1 ml enzyme solution

Results (Table 48) show that preliminary washing of disks in NaCl up to 0.2M did not much decrease release of acid phosphatase or malic dehydrogenase when disks were later treated with PTE in a plasmolyticum. Jansen, Jang and Bonner (1960) found that adsorbed enzyme could be removed from cell walls by washing with 0.15M NaCl and Hall and Butt (1968) showed that washing with 0.125M NaCl considerably reduced β -glycerophosphatase activity of cell wall preparations.

6. Release of RNA

Although PTE treated normal disks are killed within 3h and release to the ambient solution electrolytes, protein and enzymes there is also a substantial release of enzymes from PTE treated plasmolysed disks which are not killed. An attempt was made to detect RNA in the ambient solutions from PTE treated disks, as it was considered unlikely that release of RNA would occur from plasmolysed PTE treated disks in which the protoplasts remained alive and intact.

A. Gel electrophoresis

The ambient solution from normal or plasmolysed potato disks treated with PTE were collected after 3h and protein content estimated by the Folin-Ciocalteu method. The concentration of protein was adjusted to be approximately equal in each sample. Each was made dense by the addition of sucrose and then subjected to gel electrophoresis on 7.5% polyacrylamide gel at pH 2.3. The gels were stained with 1% w/v toluidine blue in 15% v/v ethanol for 1h and then destained with fresh solvent. No bands of RNA became stained.

This procedure was repeated using 5% gel at pH 9.5. After staining, a single band was present on each gel. This indicates that RNA is apparently released from both normal and plasmolysed PTE treated potato disks and that RNA is excluded from 7.5% polyacrylamide gel.

B. Spectrophotometric estimation of RNA

Samples prepared as previously and also samples from autoclaved PTE treated normal or plasmolysed disks were extracted with phenol to remove protein.

To each sample was added an equal volume of saturated phenol in 0.01M Tris-HCl buffer at pH 7.4. The protein precipitated at the interface between the phenol and the aqueous layers after centrifugation at 12,000 x g for 10 min. The aqueous solution was decanted and the procedure repeated until no further precipitate of protein was visible at the interface.

To one volume of the aqueous phase was added two volumes of ethanol and the mixture left overnight at -20°C . The precipitated RNA was collected by centrifugation at 26,000 x g for 1h. Further ethanol (100%) was added to wash the precipitate and left overnight at -20°C . The precipitate was finally collected by centrifugation as before and taken up in 2 ml of 0.15M NaCl in 0.015M sodium citrate. The protein content (by the Folin-Ciocalteu method) and absorbance of this solution at 260 and 280 nm were estimated. Results are shown in Table 49.

It was found that although some protein had been removed from the samples by phenol, removal was not complete because PTE treated normal and plasmolysed disks contained c. one quarter of the protein concentration of corresponding samples before extraction with phenol (see Table 47). All samples including the controls showed higher absorbance at 260 nm than at 280 nm indicating the presence of nucleic acid. Samples from PTE treated plasmolysed disks showed higher absorbance at 260 nm than samples from normal PTE treated disks but higher absorbance was also shown by autoclaved PTE treated plasmolysed disks. From these results it appears that nucleic acids absorbing strongly at 260 nm are released from both PTE treated normal and plasmolysed disks this release being more than double that shown by controls.

Table 49. Spectrophotometric estimation of RNA after removal of protein with phenol

Treatment	Protein μg/ml	Absorbance 260 nm	Absorbance 260 nm	μg/ml ^{**} RNA
PTE	76	0.479	0.272	19
PTE(A)	24	0.137	0.074	5
PTE [*]	80	0.487	0.260	19
PTE(A) [*]	43	0.220	0.108	9

(A) - autoclaved * - disks plasmolysed in 0.5M sucrose

Results represent means for two replicates

** - if $\frac{E_{260}}{E_{280}} \cong 2$, then 1 E_{260} unit $\equiv$ 40 μg/ml RNA

C. Orcinol method for estimation of RNA

Orcinol (BDH Ltd.) was recrystallized from benzene. 100 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was dissolved in 100 ml conc. HCl and to this was added 3.5 ml of a solution of 6% w/v orcinol in ethanol. Samples from PTE treated normal or plasmolysed potato disks extracted with phenol as described earlier were used together with controls. Two ml orcinol reagent was added to 1 ml of sample, mixed and kept for 30 min at 90°C in a water bath. After cooling, absorbance was measured at 665 nm against the corresponding controls.

Mean values for two replicates were 0.40 and 0.50 for PTE treated normal or plasmolysed disks respectively. This confirms that RNA is released from both normal and plasmolysed potato tissue treated with PTE.

7. Gel electrophoresis of protein released from PTE treated disks

Results obtained previously have shown that protein and enzymes are released from PTE treated normal or plasmolysed potato tissue. All enzymes tested for were present in both samples. As protoplasts are killed in normal tissue after exposure for 3h to PTE but remain alive in plasmolysed tissue it was thought that the former treatment would release protein from protoplast and wall whereas the latter would release only wall protein.

Samples from PTE treated disks were subjected to gel electrophoresis, after first adjusting the concentration of protein in each to be approximately equal, on 7.5% gels at pH 2.3 or 5% gels at pH 9.5.

Bands of protein, after staining with Coomassie brilliant blue, are shown in Plate 2 (a) and (b). It could be seen that two large protein bands were resolved with both samples. Several other bands were present but no differences could be seen with either sample. Similar results were obtained with 5% gel at pH 9.5.

Absorbance of Coomassie brilliant blue at 550 nm was measured by scanning the gels as described in 'Materials and Methods'. Results are shown in Figures 16 and 17. Although differences in levels of absorbance were obtained this was probably due to inaccuracies during loading of gels or to uneven destaining. The general patterns of absorbance for both samples were very similar. One large double peak and several smaller peaks were detected on 7.5% gel (Figure 16 (a) and (b)) whereas greater separation was obtained on 5% gel (Figure 17 (a) and (b)).

These results demonstrate that components of protein released from PTE treated normal or plasmolysed potato tissue are essentially similar.

8. Respiratory changes in enzyme treated potato tissue

Plasmolysed disks treated with PTE remain alive in the sense that they retain neutral red in hypertonic solution and do not release electrolytes. However, losses of protein, enzymes and RNA have been demonstrated. An

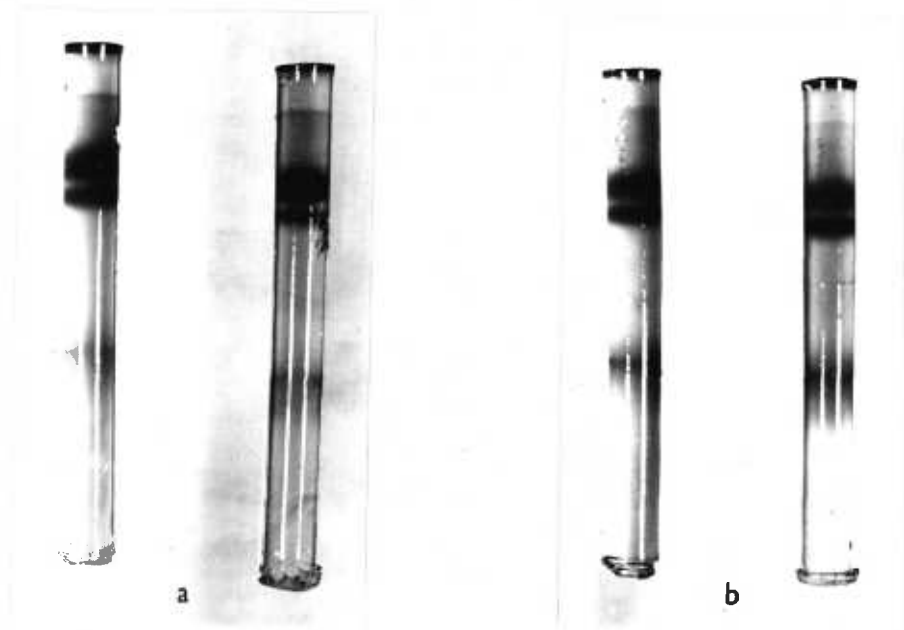


Plate 2. x 1. Electrophoresis on 7.5% gel at pH 2.3 of protein released from PTE treated potato tissue (a) Normal tissue (b) Plasmolysed tissue (0.5M sucrose)

Figure 16. Absorbance at 550nm of bands of protein stained with Coomassie brilliant blue after electrophoresis on 7.5% gel at pH 2.3 of samples from PTE treated potato tuber tissue

