SUMMARY

The roles of various physiological systems in the ability of Erwinia atroseptica to cause soft rots of potato tuber tissue were investigated by studying isolates, mutant for these different characters.

One of these characters was pectic enzyme production where it was shown that the only detectable pectic enzyme produced both in vitro and in vivo was endo-Polygalacturonate trans-eliminase. Mutants which were selected out on their inability to liquefy pectate gels proved to be abnormal for the production of this enzyme as well as having greatly reduced virulence. Culture filtrates of these different mutants caused maceration and browning of potato tissue in amounts corresponding approximately to the levels of enzyme activity they contained.

Different types of soft rots were observed for isolates with different colony appearance on glucose minimal medium agar. It was possible to select from one single colony isolate of one colony type, creamy, mutants which had the appearance of the other colony type, diffuse. These isolates caused soft rots characteristic of the other diffuse isolates. Various diffuse isolates differed from one another, and the creamy isolates, in different characteristics according to the particular isolate under study. However, they always differed from the creamy isolates in the type of soft rot they caused, the levels of endo-P.G.T.E. produced in vitro, and their colonial morphology on glucose containing media.

Changes in pH occurring in infected host tissue might explain the differences in the soft rots produced by creamy and diffuse isolates.

The higher pHs found on infection with creamy isolates may result in the prevention of the browning reaction in infected host tissue by reversing the effect of the host's polyphenoloxidase through the action of bacterial dehydrogenases. Differences found in the pH of infected tissues did not occur *in vitro*.

Other studies on colonial morphology produced evidence to suggest that the metabolism of pectic substances and their breakdown products by the bacterium may be associated with its metabolism of galactose and lactose. However, this does not appear to be directly related to the ability of an isolate to cause soft rots.

Similarly evidence is presented to show that the motility and the production of proteolytic enzymes by this organism do not appear to be directly involved in the production of disease symptoms by an isolate.

It would appear that the characters most closely associated with an isolate's virulence are its ability to produce endo-P.G.T.E., and its colonial morphology, which is possibly related to an ability to raise the pH of the host tissue being infected.

Mutational Studies on Virulence in *Erwinia carotovora*.

Thesis presented by

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for the degree of

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CONTENTS

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GENERAL INTRODUCTION

The use of biochemical mutants has greatly helped in the elucidation of metabolic pathways in intermediary metabolism, starting with the work on biochemical mutants of Neurospora crassa, Beadle and Tatum (1945). In similar fashion, work with mutants of animal pathogens has helped in an understanding of factors associated with their virulence, Stocker (1959).

The basic concept in such studies involves the isolation of mutant strains differing from the wild type in a single physiological character. If there are differences in virulence between such strains, then these must be a direct consequence of the change that has occurred.

The complement of this approach is to isolate and study strains differing in virulence from the original parent type. These isolates can be examined to see what physiological differences exist between them and the parent type. In this way, characters can be assessed for their importance in virulence.

It may prove difficult to obtain mutant isolates differing from the wild type in one character only, but the use of numbers of different isolates which may differ for some characters but which are identical for the particular character under study, should overcome this problem. It should also be possible, at least theoretically, to use back mutations or crossing experiments to show that the lines are isogenic except for the character under study. This would provide a definite proof that what is involved is, in fact, a single 'character'.

Using these approaches it has been possible to demonstrate the

importance of capsular structure in Pneumococcus as a factor in its virulence. The fact that mutation from encapsulation to non-encapsulation is accompanied with a loss of virulence coupled with studies of genetic transformation in the opposite direction, illustrates the importance of capsular structures in a clear-cut, unequivocal manner, Stocker (1959).

A similar situation with regard to reduction in virulence with mutation from smooth to rough colony appearance, occurs in Salmonella species, Roantree (1967).

Studies on phytopathogenic microorganisms are rather limited, not least because of the general difficulties in handling fungi, which are of greater importance in plant diseases than are bacteria. However, there have been several intensive studies done on both bacteria and fungi using nutritional mutants. It has been demonstrated by Garber, (1959 and 1960) and Garber and Shaeffer (1957) in their work with the bacterial plant pathogens, Erwinia aroideae and Pseudomonas tabaci, that some nutritional requiring mutant strains derived from a virulent wild type are, in fact, avirulent. This is similar to the situation found by Boone et al. (1957) working with nutritional mutants of the apple scab pathogen Venturia inaequalis. Elaboration of these results leads to the 'nutrition-inhibition hypothesis' of Garber (1956) to explain virulence, avirulence, susceptibility and resistance. Essentially the idea is that if a substance is absent from a host in a free, available form, then it is likely that any pathogen requiring that substance for growth, would be unable to parasitize it. It is interesting to note

that avirulent nutritional mutants isolated by Kline et al. (1964) did not induce the hypersensitive reaction of the host. Addition of the growth requirement after some time allowed parasitism.

Although such nutritional aspects may be important in some diseases, e.g. Xanthomonas vesicatoria bacterial spot of tomato, Nayudu and Walker (1961), many workers feel that a nutritional concept is too simple a view of all that occurs in pathogenesis, Wood (1966). In the case where the wild type pathogen can readily grow on a defined carbon source/mineral salts medium, then it would appear to be of little relevance to consider purely nutritional markers. It is possible, of course, that the host may create conditions where materials cannot be utilised by the pathogen, but it would seem logical to consider such a position as a host response to infection rather than consider it to be a nutritional situation.

Other studies with plant pathogens have concentrated on using non-nutritional characters and there are several reports in the literature involving pectic enzymes. Mann (1962) and McDonnell (1962) working with Fusarium oxysporum f.sp. lycopersici tested all survivors after mutagenic treatments and isolated strains differing in pectic enzyme production, but as close to wild type as possible regarding nutrition. They then tested these for their virulence and compared that with the virulence of wild type. Thus, they employed the approach of getting mutant isolates for specific systems supposedly involved in virulence. Friedman and Ceponis (1959) carried out the opposite of this by testing avirulent mutants of Pseudomonas marginalis, a pathogen of lettuce, for their abilities to produce pectic enzymes in vitro. Friedman (1962), employed a somewhat similar tech-

nique when studying Erwinia carotovora on whitloof chicory.

Colony appearance on agar media containing triphenyl tetrazolium salts has been studied for Erwinia carotovora by Friedman (1964), and for Pseudomonas solanacearum, by Kelman (1954). The fact that differences in virulence are associated with changes in colony morphology gives another example of the approach of isolating mutants for specific characters and then studying the effect of these changes on the virulence of the organism. The effect of changes in colonial morphology on virulence appears to be a problem particularly suited to this approach.

Other studies with bacterial species related to phytopathogens only serve to illustrate the possible benefits that mutants could provide. For example, Klement et al. (1964) using non-pathogenic and pathogenic species of Pseudomonas, have investigated the response of tobacco plants to infection and highlighted the differences between their abilities to grow in the host. Similarly a yellow Erwinia-like bacterial species as well as other bacterial species have been used in studies concerned with the physiology of the fireblight pathogen Erwinia amylovora and in the events occurring during infection, Klement and Goodman (1966) and Goodman (1967). Lapwood (1957) has also made use of related non-pathogenic bacterial species in comparative studies of pectic enzyme production by phytopathogenic bacteria. In such studies it is certain that the related species differ by more than one character from the pathogenic species. The rather gross differences in the physiological

tems which must exist between two distinct bacterial species must

limit any conclusions that can be drawn from comparative studies of this nature. This limitation would not, however, apply to the same extent to studies using bacterial isolates derived from the same source. In this way, a large range of problems would appear to be suited to study using mutant strains.

This study has attempted to elucidate some of these problems using the following approaches.

(i) Studying mutant isolates which possess different characteristics from the wild type, particularly with respect to the ability to produce certain enzyme systems.

(ii) Studying avirulent isolates to see how their physiology has been altered.

The organism used in this study was Erwinia atroseptica. It is a gram negative coliform bacterium which causes blackleg symptoms on potato plants in the field, and soft-rot of potato tubers both in the field and under storage, Butler and Jones (1949). Several important factors make it a suitable organism for such a study,

(1) Bacteria tend to be more easily handled, lending themselves to standardised techniques. It is possible, for example, to use replica plating and other techniques developed with Escherichia coli to screen large numbers of colonies in the isolation of specific mutant types.

(ii) Since it is a gram negative coliform bacterium, it was thought that any results concerning its genetics would be of interest in comparison with the known genetics of other gram negative coliforms, e.g. Escherichia coli.

(iii) It has no exacting growth requirements, growing readily on a glucose/mineral salts medium.

(iv) On infection, it produces soft-rot symptoms, and standardised infection can readily be obtained in the laboratory.

CHAPTER I - GENERAL METHODS AND PROCEDURES

Section I - Cultures

1. Isolates

The isolates used in these studies were as follows.

G110)	Dr. Graham, East Craigs, Edinburgh.
G110 cr.)	Creamy type of colony derived from G110.
G110 dif.)	Diffuse-edged colony derived from G110.
N/C 434)	National Culture Collection of Plant Pathogenic Bacteria.
N/C 549)	
N/C 1042)	
N/C 1449)	

2. Maintenance of cultures

All cultures were maintained on nutrient agar slopes in 1" screw-top bottles. Sub-culturing was generally of a "mass transfer" type with a platinum loop, though occasionally, single colony isolates were selected out of each culture for continued sub-culturing.

All cultures were stored at 4-5°C in a refrigerator when not in use.

3. Media

Unless otherwise stated all media were autoclaved at 15 lbs. pressure for 15 minutes.

Nutrient broth

Bacto-peptone	10 gms.
Lab-Lemco beef extract (Oxoid)	10 "
NaCl	5 "
Glass distilled H_2O	1 litre

pH adjusted to 7.2 with 1N NaOH.

3. Media (contd)

Nutrient agar

Nutrient broth + agar (Oxoid No.3) 15 gm./litre

Section II - Growth Conditions

1. General conditions

Nutrient broth was used for growing up bacteria when no specific experimental procedures were required. Two procedures were used:

(i) 10 ml. of nutrient broth in 1" screwtop bottles. Standing cultures at 25°C.

(ii) 50 ml. of nutrient broth in 100 ml. Erlenmeyer flasks at 26°C on a rotary shaker of amplitude 3.5 cm. at 150 revs. per minute.

Inoculation into these cultures was generally done with a platinum loop. The shaken cultures were always used where standard inocula were needed as in the virulence tests and the *in vitro* pectic enzyme production tests.

After about 24 hours, the density of cells in tube cultures was generally in the range of about 10^7 - 10^8 cells per ml. Shaken cultures usually ranged between 1-5 x 10^9 cells per ml. after 24 hours.

2. Minimal medium (MM)

The minimal medium was that used by Starr (1946). It consisted of the following:

Glucose	0.5 gms. (added seperately)
KH_2PO_4	0.2 "
NH_4Cl	0.1 "
$MgSO_4$	0.02 "
Trace solution *	1 ml.
Glass distilled H_2O	100 ml.

For a solid medium, 1.5 gms. of agar (Oxoid No.3) was added. The pH was adjusted to 6.8 with 1N NaOH added dropwise before adding agar or autoclaving. Liquid media was used in 50 ml. batches incubated under exactly similar conditions as nutrient broth. In specific cases, the glucose was replaced by the following sugars, fructose, galactose and lactose. In such cases, the medium will be referred to as fructose MM, galactose MM and lactose MM respectively.

* Trace solution

B (H_3BO_3)	0.5 µg.
Ca ($CaCO_3$)	10.0 "
Cu ($CuSO_4.5H_2O$)	1.0 "
Fe ($FeSO_4[NH_4]_2SO_4.6H_2O$)	10.0 "
Mn ($MnSO_4.H_2O$)	1.0 "
Mo (MoO_3)	1.0 "
Zn ($ZnSO_4.7H_2O$)	5.0 "

3. Eosin Methylene blue agars (EMB agars) Difco Manual (9th Edition, 1953).

These consisted of the following:

Sugar	10 gms.
Bacto-peptone (Difco)	10 "
K_2HPO_4	2 "
Eosin Y	0.4 "
Methylene blue	0.065 "
Agar (Oxoid No.2)	15 gms.

When the sugar was lactose, Difco's Levine EMB agar was used for convenience. Other sugars used were glucose, fructose and galactose.

4. Triphenyltetrazolium chloride (TTC) agars

These consisted of:

Oxoid Nutrient agar (C.M.3)	28 gms.
Sugar	5 "
TTC solution (0.5% w/v solution)	10 ml.
Distilled water	1 litre

Carbon source and TTC solution autoclaved separately at 10 lbs. pressure for ten minutes. The same sugars were used as for the EMB agars. This was the same as that of Logan (1966), minus the yeast extract.

5. Peptone-sugars

These consisted of the following:

Bacto-peptone (Difco)	5 gms.
K_2HPO_4	2 "
Sugar or other carbon source	5 "
Glass distilled H_2O	1 litre
1.6% w/v, 50% w/v ethanolic solution of bromo-cresol purple	1 ml.

adjusted to pH 7.0 with 1N naOH.

The solutions were dispensed in test tubes with an enclosed Durham tube to test for gas production. They were steamed in an autoclave for twenty minutes on each of three consecutive days and stored at 4-5°C in a refrigerator. The various carbon sources used are given in the appropriate experiments. Where ethanol was used, it was added to the sterile tubes after steaming, just before use.

The tubes were inoculated from standing 24 hour old nutrient broth cultures by adding one drop of bacterial suspension. The tubes were incubated at 25°C and examined at various intervals to check for acid (a yellow colour) and gas formation.

Acid production was classified into five categories as given in the following table.

(i)	-	=	No reaction
(ii)	$\overline{+}$	=	Possible, very small reaction
(iii)	+	=	Definite but incomplete reaction
(iv)	++	=	Complete reaction
(v)	d	=	Delayed reaction

6. Nutrient gelatine

This medium was routinely used for gelatine liquefaction. It was prepared to the following formula modified from the Difco Manual (9th Edition, 1953).

Bacto-peptone (Difco)	5 gms.
Lab-Lemco Beef extract (Oxoid)	3 "
Gelatine (B.D.H.)	120 "
Glass distilled H_2O	1 litre

This gelatine was dissolved by heating in the water and then the other constituents were added. The medium was adjusted to pH 6.8 with 1N NaOH and dispensed in bottles.

7. Examination of cultures for motility

Motility was determined by examining a drop of bacterial suspension (nutrient broth 24 hours) on a microscope slide without cover-slip under the light microscope. The degree of motility was estimated as follows:

(i) - = non-motile

(ii) + = slightly motile, very little movement out of a small localised area

(iii) ++ = about 50% of the cells moving freely

(iv) +++ = all or nearly all cells moving freely

The greatest difference exists between categories (ii) and (iii), there being possible overlaps between categories (i) and (ii) and also between (iii) and (iv).

8. Measurement of cell numbers and densities

Estimates of cell numbers in liquid culture were generally made using a colorimeter as a densitometer. In this study, optical densities (O.D's) were taken using either:

(i) an EEL portable colorimeter with red filter No.608, or

(ii) a Hilger-Watt colorimeter with a red filter No.700.

With the EEL, dilution of 24 hour-old cultures was not necessary to get accurate readings. However, on the more sensitive Hilger-Watt machine, 1/10th dilutions were generally needed to get accurate readings. Reference solutions used, consisted always of the solutions in which the bacteria had been grown. These were diluted if the culture had been diluted.

In addition to this, viable counts were often taken by plating out

dilutions of cultures onto Oxoid nutrient agar (C.M.3.)

Inocula prepared from nutrient broth cultures were estimated to give an O.D. of 0.1 when inoculated into 9 ml. of nutrient broth. This inoculum was used to inoculate shaken cultures and for the preparation of inocula for virulence tests.

In a few specific cases, estimates of cell numbers were made by haemocytometer counts. Where such cases occur, they will be clearly indicated.

Section III - Virulence Tests

Healthy Pentland Dell tubers were washed under a cold tap to remove dirt and then immersed in a 2% calcium hypochlorite bleach solution for thirty minutes. Thereafter, they were rinsed and dried with paper tissues. After being quickly flamed with ethanol, they were cut into slices approximately 3-5 mm. thick with a knife which had been flamed previously. These slices were washed four times with glass distilled H_2O to remove debris and finally washed twice with sterile glass distilled water.

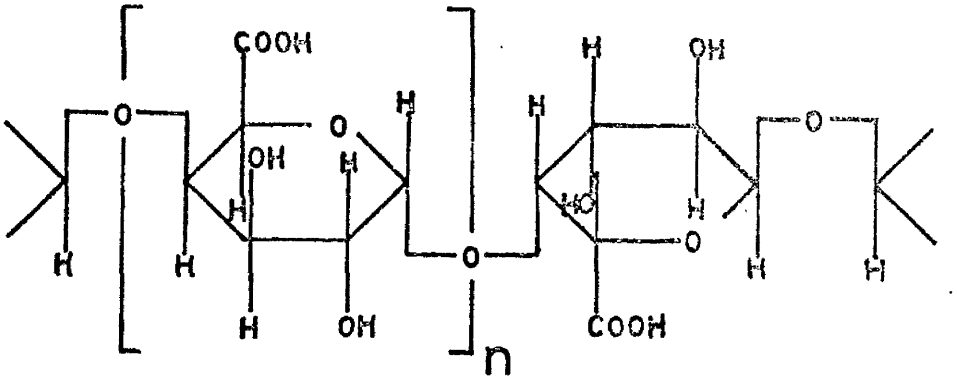
After allowing the excess water to drain off, each slice was placed in a separate, sterile petri dish containing an 11 cm. Postlip filter paper in its lid, to which 3 ml. of sterile glass distilled H_2O had previously been added. As soon as possible after placing in the dishes, the slices were inoculated with a suspension of the bacterium under test which was prepared as follows.

The bacterium was grown up first of all in 50 ml. of shaken nutrient

broth for 24 hours. Its optical density was measured against sterile nutrient broth (page 12) and an inoculum made from the culture into 9 ml. of sterile glass distilled H_2O. This inoculum was calculated to give an O.D. of 0.1 if it had been added to 9 ml. of sterile nutrient broth. The suspension thus prepared, was then inoculated onto the tuber slices, a 0.5 ml. portion for each tuber slice. Controls consisted of inocula prepared in an exactly similar fashion with sterile nutrient broth.

The slices were then incubated at $20^{o}C$ in petri dishes. By way of keeping the humidity of the atmosphere high, the petri dishes were placed in polythene bags. Control slices were always incubated in the same bags as those under test.

Figure 1. Basic structure of pectic substances.



alpha 1,4 polygalacturonic acid.

CHAPTER II - PECTIC ENZYMES

Section I - Introduction

This section is concerned with the production of pectic enzymes by the organism *in vivo* and *in vitro* and the part that these enzymes play in the host-parasite relationship.

Pectic enzymes have been implicated in soft-rot diseases by numerous workers. A comprehensive list of organisms producing pectic enzymes is given by Wood (1966). Tissue maceration is considered to be mainly due to the action of these enzymes on the middle lamella of plant cells where pectic substances occur in considerable amounts, Albersheim *et al.* (1960a); McClendon (1964). The middle lamella binds cells together and the destruction of it results in the disruption of the affected tissue, Hancock (1968); Wood (1966).

Chemistry:

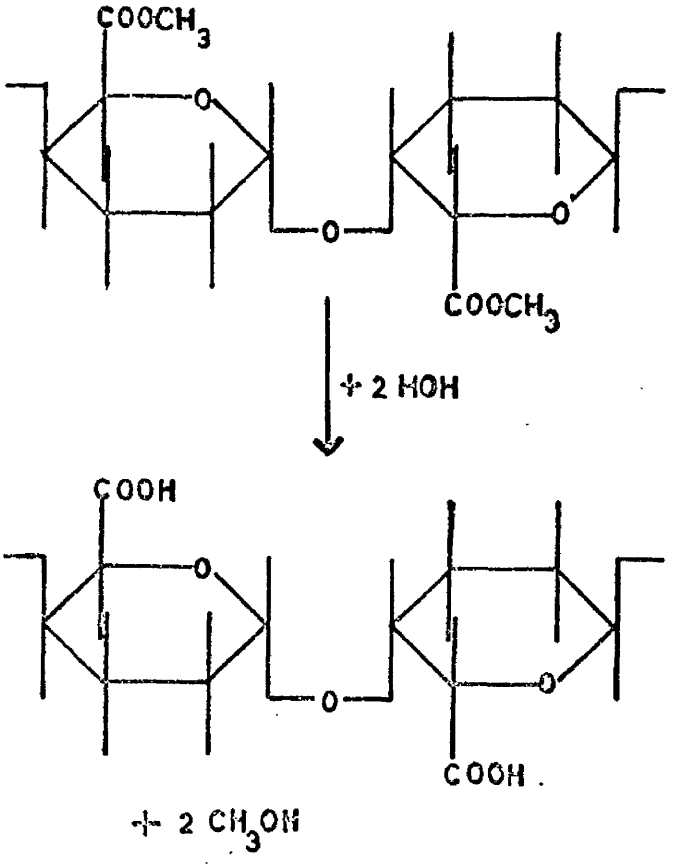
The pectic substances which are the main components of the middle lamella, have the basic structure given in figure 1. They consist of polymers of Galacturonic acid units linked by α1:4 glycosidic bonds. The carboxyl side groups of the units can be esterified generally with methanol. The degrees of esterification which vary with age, species etc., are used to classify the pectic substances. Using Bateman and Miller's classification (1966), three types are recognised:

(i) Pectin, esterification > 75%

(ii) Pectinic acid, esterification <75%

(iii) Pectic acid or polygalacturonic acid, no esterification

Figure 2. Action of pectin methyl esterase (P.M.E.) on pectin.



Although galacturonic acid appears to be the main sugar unit, other sugars and substances have been found associated with pectic substances. These include the sugars arabinose and galactose as well as proteins, and calcium and magnesium ions.

Most work has been concentrated on those enzymes attacking the α1:4 linkages of the galacturonic acid residues and those removing the methyl groups from the pectin. Hancock (1968) has suggested that although other enzymes may be necessary for the complete breakdown of pectic substances, even a limited breakdown of the polymeric structure could greatly affect their properties.

Bateman and Miller (1966) have produced a classification of these pectic enzymes which is both ordered and comprehensive. They first of all separate the enzymes on the basis of whether they attack the ester linkage or the glycosidic linkages, i.e.

(i) Pectin Methyl Esterase (P.M.E.)

(ii) Hydrolases and Trans-eliminases

(i) Pectin Methyl Esterase (P.M.E.)

There are no reports in the literature of any other type of esterase. P.M.E. has been reported to occur in a wide range of organisms. It is present in substantial amounts in higher plants, e.g. the common source for the commercial preparation of this enzyme is tomato fruits. It is present in appreciable amounts in healthy potatoplants, Lineweaver and Jansen (1951). Its action on pectin is given in Figure 2.

The reports of the production of this enzyme by Erwinia species are rather confusing. In some cases, the enzyme can be detected in small quantities, while in others it cannot be detected at all.

Graham (1964) has suggested that the production of this enzyme may be correlated with pathogenesis, this suggestion being reached from the data of Smith (1958). Smith showed activity in culture supernatants, such activity, however, having been measured over a fairly long period (24 hours) with the result that the quantities of the enzyme may have been very small.

Other reports of P.M.E. production by Erwinia spp. have come from Kraght and Starr (1953) and Nasuno and Starr (1966). Kraght and Starr judged activity to be present from acid production in a growth medium containing pectin. Nasuno and Starr produced good evidence of some pectin methyl esterase (P.M.E.) production by Erwinia carotovora.

There are other reports in the literature where little or no enzyme has been shown to be produced. Thus, Echandi et al. (1957) found that E. carotovora, E. aroideae and E. atrosephia only produced a depolymerase in culture and did not produce P.M.E. or polygalacturonase (P.G.). Similarly Walton and Capellini (1962) found that E. carotovora produced no P.M.E. in vitro.

After studying in vitro production of pectic enzymes by E. aroideae, Wood (1955) concluded "that crude solutions, i.e. crude filtrates from cultures of Bacterium aroideae, are practically free of pectinesterase". He was also able to demonstrate rapid reduction in the viscosity of pectin and pectate solutions over short periods of time (20 mins) which were accompanied by little, if any, drop in acidity due to the action of P.M.E. It would appear that no definite correlation exists between P.M.E. production in vitro and the pathogenicity of Erwinia soft-rot species, although this throws little light on the in vivo relationship.

Figure 3. Action of hydrolases on pectic substances.

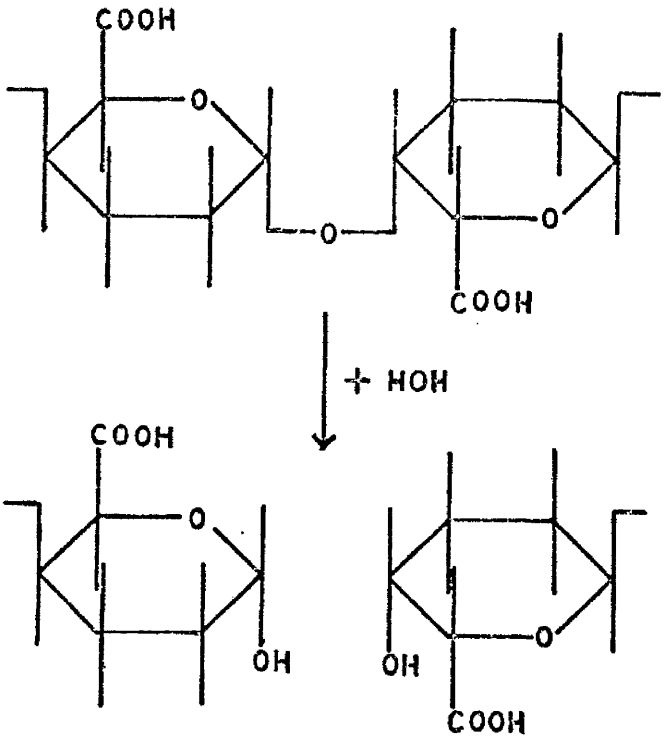
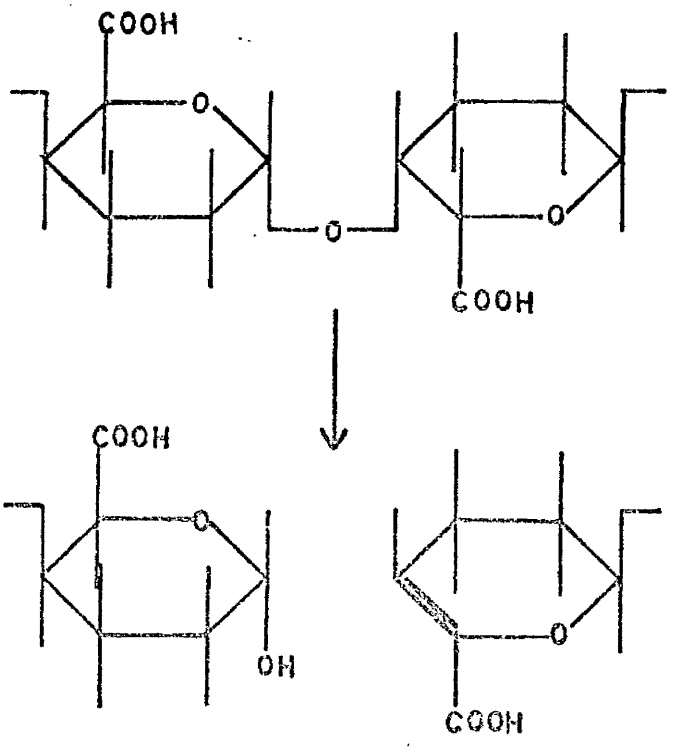


Figure 4. Action of trans-eliminases on pectic substances.



Of studies involving mutant strains, it appears that only one previous report with specific regard to P.M.E. is available for Erwinia. Using a mutant with reduced virulence on witloof chicory Friedman (1962) found that there was no change in the level of P.M.E. produced by this isolate in vitro whereas it had reduced ability to produce other pectic enzymes.

Mann (1962) and McDonnell (1962) studying Fusarium oxysporum f.sp. lycopersici found that mutant strains lacking the ability to produce this enzyme, or those producing less than wild type, were still as pathogenic as wild type. It would appear, therefore, that in the context of this disease at least, P.M.E. production in vitro is of little importance.

It should be noted, however, that P.M.E. may act as a 'primer' for the action of other pectic enzymes, and that its action on pectinaceous substances in the host may result in a greater ability of other enzymes to degrade them, i.e. a synergistic effect.

It is also possible that in some cases, alkaline saponification or demethylation of pectin can occur, and in such cases, it would be more difficult still to decide if pectin esterase was closely involved in pathogenesis, Hancock (1968). It is clear, however, that P.M.E. does not cause breakage of the α1:4 linkage in pectic substance. It is this linkage that is now considered to be important from the standpoint of maceration.

(ii) Hydrolases and Trans-eliminases (lyases)

These two groups of enzymes attack the α1:4 linkages of pectic and related polymers. Their action is thought to be as shown in figures 3 and 4.

In effect, both these enzymes bring about the cleavage of the α1:4 linkage. Further sub-divisions of these enzymes can be made along the lines suggested by Bateman and Miller (1966), whose classification is reproduced here.

A. Hydrolases

(i) Random mechanism of hydrolysis.

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

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

(ii) Terminal mechanism of hydrolysis.

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

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

B. Trans-eliminases

(i) Random mechanism of trans-eliminative degradation.

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

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

(ii) Terminal mechanism of trans-eliminative attack.

(a) Pectin attacked in preference to pectic acid.....
..... exo-pectin-methyl-trans-eliminase (exo-P.M.T.E.)

(b) Pectic acid attacked in preference to pectin .....
..... exo-polygalacturonate-trans-eliminase (exo-P.G.T.E.)

From this classification, it is possible to make several useful observations on pectic enzymes:

Exo-enzymes lead to the release of large amounts of reducing groups, with little effect on viscosity, while endo-enzymes also release reducing groups, but have a much greater effect on the viscosity of pectin and pectic acid solutions.

Trans-eliminases and hydrolases can be distinguished by the type of coloured compound formed by the reaction of the product and thiobarbituric acid. The products of hydrolases give a peak at 517 mμ while those of trans-eliminases give a peak at 547-550 mμ (Albersheim et al., (1960); Sherwood (1967). Further, the formation of double bonds by trans-eliminase action can be followed spectophotometrically by measuring the increase in absorbance at 230-235 mμ where these compounds absorb ultra-violet (U.V.) light.

