I DEDICATE THIS THESIS TO MY

MOTHER AND FATHER

Посвящаю это тесис
к профессору Вуд.
Благодарю вас для всех.

Алан Слюсаренко.

1981

INTERACTIONS BETWEEN *PHASEOLUS VULGARIS* AND *PSEUDOMONAS PHASEOLICOLA*

By

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ABSTRACT

Two races of Pseudomonas phaseolicola can be distinguished using Phaseolus vulgaris cv. Red Mexican (RM) which is resistant to Race 1 but susceptible to Race 2 isolates. Cv. Canadian Wonder (CW) is susceptible to both races. The possibility that the different responses of RM to Races 1 and 2 were linked with differential binding to plant cell walls was investigated.

Cells of avirulent Race 1 isolates were agglutinated more actively than were cells of virulent Race 2 isolates by a lectin containing extract from RM cotyledons. No activity was detected in a similar lectin preparation from CW cotyledons. Agglutination of bacteria was caused by pectic polysaccharide which had been fortuitously extracted with the lectins. Specific extraction of pectic polysaccharides yielded active fractions from leaves and cotyledons of both cultivars. The active polysaccharides from RM and CW had similar compositions.

Cells of both races attached to bean cell walls in vivo showing that specific attachment of avirulent Race 1 isolates to RM cell walls was not the decisive recognition event which enabled RM to react hypersensitively. The hypersensitive reaction (HR) was altered by pretreatment of bacteria to occupy surface binding sites, which suggests attachment to plant cell walls was necessary for induction of HR.

Acidic pectic polysaccharide from a selection of higher plant species agglutinated phytopathogenic and non-phytopathogenic bacteria from diverse genera. This suggests that attachment of bacteria to pectic polysaccharide in plant cell walls is a widespread phenomenon.

LIST OF ABBREVIATIONS

A_{xxx}	absorbance at the given wavelength
BPB	bromophenol blue
c.	circa
cm	centimetre
CW	Canadian Wonder
d	day
disc-PAGE	discontinuous polyacrylamide gel electrophoresis
EDTA	ethylenediaminetetra-acetic acid (dissodium salt)
EPS	extracellular polysaccharide
g	gram
GLC	gas-liquid chromatography
h	hour
HR	hypersensitive reaction
Kg	kilogram
LPS	lipopolysaccharide
M	molar (concentration)
mA	milliampere
min	minute
MW	molecular weight
nm	nanometre
P	ammonium persulphate
PAS	periodate acid Schiff's
PG	polygalacturonase
PHA	*Phaseolus vulgaris* lectin
PME	pectin methyl esterase
RB	running buffer
RGB	resolving gel buffer

RM	Red Mexican
s	second
SDS	sodium dodecylsulphate
SGB	spacer gel buffer
TEMED	NNN´N´-tetramethyl-1,2-diaminoethane
TFA	trifluoroacetic acid
UV	ultra violet
V	volt
v/v	volume for volume
w/v	weight for volume

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REVIEW OF THE LITERATURE

Interactions between higher plants and bacteria

The interactions between plants and bacteria can be classified into three categories (Klement, 1971a).

1. Plants exposed to saprophytic bacteria
2. The compatible or susceptible reaction
3. The incompatible or resistant reaction

In leaves the general characteristics of interactions in the first category are little bacterial multiplication and no apparent macroscopic damage to the leaf cells, or bacteria may multiply to some degree, but not cause visible damage to the leaf tissues (Lyon (neé O'Brien)and Wood, 1975). Some phytopathogenic bacteria in their natural hosts show only limited multiplication and cause no apparent disease, for example a pseudomonad pathogenic to soybean was isolated from buds, pollinated flowers and young pods of healthy field-grown soybean plants (Leben and Miller, 1973). Latent bacterial infection was reviewed by Hayward (1974).

In the second category virulent phytopathogenic bacteria gain access to a susceptible host under suitable conditions for growth. The bacteria multiply rapidly and reach high levels, for example Pseudomonas phaseolicola inoculated into leaves of susceptible French bean plants increased to 10^6 times the original population after 5 - 6 days (Omer and Wood, 1969). Typical symptoms of bacterial leaf spot diseases are the production of greasy-looking water soaked lesions which gradually increase in size and become necrotic in the centre, often associated with toxin induced systemic and/or localized chlorosis (Agrios, 1978). These are characteristic symptoms of Halo Blight of French beans caused by Pseudomonas phaseolicola, along

with growth distortions of newly forming leaves and a general stunting of the whole plant. Bacterial mobility by means of flagella is important in the invasion of bean leaves by P. phaseolicola (Panopoulos and Schroth, 1974). Conditions of high humidity in leaves are essential for infection and multiplication of bacteria, and it was suggested that P. phaseolicola creates a suitable environment within leaves by means of a high molecular weight polysaccharide exotoxin which increases humidity by inducing water-soaking of the leaf tissues (El-Banoby and Rudolph, 1979a, 1979b; Rudolph, 1980). A lipomucopolysaccharide from P. lachrymans may function similarly (Keen and Williams, 1971).

The involvement of the chlorosis inducing toxin produced by P. phaseolicola in the establishment of compatibility is obscure at present. Gnanamanickam and Patil (1977a) suggested that the toxin suppressed the hypersensitive reaction and was necessary for the successful establishment of the pathogen in the host. However, a non-toxin producing mutant strain of P. phaseolicola induced water soaked local lesions but did not spread systemically, which suggested that the toxin in some way facilitated systemic spread of the cells (Patil et al., 1974). Rudolph (1972) showed that the toxin could break the resistance of bean leaves towards P. phaseolicola and elicit a susceptible reaction. Rasche (1977) suggested this effect was due to increased humidity in the intercellular spaces of leaf parenchyma caused by toxin induced closure of stomata.

In the third category, the numbers of bacteria increase over a short period, then decrease or remain more or less constant (Lyon (neé O'Brien) and Wood, 1975), often accompanied by rapid necrosis of plant cells in the region of inoculation - the characteristic hypersensitive reaction (HR). HR occurs when cells of a phytopathogenic bacterium are introduced into tissues of a non-host plant e.g. Pseudomonas pisi in tobacco; or when cells of an avirulent strain of a pathogen are introduced into a resistant host, e.g. Race 1 isolates of P. phaseolicola in Phaseolus vulgaris cv. Red

Mexican. Although the resulting reactions from these two types of combination are visually identical, the induction and mechanism of the response may be different (Wood, 1976).

The HR of plants to phytopathogenic bacteria can be divided into three phases (Klement, 1971a, b).

1) The induction phase, which requires the presence of living bacterial cells.

2) The latent phase, during which living bacteria are no longer required. No macroscopic symptoms occur, but the permeability of the cells increases, as evinced by the increased leakage of ions into the intercellular spaces (Cook and Stall, 1968; Goodman, 1968) accompanied by ultrastructural changes (Goodman and Plurad, 1971; Roebuck et al., 1978).

3) The presentation or collapse phase, during which host cells in the inoculated region collapse and desiccate, taking on firstly a silvered then a bronzed appearance. The accumulation of antibacterial isoflavonoids associated with the HR is thought to restrict the growth of bacteria in the lesion (Lyon and Wood, 1975; Keen and Kennedy, 1974; Gnanamanickam and Patil, 1977a, b; Holliday et al., 1981).

The temporal relationship of these phases in P. vulgaris cv. Red Mexican are summarised in Figure 1.

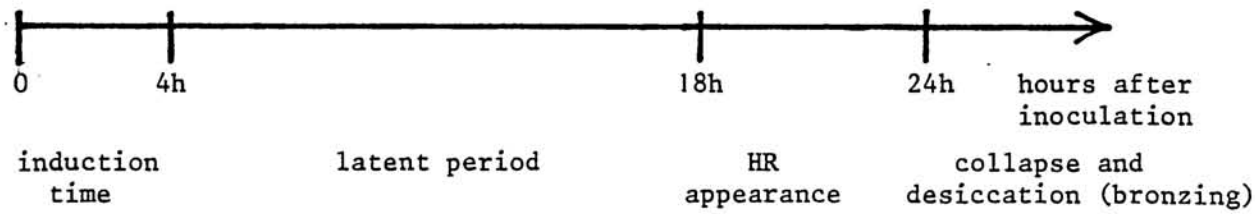


Figure 1 Development of HR in leaves of P. vulgaris cv. Red Mexican in response to inoculation with cells of a Race 1 isolate of P. phaseolicola (after Roebuck et al., 1978).

Lyon (neé O'Brien) and Wood (1975) reported that cultures of a Race 1 isolate of P. phaseolicola more than 8 days old failed to induce a HR in P. vulgaris cv. Red Mexican, which is normally resistant. This is interesting in view of the delay in response of tobacco leaves following the use of cultures of P. phaseolicola of increasing age (Sülle and Klement, 1971), summarised in Figure 2.

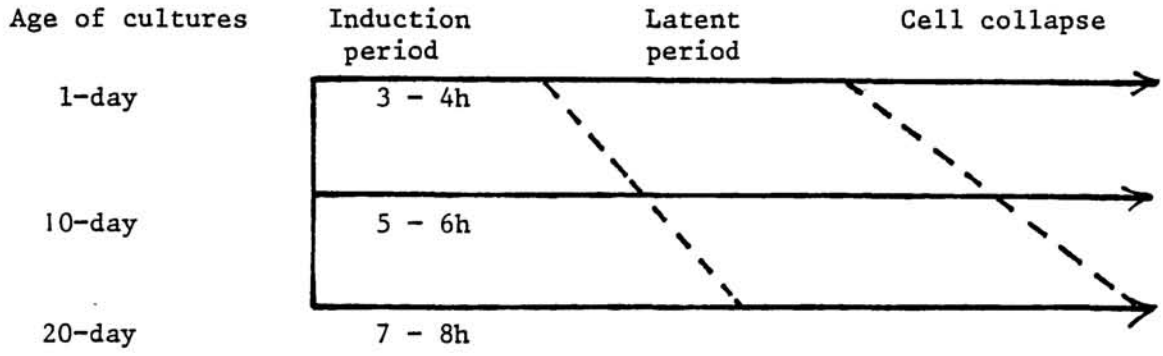


Figure 2 Effect of age of P. phaseolicola culture on development of HR. HR presentation was delayed 3 - 4h when old cultures were used (After Sülle and Klement, 1971).

Seemingly rather high inoculum concentrations are needed to induce the confluent necrosis associated with macroscopically visible HR : c. 5×10^6 cells cm^{-3} of Pseudomonas syringae in tobacco (Klement, 1967), c. 2×10^6 cells cm^{-3} of P. mors-prunorum and P. phaseolicola in Phaseolus vulgaris cv. Red Mexican (Lyon (neé O'Brien) and Wood, 1976). In the latter case this corresponds to about 2 bacterial cells for each necrotic leaf cell; viewed in these terms the initial inoculum concentration does not seem so unrealistic compared to natural infections in the field. At inoculum concentrations below the threshold necessary to induce confluent necrosis, P. pisi introduced into tobacco gave dead leaf cells at a ratio of 1:1 to the number of bacteria introduced (Turner and Novacky, 1974).

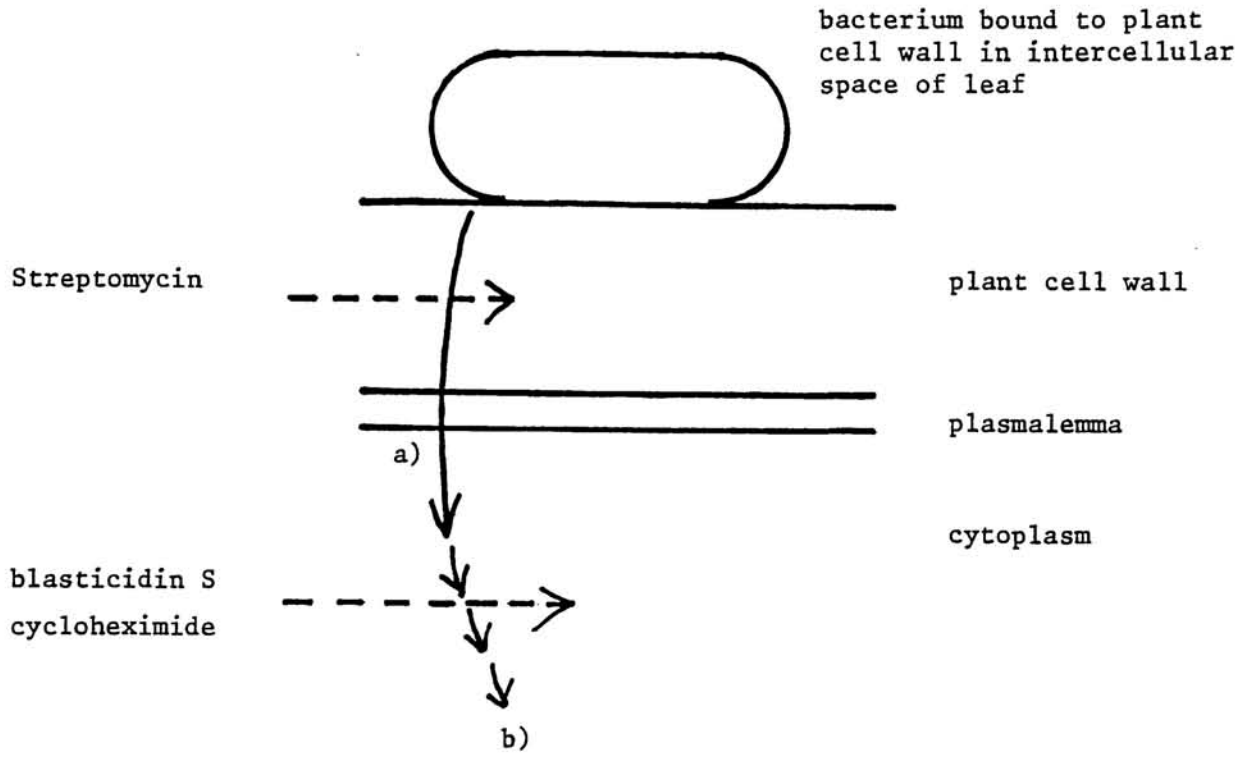
Similarly in French bean with low inoculum concentrations of an incompatible P. phaseolicola isolate small groups of killed protoplasts, often beneath stomata were readily seen by light microscopy (Lyon (neé O'Brien) and Wood, 1975).

The different effects on HR's in different hosts to various treatments suggests that, although of similar final appearance, dissimilar processes may be involved. The delay or suppression of HR in tobacco following high temperature treatment (Sule and Klement, 1971) was not observed in French bean (Lyon (neé O'Brien) and Wood, 1975). In tobacco incubation in the dark after inoculation with a normally incompatible isolate of P. solanacearum led to a "susceptible-type" response (Lozano and Sequeira, 1970a). Also HR could be prevented with heat killed cells (Lozano and Sequeira, 1970b), whereas in French bean (Lyon (neé O'Brien) and Wood, 1975) and soybean (Kennedy and Mew, 1971) HR was independent of light and pretreatment of beans with heat killed cells did not affect HR development(O'Brien, 1973). Also, in tobacco high humidity suppresses HR (Klement and Goodman, 1967) whereas in soybean and French bean this is not the case (Kennedy and Mew, 1971; Lyon (neé O'Brien) and Wood, 1975).

The many investigations to define the events in the early and rapid induction of HR have not met with much success. Attempts to isolate a discrete "inducer" from several species of bacteria have all failed (Sequeira, 1976). A compound from osmotic shock preparations of Erwinia rubrifaciens which reproduced the symptoms of HR was reported by Gardner and Kado (1972), and high speed grinding of cells of Pseudomonas solanacearum yielded a fraction which gave a reaction similar to HR in tobacco leaves (Sequeira and Ainslie, 1969). However, it is difficult to distinguish a typical HR from the non-specific toxicity of bacterial fractions containing very high concentrations of proteins (Sequeira, 1976). HR is induced by living bacteria in close contact with the host cell wall (Ibid; Stall and Cook, 1979) and is dependent upon de novo synthesis of bacterial protein (Lallyett, 1977). It

is postulated that the "inducer" is highly unstable or readily degraded, hence the necessity for close proximity of bacterial cells to host cells and the inability of culture filtrates or cell-free fluid from intercellular spaces of leaves (after inoculation with avirulent bacteria) to induce HR (Sequeira, 1976). Using P. pisi in tobacco Klement (1977) failed to demonstrate an inducer by an indirect method which did not involve extraction of the intercellular fluid. He concluded that contact between the bacterial and plant cells was essential in recognition of incompatibility and induction of the HR. In the same paper he suggests that different factors are responsible for HR induction and establishment of cell collapse. This suggestion is also made in the review by Sequeira (1976). Recent work (Keen et al., 1981) supports this view. Working with heterologous Pseudomonas spp. in soybean they showed that infiltration of streptomycin within 2h of inoculation prevented HR and glyceollin accumulation via an effect on bacterial metabolism, and blasticidin S, a protein synthesis inhibitor in soybean blocked HR and glyceollin accumulation up to 9h after inoculation, thereby suggesting that the processes leading to cell death are dependent on translation in the host. O'Brien and Wood (1973) reported that treatment of French bean leaves inoculated with incompatible bacteria with cycloheximide, which inhibits protein synthesis in eucaryotic systems by inhibiting the transfer reaction in peptide bond formation, suppressed electrolyte leakage and tissue collapse. In contrast Lallyett (1977) states that cycloheximide did not inhibit HR in bean.

Current concepts of HR to phytopathogenic bacteria are summarised in Figure 3.



a) Unstable HR inducing factor dependent upon de novo bacterial protein synthesis reaches resistant host protoplast.

b) Production of factor(s) by de novo host protein synthesis during the latent period which cause cell collapse.

Figure 3 Summary of the current concepts of HR induction and presentation in response to phytopathogenic bacteria

Pseudomonas phaseolicola and French bean (Phaseolus vulgaris)

P. phaseolicola causes Halo blight of French beans. The organism is seed borne and planting of infected seed is thought to be the most important source of new infection (Taylor, 1970a). Infection of surrounding healthy plants occurs via rainsplash of bacterial ooze which issues from stomata or wounds on an infected plant (Agrios, 1978).

All cultivars of French bean tested by Burkholder and Zaleski in 1932 were susceptible to two American and one European isolate of

P. phaseolicola. In 1942 Jensen and Goss reported that cultivars Red Mexican and Schwert were resistant to halo blight caused by P. phaseolicola. In 1955 Fergusson et al. reported that Red Mexican grown in Ottawa, Canada was susceptible. This confused situation was resolved when in 1964 Patel and Walker identified two races of the pathogen, Race 1 and Race 2. Race 1 isolates were avirulent on the resistant cultivars whereas Race 2 isolates were virulent.

Resistance of Red Mexican to Race 1 isolates of P. phaseolicola is monogenic and dominant (Taylor, 1978). Red Mexican is used as differential host in reacting hypersensitively to Race 1 isolates of P. phaseolicola, whereas under suitable conditions Race 2 isolates cause the susceptible reaction.

Recognition, specificity and binding

"A cell that reacts in a special way in consequence of association with another must do so because it acquires "information" from that other, information that must be conveyed through chemical or physical signals. Phenomena of this kind are termed "recognition phenomena" and their examples in nature are manifold" (Heslop-Harrison, 1978). Four ways in which recognition phenomena are mediated have been recognized :-

a) Receipt of diffusible signals from one cell or tissue by the receptors of another cell or tissue, for example aggregation in the cellular slime moulds in the transition from myxamoebae to a plasmodium; pheromones in sexual reproduction in algae (Chlamydomonas) and fungi (Mucurolales) (Stegwee, 1976).

b) Cell wall contacts, for example opposite mating types of heterothallic yeasts agglutinate when mixed in suspension cultures, after which tubes are developed establishing continuity of the

cytoplasms (Wickerham, 1958; Yanagishima et al.,1976). Other examples are incompatibility systems in flowering plants, where interactions are mediated via pollen/stigma interactions (reviewed by Heslop-Harrison, 1975).

c) Contact between cell membranes, for example gamete fusion in Chlamydomonas is mediated by the membranes of the flagellar tips (Wiese, 1974), and fertilization in Fucus is determined by surface carbohydrates on the membrane of the ovum interacting with complementary carbohydrate binding proteins on the membrane of the spermatozoan (Callow et al., 1978; Bolwell et al., 1979). Morphogenesis in slime moulds appears to be mediated by lectin-like molecules able to bind with complementary saccharide groups of glycoproteins on the surfaces of partner cells (Heslop-Harrison, 1978).

d) Cytoplasmic contacts, for example plasmogamy in the fungal genus Coprinus, in which fusion of hyphae is a random process and can occur between different species; however when certain pairs of species interact death of the hybrid cytoplasm occurs, such that karyogamy does not occur (Ibid).