(a) Normal

(b) Plasmolysed

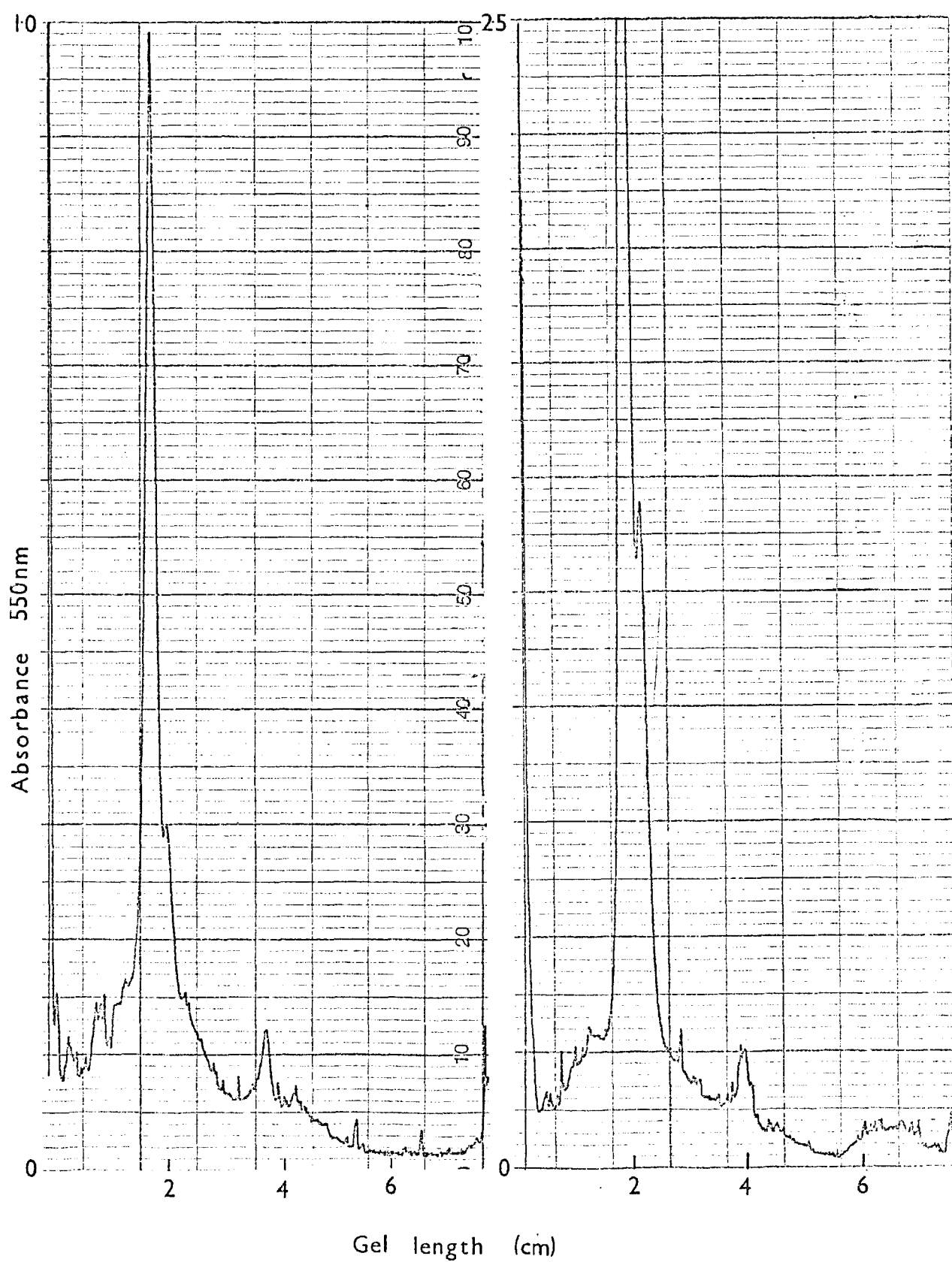
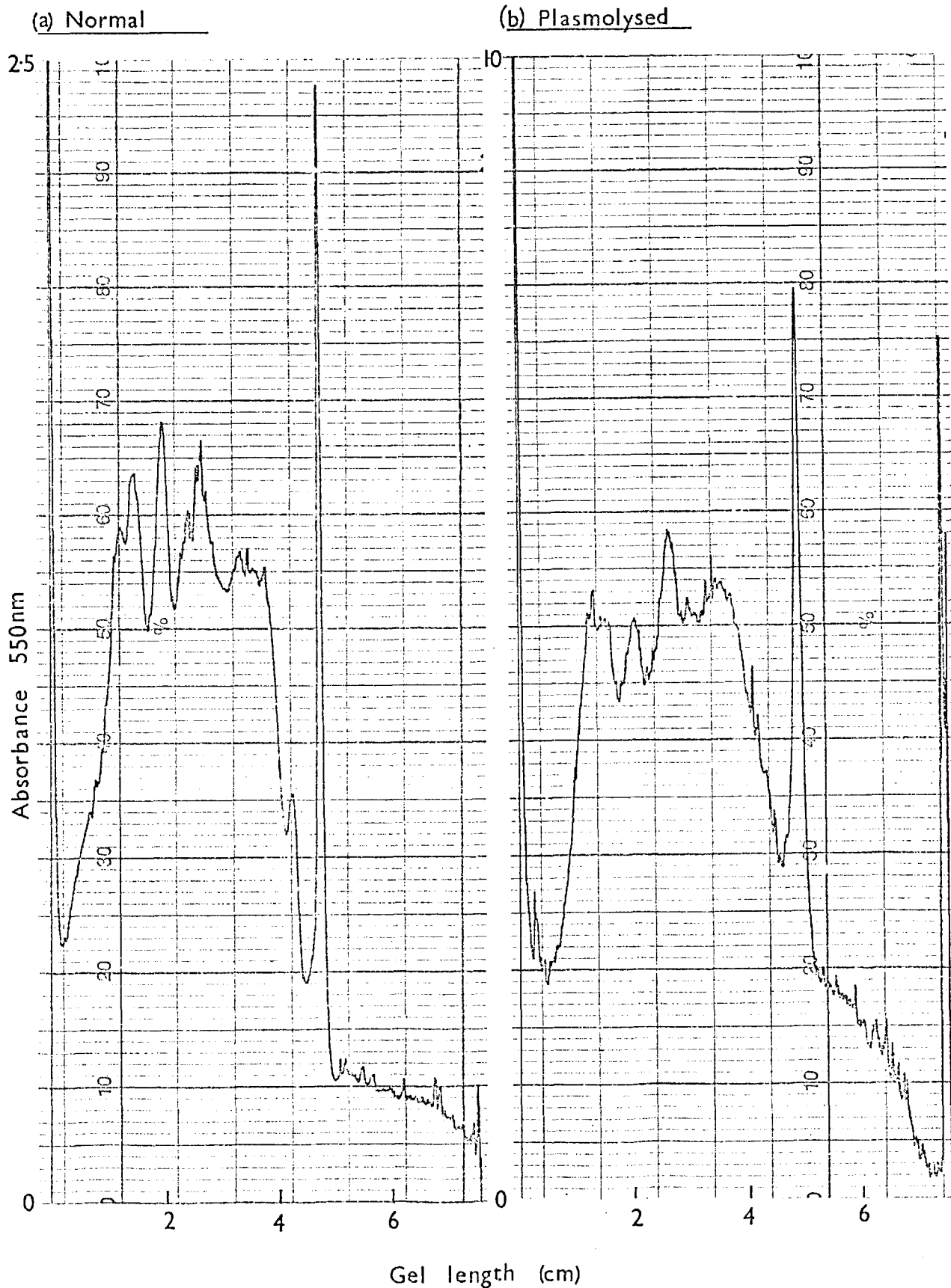


Figure 17. Absorbance at 550nm of bands of protein stained with Coomassie brilliant blue after electrophoresis on 5% gel at pH 9.5 of samples from PTE treated potato tuber tissue



attempt was made to study respiratory changes in disks treated with cucumber rot extract or PTE.

Standard reaction mixtures (6 ml) containing 50 normal or plasmolysed potato disks (4 mm x 0.5 mm) were placed in an oxygen electrode; respiration rate was measured and expressed as uptake of oxygen (μ moles/50 disks). Results are shown in Figure 18 (a) and (b).

Disks in autoclaved rot extract or autoclaved PTE showed a respiration rate similar to that of disks in water and so are omitted. Normal disks in rot extract showed an increased respiration rate in the first 10 min which then declined after 30 min. This is consistent with killing of protoplasts of disks by rot extract. The fact that the respiration rate did not completely stop may have been due to post-mortem oxidation reactions in the reaction mixture. PTE treated normal disks did not show a greatly increased respiration rate in the first 30 min but the rate did decline steadily in much the same way as for disks in rot extract.