At this point, it is necessary to say a few words about methods of estimating pectic enzyme activity. In the past, it has been usual to measure reduction in viscosity of pectin/pectic acid solutions and/or the release of reducing groups by enzyme action. It is now clear that such systems cannot give completely accurate information on enzyme activity in those circumstances where more than one enzyme attacking the α1:4 linkage is present. Measurement of enzyme activities separated on the basis of optimum pH alone is also of doubtful value, since it is possible that the range of action of different enzymes with different pH optima may overlap (page 38). Therefore, unless the enzymes in the system have been specified, the use of viscosity and reducing group measurements should be avoided, unless, of course, a composite assessment of all the enzymes present is required.

Other enzymes or terms used in studies include protopectinases and depolymerases.

Bateman and Miller (1966) state that the term protopectinase has been applied to those enzymes causing tissue maceration. Wood (1955 and 1966), suggests that protopectinases attack the ill-defined substance, protopectin, present in the middle lamella, and so cause maceration. In this study, the term will be avoided, and use made of the term 'macerating enzymes' as suggested by Bateman and Miller (1966), i.e. as long as the enzymes causing maceration remain unidentified.

The term depolymerase also occurs frequently in the literature, and has been applied to those situations where rapid drops in viscosity of pectin/pectic solutions have not been accompanied by a great rise in reducing groups. Increases in reducing groups do occur, however, as is witnessed by the work of Deese and Stahmann (1962) who used reducing group formation as a measure of 'pectic depolymerase' produced by F. oxysporum f.sp. cubense. In such cases, galacturonic acid appears not to be formed from the action of these enzymes. Bateman and Miller (1966) suggest that enzymes described by this term are probably trans-eliminases.

It appears possible, however, that depolymerases could include both trans-eliminases and hydrolases which have an endo-action, since the action of such enzymes would probably be very similar in respect to viscosity and reducing group assays. It is possible that endo-hydrolases may not give rise to galacturonic acid directly.

There are numerous and conflicting reports of different pectic

enzymes being produced by soft-rot Erwinias. Echandi et al. (1957), report only the production of a 'depolymerase' for the three soft-rot species E. carotovora, E. aroideae and E. atrosephia. Wood (1955), found large amounts of 'depolymerase' produced in vitro as well as large amounts of 'protopectinase' produced in vitro and in vivo. The similarity of both these enzymes is extremely close, and it appears likely that they are one and the same. Walton and Capellini (1962) report the production of 'depolymerase, protopectinase' and polygalacturonase by E. carotovora, the optimum pH of the latter enzyme being at pH 9. However, the methods used are not described and it would be of interest to know which assay was used for these enzymes, particularly the polygalacturonases.

In 1962, Starr and Moran reported trans-eliminase activity in culture supernatants of E. carotovora. The optimum pH for activity of this enzyme is pH 8.5. They concluded that this enzyme had an endo-action and attacked pectic acid or polygalacturonate in preference to pectin, i.e. it was an endo-polygalacturonate-trans-eliminase (endo-P.G.T.E.) on the basis of Bateman and Miller's classification. The properties of this enzyme agree remarkably with those of the 'depolymerase' described by Wood for E. aroideae i.e. calcium stimulation, pH optimum, fast reduction in viscosity of pectic solutions and no formation of monogalacturonic acid as detected by paper chromatography.

Thus, it would appear that Wood's 'depolymerase' and endo-P.G.T.E. are one and the same enzyme. It is interesting to note that Smith (1958) reported the presence of 'γ-pectinglycosidase' (i.e. an enzyme active at pH 7.5) in culture supernatants which attacked pectic acid in

preference to pectin. In some cases he detected α-pectinglycosidase activity, (i.e. pectic enzyme activity at pH 5.5), but this was always accompanied by very high activity at pH 7.5.

It is generally accepted that many reports of such enzymes with high optima pH's are probably reports of trans-eliminases. There are also reports in the literature of cell-bound exo-polygalacturonate trans-eliminases, Okamoto et al (1963; 1964). Since cell free filtrates of this and other soft rot bacteria can cause maceration, it is unlikely that such cell-bound enzymes are greatly involved in maceration, although it is conceivable that they may be involved in the utilisation of pectic substances as carbon sources.

Reports of hydrolases are less easy to find, since it is now clear that many previous reports were, in actual fact, reports of trans-eliminases. However, Nasuno and Starr (1966) have clearly and unequivocally demonstrated the production of an endo-polygalacturonase by Erwinia carotovora with a pH optimum of 5.4. It would be of interest to see if future reports for other Erwinia sp. and varieties also gave reports of such an enzyme.

Of pectic enzymes present in infected tissues, so called 'protopectinase' appears to be the most commonly detected enzyme, Wood, (1955), Friedman (1962) and Lapwood (1957). Lapwood demonstrated highest macerating activity at pH 8-10, which is similar to that of Wood (1955). The close similarity of 'protopectinase' to endo-P.G.T.E. has already been remarked on. Hancock (1968) working on Fusarium solani concluded that the enzyme mainly responsible for maceration was an endo-P.G.T.E. Turner and Bateman

(1968) produced evidence in a study of Erwinia carotovora and Erwinia debaryanum on carrot, cucumber and onion, that endo-P.G.T.E. was probably responsible for tissue maceration in all cases.

The use of mutant strains to elucidate the relationship of pectic enzymes to disease is, to say the least, limited. However, Friedman and Ceponis have used such a procedure. They found that five avirulent isolates of Pseudomonas marginalis selected at random, failed to produce detectable pectic enzymes in culture, whereas the wild type produced P.M.E., pectic depolymerase and protopectinase. Those strains differed from wild type only in characteristics associated with the metabolism of pectic substances and in one other character, namely acid production from sucrose. Supplementation with unheated pectin to one of the isolates did not promote its growth. They concluded that loss of ability to synthesise pectic enzymes by some strains was a genetic effect and was the cause of the loss of virulence of those isolates. This study did not indicate, however, which particular enzyme or enzymes were involved in loss of virulence. However, Friedman and Jaffe (1960) showed, using these mutants, that tissue injury (as measured by electrical conductance) was probably due to pectic enzymes, since the culture filtrates of a mutant strain did not cause any increase in conductance, whereas those of other Pseudomonas species and Erwinia species did.

Using ultra-violet light, Friedman (1962), isolated a weakly virulent strain of Erwinia carotovora from a virulent parent wild type. This strain differed from wild type only in having a reduced ability to decrease viscosity of pectin solutions and cause increases in reducing groups of

of sodium pectate. No differences in P.M.E. activities were noted, and the only other difference involved gelatine liquefaction and other tests associated with proteolytic activity. However, later studies on changes in virulence of cultures, growth rates *in vivo* and acid production *in vivo* and *in vitro* suggest that changes in other physiological systems may have occurred complicating any straightforward assessment. All this work implicates pectic enzymes as important in pathogenicity, at least in bacterial soft-rots. It must be noted, however, that there is some evidence that this may not be the case in many other diseases. Mann (1962) and McDonnell (1962) found that differences in P.M.E. and P.G. production *in vitro* by mutant strains of *Fusarium oxysporum* f.sp. *lycopersici* were not correlated to any great extent with differences in pathogenicity on tomato.

To conclude, there is some evidence that pectic enzymes are important, at least in soft-rot diseases. Which precise enzymes, however, are responsible for maceration is not clear, and this must be allowed for in any study of pectic enzymes in disease.

Section II - Methods

A. Cultural Conditions

Pectin and Sodium polypectate media

The media for the production of pectic enzymes are modifications of that of Nasuno and Starr (1966). They were prepared in two parts as follows:

Solution 1

Pectin (B.D.H.) or exchange sodium polypectate (S. & S. Services Ltd) washed with acidified ethanol to remove sugars and other low molecular weight compounds, Wood (1961)	5 gms.
Bromothymol blue indicator solution (Hopkin & Williams)	2 ml.
Glass distilled H_2O	400 ml.

The pectin or sodium polypectate was dissolved by continuous stirring in the water at a temperature of 80-90°C. The bromothymol blue was added and the pH adjusted to approximately neutral (a green colour) with 1N NaOH. The solution was dispensed in 20 ml. portions and autoclaved at 10 lbs. pressure for 10 minutes.

Solution 2

KH_2PO_4	2.4 gms.
Na_2HPO_4	0.8 gms.
$MgSO_4.7H_2O$	0.2 gms.
$(NH_4)_2SO_4$	3.0 gms.
Sodium monoglutamate	5.0 gms.
Glass distilled H_2O	600 ml.

Table 1. The cell numbers and O.Ds. of various cultures of isolate G110 dif.

Medium	Estimated numbers of cells ($X10^9$) per ml. of cultures with an O.D. = 1.0 on the EEL colorimeter	Estimated numbers of cells ($X10^9$) per ml. of cultures with an O.D. of 1/10 dilution = 0.10 on Hilger-Watt colorimeter
Nutrient Broth	1.10	1.86
Liquid minimal medium	0.93	1.74
Sodium polypectate medium	1.10	2.01
Pectin medium	1.30	2.74

The mineral salts were dissolved in the water and the pH was adjusted to 6.8 with 1N NaOH. The solution was dispensed in 30 ml. portions in 100 ml. flasks and autoclaved at 15 lbs. pressure for 15 minutes.

The medium was finally prepared by adding one portion of Solution 1 (20 ml.) to one part of Solution 2 (30 ml.).

Inocula

Inocula were generally obtained from 24 hour-old shaken nutrient broth cultures (page 13). The inoculum was calculated so as to give an optical density of 0.1 if inoculated into 9 ml. of nutrient broth. Table 1 gives the cell numbers against O.Ds. of nutrient broth and other cultures; it will be seen that cell numbers in the inocula lay between 0.5-2.0 x 10^9 cells or about 1-4.0 x 10^7 cells per ml. in the inoculated pectic media flasks. In some cases, a smaller inoculum was used and the details are reported in the appropriate experiment in a later section.

Growth conditions

The inoculated cultures were grown at 26°C on a rotary shaker at 150 rotations per minute of 3.5 cm. amplitude.

Estimation of growth

Growth was generally estimated from O.Ds. of the cultures after the desired incubation period. These were read in exactly the same way as the O.Ds. of N. broth cultures, except that the reference solution consisted of the appropriate pectic medium used in the experiments. In many cases, nutrient agar dilution counts were also taken to provide estimates of viable cell counts for the cultures.

E. Enzyme Assays

Preparation of solutions for testing for pectic enzymes

1. Culture supernatants

The culture supernatants were obtained by centrifuging at 4,000 r.p.m. in a Gallenkamp bench centrifuge for 20 minutes to remove the bacteria. The supernatants were decanted off and passed through a membrane filter. Generally, they were used the same day but if not, were stored under toluene at 3°C. In some cases, they were stored without toluene at -20°C. Results did not seem to vary with these two different procedures.

2. Extracts from blackleg infected plants from the field

Plants were collected from a heavily infected plot in the garden of the Botany Research Laboratories, Garscube Estate, in mid-August, 1968. These plants displayed all the characteristic symptoms of heavily infected plants, i.e.

(i) Separation of the haulms from the underground parts of the plant when pulled gently.

(ii) Blackening and rotting of the haulm near the soil.

(iii) Curling of the leaves and drying up of the stems.

(iv) In some cases, complete wilting of the plant had occurred, presumably because of disruption of the water-conducting elements of the plant.

(v) After collection, masses of bacteria could be seen if small pieces were macerated with water.

It was therefore decided to use these plants as representative samples of the infection in the field.

The collected haulms were gently washed with distilled water and dried by careful blotting with paper tissues. The blackened parts of the haulms which occurred at, and just above ground level, were cut off and the rest of the plants discarded. 10 gms. of these blackened sections were weighed out and added to 50 ml. of distilled water in a Waring blender where they were blended for 1-2 minutes until only the fibrous material of the stems was left. The fluid was then poured off and was seen to be full of a dense suspension of bacteria which proved, on examination, to be a motile gram negative rod. The suspension was then treated in exactly the same way as pure bacterial cultures to remove the bacteria and debris.

3. Extracts from laboratory infected potato tuber slices

Potato tuber slices were inoculated in the manner described for tests of virulence with a suspension of bacteria (page 14). After 48 hours, 10 gms. of rotted material were removed and blended in 50 ml. of glass distilled water as described for the blackleg stem extracts. The procedure after this point was exactly similar to that of the pure cultures and blackleg haulms.

In those cases where direct assays of P.G.T.E. in infected discs were made, the extraction was done with TRIS-HCl buffer 0.1 M (pH 8.5).

Pectin Methyl Esterase (P.M.E.)

A 1.25% solution of pectin (B.D.H.) was prepared by heating and stirring. This solution contained 0.5 ml. of a 1.6% w/v solution of bromo-cresol purple in ethanol for every 100 ml. of solution. The pH of the solution was adjusted to 7.0 with N/10 and N/100 NaOH.

The assay mixture for the enzyme consisted of the following:

24 ml. of the pectin solution.

3 ml. of 1.0 M NaCl.

3 ml. of culture filtrate under test.

The pH of the mixture was readjusted to 7.0 with N/100 NaOH after addition of the enzyme solution. Control solutions consisted of incubation mixtures with culture filtrates which had been heated in a boiling water bath for 15 minutes. Other controls consisted of mixtures with the enzyme replaced by distilled water and the pH adjusted to 5.5, 6.0 or 6.5 with N/10 NaOH. After incubation for one hour the absorption spectra of the solutions were read in an SP 800 spectrophotometer at 590 mμ. Since there is a change in the absorbance of the bromo-cresol purple at 590 mμ over the pH range 5.0-7.0 then this can be used as a measure of the change in the pH due to the action of P.M.E. N/100 NaOH was added to 3 ml. samples in increasing quantities until the absorbance was restored to the original value. Checks were also taken of the solutions with a pH meter. The amount of NaOH needed to restore the solutions to the original pH was calculated and used as a measure of the activity of P.M.E.

For the assay of P.M.E. the cultures were always grown up in a pectin medium.

Hydrolases and Trans-eliminases for Thiobarbituric Acid Assay

Albersheim et al. (1960b) showed that the products of hydrolases, i.e. galacturonic acid and the oligosaccharides of it, gave products with a maximum absorbance at 517 mμ, while those of trans-eliminases, i.e. unsaturated galacturonic acid and oligosaccharides containing this group, had a maximum absorbance at 547-550 mμ.

Procedure: The following procedure is a modification of that by Sherwood, (1967).

A solution containing sodium polypectate or pectin, buffer, enzyme solution and other minerals reportedly necessary for the enzyme's activity, was incubated at 30°C in a water bath. 0.1 ml. samples were taken from this and added to a test tube 3/8" in diameter and 3" long, containing the following mixture.

1 ml. 0.01 M Thiobarbituric acid

0.5 ml. 0.50 M HCl.

These tubes, two per sample, were carefully plugged with cotton wool, care being taken to avoid getting solutions onto the cotton wool, and heated in a boiling water bath for one hour. After cooling, their spectrum was measured in an SP 800 Unicam spectrophotometer over the range 500-650 mμ . in an 0.5 cm. cell. The zero for all readings was taken at 650 mμ because experience showed that presence or absence of absorbance in the range 500-550 mμ little affected absorbance at 650 mμ. Controls in all cases, employed boiled enzyme solutions.

The incubation mixtures consisted of the following:

Buffer	5 ml.
2.5% w/v Sodium polypectate or pectin	2 ml.
1 M NaCl or 0.001 M $CaCl_2$	1 ml.
2% w/v Phenol	1 ml.
Enzyme solution	1 ml.
Total volume	10 ml.

In each case, the test was carried out with the following buffer solutions.

Citric acid/sodium citrate (0.1 M)	pH 3
" " " " "	pH 4
" " " " "	pH 5
" " " " "	pH 6
Tris-Maleate (0.1 M)	pH 6
" " "	pH 7
" " "	pH 8
Tris-HCl (0.1 M)	pH 8
" " "	pH 9

This combination of buffer, substrate etc. gave 36 test incubations per sample tested. In some cases, sample sizes were increased by a factor of 5, and then readings taken with 1 cm. spectrosil cells. This gave larger curves in specific cases, if required.

Assay of reducing groups by Nelson's Reagent

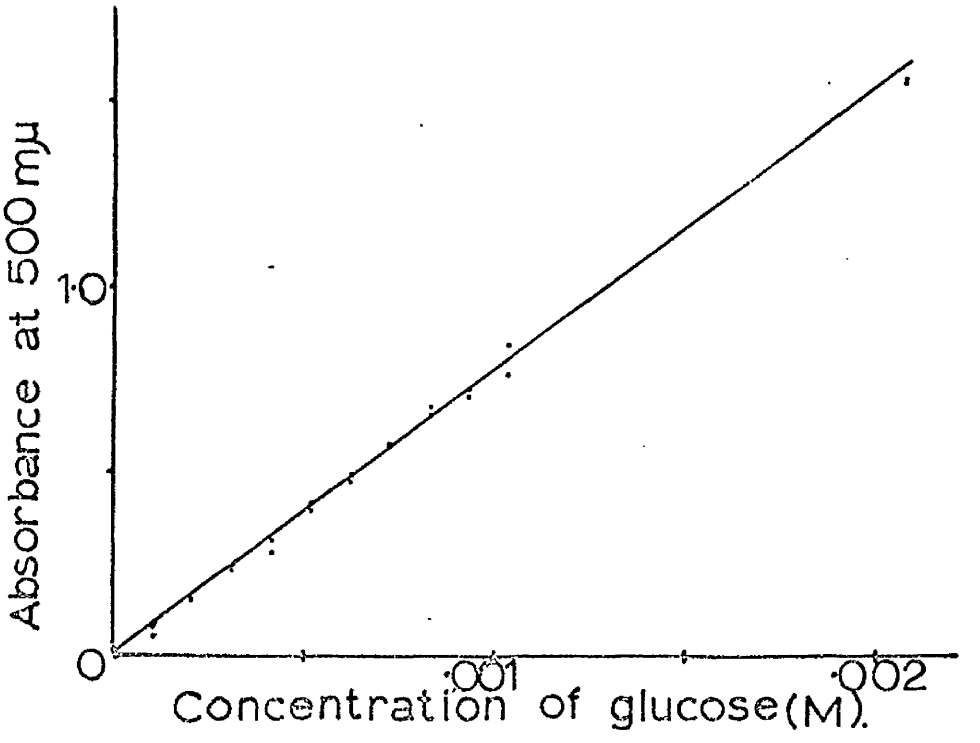
The reagents were prepared according to the procedure of Nelson (1944) as follows:

Reagent A

Solution 1:	Na_2CO_3 (anhydrous)	25 gms.
	Rochelle salt	25 gms.
	$NaHCO_3$	20 gms.
	Na_2SO_4 (anhydrous)	200 gms.

Dissolved in 800 ml. of glass distilled H_2O and made up to 1 litre. Filtered if necessary and stored above 20°C.

Figure 5. Standard curve for the measurement of reducing groups (glucose) using Nelson's procedure.



Reagent A (contd)

Solution 2: 15% w/v $CuSO_4.5H_2O$ with 1 or 2 drops of concentrated H_2SO_4 per 100 ml. of solution.

The reagent was prepared finally for use by adding 25 parts of Solution 1 to one of Solution 2.

Reagent B

25 gms. of ammonium molybdate in 450 ml. of glass distilled H_2O. 21 ml. of concentrated H_2SO_4 were added and then 3 gms. sodium arsenate dissolved in 25 ml. of glass distilled H_2O. This solution was stored for at least 48 hours after preparation at 37°C in a brown, glass-stoppered bottle. After this, it was kept at room temperature.

The procedure for testing for reducing groups was as follows: 1 ml. of the solution under test was added to 1 ml. of Reagent A and heated in a boiling water bath for 20 minutes. After cooling, 1 ml. of Reagent B was added. The solution was left for 20 minutes before reading in the SP 800. It was general procedure to dilute the treated solution to 10 ml. with glass distilled H_2O. When standard solutions of glucose were prepared, the blank control solution consisted of glass distilled H_2O. The control solution in enzyme assay mixtures consisted of treated samples of the mixtures removed immediately the enzyme was added, i.e. at time 0. The concentration of reducing groups released in such mixtures was estimated from absorbance at 500 mμ in 1 cm. silica glass cells in the SP 800 Unicam spectrophotometer. Figure 5 shows the standard curve obtained by reacting glucose with Nelson's reagent.

Assay of Polygalacturonate trans-eliminase (P.G.T.E.) using absorbance measurements at 235 mμ.

The assay used was a modification of that of Nasuno and Starr (1966). Activities were measured as follows:

An SP 800 U.V. spectrophotometer (Unicam) was set at 'fixed wavelength' at 235 mμ with constant temperature cell holders at 30°C. Using 0.5 cm. silica glass cells, the assay mixture consisted of the following:

Tris Buffer (0.1 M) pH 8.5	0.75 ml.
1.5% w/v Sodium polypectate	0.25 ml.
0.001 M $CaCl_2$	0.15 ml.
Enzyme solution	0.35 ml.
Total volume	1.50 ml.

In some early experiments, 0.01 M $CaCl_2$ was used, but this led to gel formation in the control cells where boiled enzyme solution was used in place of the fresh enzyme solution. The use of 0.001 M $CaCl_2$ stopped gel formation but still appeared to give the same enzyme activity as the 0.01 M $CaCl_2$.

The increase in absorbance at 235 mμ was taken as a measure of enzyme activity. As the carriage of the spectrophotometer moved sideways at a fixed rate, it was possible to get a graph of increase of absorbance against time; in other words, a direct measure of activity.

Activity was described arbitrarily as:

1 unit of activity = an increase of 0.5 absorbance units per minute (using 0.5 cm. cells in above conditions).

Since most curves tend to be sigmoid in shape with a fairly long, straight middle section, the activities were measured from this middle, straight line part of the curve.

Estimation of Macerating Activity

Macerating activity was measured using the procedure of Brown (1915). 1 cm. cores of potato tuber were cut from healthy tubers using a 1 cm. diameter cork borer. These were washed, and then slices approximately 0.5 mm. thick, were cut with a razor and bench microtome. The slices were also washed thoroughly. After washing, sets of 5 discs were placed in petri dishes and 10 ml. of the solution under test were added. At appropriate intervals attempts were made to tease the discs apart gently with a pair of mounted needles. Maceration was judged to have occurred if the disc broke under very little force. Control solutions consisted of the test solution heated in a boiling water bath for 15 minutes.

Assay of Polyphenoloxidase

The assay used was essentially that given by Lovrekovich *et al.* (1967). The reaction mixture consisted of the following:

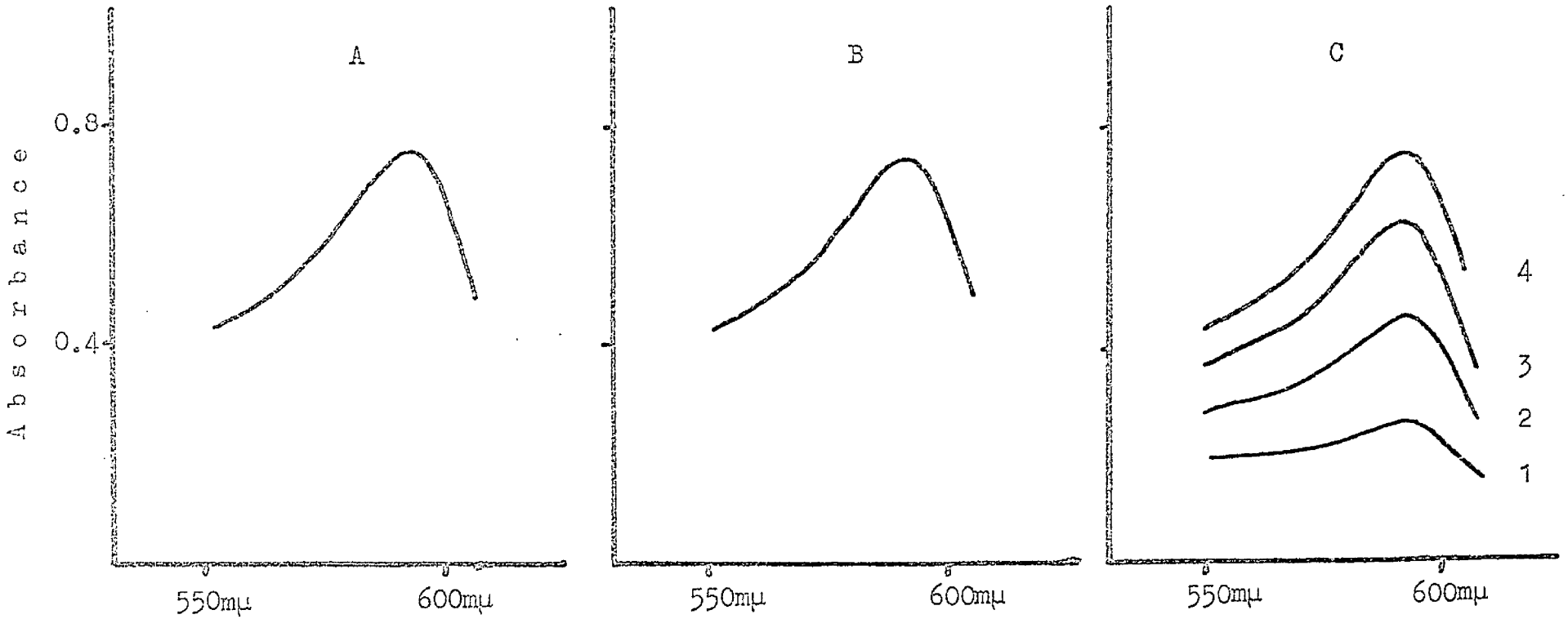
Phosphate buffer (0.1 M) pH 6.0	1.0 ml.
Catechol solution (2 mg./ml.)	0.5 ml.
Proline solution (5 mg./ml.)	0.5 ml.
Enzyme solution	1.0 ml.

The enzyme solution was added last to the test tube containing the mixture. The test tube was then shaken vigorously on a test tube shaker for 3 minutes

to provide aeration. After shaking, the absorbance of the solution was measured at 520 mμ in an SP 800 Unicam spectrophotometer. Control solutions, using enzyme preparations heated for 15 minutes in a boiling water bath, were used in the reference cells. No differences could be noticed if water was substituted for the boiled solutions in the controls.

The absorbance at 520 mμ was taken as a direct measure of polyphenoloxidase activity. In some cases absorbance of some solutions had to be calculated using dilutions of the original ones.

Figure 6. Spectra of pectin solutions containing bromocresol purple after incubating for one hour at 30°C with the culture supernatants.



Culture supernatant of G110 cr.str-r.

Culture supernatant of G110 dif.

Controls

1. pH 5.5
2. pH 6.0
3. pH 6.5
4. pH 7.0

Section III - Results

A. Pectic enzyme production in vitro

The production of P.M.E. in vitro

The following isolates were used in this experiment. G110 cr.str-r. (a streptomycin resistant creamy isolate) and G110 dif. They were grown up in the pectin medium for 48 hours, after which the culture supernatants were tested for P.M.E. activity.

The results are given in Figure 6. It is clear that no change in the absorbance at 590 mμ occurs after incubation for one hour when compared with the control cells, indicating that no change in pH had occurred. No change in pH could be detected using the pH meter. Thus, no detectable P.M.E. activity was present in the culture supernatant of these two isolates.

Qualitative determination of hydrolase and trans-eliminase activity in vitro

The isolates used were the same ones used in the previous experiment. They were grown up in the pectin and sodium polypectate media and after 24 hours growth culture supernatants were prepared and tested for pectic enzyme activity using the thiobarbituric acid assay.

The results using one isolate, G110 cr.str-r., are given in figures 7 a, b, c and d. The other isolate gave similar results.

All the reaction mixtures had an an absorption peak at 550 mμ. This indicates the presence of trans-eliminase activity. Little or no absorption occurred at 517 mμ, indicating the absence of hydrolase activity. Absorbance at 550 mμ was generally greater with polypectate in the reaction mixtures instead of pectin and calcium chloride instead of sodium chloride. This suggests the possibility of the trans-eliminase being polygalacturonate trans-eliminase (P.G.T.E.).