To the above examples can be added :- recognition of innumerable antigenic substances highly specifically by the mammalian immune system, interactions between cells and viruses - both animal viruses and bacteriophages attach specifically to surface components of host cells. Hormones and toxins often have cell surface associated sites of interaction. Recognition phenomena are shown by the species-specific reaggregation of separated cells of certain sponges and by the reaggregation of separated cells of certain embryonic tissues. For more details and examples see Callow (1977).

In a recent review Sequeira (1978) suggests that specificity in plant diseases depends on recognition mediated by the interaction of complementary macromolecules present at the surfaces of the two organisms, and that recognition results in a host response that either prevents or facilitates multiplication of the potential pathogen.

Specificity in plant diseases is apparent at many levels, from pathogens with extremely wide host range for example Phymatotrichum omnivorum which can infect more than 2000 plant species, pathogens restricted to a single host species to where physiologic races of the pathogen occur which are restricted to a few genotypes of a host species (Brian, 1976). At these higher levels of specificity the outcome of the interaction between host and parasite appears to be established very soon after contact between pathogen and potential host. For many fungal infections, e.g. Phytophthora infestans and potato the hypersensitive response is established very soon after germ tubes or haustoria come into contact with host tissues (Tomiyama, 1967). For many rusts, however, resistance/susceptibility seem to be controlled at a number of "switching points" during the penetration and early infection process, e.g. with Uromyces phaseoli var. vignae and cowpea (Heath, 1974). Many of these highly specialized interactions appear to be controlled by the gene for gene system proposed by Flor (1971) in which the host has a number of resistance genes and in order to induce the susceptible reaction in the host, the pathogen must have each of the corresponding virulence genes. Resistance genes and genes for avirulence are often dominant, i.e. resistance and avirulence are properties due to functional gene products whose interaction (recognition event) leads to a resistant response. On this hypothesis alteration of the avirulence gene to a gene for virulence is a change after which the gene product is no longer able to interact with the product of the host's resistance gene. That is recognition goes with resistance.

The products of genes for resistance or virulence/avirulence have not been identified, but it is worthwhile to consider which chemical classes of substances may have the properties necessary for mediators in these interactions. They will be macromolecules with variable enough structures to account for the many interactions, and likely candidates are proteins,polysaccharides, either alone or with protein or lipid. It has been calculated that a polysaccharide of three different sugar residues allows for over 1000 forms by permutation of the sugar sequences and differences in the pattern of branching (Heslop-Harrison, 1978). One class of proteins which may be important in these interactions are the lectins. Liener (1976) proposed that the term "lectin" be used as a generic term to denote all sugar-specific proteins or glycoproteins, whatever their origin. Particular lectins bind specifically to particular sugar residues or groups of residues, and where they are di- or multivalent (i.e. have more than one binding site) and when the specific sugars are those found on the surfaces of erythrocytes,then these are agglutinated. Since lectins were first isolated from plants they were called "phytohaemagglutinins". For a more complete treatment of lectins, their distribution, biological effects and properties see Lis and Sharon (1973), Liener (1976), Sharon (1977) and for implied roles in plant-microorganism interactions the reviews by Callow (1977), Sequeira (1978) and Etzler (1981).

A number of different systems have been proposed for interactions in gene for gene systems :-

1. The genes may code for complementary macromolecules located at the interacting surfaces of the host and pathogen.

a) Directly - by giving rise to a protein with lectin-like properties (Callow, 1977) or a specific glycosyl transferase enzyme, which may be surface localized and will recognize and bind certain terminal saccharide residues of surface

carbohydrates (Roseman, 1970; Albersheim and Anderson-Prouty, 1975).

b) Indirectly - for example a specific glycosyl transferase might be coded for, whose activity results in a carbohydrate with a novel sequence of saccharide units or terminal groups which can be recognized by a complementary macromolecule in the other partner.

One view is that the products of avirulence genes are so called "elicitors", which interact specifically with receptors in the host, this interaction then leading to induction of phytoalexin synthesis which limits spread of the potential pathogen. This model assumes that differential accumulation of phytoalexins is the most important determinant of resistance, and this may be an oversimplification (Dixon and Lamb, 1980). With only one exception race specific high molecular weight glycoproteins solubilized from cell envelopes of Pseudomonas glycinea by detergent showed the same specificity in eliciting accumulation of the phytoalexin glyceollin in cotyledons of two varieties of soybean as the original parent races (Brugger and Keen, 1979). A glucan has been isolated from the cell walls of Phytophthora megasperma var. sojae that is an extremely potent elicitor of glyceollin accumulation in soybean seedlings. However the elicitor can be extracted from both compatible and incompatible races of the fungus, and thus lacks the specificity expected for the product of an avirulence gene (Ayers et al., 1976).

In the systems proposed above recognition leads to incompatibility.

2. The genes may code for a toxin/toxin receptor system. Where a host selective toxin is produced, recognition would result in susceptibility rather than resistance. It follows that resistance

and avirulence would be recessive traits. This appears to be the case for some systems, for example introduction of a gene for resistance to crown rust into oat cultivars rendered them very susceptible to toxin producing isolates of Helminthosporium victoriae. Toxin production is determined by a single dominant gene (Scheffer, 1976).

3. Vanderplank (1976, 1978) suggested that the products of resistance genes are enzymic and may be associated with wound responses. He suggested the protein products of the pathogen's virulence genes were able to dimenize with the products of the corresponding host resistance genes. Thus, disarming the plants defensive response and providing a source of food for the pathogen which would not interfere with the normal metabolism of the host. Since most gene for gene diseases have biotrophic association of host and parasite at least in the early stages of infection, the latter point is very important. In this system recognition, that is the specific dimerization of complementary macromolecules, would be associated with compatibility.

Attachment of bacteria to plant surfaces

a) Rhizobium-legume interactions

Rhizobia are very specific to their legume hosts and are classified on this basis, for example Rhizobium trifolii forms root nodules with clover (Trifolium repens) but not other plants.

Infection occurs via the root hairs and involves initial attachment of the rhizobia to the tips. The suggestion that plant lectins were involved in this attachment was made by Hamblin and Kent (1973), who reported that bean lectin combined specifically with R. phaseoli. Bohlool and Schmidt (1974) found that a lectin from soybean (SBL) bound to 22 out of 25 strains of Rhizobium capable of nodulating the host, but did not bind

to any of 23 non-nodulating strains. However, Chen and Phillips (1976) on testing 7 strains of incompatible rhizobia found that SBL bound to 5. However, in this case specificity is probably determined at a later stage in the infection process.

White clover root hair cells and Rhizobium trifolii have surface antigens which are cross reactive to antibodies raised against them (Dazzo and Hubbell, 1975). Trifoliin, a lectin from clover roots, binds to both root hair and bacterial cell surfaces, presumably via the common antigens. It is postulated that the lectin forms cross-bridges which bind the rhizobia to the root hairs. Trifoliin did not bind to the roots of alfalfa, trefoil, vetch and other legumes. This work has been recently reviewed by Dazzo (1980).

There is a controversy over the nature of the carbohydrate receptor for lectin binding on rhizobia. An acidic polysaccharide of the extra cellular polysaccharide material (EPS) was suggested by Dazzo and Hubbell (1975). The "O" antigen region of lipopolysaccharide (LPS) was suggested by Wolpert and Albersheim (1976), and a glycan which is not a component of EPS or LPS was proposed by Planque and Kijne (1977). It seems likely that different receptors mediate binding in different systems.

Writing on the suggestion that lectins are primary determinants in the specificity shown by rhizobia toward legumes Sequeira (1978) concluded that sufficient exceptions were known to question the validity of the models proposed.

b) Erwinia amylovora in apple

Huang et al. (1975) reported that avirulent cells of E. amylovora were agglutinated in xylem vessels of apple shoots by granular particles. The particles were produced by adjacent xylem parenchyma in response to avirulent cells and were not observed with virulent inocula. Agglutination prevented spread of the pathogen. The avirulent strain had moved no more

than 5 mm from the inoculation site after 72h, while virulent cells had moved up to 120 mm. Goodman (1978) reported that the agglutinating substance from apple shoots was a glycoprotein and suggested it could have lectin-like properties. The avirulent strains of E. amylovora produced minimal amounts of EPS, and it was suggested this made the cells more easily agglutinated or more effective at inducing agglutinin production than the EPS covered virulent cells.

Buddenhagen and Takata (1969) reported a similar phenomenon in which avirulent cells of P. solanacearum were trapped by globular material in vessels in banana roots. In vivo localization of Xanthomonas oryzae in rice leaf xylem vessels was reported by Horino (1976).

c) Pseudomonas pisi in tobacco

20 minutes after infiltration of the incompatible pathogen P. pisi into tobacco leaves, what was presumed to be a thin internal cuticle began to blister and rupture where bacterial cells were close to it. The "cuticle" became progressively thicker as more material was added to it and after 6h a well developed wall "apposition" of fibrillar material ensheathed the bacterial cells. Membrane bound vesicles originating from the convoluted plasma membrane appear to carry and deposit the microfibrillar materials that form the cell wall appo sition. Externally the typical early symptoms of HR were apparent (Goodman et al., 1976a, b; 1978; Goodman and Politis, 1978). The saprophyte P. fluorescens induced the separation of wall "cuticle" which appeared to envelop the bacterial cells, but the ensheathment did not become as heavy as with P. pisi. The compatible pathogen P. tobaci was loosely attached by fibres, apparently of host origin, but was not ensheathed in a "cuticle".

Scanning electron micrographs of cells of P. pisi enveloped in fibrils on tobacco callus tissue were published by Huang and Van Dyke (1978).

d) Pseudomonas solanacearum in tobacco

Sequeira et al. (1977) reported that avirulent cells of P. solanacearum readily attached to the walls of tobacco mesophyll cells. By 4h after infiltration the attached bacteria were enveloped by a "pellicle" consisting of fibrillar and granular material extruded from the host cell walls. Below the point of attachment the host plasma membrane was convoluted and membrane bound vesicles were produced. The typical symptoms of HR had developed. In contrast cells of virulent strains did not attach to cell walls, or give rise to ultrastructural changes in the first 12h after infiltration. Heat killed cells of an avirulent strain attached in the same manner as living cells, and there were initial stages of envelopment. Subsequently infiltrated living cells did not attach and HR did not develop. The authors suggested that prevention of HR was related to lack of attachment of the avirulent cells to host cell walls. Sequeira (1978) concludes that although initial recognition/attachment of bacteria must occur very rapidly, envelopment of bacteria was dependent upon increased synthesis of plant cell wall components.

A potato lectin was shown by Sequeira and Graham (1977) to agglutinate 34 avirulent but did not, or only weakly agglutinate 55 virulent isolates of P. solanacearum. All the virulent isolates possessed EPS whereas avirulent isolates did not, and removal of EPS by repeated washing and centrifugation rendered virulent cells agglutinable by the lectin. Agglutination of avirulent, or washed virulent cells could be prevented by addition of EPS before the lectin. A lectin with identical properties was isolated from tobacco leaves and roots.

Sequeira (1978) suggests that readily soluble EPS diffuses into the medium surrounding the invading bacteria, occupies binding sites of the host lectin, thus leaving the bacterium free to multiply in the intercellular spaces. However, he goes on to say that there are EPS producing incompatible strains of P. solanacearum which attach to tobacco cell walls, cause HR

and are unable to multiply.

Sequeira and Graham (1977) suggest that part of the lipid A portion of bacterial LPS in rough, avirulent isolates binds to the lectin, since rough strains of P. solanacearum lack the "O" antigen component of LPS but have the lipid A component, and isolated lipid A readily precipitates with potato lectin.

e) Agrobacterium tumefaciens

A tumefaciens causes crown gall in many dicotyledonous hosts. Lippincott and Lippincott (1969) showed that certain avirulent strains of Agrobacterium as well as killed cells of virulent strains, when pre-inoculated or simultaneously inoculated with virulent strains into bean leaves could inhibit tumour formation. If added only 15 min after inoculation with virulent cells no inhibition occurred. Cells of other bacterial genera were non-inhibitory, suggesting a degree of specificity between the host and pathogen in this response. From these data the authors proposed that an initial step in Agrobacterium infection was the attachment of the bacterium to a site in the host exposed by wounding.

The part of the bacterial surface involved in attachment was shown to be part of the LPS (Whatley et al., 1976). LPS inhibits tumour formation when pre- or simultaneously inoculated into leaves. Concentrations as low as 1 ng cm^{-3} were sufficient to inhibit tumour induction by 35% compared to controls. LPS from non-inhibitory strains of Agrobacterium had no effect on tumour induction by virulent strains. It was recently reported that the O-antigen portion of LPS was as inhibitory as the intact molecule, whereas the lipid A portion was non-inhibitory. It was not known which part of the O-antigen region was involved (Dazzo, 1980; Lippincott and Lippincott, 1980).

The nature of the host attachment site was investigated by Lippincott et al. (1977) and Lippincott and Lippincott (1977, 1978). They showed that host cell wall fragments attached to bacteria and blocked binding to

wound exposed sites in the host necessary for tumour induction. Host cell membrane preparations were inactive. Cell wall fragments from a number of dicotyledonours hosts tested were all able to bind bacteria whereas those from 10 monocotyledonous plants were not. Since monocots rarely develop crown gall, whereas most dicots are susceptible it was suggested that the presence of binding sites in the cell wall could determine the host range of the pathogen. Cell wall fragments from embryonic and tumourous tissues were unable to bind cells of Agrobacterium. Polygalacturonic acid, sodium polypectate, "pectic" polysaccharide and arabinogalactan all inhibited tumour formation. Polygalacturonic acid was a very potent inhibitor, giving total inhibition of tumour formation at 10 mg cm^{-3} and 50% inhibition at 1 µg cm^{-3}. Pueppke and Benny (1981) reported that carbohydrates containing uronic acid residies, including several thought to competitively inhibit bacterial attachment to the host, greatly inhibited tumourigenesis in potato discs, as did a crude "pectin" preparation from potato tubers. The ability of cell wall fragments to bind Agrobacterium is related to the methyl ester content since treatment with pectin methyl esterase increases the inhibitory effect, whereas pectin methyl transferase decreases the inhibitory effect. Pectic polysaccharides from monocots and embryonic tissues have a high methyl ester content, and cell wall fragments from these normally inactive tissues treated with pectin methyl esterase become inhibitory to tumour formation.

Various plant lectins were all found non-inhibitory to tumour formation, and a recent review (Lippincott and Lippincott, 1980) stated that no evidence for lectin participation in adherence of Agrobacterium to its hosts had been found, and a polysaccharide-polysaccharide recognition event was suggested by all the available evidence.

f) Xanthomonas sp.

Another example of specific carbohydrate-carbohydrate interaction between a plant pathogen and its host was reported by Morris et al. (1977) and Dea et al. (1977). Extracellular polysaccharides of Xanthomonas exist in ordered configuration in solution and bind strongly to unsubstituted plant glycans to form a stable gel. It was suggested that this interaction may be a major determinant of Xanthomonas specificity. Polysaccharides produced by other micro-organisms e.g. Erwinia carotovora do not maintain ordered configuration in solution and do not form stable gels.

g) Attachment of bacteria to cucumber leaves

Leben and Whitmoyer (1979) reported non-selective adherence of phytopathogenic and other bacteria to the outer surfaces of cucumber and other leaves. They suggested this may be of ecological significance for the survival of bacteria in natural environments. Electron micrographs of bacteria binding to cells released by grinding from the leaf interior were presented. Ruthenium red staining showed polymeric material on the bacterial surface, associated with material at the plant cell surface. Ruthenium red stains negatively charged anions, and has been used as a stain for bacterial extracellular polysaccharide (EPS) (Politis and Goodman, 1980) and pectic polysaccharides (Rougier, 1970). Possibly these substances are involved in the binding to internal surfaces. The external surfaces however are bound by a waxy cuticle, so a different interaction may be inferred.

h) Attachment of bacteria to cell walls of bean

Sing and Schroth (1977) reported attachment and active encapsulation of the saprophytic bacterium Pseudomonas putida in intercellular spaces of Phaseolus vulgaris cv. Red Kidney. Cells of P. phaseolicola virulent on

Red Kidney and cells of P. tomato avirulent on bean were not attached or encapsulated. Leaves responded to P. tomato with a typical hypersensitive reaction. Numerous bacteria were observed in contact with the plant cell walls, but did not appear to be attached. This contrasts with the work of Sequeira with P. solanacearum and tobacco (see earlier in this review) and Stall and Cook (1979) with Xanthomonas vesicatoria, who concluded that attachment of bacteria to host cell walls was necessary for induction of the hypersensitive reaction. Roebuck et al. (1978)showed the attachment of cells of an avirulent Race 1 isolate of P. phaseolicola to cell walls in the intercellular spaces of P. vulgaris cv. Red Mexican.

Hildebrand et al. (1980) reported that envelopment of bacteria in leaves of P. vulgaris cv. Pinto was a purely physical process. They reported no difference between responses to compatible or incompatible strains of P. syringae, to the incompatible pathogens, P. marginalis, P. pisi, and P. tomato, or to the saprophyte P. fluorescens. This contrasts with earlier work (Hildebrand and Schroth, 1968) which reported recovery from inoculated cowpea leaves of c. 0.01 - 0.1% of the initial inoculum of saprophytes P. putida and Serratia marcescens compared to c. 1.0% recovery of the pathogen P. syringae, using watersoaking and centrifugation to collect the intercellular fluid. This difference was maintained even when mixed inocula of saprophytic and pathogenic bacteria were used. The results suggest the leaves can distinguish in some way between cells of the saprophyte and cells of the pathogen.

The physical mechanism they propose is that water infiltrated into the bean leaves dissolves materials from the cell wall surfaces, transpiration causes intercellular fluid to recede until amorphous and/or fibrillar films, condensing from the dissolved material form at air-water interfaces, trap the bacterial cells. Cells of both pathogens and saprophytes remained suspended in the intercellular fluid and did not attach to cell walls if the leaves were kept watersoaked for 12h after infiltration. They state

that "bacterial attachment should not be prevented by the presence of water if this is an active process such as that evidenced by the attraction (sic) of Agrobacterium to plant cells (Lippincott et al., 1977) or root hairs (Schroth et al., 1971)." They appear to be confusing two distinct events - attachment which is suggested as the recognition event, and would therefore be expected to be passive, and envelopment (which no one has suggested occurs with Agrobacterium) which was regarded by other authors e.g. Sequeira and Goodman as the active host response to the passive recognition.

There are numerous reports of extracts from bean tissues which agglutinate bacterial cells. Possibly the substances causing agglutination are important in binding bacteria to cell walls in intercellular spaces.

Anderson and Jasalavich (1979) reported a protease and trypsin stable agglutinin in water extracts of leaves of P. vulgaris cv. Dark Red Kidney. Cells of the saprophytes P. fluorescens and P. putida but not cells of the pathogen P. phaseolicola or P. syringae were agglutinated. Agglutination of cells of P. tabaci was variable. A divalent metal ion was required for activity.

A different agglutinin was released by treatment of cell walls with 0.3M NaCl. This agglutinin was protease and trypsin labile and sensitive to the presence of metal ions. Variable agglutination of both saprophytic and pathogenic pseudomonads occurred. Using commercially obtained cell wall polysaccharides not extracted from bean they showed that "pectin" and galacturonic acid agglutinated cells of both saprophytic and pathogenic pseudomonads, whereas mannan, xylan and galactan solutions were inactive.

Slusarenko and Wood (1981) reported a fraction extracted from cotyledons of P. vulgaris cv. Red Mexican which differentially agglutinated cells of Race 1 and 2 isolates of P. phaseolicola; cells of avirulent Race 1 isolates were agglutinated more actively than cells of virulent Race 2 isolates.

El-Banoby and Rudolph (1980) reported an extract from leaves of a halo blight resistant breeding line "02" which agglutinated cells of Race 1 and 2 isolates of P. phaseolicola. Extracts from the susceptible cultivar Red Kidney did not cause agglutination.

i) Adhesion of rumen bacteria to plant cell walls

There appears to be a selective adhesion of some of the species of the rumen microflora to the plant solids in the rumen. As much as 70% of the microbial biomass in the rumen can be associated with particulate material. The subject was recently reviewed by Latham (1980).

The adhesion process

Due to their small size most bacteria behave as colloidal particles (Marshall, 1980). The bacterial cell surface and most of the substrates with which they come into contact in nature (certainly plant cell walls) are negatively charged (Fletcher et al., 1980). In aqueous environments a charged surface will tend to collect around itself oppositely charged ions, giving what is known as the "electrical double layer" (Figure 4). This effect plays an important part in colloidal stability as outlined by the DLVO theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948).