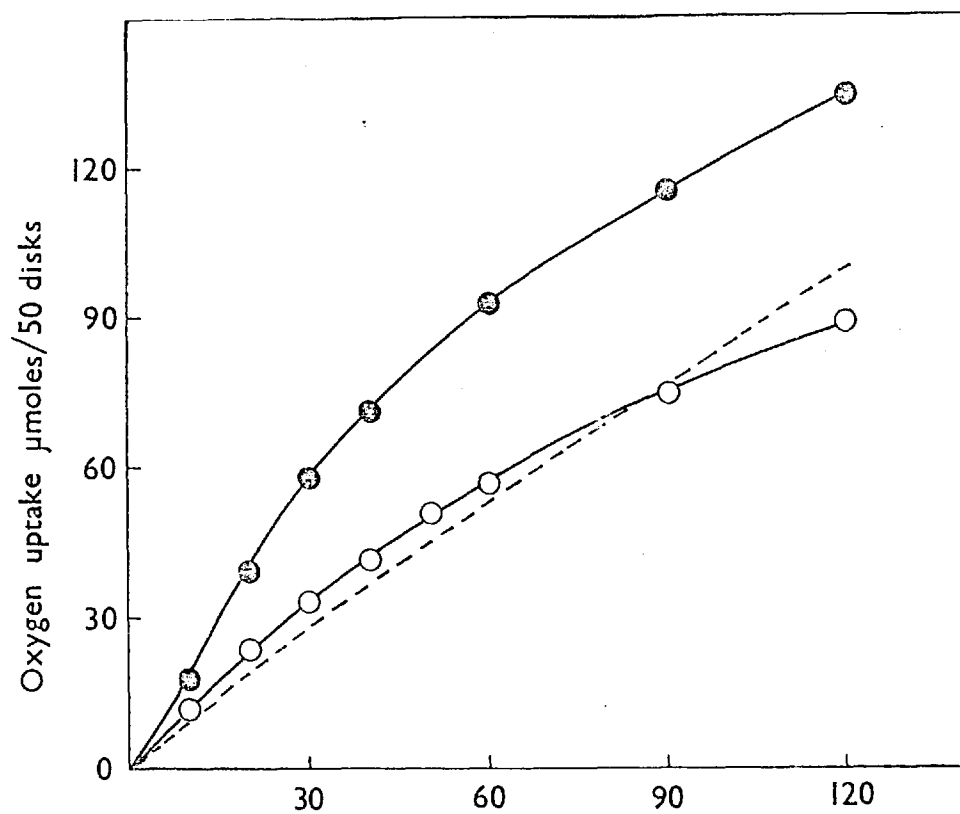
Plasmolysis of disks in 0.5M sucrose increased the respiration rate. Rot extract treated plasmolysed disks showed an increased respiration rate which declined after 40 min and was almost stopped after 120 min. This is very similar to the result obtained with normal disks. PTE treated plasmolysed disks showed no increase and very low decrease in respiration rate in 120 min. It was thought that slight decrease in respiration rates may have been due to cells being broken by contact with the magnetic

stirrer, especially after PTE treatment. Although attempts were made to separate disks from the stirrer in early experiments using baskets of gauze, it was found that very variable respiration rates were obtained, possibly because of uneven diffusion of oxygen throughout the reaction mixture.

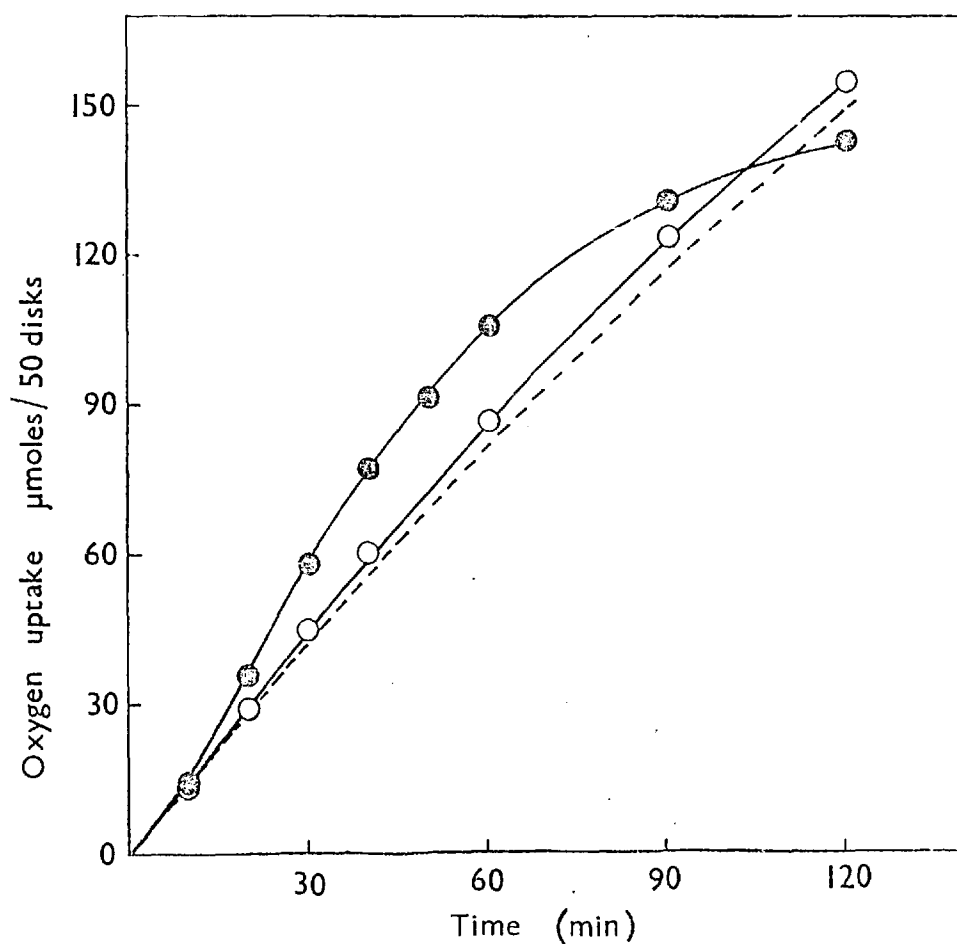
These results demonstrate that rot extract increases the respiration rate of potato tissue, this increase being due to factors other than PTE content. Similar results were obtained by Hall (1971) for extracts of

Figure 18. Uptake of oxygen by rot extract and PTE treated potato tissue

(a) Normal tissue



(b) Plasmolysed tissue



rots of potato caused by E. carotovora var. atroseptica and partially purified PTE.

These results also confirm that PTE treated plasmolysed disks in contrast to PTE treated normal disks or rot extract treated normal or plasmolysed disks, show respiration rates very similar to that of controls and are presumably not killed by the PTE.

In summary, low molecular weight toxins, particularly H_2O_2 were not released from potato tissue by PTE of cucumber rot extract.

Protoplasts of plasmolysed tissue treated with PTE but not autoclaved PTE were killed on deplasmolysis.

Normal tissue treated with rot extract or PTE released electrolytes, protein and enzymes. The protein was shown to be predominantly high molecular weight.

PTE treated plasmolysed tissue, although remaining alive as indicated by retention of neutral red and electrolytes, and uptake of oxygen, released protein, enzymes and RNA. The protein released was apparently similar in composition, as shown by gel electrophoresis, to that released by normal PTE treated tissue.

VIII. Light and electron microscopy

1. Potato tuber tissue

Light microscopy of potato tuber tissue treated with rot extract for 2h showed that cell separation was complete in this time. The coherent untreated tissue (Plate 3) was transformed into a suspension of individual cells which however, retained their identity and starch granules (Plate 4). This confirms that the walls were essentially intact at the time of cell separation. The cells shown in Plate 4 would have been killed within this time as shown by their inability to plasmolyse and retain

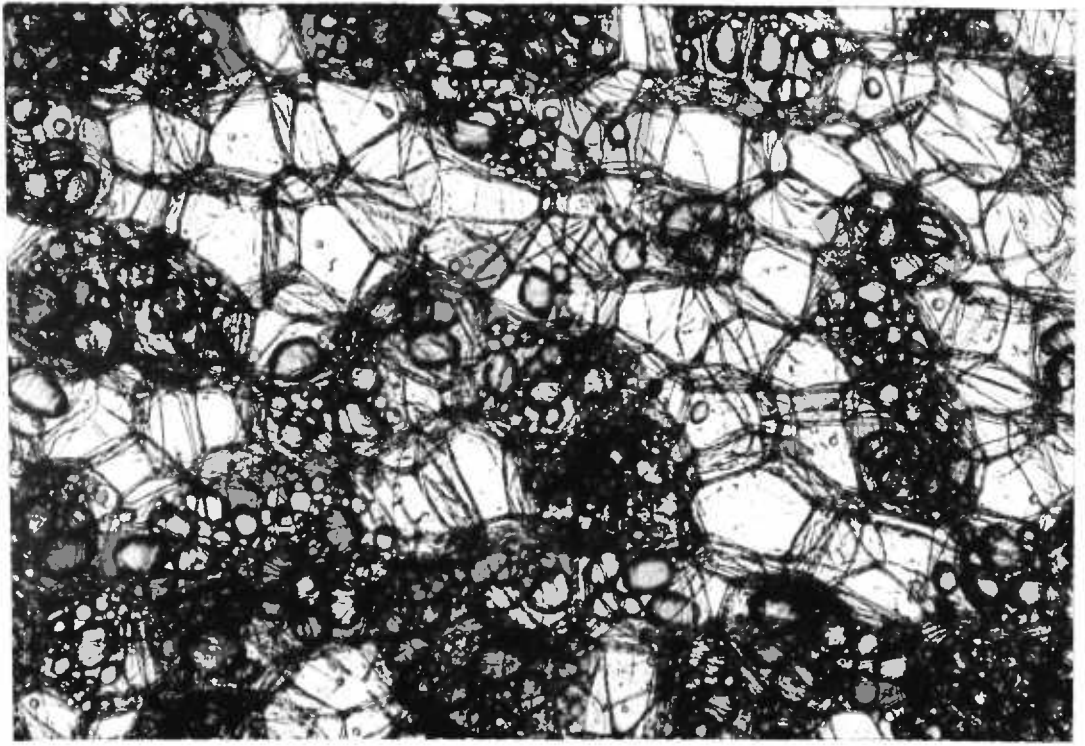


Plate 3. x 1,760. Untreated potato tuber tissue fully coherent.