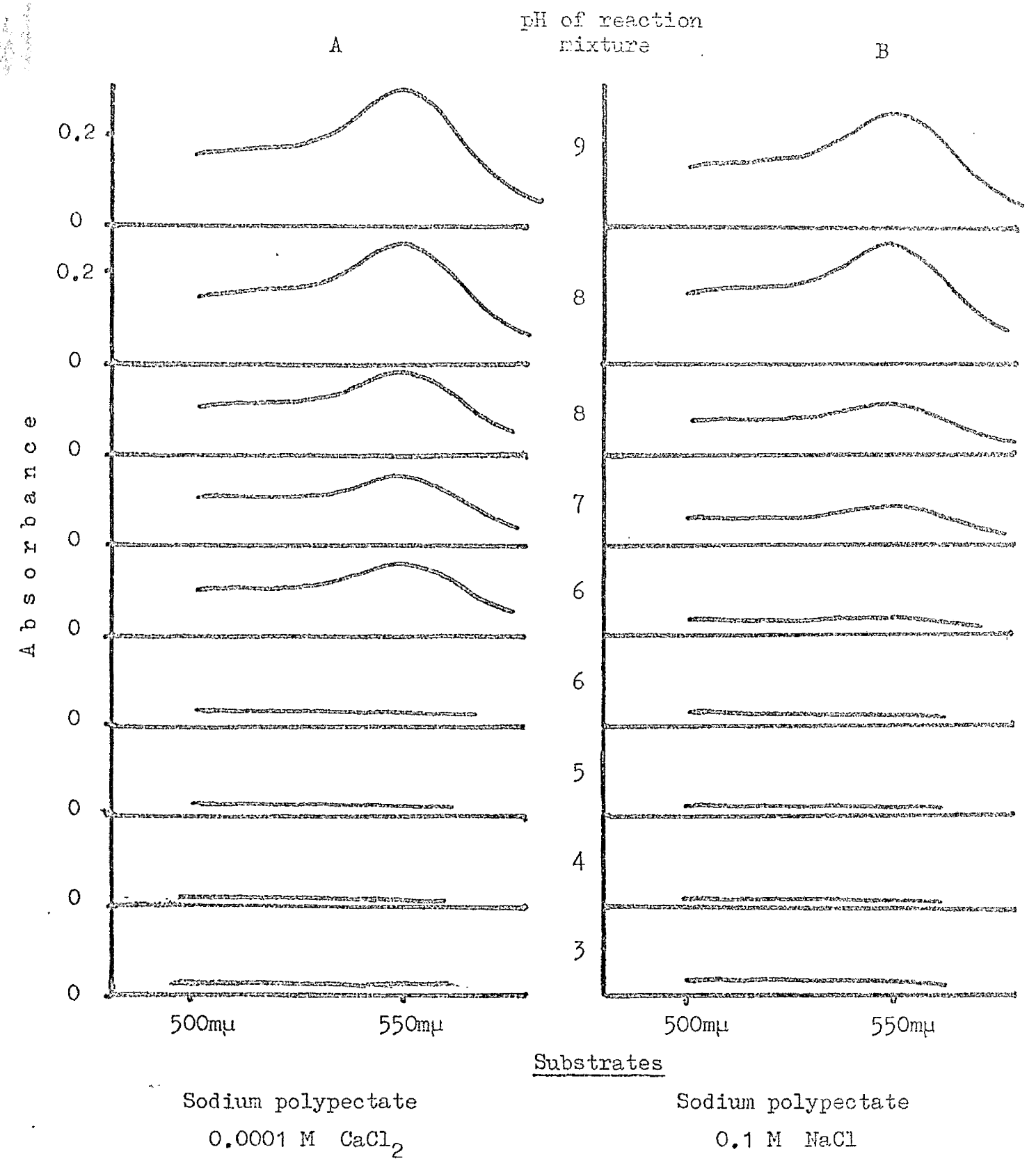
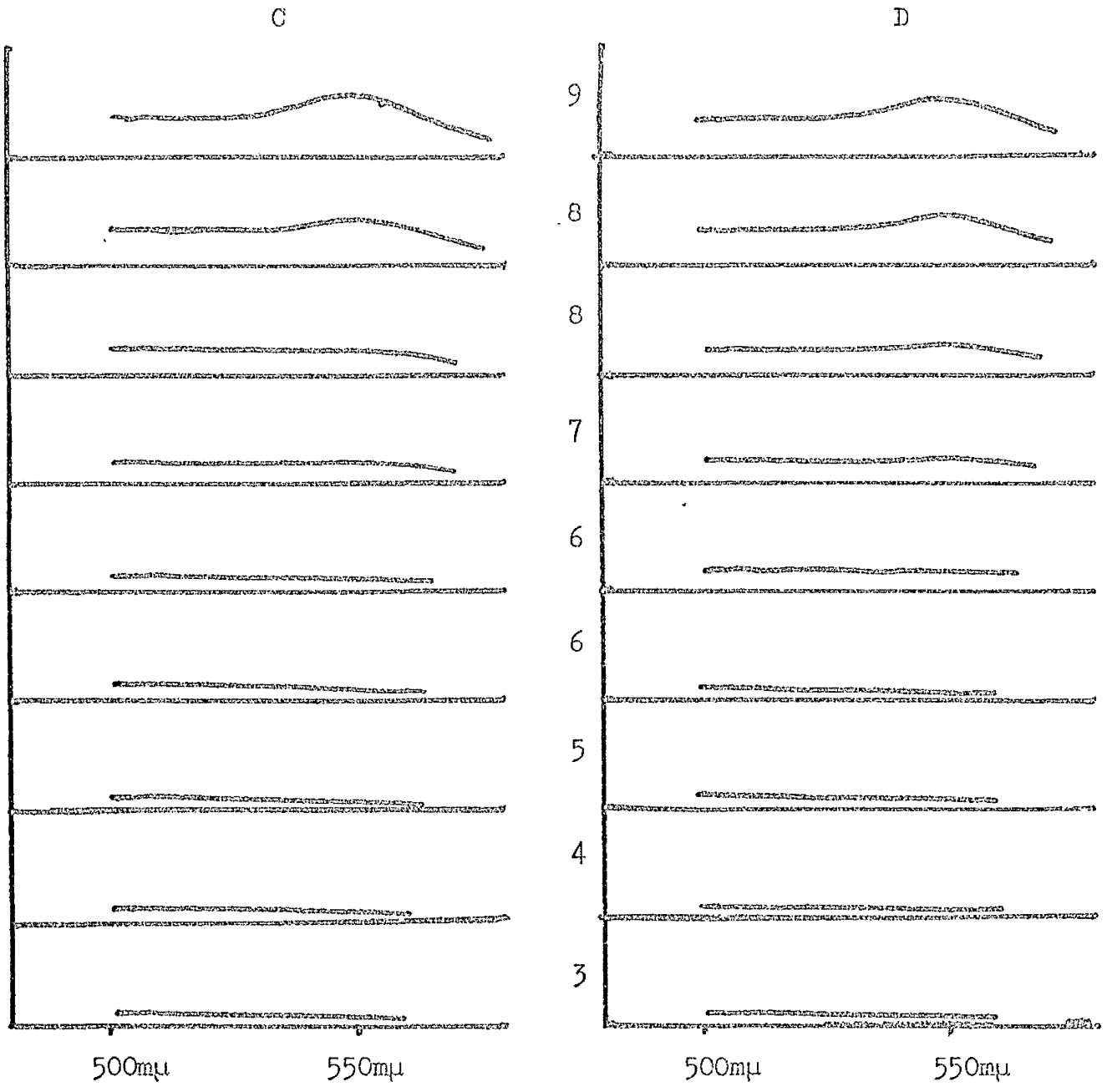


Figure 7. Spectra of reaction mixtures containing culture supernatant of G110 cr.str-r., incubated for 24 hours at 30°C and then reacted with thiobarbituric acid.

pH of reaction mixture



Substrates

Pectin

0.0001 M $CaCl_2$

Pectin

0.1 M NaCl

Thus trans-eliminase activity (possibly due to P.G.T.E.) was readily demonstrated in all cultures, but there was no indication that hydrolases were also produced. The following experiment, using a commercial sample of pectinase, was carried out to determine whether hydrolase activity could in fact be detected using this procedure.

The determination of enzyme activities in a commercial pectinase sample using the thiobarbituric acid assay

The commercial preparation of pectinase was obtained from Sigma and was prepared for testing as a 1% w/v solution.

The results obtained after 24 hours incubation are given in Figure 8a, b, c and d. These results are for the large sample size and large cuvette size mentioned in the methods. However, they agree completely with the results for the usual sample size and the 0.5 cm. cuvettes. It is clearly seen that both hydrolase and trans-eliminase activities are present. Since the trans-eliminase is active only when pectin is the substrate it would appear to be pectin methyl trans-eliminase. This is supported by the fact that the optimum pH for its activity occurs at about pH 5-6.

Hydrolases are present which degrade pectin and sodium polypectate with highest activity in the lower pH range 3-5. It is noticeable that NaCl seems to give a slight shift in absorption maxima from 517 mμ to 510 mμ.

The technique is clearly capable of differentiating between the different types of enzyme produced. These results would indicate that the failure to detect hydrolase activities in culture supernatants was due to the absence of active forms of enzymes in culture supernatants. Enzyme could be produced but could be inactive or inactivated.

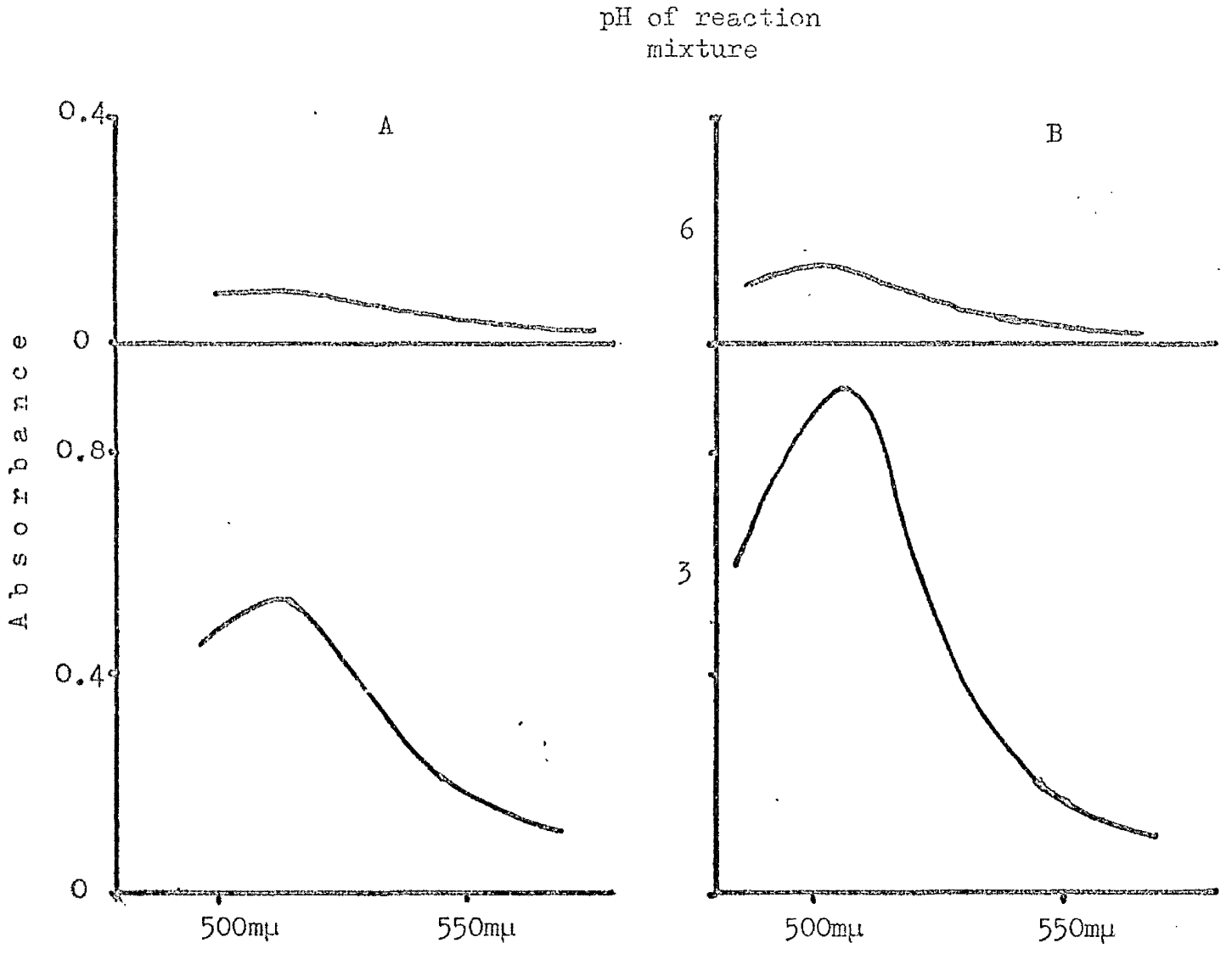
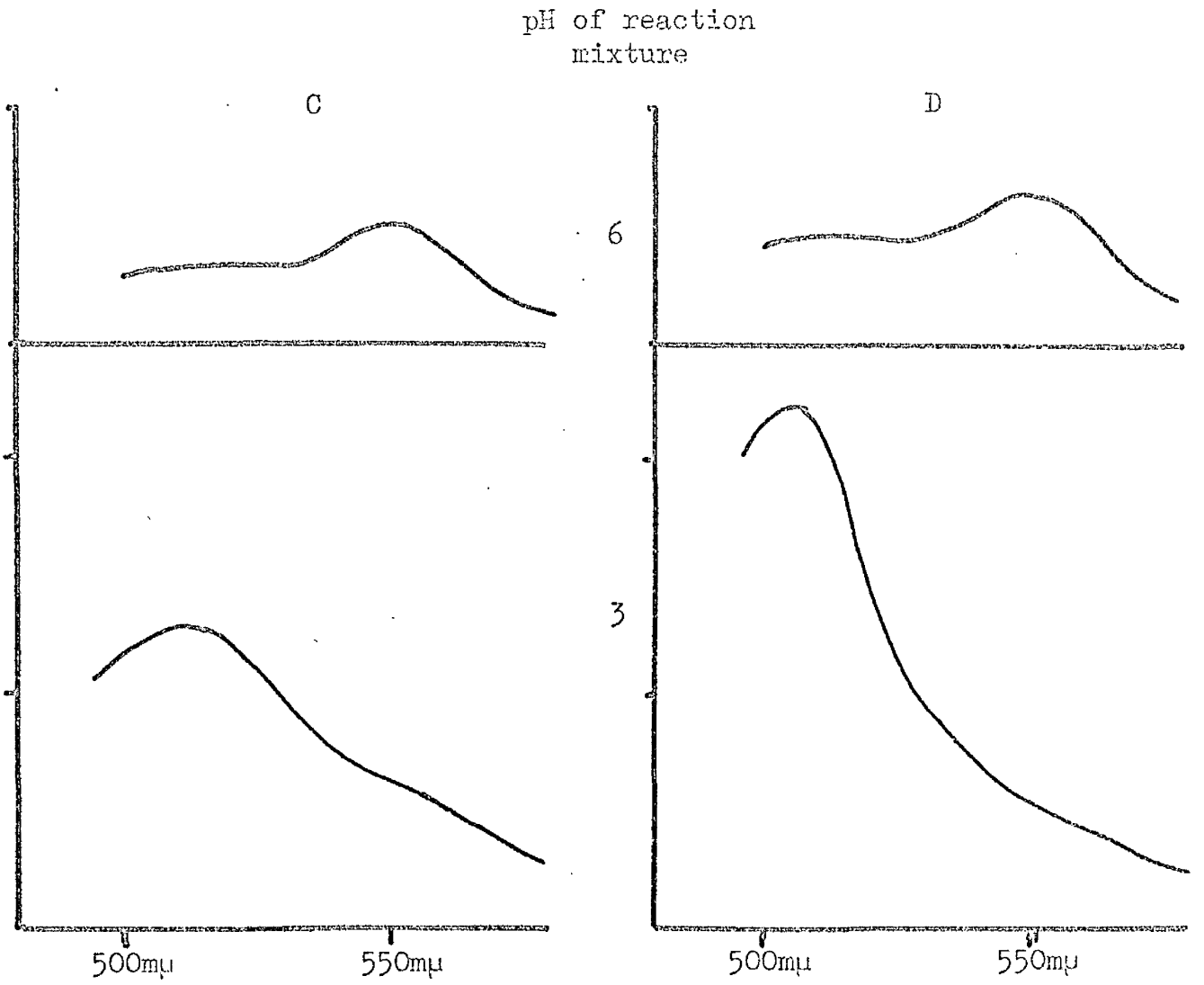


Figure 8. Spectra of reaction mixtures containing a commercial pectinase, incubated for 24 hours at 30°C and then reacted with thiobarbituric acid.



Substrates

Pectin
0.0001 M $CaCl_2$

Pectin
0.1 M NaCl

Figure 9. Effect of $CaCl_2$ on trans-eliminase activity.

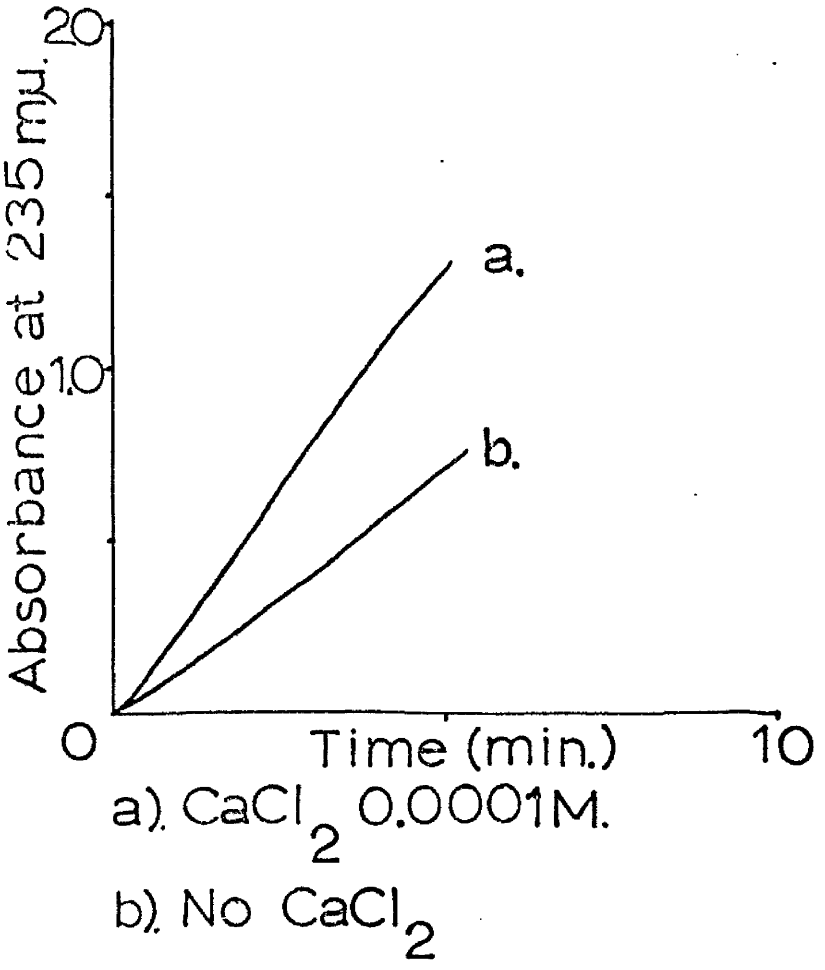
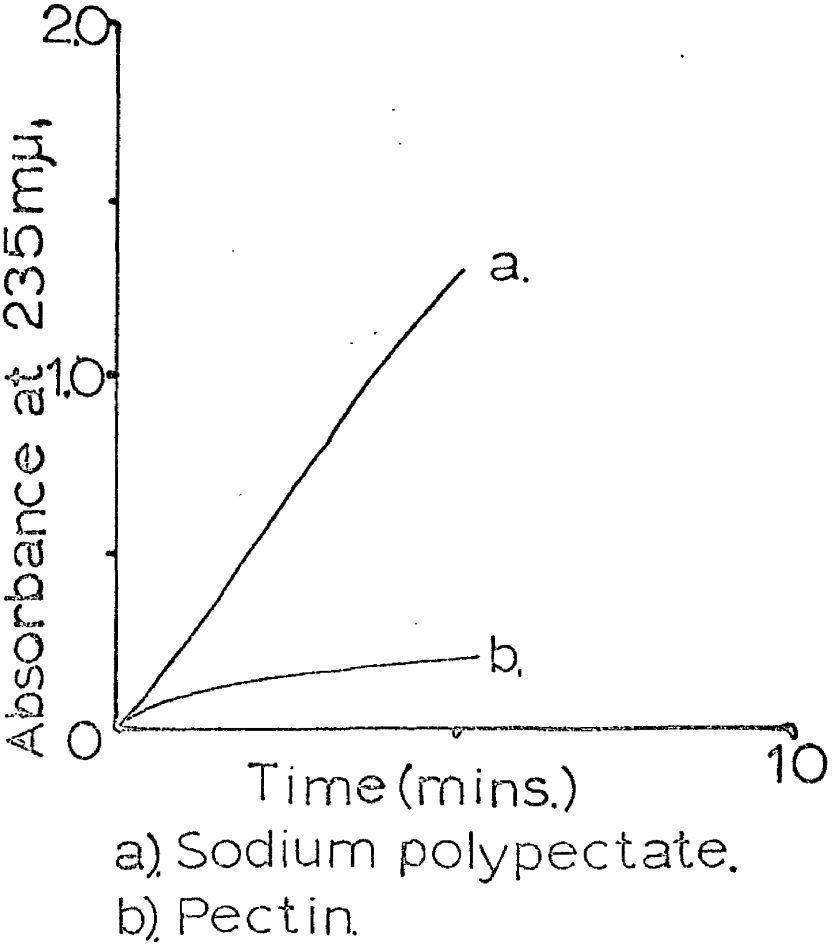


Figure 10. Effect of substrate on trans-eliminase activity.



The following experiments were carried out to investigate further the properties of the trans-eliminase produced by E. atroseptica in more detail.

The effect of calcium chloride on trans-eliminase activity

G110 dif. was grown up for 24 hours in polypectate medium. Culture supernatants were assayed for trans-eliminase activity by measuring changes in absorbance at 235 mμ in the presence of 0.001 M calcium chloride and in its absence.

The results are given in Figure 9. It will be seen that the omission of calcium chloride from the assay mixture gives a reduced rate of increase in absorbance, indicating a reduction in the activity of the enzyme.

It would therefore appear that calcium chloride stimulates enzyme activity. Other isolates gave similar results when tested.

The effect of different substrates on trans-eliminase activity

G110 dif. was grown up for 24 hours in a sodium polypectate medium and the culture supernatant was tested for trans-eliminase activity. This was done by measuring the change in absorbance at 235 mμ using 1.50% w/v polypectate or 1.50% w/v pectin as the substrate. The results are given in Figure 10.

It clearly shows the marked stimulatory effect of sodium polypectate on the activity of the enzyme. Pectin is thus less suitable as a substrate for the trans-eliminase. Other isolates gave similar results.

The effect of pH on trans-eliminase activity

All isolates had similar pH activities but the results using G110 cr. str-r. are presented because of the initial high concentration of activity in the culture supernatants of this isolate. Such activities allowed more

Table 2. showing the effect of pH on trans-eliminase activity

Buffer	pH	Trans-eliminase Activity
TRIS-HCl	9.0	4.816
" "	8.5	6.163
" "	8.0	3.780
Phosphate	8.0	1.204
"	7.5	0.812
"	7.0	0.399
"	6.5	0.162
"	6.0	0.080
Citrate	6.0	~ 0.002
"	5.5	~ 0
"	5.0	~ 0

accurate determination of lower activities at other pHs.

G110 cr.str-r. was grown up in the sodium polypectate medium and after 48 hours the culture supernatants were tested for trans-eliminase activity by measuring the change in absorbance at 235 mμ. The following buffers were employed to vary the pH of the reaction mature.

Buffer	pH
TRIS-HCl (0.1 M)	9.0
" " "	8.5
" " "	8.0
KH_2PO_4/Na_2HPO_4 (0.1 M)	8.0
" "	7.5
" "	7.0
" "	6.5
" "	6.0
Citric Acid/Sodium Citrate (0.1 M)	6.0
" " " " "	5.5
" " " " "	5.0

The results are given in Table 2 .

The activity increased with pH, the highest activity being present at pH 8.5. Only very small activities were detected when citrate buffers were used.

Thus the evidence suggest that the trans-eliminase produced by this organism is a polygalacturonate trans-eliminase (P.G.T.E.), by virtue of the fact that it is most active against polypectate, is stimulated by calcium and has an optimum pH of 8.5. These agree well with the properties of endo-P.G.T.E. reported for Erwinia carotovora by Starr and Moran (1962).

It is also worth noting here that concentrations of calcium chloride higher than that normally used in the absorbance assay give gel formation in the U.V. cuvettes. This gel is rapidly liquefied when detectable concentrations of P.G.T.E. are present but not in the control cuvettes. Since rapid liquefaction of gels is a characteristic of endo-enzymes then it is likely that the enzyme in culture supernatants is endo-P.G.T.E.

A characteristic of endo-enzymes is that they do not give high levels of reducing groups in reaction mixtures unlike exo-enzymes which give release of 90% or more of reducing groups in reaction mixtures.

It was decided to investigate the production of reducing groups at pH 8.5 and 5.0 to determine whether this was an endo- or exo-enzyme and also to see if any reducing group release could be detected at the lower pH where increase of reducing groups has regularly been used as a measurement of polygalacturonase activity.

Increases in reducing groups with trans-eliminase activity

G110 cr.str-r. was grown in a sodium polypectate medium for 48 hours. Using 1/50 dilutions of the culture supernatant P.G.T.E. measurements were determined by measuring the change in absorbance at 235 mμ, at pH 8.5, and in the presence of calcium chloride (0.0001 M). P.G.T.E. activities in undiluted culture supernatant were also determined by the same method but using 0.1 M citric acid / sodium citrate buffer (pH 5.0), in the presence of sodium chloride (0.1 M).

In order to measure the production of reducing groups two sets of reaction mixtures were prepared. These consisted of four tubes per set. They were prepared with exactly the same constituents as the reaction mixtures used for P.G.T.E. determinations by absorbance at 235 mμ except that

Figure 11. Increase in reducing power of reaction mixtures at pH 8.5, 1/50 diluted culture supernatant.

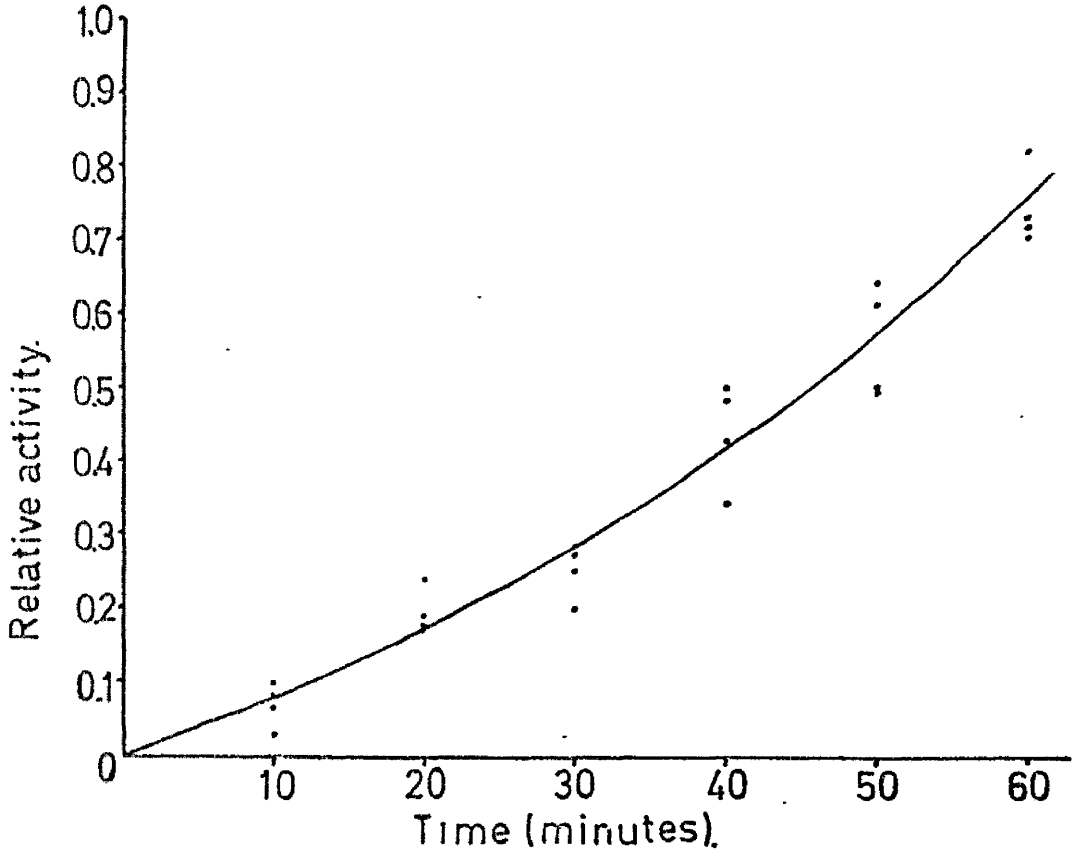
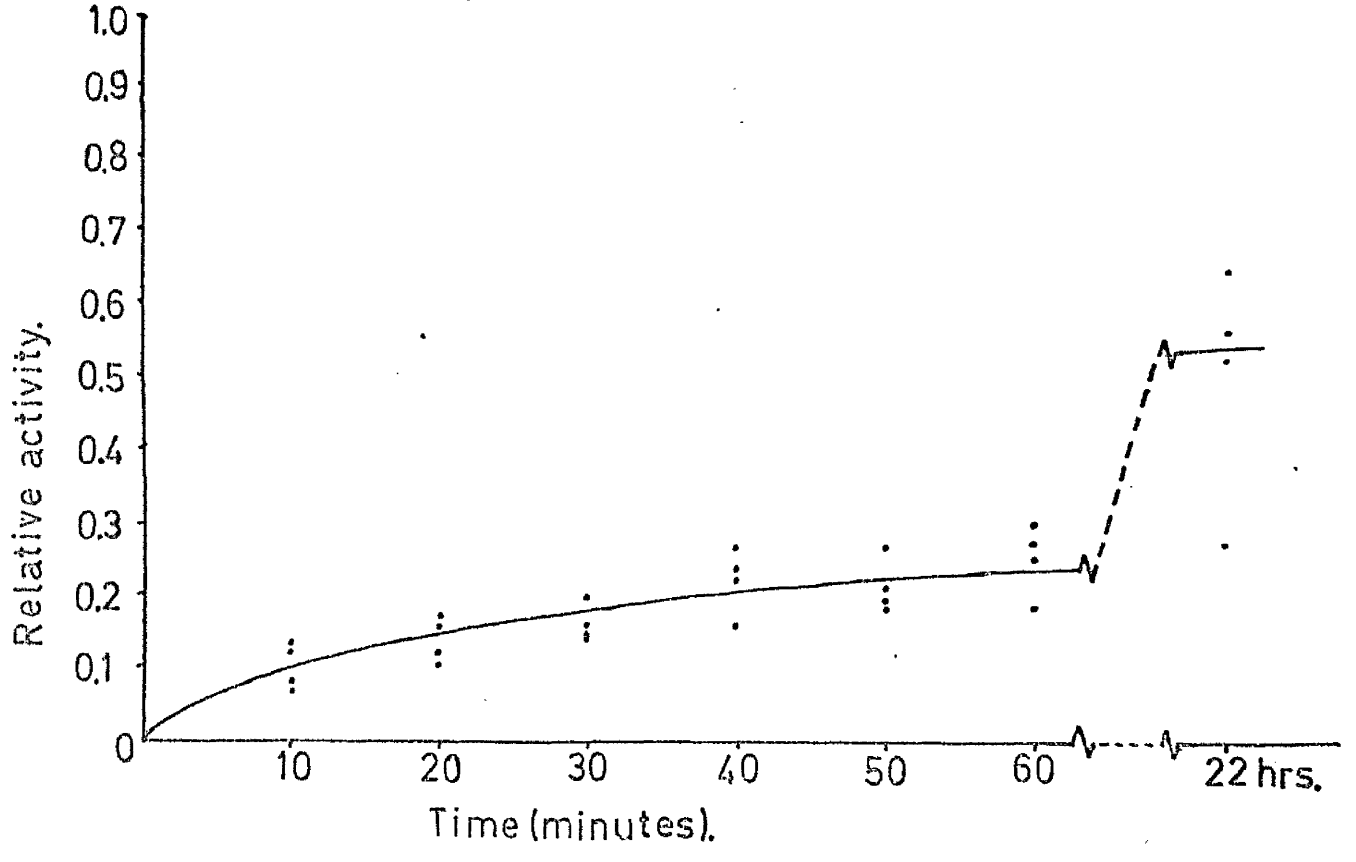


Figure 12. Increase in reducing power of reaction mixtures at pH 5.0, undiluted culture supernatant.