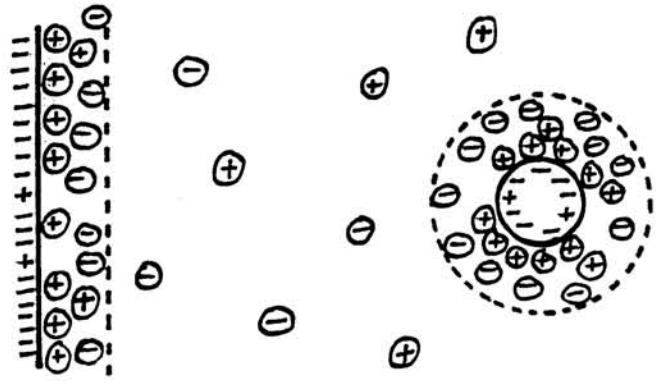


Figure 4 The electrical double layer associated with a planar surface and a spherical particle. The distance from each surface to the dotted line represents the thickness of the diffuse double layer

When two surfaces of similar charge approach, there is a tendency for mutual repulsion. However, van der Waals and London attraction energies tend to counteract the double layer repulsion energies. The degree of attraction and repulsion is affected by the concentration and valency of electrolytes in the bathing solution. Marshall et al. (1971) referred to this phenomenon as the phase of reversible sorption, since the bacteria are held at a finite distance away from the substratum by close distance repulsive forces, and are easily removed by jets of water or possibly flagellar action.

These early electrical effects are fairly non-specific, although obviously inherent differences in surface charge distribution and concentration will give rise to differences in the way various bacteria interact with the same surface and vice versa.

For the bacterium to form a more stable attachment to the substrate the repulsion energy barrier must be overcome. Heckels et al. (1976) studied adhesion of Neisseria gonorrhoea to human cells and observed that pili on the surface of the bacteria conferred an advantage for adhesion purposes when an electrostatic barrier existed. They suggested that pili might facilitate adhesion by providing an extension of the cell wall with a small tip radius which would only suffer a small degree of electrostatic repulsion as it approached the substrate (Pethica, 1961). Pili are structurally organized fibres of high molecular weight polymers extending away from the cell wall. Tenney and Stumm (1965) and Busch and Stumm (1968) showed that synthetic non-ionic and anionic polymers were able to bridge between bacteria under suitable conditions of pH and ionic strength. This suggests that fine polymer fibrils can overcome the repulsive force in some way. In a similar way extracellular polymers produced by bacteria provide a means for bridging to the substrate, and may interact with complementary structures giving rise to the specific attachment so far found only in organism-organism interactions (Marshall, 1980).

Interaction between the surface polymers can occur in two ways :-

a) by mutual exclusion of incompatible molecules to give an increased local concentration of both polymers,

b) by energetically favourable associations of structurally compatible chain segments, as occurs in the interaction of Xanthomonas extracellular polysaccharides with unsubstituted glycans of the host plant cell wall (Dea et al., 1977; Morris et al., 1977).

Non-specific attachment, as seen in some marine pseudomonads attaching to non-living surfaces (Fletcher, 1980), is achieved when the bridging polymer has no complementary interaction, but acts as a simple adhesive "gluing" the cell to the surface. This involves both energy production and protein synthesis by the bacteria and is thus an active process.

The Gram-negative bacterial cell surface

In Gram-negative bacteria the peptidoglycan layer which gives the cell its shape and osmotic stability due to its mechanical strength is not exposed to the outer environment. It is covered by the outer membrane which is made up of proteins, phospholipids and lipopolysaccharides (LPS). The phospholipids form the matrix in which the protein and LPS are embedded (Figure 5).

There are few major proteins present in the outer membrane, however, "porins" are present. These proteins form hydrophilic channels and have a relatively non-specific role in the passage of small molecules across the membrane. Lipoprotein, which attaches the outer membrane to the peptidoglycan layer is also present. Minor proteins include those involved in high affinity transport of nutrients and those which form attachment sites

for certain bacteriophages and colicins. Eshdat et al. (1978) reported the isolation of a mannose specific lectin from the surface of E. coli which was involved in adhesion of the bacteria to epithelial cells.

Lipopolysaccharides which are complex molecules with molecular weights over 10000 form a major part of the outer surface. They vary widely in chemical composition within and between different Gram-negative groups. The structure of LPS from Salmonella spp. has been worked out in great detail (Figure 6). The molecule can be divided into three distinct regions. The O side-chain composed of repeating units of neutral sugar tetramers or pentamers may bear O-acetylated groups and extends up to 150 nm from the wall surface (Shands, 1966). The O side-chain is joined to the glucosamine containing lipid A region by an oligosaccharide containing in the inner region two sugars unique to LPS - L-glycerol-D-mannoheptose and 2-keto-3-deoxyoctonate (KDO). The lipid A region anchors the LPS into the outer membrane. Meadow (1975) reported that in P. aeruginosa as in the Enterobacteriaceae the LPS contained a region common to all strains, but that there were strain specific side chains.

Bacterial capsules or slime layers can be broadly classified into three types (Ward and Berkeley, 1980) :- a loose association of extracellular polysaccharide (EPS) easily removed by centrifugation; microcapsules not visible under the light microscope and lastly macrocapsules easily visualized under the light microscope by suspending the cells in Indian ink. Most EPS is made up of more than one sugar and uronic acid and other negatively charged substituents are often present. Some streptococci produce large amounts of hyaluronic acid which contains N-acetyl glucosamine. However, this does not prevent induction of the formation of antibodies to seemingly underlying wall components. This suggests that either the capsule does not cover the entire surface and "underlying" components are exposed through gaps, or that the surface antigens are long enough to extend beyond the capsule. This may apply to antigenic determinants of other capsulated

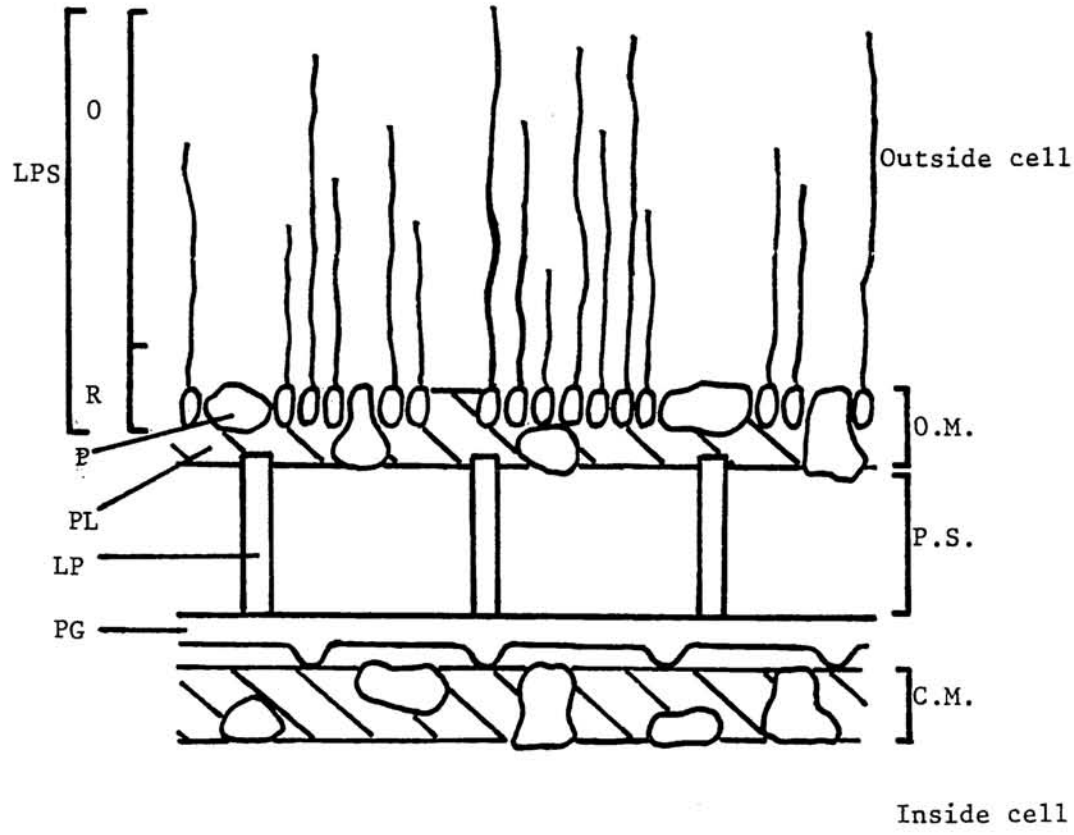


Figure 5 The currently accepted model for the arrangement of the major components in the outer layers of Gram-negative bacteria. (C.M., cell membrane; LP, lipoprotein- LPS, lipopolysaccharide; O, O-region; O.M., outer membrane; P, protein; PG, peptidoglycan; PL, phospholipid; P.S., periplasmic space; R, R-core)(not to scale)

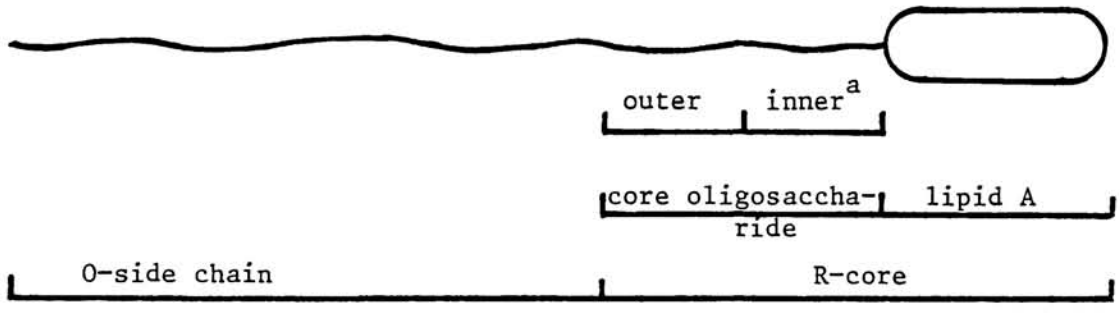


Figure 6 Subdivisions of lipopolysaccharide molecule (not to scale)
a the inner region contains two sugars unique to LPS-L-glycerol-D-mannoheptose and 2-keto-3-deoxyoctonate (KDO)

organisms (Ibid).

Surface structures such as pili and flagellae may occur. They are composed of protein, are anchored in the cytoplasm and traverse the membrane and wall to the exterior of the cell. Pili are straight threads from 4 - 35 nm in diameter and up to 1 - 2 μm in length. They are reported mainly in enteric bacteria, but they also occur in many Gram-negative genera and have been reported in Pseudomonas spp. (Ottow, 1975). There are two groups of pili. Those in Group 1 are regarded as adhesive organelles; pili from E. coli, Salmonella typhimurium, Klebsiella and Proteus spp. have been reported to adhere to fungal and plant cell walls (Ibid). Group 2 pili are termed sex pili and are involved in bacterial conjugation. They may also serve as receptors for male specific bacteriophages.

Flagellae are helical threads 12 - 18 nm in diameter and a few μm in length.

The plant cell wall

The major fractions making up the plant cell wall are defined in terms of the method used to extract them.

The pectic fraction is extracted by hot water, dilute hydrochloric acid or treatment with Ca^{++} chelating agents such as ethylenediaminetetra-acetic acid (EDTA) or citrate-oxalate buffer. Various heteropolymers make up what are known as pectic polysaccharides. A major component is a rhamnogalacturonan to which neutral side chains are attached (Talmidge et al., 1973). A proportion of the D-galacturonic acid residues are present as carboxymethyl esters. Various proportions of other sugars are usually present, such as; D-galactose, L-arabinose, L-fucose. Other sugars for example 2-O-methyl-D-xylose and 2-O-methyl-L-fucose have been reported as very minor constituents of pectic polysaccharide from some sources (Aspinall, 1970a). Fractional precipitation of a pectic polysaccharide extract from apple gave neutral and acidic polysaccharide fractions (Barrett and Northcote,

1965). The acidic fraction was a pectinic acid containing galacturonic acid, arabinose, galactose, rhamnose, xylose and certain trace sugars. A pectinic acid is defined as a pectic polysaccharide in which a proportion of galacturonic acid residues are esterified with methanol, whereas a pectic acid is devoid of ester groups (Aspinall, 1970b). It was suggested by Jarvis et al. (1978) that when applied to primary plant cell walls the term "pectic substances" should be used to denote polysaccharides based on a rhamnogalacturonan core irrespective of the extraction method. 60% of the rhamnogalacturonan from potato is extracted only after treatment under mild alkaline conditions (0.1M Na_2CO_3 at 4^oC). A suggested structure for primary cell walls of suspension cultured sycamore cells showing the relationship of pectic polysaccharide to other wall components was proposed by Keegstra et al. (1973).

Fibrillar modified polygalacturonic acid has been reported to occur outside, between and sometimes within the cell walls of suspension cultured cells of Daucus, Ipomea and Phaseolus (Colvin and Leppard, 1973; Leppard et al., 1971). It was suggested the fibrils may serve as a filler material within cell walls and serve to cement cells together, also as a carrier of ligninprecursors and a "promoter of symbiosis" (sic).

The plant cell wall residue left after extraction of the pectic polysaccharides, on treatment with 4% w/v NaOH gives the hemicellulose A fraction. Subsequent treatment of the residue with 16% w/v NaOH gives the hemicellulose B fraction. When no lignin is present, the residue left after this treatment is the cellulose fraction.

The hemicelluloses are principally made up of xylans, gluco- and galactomannans with arabinogalactans present in some cases.

A hydroxyproline rich glycoprotein termed extensin by Lamport (1965) makes up a major part of the protein content of primary cell walls. Many of the hydroxypoline residues carry glycosidically attached arabinose

complexes (Heath and Northcote, 1971; Keegstra et al., 1973) and seryl residues may carry arabinogalactan complexes (Keegstra et al., 1973). Enzymic proteins are present in cell walls for example pectin methyl esterase, ascorbic acid oxidase, phosphatase and invertase (Lamport, 1965). A glycoprotein with lectin like properties was present in the cell walls of P. vulgaris hypocotyls, tomato stems and suspension cultured sycamore cells (Albersheim and Anderson, 1971). β-lectins with specificity for β-glycosidic linkages were reported to be present in cell walls and intercellular spaces of legume cotyledons (Clarke et al., 1975).

In land plants the external surfaces are covered with a layer of waxy material called the cuticle, which restricts water loss from the aerial organs. The cuticle has been shown to extend as a much thinner layer into the substomatal space (Martin and Juniper, 1970; Scott, 1949 - 50). Goodman et al. (1976a) suggested that material they observed lining their cells as a thin osmiophilic layer contiguous with the middle lamella was an internal cuticle. However, they did no chemical tests to define the nature of the material. Sargent (1976) reports osmiophilic fibrils of pectic material extending through the external cuticle, and believes (personal communication) that the material observed by Goodman et al. may itself be pectic in nature.

MATERIALS AND METHODS

1. Biological

A. Higher plants

a) Cultivars

Seeds of *Phaseolus vulgaris* cv. Red Mexican UI 34, cv. GN Nebraska No. 1 selection 27 and cv. PI 150414 were kindly supplied by Dr J D Taylor (National Vegetable Research Station, Wellesbourne) and multiplied by the Botanical Supply Unit, University of London. Seeds of *P. vulgaris* cv. Canadian Wonder, *P. mungo*, *Vicia faba* cv. The Sutton and *Pisum sativum* cv. Onward were obtained from Sutton & Sons Ltd., Reading.

b) Growth

All seeds of *Phaseolus* spp. were soaked on moist tissue paper for 24h at 25°C before potting in John Innes No. 2 potting compost and kept in a glasshouse with incandescent mercury vapour lighting on a 16h day length cycle giving 4,290 lux at plant level. Seeds of *P. sativum* and *V. faba* were soaked for 3d at 25°C before potting. The age of seedlings is given from the time at which soaking was started. In some experiments seedlings were removed from the glasshouse and kept in a growth cabinet at *c.* 20°C supplemented with fluorescent lighting on a 16h day length cycle giving 4,200 lux at plant level.

c) Inoculation

Bacterial suspensions were injected into the intercellular spaces of leaves with a hypodermic syringe fitted with a No. 30 needle to give a well defined watersoaked area which became normal in appearance again

after c. 1h. The usual site for inoculation was the junction of the lamina with the petiole.

Alternatively bacterial suspensions were forced through stomata on the abaxial leaf surface from a "Quickfit" atomiser by compressed air at a pressure of 0.2 kg cm^{-2} to give a watersoaked area which became normal in appearance again after c. 1h. The usual site for inoculation was the central portion of the leaf lamina, either side of the midrib.

B. Bacteria

a) Cultures

Pseudomonas phaseolicola isolates 31A, 710A (Race 1), 777A and 882 (Race 2) were kindly supplied by Dr J D Taylor (National Vegetable Research Station, Wellesbourne), whose codes are the ones given. P. phaseolicola RI(X) (Race 1), P. mors-prunorum, P. fluorescens, Erwinia atroseptica, E. carotovora, Agrobacterium tumefaciens, A. radiobacter, Corynebacterium fascians, Sarcina subflava came from the culture collection, Imperial College. The Escherichia coli isolate was kindly supplied by Dr M Gasson (National Institute for Research in Dairying, Reading).

b) Growth

E. coli was grown at 37°C on minimal medium M9 (Clowes & Hayes, 1968) containing 100 cm^3 of M9 salts (6.0% w/v Na_2HPO_4, 3.0% w/v KH_2PO_4, 0.5% w/v NaCl, 1.0% w/v NH_4Cl), 20 cm^3 of 20% w/v glucose, 10 cm^3 of 2.5% w/v $MgSO_4.7H_2O$, 10 cm^3 of 0.15% w/v $CaCl_2.2H_2O$, 500 cm^3 of 3.0% w/v "Bacto" agar (Difco), all autoclaved separately for 15 min at 121°C mixed aseptically and brought to a final volume of 1 dm^3 with sterile distilled water. The stock culture was stored at 4°C and subcultured every 3 weeks.

All other bacterial isolates were maintained on slopes of King's B agar (King et al., 1954) at 4°C and subcultured every 3 weeks. King's B agar contains the following :- 2% w/v Proteose peptone No. 3, "Bacto" agar 1.5% w/v, 1.0% w/v glycerol, 0.2% w/v $K_2HPO_4.3H_2O$ and 0.15% w/v $MgSO_4.7H_2O$, autoclaved for 15 min at 121°C and the pH adjusted to 7.2 after autoclaving.

For experimental work, bacteria were grown for 24h at 25°C either on slopes of King's B agar or in King's B broth, prepared as above, leaving out the "Bacto" agar. In some experiments bacteria were grown in nutrient broth containing 13g dm^{-3} Oxoid Nutrient Broth No. 2. Before use, all bacteria were washed by centrifuging in sterile distilled water at 8000g for 10 min, before resuspending the pellet in the required medium for the experiment.

In one experiment P. phaseolicola isolates were grown in a defined liquid medium consisting of 19g glucose, 1 mg biotin, 1.0g NH_4Cl, 0.2g $MgSO_4$, 4.1g KH_2PO_4, 3.6g K_2HPO_4 dm^{-3}. All autoclaved separately at 121°C for 15 min (except the biotin which was sterilized by filtration) then mixed aspetically (Hointink & Sinden, 1970).

All broth cultures were grown for 24h at 25°C in a "Gallenkamp Orbital Incubator" at c. 150 cycles per minute.

Lyophilized stock cultures of P. phaseolicola isolates R1(X),31A, 710A, 777A and 882 were also prepared, and new working stock cultures taken from these at intervals to avoid loss of pathogenicity which might occur through repeated subculturing on agar media. The following method recommended by Dr J D Taylor was used. A 24h culture was washed from a slope of King's B agar with sterile distilled water and centrifuged at 8000g for 10 min. The pellet was resuspended in a freeze drying medium containing 7% w/v peptone and 7% w/v glucose, autoclaved separately for 15 min at 121°C as 14% w/v solutions, then mixed aseptically. Two drops of the suspensions were placed on a cotton wool plug in a glass ampoule,

previously heat sterilized in an oven, then freeze dried overnight using an Edwards "Modulyo" freeze drier, and sealed under vacuum with an oxy-acetylene blowlamp. The lyophilized cultures were subsequently stored at $4^{o}C$.

c) Standardization of inocula

Washed 24h bacterial cultures were adjusted to give readings at suitable points on a nephelometer (Eel "Unigalvo").Serial dilutions were then made and 0.1 cm^3 aliquots spread on to 9 cm diameter petri dishes containing King's B agar. Colonies were counted after 48h incubation of the plates at $25^{o}C$. A calibration plot was made using the data obtained. A reading of 9 on the linear scale was found to correspond to 1.5×10^8 colony forming units (CFU) cm^{-3}.

2. Chemicals

A. General

All chemicals routinely used were "AnalaR" grade, and were obtained from British Drug Houses (BDH) Ltd., Poole, Dorset.