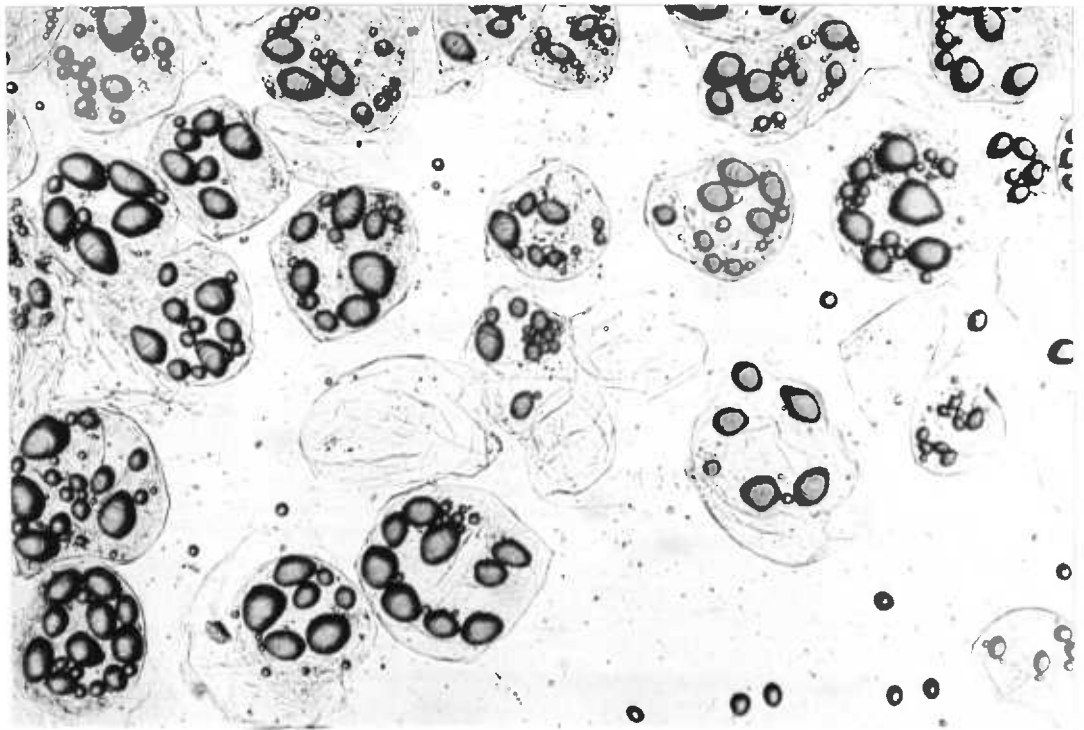


Plate 4. x 1,760. Potato tuber tissue treated for 2h with cucumber rot extract showing separation of cells still surrounded by wall and containing starch granules.

neutral red. The changes that occurred in cell walls and membranes were studied by electron microscopy, especially the association of the plasmalemma with the cell wall as affected by the action of PTE because earlier results suggested that protoplasts are killed because they rupture under turgor pressure.

Plates 5 and 6 show untreated potato tuber cell walls. The very large size of these cells was a disadvantage for a study of this kind. Intercellular spaces were surrounded by walls with smooth surfaces and contained little debris. A region, probably the middle lamella, was visible as a slightly more electron-dense layer. The walls appeared to be layered probably because of the fibrillar nature of the cellulose. Each cell contained a very large vacuole within cytoplasm adhering closely to the walls. Several vesicles, some of which might be mitochondria were visible together with crystals apparently contained in vesicles.

Plate 7 shows the wall after 30 min treatment with PTE. There appears to be degradation of the wall in the intercellular space as indicated by the debris.

In plate 8 the wall appears similar to the controls and separates a living cell with intact membranes on the left from a cell where the tonoplast but perhaps not the plasmalemma is ruptured. Also in this cell is a large starch granule.

Plate 9 shows a part of the wall after 120 min treatment with PTE. The wall did not stain and was electron translucent. No cell contents are visible in the lower cell and in the upper cell there are only disorganized membranes. These cells would have been killed within this time and cell separation would have been complete.

Samples of potato tissue treated for 45 and 60 min were also examined but no further information could be gained. The tissue was very difficult to section because of the starch grains and because after treatment with PTE walls were very difficult to stain. The cells were also too large to observe the plasmalemma in its entirety so that

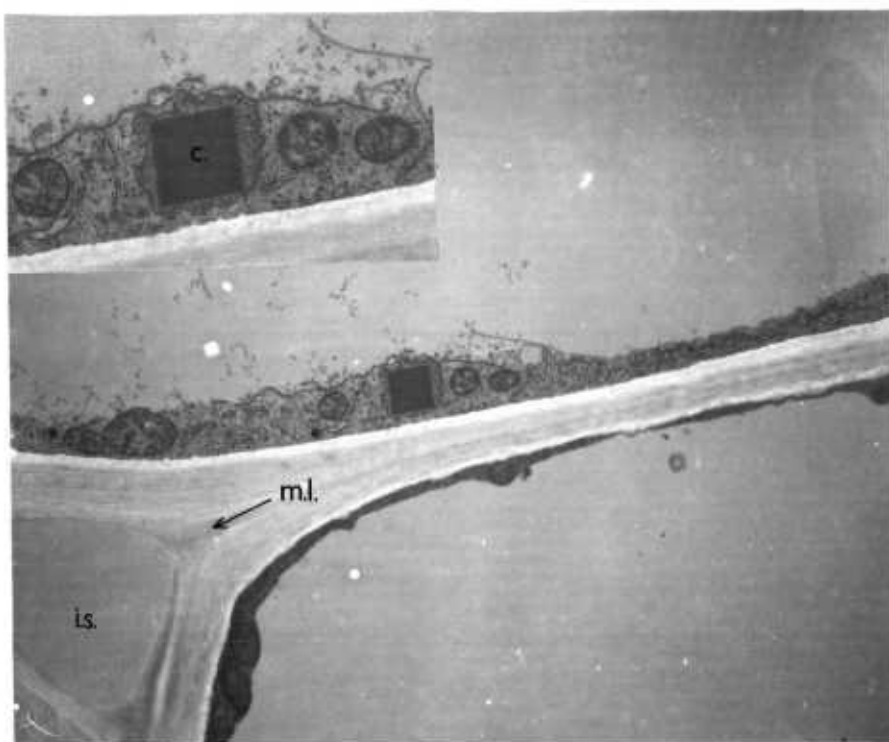


Plate 5. x 6,150. Transmission electron micrograph of untreated potato tuber tissue showing intercellular space (i.s.), middle lamella (m.l.) and in inset, crystal in vesicle (c.) and other cytoplasmic inclusions.

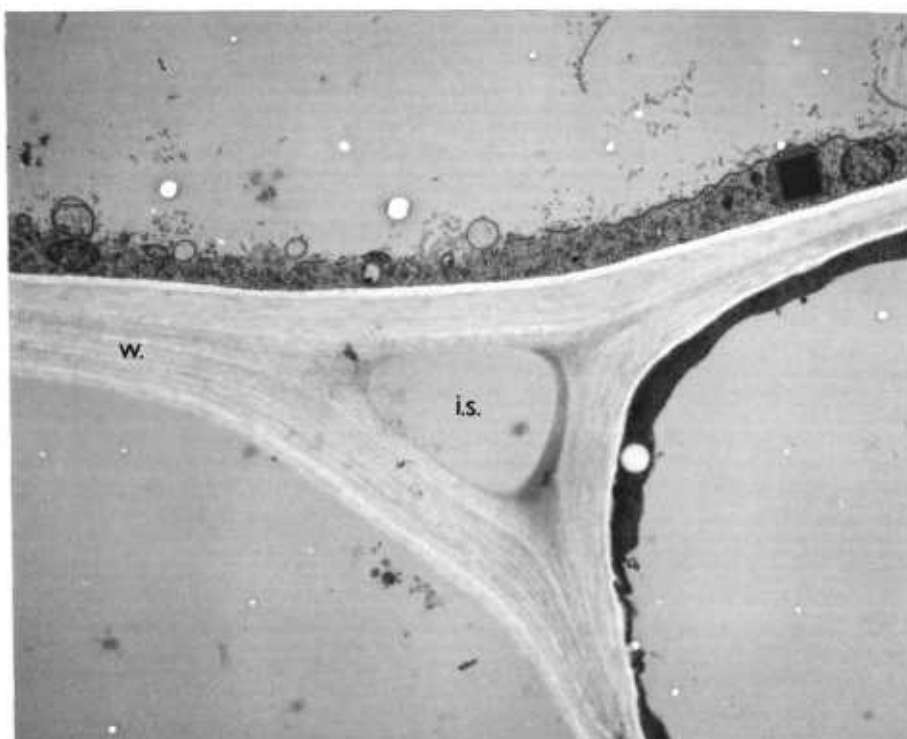


Plate 6. x 7,960. Transmission electron micrograph of untreated potato tuber tissue showing intercellular space (i.s.), fibrillar wall (w) and cytoplasmic inclusions.