1 Unit of activity ≡ 0.001 Moles of Reducing Groups

each had a total volume of 15 ml., i.e. ten times the volume of the absorbance assay mixtures. They were incubated at 30°C and 1 ml. samples removed at time 0, 10, 20, 30, 40, 50 and 60 minutes and 22 hours after the addition of the enzyme. The samples were treated with Nelson's reagent and the quantities of reducing groups released were estimated. The time zero sample was used as a reference solution for the other solutions.

As an additional control, samples were removed from the reaction mixtures after three hours and tested using the thiobarbituric acid assay. These samples were five times larger than the samples normally used in this assay. The spectra were also scanned in 1.0 cm. silica glass cells (as opposed to the normal 0.5 cm. cells) to provide larger peaks of absorbance. This procedure was adopted to detect, qualitatively, any very low levels of P.G.T.E. or hydrolase that might be present at pH 5.0.

The activities of P.G.T.E. as estimated by absorbance at 235 mμ are given in Table 3.

Table 3. P.G.T.E. activity of culture supernatants

1/50 Diluted Supernatant at pH 8.5	Undiluted Supernatant at pH 5.0
0.074	~0.002

It can be seen that little P.G.T.E. activity, if any, is detectable at pH 5.0 even when undiluted culture supernatant is used.

The increase of reducing groups is shown in Figures 11 and 12.

The thiobarbituric acid tests gave characteristic absorption maxima

for pH 8.5. Only a very small absorption maxima was detectable at 550 mμ at pH 5.0 and none at 517 mμ.

It would appear from these results that increases in reducing groups at both pHs are due to P.G.T.E. activity. It supports previous observatIn addition it is worth looking at the percentage release of reducings.

In addition it is worth looking at the percentage release of reducing groups. The concentration of sodium polypectate in the reaction mixtures is 0.25%. Assuming that polypectate consists only of 1.4 alpha linked galacturonic acid residues then the maximum possible amount of reducing groups that could be released would be about 0.02 M. At 22 hours the reaction mixture at pH 8.5 has released approximately 0.002-0.003 M of reducing groups or 10-15% of the theoretical total. This would be in agreement with the suggestion that the P.G.T.E. enzyme is an endo type enzyme, i.e. endo-P.G.T.E.* There is a small release of reducing groups at pH 5.0 and most of this occurs in the first hour. It is likely that the high concentration of P.G.T.E. present (the culture supernatant at this pH is undiluted) allows some release of reducing groups. It is obvious that much higher enzyme activities occur at pH 8.5.

The variation in the production of P.G.T.E. in vitro by different isolates

The following isolates were used. G110 dif., N/C 434, N/C 549, N/C 1042 and N/C 1449. An inoculum of 1×10^9 cells was used to inoculate pectin and sodium polypectate media. 5 ml. samples were removed at 24 hrs. and 48 hrs. for O.D. measurements to determine cell density, and to provide culture supernatants for the determination of P.G.T.E. When culture supernatants had above 0.6-0.7 units of activity dilutions had to be made so as to get more accurate measurements of activity.

* Endo-P.G.T.E. after this will often be referred to simply as P.G.T.E.

Results

The results are given in Tables 4 and 5.

Table 4. Approximate cell numbers ($X10^9$) per ml. of sodium polypectate (A) and pectin (B) media cultures

Age of Culture	G110 dif.		N/C 434		N/C 549		N/C 1042		N/C 1449	
	A	B	A	B	A	B	A	B	A	B
24 hours	2.68	1.52	2.92	1.95	3.25	2.15	3.63	2.56	3.17	2.33
48 hours	2.62	3.15	3.06	3.25	3.25	-*	3.15	5.14	3.07	4.25

* Sample not taken.

Table 5. P.G.T.E. activities in sodium polypectate (A) and pectin (B) media culture supernatants

Age of Culture	G110 dif.		N/C 434		N/C 549		N/C 1042		N/C 1449	
	A	B	A	B	A	B	A	B	A	B
24 hours	0.416	0.096	2.320	1.848	1.540	0.576	2.212	2.100	0.416	0.032
48 hours	0.556	0.523	1.988	2.912	1.120	-*	2.660	2.408	0.383	0.037

* Sample not taken.

All the isolates produced appreciable quantities of P.G.T.E. although there were considerable variations between isolates.

In conclusion then, there is evidence that the isolates of Erwinia

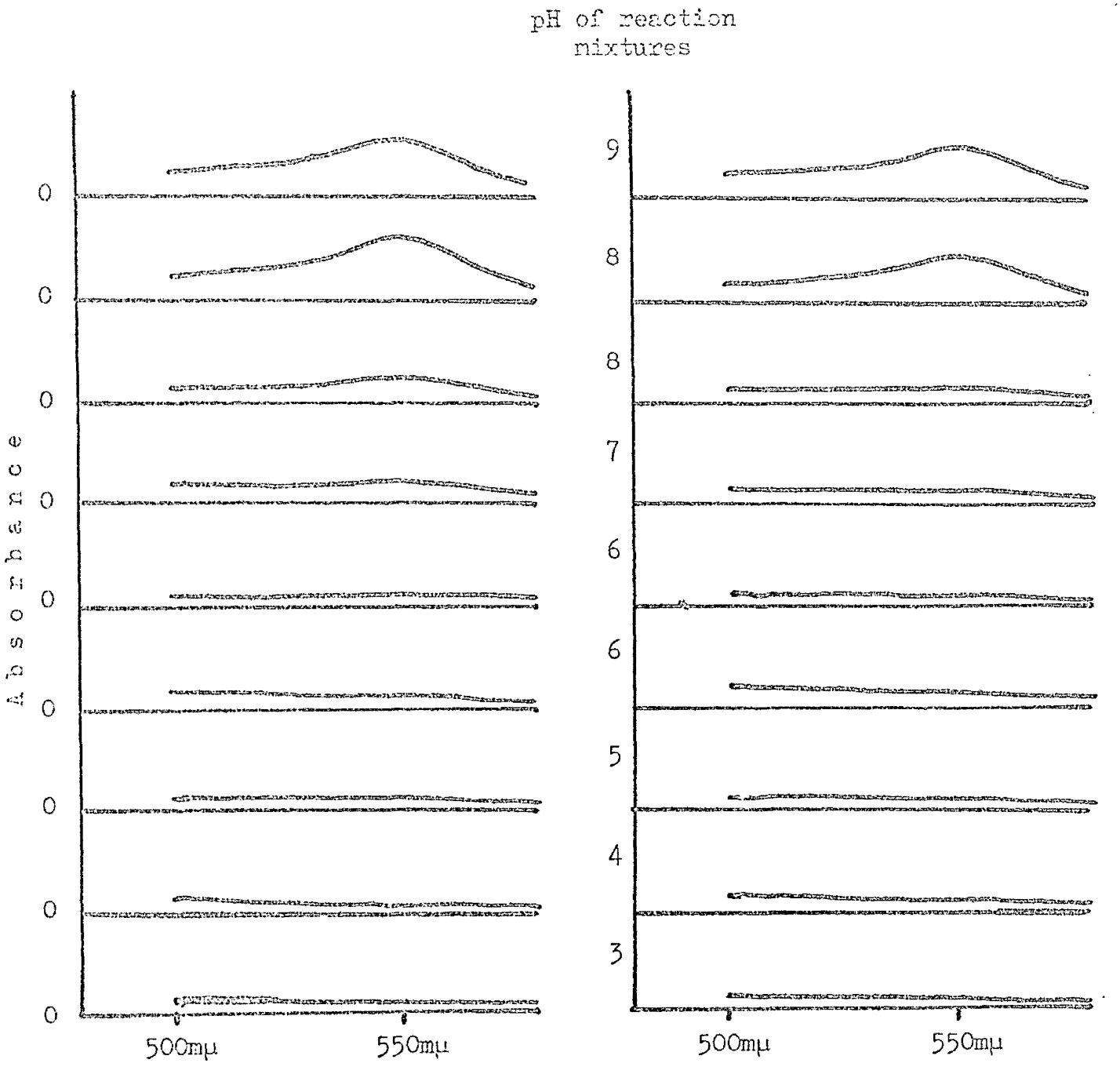


Figure 13. Spectra of reaction mixtures containing an extract of blackleg infected potato haulms from the field, incubated for 24 hours at 30°C and then reacted with thiobarbituric acid.

atroseptica examined in this study produce abundant P.G.T.E., but no P.M.E. or Polygalacturonase, in vitro. It is necessary, of course, to see if similar results can be found for the pectic enzymes present in infected potato tissue. This would provide some evidence that events in vitro are related to some degree with events in vivo.

B. Pectic enzymes produced in vivo

The production of P.M.E. in vivo

Since no P.M.E. was detected in vitro and since P.M.E. is present in healthy potato tissue, Lineweaver and Jansen (1951); Holden (1946), no attempts were made to investigate whether or not this enzyme was produced by E. atroseptica in infected tissues.

Qualitative determination of hydrolase and trans-eliminase activity in vivo

(1) Blackleg infected potato haulms

Extracts were made of blackleg infected stems from the field, (see page 28). These extracts were incubated with sodium polypectate and pectin at different pH's and in the presence of NaCl. or $CaCl_2$. Enzyme activity was assayed using thiobarbituric acid.

The results, shown in Figure 13, were very similar to those obtained with the culture supernatants. Again sodium polypectate gives bigger peaks of absorption than pectin. Similarly, calcium chloride gives bigger peaks than sodium chloride. Thus the main enzyme present would appear to be P.G.T.E.

(2) Laboratory infected tuber slices

The extracts were prepared from infected tuber slices 48 hours after

inoculation, as described in the methods. Enzyme activities were determined using thiobarbituric acid. The results agree exactly with the results for blackleg haulm extracts and culture supernatants.

These results indicate that P.G.T.E. is present in quantity in infected tissue both in field and laboratory infected material. It is of interest to consider the actual levels of P.G.T.E. present in infected and healthy potato tissue.

Levels of P.G.T.E. present in infected and healthy tuber slices

Tuber slices were infected with G110 cr.str-r. and incubated for 4 days. Uninfected control slices were prepared by inoculation with sterile broth and then incubated under the same conditions for the same period of time. Macerates were made of the slices using 0.1 M TRIS-HCl buffer at pH 8.5 and assayed for P.G.T.E. activity using the direct spectrophotometric assay.

The results for sets of six tuber slices are shown in Table 6.

Table 6. Levels of P.G.T.E. detectable in extracts of infected and healthy tuber slices

Healthy Slices	Infected Slices (Mean of 2 readings)
0	1.057
0	0.991
0	0.826
0	0.571
0	0.869
0	1.043

It is clear that considerable P.G.T.E. activity was present in the infected slices, while no activity was found in the healthy slices. Since P.G.T.E. is produced in quantity *in vitro* it is likely that the presence *in vivo* is due to secretion of the enzyme by the bacterium rather than by the host.

In summary then, it is clear that different isolates produce abundant P.G.T.E. *in vitro* but no other pectic enzymes in readily detectable amounts. In addition, this is also the only enzyme attacking the α1:4 linkage that can be detected in infected plant material. All this suggests that P.G.T.E. is indeed the main pectic enzyme produced *in vitro* and *in vivo* by this organism.

The next section describes the work using mutant isolates which was designed to investigate the importance of this enzyme in pathogenesis by this organism.

C. Isolation of mutant strains

The mutants were selected for their inability to liquefy, or their ability to liquefy at a slower rate than the wild type, a solid sodium polypectate medium. This medium is a modification of that used by Logan (1963) for isolating *Erwinia* species.

Isolate

The isolate used in these studies was G110 dif. which was generally found most suitable as it was non-motile and did not cause too rapid fusion of the areas liquefied by the individual colonies on the isolation medium.

Isolation medium

This medium was prepared in two stages:

(i) Petri dishes were prepared with distilled water agar (Oxoid No.3) 1.5% w/v, containing 0.3% w/v anhydrous calcium chloride. Since this medium has a tendency to 'bump', it was autoclaved in quantities no greater than about one third of the volume of the containing flask.

(ii) After storage overnight, a layer of liquid sodium polypectate medium was poured over the agar layer. The constituents of this part were as follows:

Sodium polypectate	15 gms.
Yeast extract (Difco)	10 gms.
Bromothymol blue (Hopkin & Williams)	2 ml.
Glass distilled H_2O	1 litre

The polypectate was dissolved in the water by heating and constant stirring. The yeast extract and bromothymol blue were added after all the polypectate had dissolved, and the pH was adjusted to approximately neutral by adding 1N NaOH dropwise while continuing to stir. This was then autoclaved at 10 lbs. pressure for 10 minutes and poured at either normal agar pouring temperature or after cooling to room temperature. The plates were left for 2-3 days at room temperature. After this time, they were examined for the presence of free liquid on the surface of the pectate gel which had formed by the diffusion of calcium ions from the agar layer. If such liquid was present, it generally could be poured off or solidified by gently rotating the dish and spreading it over the surface of the gel.

Plate 1.

Growth of G110 dif. (wild type) on yeast extract/sodium polypectate gel.

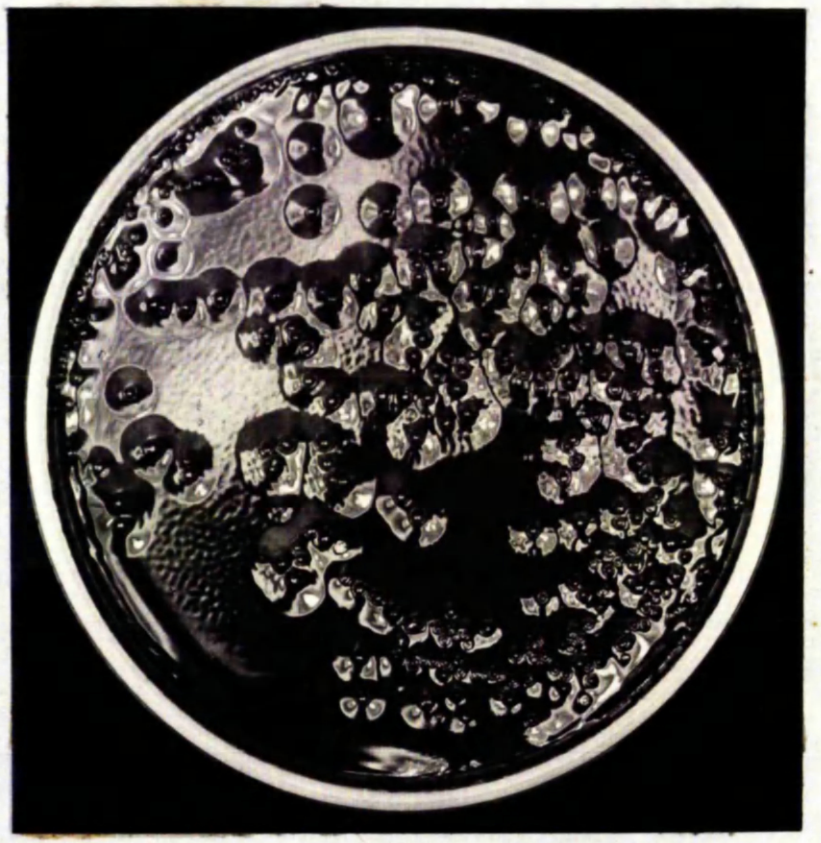
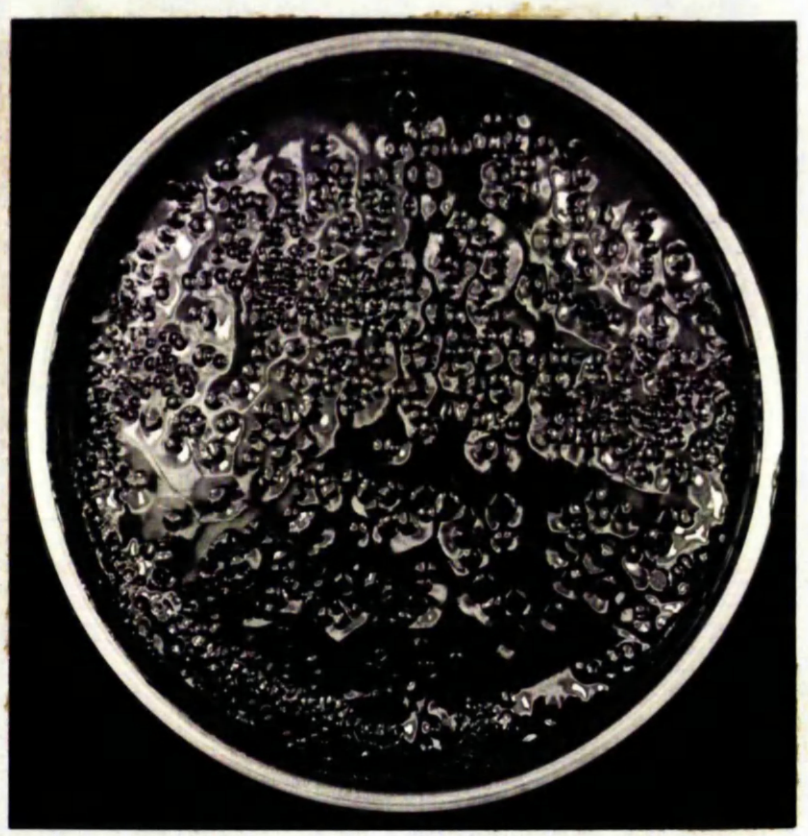


Plate 2.

Growth of mutant, G110 dif. S10, on yeast extract/sodium polypectate gel.



When the gel surface had no free liquid on it, it was ready for use. The wild type, when grown on this medium, caused 'pitting' and depression of the medium, due to the local liquefaction of the gel around the colonies. An inoculum was used which gave several hundred, but well spaced, colonies per plate. These plates were then incubated for about 2-3 days after which they were examined for colonies which did not sink into the gel. Failure to sink in was due to the fact that no liquefaction of the gel had occurred, probably due to the failure to produce P.G.T.E. Such colonies were removed and sub-cultured into nutrient broth.

Each isolate was then re-tested using the same procedure and if a large number of the colonies did not sink into the pectate gel, single colony isolates were then taken, sub-cultured onto nutrient agar slopes and investigated further.

Of sixteen strains isolated only three, G110 dif. S1, S2 and S10 have proved to differ markedly from wild type after re-testing.

These three isolates were compared with the wild type in as many characteristics as possible to ensure that they were as nearly identical as possible with the wild type except for the ability to liquefy pectate gels.

Nutritional requirements

It was important to ensure that all three mutants had no other nutritional requirements than the wild type. This was to rule out the possibility that differences in virulence were due primarily to the absence of specific growth factors from the host. All mutants grew as readily as wild type on minimal medium.

Table 7. Acid Production

Carbon Source	G110 dif. (Wild type)		G110 dif. S1		G110 dif. S2		G110 dif. S10	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
Arabinose	+	+	+	+	+	+	+	-
Dulcitol	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-
Fructose	++	+	++	+	++	+	++	-
Galactose	+	-	+	-	+	-	+	-
Glucose	++	+/-	++	+	++	+	++	-
Glycerol	d	-	d	-	d	-	d	-
Lactose	++	+	++	+	++	+	++	-
Maltose	d	-	d	-	d	-	d	-
Mannose	++	+	++	+	++	+	++	-
Raffinose	++	+	++	+	++	+	++	-
Salicin	++	+	++	+	++	+	++	-
Sucrose	++	+	++	+	++	+	++	-
Tartrate	-	-	-	-	-	-	-	-
Xylose	+	+/-	+	+	+	+	+	-

Table 8. Solid media used to test colony appearance

Glucose	MM	TTC	EMB
Fructose	MM	TTC	EMB
Galactose	MM	TTC	EMB
Lactose	MM	TTC	EMB

MacConkey's agar (a lactose containing agar)

Nutrient agar

Motility and gelatine liquefaction

The three mutants and the wild type were non-motile and did not liquefy gelatine to any extent in less than several weeks.

Colonial appearance on various media

When grown on the various MM, EMB and TTC agars described in Table 8, the mutants appeared identical with the wild type except for G110 dif. S2 which did not give the greenish black sheen on galactose EMB agar, which is characteristic of wild type. It appeared identical on all other galactose media.

No differences were noticed between the mutants and wild type on nutrient agar and MacConkey's agar.

Acid production

The mutants and the wild type were compared for acid and gas production against a range of carbohydrates (see Table 7).

There were no apparent differences in acid production from the carbohydrates used. In the case of maltose and glycerol a very small positive reaction was noticeable after more than one week. Gas production was not great in any test but G110 dif. S10 did not appear to produce gas with any of the carbohydrates tested.

The wild type appeared to give less gas overall with glucose and xylose than did G110 dif. S1 and S2, in some tests no gas apparently being evolved.

P.G.T.E. production

The mutant isolates were tested on several occasions along with the wild type for the production of P.G.T.E. in vitro. It was usual to grow each isolate up in two flasks of each medium under test. Tables 9a and 10

show the results for an experiment with wild type and all three mutants. Table 9b. shows viability counts obtained for the cultures after 72 hrs. of growth and is included to indicate the numbers of viable cells in the cultures.

Table 9a. Approximate cell numbers ($X10^9$) per ml. of sodium polypectate (A) and pectin (B) media cultures

Age of Culture	Flask	G110 dif. (Wild type)		G110 dif. S1		G110 dif. S2		G110 dif. S10	
		A	B	A	B	A	B	A	B
24 hours	1	4.2	2.4	4.1	1.9	1.5	2.1	0.02	0.2
	2	4.0	2.1	4.1	1.9	1.5	2.0	0.06	0.08
48 hours	1	4.0	4.6	4.6	2.7	4.7	3.2	3.8	1.9
	2	4.7	4.2	4.6	2.5	5.1	3.2	4.1	1.8
72 hours	1	4.6	8.3	4.6	4.6	4.8	4.8	4.6	2.9
	2	4.9	7.6	4.6	4.3	4.8	4.7	4.2	3.1

Table 9b. Estimated numbers of viable cells ($X10^9$) in cultures per ml. after 72 hours growth

Flask	G110 dif. (Wild type)		G110 dif. S1		G110 dif. S2		G110 dif. S10	
	A	B	A	B	A	B	A	B
1	2.7	7.9	9.2	3.9	7.8	3.6	6.4	3.4

Figure 14. Endo-P.G.T.E. activities in sodium polypectate medium culture supernatants of G110 dif. (Wild Type).

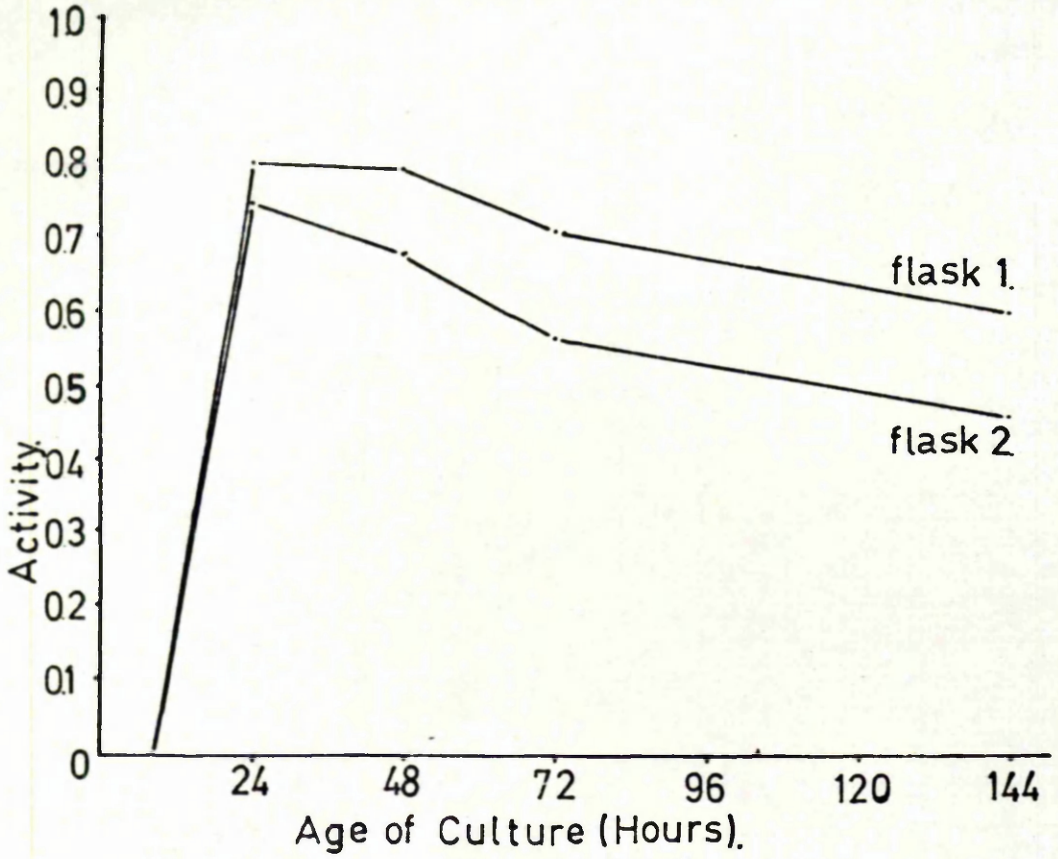


Figure 15. Endo-P.G.T.E. activities in sodium polypectate medium culture supernatants of Mutant G110 dif. S1.

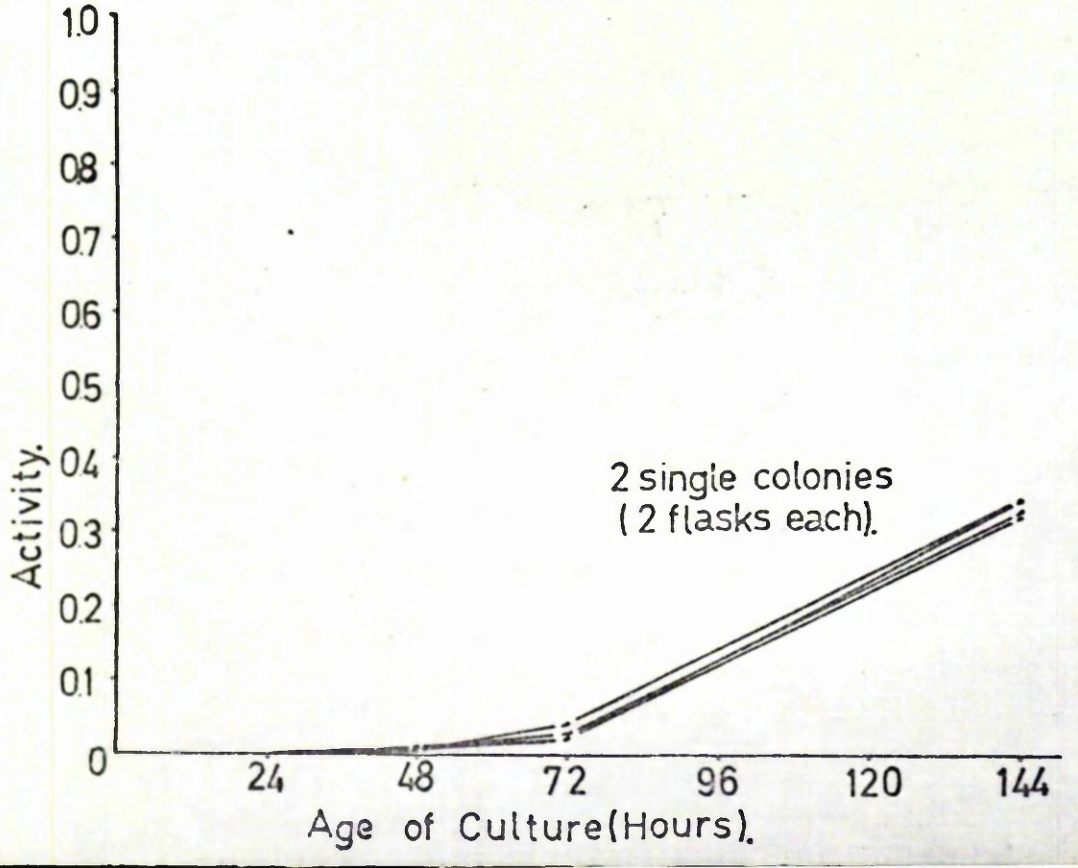


Figure 16. Endo-P.G.T.E. activities in sodium polypectate medium culture supernatants of Mutant G110 dif. S2.