Methyl(methyl-α-D-galactopyranoside) uronate, methyl-D-galacturonate and the series of polymethyl polygalacturonate derivatives used in section C. b) were kindly supplied by Dr S A Archer, Imperial College, London.

B. Pectic enzymes

a) Polygalacturonase enzymes (E.C. 3.2.1.15)

i) "Pectinase" (purified), containing exo- and endo-activity and also pectin methyl esterase (PME activity) was obtained

from Sigma (London) Chemical Co., Kingston upon Thames, Surrey.

ii) Polygalacturonase (PG) II, III and IV

(kindly supplied by Dr S A Archer, Botany Department, Imperial College, London).

11-day culture filtrates from Monilinia fructigena grown on pectate-tartrate medium (1% w/v sodium pectate, 1.5% w/v ammonium tartrate, 0.1% w/v KH_2PO_4, 0.05% w/v $MgSO_4.7H_2O$) was used as source material for the enzymes. The culture filtrate was dialysed against distilled water at $2^{\circ}C$ then concentrated by freeze drying. Enzymes were initially resolved by isoelectric focussing over a pH 3.5 - 10 ampholine pH gradient, which gave four isozymes which were designated PG I, II, III and IV. Fractions containing active peaks were bulked, dialysed against 0.05M potassium phosphate buffer pH 7.0 containing 0.1M KCl and 0.02% w/v sodium azide, then filtered on Sephadex G-100, eluting with the same buffer. Preparation PG III (Archer and Fielding, 1979) contained low activity of PG II (<10%), however both had endopolygalacturonase activity.

The fractions from the isofocussing column containing PG IV were bulked, the pH adjusted to 5.8 and dialysed against 0.05M sodium acetate buffer pH 5.8, and purified by ion exchange chromatography on CM-Sephadex, eluting with a NaCl gradient from 0 up to 0.25M. Fractions containing endo- PG activity were bulked and filtered on Sephadex G-100. The preparation was free of other pecteolytic and plant cell wall degrading enzymes.

Prior to use, PG II, III and PG IV preparations were dialysed against 0.02M sodium phosphate buffer pH 5.8.

b) Pectin methyl esterase (PME) (E.C. 5.1.1.11.)

i) Apple PME (kindly supplied by Dr S A Archer, Botany Department, Imperial College, London).

Acetone powder of apple tissue (cv. Cox's Orange Pippin) was extracted with 1M KNO_3 containing 0.1M potassium phosphate, pH 7.0, filtered and the filtrate centrifuged and dialysed against distilled water. Isofocussing followed by gel filtration on G-100 was carried out as in A ii). Three isoenzymes were detected and the one used in these experiments had isoelectric point 2.7

ii) Orange PME

This was obtained from orange peel which has no PG activity (MacDonnell, Jansen & Lineweaver,1945). The method used was that of Barrett & Northcote (1965). 120g of fresh orange peel was comminuted in 400 cm^3 0.2M sodium acetate at 4°C the pH adjusted to 7.6 and the slurry incubated for 12h at 4°C. The slurry was filtered, centrifuged (19000g for 10 min) and brought to 40% saturation with solid $(NH_4)_2SO_4$, left for 2h, centrifuged (19000g for 10 min), the pellet discarded and the supernatant brought to 70% saturation with solid $(NH_4)_2SO_4$. After a further 2h at 4°C, followed by centrifugation (19000g for 10 min) the pellet was dissolved in 0.1M NaCl in 0.01M sodium phosphate buffer pH 7.0, dialysed against distilled water containing a trace of 2-mercaptoethanol then passed through a column of DEAE cellulose, which had been previously equilibrated with 0.01M tris-HCl buffer pH 8.9. The PME passed through unretarded while almost all the other protein and the orange pigment were adsorbed. The enzyme solution was stored at -20°C until required for use.

c) PME assay (after Kertesz, 1951)

0.5 cm^3 of enzyme sample was added to 5 cm^3 of substrate mixture containing 0.5% w/v pectin (Sigma citrus pectin), 0.2% w/v phenol, 0.58% w/v NaCl, 0.01% w/v bromocresol purple. The pH was adjusted to 6.2 and the mixture incubated at room temperature. A colour change from blue-purple to orange-red within 12h indicates PME activity, the rate of change giving a crude indication of relative activities of different preparations.

PG II, III was found to have low PME activity, apple PME had intermediate activity and orange peel PME was highly active giving a colour change within 30 min. PG IV had little, if any, measurable PME activity.

C. Protease

a) Protease V (Pronase AS) (E.C. 3.4.24.4.)

Obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, is an unusually non-specific protease obtained from Streptomyces griseus.

3. Preparation of plant extracts

A. Seed

Seeds of RM and CW were soaked for 24h on moist paper at 25°C in the dark. The testas were removed and extracts prepared in the following ways:-

a) Lectins

i) pH 4.2 (after Moreira & Perrone, 1977)

100 cm^3 distilled water was added for every 40g fresh weight of

cotyledons which were then comminuted for 2 min in a homogenizer. The mixture was adjusted to pH 4.2 with 0.5M HCl and left for 6 to 8h at room temperature with occasional stirring; it was then centrifuged at 16000g for 15 min and the pellet discarded. The supernatant was brought to 50% saturation with solid $(NH_4)_2SO_4$, left overnight at 4°C, centrifuged at 16000g for 10 min, the pellet discarded and the supernatant brought to 100% saturation with solid $(NH_4)_2SO_4$ and left for 4h at 4°C. The sample was centrifuged at 16000g for 15 min and the surface film taken up in a small volume of 0.005M sodium phosphate buffer pH 6.8 and dialysed against 1 dm^3 of buffer overnight at 4°C with one change of buffer. The dialysate was divided into 3 cm^3 samples and stored at -20°C.

Cotyledons taken from 7-day old seedlings were also extracted in this way.

ii) 0.9% w/v saline

Each 40g fresh weight of 1-day cotyledons was comminuted with 100 cm^3 0.9% w/v NaCl solution; the subsequent procedure was as in a) i).

b) Pectic polysaccharides

i) pH 3.0 (after McCready, 1965)

1-day cotyledons were finely sliced and washed twice for 2 x 30 min with 100 cm^3 acetone for each 40g fresh weight tissue. The tissue was air dried then placed in boiling absolute alcohol, 100 cm^3 for each 40g fresh weight (equivalent) of tissue, for 5 min. The tissue was dried and comminuted with 100 cm^3 of distilled water and adjusted to pH 3.0 with 0.5M HCl. The slurry was heated in a boiling water bath for 30 min and centrifuged at 16000g for 10 min, the pellet discarded and the supernatant mixed with 1.5 volumes of absolute alcohol. The precipitate was collected

and dried at 50°C for 12h, ground with a pestle and mortar, and stored in a desiccator.

ii) EDTA

The procedure was as in b) i) but the tissue was comminuted with 200 cm^3 0.02M EDTA in 0.05M sodium citrate buffer pH 3.0 for every 40g, and the final extract was precipitated with 2 volumes of absolute alcohol.

B. Leaves

a) Acetone powders

2 to 3 week old seedlings were placed in a cupboard in the dark for 48h to destarch the cells. The primary and secondary leaves were comminuted in 300 cm^3 absolute alcohol for each 100g fresh weight of tissue. The homogenate was filtered through a double layer of muslin and resuspended in 300 cm^3 of acetone. The filtration and acetone washing was repeated a further 3 times and the final residue was air dried until the odour of acetone was not detectable. The acetone powder was then extracted in the following ways :-

b) Lectins

i) pH 4.2 (after Moreira & Perrone,1977)

Each 10g of acetone powder was suspended in 100 cm^3 distilled water and adjusted to pH 4.2 with 0.5M HCl. The subsequent procedure was as in A a) i).

c) Pectic polysaccharides

i) pH 3.0

Each 10g of acetone powder was suspended in 100 cm^3 distilled water and adjusted to pH 3.0 with 0.5M HCl and heated in a boiling water bath for 30 min. The slurry was cooled and centrifuged at 16000g for 10 min. The supernatant was precipitated with 1.5 volumes of absolute alcohol. The precipitate was collected and dried at 50°C for 12h, ground with a pestle and mortar, and stored in a desiccator.

ii) EDTA

Each 10g of acetone powder was suspended in 200 cm^3 0.02M EDTA in 0.05M sodium citrate buffer pH 3.0 and heated in a boiling water bath for 30 min. The slurry was cooled and centrifuged at 16000g for 10 min. The supernatant was precipitated with 2 volumes of absolute alcohol. The precipitate was collected and dried at 50°C for 12h, ground with a pestle and mortar, and stored in a desiccator.

iii) Citrate-oxalate buffer, pH 4.0 (after Jarvis et al., 1978)

Each 10g of acetone powder was suspended in 200 cm^3 0.1M citrate-oxalate buffer pH 4.0 and heated in a boiling water bath for 15 min. The slurry was cooled and centrifuged at 16000g for 10 min. The supernatant was precipitated with 2 volumes of absolute alcohol to yield a gelatinous precipitate corresponding to the acidic pectic fraction of Barrett & Northcote (1965). The precipitate was collected and dried for 12h at 50°C. A further 2 volumes of absolute alcohol were added to the supernatant to precipitate the neutral pectic fraction (Barrett & Northcote, 1965), a

powdery precipitate, which was collected and dried for 12h at 50°C. Both precipitates were ground with a pestle and mortar and stored in a desiccator. The neutral pectic polysaccharide ground more easily than the acidic pectic polysaccharide.

iv) 0.1M Na_2CO_3 (after Jarvis et al., 1978)

The residue after citrate-oxalate extraction was resuspended in 200 cm^3 0.1M Na_2CO_3 for each initial 10g of acetone powder, and incubated at 4°C for 30 min. The slurry was centrifuged at 16000g for 10 min and the supernatant was precipitated with 4 volumes of absolute alcohol. The precipitate was collected and dried at 50°C for 12h, ground with a pestle and mortar, and stored in a desiccator.

4. Agglutination assay

Unless otherwise stated the procedure was as follows. Micro-haemagglutination trays with 12 rows of 8 wells were used. Numbering successive wells from 1 to 8,0.05 cm^3 of sodium phosphate buffer pH 6.8 (0.005M for extracts from 3 A, a), and 0.02M for the other procedures) was placed in each except well 2, and 0.05 cm^3 of the test sample was added to wells 2 and 3. The contents of well 3 were mixed and 0.05 cm^3 of the mixture pipetted into well 4 and so on to well 8 from which, after mixing, 0.05 cm^3 was removed leaving a final volume in each well of 0.05 cm^3 with a 50% dilution series from wells 2 to 8. Well 1 was the control without sample. 0.05 cm^3 of bacterial suspension containing c. 1.5×10^{10} cells cm^{-3} was then placed in wells 1 to 8 and thoroughly mixed before removing 0.05 cm^3 to give a final concentration of 0.75×10^{10} cells cm^{-3} in each well. The trays were covered and incubated at 25°C overnight.

In bacterial agglutination assays after periodate oxidation of the sample, the dilution series was prepared with 0.1M sodium acetate buffer

pH 4.7. In haemagglutination assays the test samples were brought to 0.9% (w/v) solution with NaCl and 0.9% (w/v) saline was used as a diluent with a 2% (v/v) suspension of human erythrocytes (group 0, Rhesus positive) in 0.9% (w/v) saline.

5. Protein estimation

A. Folin and Ciocalteu's phenol reagent (after Lowry et al., 1951)

a) Reagents

Solution A - 2% w/v Na_2CO_3 in 0.1M NaOH

Solution B - 0.5% w/v $CuSO_4.5H_2O$ in 1% w/v trisodium citrate

Solution C - was made by mixing 50 cm^3 of A with 1 cm^3 of B just before use

Folin and Ciocalteu's phenol reagent was diluted 1:1.5 with distilled water to make a 1M acidic solution. The quantity of water necessary was determined by titration against 1M NaOH with phenophthalein as indicator.

b) Procedure

5 cm^3 of solution C was mixed with 1 cm^3 of sample solution and incubated at room temperature for 10 min. 0.5 cm^3 of diluted Folin and Ciocalteu's reagent was mixed in and the tubes incubated for 30 min at room temperature in the dark. The absorbance at 500 nm was then measured. The protein concentration was read from a standard plot prepared using known concentrations of bovine serum albumin (Sigma, London).

B. Ninhydrin

3 drops of a freshly made 2% w/v solution of ninhydrin in water were added to 1 cm^3 of test solution and heated in a boiling water bath for 2 min. Protein gives a purple colouration; a yellow colouration suggests the presence of hydroxyproline residues.

6. Ion exchange chromatography

DEAE cellulose (DE 52, Whatman, Maidstone) equilibrated in 0.005M sodium phosphate buffer pH 6.8 was degassed under vacuum and packed into a 30 cm x 1.5 cm internal diameter column. The sample was loaded on to the column in buffer and eluted with a gradient of 0 → 1M NaCl in buffer. The eluent was monitored using an LKB uvicord set at 280 nm for protein.

7. Isoelectric focussing

Isoelectric focussing was carried out using an LKB 110 cm^3 isofocussing column with a 1% ampholine solution over a 1 - 66% w/v sucrose gradient cooled to 4°C. The sample, and the ampholine (pH range 3.5 - 10) were applied with the light solution in the gradient mixer. The cathode solution of 0.014% w/v NaOH in 86% w/v sucrose solution was at the bottom of the column. The anode of 0.014% v/v H_2SO_4 was at the top of the column. The separation was carried out over 3 days with a starting voltage of 300 V. 5 cm^3 fractions were collected from the column, the pH determined using a pH meter, and the absorbance at 280 nm recorded as a measure of protein content.

8. Electrophoresis

A. Polyacrylamide gel electrophoresis (PAGE)

a) disc-PAGE (after Davis, 1964)

i) Reagents

Acrylamide stock - a 30% w/v solution of acrylamide in 0.8% w/v solution of methylene-bis acrylamide. Stored at $4^{o}C$ for up to 1 month.

Spacer gel buffer (SGB) - 5.98g Tris (Tris(hydroxymethyl)aminomethane) dissolved in 40 cm^3 of water then adjusted to pH 6.7 with 1M HCl, then the volume brought up to 100 cm^3 with distilled water.

Resolving gel buffer (RGB)-36.3g Tris dissolved in 48 cm^3 1M HCl, adjusted to pH 8.9 then the volume brought up to 100 cm^3 with distilled water.

Ammonium persulphate (P) - 10% w/v solution of ammonium persulphate (Bio-Rad, electrophoresis purity reagent) in distilled water.

TEMED - NNN´N´-tetramethyl-1,2-diaminoethane. (BDH Laboratory reagent).

Tris-glycine running buffer - 14.4g glycine and 3.0g Tris made up to 500 cm^3 with distilled water, then adjusted to pH 8.3.

Bromophenol blue (BPB) - 0.12% w/v bromophenol blue in 20% w/v sucrose in running buffer.

ii) Procedure

The 10% w/v acrylamide resolving gel was prepared by mixing 10.0 cm^3 of acrylamide stock with 3.75 cm^3 RGB, 0.015 cm^3 TEMED, 16.1 cm^3 distilled water and finally 0.15 cm^3 P. This mixture was then pipetted into 7.5 cm cylindrical gel tubes, internal diameter 0.5 cm, previously rinsed in 2% v/v dimethyldichlorosilane in carbon tetrachloride then dried. 0.5 cm depth of distilled water was then carefully layered on with a syringe to prevent meniscus formation as the gels solidified. The water was removed and a small amount of spacer gel layered on to the resolving gel. The 3% acrylamide spacer gel was prepared by taking 1 cm^3 of acrylamide stock and mixing it with 1.25 cm^3 SGB, 0.005 cm^3 TEMED,7.64 cm^3 distilled water and finally 0.1 cm^3 P.

Electrophoresis was carried out using a Shandon kit at 4°C. The test sample had sucrose added to make a 20% w/v solution and 0.025 or 0.050 cm^3 applied along with 0.010 cm^3 of BPB to the top of the tubes. An initial current of 2mA per tube was applied until the BPB had migrated 2 - 3 mm into the resolving gel, whereupon the current was increased to 4mA per tube. After separation the gels were stained with either Kenacid blue (formerly Coomassie blue) for protein, or periodate acid-Schiff's (PAS) for carbohydrate.

iii) Staining

Kenacid blue - Gels were left overnight in a 0.03% w/v solution in methanol: acetic acid:water, 5:1:5, destained in 10% v/v acetic acid and scanned at 550 nm with a Gilford recording spectrophotometer fitted with a gel-scanning attachment.

PAS (After Zacharius et al., 1969) - Immediately on removal from the glass tubes the gels were nicked with a sharp scalpel at the position of the BPB, since this is decolourized by the staining procedure. The gels were then

immersed in 12.5% w/v trichloroacetic acid for 30 min, then rinsed in distilled water and immersed in 1% w/v periodic acid in 3% v/v acetic acid for 50 min and subsequently washed overnight through several changes of distilled water, using at least 200 cm^3 per gel. The gels were then submerged in fuchsin-sulphite stain in the dark for 50 min. The stain was prepared by making a 4% w/v solution of basic fuchsin in an 8% w/v solution of sodium metabisulphite in 1M HCl. After standing for 4h the faintly pink solution was decolourized using a small amount of acid washed activated charcoal, and the mixture filtered within 15 min. The gels were then washed 3 times for 10 min in freshly prepared 0.5% w/v potassium metabisulphite, then washed in large volumes of distilled water with several changes for 2 - 3 days until destained. The gels were stored in 7% v/v acetic acid and scanned at 520 nm with a Gilford recording spectrophotometer fitted with a gel-scanning attachment.

b) Molecular weight determination by continuous-PAGE

The samples were mixed 1:1 with a disrupting buffer (0.02M sodium phosphate buffer pH 7.2 in 1M sucrose containing 2% v/v 2-mercaptoethanol and 2% w/v SDS) and boiled for 5 min in a water bath to disrupt the quaternary and tertiary protein structure. 0.010 cm^3 BPB in running buffer (RB-0.03M sodium phosphate buffer pH 7.2 containing 0.1% w/v SDS was added for each 0.1 cm^3 disrupted sample. The 12% w/v acrylamide gel was prepared by mixing 9.95 cm^3 of acrylamide stock with 12.45 cm^3 of double strength RB, 0.013 cm^3 TEMED, 2.36 cm^3 distilled water and finally 0.12 cm^3 P. This mixture was pipetted into the slab gel former to make 8 cm x 8 cm slab gels. The 3% acrylamide spacer gel was prepared by taking 1 cm^3 acrylamide stock and mixing it with 5 cm^3 double strength RB, 0.005 cm^3 TEMED, 3.9 cm^3 distilled water and finally 0.1 cm^3 P.

After running for 4h at c. 100 v the gels were stained in 0.25% w/v Kenacid blue in water:acetic acid:ethanol 5:1:5 for 24h and destained

in 7% v/v acetic acid, sliced with a scalpel and scanned at 550 nm.

Bovine serum albumin, MW 6.7×10^4; γ-globulin (heavy), MW 5.5×10^4; ovalbumin MW 4.7×10^4 and cytochrome c, MW 1.24×10^4 were run as markers and the $\log_{10}$ MW plotted against their relative mobilities compared to BPB to give a calibration plot (Figure 13).

B. Glass-fibre paper electrophoresis of pectic polysaccharides (after Jarvis et al., 1977)

a) Procedure

Whatman GF/C glass fibre paper was cut into 15 cm x 10 cm strips and heated at 400°C for 2h to burn out organic material, then immersed for 18h in 2% v/v dimethyldichlorosilane in carbon tetrachloride, rinsed in toluene and dried. The strips were wetted by floating for 18h in 0.1M sodium acetate buffer pH 5.0 containing 0.02M EDTA to which Tween-20 had been added to make a 0.2% v/v solution. Electrophoresis was carried out in a standard Shandon tank designed for cellulose acetate membrane electrophoresis. 0.1M sodium acetate buffer pH 5.0 containing 0.02M EDTA was used as running buffer. The paper was equilibrated by a 15 min pre-run at the running voltage. 0.020 cm^3 of 3mg cm^{-3} solutions of the samples in running buffer were applied at the origin. Samples were run for 1.5 - 2h at an initial current of 22mA and potential difference of 200 V, which became c. 34mA and 175 V by the end of the run.

b) Sample location

After electrophoresis the strips were immersed in absolute alcohol to precipitate and thus immobilize the samples. The strips were then dried in hot air. Samples were located by spraying with sulphonated α-napthol

reagent prepared by dissolving 200 mg α-napthol and 1 cm^3 H_2SO_4 in 50 cm^3 methanol (modified after Barrett and Northcote, 1965) and heating for 10 min at 110°C.