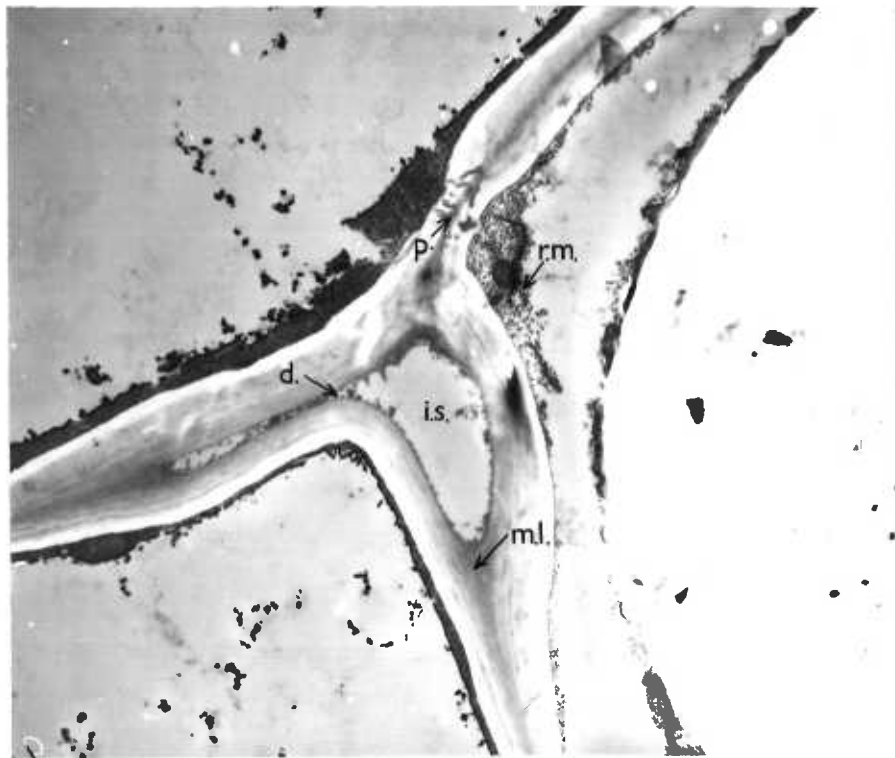


Plate 7. x 3,480. Transmission electron micrograph of potato tuber tissue treated with PTE for 30 min showing wall degradation releasing debris (d.) into intercellular space (i.s.). Also visible is middle lamella region (m.l.) plasmodesmata (p.). The cytoplasm of the cells appears disorganised with apparent rupture of membranes (r.m.)

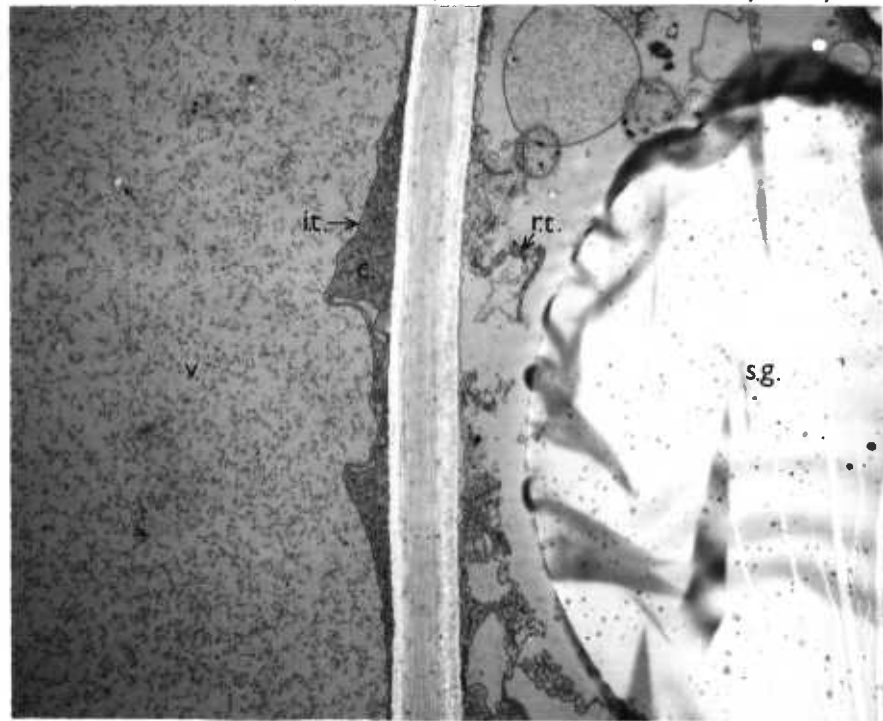


Plate 8. x 6,150. Transmission electron micrograph of potato tuber tissue treated with PTE for 30 min. Cell on left shows cytoplasm (c.) bounded by intact tonoplast (i.t.) and a large vacuole (v.) with much enclosed material. Cell on right has a ruptured tonoplast (r.t.) and contains a large starch granule (s.g.).

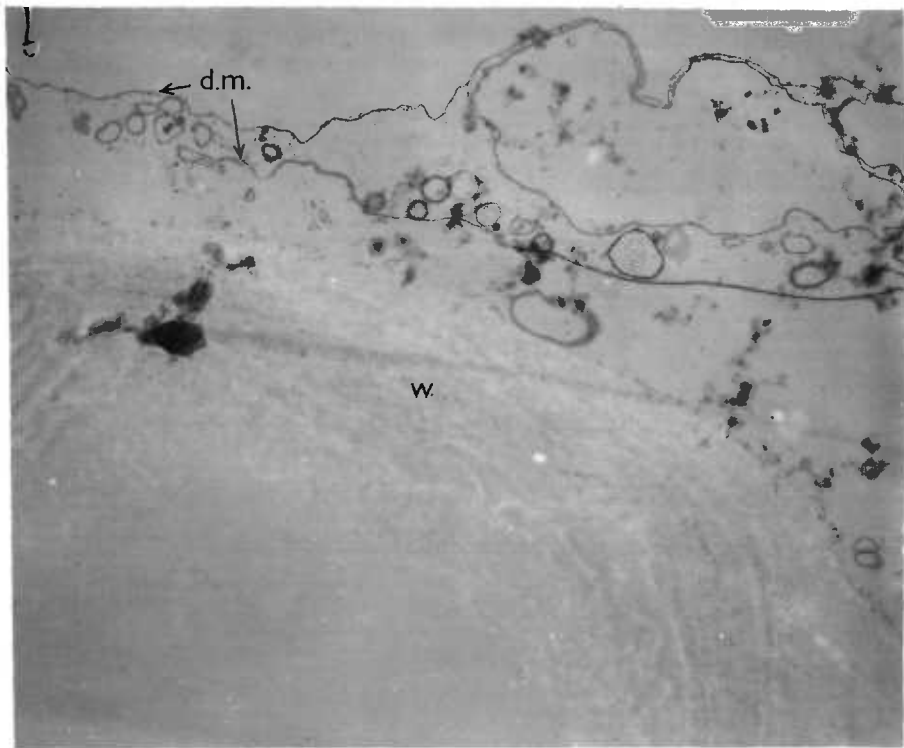


Plate 9. x 10,095. Transmission electron micrograph of potato tuber tissue treated with PTE for 120 min showing electron translucent wall (w.) and disorganized membranes (d.m.).

significant changes might have occurred in parts not under examination.

This study failed to contribute to the understanding of how PTE by its action on the wall can cause the disruption of membranes as seen in Plate 9.

2. Carrot callus cells

These were studied by scanning electron microscopy for changes visible on treatment with PTE for 2h. Protoplasts were killed in this time.

Plates 10 and 11 show that untreated cells in water are fully expanded with a relatively smooth intact surface and are accompanied by little or no debris except that from mechanically damaged cells (Plate 11).

Plate 12 shows that after PTE treatment the wall becomes wrinkled and associated with much debris. There are also discontinuities in the cell surface which could be interpreted as local extrusion of the protoplast through the cell wall.