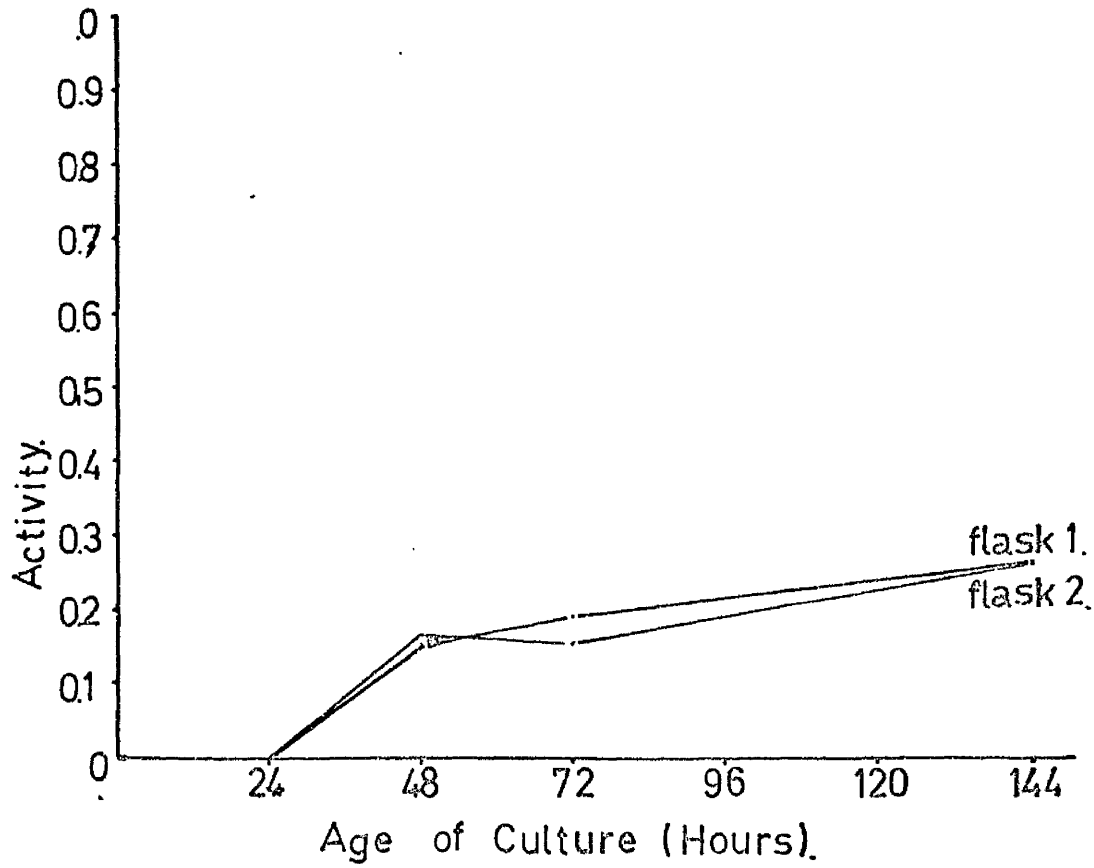


Figure 17. Estimated numbers of viable cells ($X10^9$) per ml. of sodium polypectate cultures of G110 dif. (Wild Type) and Mutant G110 dif. S2.

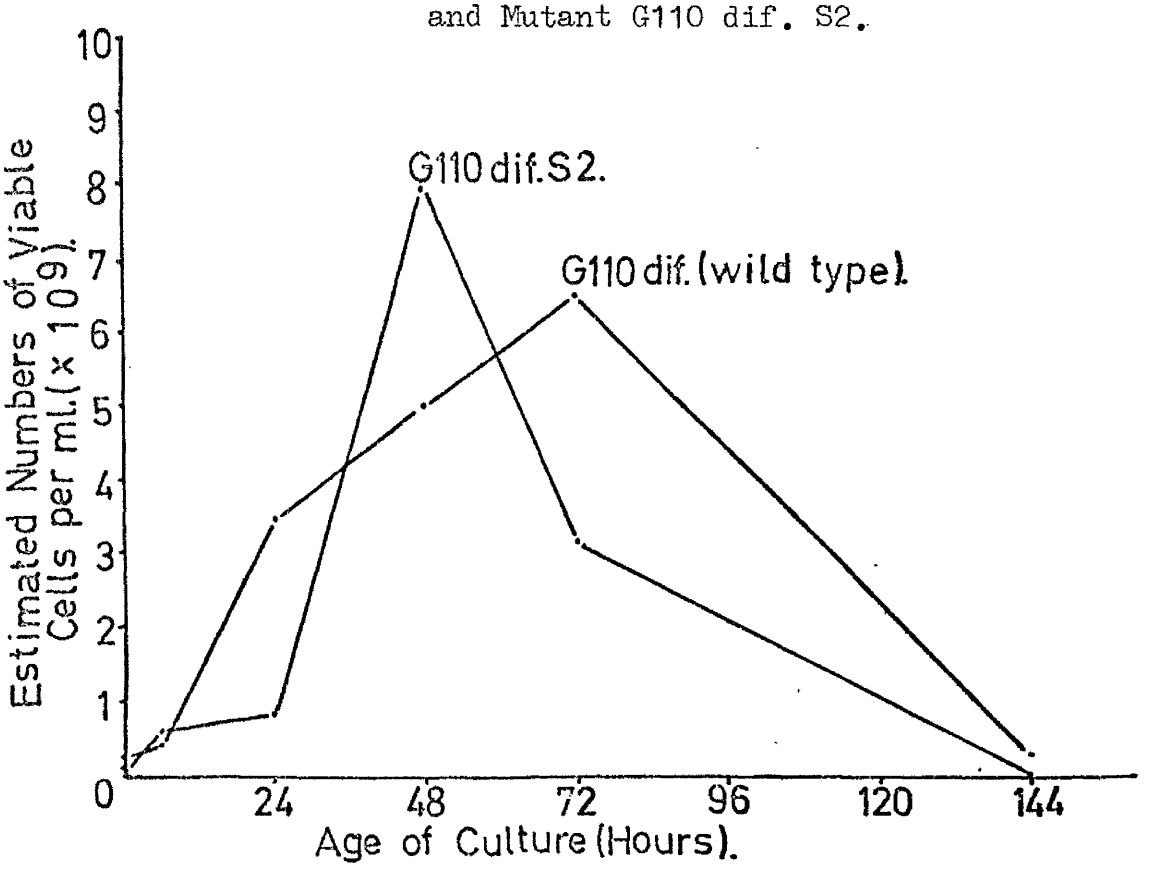


Figure 18. Estimated numbers of viable cells ($X10^9$) per ml. of sodium polypectate cultures of Mutant G110 dif. S1.

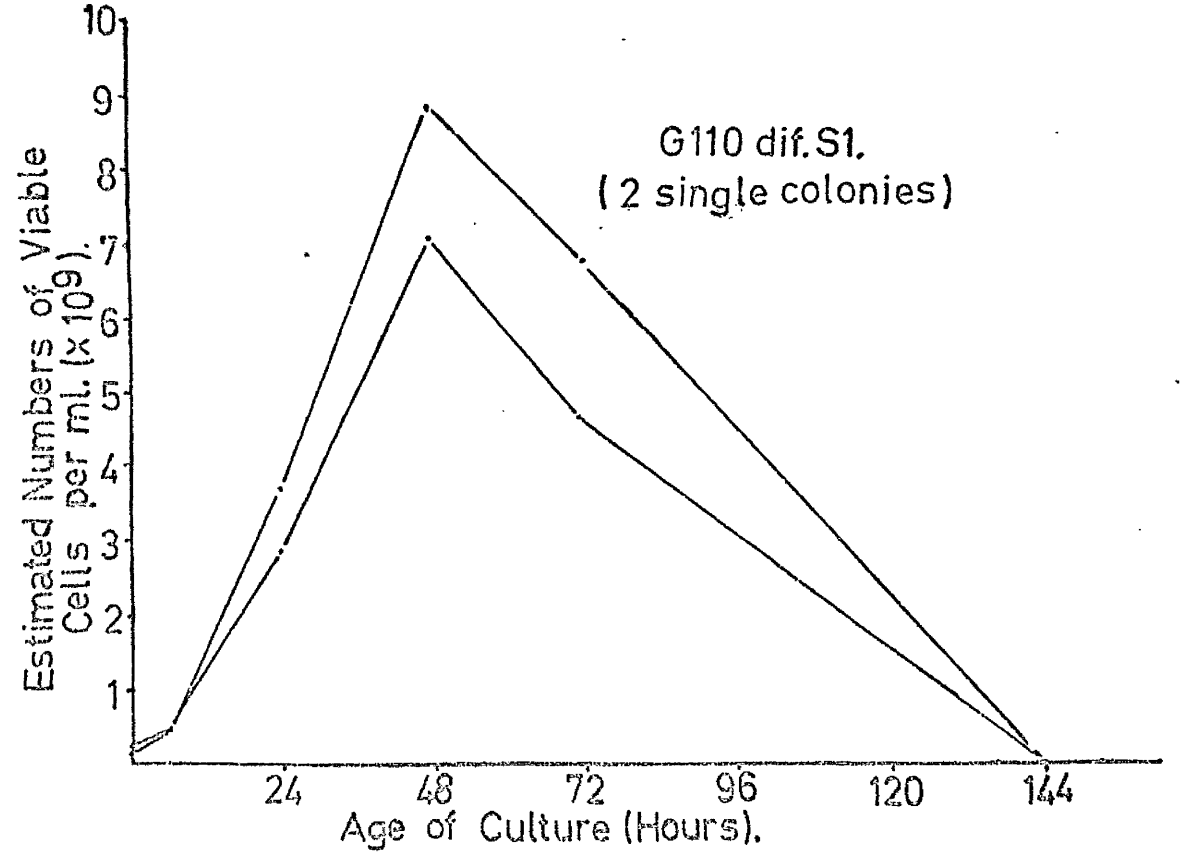


Table 10. Activities of P.G.T.E. in pectin (B) and sodium polypectate (A) media

Age of Culture	Flask	G110 dif. (Wild type)		G110 dif. S1		G110 dif. S2		G110 dif. S10	
		A	B	A	B	A	B	A	B
24 hours	1	1.208	0.088	0.002	~0	0.016	~0	~0	~0
	2	1.271	0.068	0.002	~0	0.018	~0	~0	~0
48 hours	1	1.183	0.604	0.004	~0	0.252	0.044	0.226	0.002
	2	1.190	0.628	0.004	0.002	0.246	0.048	0.214	0.008
72 hours	1	1.155	1.519	0.007	~0	0.242	0.096	0.214	0.006
	2	1.173	1.666	0.005	~0	0.220	0.096	0.218	0.005

Also given in Figures 14, 15, 16, 17, and 18 are the results for an experiment with the wild type and the two mutants G110 dif. S1 and G110 dif. S2. In this experiment a complete set of viability counts for the cultures was obtained by plating on nutrient agar. Two different single colony isolates of G110 dif. S1 were used, hence the two sets of results for this mutant.

The results clearly show the much higher levels of P.G.T.E. activity produced in the culture supernatants of the wild type compared to the mutants. There is also a marked tendency for the isolates not to produce the maximum levels of P.G.T.E. activity till a considerably later stage in incubation.

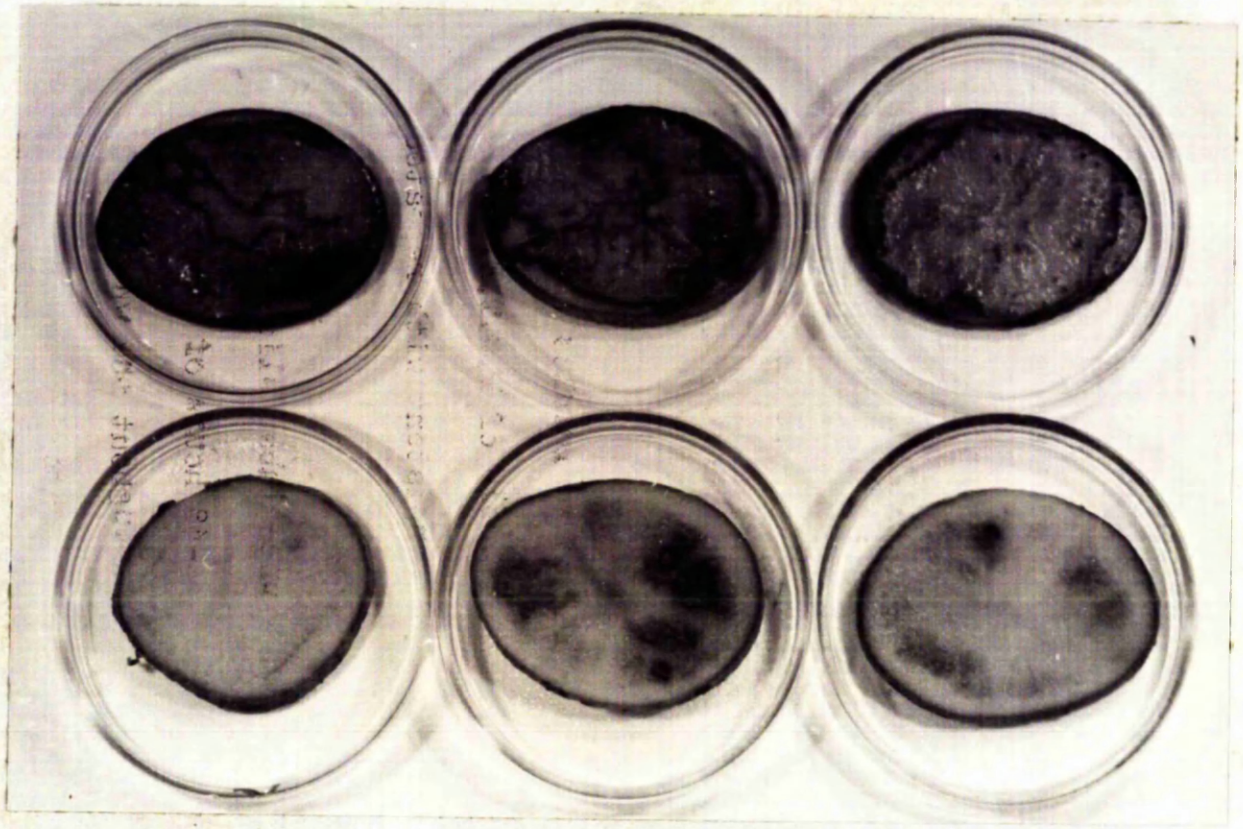
The wild type shows good correlation between growth of the organism and production of the enzyme as do G110 dif. S2 and G110 dif. S10 where growth tends to occur at a later stage, than in wild type cultures. There

Plate 3. Appearance of infected potato tuber slices

Isolates

G110 dif.

G110 dif. S1



is generally no further increase in P.G.T.E. activity after growth of the organism levels off; in fact small drops in activity often occur after several days incubation. No such correlation can be found for G110 dif. S1 which appears to grow at much the same rate as wild type, but does not produce the enzyme in any appreciable quantities till the viability of the culture has fallen below 10^6 cells/ml.

Pectin is a poorer inducer of P.G.T.E. activity over the first 48 hrs. of growth, but in the wild type at least, the level of the enzyme activity in pectin medium cultures reaches that in the sodium polypectate cultures after about 72 hours.

It is clear from these results that the mutants are very different from wild type in the levels of P.G.T.E. activity that they produce in vitro. These differences are consistent with repeated testing.

Virulence

When inoculated onto tuber slices, G110 dif. (wild type) gave a brownish black rot within 24-48 hours of inoculation. This rot, though extensive, did not spread throughout the whole of the tuber slice. In many cases, the cortical area outside the vascular bundle as well as a small area in the region of pith, did not rot. A brown necrotic zone often developed around the rotted areas and appeared to limit further spread of the rot.

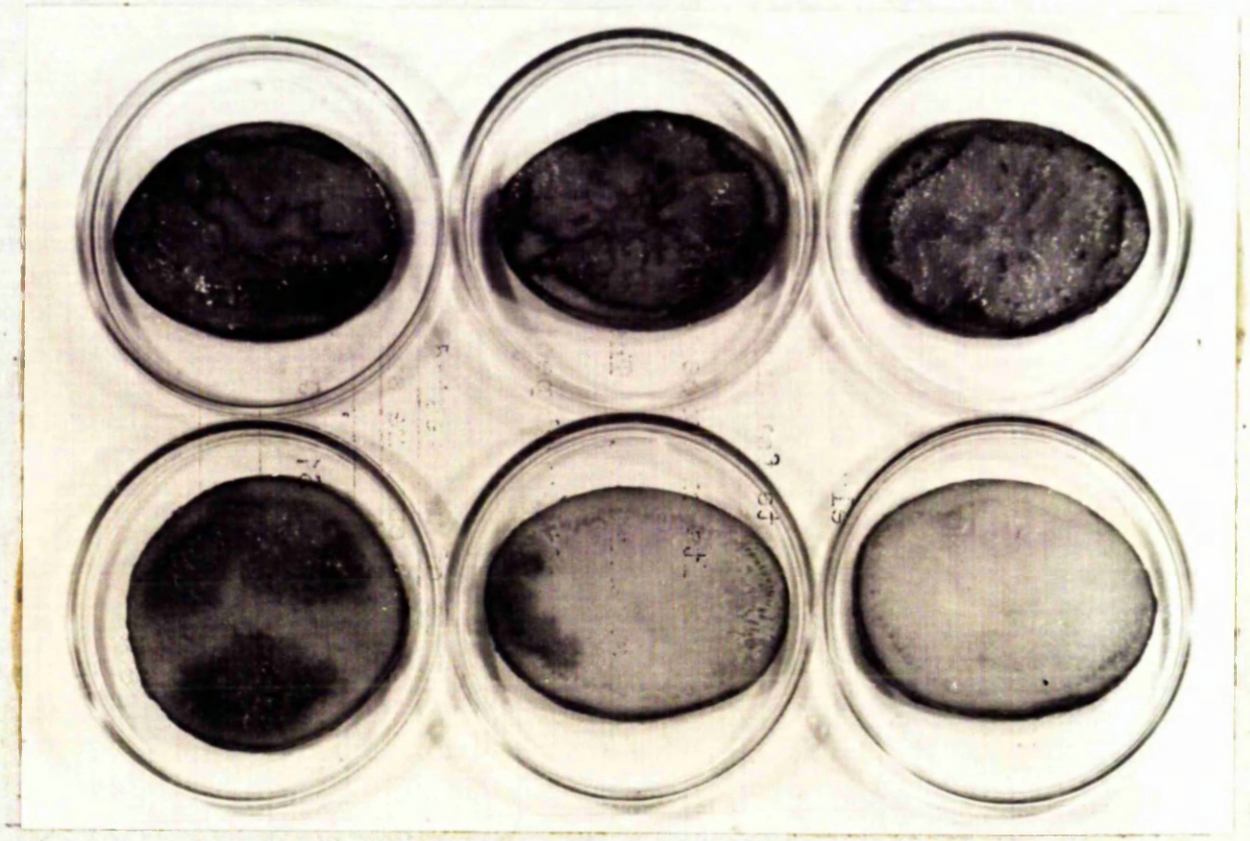
During repeated tests, the two mutants G110 dif. S1 and G110 dif. S2 never produced extensive rots on potato slices. In a few cases, very small lesions developed, but these did not appear to spread and usually dried up within a few days. It would appear that these isolates were unable to produce lesions comparable to G110 dif. and have, in fact, reduced virulence as judged by this test.

Plate 4. Appearance of infected potato tuber slices

Isolates

G110 dif.

G110 dif. S2



In one comprehensive test with mutant G110 dif. S10, similar results were found.

Maceration Activity

Twenty-four hour old sodium polypectate and pectin media culture supernatants having the P.G.T.E. levels shown in Table 10 were tested for the ability to macerate tuber tissue using Brown's method. The supernatants were also tested for the presence of undigested higher molecular weight pectic substances, as it was felt that the presence of such material may provide an alternative substrate for any pectic enzymes and so adversely affect maceration rates. Its presence was determined by adding absolute alcohol to the culture supernatant. The precipitation of gelled material indicated the presence of undigested pectic substances as shown in Table 11.

Table 11. Presence of undigested pectic materials in culture supernatants

G110 (Wild type)		G110 dif. S1		G110 dif. S2		G110 dif. S10	
A	B	A	B	A	B	A	B
-	+	-	+	+	+	+	+

The degree of maceration after 40 minutes and 2 hours are given in Tables 12a and 12b respectively.

Table 12a. Maceration of potato tissue after 40 minutes by culture supernatants

G110 (Wild type)		G110 dif. S1		G110 dif. S2		G110 dif. S10	
A	B	A	B	A	B	A	B
++	-	+	-	+	-	-	-

Table 12b. Maceration of potato tissue after 2 hours by culture supernatants

G110 (Wild type)		G110 dif. S1		G110 dif. S2		G110 dif. S10	
A	B	A	B	A	B	A	B
++	+	++	+	++	+	+ −	+ −

It is clearly seen that the culture supernatant of the wild type macerates faster than the mutants. However, the mutants culture supernatants do macerate even when little P.G.T.E. activity is detectable.

Maceration is measured after a much longer period of incubation than is used for the estimation of P.G.T.E. activity. Thus more maceration may be recorded than one would expect from the levels of P.G.T.E. activity, if maceration was the result of the activity of P.G.T.E. It must be admitted, however, that no direct evidence has been found to indicate that maceration is definitely caused by the P.G.T.E.

In those cases where undigested material is present there appears to be a reduction in the ability to cause maceration, even where the levels of P.G.T.E. are higher than in solutions where there is no undigested material. This result has been found on several occasions, i.e. the presence of undigested material is associated with slower rates of maceration.

In general it can be said, however, that macerating ability correlates to some degree with the levels of P.G.T.E. in the culture supernatants.

The effect of culture supernatants on whole tuber slices

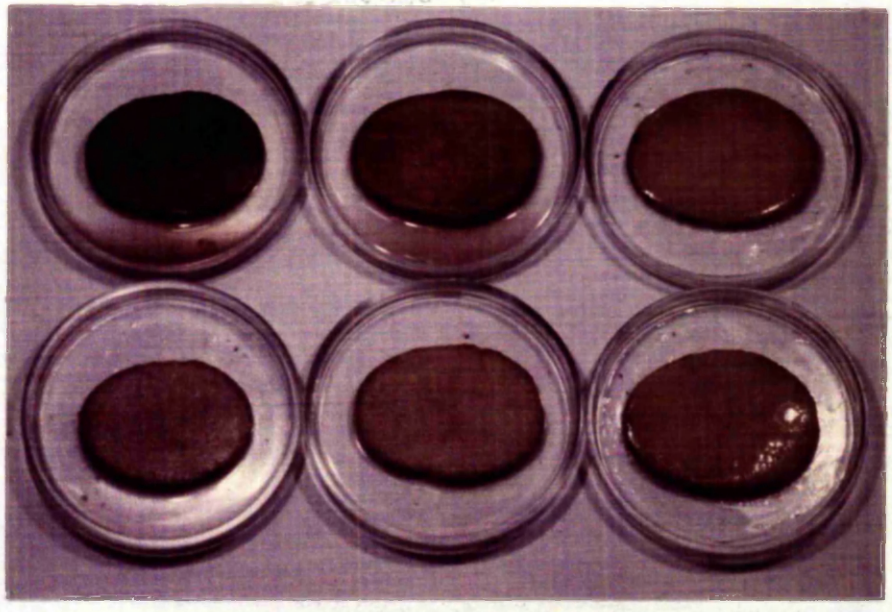
Culture supernatants of the wild type and the mutant G110 dif. S1 were tested for the effect they had on whole tuber slices. Whole tuber slices

Plate 5. Potato tubes slices treated with sterile sodium polypectate culture supernatants.

Isolates

G110 cr.str.r. G110 dif. G110 dif. S1

Tests



Controls

were prepared as for the virulence tests. They were treated with 2.5 ml. of filter sterilised 24 hour old polypectate culture supernatants, by inoculating in 0.5 ml. portions at 30 minute intervals. Control slices were prepared by treating slices with supernatants autoclaved for 15 mins. at 15 lbs. pressure. 0.5 ml. portions of the supernatants were also inoculated into nutrient broth tubes and these were incubated for three days at 25°C to check for any bacterial growth. No such growth occurred, indicating that the culture supernatants were indeed sterile. After 24 hours incubation the tuber slices were examined for any changes. The results are illustrated in Plate 5.

The slices showed definite responses to the supernatants. The slices treated with the culture supernatant of the wild type were a brown colour and their surfaces were macerated since the tissue could be removed by lightly dragging a spatula across their surfaces. No colour changes had occurred with the culture filtrates of G110 dif. S1 and no maceration either. This last treatment was indistinguishable from the slices treated with autoclaved supernatants of both types.

After 24 hours some leakage of liquid had appeared to have occurred from all slices, including the controls. Since this fluid was similar in colour to the treated tuber slices, i.e. brown if the slices were brown or clear if they were unaffected, it was thought that measurement of the polyphenoloxidase in this liquid would be of interest. Polyphenoloxidase is responsible for the browning reaction of damaged tuber tissue. Increases in the activity of this enzyme in host tissue as well as darkening of host tissue have been recorded after treatment with pectic enzymes, Lovrekovich et al. (1967); Tomiyama and Stahmann (1964).

Levels of Polyphenoloxidase in free liquid from [illegible] tuber slices

The assay was carried out using 0.5 ml. of the free liquid diluted with 0.5 ml. of glass distilled water as the enzyme solution. The enzyme activity of the liquid in each dish was assayed and the results are given in Table 13.

Table 13. Absorbance at 520 mμ as measurement of polyphenoloxidase activity of free liquid in petri dishes

Filter Sterilised Culture Filtrates		Autoclaved Culture Filtrates	
G110 dif. (Wild type)	G110 dif. S1	G110 dif. (Wild type)	G110 dif. S1
3.24	1.37	0.25	0.18
3.02	1.10	0.16	0.35
2.66	0.95	0.11	0.15
2.32	0.78	0.22	0.15
2.20	0.78	0.04	0.07
2.82	0.95	0.09	0.08
2.14	0.56	0.11	0.10
2.40	0.58	0.11	0.11
2.30	0.68	0.10	0.10
2.78	0.54	0.13	0.11

P.G.T.E. activities in culture supernatants (absorbance at 235 mμ)

G110 dif. (wild type) 0.441 units

G110 dif. S1 0.002 units

It is clear that the wild type culture supernatant treated slices

have much higher levels of polyphenoloxidase present in the free liquid than those treated with culture supernatant of G110 dif. S1 or with autoclaved culture supernatants. If this measure of polyphenoloxidase can be used as a crude estimate of damage then this would suggest much more damage caused by the culture supernatants of wild type than by the mutant, although some does appear to occur with the latter isolate in comparison with the control treatments. The control treatments suggest that whatever is causing the effect in culture supernatants it is destroyed by heating. This would indicate that the substance was probably a protein and since the supernatants were shown to be free from bacteria that it is an extracellular protein. Since the two isolates, as far as could be shown, differed physiologically solely with respect to the ability to produce P.G.T.E. then it would be reasonable to suppose that the protein responsible for this effect was probably this enzyme.

If studies with back mutations were carried out it would be expected that restoration of production of P.G.T.E. activity <u>in vitro</u> would be accompanied by an increase in the browning and polyphenoloxidase effects. In fact back mutation for P.G.T.E. production should also restore virulence to the pectic enzyme type mutants if it is this character which is responsible for their greatly reduced virulence. If any other character were responsible for virulence and the browning effect then back mutation for P.G.T.E. production would not restore the mutant's virulence or ability to cause browning to that of the parent wild type.

It is interesting to note in passing that the polypectate culture filtrates of another wild type isolate, G110 cr.str-r., with even higher P.G.T.E. levels than G110 dif. caused more discolouration of slices which

as a result were nearly black after 24 hours. In this case there were also higher levels of polyphenoloxidase in the free fluid from the tuber slices.

There are at least three possible explanations of these results.

(1) The culture supernatants induce enzyme formation.

(2) The culture supernatants activate an inactive enzyme.

(3) The culture supernatants damage cells causing the release of more enzyme.

Lovrekovich et al. (1967) report that polyphenoloxidase activity increases after treatment of potato tissue with 'pectinase'. Their study would suggest either of the first two possibilities. However, observation on treated tuber slices in this study suggested that the last possibility mentioned may also occur. This is supported by the observation that the browning only occurs in the areas macerated by the culture supernatant. If the macerated surface layers are removed the tissue underneath is still intact and not discoloured. It is possible that death or damage of the cells in the macerated area leads to a release of polyphenoloxidase from the cells with a consequent browning reaction. It is, of course, also possible that all three systems or combinations of them are in operation at the same time. At any rate there appears to be a good correlation between the levels of polyphenoloxidase present in the free liquid and the P.G.T.E. levels in the culture supernatants.

Summary

The following points are worthy of note. Isolates differing from the wild type in their abilities to liquefy pectate gels are unable to cause the same rot symptoms as wild type. They are also different from wild type in the levels of P.G.T.E. they produce in culture. Also, different culture

supernatants containing different quantities of P.G.T.E. macerate potato tissue at rates corresponding approximately to the levels of P.G.T.E. activity they contain. In addition, there exists a correlation between the browning and release of polyphenoloxidase after treatment with culture supernatants and the level of P.G.T.E. in those culture supernatants.

All this, in conjunction with the in vitro and in vivo studies, would suggest that P.G.T.E. plays an important role in pathogenesis by this organism.

CHAPTER III - STUDIES ON COLONIAL MORPHOLOGY

Section I - Introduction

There are reports of a reduction in virulence associated with changes in morphology for a number of bacterial animal pathogens. For example, the change from smooth to rough associated with a reduction in virulence for Salmonella spp., Stocker (1959). These changes are thought to involve changes in antigen and capsular structures and often result in the bacteria having much reduced virulence or even being avirulent.

Goodman et al (1962) have reported a change from an avirulent rough colony type of Erwinia amylovora to a virulent smooth form when the rough isolate was treated with aphid extracts. This situation may exist among other plant pathogens where there have been reports of similar types of changes in colonial morphology, for example Goto and Okabe (1957a) have reported a mucoid mutation in Erwinia carotovora.

The precise changes in such mutants are not at all clear. They are not directly associated with the nutritional status of the organism since most variants of this type appear to grow readily on a minimal medium.

In the case of animal pathogens, antigenic and capsular structures are changed along with the alterations from the smooth to the rough type, Roantree (1967). The situation has not been as widely investigated for plant pathogenic bacteria but it is interesting to note that Goto and Okabe (1957b) have demonstrated some correlation between the virulence and the serotype of Erwinia carotovora.

In preliminary investigations in this bacterial species it was noticed

Plate 6. Appearance of creamy isolates on glucose minimal medium.