C. Column electrophoresis of pectic polysaccharides on a urea gradient (after Jarvis et al., 1977)

a) Procedure

A 110 cm^3 LKB column designed for isoelectric focussing was used. The central anode solution was 0.3M sodium acetate buffer pH 6.5 made up to 50% w/v solution of urea. A linear gradient from 50 - 25% w/v urea in 0.03M sodium acetate buffer pH 5.0 was applied with a gradient mixer. 10 mg of sample dissolved in 10 cm^3 buffer containing 18% w/v urea was layered on to the gradient followed by c. 10 cm^3 spacer solutions of buffer containing 12% w/v urea. The upper cathode solution was 0.5M acetic acid. A run time of c. 15h was routinely used with an initial current of 10mA and a potential difference of 100 V. At the end of the run the current had fallen to c. 8mA. 5 cm^3 fractions were collected.

b) Carbohydrate assays

i) Phenol-sulphuric acid (after Dubois et al., 1951)

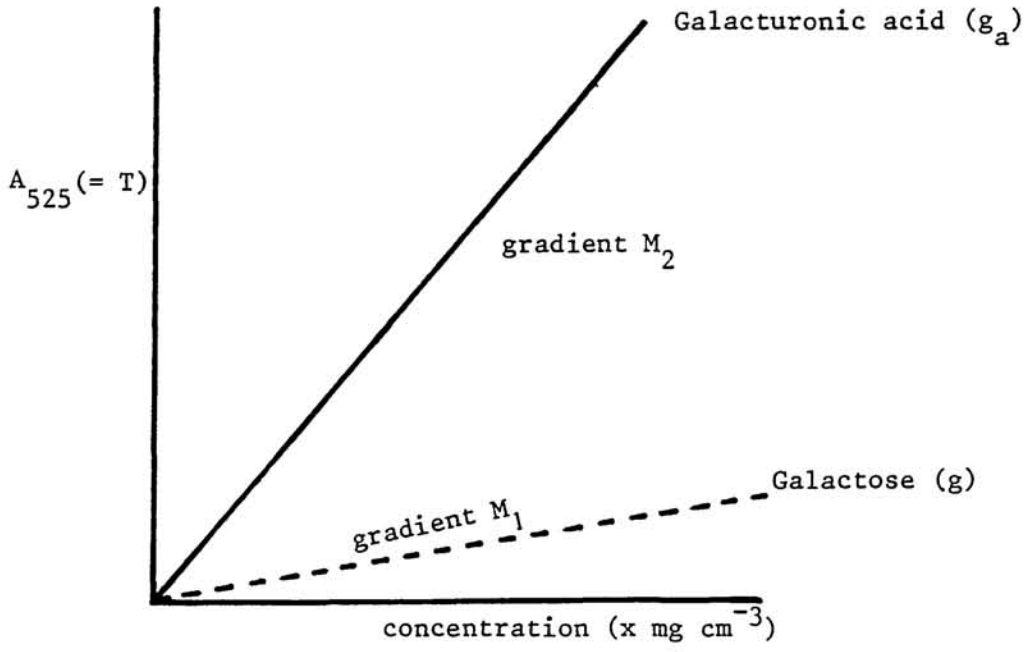
0.5 cm^3 sample was mixed with 1 cm^3 5% w/v phenol solution and 5 cm^3 of AnalaR H_2SO_4 added rapidly. The boiling tubes were shaken to complete mixing and allowed to cool for 30 min. The absorbance at 485 nm was measured and the concentration of neutral sugar as galactose equivalent calculated taking into account the presence of uronic acid residues (see Section iii) for method).

ii) Carbazole-sulphuric acid (after Dische, 1947)

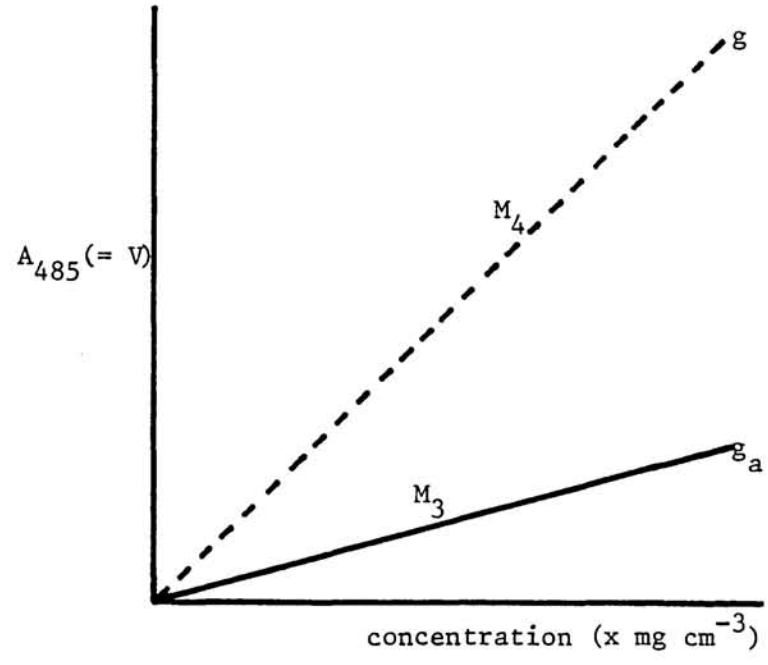
1 cm^3 sample was mized with 0.2 cm^3 0.125% w/v carbazole in absolute alcohol and 5 cm^3 0.025M sodium tetraborate in AnalaR H_2SO_4 was added rapidly. The tubes were shaken to complete mixing and allowed to cool for 30 min. The absorbance at 525 nm was recorded and the concentration of uronic acid as galacturonic acid equivalent calculated taking into account the presence of neutral residues (see Section iii) for method).

iii) Treatment of results from i) and ii)

Using solutions of known concentrations of galactose and galacturonic acid in both carbazole and phenol sulphuric acid procedures calibration plots were obtained (see Appendix I for data). The carbazole sulphuric acid procedure gives a better colour reaction with uronic acids than neutral sugars, whereas the phenol sulphuric acid procedure gives a better colour reaction with neutral sugars than uronic acids. The calibration plots are all straight lines. Thus by solving simultaneous equations expressions can be derived into which one can substitute directly the spectrophotometer readings for the absorbances at 525 and 485 nm and calculate the concentration of either uronic acid or neutral sugar equivalent taking into account the degree of cross reaction which occurs with the reagents when a mixture of the two substances are present, as in pectic polysaccharide (see Figure 7).



i) Carbazole-sulphuric acid procedure



ii) Phenol-sulphuric acid procedure

Figure 7. Representation of the calibration plots determined for reaction of solutions of known concentrations of galacturonic acid (g_a) and galactose (g) with i) carbazole-sulphuric acid and ii) phenol-sulphuric acid

M_1, M_2, M_3 and M_4 are the gradients of the lines where $y = Mx + C$, but since the plots go through the origin $C = 0$, $\therefore$ $y = Mx$, $M = \frac{y}{x}$

For i)

From the calibration pots it can be seen that :-

$$T_g = M_1 x_g \quad 1)$$ (where x_g = a given concentration of galactose)

$$T_{ga} = M_2 x_{ga} \quad 2)$$ (where x_{ga} = a given concentration of galacturonic acid)

$\therefore$ in a mixture containing both galacturonic acid and neutral sugar residues the total reading for A_{525} given by the carbazole-sulphuric acid procedure is :-

$$T_g + T_{ga} = T$$

Substituting from 1) and 2)

$$T = M_1 x_g + M_2 x_{ga} \quad 3)$$

Similarly for the phenol sulphuric acid procedure

$$V_{ga} = M_3 x_{ga}, \quad V_g = M_4 x_g$$

$\therefore$ Total reading $V_g + V_{ga} = V$

$$V = M_4 x_g + M_3 x_{ga}$$

multiply 3) by M_4 and 4) by M_1

$$M_4 T = M_4 M_1 x_g + M_4 M_2 x_{ga} \quad 5)$$

$$M_1 V = M_4 M_1 x_g + M_1 M_3 x_{ga} \quad 6)$$

subtract 6) from 5)

$$M_4 T - M_1 V = (M_4 M_2 - M_1 M_3) x_{ga}$$

$$x_{ga} = \frac{M_4 T - M_1 V}{M_4 M_2 - M_1 M_3}$$

i.e. the concentration of galacturonic acid (x_{ga}) in mg cm^{-3} can be calculated from the readings T and V for the carbazole and phenol-sulphuric acid procedures performed on an unknown mixture of neutral and uronic acid residues.

The calibration plots used in this work had $M_1 = 0.84$, $M_2 = 9.35$, $M_3 = 2.73$, $M_4 = 4.95$

the expression thus becomes uronic acid as galacturonate equivalent

$$x_{ga} = \frac{(4.95T)-(0.84V)}{43.99}$$

Similarly for neutral sugar as galactose equivalent

$$x_{g} = \frac{(9.35V)-(2.73T)}{43.99}$$

Empirical testing of these calculations gave good agreement (see Appendix II).

9. Periodate oxidation

One cm^3 of sample was mixed with 1 cm^3 of 0.05M periodic acid in 0.1M sodium acetate buffer pH 4.7 and incubated in the dark for 2h at 20°C. Two drops of glycerol were then added and the incubation continued for 30 min to reduce unreacted periodic acid.

10. Viscometry

9 cm^3 of 20 mg cm^{-3} solution of acidic pectic fraction from citrate oxalate extract of RM leaves was pipetted into a viscometer. 1 cm^3 of 1 mg cm^{-3} PG IV was added and thoroughly mixed. The time in seconds taken for the reaction mixture to flow from the upper mark to the lower on the arm of the viscometer was recorded at various time intervals. The flow time is proportional to the viscosity of the mixture. The incubation was carried out in a water bath at 30°C and the solutions were in 0.02M sodium phosphate buffer pH 5.8.

11. Hydrolysis and gas-liquid chromatography (GLC) of pectic polysaccharide (Modified after Mankarios et al., 1979)

a) Hydrolysis

10 mg pectic polysaccharide were suspended in 3 cm^3 2M trifluoro-acetic acid (TFA) in glass vials which had been cleaned thoroughly with concentrated sulphuric acid and rinsed several times with distilled water. The suspension was heated for 1h at 100°C in an oven and the hydrolysate evaporated to dryness in a stream of nitrogen at 40°C using an SC-3 Sample Concentrator (Techne, Dri-Block DB-3).

b) Preparation of alditol acetates

After hydrolysis the residue was dissolved in 0.25M NH_4OH at 4°C to hydrolyse any uronolactones or methyl esters and prevent them being converted to interfering products. Epimerisation of neutral monosaccharides by the Lobry de Bruyn-von Eckenstein reaction was not significant under these conditions. 2 mg $NaBH_4$ was added to reduce the sugars to their respective alditols. Borate was removed by repeated evaporation with methanol. The alditols were acetylated in 0.3 cm^3 acetic anhydride at 100°C for 2.5h. The acetic anhydride was removed as an azeotrope with toluene. The alditol acetates were dissolved in acetone and inositol hexa-acetate added as internal standard prior to GLC.

c) GLC

A flame-ionization detection (FID) instrument with a glass column was used. The 2.8 x 2 mm column was packed with 0.3% Reoplex-400 and 0.6% OV-275 coated on acid-washed Chromosorb W, 80 - 100 mesh by the fluidiser method (Kruppa et al., 1967). Nitrogen at 10 $cm^3 min^{-1}$ was used as the

carrier gas. Separations were carried out under isothermal conditions at 200°C. The retention times of the alditol acetates relative to inositol hexa-acetate were rhamnose 0.19, fucose 0.20, arabinose 0.26, xylose 0.33, mannose, 0.69, galactose 0.78 and glucose 0.86, determined using standards. The peak areas were determined by electronic integration and the quantities of sugar printed out in terms of the quantity of inositol hexa-acetate allowing for the different responses given by the individual alditol acetates.

12. Bacterial cell surface components

Bacteria were grown in shake culture for 24h at 25°C in 2 dm^3 conical flasks each containing 250 cm^3 of King's B broth. Flasks were inoculated with 5 cm^3 of a 24h King's B broth culture. The bacterial suspension was centrifuged at 8000g for 10 min, the supernatant retained and the pellet of bacterial cells resuspended in distilled water. The suspension was centrifuged at 8000g for 10 min the supernatant discarded and the pellet of bacterial cells lyophilized using an Edwards "Modulyo" freeze drier, and stored desiccated.

a) Extracellular polysaccharide (EPS)

EPS was isolated from the culture filtrate after the method of Husain and Kelman (1958). The supernatant after centrifugation of the broth culture was rotary evaporated at 35°C to 5% of the original volume. The concentrate was dialysed against several changes of distilled water for 3d at 4°C. The dialysate was mixed with 2 volumes of absolute alcohol and left for 12h. The supernatant was decanted off from the precipitate and mixed with a further 2 volumes of absolute alcohol. After 12h the supernatant was decanted off. Both precipitates were dried at 50°C for 12h and stored

desiccated. The two precipitates were designated EPS1 and EPS2.

b) Lipopolysaccharide (LPS)

LPS was extracted from cells after the method of Westphal and Jann (1965). 20g of lyophilized bacteria were suspended in 350 cm^3 of distilled water at 65°C in a water bath and mixed with 350 cm^3 90% w/v phenol, preheated to 65°C. The mixture was incubated for 15 min then cooled in an ice-bath to c. 10°C. The emulsion was centrifuged at 1000g for 45 min. The water phase which contained the LPS was pippetted off and the remainder mixed with a further 350 cm^3 of water and the extraction procedure repeated. The combined water phases were dialysed for 4 days against distilled water at 4°C. The dialysates were concentrated by rotary evaporation to half the original volume and the opalescent solution lyophilized. Crude preparations of this type contain 50 - 60% w/w bacterial ribonucleic acid. The crude LPS preparation was dissolved to make a 3% w/v solution in distilled water and centrifuged at 80000g for 6h. The pellet was resuspended in distilled water and centrifuged twice at 105,000g for 3h. The pellet was taken up in a minimal amount of distilled water and the solution lyophilized to give a preparation containing c. 3% w/w nucleic acid as a contaminant.

13. Ouchterlony double diffusion assay

A thin layer of 1.0% w/v Bacto-agar (Difco Laboratories) or 0.8% w/v agarose (LKB EF 1802-300) in 0.04M sodium phosphate buffer pH 6.8 containing 0.01% w/v sodium azide was used as a support. Solutions were prepared by heating in a boiling water bath for 30 min and were kept molten until required in a water bath at 45°C. Microscope slides were cleaned in soapy water, rinsed thoroughly in distilled water, wiped with muslin soaked in absolute alcohol and air dried. Solution was pippetted on to the slides and allowed to solidify. Holes were made with a cork borer

(3 mm diameter) c. 3 mm apart using a hexagonal template with a central well. Acidic pectic polysaccharide from citrate-oxalate buffer extractions of leaves of P. vulgaris cv. Red Mexican was dissolved in 0.04M sodium phosphate buffer containing 0.01% w/v sodium azide and placed in the central well. Various test substances dissolved in the same buffer were placed in the surrounding wells. The slides were placed on moist filter paper in 9.0 cm diameter petri dishes, incubated for 5 days at room temperature and checked for precipitation lines every 24h.

14. In vivo attachment and recovery of bacteria

a) Procedure

10-14 day old glasshouse grown seedlings of P. vulgaris cvs. Red Mexican and Canadian Wonder were used. Plants were placed in a moist chamber for 24h before inoculation to open the stomata. 24h cultures of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) grown on King's B agar slopes were used to prepare inocula. Bacteria washed by centrifugation were suspended in sterile distilled water and adjusted to the desired concentration by nephelometry. One primary leaf of each seedling was inoculated, and the other used to determine the intercellular volume for a given area of leaf tissue. Bacterial suspensions were infiltrated into the leaves using a "Quickfit" atomiser and compressed air at a pressure of 0.2 kg cm^{-2} and the inoculated region was outlined with water-insoluble ink. The opposite primary leaf was detached from the plant and weighed. The plants were returned to the moist chamber and placed in a growth cabinet at $20^{o}C$ with fluorescent lighting for 1.5h after which time the inoculated regions appeared normal.

The detached leaves were completely watersoaked over their whole area with distilled water. Surface water was removed with a paper tissue and the leaves reweighed. The difference in weights gives directly in cm^3

the intercellular volume of the leaf. The leaves were flattened on to a piece of paper and the outlines drawn, the shapes were cut out and weighed and the area read from a calibration plot of weight of paper against area in cm^2. The intercellular volume of the leaf divided by its area thus gives the approximate intercellular volume cm^{-2} (V) of leaf lamina. The values are approximate because leaf veins and irregularities at the leaf margins were not allowed for.

The inoculated leaves were washed in tap water and rinsed in sterile distilled water to remove free bacteria from the surface. The inoculated region was watersoaked with sterile distilled water and the surface dried with heat sterilized paper tissue. Three 1.3 cm diameter leaf discs were taken from the inoculated region with a sterile cork borer. The intercellular fluid containing bacterial cells not attached to plant cell walls was recovered after the modified method of Klement (1965). The leaf discs were placed in a small plastic vial with holes drilled in its base. The vials were placed in centrifuge tubes containing 0.2 cm^3 sterile distilled water and centrifuged at 550g for 10 min in a bench top centrifuge. The vials retain the leaf discs while allowing the intercellular fluid to pass through the holes and collect in the sterile distilled water. After mixing on a vortex mixer to ensure a homogeneous distribution of bacteria 0.05 cm^3 aliquots were spread on to King's B agar in Petri dishes. A 10^{-2} dilution was made by making 0.05 cm^3 up to 5 cm^3 with sterile distilled water and 0.05 cm^3 aliquots were spread on to King's B agar in Petri dishes. After 48h incubation at 25°C colonies were counted. Colonies of P. phaseolicola on King's B agar are a translucent off-white and develop lobed edges on aging and secrete a yellow-green pigment into the medium which fluoresces bright blue under long wave (366 nm) UV radiation. The identities of the colonies were confirmed by reinoculation into bean seedlings which responded as expected to cells of each Race.

b) Treatment of results

The area of leaf lamina in 3 x 1.3 cm diameter circular leaf discs $= 3 \times \pi (\frac{1.3}{2})^2 = 4.0\ cm^2$.

The volume of bacterial suspension infiltrated into the leaf discs = 4V, where V is the intercellular volume of the leaf cm^{-2} leaf lamina, which was measured for each leaf.

Thus the number of bacterial cells (B) infiltrated into the leaf discs = $C_i\ 4V = B$, where C_i = the inoculum concentration in cells cm^{-3}.

The volume of bacterial suspension collected after centrifugation to remove intercellular fluid = $0.2 + 4V\ cm^3$ ($0.2\ cm^3$ in bottom of tube), assuming that centrifugation removes the same volume as was initially infiltrated. The number of colonies counted on the plates after spreading $0.05\ cm^3$ aliquots of the undiluted and 10^{-2} dilutions can be used to calculate the concentration of cells cm^{-3} in this suspension (C_r). The total number of cells recovered = $C_r\ (0.2 + 4V) = D$.

Thus, the number of cells attached to plant cell walls in the leaf discs = B-D, i.e. $(C_i\ 4V) - C_r(0.2 + 4V) = G$.

Thus in a given experiment the percentage of cells of the initial inoculum which attaches to cell walls = $\frac{G}{B}.100$. Consideration of this value for different experiments allows comparison of the relative degree of attachment of cells of isolates of the two Races of P. phaseolicola within a given cultivar of P. vulgaris and of the relative degree of attachment of each race in different cultivars.

If attachment of bacterial cells to plant cell walls was a reversible process it could be summarized as follows :-

$$E + F \rightleftharpoons G$$

Where E = bacteria, F = attachment sites on plant cell wall and G = the complex formed. Under constant conditions reversible processes reach an equilibrium, which in this case would be when the rate of bacterial cells

attaching to plant cell walls equals the rate at which already attached bacteria become detached. At equilibrium :-

$$\frac{E_{eq} F_{eq}}{G_{eq}} = K$$

Where E_{eq} = the concentration of bacterial cells not attached at equilibrium, F_{eq} = the concentration of unoccupied attachment sites on the plant cell walls at equilibrium, G_{eq} = the concentration of bacterial cells attached at equilibrium, and K = a constant, here called the binding coefficient (see figure 8). The value of K is a measure of the affinity of bacteria for the attachment sites on the plant cell walls; the smaller the value of K the greater the affinity.

Except at extremely high inoculum concentrations F_{eq} can be assumed to be in such excess that its value is constant. If the number of bacterial cells per unit area of leaf lamina (i.e. the 4.0 cm^2 of the 3 leaf discs) is taken to be the unit of concentration then $E_{eq} = D$ and $G_{eq} = G$.