Plate 13 shows that cells are often collapsed after PTE treatment. The surface of this cell is also covered with debris which is not present on the surrounding stub.

Plates 14 and 15 also show the wrinkling of the cell surface and the associated masses of debris. Plate 15 shows three cells with some separation visible which is probably caused by the PTE treatment.

This study provided more information as to the manner in which PTE causes death of protoplasts. The photographs of PTE treated cells indicated that death of protoplasts is associated with collapse of the walls and although no definite points of rupture were seen, much debris, which might be extruded cytoplasmic material was associated with these cells but absent from untreated cells.

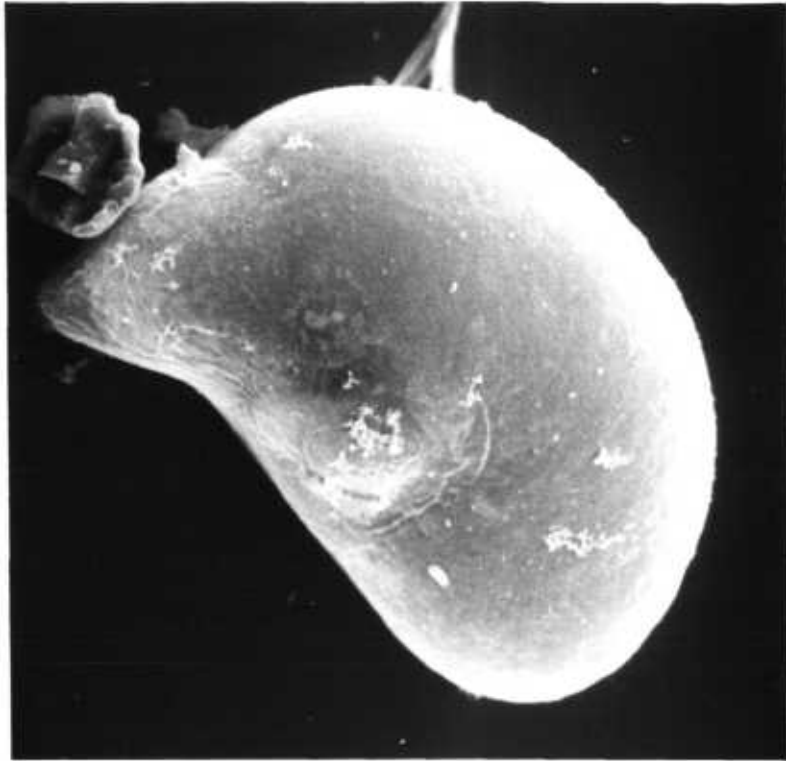


Plate 10. x 2,420 (10KV) Scanning electron micrograph of untreated carrot callus cell showing smooth intact surface.

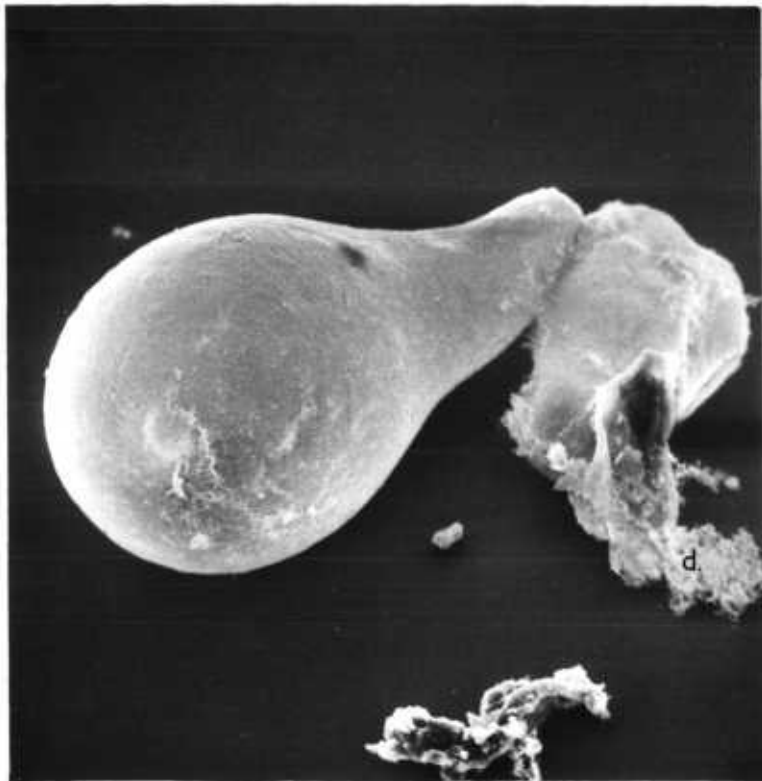


Plate 11. x 1,200 (5KV) Scanning electron micrograph of untreated carrot callus cell showing smooth intact surface with lack of associated debris. Debris (d.) is present with damaged cell which also has smooth surface.

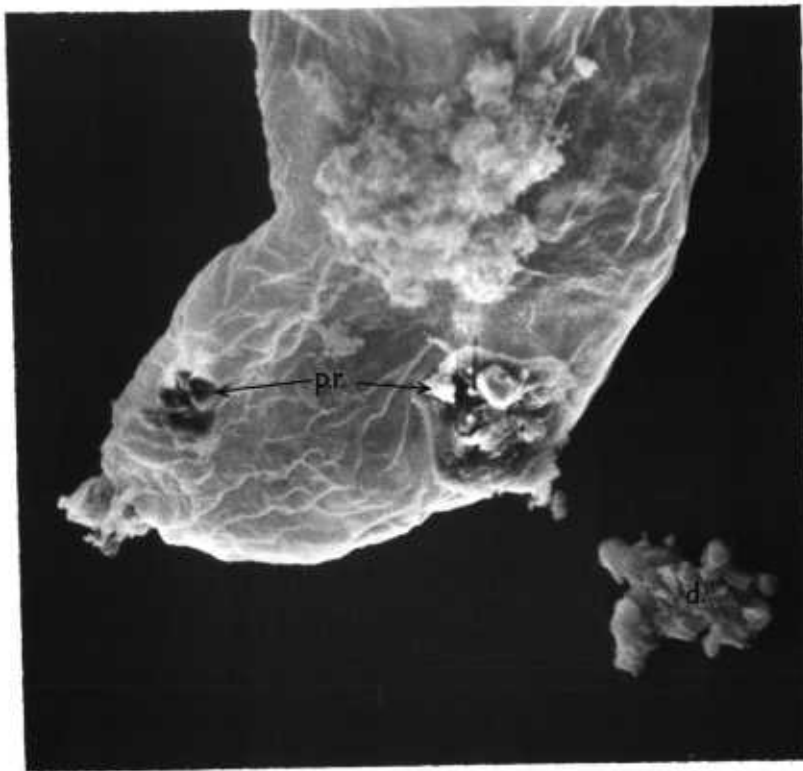


Plate 12. x 2,420 (10KV) Scanning electron micrograph of carrot callus cell treated with PTE for 2h showing wrinkled cell surface and possible points of rupture (p.r.) of wall with debris (d.) as possible extrusion of protoplast.



Plate 13. x 2,800 (10KV) Scanning electron micrograph of carrot callus cell treated with PTE for 2h showing complete collapse of cell and associated debris (d.) but lack of debris on stub (s.). Debris on cell could be extruded contents.

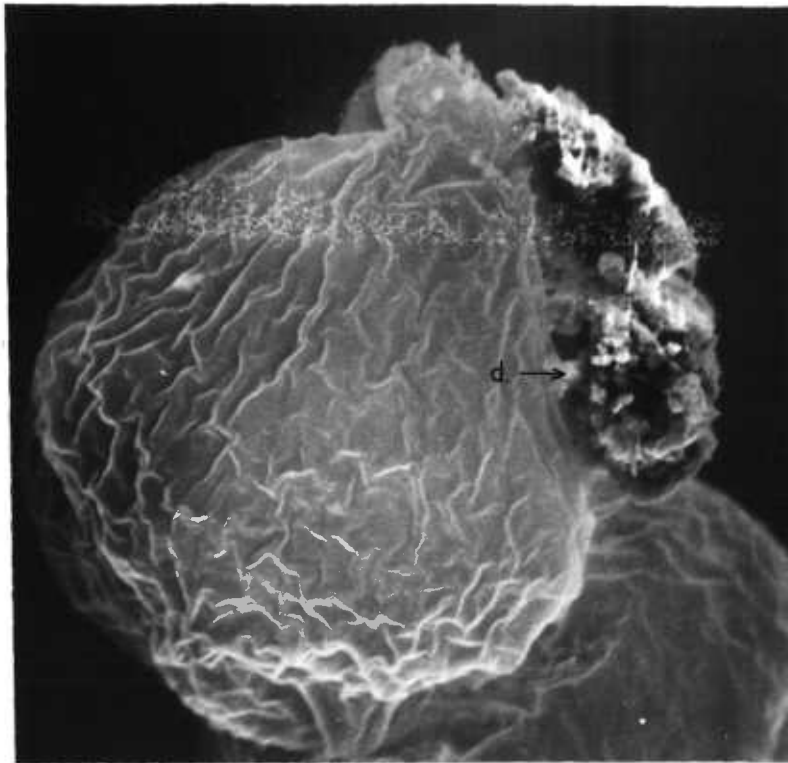


Plate 14. x 2,550 (10KV) Scanning electron micrograph of carrot callus cell treated with PTE for 2h showing wrinkled surface and debris (d.).