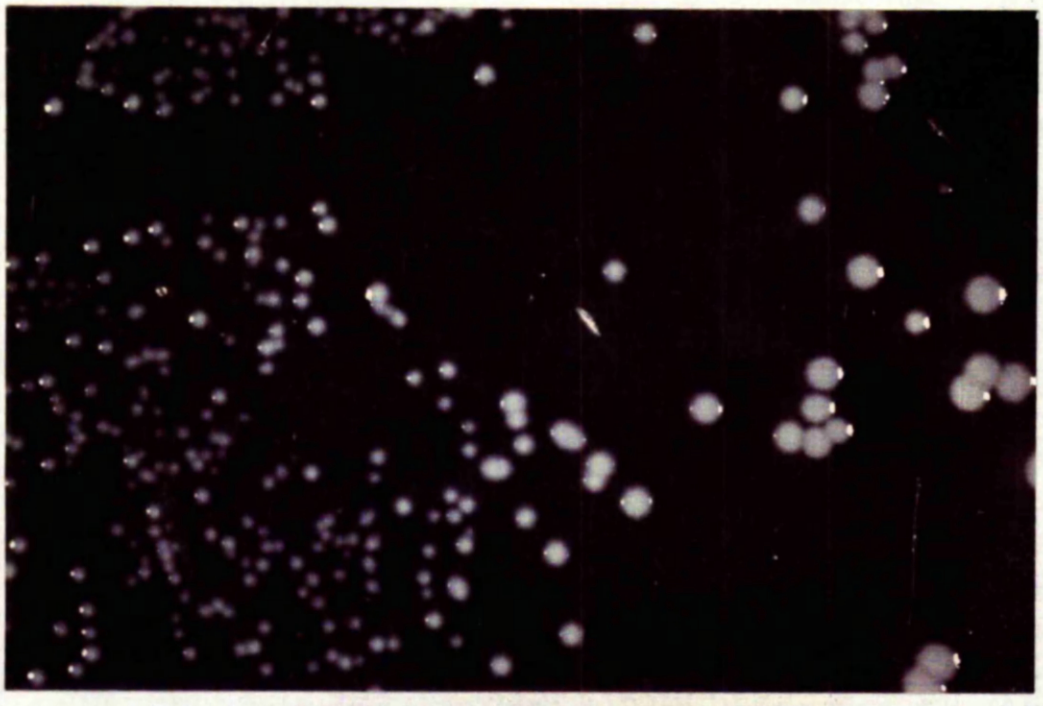
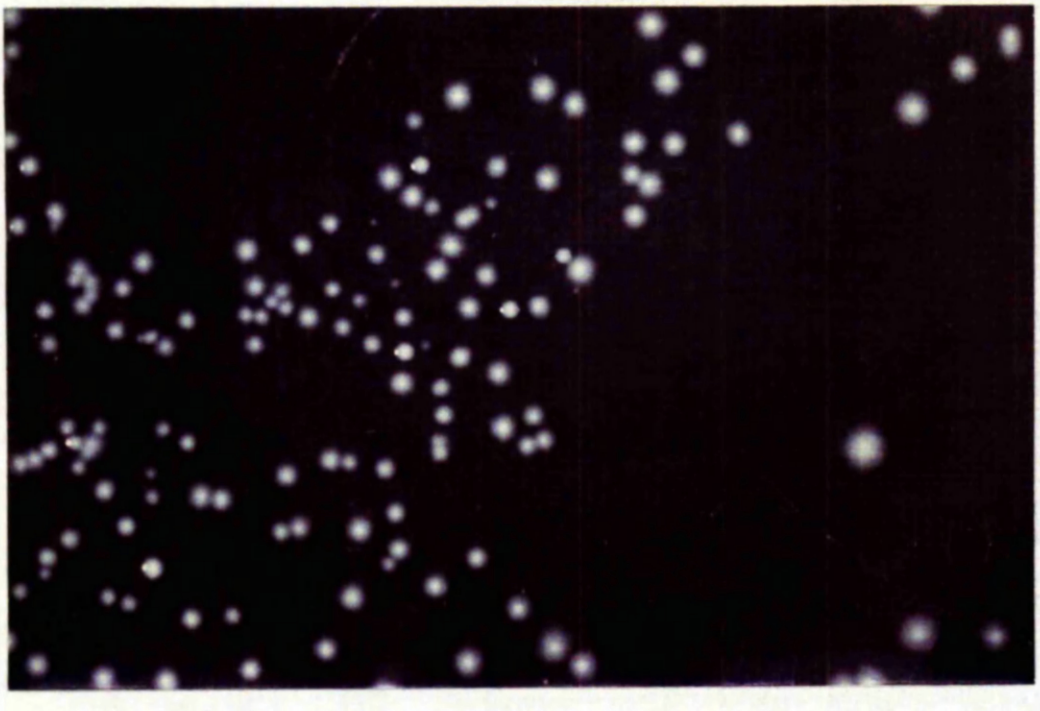


Plate 7. Appearance of diffuse isolates on glucose minimal medium.



that variation in colonial morphology was apparently associated with the type of soft-rot produced. It was therefore decided to investigate the effect of colonial morphology on virulence more completely.

Section II - Methods and Results

Isolate G110 was used in this study. In specific instances a streptomycin resistant isolate G110 cr.str-r. was used.

The isolation of morphologically distinct strains

Different colony morphologies were first observed on plating suspensions of G110 onto glucose MM agar. Since these different types were found in two separate G110 cultures which had been received at different times, it was decided to look further at these isolates. Close examination of the plates revealed two distinct forms of colonial morphology.

(i) Creamy morphology, labelled G110 cr. Isolates of this type have a rather smooth, creamy appearance on glucose MM agar. The edges of the colonies are clearly defined (Plate 6).

(ii) Diffuse morphology, labelled G110 dif. Colonies of this isolate generally tend to be smaller on glucose MM agar. They are less dense than the creamy isolates, being whitish-grey with a rather rough appearance. The edges of the colonies are rather fuzzy or diffuse (Plate 7).

It was decided to make a detailed comparison of the virulence and physiological characters of these two types. Three single colony isolates of each type were isolated from MM agar and used in further studies.

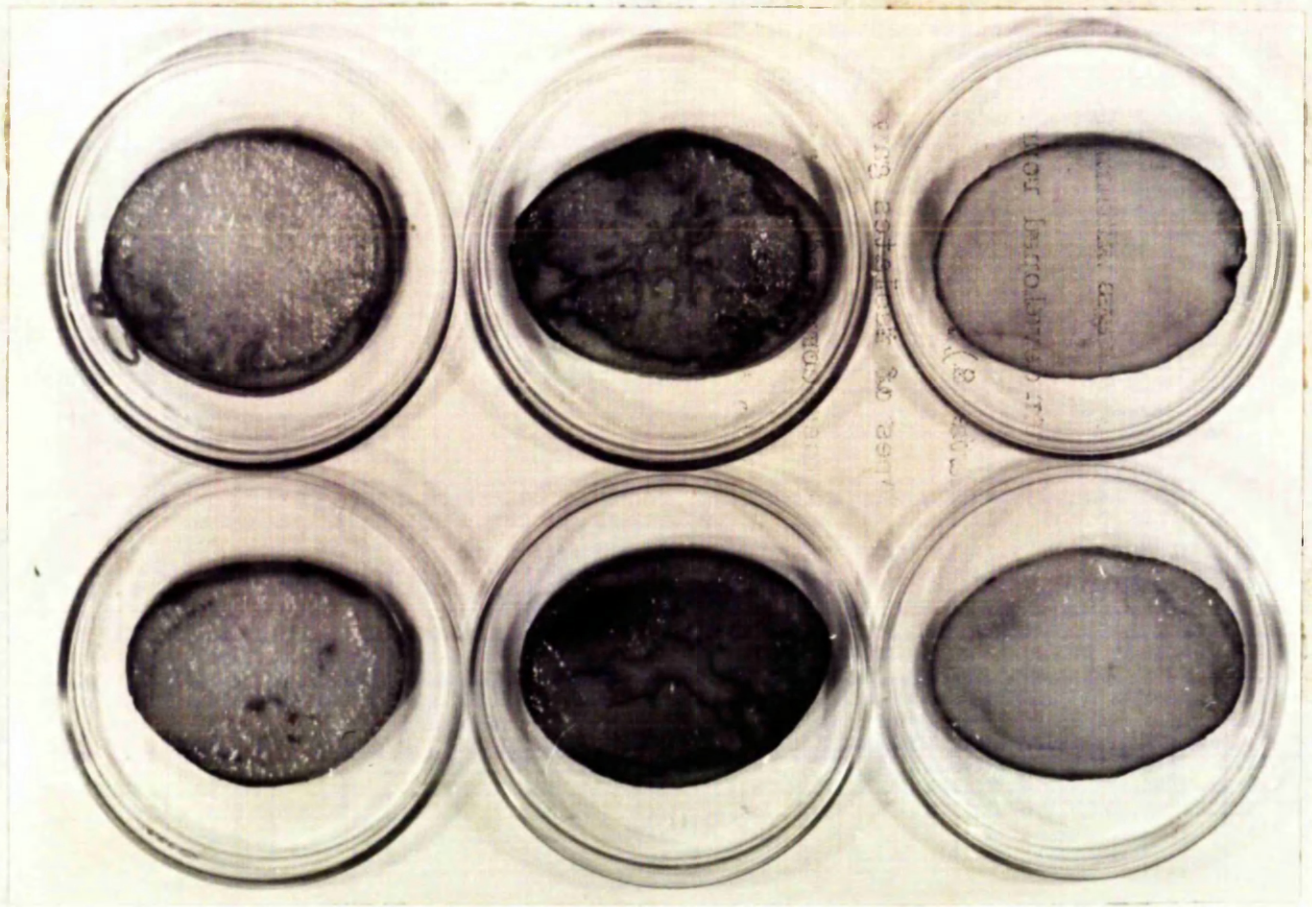
Comparison of virulence

The G110 cr. isolates completely rotted the tuber slices within forty-

Plate 8. Appearance of infected tuber slices after 48 hours.

Isolates

G110 cr.str-r. G110 dif. Controls



eight hours of inoculation. The rots were rather creamy in appearance, tending to remain this colour for several days before starting to turn brown (Plate 8).

The G110 dif. isolates often gave incomplete rots. In particular, the cortical tissue outside the vascular bundle, and tissue in a position corresponding to the pith, often remained intact. The rots turned brown or brownish-black nearly always within forty-eight hours of inoculation. Dark necrotic zones often developed round the lesions, appearing to limit further expansion (Plate 8).

Since the two types of isolates gave different symptoms on potato slices, they were further compared for any other physiological differences which could be related to their different patterns of virulence.

Motility

The G110 cr. isolates, at least in young cultures not more than four or five days old, were always motile in nutrient broth, sodium polypectate and pectin medium cultures. Motility was observed in the first 24-48 hrs. of growth in glucose MM but seemed to be lost rapidly after this. G110 dif. isolates never showed motility at any stage in any of the above media.

Cultures were therefore examined using the electron microscope to determine whether differences in motility and colonial morphology could be related to differences in cell structure.

The E.M. examination was kindly performed by Mr. D. Hunsley. Twenty-four hour old sodium polypectate cultures of G110 cr. str-r. and G110 dif. were spun down and re-suspended in distilled water. The suspensions were dropped onto copper grids which had been covered previously with FORMVAR and carbon. After drying, the grids were shadowed with Palladium-gold at

Plate 9. Morphology of isolate G110 cr.str-r. as revealed by Electron Microscope (X20,000)

Plate 10. Morphology of isolate G110 dif. as revealed by Electron Microscope (X50,000)

an angle of 22°. The prepared material was examined in an A.E.I. Electron Microscope 6B using an accelerating voltage of 60K volts. The different cell types are given in plates 9 and 10.

G110 cr. str-r. was peritrichously flagellate having from one to four flagella. This agrees with the description given in Bergey's Manual of Determinative Bacteriology (1957). Further, the size of the cells, 0.5-0.7 μ in width by 1-2 μ in length agreed with the data on cell size.

The diffuse isolate, while similar as regards cell size, did not appear to be flagellated. This observation naturally fits in with the observation made about motility, namely that the diffuse isolates are non-motile. In addition, it appeared that in both strains, small cap-like structures may be present at the ends of the cells. Thus the two isolates appeared to be similar in structure except in the possession of flagella.

Gelatine liquefaction

When inoculated into nutrient gelatine tubes, it was found that the three isolates of G110 cr. gave marked liquefaction within 3-4 days, while G110 dif. isolates produced no liquefaction in this time. After incubation of G110 dif. for at least one week a small amount of liquefaction occurred, in some cases, at the surface round the point of inoculation.

Thus the two isolates differed markedly in their abilities to liquefy gelatine. This is interesting since in his study on Erwinia carotovora Friedman (1962) found a similar difference between the virulent wild type and the partially virulent mutant isolate, the latter being less able to produce proteolytic enzymes as judged by gelatine liquefaction and milk clotting.

Table 15. P.G.T.E. activities in sodium polypectate (A) and pectin (B) media culture supernatants

Age of Cultures	Creamy Isolates						Diffuse Isolates					
	1		2		3		1		2		3	
	A	B	A	B	A	B	A	B	A	B	A	B
24 hrs	3.205	1.120	2.267	1.001	2.177	1.211	0.739	0.200	0.711	~0	0.748	0.236
	-	-	2.467	0.959	2.191	1.211	0.648	0.110	0.721	~0	0.770	0.172
48 hrs	2.248	1.288	1.813	1.078	1.879	1.057	0.662	0.230	0.669	0.006	0.732	0.170
	2.678	0.589	1.785	1.064	1.698	1.239	0.627	0.134	0.658	~0	0.725	0.308
72 hrs	1.841	0.966	1.435	1.491	1.568	1.365	0.644	0.208	0.739	0.128	0.756	0.190
	1.813	0.882	1.554	1.442	1.414	0.846	0.609	0.122	0.693	0.112	0.784	0.264

Production of P.G.T.E. in vitro

The three isolates of each type were grown in liquid sodium polypectate and pectin media, and the levels of P.G.T.E. in the culture supernatants determined at various times after inoculation. The results are given in Tables 14 and 15.

Table 14. Approximate cell numbers ($X10^9$) per ml. of sodium polypectate (A) and pectin (B) media cultures

Age of Cultures	Creamy Isolates						Diffuse Isolates					
	1		2		3		1		2		3	
	A	B	A	B	A	B	A	B	A	B	A	B
24 hrs	5.4	4.1	5.1	4.1	5.2	4.0	4.8	4.4	4.5	3.1	4.9	3.8
	5.7	3.2	5.4	3.9	5.2	4.2	4.8	4.0	4.6	3.0	5.3	4.1
48 hrs	5.2	6.7	4.9	6.7	5.2	6.7	4.9	7.1	5.5	5.3	5.3	6.7
	5.2	5.1	5.0	6.9	5.1	6.6	5.2	6.2	5.3	5.4	5.4	7.2
72 hrs	2.7	8.4	3.2	8.5	2.6	6.0	3.2	6.5	2.7	7.5	2.4	6.2
	2.4	8.8	3.4	7.7	2.8	8.4	3.0	6.8	2.2	8.3	2.2	7.4

It is clear from this study that there is a considerable difference in amounts of P.G.T.E. produced by the two morphological types in culture. There is little difference in the levels of P.G.T.E. between single colony isolates of each type except perhaps for G110 cr. isolate 1 which appeared to produce slightly more P.G.T.E. than the other two isolates of this type. It is clear, however, that the levels of activity of G110 cr. isolates is about 2-3 times that of G110 dif. isolates.

Plate 11. Appearance of isolates G110 cr.str-r. and G110 dif. on glucose TTC agar.

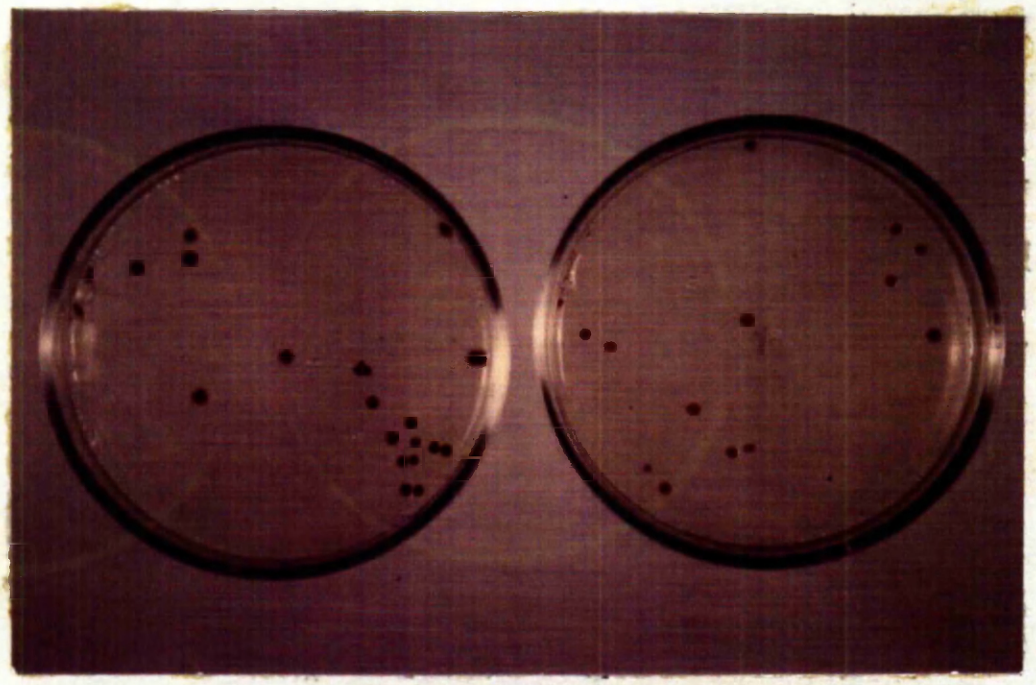
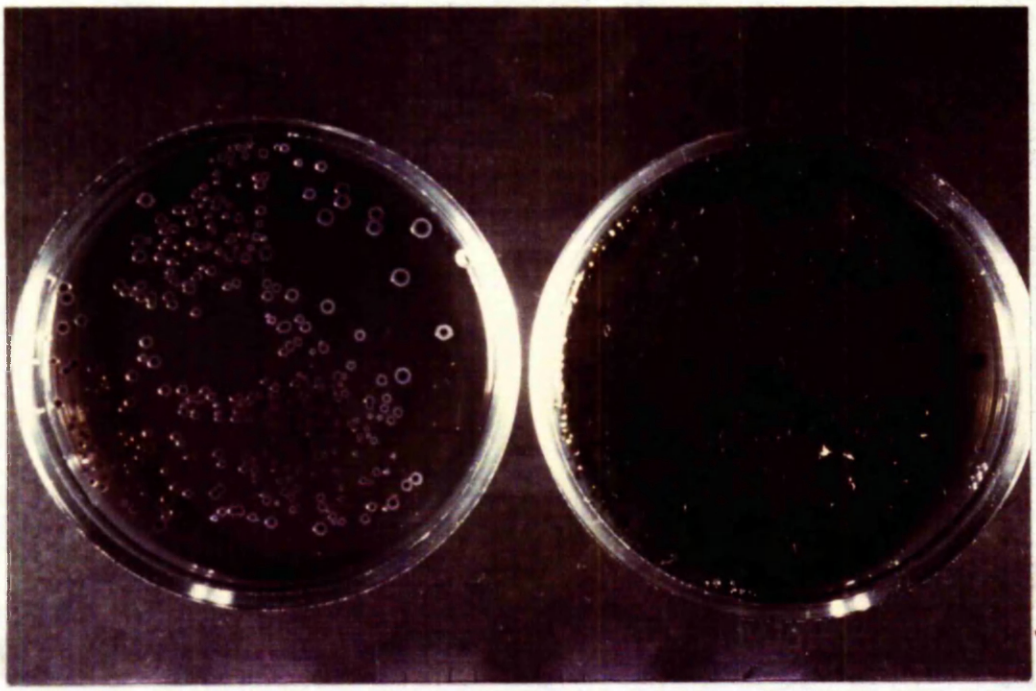


Plate 12. Appearance of isolates G110 cr.str-r. and G110 dif. on glucose EMB agar.



Colony appearance on various solid media

The isolates were grown on the various solid media given in Table 8. The appearance of the colonies on these plates was determined after 4-5 days growth.

Differences occurred between the two types on all glucose containing media. On glucose TTC agar the diffuse isolates always gave colonies with large white borders and pink centres, while the creamy isolate colonies had wider, deep red centres (Plate 11). On glucose EMB agar the diffuse colonies had a greenish black sheen, while the creamy isolates were bluish-pink with no sheen (Plate 12). The appearance on glucose MM was as described on page 62.

The results appeared to be variable on fructose media. In particular, the creamy isolates gave variable results on fructose MM with, in some cases, marked inhibition of growth. However, on those plates where growth did occur creamy isolates always gave rise to creamy colonies and diffuse isolates gave rise to diffuse colonies. The morphological differences between the two types were, however, not as clearly defined on this medium as they were on the glucose MM.

There were slight differences on fructose EMB agar where the greenish-black sheen of the creamy isolates was less marked than that of the diffuse isolates. No differences were found on fructose TTC agar, where the colonies of both isolates were deep red in colour.

Thus the differences apparent on the glucose media were much less marked when the isolates were grown on the fructose containing media.

In contrast no morphological differentiation occurred when creamy and diffuse isolates were grown on galactose and lactose containing media (including MacConkey's agar), or on nutrient agar.

Acid and gas production

Acid and gas production from various carbohydrate sources were compared. The results are given in Table 16.

Table 16. Acid and gas production from various carbohydrate sources

Carbon source	G110 dif.		G110 cr.str-r.	
	Acid	Gas	Acid	Gas
Arabinose	++	+ -	++	+
Dulcitol	-	-	-	-
Ethanol	-	-	-	-
Fructose	++	+	++	+
Galactose	+	-	+	-
Glucose	++	-	++	+
Glycerol	d	-	d	-
Lactose	++	+	++	+
Maltose	d	-	d	-
Mannose	++	+	++	+
Raffinose	++	+	++	+
Salicin	++	+	++	+
Sucrose	++	+	++	+
Tartrate	-	-	-	-
Xylose	+	+	+	+

The only difference appeared to be in gas production from glucose, but this difference was only slight and did not occur in all tests.

The two types of isolate differed in the characteristics given in Table 17.

Table 17. Comparative list of characters of creamy and diffuse isolates

Character	Creamy Isolates	Diffuse Isolates
Appearance on Glucose MM agar	Creamy colony	Duffuse edged colony
Glucose EMB agar	Bluish pink colonies	Greenish black colonies
Glucose TTC agar	Deep red colonies	Pink colonies
Frucoste media	Similar to above but less marked	
Galactose media	No differences	
Lactose media (including MacConkey's agar)	No differences	
Nutrient agar	No differences	
Virulence	Creamy extensive rots	Brown limited rots
Motility	Very motile	Non-motile
Flagella	1 - 4	None
Gelatine liquefaction	Rapid	Very slow
P.G.T.E. activity in vitro	2-3 units	0.5-1.0 units

In view of these differences it was decided to attempt to isolate diffuse colony types from a creamy isolate, to determine whether the change in colonial morphology was always associated with changes in the other physiological characters including virulence.

The isolation of morphological variants

G110 cr.str-r. was grown up on glucose MM, EMB and TTC agars on which

the distinction between the diffuse and creamy isolates was most marked. Two isolates were obtained from glucose EMB agar which had the greenish-black sheen characteristic of the diffuse isolates.

They were both shown to be streptomycin resistant indicating that they were derived from the parent isolate G110 cr.str-r. and were not chance contaminants.

These isolates were labelled G110 cr.str-r. gl.1 (hereafter labelled Mutant dif. 1) and G110 cr.str-r. gl.2 (hereafter labelled Mutant dif. 2). They were tested for all the characters in which the original diffuse and creamy isolates differed.

Virulence

Both these isolates consistently produced rots similar to those produced by all wild type diffuse isolates and not like that of the parent G110 cr.str-r. isolate.

Motility

Both isolates were motile in a variety of media and were thus similar to the parent isolate. Therefore the two mutants differed from the wild type diffuse isolate with respect to this character.

Gelatine liquefaction

Both isolates liquefied gelatine. However they liquefied the gelatine to a uniform depth over the whole surface and did not produced the funnel shaped liquefaction characteristic of the parent isolate. Thus, although different from both creamy and diffuse wild types, the speed of gelatine liquefaction was approximately that of the creamy wild type.

Colony appearance on various solid media

Growth of the mutants and the wild types were compared on the range of solid media listed in Table 3.

Both mutants were identical with wild type dif. isolates on all glucose containing media including glucose MM. Growth on this medium indicated that they both had wild type nutrition.

On media containing fructose the mutants appeared very similar in appearance to all wild type diffuse isolates, although there was again some variation in growth especially on the fructose MM.

Mutant dif. 2 was similar to the diffuse isolates on all other media. However Mutant dif. 1 although similar to all other isolates on nutrient agar was abnormal on the galactose and lactose containing media.

Only very small colonies grew on the galactose MM and none at all on lactose MM. On all other galactose and lactose media only colonies with a very rough abnormal appearance grew. There was no greenish-black sheen produced on galactose or lactose EMB agars and little red colour on the corresponding TTC agars. It would therefore appear that this isolate had a reduced ability to utilise galactose and lactose as carbon sources.

Acid and gas production

The range of carbohydrates given in Table 7 was used. Mutant dif. 2 was identical with both wild types. Mutant dif. 1 produced acid at a much slower rate from galactose and lactose than any of the other isolates. This agrees with the growth characteristics of this isolate on media containing these sugars.

Production of P.G.T.E. in vitro

Three single colony isolates of each mutant were tested for P.G.T.E. production and compared with the wild types G110 cr.str-r. and G110 dif. The results are given in Tables 18 and 19.

Table 18. Approximate cell numbers ($X10^9$) per ml. of sodium polypectate medium cultures

Age of Cultures		G110 cr.str-r.	G110 dif.	Mutant dif. 1 isolates			Mutant dif. 2 isolates		
				1	2	3	1	2	3
24 hrs	1	3.3	3.8	0.08	0.04	0.08	2.8	2.6	2.8
	2	-	-	0.08	0.07	0.07	2.6	2.5	2.7
48 hrs	1	3.8	4.7	0.18	0.20	0.57	4.1	3.9	3.9
	2	-	-	0.20	0.26	0.97	4.0	3.8	3.9

Table 19. P.G.T.E. activities in sodium polypectate culture supernatants

Age of Cultures	G110 dif.	G110 cr.str-r.	Mutant dif. 1.			Mutant dif. 2		
			1	2	3	1	2	3
24 hrs	0.868	2.156	~0	~0	~0	0.511	0.420	0.644
			~0	~0	~0	0.483	0.406	0.581
48 hrs	0.777	1.843	~0	0.016	0.044	0.533	0.553	0.581
			0.016	0.020	0.068	0.483	0.385	0.553

The supernatants from two flasks of each of the isolates of each mutant were tested.

It is clear that Mutant dif. 2 produced P.G.T.E. levels only slightly less than those of G110 dif. but about one third of that of the wild type creamy from which it was derived.

On the other hand, Mutant dif. 1 produced very little activity in culture and also grew slowly in the polypectate medium.

This observation is extremely interesting because this isolate readily produces soft rot symptoms on potato tuber slices, and also liquefies yeast extract pectate gels in a similar fashion to the parent creamy wild type. Since this liquefaction is apparently due to the production of P.G.T.E. it suggested that adding yeast extract to liquid sodium polypectate medium would enhance the production of enzyme by this isolate. It was therefore decided to test the effect of adding yeast extract on the growth and P.G.T.E. production in liquid sodium polypectate cultures of Mutant dif. 1.

Effect of the addition of yeast extract to sodium polypectate medium on the growth of Mutant dif. 1.

Five ml. of 2.0% w/v autoclaved yeast extract were added to 50 ml. of the sodium polypectate medium to give a final concentration of approximately 0.20% w/v. Other flasks with 5 ml. of sterilised glass distilled water instead of yeast extract were prepared in a similar manner. All the flasks were then inoculated with the same volume of the same inoculum. They were tested for P.G.T.E. activity after 24 hours and 48 hours of growth. The results are shown in Tables 20 and 21.

Table 20. Approximate cell numbers ($X10^9$) per ml. of sodium polypectate cultures

Age of Cultures	Flask	Unsupplemented Cultures	Yeast Extract Supplemented Cultures
24 hrs	1	0.06	1.3
	2	0.07	1.5
48 hrs	1	0.68	3.1
	2	0.54	3.1

Table 21. P.G.T.E. activities in sodium polypectate medium culture supernatants

Age of Cultures	Flask	Unsupplemented Cultures	Yeast Extract Supplemented Cultures
24 hrs	1	~0	0.085
	2	~0	0.100
48 hrs	1	0.032	0.336
	2	0.040	0.306

Thus the addition of yeast extract to the sodium polypectate medium increases the growth of Mutant dif. 1 and also its production of endo-P.G.T.E.

Since this isolate can readily grow on glucose minimal medium it is unlikely that the yeast extract is acting as the source of some basic nutritional requirement such as an amino acid. More likely explanations

would appear to be that either the yeast extract is being used as an alternative carbon source to the sodium polypectate or it is in some way promoting the utilisation of the sodium polypectate as a carbon source.

As was reported in Chapter II, mutants producing very little endo-P.G.T.E. are able to grow as readily as the wild type on a sodium polypectate medium, for example G110 dif. S1. This suggests that the utilisation of sodium polypectate for growth is not directly associated with the production of endo-P.G.T.E. One may therefore consider the metabolism of pectic substances in two parts.

(1) Production of endo-P.G.T.E. which breaks down the pectic substances to oligosaccharides and which is believed to be responsible for tissue maceration in the host.

(2) The breakdown of pectic substances to smaller saccharide units which would then be utilised by the bacterium. This breakdown could be the result of cell bound exo-P.G.T.E. activity. Such enzymes have been reported to be produced by Erwinia aroideae, Okamota et al (1963; 1964), but have not been looked for in this study.

Thus G110 dif. S1 although it does not produce endo-P.G.T.E. does grown on sodium polypectate, suggesting that it does breakdown the polypectate, possibly through the action of cell bound exo-P.G.T.E.s and also utilises the saccharide units formed by the action of these enzymes. On the other hand it is possible that Mutant dif. 1 either does not produce these cell bound exo-P.G.T.E.s or cannot utilise their products.