Hence $\frac{D}{G}$. constant = K

i.e. the ratio of unattached to attached cells should be constant for a range of inoculum concentrations for given environmental conditions, if i) attachment is reversible, ii) inoculum concentrations used are not so high as to make binding sites on the plant cell walls limiting (see Figure 9).

Attachment of bacteria to plant cell walls occurs at the surfaces of the intercellular spaces. The volume of the intercellular spaces of the leaves cm^{-2} of leaf lamina varies, and with it varies the surface area over which attachment can occur. For a body of constant shape varying only in size, the relationships of change in lengths to change in surface area to change in volume is as follows :- If the length (l) is increased by a factor p i.e. new length = p l, then the surface area is increased by a factor p^2 and the volume by p^3. It follows that if the volume increases by p^3 the surface area increases by p^2. Since the intercellular volumes of

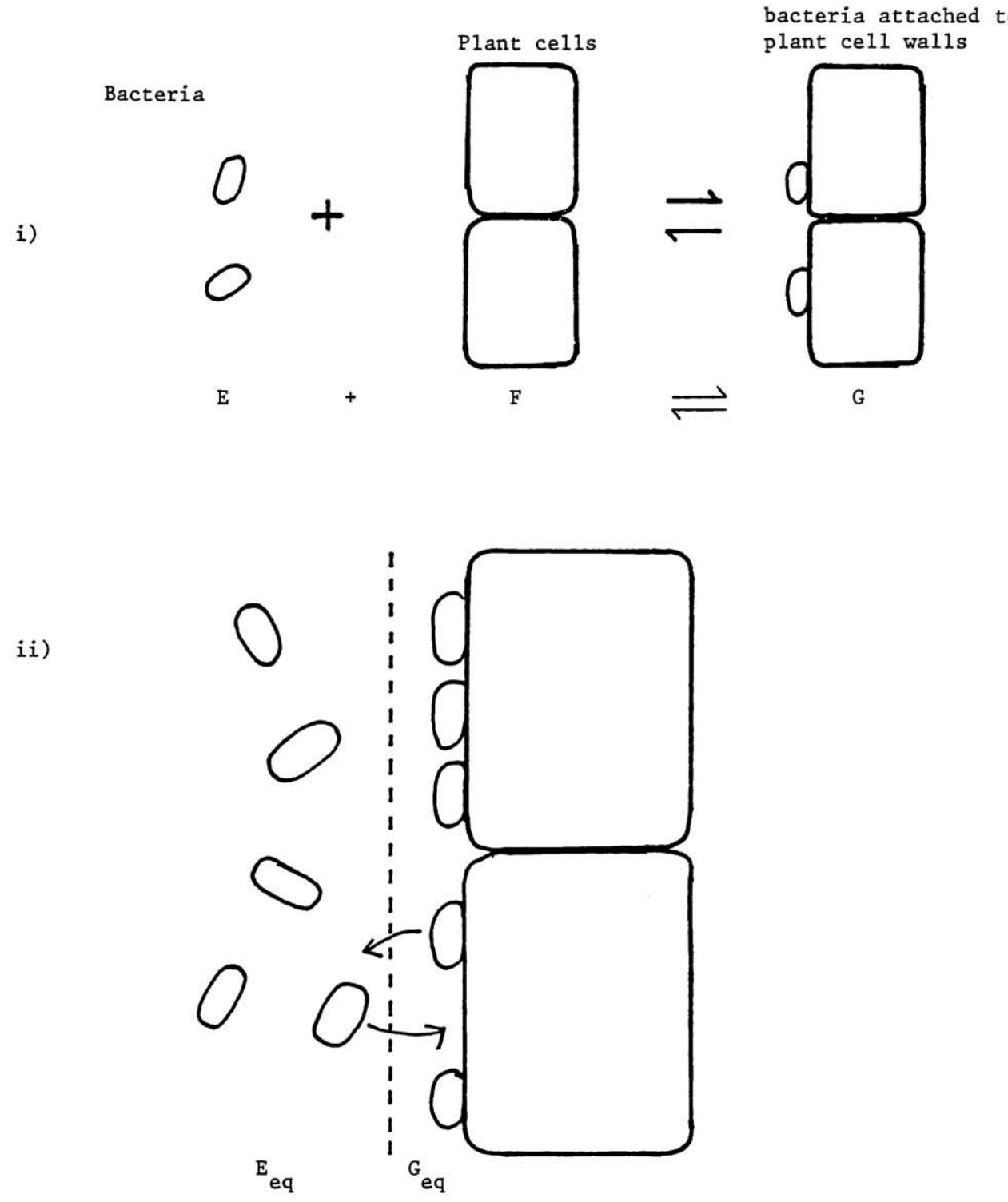


Figure 8. Representation of i) attachment of bacteria to plant cell walls, ii) equilibrium where the rate of attachment of new bacterial cells equals the rate of detachment of already attached cells. E = unattached bacteria, E_{eq} = concentration of unattached bacteria at equilibrium, F = attachment sites on plant cell surfaces, G = attached bacteria, G_{eq} = concentration of attached bacteria at equilibrium.

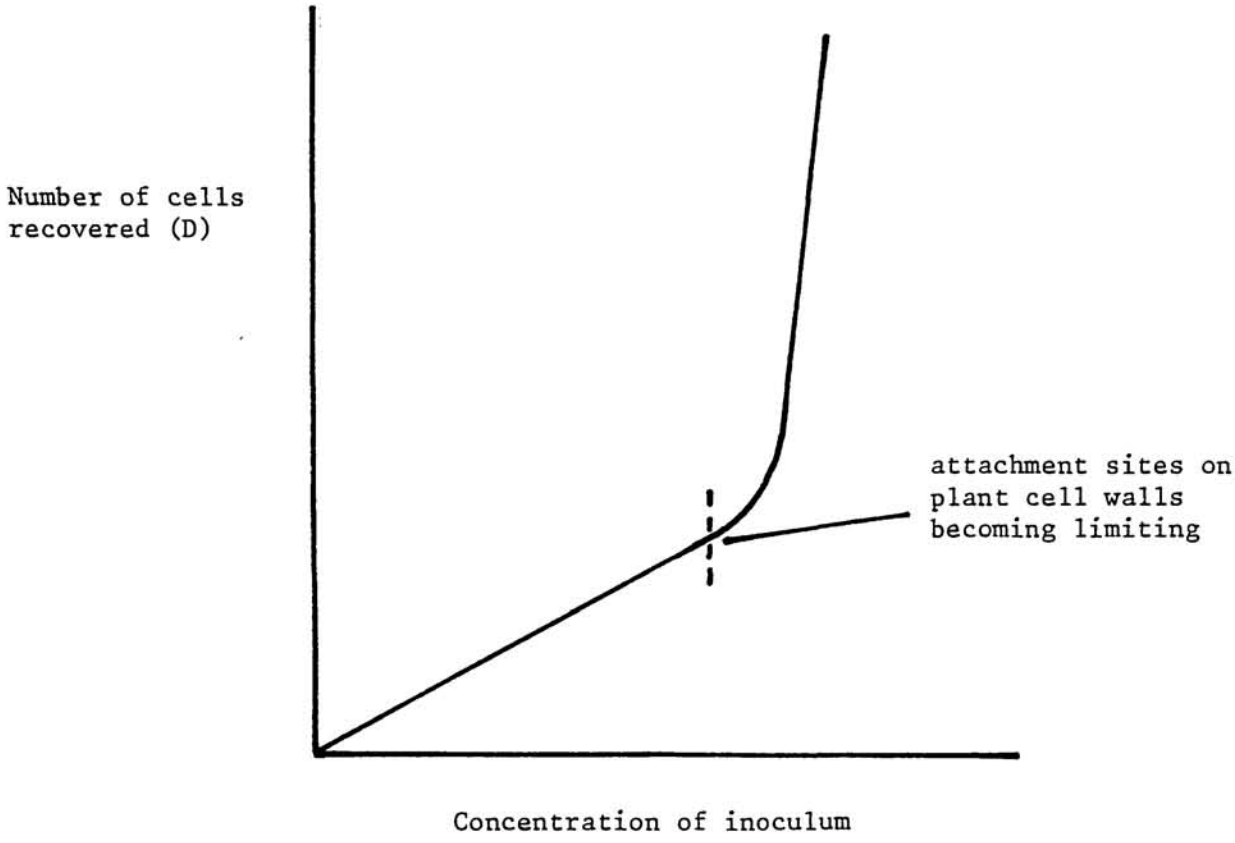


Figure 9. When attachment sites on plant cell walls become limiting increase in concentration of inoculum would yield a greater proportion of cells recovered on centrifugation.

the leaves cm^{-2} leaf lamina can be measured, if one takes the smallest value and gives it an arbitrary unit of surface area equal to 1, then the surface areas over which attachment can occur in the other leaves can be calculated in terms of this, viz.

let p_z^3 = smallest measured volume of intercellular space cm^{-2} leaf lamina

p_y^3 = intercellular volume of another leaf

Therefore, the factor by which the volume is greater,

$$p^3 = \frac{p_y^3}{p_z^3}$$

Therefore, p^2 which is the factor by the surface area is increased =

$$\frac{p^3}{\sqrt[3]{p^3}} = p^2$$

Thus the relative internal surface area of the leaf compared to the standard =

$$1\ (p^2) = p^2$$

With the inoculum concentrations used for the experiments reported in this thesis it was assumed that the surface areas and therefore the numbers of attachment sites were in such large excess to the numbers of bacterial cells that comparisons such as the above were unnecessary. However, the calculation is included for completeness and would be of use at higher inoculum concentrations where saturation of surfaces available for attachment became apparent.

EXPERIMENTAL AND RESULTS

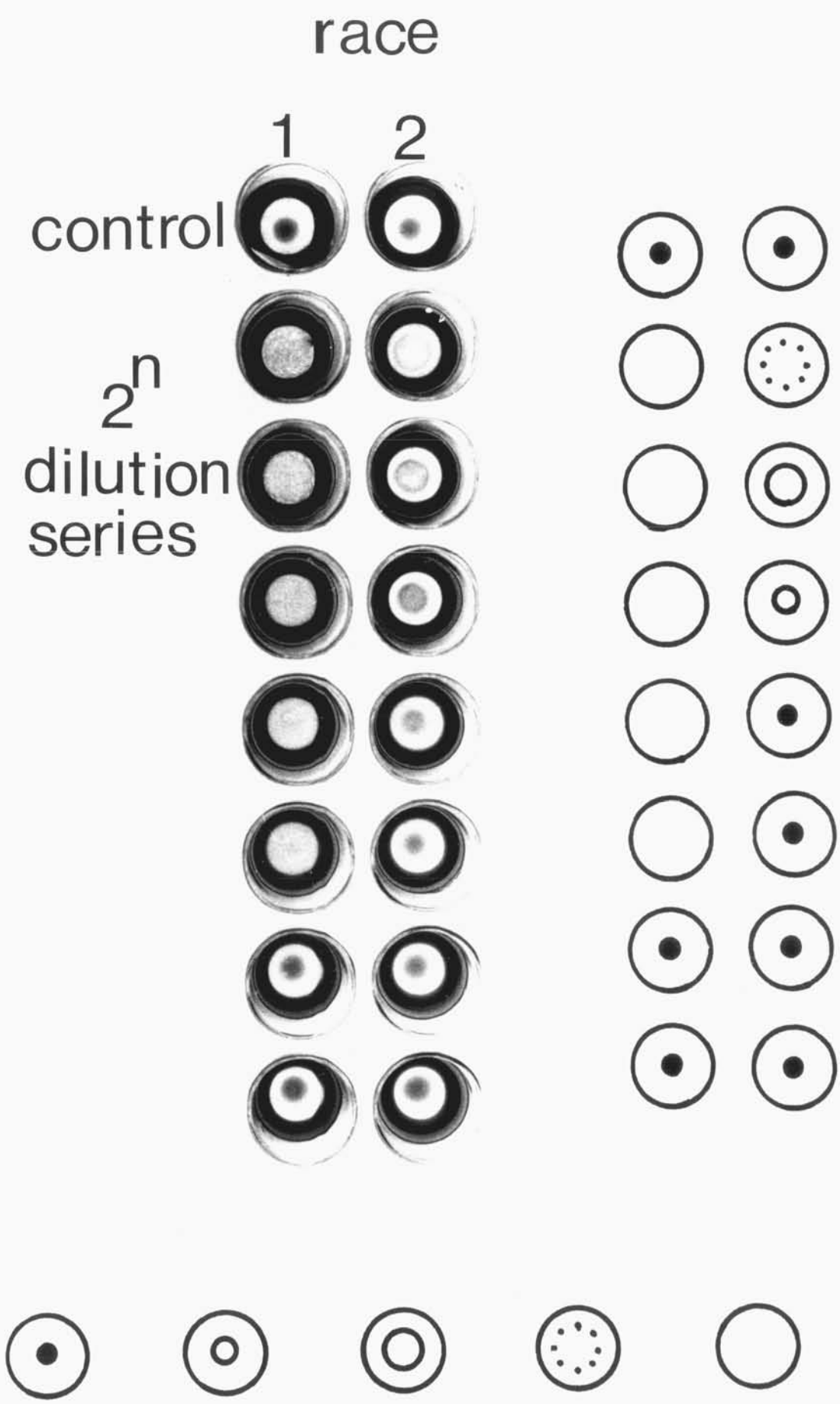
A. Agglutination of bacteria and erythrocytes by plant extracts

Crude extracts from bean cotyledons and leaves were made and their ability to agglutinate cells of P. phaseolicola and human erythrocytes was studied.

a) Cotyledon and leaf pH 4.2 lectin extracts

1 and 7-day cotyledons and 2 - 3 week old leaves of P. vulgaris cv. Red Mexican (RM) and cv. Canadian Wonder (CW) were extracted for lectins as in Materials and Methods 3A a) i) and 3B b) i). The precipitates from the $(NH_4)_2SO_4$ fractionation procedure and the supernatant from the 100% saturation step, after dialysis were tested for ability to agglutinate cells of different isolates of the two races of P. phaseolicola and human erythrocytes. The precipitates collected by centrifugation of the 50% $(NH_4)_2SO_4$ saturated extracts from 1 and 7-day RM and CW cotyledons did not agglutinate cells of P. phaseolicola Race 1 or 2. 100% $(NH_4)_2SO_4$ saturation of the extracts from RM cotyledons gave two fractions on centrifugation; a film which collected at the surface and a pellet at the bottom of the centrifuge tube. The pellets were inactive. However material from the surface films of extracts from 1 and 7-day RM cotyledons differentially agglutinated cells of Race 1 and Race 2 isolates of P. phaseolicola. Cells of Race 1 isolates were agglutinated to a greater degree than cells of Race 2 isolates (Plate 1 and Table 1). This is shown by the dilution end points (Table 2) and by the relative degree of agglutination in corresponding wells. Much less of this surface film could be obtained from CW cotyledons. Indeed from 7-day cotyledons there was insufficient material for collection and assay. The film from 1-day CW cotyledons was less active in agglutination and did not differentiate between cells of the two races (Tables 2 and 3).

Plate 1. Differential agglutination of cells of avirulent Race 1 and virulent Race 2 isolates of Pseudomonas phaseolicola by surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts from 7-day old cotyledons of Phaseolus vulgaris cv. Red Mexican. Photograph and key showing agglutination index.


race
1
2
control
2^n
dilution
series


0
1
2
3
4
agglutination index

Table 1 Differential agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of 1 and 7-day old cotyledons of P. vulgaris cv. Red Mexican

	Race 1 isolates						Race 2 isolates			
	R1(X)		31A		710A		777A		882	
Age of cotyledons	1-day	7-day	1-day	7-day	1-day	7-day	1-day	7-day	1-day	7-day
Dilution										
Control	0[a]	0	0	0	0	0	0	0	0	0
2^1	4	4	4	4	4	4	4	4	4	3
2^2	4	4	4	3	4	4	1	2	2	2
2^3	4	4	2	3	3	3	0	0	0	1
2^4	0	3	0	1	0	3	0	0	0	0
2^5	0	2	0	0	0	2	0	0	0	0
2^6	0	0	0	0	0	0	0	0	0	0

[a] agglutination index - see Plate 1. All agglutination indexes were compiled from at least 5 replicates in this and other tables

Table 2 Dilution end-points for the agglutination of cells of Races 1 and 2 of *P. phaseolicola* by surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of cotyledons of *P. vulgaris* cvs. Red Mexican and Canadian Wonder

	Dilution end-point[a]				
	Race 1 isolates			Race 2 isolates	
Agglutinating sample	R1(X)	31A	710A	777A	882
Fraction from 1-day old Red Mexican cotyledons	2^{3}[b]	2^{3}	2^{3}	2^{2}	2^{2}
Fraction from 7-day old Red Mexican cotyledons	2^{5}	2^{4}	2^{5}	2^{2}	2^{3}
Fraction from 1-day old Canadian Wonder cotyledons	2^{1}	-	-	-	2^{1}

[a] greatest dilution showing activity

[b] data from Tables 1 and 3

Table 3 Agglutination of cells of Races 1 and 2 of P. phaseolicola by surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of 1-day old cotyledons of P. vulgaris cv. Canadian Wonder

Dilution	Race 1 R1(X)	Race 2 882
Control	0[a]	0
2^1	3	3
2^2	0	0
2^3	0	0

[a] agglutination index - see Plate 1

The fractions from the 100% $(NH_4)_2SO_4$ saturated extracts, which pelleted at the bottom of the centrifuge tubes caused some bacterial agglutination, but did differentiate between cells of Race 1 and Race 2 isolates (Table 4).

All precipitates from the 50 and 100% $(NH_4)_2SO_4$ saturated extracts from RM and CW cotyledons agglutinated human erythrocytes (Table 5).

No surface film was detected in the 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of RM or CW leaves. No bacterial or erythrocyte agglutinating activity was detected for any of the precipitates or for the supernatant from the 100% $(NH_4)_2SO_4$ saturated fractions.

From these results it was concluded that a fraction could be obtained from cotyledons of P. vulgaris cv. Red Mexican which differentially agglutinated cells of Race 1 and 2 isolates of P. phaseolicola and also showed erythrocyte agglutinating activity. Since a fraction with similar

Table 4 Agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by pelleted material from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of 7-day old cotyledons of P. vulgaris cvs. Red Mexican (RM) and Canadian Wonder (CW)

	RM					CW				
	Race 1 isolates			Race 2 isolates		Race 1 isolates			Race 2 isolates	
Dilution	R1(X)	710A	31A	777A	882	R1(X)	710A	31A	777A	882
Control	0[a]	0	0	0	0	0	0	0	0	0
2^1	4	4	4	4	3	3	2	3	2	2
2^2	0	0	0	0	0	0	0	0	0	0
Dilution end-point	2^1	2^1	2^1	2^1	2^1	2^1	2^1	2^1	2^1	2^1

[a] agglutination index - see Plate 1

Table 5 Agglutination of erythrocytes by fractions from pH 4.2 extracts of 1 and 7-day old cotyledons of P. vulgaris cvs. Red Mexican (RM) and Canadian Wonder (CW)

	CW			RM			
Age of cotyledons	7-day	7-day	1-day	7-day	7-day	1 and 7-day	
% $(NH_4)_2SO_4$ saturation	50	100	100	50	100	100	
Nature of precipitate	pellet	pellet	surface film	pellet	pellet	surface film	
Dilution							
Control	0[a]	0	0	0	0	0	0
2^1	4	4	4	4	4	4	4
2^2	3	4	4	2	3	4	4
2^3	2	4	4	0	2	4	4
2^4	0	3	4	0	0	3	4
2^5	0	3	4	0	0	3	4
2^6	-	-	4	-	-	3	2
2^7	-	-	4	-	-	3	1

[a] agglutination index - see Plate 1

properties was not obtained from P. vulgaris cv. Canadian Wonder it was concluded that the active substance(s) was either absent or present in too small a quantity for the assay to detect. It was speculated that the activity might be due to a protein with lectin like properties, and it was decided to extract RM cotyledons in the more conventional manner for lectin preparation - by using dilute saline solution.

b) 0.9% w/v saline extract for lectin

pH 4.2 and 0.9% w/v saline extracts were made from the same batch of soaked RM seed (24h at 25°C). The procedures were as in Materials and Methods section 3 A a) i) and ii). Surface film from the 100% $(NH_4)_2SO_4$ saturated preparation was collected. Folin-Lowry estimation of the protein contents of the extracts showed that saline extraction gave twice the yield of protein compared to pH 4.2 extraction, but the saline extract was almost inactive in bacterial agglutination. Both extracts had high haemagglutinating activity (Tables 6 and 7).