Plate 15. x 1,430 (10KV) Scanning electron micrograph of carrot callus cells (1, 2, 3) treated with PTE for 2h showing wrinkled surfaces and debris (d.) and separation of cells 2 and 3.

DISCUSSION

Erwinia carotovora, strain 1747, was found to cause a soft rot of cucumber, potato, carrot and onion. With the exception of carrot, the pH of the rotted tissues was higher than that of healthy tissues. The endo-PTE present in all rot extracts was most active at high pH. The shift in pH was similar to that noted for alfalfa infected with Colletotrichum trifolii which also contained TE (Hancock and Millar, 1965).

The pH optimum for cell separation and toxicity of cucumber rot extract was 9 - 10. Cell separating activity and toxicity were greatly reduced at the pH of the tissue. However, this value represents a mean for a large mass of tissue and may not be the pH at the sites of enzyme action.

Cucumber rot extract contained only one pectic enzyme, endo-PTE. A purified preparation of PTE caused leakage of electrolytes, cell separation and toxicity in much the same way as crude rot extract. Rot extract showed slightly higher permeability increasing activity, cell separating activity and toxicity when used at equivalent PTE concentrations but this might have been due to the action of the many other enzymes present in the crude extract. Preparations of PTE were free of a large number of enzymes that were present in rot extract and gave a single band after electrophoresis on polyacrylamide gel. This is in agreement with the results obtained by Mount et al. (1970) using filtrates of cultures of E. carotovora and those of Garibaldi and Bateman (1971) using purified PTEs from E. chrysanthemi.

A close association of cell separating activity and toxicity was obtained both with rot extract and PTE as noted by Brown (1915) and subsequently by many other workers.

Ions inhibited cell separation in potato tissue caused by both rot extract and PTE with a parallel reduction in toxicity. Inhibition was less with rot extract and may have been overcome by the action of the other enzymes present in the extract. A similar reduction in toxicity was found

when single carrot cells were treated with rot extract or PTE in the presence of ions. Divalent cations were most effective and although some effect on NaPP degradation was demonstrated, tissues washed free of ions regained their sensitivity to rot extract or PTE. This agrees with certain findings of Spalding (1969). He found however, that anions were more effective than cations in inhibiting the PG of Rhizopus stolonifer.

Toxicity but not cell separation in potato tissue treated with rot extract was considerably delayed when the tissue was treated in hypertonic concentrations of sucrose as found earlier by Tribe (1955). The slight effect on cell separation was related to concentration of plasmolyticum, and may have been caused by a dehydration of substrate in the walls. Permeability increasing activity of rot extract was also delayed in 0.5M sucrose. In striking contrast, when potato tissue was treated with PTE in a plasmolyticum almost all protoplasts were alive after 24h whereas rot extract killed all protoplasts within 6h under these conditions. PTE caused complete cell separation but permeability increasing activity was also abolished in 0.5M sucrose. The permeability increasing activity of ethanol or Triton X-100 was not delayed in hypertonic solution.

These results suggest that rot extract contains another heat labile factor not PTE, toxic to plasmolysed protoplasts. Similar effects were found with carrot and cucumber tissues. Single carrot cells were killed by both preparations in hypotonic solution but were unaffected by PTE in 0.5M sucrose and killed by rot extract. Isolated cucumber protoplasts in a plasmolyticum were also rapidly ruptured by rot extract but not by PTE.

Proteinase and phosphatidase purified from rot extracts were together almost as effective as rot extract in rupturing isolated cucumber protoplasts when used at concentrations equivalent to those in rots. A recent paper has reported that purified proteinase and phosphatidase from filtrates of cultures of E. carotovora are able to rupture isolated cucumber protoplasts whereas purified PTE is not (Tseng and Mount, 1974).

In the present work phosphatidase and proteinase did not kill

plasmolysed protoplasts of single cells or of tissues within 6h when used at concentrations equivalent to those in rot extract which completely killed plasmolysed protoplasts within this time. Even when used with PTE or PTE in combination with CMCase from rot extracts or with equivalent concentrations of commercial cellulases no killing occurred within 6h. Thus, either proteinase and phosphatidase are not the factors causing death of plasmolysed protoplasts or the cell wall is insufficiently degraded to allow access of these enzymes to the protoplasts. However, it was found that potato tissue treated with PTE released protein 70% of which had a molecular weight in excess of 15 - 25,000. The molecular weight of the phosphatidase was slightly less than that of PTE, known to be c. 31,000. This would seem to indicate that the wall after PTE treatment is permeable to high molecular weight proteins and thus it is surprising that proteinase and phosphatidase cannot enter, even after pretreatment with PTE or PTE plus cellulase.

Tseng and Mount (1974) found that phosphatidase and proteinase were ineffective against potato but killed some protoplasts of cucumber tissue within 4h. No cell separation was observed with either preparation which is in agreement with the results presented here.

The nature of the factor or factors in rot extracts toxic to plasmolysed protoplasts was not identified. When rot extract was fractionated on Sephadex G75 Superfine factors toxic to plasmolysed protoplasts of potato tissue were eluted together with PTE and cell separating activities. Phosphatidase and RNase were also eluted with these factors but could not alone wholly account for the toxicity observed. It is possible that a complex of enzymes are responsible for this toxicity but further work will be necessary to establish their identity.

Although phosphatidase and proteinase are toxic to isolated protoplasts and Tseng and Mount (1974), have shown that they can kill protoplasts of cucumber tissue their role in pathogenesis must be secondary if they occur with PTE which is able to cause death of protoplasts much more rapidly than either phosphatidase or proteinase in hypotonic solution.

Pectic enzymes are highly substrate specific and rupture the α -1,4 glycosiduronic linkages of pectic substances believed to be confined largely to middle lamella and matrix of primary cell wall. No compelling evidence has been presented which suggests that pectic substances occur in cell membranes or cytoplasm. The problem is, therefore, how purified pectic enzymes can cause death of protoplasts. Mount et al. (1970), and Garibaldi and Bateman (1971), considered that there is a direct toxic effect upon cell membranes and protoplasts. The observation originally made by Tribe (1955) that plasmolysed tissues are not as rapidly killed led Mount et al. (1970), to suggest that either ;

- i) a substrate for the enzymes resides in the cell membrane and is less exposed when the membrane is in a contracted condition ; or
- ii) a substrate is within the protoplasm, and penetration of the contracted membrane by the enzyme is greatly retarded.

However, the difficulty of this hypothesis, as pointed out by Hall and Wood (1974) is that killing is greatly delayed by concentrations of solutes that just cause plasmolysis and therefore little contraction of the surface membranes. Also, that PTE is not toxic to isolated protoplasts which is demonstrated by the fact that when these are prepared by the use of wall degrading enzymes they remain viable for long periods and can even regenerate a cell wall (Pojnar et al., 1967).

Ruesink and Thimann (1965), have suggested that the basic properties of some proteins enable them to disrupt critical anionic sites on the membrane surface leading to protoplast bursting. In the present work phosphatidase (pI 7.7) caused protoplast bursting but PTE (pI 10.4) did not. Similar results were found by Tseng and Mount (1974), although they pointed out the possibility that plasmolysis could stabilise the ionic condition and, therefore, prevent this action.

The present work has shown that toxins are not released from cell

walls by the action of rot extract which is in agreement with the earlier work by Fushtey (1957).

Wood (1972) has suggested that killing may be due to an osmotic effect and that once certain barriers in the cell wall have been removed by the action of the pectic enzymes, then the protoplasts can expand under turgor pressure and burst at points of minimum resistance. Thus the lethal action would be a consequence of action on the wall.

It has been suggested that plasmodesmata might be involved (Hall and Wood, 1973; 1974). As pectic enzymes degrade the middle lamella presumably the plasmodesmata become successively exposed to the solution that replaces the gel between cells. This means that the plasmalemma which is continuous through the plasmodesmata also becomes exposed to this solution. If this solution were hypotonic to the protoplasts then the plasmalemma at these points might be ruptured by osmotic forces. Expansion of plasmodesmata have been seen even in hypertonic solution once enzymic degradation of the cell wall removes constrictions on the plasmodesmata (Withers and Cocking, 1972).

Although this hypothesis explains much of the data it does not explain why PTE causes increased permeability to electrolytes after 15 min when cell separation is not complete until 120 min. Friedman and Jaffe (1960), have pointed out that some of the increased conductivity may be due to ions released from the walls. However, potato disks killed in alcohol and then leached in water did not leak more electrolytes when treated with rot extract showing that the electrolytes released are from the protoplast. This has also been demonstrated for extracts of rots of potato caused by Corticium praticola (Hall and Wood, 1974).