It is interesting to note that Mutant dif. 1 does not appear to readily utilise galactose or lactose. Perhaps galacturonic acid together with the

Table 22. Comparative list of characters of creamy and mutant diffuse isolated

Character	Isolates		
	G110 cr.str-r.	Mutant dif. 2	Mutant dif. 1
Appearance on various solid media.			
1. Glucose M.	Creamy colonies	Diffuse edged colonies	Diffuse edged colonies
2. Glucose EMB.	Bluish-pink colonies	Greenish-black colonies	Greenish-black colonies
3. Glucose TTC.	Deep red colonies	Pink colonies	Pink colonies
4. Fructose media	Similar to above but less marked		
5. Galactose media	Similar to all other wild type isolates		Abnormal rough colonies
6. Lactose media	Similar to all other wild type isolates		Abnormal rough colonies
7. Nutrient agar	No differences		
Growth on media containing 100 p.p.m. streptomycin sulphate	Good	Good	Good
Motility	Very motile	Very motile	Very motile
Gelatine liquefaction	Rapid, funnel shaped	Rapid, to uniform depth	Rapid, to uniform depth
P.G.T.E. activity in unsupplemented sodium polypectate	2-3 units	0.4-0,6 units	~0

other breakdown products of sodium polypectate are metabolised through the same metabolic pathways as galactose and lactose. Thus any blockage in this pathway would obviously affect the utilisation of all these compounds and would explain the inability of Mutant dif. 1 to grow on unsupplemented sodium polypectate medium.

Whatever the cause of the inability of the mutant to grown on this medium, it is clear that the addition of yeast extract allows growth to occur and also results in an increase in endo-P.G.T.E. activity. In all studies of wild type isolates it has been found that the greatest increases in endo-P.G.T.E. activity are associated with rapid growth. The limiting factor then in the production of endo-P.G.T.E. by Mutant dif. 1 in unsupplemented sodium polypectate culture could be the slow growth rate of the isolate, due to the lack of a suitable carbon source, and as a result the inability to synthesize the enzyme.

Table 22 gives a comparison of the characters of the wild type creamy isolate and the two mutants derived from it.

It would appear that motility and gelatine liquefaction are not closely associated with virulence, since both mutants are similar to the original creamy wild type with regard to these characters.

No variations in acid and gas production on any medium, except in the case of Mutant dif. 1 on galactose and lactose containing media, could be found that could account for the differences in virulence.

It is possible that creamy morphology and the creamy type of rot are in some way associated with high levels of P.G.T.E. production since both mutant diffuse isolates and all wild type diffuse isolates produce much less enzyme than the creamy isolates. Changes in morphology may be due to

changes in cell wall properties, which could affect the synthesis and release of exo-cellular enzymes. If this is so then a single mutational event could bring about a change in colonial morphology and at the same time affect P.G.T.E. production.

In effect a good correlation exists between virulence and colonial morphology since even with Mutant dif. 1 where various physiological systems differ considerably from diffuse wild types, its degree of virulence is nevertheless still identical with that characteristic of the isolates.

Further support for this is provided by the fact that of two different mutants isolated from other creamy wild types, both had the degree of virulence characteristic of the wild type diffuse isolate. However, neither of these has been studied as extensively as have Mutants dif. 1 and dif. 2.

One of these mutants G110 cr.str-r. Gel 6 was isolated while studying motility and gelatine liquefaction (see Chapter IV). This isolate has been tested for its motility, its ability to liquefy geltatine, colony appearance on various solid media and acid and gas production, but not for P.G.T.E. production. It appears to be identical with Mutant dif. 1 for all these characters. The second, G110 cr.Av-14 was isolated during attempts to isolate an avirulent mutant from G110 cr. This isolate has only been tested for virulence and its colonial morphology on glucose MM agar. It is identical with G110 dif. for both these characters.

Thus all four diffuse mutants which have been isolated from parent creamy isolates cause soft-rots identical with those produced by all wild type diffuse isolates. This would cearly indicate that colonial morphology and the type of soft rot produced are related in some way.

Section III - Studies on dehydrogenases and acid production

It has been demonstrated that the diffuse and creamy isolates produce limited brownish-black and extensive creamy rots respectively on potato slices. The phenomenon was investigated further by studying those aspects of their physiology which might explain such differences.

While studying the infection of potato tissue by Erwinia carotovora Lovrekovich et al (1967) noticed that the bacteria were able to prevent the browning of potato sap, which normally occurs as the result of polyphenoloxidase activity. Furthermore, they also showed that this organism, when added to oxidised potato sap could reduce the coloured quinones present to colourless compounds, supporting the concept that the bacteria can reduce these compounds back to their original form as rapidly as they formed. They suggested that this effect was related to the high dehydrogenase activity of the bacteria, and concluded that a cell bound bacterial dehydrogenase requiring glucose for activity was responsible.

If this were so, then it would be reasonable to propose that differences in the colour of the two types of rot caused by creamy and diffuse isolates could be due to their having qualitatively or quantitatively different dehydrogenase systems. That this may be so is suggested by the observation that the colonies differ in appearance when grown on glucose TTC agar. Tetrazolium salts have commonly been used to demonstrate dehydrogenase activity in a wide range of organisms, Lederberg (1948), Jensen et al (1951), and so differences in appearance on this medium suggest differences in the dehydrogenase systems between the two isolates.

Other workers have used differences in appearance on media containing

tetrazolium salts to isolate strains of phytopathogenic bacteria differing in virulence from one another. Thus, Kelman (1954), was able to select out weakly virulent or non-virulent isolates of Pseudomonas solanacearum from a virulent parent stock culture, on the basis of their appearance on such a medium. Similarly, Smale and Worley (1956) have used a tetrazolium medium to isolate highly pathogenic isolates of Pseudomonas phaseolicola and Xanthamonas phaseoli from infected bean plants. They were also able to pick out weakly virulent isolates from stock cultures of Pseudomonas phaseolicola using the same medium but not from stock cultures of Xanthamonas phaseoli. A medium containing tetrazolium salts was also used by Friedman (1964) to distinguish between colonies which differed in their virulence. All these differences of all these isolates on the tetrazolium media could well be due to differences in their dehydrogenase systems.

Attempts were therefore made to investigate the role of the dehydrogenase systems of Erwinia atroseptica in its mechanism of virulence.

The rate of decolourisation of oxidised potato sap

The oxidised potato sap was prepared from healthy Pentland Dell tubers. These were thoroughly washed and peeled. The peeled tubers were washed again before macerating 100 gm. of the tissue with 300 ml. of distilled water in a Waring blender for two minutes. After filtering through Whatman No.1 filter paper using a Buchner funnel, the supernatant was spun in an M.S.E. refrigerated centrifuge at 4,000 r.p.m. for thirty minutes, to remove most of the cell debris.

The G110 cr.str-r. and Mutant dif. 2 were used to compare the abilities of the two types of isolate to decolourise potato sap. They were grown up in shaken nutrient broth cultures, harvested after 24 hours growth, centri-

Figure 19. Decolourisation of potato sap by Mutant dif. 2.

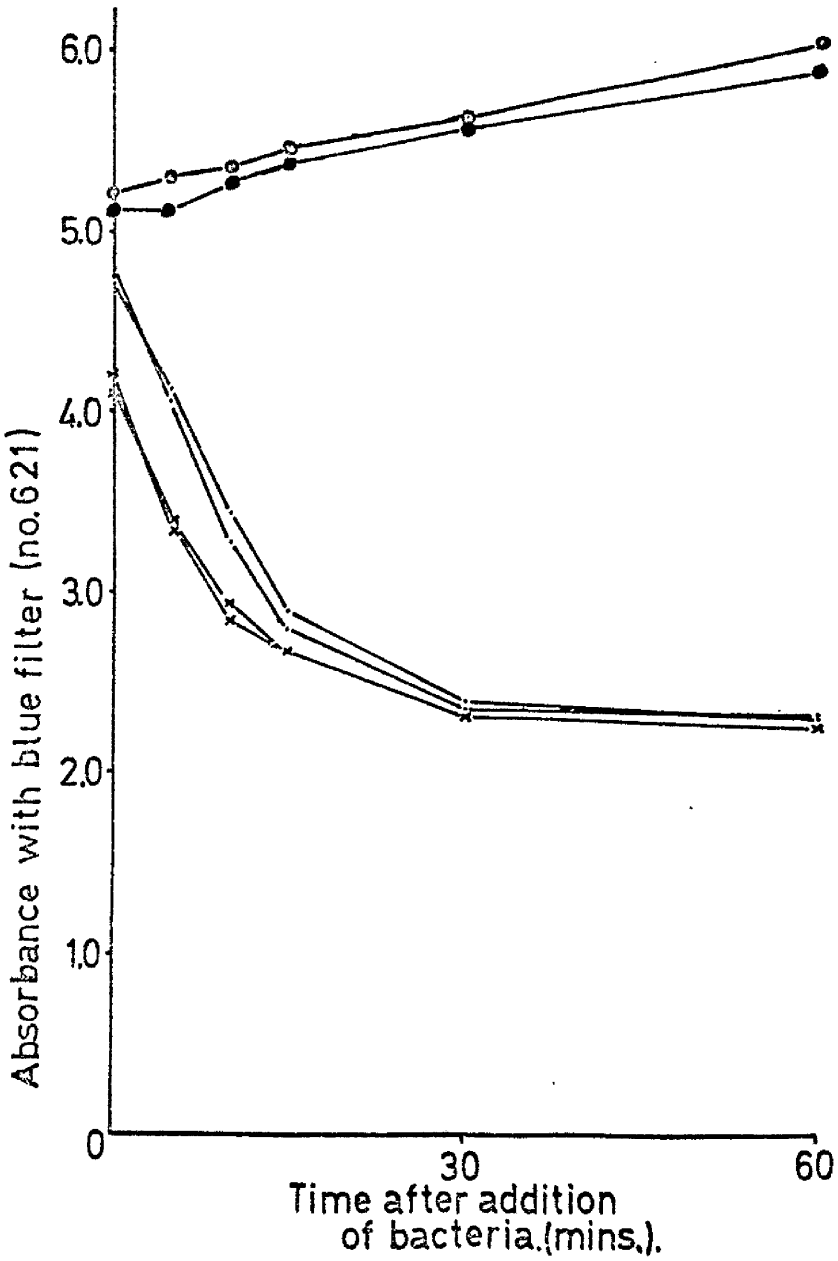
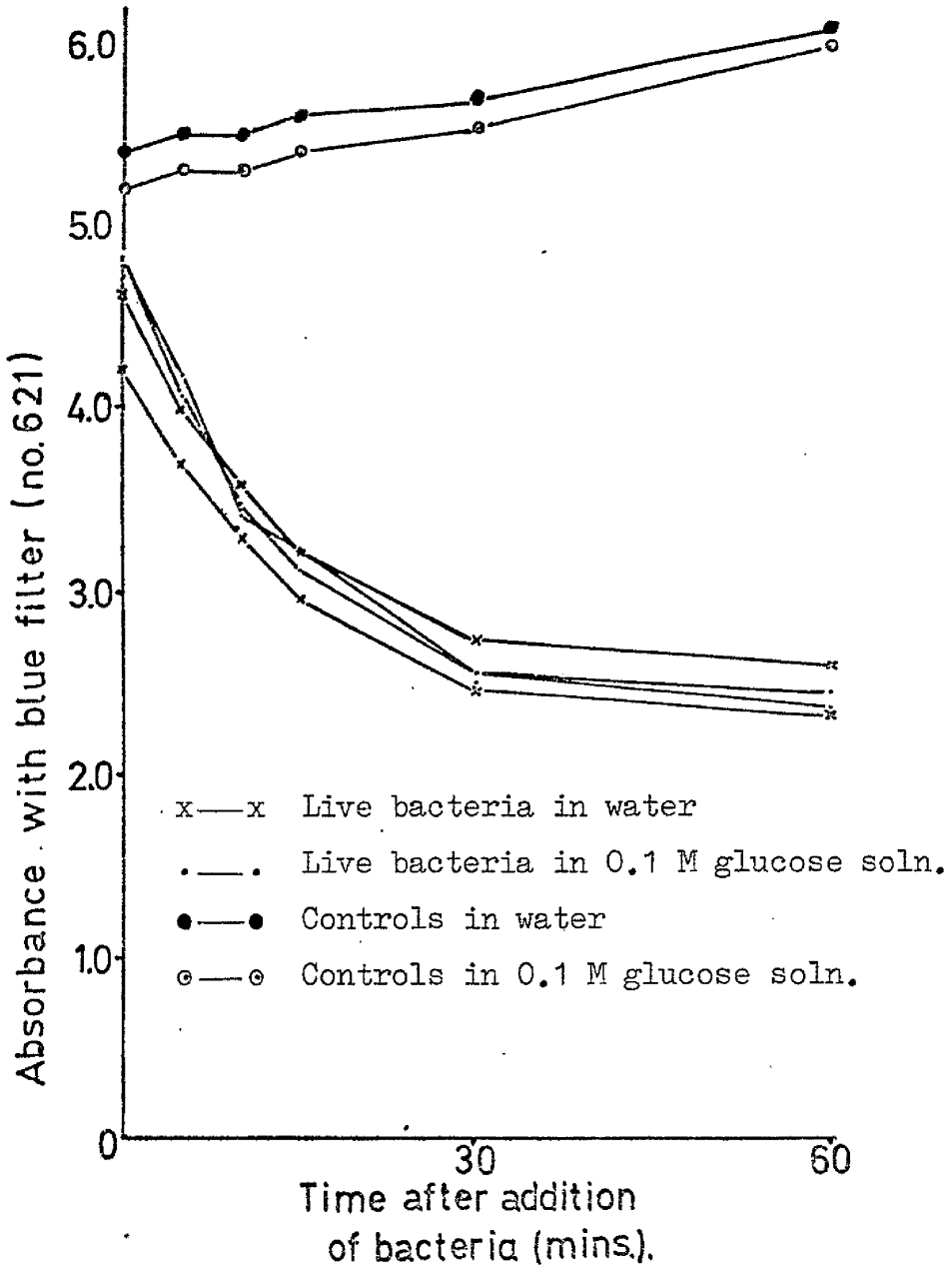


Figure 20. Decolourisation of potato sap by G110 cr.str-r.



fuged down and resuspended in water. After centrifuging again, half the sample was resuspended in water and the other half was suspended in a 0.8 M glucose solution, giving 4 x 10^9 cells per ml. in each case.

1 ml. of the bacterial inoculum was added to 7 ml. of the potato sap in glass colorimeter cells. After inoculation the final concentration of cells in all cases was about 5 x 10^8 cells/ml and in those tubes where the bacteria were added in glucose solution, the final concentration of the glucose was 0.1 M.

The absorbance of the solutions was measured in the EEL colorimeter, using blue filter No.621. Reference solutions consisted of the bacterial suspensions inoculated into water. Controls consisted of cells containing potato sap, inoculated with suspensions of the bacteria which had been heated in a boiling water bath for 15 minutes.

The results are given in Figures 19 and 20.

It is clear that both the diffuse and creamy isolates have a pronounced effect, for after one hour all tubes where the living bacteria had been added had been decolourised. The rate of decolourisation was similar for both isolates, and was also similar whether or not glucose was present in the reaction mixture. All the control tubes on the other hand became darker.

Thus the different types of rot produced by the two isolates could not be correlated with different abilities of the two isolates to decolourise potato sap <u>in vitro</u>. However, this experiment did not rule out the possibility of differences in the activities of different dehydrogenases and this aspect was then investigated.

Table 23. Dehydrogenase activities as estimated by formazan formation

Substrate	G110 cr.str-r.	G110 dif.	Mut. dif. 2	Controls
Ethanol	1	1	1	1
Fructose	3	3	3	1
Galactose	3	3	3	1
Glucose	3	3	3	1
Sodium glutamate	1	1	1	1
Glycerol	2	2	2	1
Sodium glycerophosphate	1	1	1	1
Glyoxyllic acid *	1	1	1	1
Sodium lactate	4	4	4	1
Lactose	1	1	1	1
Malic acid *	4	4	4	1
Sodium succinate	2	2	2	1
Water	1	1	1	1

* These substrates were adjusted to pH 7.5. with 1N NaOH before use.

1. Very slight red colour after 2 hours' incubation.

2. Red colour noticeable after 1 hour's incubation.

3. Deep red cclour noticeable only after 20 minutes or more incubation.

4. Very deep red colcur noticeable within 10 minutes of incubation.

Patterns of dehydrogenase activity

The two wild type isolates G110 cr.str-r. and G110 dif. and Mutant dif. 2 were grown up in shaken nutrient broth and tested for dehydrogenase activity in the presence of the substrates listed in Table 23.

The cultures were centrifuged down and then resuspended in phosphate buffer (0.1 M) at pH 7.5. This process was repeated and the O.Ds. of the cultures adjusted with buffer to give cell densities of about 4×10^9 cells/ml. The reaction mixtures were prepared by adding 1 ml. of the bacterial suspension to tubes containing the following.

0.1 M Phosphate buffer (pH 7.5)	4 ml.
0.4 M Substrate	2 ml.
0.4% w/v Triphenyl tetrazolium salts	1 ml.

Controls consisted of the above mixture inoculated with 1 ml. of a bacterial suspension which had been heated for 15 minutes in a boiling water bath.

The amount of formazan produced and thus the depth of colour of the reaction mixture gives a measure of dehydrogenase activity. The depth of red colour of the different reaction mixtures was judged by eye on a scale from 1 - 4 over a period of two hours after which even the controls had turned a slight red colour. The results are given in Table 23.

Sodium lactate appears to be the best substrate, followed by malate, then glucose, fructose and galactose, the last three being more or less the same. Succinate and glycerol appeared to be utilised to a small extent but the others were utilised little, if at all.

There appeared to be no obvious differences between the three isolates in their ability to dehydrogenate these substrates. This experiment has

Table 21. [illegible] of diameter of infected tuber slices

	G110 cr.str-r.	G110 dif.	Controls
1	6.56	6.14	5.82
2	6.78	6.10	5.74
3	6.26	5.98	5.70
4	6.82	5.84	5.90
5	6.40	6.04	5.72
6	6.46	6.08	5.78
7	6.30	6.04	5.78
8	6.52	5.88	5.92
9	6.42	5.70	5.86
10	6.52	6.00	5.82
Mean	6.50(6)	5.98(0)	5.80(4)

been repeated four times and the same result was obtained on each occasion.

Thus the differences in virulence between diffuse and creamy isolates cannot readily be explained by any differences in any of the dehydrogenase systems investigated *in vitro* so far. However, this investigation has covered only a minor part of the dehydrogenase activities of the bacterium, and whether or not dehydrogenases are involved in the differences between the two types of isolates awaits a more complete investigation.

For instance other work suggests that the optimum pHs of dehydrogenases *in vitro* is around 7.0 and above, Biochemists' Handbook (1961), and this may also be the case *in vivo*. Thus it may well be that the isolates differ in their abilities to change the pH of the infected tissue which in turn affects the activities of their dehydrogenases. For example, Friedman and Ceponis (1964) found differences in acid production in infected witloof chicory between a partially virulent mutant of *Erwinia carotovora* and the virulent parent wild type. The mutant infected tissue had a lower pH than that infected by the wild type.

The pH of the macerates of infected tissues

Sets of potato tuber slices were inoculated with isolates G110 cr.str-r. and G110 dif. Control slices were spread with sterile nutrient broth.

After incubation for 48 hours the slices were individually weighed and macerated in a Waring blender with glass distilled water. For each 10 gm. of tissue 50 ml. of water were used. The pH of the macerates was measured immediately afterwards. The results are given in Table 24.

An analysis of variance reveals that the pH of the macerates of the creamy infected discs is significantly higher ($P = 0.001$) than the pHs of the macerates of the control discs or those infected with the diffuse isolate.

The discs infected with the diffuse isolate, however, also have a significantly higher pH (P = 0.01) than the macerates of the control discs.

Table 25 gives the results for another experiment in which Mutant dif.2 was included. Six discs were inoculated with each isolate. After 48 hrs. the discs were individually weighed and macerated as before and the pHs of the macerates were measured.

Table 25. pH of macerates of infected tuber slices

	G110 cr.str-r.	Mutant dif. 2	G110 dif.	Controls
1	6.40	5.70	6.05	5.95
2	6.28	6.04	6.04	6.16
3	6.20	5.66	6.18	6.16
4	6.76	5.82	6.20	5.84
5	6.58	5.96	5.92	5.86
6	6.28	5.82	6.06	5.86
Mean	6.41(7)	5.83(3)	6.07(5)	5.97(1)

In this case an analysis of variance again showed the pH of the discs infected with the creamy isolate was significantly higher than all other treatments (P = 0.01). The pH of the macerates of discs infected with G110 dif. and Mutant dif. 2 did not differ significantly from the controls. However, the discs infected with G110 dif. had significantly higher pH than those infected with Mutant dif. 2 (P = 0.01). The lower levels of significance obtained with the second set of results was probably due to the use of smaller samples.

It would appear then that infection of potato tuber slices with the creamy isolates leads to an increase in the pH of the infected tissue.

Infection with the diffuse wild type may produce a rise in the pH of the infected tissue, but in no case was it nearly so marked as that produced by the creamy isolate, while Mutant dif. 2 did not alter the pH of the tissue at all.

The rise in the pH of the slices infected with G110 cr.str-r. would help explain why this isolate causes rapid extensive rots of the tuber slices without any noticeable browning. Such a rise may affect the dehydrogenase activities of the bacterium *in vivo* as well as the polyphenoloxidase activity of the host. The result may be that bacterial dehydrogenases under such conditions are more able to prevent or reverse the action of polyphenoloxidase so preventing the formation of possibly inhibitory or toxic compounds.

There are reports that some of the products of polyphenoloxidase activity inhibit a wide range of enzymes. Williams (1963) has demonstrated that various phenolic compounds such as chlorogenic acid and catechol, after oxidation, in fairly low concentrations are inhibitory to the polygalacturonase and macerating enzymes of *Sclerotina fructigena*.

If such products were not formed in the infected host then the exocellular enzymes of the bacterium attacking the host tissue e.g. endo-P.G.T.E. would be more effective in disrupting the tissue and allowing the organism to spread.

It is interesting to note that other enzyme systems may be affected by such a pH rise. Thus Hancock (1968) has noted a rise in pH of cucumber

tissue infected with Fusarium solani f.sp. cucurbitae. He concluded that this rise in pH allowed greater activity of the endo-P.G.T.E. produced by the fungus, and probably increased alkaline degradation of the pectic substances present in the host. Such a situation could exist in this disease since the pH optimum of the endo-P.G.T.E. produced by Erwinia atroseptica has been shown to be about pH 8.5.

Since it was demonstrated that differences existed between the pH of tissue infected with the two different morphological types it was decided to determine whether any of the differences could be related to differences in acid production from glucose in vitro.

Although no differences were noticeable in the acid/gas tests which were earlier carried out on these isolates, the results of which were reported in Table 16 it was felt that quantitative differences may be present. Meynell and Meynell (1965) state that the type of acid/gas test used here, although giving good indications as to what substrate an isolate will or will not utilise, is unlikely to be very sensitive in any quantitative study. It is therefore possible that two isolates could give a similar reaction in the usual acid/gas test procedure but differ in the actual amounts of acid produced.

In addition, it must be noted that both these colony types gave different growth appearance on glucose EMB agar. G110 cr.str-r. was bluish-pink, while G110 dif. was greenish-black. The appearance of the latter isolate is indicative that a drop in the pH of the medium has occurred. This particular medium is generally used in studies where the effect of an isolate on pH is being investigated, that is, it is a pH

Table 26. Approximate cell numbers ($X10^9$) per ml. of shaken (A) and unshaken (B) liquid minimal medium cultures

	G110 cr.str-r.				Mutant dif. 2				G110 dif.			
	1		2		1		2*		1		2	
Age of Culture	A	B	A	B	A	B	A	B	A	B	A	B
24 hours	1.00	0.15	1.00	0.18	0.57	0.11	-	0.002	0.90	0.23	1.03	0.23
48 hours	1.47	0.16	1.38	0.23	0.87	0.211	-	0.16	1.21	0.27	1.37	0.29
72 hours	1.76	0.39	1.81	0.41	1.20	0.41	-	0.31	1.53	0.44	1.64	0.42

* In this series the inoculum was a tenth of that used in all the others; (final concentration of 2×10^6 cells/ml. of medium). All ten flasks were left unshaken so that the results for this series are the averages for 10 flasks. All other results are the means of 5 flasks.

Table 27. pHs of shaken (A) and unshaken (B) liquid glucose minimal medium cultures

	G110 cr.str-r.				Mutant dif. 2				G110 dif.			
	1		2		1		2		1		2	
Age of Culture	A	B	A	B	A	B	A	B	A	B	A	B
0 hours	6.8	6.8	6.8	6.8	6.8	6.8	-	6.8	6.8	6.8	6.8	6.8
24 hours	4.47	5.07	4.44	5.04	4.82	5.18	-	6.76	4.39	4.96	4.33	4.98
48 hours	4.50	4.62	4.48	4.58	4.49	4.56	-	6.08	4.47	4.62	4.50	4.55
72 hours	4.52	4.54	4.52	4.47	4.52	4.51	-	4.66	4.52	4.49	4.56	4.48

indicator medium, Meynell and Meynell (1965).

Since the difference in the appearance of the two types of isolate was most marked on all media containing glucose it was decided to investigate the changes in pH which occurred during growth in liquid glucose minimal medium.

Acid production in vitro

The isolates used in this experiment were G110 cr.str-r., G110 dif. and Mutant dif. 2. The medium used was the glucose MM.

The isolates were grown in shaken nutrient broth cultures to provide the inoculum. An inoculum giving a final concentration of 2×10^7 cells/ml. of culture medium was used.

Two different series of inocula from different nutrient broth flasks were prepared for each isolate under test. Each inoculum series was inoculated into 100 ml. Erlenmeyer flasks containing 50 ml. of medium. All flasks were incubated at $26^{\circ}C$.

Each set of ten flasks was split into two groups of five. One group was shaken on a rotary shaker at 150 r.p.m. and amplitude 3.5 cm., while the other group was left unshaken in the constant temperature room.

At appropriate intervals 10 ml. portions of the cultures were removed and the cell numbers (estimated from O.D. readings) and pHs were taken. The results are given in Tables 26 and 27.

There were no clear cut differences between the isolates. The final pH achieved was about the same for all three. With the exception of Mutant dif. 2 this pH was achieved within 24 hours in the shaken cultures. The second inoculum series for this isolate demonstrates that different inocula may affect the growth rate and the fall in pH. In this case

where a much smaller inoculum was used the growth and fall in pH seem to lag behind all the other isolates although it appears to catch up in 2-3 days.

The results of this experiment and observations on the pH of liquid glucose MM cultures on other occasions indicated that no great differences existed between diffuse and creamy morphological types when compared for acid production in vitro. It would appear that there is little relationship between the effect of the isolates on the pH of the tissue that they infect and the drop in pH that occurs when they are grown in a medium containing glucose.

In summary then, it would appear that the only detectable difference between the different colony types, other than those discussed in Section II of this chapter, and summarised in Tables 17 and 22 , is the effect that each has on the pH of the tissue infected by them.

CHAPTER IV - MOTILITY AND GELATINE LIQUEFACTION STUDIES

Section I - Introduction

As was reported in Chapter III, differences in motility and gelatine liquefaction were observed between the two morphological types, described as creamy and diffuse. It was decided to study further the importance of these differences on pathogenicity.

It is worth noting that among various differences between a partially virulent mutant isolate of Erwinia carotovora and the virulent wild type, Friedman (1962) found differences in proteolytic activities as judged by gelatine liquefaction and the clotting of litmus milk.

As regards motility it is possible that it may have important effects on growth and survival of the organism. Thus it has been shown that motile strains of Pseudomonas fluorescens grow better under conditions of low nutrient status than non-motile ones, Smith and Doetsch (1969).

Section II - Procedures and Results

Isolate

The isolate used was G110 cr.str-r. since it was a motile isolate causing fairly rapid liquefaction of gelatine.

Isolation of gelatine non-liquefiers

The medium used for this isolation procedure was nutrient gelatine (page 11) with 0.5% w/v Agar (Oxoid No.3) autoclaved at 15 lbs. pressure for 15 minutes. Suspensions of bacteria were spread over plates of this medium so as to produce less than 100 colonies per plate. The plates were incubated at 25°C and examined after 2-3 days for colonies which did

not sink into the medium. These colonies were then isolated into tubes of nutrient broth and the procedure repeated as a check on the isolates. Of six isolates, five were found on re-testing not to sink into the nutrient gelatine/agar medium. These isolates were then used in an experiment aimed at checking whether differences in ability to liquefy gelatine were associated with changes in virulence. They were named as follows.

G110 cr.str-r.	Gel - 1
" "	Gel - 3
" "	Gel - 4
" "	Gel - 5
" "	Gel - 6

Comparison of wild types and mutants for gelatine liquefaction, motility and virulence

The two wild types G110 cr.str-r. and G110 dif. and the five mutants were subbed into nutrient broth tubes and grown for 24 hours at 25°C. These suspensions were spread over nutrient agar plates, from which single colonies were isolated after 48 hours incubation. Nine single colonies were selected for each isolate. Each of these was grown up in a test tube containing 1.1/2 ml. of nutrient broth.

After 24 hours incubation at 25°C the suspensions were used in the following tests.

(1) Drops of suspensions were examined under the light microscope and motility assessed according to the method given in Chapter I.

(2) Each suspension was stabbed into a nutrient gelatine tube and incubated at 25°C. After 8 days the liquid formed by the

(2) (contd)

action of the bacteria was poured off by inverting the tubes for 10 seconds. The weight of this liquid was taken as a crude measure of liquefaction.

(3) The cultures were spotted onto glucose MM and nutrient agar plates and these were incubated at 25°C to ensure that all isolates had wild type nutrition.

(4) The rest of the bacterial suspension was spread over potato slices in petri dishes to check virulence. The potato slices were incubated at 20°C and examined after 48 hours when the type of rot was assessed.

The results are summarised in Table 28, a, b, c, d, e, f and g.

All isolates grew on nutrient agar, even the three not growing on MM.