Table 6 Comparison of protein yields in the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 and 0.9% w/v saline extracts of 1-day RM cotyledons

Extraction method	pH 4.2	0.9% w/v saline
Mass of cotyledon tissue	37.3 g	37.3 g
Total protein yield	129.6 mg	270.4 mg

The results suggested that erythcrocyte agglutination might be caused by a substance different from that causing bacterial agglutination, or alternatively that more than one haemagglutinin was being extracted, but that saline did not solubilize the substance with bacterial agglutinating

Table 7 Comparison of agglutination of cells of Race 1 and 2 isolates of P. phaseolicola and of erythrocytes by fractions from pH 4.2 and 0.9% w/v saline extracts of 1-day old cotyledons of P. vulgaris cv. Red Mexican

		pH 4.2 extract						0.9% w/v saline extract					
		Race 1 isolates	Race 2 isolates					Race 1 isolates	Race 2 isolates				
Dilution	Protein concentration mg cm^{-3}	R1(X)	31A	710A	777A	882	erythrocytes	R1(X)	31A	710A	777A	882	erythrocytes
Control	0	0[a]	0	0	0	0	0	0	0	0	0	0	0
2^1	3.60	4	4	4	4	4	4	1	0	0	0	0	4
2^2	1.80	4	4	4	1	2	4	0	0	0	0	0	3
2^3	0.90	4	2	3	0	0	4	0	0	0	0	0	3
2^4	0.45	0	0	0	0	0	3	0	0	0	0	0	3
2^5	0.23	0	0	0	0	0	3	0	0	0	0	0	3
2^6	0.12	0	0	0	0	0	3	0	0	0	0	0	3
2^7	0.06	0	0	0	0	0	3	0	0	0	0	0	3

[a] agglutination index - see Plate 1

[b] protein concentrations in the wells containing erythrocytes were 3.2, 1.6, 0.8 mg cm^{-3} etc. as the samples were brought to 0.9% w/v saline concentration by addition of 9.0% w/v saline

properties. To resolve this problem it was decided to isolate and purify the active substance from the crude extract in a number of ways.

c) Isolation of active substance from crude extracts

i) Ion-exchange chromatography

The surface film from the 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract of 7-day RM cotyledons was chromatographed on DEAE cellulose as in Materials and Methods section 6. Three minor peaks and 1 major peak were obtained (Figure 10). However, the resolution was not good, and rather than alter the parameters of the system to achieve a better separation it was decided to carry out isoelectric focussing. The small number of proteins in the sample suggested that it would be suitable for this technique which can be used on a preparative scale.

ii) Isoelectric focussing

3 cm^3 of a solution of the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract from 7-day RM cotyledons were focussed on a broad ampholine pH range over sucrose gradient as in Materials and Methods section 7. The 3 cm^3 of solution contained 21.6 mg of protein determined by Folin-Lowry estimation. Four minor peaks and 1 major peak were obtained with isoelectric points at pH 11.3, 7.5, 6.3, 4.5 and 2.5 (Figure 11). The fractions containing the peaks were bulked and dialysed for 36h against 0.05M sodium phosphate buffer pH 6.8 at 4^oC. Dialysates were concentrated by placing the dialysis tubes in polyethylene glycol (PEG 6000) until the required reduction in volume had occurred. Then after adjusting so that protein concentrations were equal, agglutination assays were carried out with bacteria and erythrocytes. Fractions 1 - 3 (Figure 11) caused no

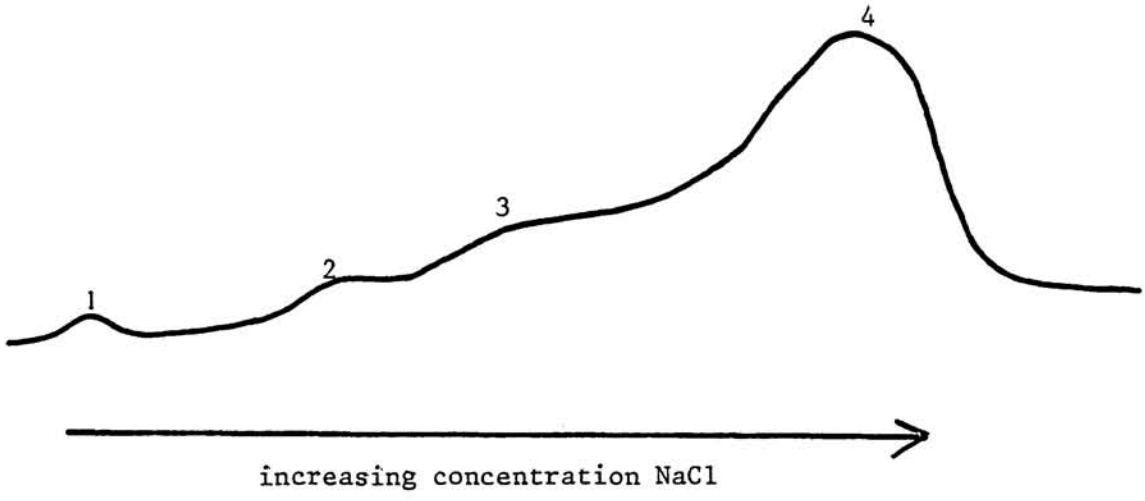
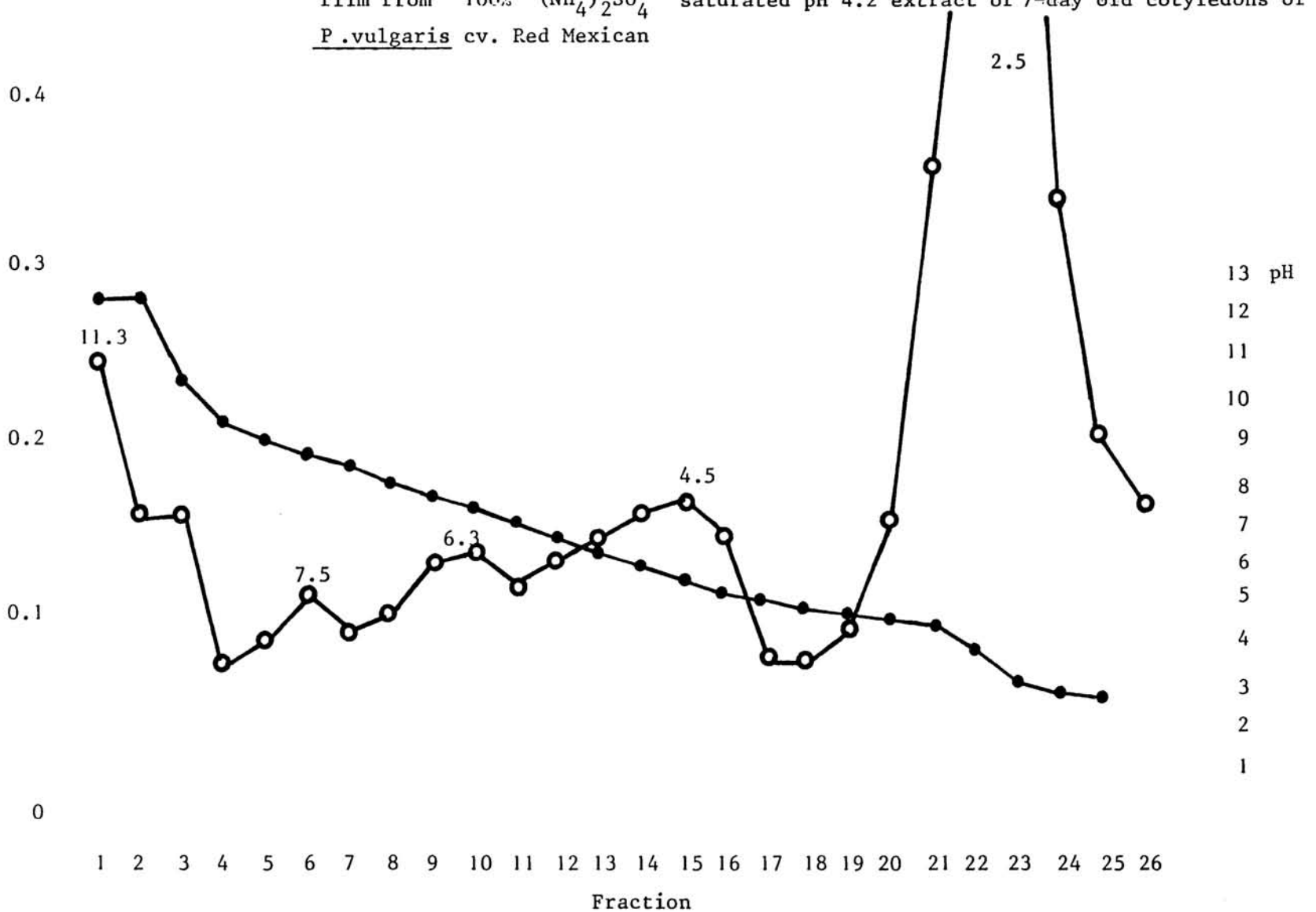


Figure 10. Absorbance at 280 nm of the eluent from ion exchange chromatography of the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of 7-day old Red Mexican cotyledons. DEAE cellulose equilibrated in 0.005M sodium phosphate buffer pH 6.8 was used and elution was with a linear gradient of sodium chloride from 0 to 1M in buffer

Figure 11. Absorbance (o-o-o) and pH (●-●-●) of the fractions after isofocussing of the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract of 7-day old cotyledons of P.vulgaris cv. Red Mexican



detectable agglutination of bacteria or erythrocytes. Fractions 5 - 7 agglutinated erythrocytes, but not bacteria. Fractions 8 - 11 and 12 - 16 agglutinated erythrocytes and caused some agglutination of cells of a Race 1 isolate of P. phaseolicola, but not cells of a Race 2 isolate. The combined fraction 19 - 26 agglutinated erythrocytes and showed the greatest differential agglutinating activity for cells of the two races of P. phaseolicola (Table 8). The results suggested that the major protein component of the pH 4.2 extract of 7-day RM cotyledons had both erythrocyte and bacterial cell agglutinating properties. However, it is also possible that another substance coincident with the major protein peak is responsible for some or all of the activity. The presence of a protein with isoelectric point 7.5 which agglutinates erythrocytes but not bacteria is in accordance with the results from section b) which suggested that possibly the component with bacterial agglutinating activity was not solubilized by 0.9% w/v saline whereas other compoent(s) with erythrocyte agglutinating activity alone were. The results suggested that the proteins with isoelectric points 6.3 and 4.5 had some differential agglutinating activity for the two races of P. phaseolicola. However later work indicated that these peaks were probably contaminated with the active substance present mainly in fractions 19 - 26.

A molecular weight determination of the protein with isoelectric point 2.5 was performed, and discontinuous polyacrylamide gel electrophoresis (disc-PAGE) of the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 and 0.9% w/v saline extracts to compare the proteins extracted.

d) Molecular weight determination by continuous PAGE

The dialysed, concentrated sample from the isoelectric focussing column containing the major protein peak was mixed 1:1 with disrupting buffer and boiled for 5 min, as in Materials and Methods 8 A b). BPB marker (Materials and Methods section 8 A a)) was added and 0.05 cm^3 of the mixture applied to gel slots. The combinations applied were : sample + BPB; sample +

Table 8 Agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) and erythrocytes by the 5 protein peaks from the isofocussing of the surface film from 100% $(NH_4)_2SO_4$ saturation of pH 4.2 extracts of 7-day old cotyledons of P. vulgaris cv. Red Mexican

		Bacteria									Erythrocytes				
Isoelectric point		11.3	7.5	6.3		4.5		2.5			11.3	7.5	6.3	4.5	2.5
Dilution	protein concentration mg cm^{-3}	R1(X)	R1(X)	R1(X)	882	R1(X)	882	R1(X)	882	protein concentration mg cm^{-3}					
Control	0	0[a]	0	0	0	0	0	0	0	0	0	0	0	0	0
2^1	0.355	0	0	1	0	2	0	4	4	0.320	0	4	4	4	4
2^2	0.178	0	0	1	0	1	0	4	2	0.160	0	4	4	4	4
2^3	0.089	0	0	0	0	0	0	3	0	0.080	0	4	3	4	4
2^4	0.044	0	0	0	0	0	0	2	0	0.040	0	4	0	4	4
2^5	0.022	0	0	0	0	0	0	0	0	0.020	0	4	0	0	0
2^6	0.011	0	0	0	0	0	0	0	0	0.010	0	0	0	0	0

[a] agglutination index - see Plate 1

BPB + calibration markers; BPB + calibration markers. Figure 12 i), ii) and iii) show the respective gel scans at 550 nm after staining with Kenacid blue. Three peaks detected from the disrupted sample had mobilities of 0.61, 0.75 and 0.80 relative to BPB which indicated molecular weights of 3.24×10^4. 2.35×10^4 and 2.10×10^4 for the sub-units of the protein with isoelectric point 2.5 (Figure 13).

e) Disc-PAGE

0.025 cm^3 samples of solutions of the surface films from 100% $(NH_4)_2SO_4$ saturated pH 4.2 and 0.9% w/v saline extracts from 1-day RM cotyledons were loaded on to cylindrical polyacrylamide gels and electrophoresis was performed as in Materials and Methods 8 A a). The samples contained 7.2 and 8.5 mg protein cm^{-3} respectively, determined by Folin-Lowry estimation. The gels were stained with Kenacid blue and after destaining were scanned at 550 nm (Figure 14). Qualitatively the proteins released by both procedures were identical, except for a minor peak with relative mobility 0.57 present in the saline but not the pH 4.2 extract. Quantitatively similar amounts of the other proteins were released by both procedures. This suggested that agglutination of bacteria was not caused by protein in the surface film from 100% $(NH_4)_2SO_4$ saturation of the pH 4.2 extracts. Alternatively, the protein with relative mobility 0.57 in the saline extract could have acted as an inhibitor preventing bacterial agglutination.

The major protein peak in the extracts had relative mobility c. 0.20 (Figure 14). This peak presumably corresponded to the major protein peak with isoelectric point 2.5 in the isoelectric focussing experiment. Disc-PAGE of the protein with isoelectric point 2.5 was performed and the gel scans compared to those obtained after disc-PAGE of the 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract from 7-day RM cotyledons. 0.05 cm^3 of each solution were loaded on to the gels, which were stained after running with Kenacid blue and scanned at 550 nm

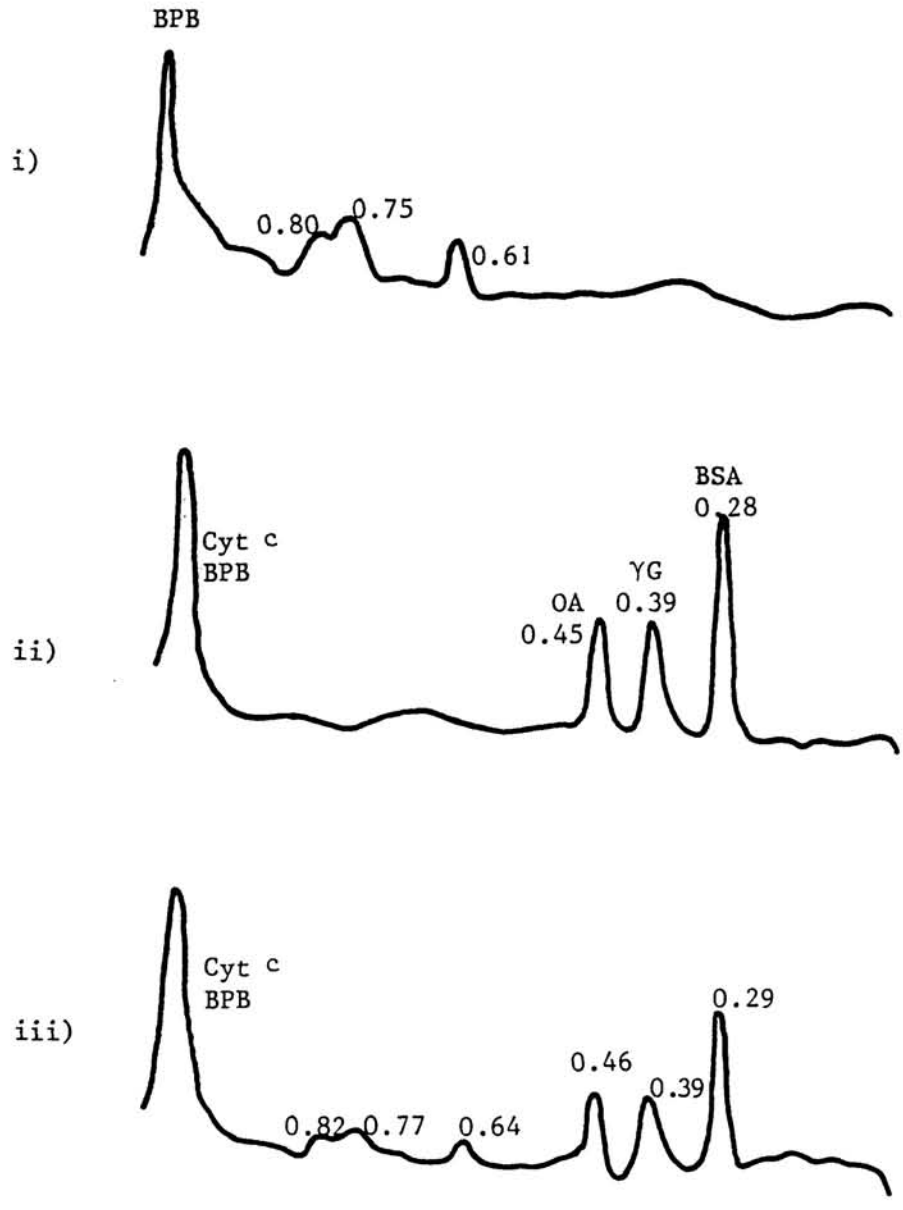


Figure 12. Gel scan at 550 nm after Kenacid blue staining of the gels from continuous PAGE of

i) the major protein from the isofocussing experiment

ii) protein markers of known molecular weight

iii) sample + markers

BPB - bromophenol blue, BSA - bovine serum albumin,

Cyt c - cytochrome c, γG - gamma globulin, OA - ovalbumin

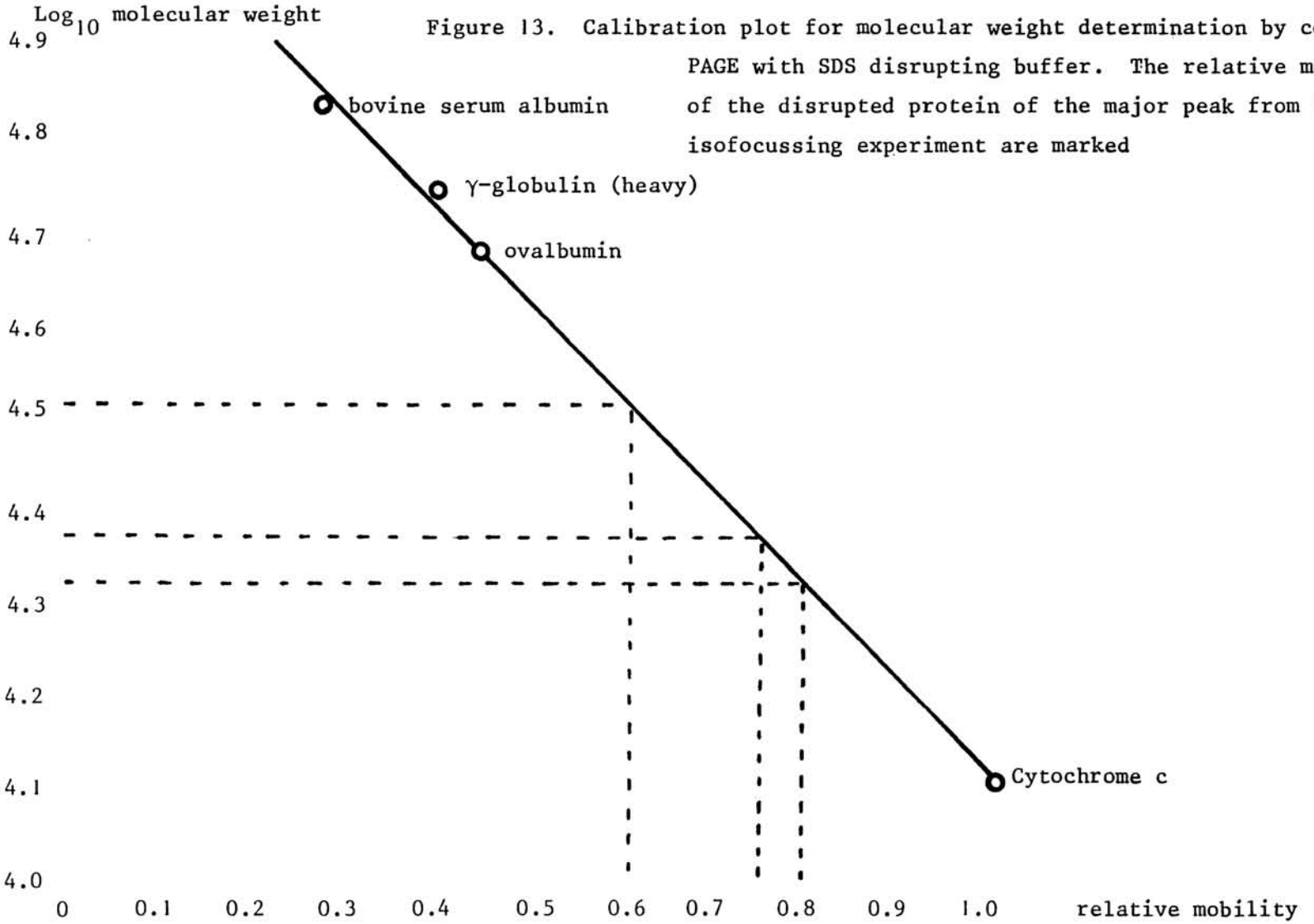


Figure 13. Calibration plot for molecular weight determination by continuous PAGE with SDS disrupting buffer. The relative mobilities of the disrupted protein of the major peak from the isofocussing experiment are marked