Leakage of electrolytes from PTE treated tissue also occurs well in advance of toxicity as estimated by the plasmolytic method. This method however, depends on the retention of neutral red which is believed to accumulate within vacuoles which are limited by the tonoplast (Stadelmann and Kinzel, 1972). Release of neutral red is, therefore, dependent on the

integrity of this membrane which has properties quite different from those of the plasmalemma upon which release of electrolytes is dependent. The tonoplast remains as a coherent semipermeable membrane in conditions in which the plasmalemma becomes non-functional (Robards, 1970).

It is not known whether the rapid increase in leakage of electrolytes depends on more or less complete loss from individual protoplasts that burst successively or on a more gradual loss more or less simultaneously from most protoplasts. Hall and Wood (1973) have tentatively suggested that if the plasmalemma at plasmodesmata is ruptured by an osmotic effect it may merely retract into the cell lumen and reseal. If this happened successively as plasmodesmata became exposed it could explain the rapidity with which pectic enzymes increased permeability to solutes culminating in loss of capacity to plasmolyse. They also pointed out that when washed disks cut from tubers are placed in water, the surfaces of these disks are covered with ruptured cells but they lose solutes only slowly. Also, that the underlying undamaged cells remain alive for long periods.

Protoplasts also remain alive when epidermis is removed mechanically from scale leaves of onion; this separation would seem to resemble closely that caused by PTE.

The structure of plasmodesmata is still unclear but some features have been resolved. Robards (1968), described a structure lining the plasmodesmata which he called a desmotubule. Burgess (1971) and Withers and Cocking (1972), suggest that this structure is membranous and in continuity with endoplasmic reticulum at each end. This structure perhaps regulates transport of materials between protoplasts. It could, however, also act as a plug if the plasmalemma at these points was ruptured.

It is known that plasmolysis causes stretching and in many cases disruption of plasmodesmata but these must reseal as isolated protoplasts remain viable for long periods (Burgess, Watts, Fleming and King, 1973).

Hall and Wood (1973; 1974) suggested that possibly these repair mechanisms do not function in the presence of cell separating enzymes.

In the present work PTE was shown to kill protoplasts of single carrot callus cells which presumably lack plasmodesmata. Thus, perhaps, plasmodesmata are not involved.

Another suggestion is that as protoplasts expand under turgor pressure elastic stretching of plasmalemma could cause rapid conformational changes and, possibly, 'holes' by which permeability of protoplasts would be increased. Later, when the limit of elasticity is reached, protoplasts would burst to give the effect measured by the neutral red technique (Alberghina, Mazzucchi and Pupillo, 1973).

Calonge, Fielding, Byrde and Akinrefon (1969), have found that Sclerotinia fructigena may kill cells without destroying the physical integrity of the plasma membrane. Also, it has been reported that the toxin victorin may cause changes in permeability without changes in ultrastructure of cell membranes (Wheeler, 1971).

With plasmolysed potato tissue treated with PTE, although cells are separated, protoplasts remain alive for long periods. When the concentration of the plasmolyticum is reduced by the addition of water until it is just about hypotonic for potato tissue there are rapid increases in release of electrolytes. This does not occur with autoclaved PTE treated tissue. This provides strong evidence that the action of PTE alters the wall in some way so that it can no longer restrain the protoplasts from bursting as they expand under turgor pressure.

Potato tissue treated with rot extract or PTE in hypotonic solution increases the conductivity of bathing solutions by release of electrolytes especially K^+ and to a lesser extent Ca^{2+} . Na^{2+} was shown not to contribute greatly to the conductivity changes measured. Protein was also released, this release following the same pattern as conductivity changes for rot extract, although with PTE, release was not so rapid which perhaps suggests a less complete degradation of the walls slowing the release

of protein. Release of protein was also detected from PTE treated plasmolysed tissue which remained alive for 24h but amounts released were not much more than with controls.

If death of protoplasts of tissue in hypotonic solution is caused by an osmotic bursting it would be expected that the contents of the protoplasts would be released to the ambient solution. The smaller amounts of protein released from plasmolysed tissue may have been due in part to the bursting of a few protoplasts but may also have been released from the wall.

Phenoloxidase activity in the ambient solution of plasmolysed disks increased on PTE treatment indicating release from walls but ambient solutions did not turn brown unless substrate was released from within protoplasts on bursting. Browning was only found in those treatments where death occurred as estimated by the plasmolytic method.

Protein released from PTE or rot extract treated normal disks showed many enzyme activities. However, these activities were also present although at lower levels in the ambient solutions of plasmolysed PTE treated disks where death did not occur. K^+ , enzymes and protein were released when tissue was frozen and then thawed and also when PTE treated plasmolysed tissue was rapidly deplasmolysed. This indicates that similar drastic changes are occurring with membranes on deplasmolysis as on freezing where complete disruption might be expected and also suggests that rapid increases in electrolyte leakage might indicate changes in limiting membranes sufficient to allow release of large molecular weight enzyme protein.

In a model of primary cell walls of suspension-cultured sycamore cells it was proposed that the rhamnogalacturonan chains of the pectic polymers are attached covalently by arabinogalactan side chains to the wall protein (Keegstra et al., 1973). Treatment with PTE is likely to disrupt this association and could release protein from the walls. Preparation of isolated protoplasts from carrot cells using Macerozyme has shown that wall

bound invertase is released to the ambient solution (Ueda, Ishiyama, Fukui and Nishi, 1974).

Release of enzymes from plasmolysed potato tissue by PTE was not reduced by prewashing in NaCl which suggested that the enzymes were not cytoplasmic contaminants adsorbed to walls during preparation of the disks. The results presented in this work have confirmed earlier work which showed that cell walls contain substantial amounts of peroxidase (Ridge and Osborne, 1971) and acid phosphatase (Lamport and Northcote, 1960b) but is unexpected for malic dehydrogenase which is known to occur as different isoenzymes in mitochondria, chloroplasts and cytoplasm (Mukerji and Ting, 1969) but not outside the plasmalemma. It is difficult to explain the function of wall bound enzymes such as malic dehydrogenase hitherto regarded as essentially cytoplasmic and further work will be needed to find other similar enzymes and to elucidate their roles.

It was expected that some differences would have been detected when protein released from normal or plasmolysed PTE treated potato tissue was subjected to electrophoresis on polyacrylamide gel. However, wall plus protoplast or wall only components respectively were not resolved with these two samples as might have been expected. Another result which contributed little to the understanding of the release of malic dehydrogenase was the release of RNA from both normal and plasmolysed PTE treated potato tissue. As leakage of K^+ was detected from normal but not plasmolysed PTE treated tissue, leakage of RNA from both was unexpected. Further work will be needed to clarify these apparently anomalous results.

Changes in respiration of potato tissue were measured as an alternative estimate of toxicity. Rot extract caused an increased oxygen uptake which later fell below that of controls indicating cell death. PTE did not increase oxygen uptake initially but uptake later fell, again indicating cell death. This shows that factors causing increased respiration are removed during purification of PTE. Similar results were obtained by Hall (1971). Spalding (1969), found that extracts of

sweet potatoes soft rotted by Rhizopus stolonifer contained PG but no phosphatidase and only slight proteinase. These extracts decreased the respiration rate of sweet potato tissue without an initial increase.

In the present work plasmolysed tissue showed increased rate of respiration. Rot extract treated plasmolysed tissue again showed an increased uptake of oxygen which then declined rapidly. PTE caused little change in oxygen uptake. The slight decline was probably due to disks being broken by agitation causing protoplast rupture. Although not conclusive these results indicated that protoplasts of plasmolysed tissue were unaffected by PTE.

Scanning electron microscopy of single carrot cells indicated a possible release of cytoplasm to the exterior accompanied by a collapse of the cells. This is an agreement with a hypothesis of osmotic rupture.

Fox, Manners and Myers (1972), have found by transmission electron microscopy of potato tissue infected by E. carotovora var. atroseptica that wall degradation also involves cytoplasmic disorganisation with enlargement of microbodies and expansion and bursting of cell membranes. The electron microscope study carried out on PTE treated potato tissue in the present work produced limited results. Staining of the walls by standard techniques was reduced considerably by PTE treatment and did not allow detailed study of changes in wall structure. Although membranes were disorganised after 120 min the relation of this with changes in wall structure was not established. Further work using tissue with smaller cells and alternative staining techniques might provide a better understanding of the relation between the plasmalemma and the inner surface of the cell walls as complete cells could be studied.

Throughout this work the importance of PTE in soft rots caused by E. carotovora and thus the importance of pectic substances was repeatedly established. Many drastic changes occur on exposure of tissues to PTE. Dissolution of pectic substances in the walls causes cell separation and the release of wall bound enzymes which emphasises the structural importance

of pectic substances in plant cell walls. Wall degradation in turn can cause rupture of the protoplasts in hypotonic solution and results presented here suggest that this occurs by osmotic forces.

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