Table 28. Gelatine liquefaction, motility and virulence of single colony isolates

(a)

G110 cr.str-r. (wild type)				
Colony Number	Motility	Virulence	Growth on M. Medium	Quantity of Gelatine Liquefied
1	+++	A	+	0.63(4) gm.
2	+++	A	+	2.50(7)
3	++	A	+	1.53(7)
4	+	A	+	2.93(4)
5	++	A	+	2.71(1)
6	++	A	+	1.52(2)
7	++	A	+	1.63(2)
8	+++	A	+	2.45(7)
9	+++	A	+	2.67(8)

(b)

G110 dif. (wild type)				
Colony Number	Motility	Virulence	Growth on M. Medium	Quantity of Gelatine Liquefied
1	-	B	+	0 gm.
2	-	B	+	0
3	-	B	+	0
4	-	B	+	0
5	-	B	+	0
6	-	B	+	0
7	-	B	+	0.16(9)
8	-	B	+	0
9	-	B	+	0

(c)

	G110 cr.str-r Gel - 1			
Colony Number	Motility	Virulence	Growth on M. Medium	Quantity of Gelatine Liquefied
1	+	A	+	0.20(9)gm.
2	+	A	+	0
3	+	A	+	0
4	+	A	+	0
5	+	A	+	0.25(7)
6	+	O	-	0
7	-	A	+	0.25(1)
8	-	A	+	0
9	-	A	+	0.02(4)

G110 cr.str-r. Gel - 3				
Colony Number	Motility	Virulence	Growth on M. Medium	Quantity of Gelatine Liquefied
1	+	A	+	0 gm.
2	+	A	+	0
3	+	A	+	0
4	+	A	+	0
5	+	A	+	0
6	+	A	+	0
7	+	A	+	0
8	+	A	+	0
9	+	A	+	0

(d)

G110 cr.str-r. Gel - 4				
Colony Number	Motility	Virulence	Growth on M. Medium	Quantity of Gelatine Liquefied
1	+	B	+	0 gm.
2	+	B	+	0
3	+	A	+	3.42
4	-	A	+	1.03(3)
5	+	O	-	0
6	+	A	+	0.54(4)
7	+	B	+	0
8	+	B	+	0
9	+	A	+	0

(e)

(f)

G110 cr.str-r. Gel - 5				
Colony Number	Motility	Virulence	Growth on M. Medium	Quantity of Gelatine Liquefied
1	+	A	+	0 gm.
2	+	A	+	0
3	+	A	+	0
4	+	A	+	0
5	+	A	+	0.01(5)
6	+	A	+	0
7	+	O	-	0
8	+	A	+	0
9	+	A	+	0

(g)

G110 cr.str-r. Gel - 6				
Colony Number	Motility	Virulence	Growth on M. Medium	Quantity of Gelatine Liquefied
1	+++	B	+	1.80(5)gm.
2	+++	B	+	1.20(3)
3	+++	B	+	1.26(6)
4	+++	B	+	0.70(3)
5	+++	B	+	0.83(7)
6	+++	B	+	0.80(0)
7	+++	B	+	1.27(0)
8	+++	B	+	1.55(7)
9	+++	B	+	0.65(5)

A = Extensive rot characteristic of creamy isolate

B = Brown rot characteristic of diffuse isolate

O = No rot or symptoms

There is obviously wide variation between these mutants and the parent wild type. In most cases a marked reduction in, or loss of motility is accompanied by a reduction or loss of the ability to liquefy gelatine. There was a lot of variation for isolate G110 cr.str-r. Gel 4 so that results for this isolate were inconclusive.

Isolate G110 cr.str-r. Gel 6 was interesting in that it was motile and gave gelatine liquefaction, but produced rots in tuber slices similar to those produced by the G110 dif. isolate.

Single colony isolates of G110 cr.str-r. Gel 6 and G110 cr.str-r. Gel 5 were used for comparison with the wild types as regards various physiological characters. These strains were selected as representative of two extremes with G110 cr.str-r. Gel 6 having the properties described and G110 cr.str-r. Gel 5 having only slight motility at best, being unable to liquefy gelatine but still producing extensive rots characteristic of the creamy isolates.

Streptomycin Resistance

All the mutants were tested for this character and all proved able to grow on media with streptomycin incorporated, indicating that their origin was from the G110 cr.str-r. isolate, thus ruling out the remote possibility that they were contaminants.

Colonial appearance on various media

The mutants G110 cr.str-r. Gel 5 and Gel 6 were grown on the various MM, EMB and TTC agars listed in Table 8. and on nutrient agar and McKonkey's agar. G110 cr.str-r. Gel 5 was similar to G110 cr.str-r. on all media. G110 cr.str-r. Gel 6 was unlike the parent isolate on galactose and lactose con-

taining media, and in fact appeared identical in all respects with isolate G110 cr.str-r. gl-1., (Mutant dif. 1).

The two mutants were tested for acid and gas production from various carbohydrates. The results are given in Table 29.

Table 29. Acid and gas production from various carbohydrate sources

Carbon source	G110 cr.str-r. Gel - 5		G110 cr.str-r. Gel 6	
	Acid	Gas	Acid	Gas
Arabinose	+	+	+	+
Dulcitol	-	-	-	-
Ethanol	-	-	-	-
Fructose	++	+	++	+
Galactose	+	-	d	-
Glucose	++	+	++	+
Glycerol	d	-	d	-
Lactose	++	+	d	-
Maltose	d	-	d	-
Mannose	++	+	++	+
Raffinose	++	+	++	+
Salicin	++	+	++	+
Sucrose	++	+	++	+
Tartrate	-	-	-	-
Xylose	+	+	+	+

Again it is noticeable that strain G110 cr.str-r. Gel 6 is much slower at producing acid from galactose and lactose, although some pro-

duction occurs after longer periods of time. This is very similar to the situation with G110 cr.str-r. gl.1.

In effect then, it must be pointed out that isolates can be found having reduced motility and reduced ability to liquefy gelatine but still able to cause extensive creamy type rots on potato tuber slices. This suggests that these characters are not of immediate importance in determining an isolate's ability to cause soft rot symptoms. More interesting is the isolation of strain G110 cr.str-r. Gel 6. This is the second isolation of a diffuse isolate which has abnormal growth on the galactose and lactose media and which causes brown limited types of rot.

It again indicates that diffuse colonial morphology which, in this case, is apparently associated with an inability to utilise galactose and lactose can affect the type of rot produced.

CHAPTER V - STUDIES ON AVIRULENT MUTANTS

The isolation of avirulent mutants in which the physiological changes associated with avirulence can be studied, offers an alternative approach to that employing isolates, mutant for specific physiological systems.

The greatest difficulty lies in determining which physiological changes have occurred, since it is possible that many physiological systems may be altered by a mutational event. As a result it is generally only possible to examine those systems previously implicated by other studies and those physiological characters which are easily determined. In this study examination has been restricted to colony appearance, acid and gas production and production of polygalacturonate trans-eliminase *in vitro*. These were selected because the foregoing chapters on pectic enzymes and colonial morphology indicated that studies on these aspects of the organism's physiology may prove useful.

Another major difficulty in this approach is the initial isolation of the avirulent mutants. This must be more difficult if the study specifically excludes the isolation of avirulent mutants having nutritional requirements, because the normal replica plating techniques cannot be applied. However, all this is a question of technique, dependent on the development of rapid assay procedures, which allow fast, accurate assessments of an isolate's virulence.

Friedman and Ceponis (1959) working with *Pseudomonas marginalis* were able to isolate avirulent mutants of this organism. These isolates produced less pectic enzymes *in vitro* when compared with the wild type. Friedman (1962) using *Erwinia carotovora* isolated a mutant of *Erwinia*

carotovora with reduced virulence. This isolate as well as having reduced abilities to produce pectic enzymes and proteolytic enzymes, also appeared to produce more acid in vivo, i.e. on infection of its host, whitloof chicory, Friedman and Ceponis (1964).

Mutants of Agrobacterium tumefaciens differing in degrees of virulence have been used in genetical studies of that organism, in which it has been possible to demonstrate the 'multi loci' nature of the genetics of its virulence, Klein and Klein (1956).

Isolation of Avirulent Mutants

Isolate: The isolate used in these studies was G110 dif.

Procedure: A 24 hour old culture in nutrient broth was treated with the chemical mutagen ethyl methane sulphonate (E.M.S.), Loveless and Haworth (1959), in the following manner. 3 ml. of the bacterial suspension were added to a solution containing 0.35 ml. of E.M.S. in 6.65 ml. of glass distilled water. After two and a half minutes 1 ml. of the treated culture was removed and added to 9 ml. of glass distilled water. Previous studies showed that this treatment killed approximately 50% of the bacteria.

0.1 ml. portions were then used to inoculate 50 ml. of nutrient broth which was then incubated on a shaker at 25°C. This step in the procedure was included because it reduced the hazards in handling the mutagen. It was thought that it would allow segregation of mutant 'nuclei' out of 'multinucleate' bacteria, as well as reducing losses of mutations, which occur if the bacteria are grown immediately on Minimal Medium, Witkin (1956).

After 24 hours dilutions of this culture were made so as to give samples containing approximately 100 viable cells. These were spread over nutrient agar or glucose MM agar plates.

After 2-3 days growth at 25°C the colonies were clearly visible and single colonies were inoculated into small tubes of nutrient broth and again incubated at 25°C. The turbid suspensions, formed after 24 hours growth, were then spotted onto nutrient agar plates. If the initial isolation had been made on nutrient agar the single colony isolates were also tested for growth on the minimal medium to check that they still had the same nutritional requirements as the wild type. The rest of the suspensions were inoculated onto tuber slices which were incubated for two days at 20°C before examining for the development of soft rots.

If a potato slice did not show any symptoms of soft rot, the isolate was retested by inoculation onto at least five slices. If this test proved negative, the isolates were kept for more comprehensive tests.

Using this procedure it has been possible to examine about 1,000 single colony isolates of G110 dif. Of these, two avirulent isolates G110 dif. Av-1.7 and Av-1.34 were kept for further study.

Virulence

Both of the mutants had completely lost their ability to soft-rot potato slices, although in some tests a slight darkening of the surface of the tuber slices occurred. This was, however, variable and no soft rot ever developed over these areas.

Motility and gelatine liquefaction

Both mutants were identical with wild type with respect to these characters.

Colony appearance on various solid media

The media used are listed in Table 8. The appearance and growth of the two mutants was identical to wild type on all media. Growth on

Figure 21. Endo-P.G.T.E. activities in sodium polypectate medium culture supernatants of G110 dif. (Wild type).

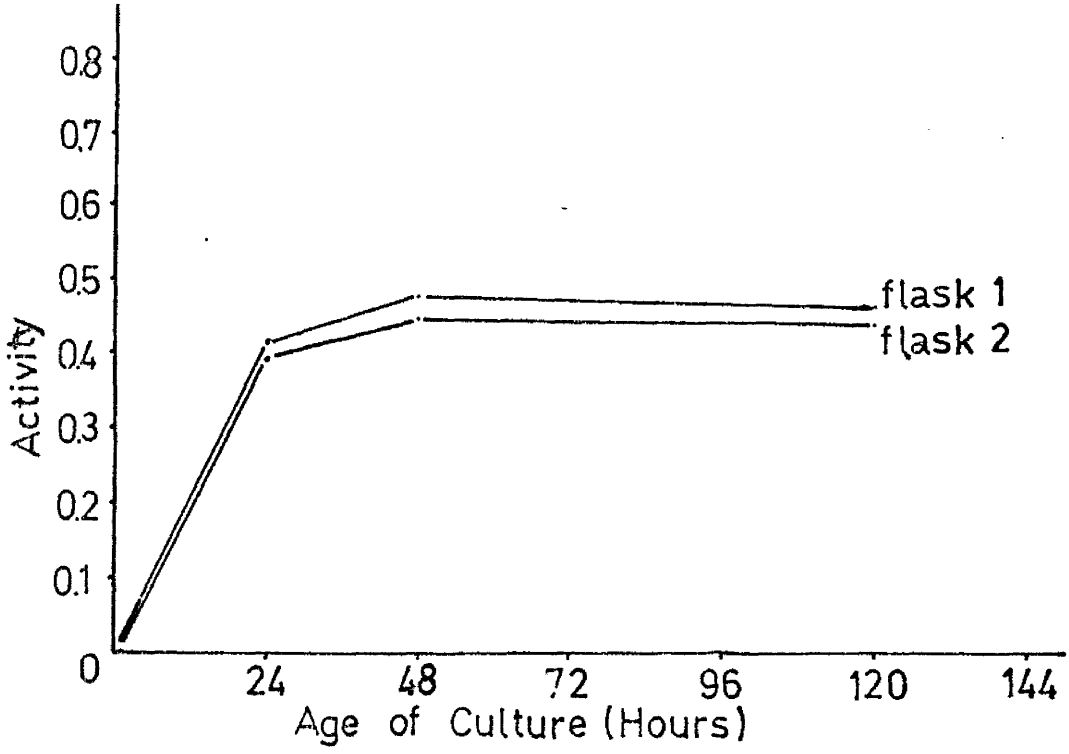
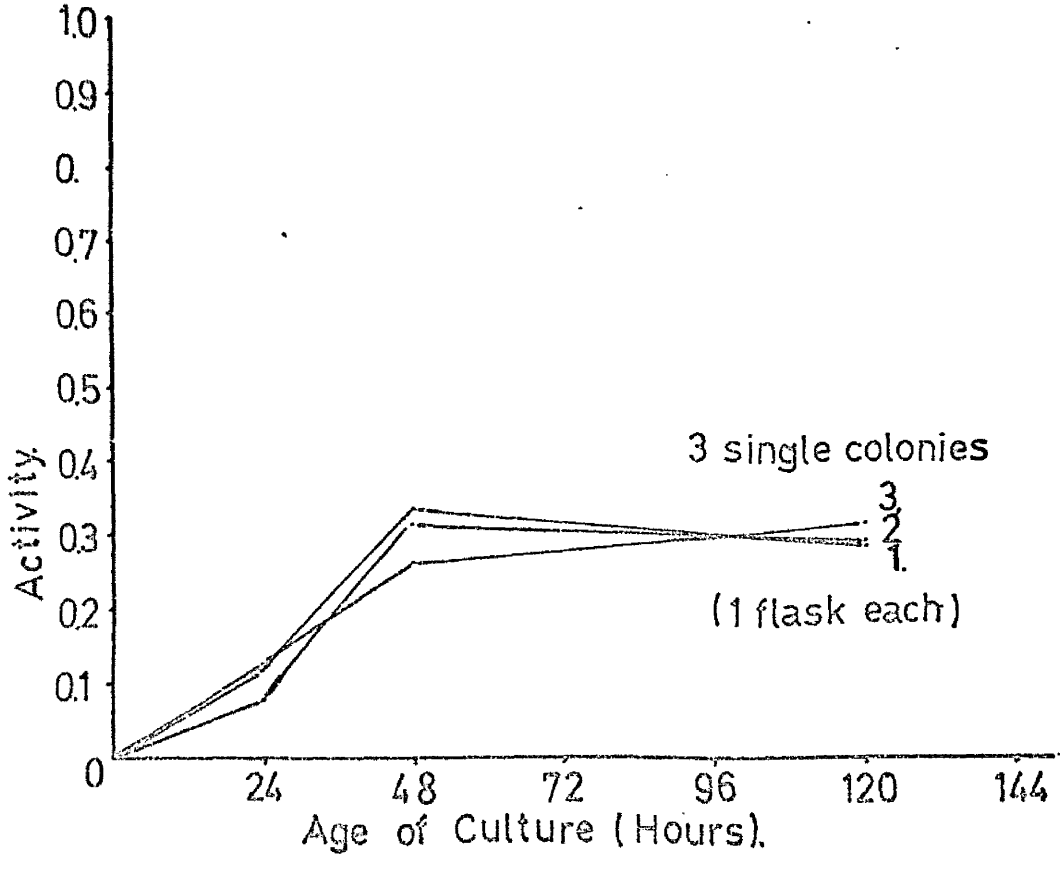


Figure 22. Endo-P.G.T.E. activities in sodium polypectate medium culture supernatants of G110 dif. Av.-1.7.



the various minimal media clearly indicated that these mutants had no specific growth requirements.

Acid and gas production from various carbohydrates

The carbohydrates used are listed in Table 7. The mutants appeared identical with the parent wild type in all these tests.

P.G.T.E. Production

When tested for P.G.T.E. activities in vitro the isolates gave the results shown in Figures 21, 22 and 23.

This experiment has been repeated several times with similar results.

Both mutants grew fairly readily in both polypectate and pectin medium.

It would appear that although the mutants are capable of producing the enzyme in vitro on a sodium polypectate medium they do not produce as much or as readily as the wild type. After 24 hours growth appreciable activity is detectable but maximum activity is not detectable until after about 48 hours growth. The final level of activity is lower than in wild type but not greatly so.

Activity in the pectin medium was not very high but appeared on measurement to be much higher for Av.1.34 than Av.1.7. (Tables 30 and 31).

Conclusions

The only difference that could be detected between the avirulent mutants and the wild type was in the amount or the activity of the P.G.T.E. produced in vitro. It is possible that this difference is the major one, if not the only one, responsible for the loss of virulence. If this is so it raises several interesting points. Both isolates did not produce any soft rot lesions, unlike the pectic enzyme mutants which occasionally produce some

Figure 23. Endo-P.G.T.E. activities in sodium polypectate medium culture supernatants of G110 dif. Av.-1.34.

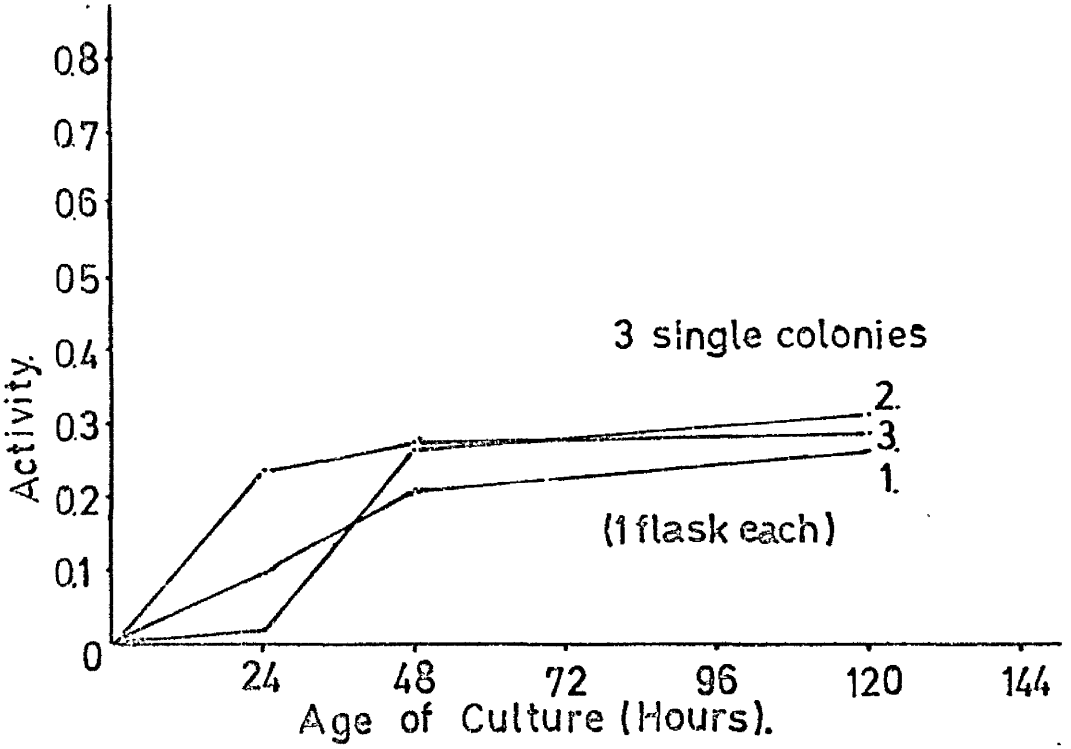


Table 30. Approximate cell numbers ($X10^9$) per ml. of sodium polypectate (A) and pectin (B) media cultures

Age of Cultures	Flask	G110 dif.		G110 dif. Av.-1.7		G110 dif. Av.-1.34	
		A	B	A	B	A	B
24 hours	1	4.2	2.4	4.1	2.4	4.4	1.9
	2	4.0	2.1	3.8	2.2	3.9	1.0
48 hours	1	4.0	4.6	4.8	2.8	3.9	3.9
	2	4.7	4.2	4.8	3.1	4.0	3.8
72 hours	1	4.6	8.3	4.9	3.2	4.4	9.0
	2	4.9	7.6	4.8	3.3	4.2	7.6

Table 31. P.G.T.E. activities in sodium polypectate (A) and pectin (B) media culture supernatants

Age of Cultures	Flask	G110 dif.		G110 dif. Av.-1.7		G110 dif. Av.-1.34	
		A	B	A	B	A	B
24 hours	1	1.208	0.088	0.151	~0	0.182	~0
	2	1.271	0.066	0.154	~0	0.160	~0
48 hours	1	1.183	0.604	0.268	~0	0.212	0.030
	2	1.190	0.628	0.276	0.008	0.186	0.022
72 hours	1	1.155	1.519	0.246	0.002	0.242	0.470
	2	1.173	1.666	0.244	0.004	0.212	0.428

lesions, albeit very small ones. These latter mutants in fact produced even less of the enzyme than the avirulent mutants.

Lapwood (1957) carried out a comparative study *in vitro* of an *Erwinia* sp. with such non-pathogenic bacterial species as *Flavobacterium*, which also produced pectic enzymes. He found that the best correlation with pathogenicity was the length of the lag phase of pectic enzyme production after inoculation into the growth medium, the shorter the lag phase the greater the pathogenicity.

This situation would not seem to apply here since the two avirulent mutants had shorter lag phases than any of the three pectic enzyme mutants. However this situation may not obtain *in vivo* where delay in production of pectic enzyme may affect further growth of an isolate. This aspect obviously requires further study.

Thus it is likely that other factors are also involved in the reduced virulence of the mutants. It is clear that many characters which could also be involved have not been tested. It may be that differences in their abilities to alter pH in the infection court are involved as appears to be the case for the creamy and diffuse isolates.

This, however, would be difficult to investigate since the isolates do not cause any soft rots and do not grow much in infected tissue. It would therefore be extremely difficult to investigate, since any effect would be very localised.

Other factors may also be involved in the loss of virulence of these isolates. Certainly these mutants indicate that other factors besides the ability to produce pectic enzymes are involved in the process of infection.

CHAPTER VI - NUTRITIONAL AND DRUG RESISTANT MUTANTS

Attempts were made to induce nutritional and drug resistant mutations in order to determine the ease with which they could be produced and then develop methods for their isolation.

Isolates carrying such mutations, if they were as virulent as the original wild type, could be usefully employed in a number of ways. Firstly, the mutation could serve as a marker and help in establishing the origin of other isolates from a particular wild type, and reduce the possibility of errors due to chance contamination. Streptomycin resistant strains were particularly useful for this purpose.

In addition, they could be employed in attempts to map the organism genetically. Mapping of mutations for virulence has been done by Klein and Klein (1956) on Agrobacterium tumefaciens, where they demonstrated that virulence is controlled by multiple loci. It would be useful in any attempts to map loci for virulence or related markers in this bacterial species to be able to use other markers not directly affecting virulence, or any of the other physiological systems under study.

Methods

A. Nutritional Mutants

Isolate: Isolate G110 dif. was used.

Production of Mutants: The procedure for the isolation of nutritional mutants employed penicillin enrichment and replica plating techniques. Sederberg (1950) gives a full description of these techniques.

Ultra-violet light in the 254 mμ region of the spectrum was used as the mutagen. A killing curve was first obtained by irradiating a suspension of the bacteria in saline for varying periods of time, until a 99% kill was achieved. Other suspensions of the bacteria were then treated to give a 99% kill, after which the penicillin enrichment technique was used to increase the percentage yield of mutants. The survivors were grown on Complete Medium and then replica plated onto glucose MM. If any isolates failed to grown on the minimal medium they were retested on the following media.

1. Vitamins medium

   Glucose MM + Vitamin stock solution — 1 ml. per litre
   (Pontecorvo et al (1953)

2. Amino acids medium

   Glucose MM + Casamino acids (DIFCO - Vitamin free) — 3 gm. per litre

3. Nucleic acid bases medium

   Glucose MM + Nucleic acid digest — 3 ml. per litre
   (Pontecorvo et al (1953)

4. Complete medium

   Glucose MM + the three supplements listed above and also yeast extract (DIFCO) — 2 gm. per litre

After determining which medium supported the growth of each mutant, further tests were carried out to determine their apecific nutritional requirements.

Vitamins No mutants were isolated which grew on the medium supplemented with vitamin stock solution.

Amino acids A total of six isolates grew on the amino acid medium

and were tested further to determine their specific requirements. They were spotted onto glucose MM agars containing individual amino acids in the concentration of 0.5 gm. per litre. Table 32 gives a list of the amino acid requiring mutants which were isolated.

Table 32. Amino acid requiring mutants

leu$^-$	(4 isolates)
met$^-$	(1 isolate)
arg$^-$	(1 isolate)

Nucleic acid bases Three isolates were tested for growth on glucose MM supplemented with the various nucleic acid bases (in the hydrochloride form), at the level of 0.2 gm/litre. Table 33 gives a list of these isolates.

Table 33. Nucleic acid base requiring mutants

ura$^-$	(2 isolates)
ade$^-$	(1 isolate)

Incorporation of additional markers

In order to incorporate two markers into any particular isolate, the process was repeated with an isolate with one of the desired markers already incorporated. The minimal medium in such instances consisted of glucose MM supplemented with the particular nutritional requirement of that mutant.

Using this procedure it has been possible to isolate several double mutants, which are listed in Table 34.

Table 34. Double nutritional requiring mutants

ura^-. cys^-	(1 isolate)
ura^-. lys^-	(1 isolate)
ura^-. arg^-	(3 isolates)
ura^-. met^-	(1 isolate)
ura^-. val^-	(1 isolate)
ura^-. leu^-	(1 isolate)
ura^-. tyr^-	(1 isolate)
ura^-. asp^-	(1 isolate)

B. Streptomycin resistance

Isolates G110 dif. and G110 cr. isolates have been used in these studies. Neither of these isolates grow on glucose MM when streptomycin sulphate is incorprated at 100 p.p.m.

Isolation of mutants

A turbid suspension of the wild type was plated onto glucose MM agar supplemented with streptomycin sulphate in the concentration of 100 p.p.m.

The few survivors which proceeded to grow on these plates were selected out and subcultured again onto the same medium, before single colonies were re-isolated onto nutrient agar slopes.

All the isolates obtained by this procedure were able to soft-rot potato tuber slices, and were indistinguishable in this respect from the original wild type.

Although various mutants have been isolated, only one has been used extensively in any of the previously described studies. This mutant was

derived from a creamy isolate G110 cr. and was labelled G110 cr.str-r. All its characters were identical with those of the wild type except for its ability to grow in the presence of 100 p.p.m. of streptomycin sulphate.

This isolate proved extremely useful in all studies in which it was used. In particular, it gave unequivocal proof that various types of mutants obtained in studies using it were in fact derived from it, and were not chance contaminants.

GENERAL CONCLUSIONS

The results of this work clearly show the value of the use of induced and spontaneous mutations in studying the physiological basis of the host-parasite relationship using *E. atroseptica*.

Using this approach it has been possible to investigate the roles of pectic and proteolytic enzymes, motility and colonial morphology and related factors in relation to the pathogen's ability to cause soft-rots of potato tissue.

A good correlation has been found between the ability to produce endo-P.G.T.E. *in vitro*, and the ability to rot tuber tissue. The amount of enzyme produced, and also the time taken for the isolate to release the enzyme in response to the inducing medium appear to be the important factors involved.

Factors related to colony morphology also appear to be associated with the type of soft rot an isolate produces since all the diffuse isolates gave brownish limited rots, while all the creamy isolates gave creamy extensive rots.

Tomiyama and Stahmann (1964) produced evidence to suggest that the pectic enzymes produced by a pathogen may be responsible for the browning reaction of infected host tissue. This is supported by the observation that bacteria free culture supernatants of the creamy isolates cause a greater increase in polyphenoloxidase activity and darkening of treated slices than culture supernatants of the diffuse isolates. Culture supernatants of the creamy isolates contain more endo-P.G.T.E. activity than

those of the diffuse isolates.

These results, however, are in contrast with the observation that slices infected with the creamy isolates do not become discoloured whereas those infected with diffuse isolates do. Since large amounts of P.G.T.E. activity are detectable in slices infected with the creamy isolates, it suggests that these isolates have some ability to reverse or inhibit the action of the host's polyphenoloxidase systems not possessed by the diffuse isolates.

Attempts were therefore made to determine if any differences could be detected between the abilities of the two different colony types to reverse the action of polyphenoloxidase or in the dehydrogenase patterns they exhibited. However, both isolates decolourised potato sap and no differences in their dehydrogenase patterns were found.

The only difference which was discovered which might explain the

The only difference which was discovered which might explain the

different types of rot produced was that the pH of tissue infected with the creamy isolate was significantly higher than the pH of the tissue infected with the diffuse isolates. Since dehydrogenases, in general, appear to be more active at higher pHs, the pH difference could well affect the ability of an isolate to reduce the products of polyphenoloxidase activity. However, no differences in physiology in vitro between the isolates could be discovered which would explain the different effects on pH in vivo.

If the characters exhibited by the creamy isolates are separate distinct one, that is high P.G.T.E. levels in vitro, creamy morphology and ability to raise the pH of infected tissue, it should be possible to get isolates, mutant for one character but not the others. For example, it

may prove feasible to isolate a creamy mutant producing very little endo-P.G.T.E. activity *in vitro*. In this way it should be possible to study the relationship of each of these factors in the ability of an isolate to cause soft rots.

This has been done, with some success, for motility and proteolytic enzymes. These do not appear to be directly involved in the ability of an isolate tc soft rot potato tuber tissue once infection has occurred. However, it is likely that motility may affect the spread and entry of this organism under field conditions.

Thus, the ability of *Erwinia atroseptica* to parasitise potato tuber tissue does not depend on one physiological factor, but appears to be the result of a complex interaction of various factors . These include the ability to produce pectic enzymes, to overcome the host's defence mechanism and to change the pH of infected tissue to one more favourable for pathogenesis.

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