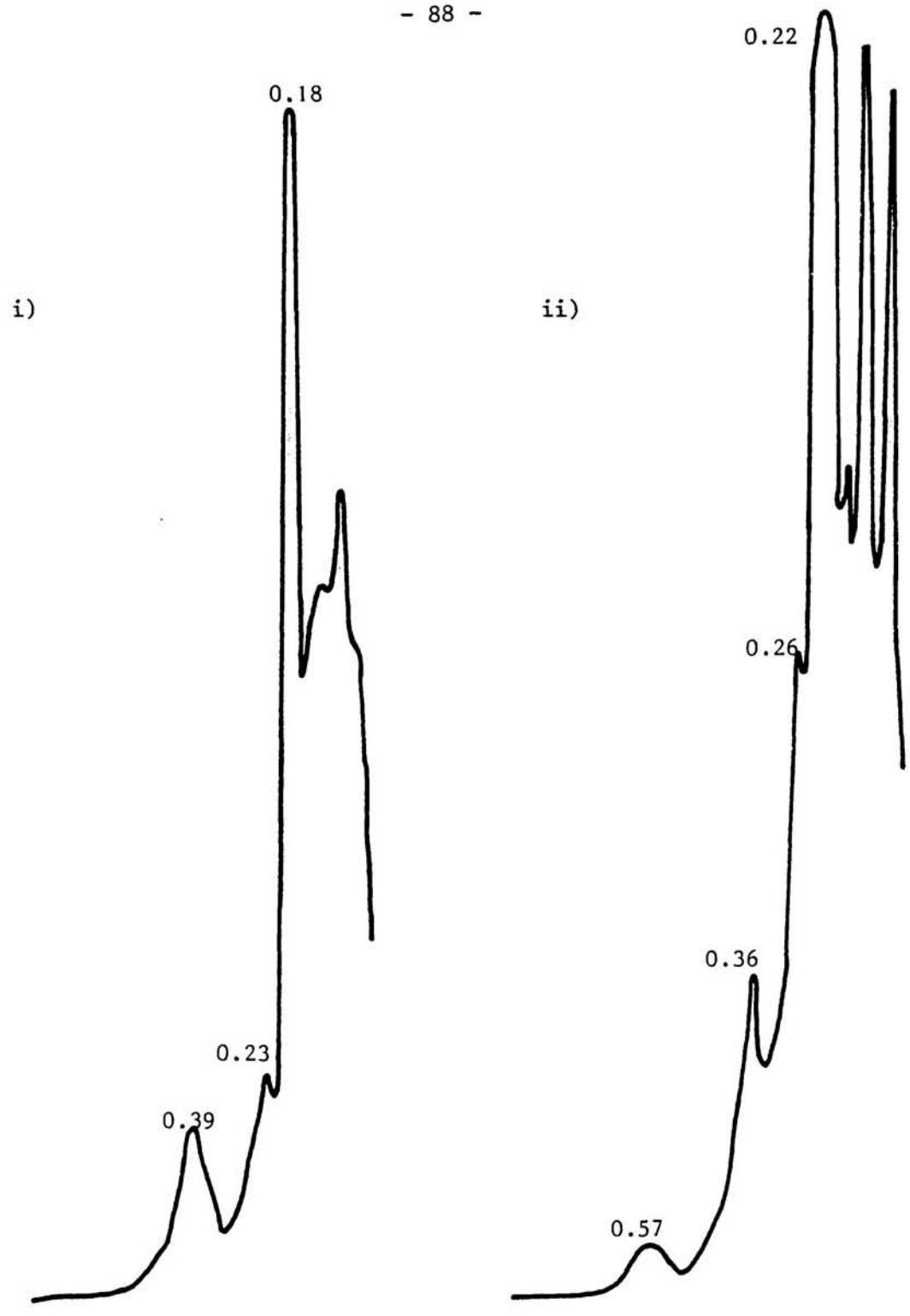


Figure 14. Gel scans at 550 nm of Kenacid blue stained gels after disc-PAGE of :

i) surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract of 1-day Red Mexican cotyledons

ii) surface film from 100% $(NH_4)_2SO_4$ saturated 0.9% w/v saline extracts of 1-day Red Mexican cotyledons

Relative mobilities were determined with reference to a bromophenol blue marker

(Figure 15). The protein with relative mobility c. 0.20 corresponded to the protein with isoelectric point 2.5, which had sub-units of MW 3.24×10^4, 2.35×10^4 and 2.10×10^4; very small quantities of the two adjacent proteins were present, possibly breakdown products of the major protein. This protein was the most likely to be the bacterial and erythrocyte agglutinin. However, since this was present in the saline extract, which caused no bacterial agglutination, some inhibitor which prevented bacterial but not erythrocyte agglutination must be postulated as present in this extract to account for the observations. Alternatively, bacterial agglutination could have been caused by another, still undetected substance.

It was decided to investigate whether bacteria adhered to one region of the gels more than any other, and to see if this region corresponded to any particular staining band. Gels were run with the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract from 7-day RM cotyledons and the purified major peak from isoelectric focussing. 0.05 cm^3 of the samples being loaded onto the gels. After electrophoresis duplicate gels were either stained with Kenacid blue for protein or periodate acid - Schiff's (PAS) stained for carbohydrate, whereas others were sliced longitudinally under aseptic conditions. Care was taken to leave the spacer gels attached to facilitate orientation when comparing sliced to stained gels. The sliced gels were immersed for 15 min in a 1×10^{10} cells cm^{-3} sterile distilled water suspension prepared from a 24h King's B slope culture of P. phaseolicola isolate R1(X). The gels were then rinsed gently through 8 changes of sterile distilled water, air dried for 20 min and laid in petri dishes on King's B agar. After 48h incubation at 25°C the plates were examined for bacterial growth in daylight and longwave ultra violet radiation (366 nm). On some plates bacteria had grown along the whole length of the gel, possibly due to insufficient drying before laying on the agar and subsequent swimming of the bacterial cells. In other gels from both samples bacteria grew mainly at the spacer gel end of the gels. In one case, a gel of the sample from the

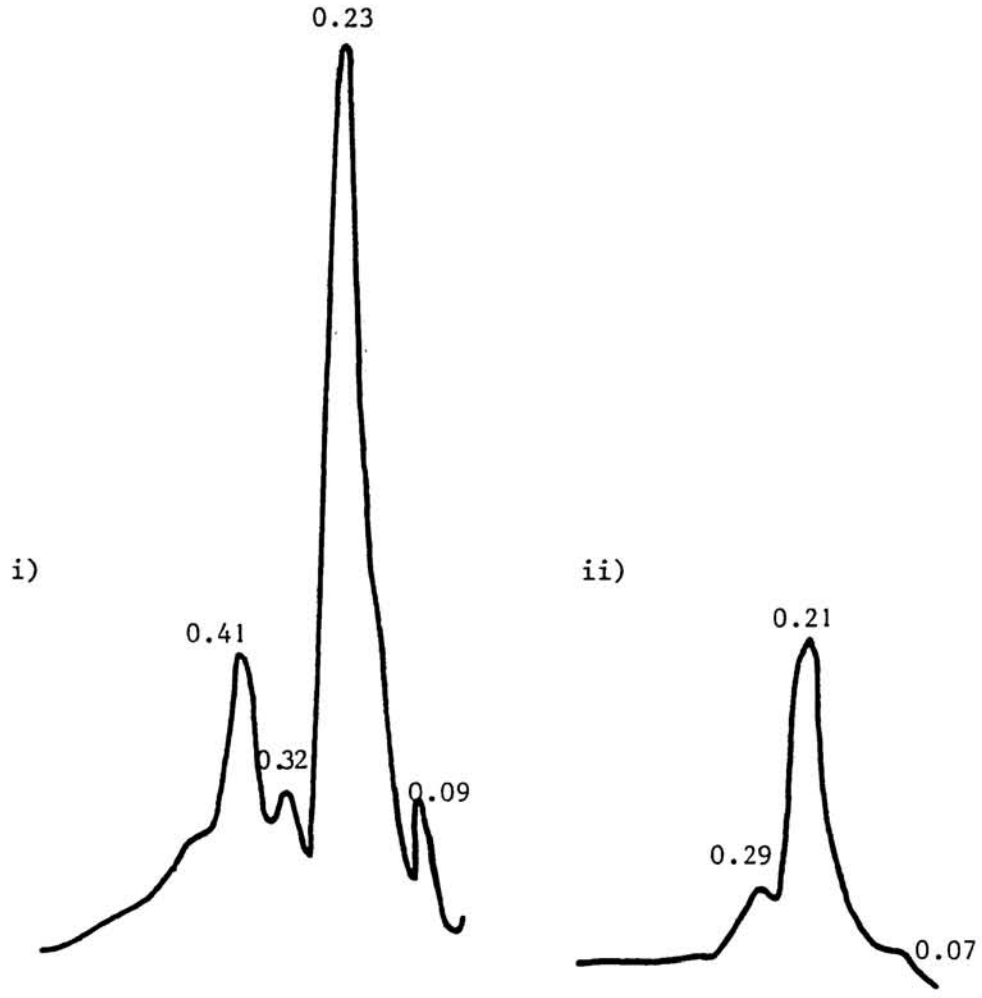


Figure 15. Gel scans at 550 nm of Kenacid blue stained gels after disc-PAGE of :

i) surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract of 7-day Red Mexican cotyledons

ii) the major protein obtained by isofocussing the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract of 7-day Red Mexican cotyledons

Relative mobilities were calculated with reference to a bromophenol blue marker

isoelectric focussing column had bacterial growth associated only with the spacer gel. Examination under longwave ultra violet light facilitated comparison of the relative amounts of bacterial growth, since diffusion of the bright blue fluorescing pigment into the agar showed very clearly where most bacterial growth had occurred. In some cases marked bacterial growth had occurred at the lower (BPB) end of the gels, possibly due to fluid droplets containing bacterial cells, which had run along the gels to their ends. The experiment cannot be regarded as unequivocally locating the region of the gels to which the bacteria adhere, although the spacer gel end can be tentatively suggested. The results are interesting when considered with the results from PAS staining of the gels from the various disc-PAGE experiments (Figures 16 and 17). Kenacid blue stains protein whereas PAS stains carbohydrate. The two procedures are used in conjunction to show the location of glycoproteins, which would be stained by both reagents, whereas proteins without attached carbohydrate would only be stained by Kenacid blue. A substance was found in the spacer gels after disc-PAGE of active fractions from pH 4.2 extracts of 1 and 7-day cotyledons and of the sample from the isoelectric focussing column, which stained with PAS. Significantly, the spacer gels from the 0.9% w/v saline extract after disc-PAGE showed much less PAS staining. The distribution of this substance mirrored the bacterial agglutinating activity of the samples, and suggested that the fuchsin staining substance may cause agglutination of bacteria. Since the fuchsin staining substance in the spacer gel did not stain with Kenacid blue, it contained little or no protein, and was probably largely carbohydrate in nature. It can be inferred that it was negatively charged in order to enter and migrate through the spacer gel, and possibly also of high moecular weight since it failed to enter the 10% acrylamide resolving gel where the pore size was much smaller than in the 3% acrylamide spacer gel. Since the carbohydrate was negatively charged it would migrate towards the anode in the isoelectric focussing experiment. This would account for the distribution of bacterial agglutinating activity seen in

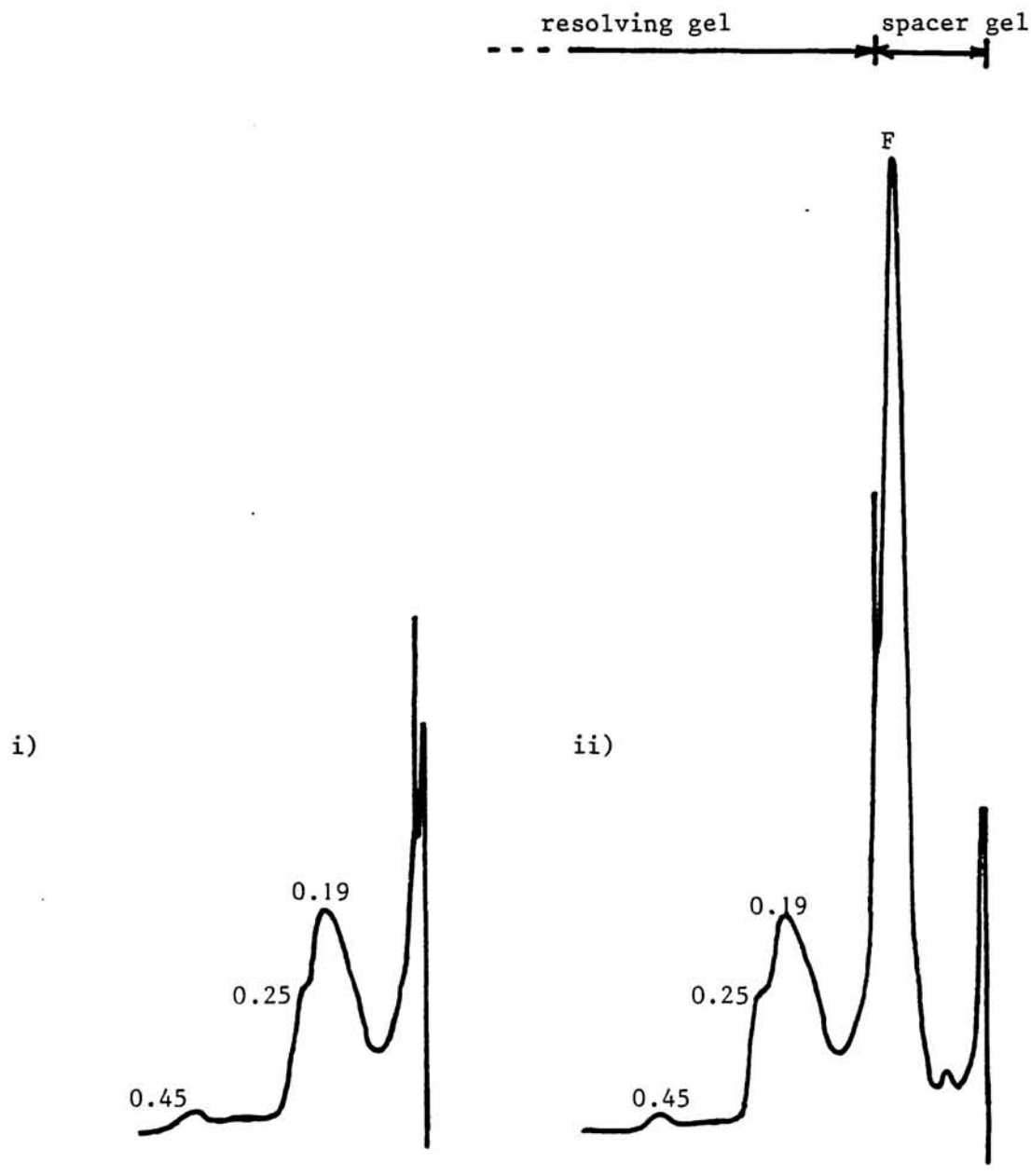


Figure 16. Gel scans at 520 nm of periodate acid-Schiff's stained gels after disc-PAGE of the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extract from 1-day Red Mexican cotyledons

i) without spacer gel

ii) with spacer gel attached to resolving gel

Relative mobilities were calculated with reference to a bromophenol blue marker

F - fuchsin staining substance in spacer gel

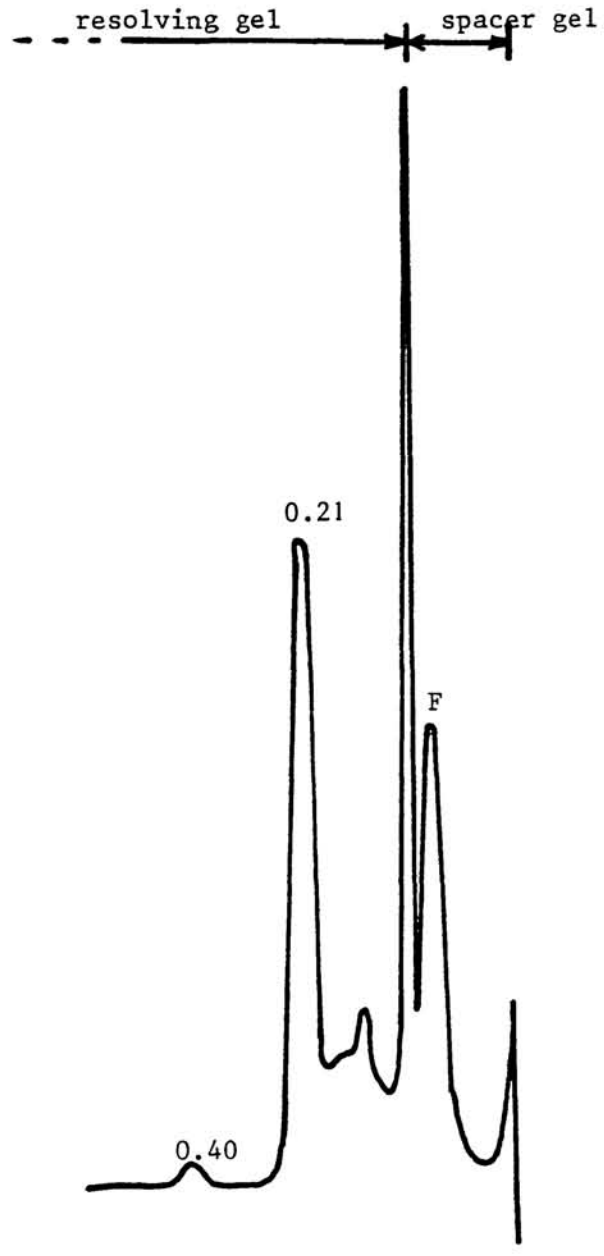


Figure 17. Gel scan at 520 nm of a periodate acid-Schiff's stained gel after disc-PAGE of the surface film from 100% $(NH_4)_2SO_4$ saturated 0.9% w/v saline extract from 1-day Canadian Wonder cotyledons. Relative mobilities were calculated with reference to a bromophenol blue marker

F - fuchsin staining substance in spacer gel

the fractions collected from the isoelectric focussing column (Figure 11, Table 8).

To investigate the possibility that carbohydrate in the samples was causing bacterial agglutination, it was decided to carry out periodate oxidation and observe any effect on the performance of samples in agglutination assays.

f) Periodate oxidation

Periodic acid will cleave the carbon-carbon bond between adjacent carbon atoms which both have hydroxyl groups. Thus under mild conditions it is fairly specific for carbohydrates, which contain many of the above groups (vicinal diols), and will not affect protein.

A solution of the surface film from 100% $(NH_4)_2SO_4$ saturation of the pH 4.2 extract from 7-day RM cotyledons was subjected to periodate oxidation. The procedure was as in Materials and Methods section 9, and the treatments used are summarised in Table 9. The 3 + 3´ treatment was included to assess whether possible products of the reaction between periodate and glycerol affect agglutination.

The results are shown in Table 10. Periodate oxidation of the sample eliminated its capacity to agglutinate bacteria. Periodate itself lyses erythrocytes, but after this effect had been removed by dilution, haemagglutination was still observed, and appeared only slightly reduced in comparison to the controls.

These results separate the two properties of bacterial and erythrocyte agglutination and suggest that bacterial agglutination was due to carbohydrate in the extracts whereas erythrocyte agglutination was due to another substance, probably protein. Legumes are known to be rich sources of lectins, many of which have haemagglutinating properties.

Table 9 Treatments used in periodate oxidation of the surface film from 100% $(NH_4)_2SO_4$ saturation of pH 4.2 extracts of 7-day old cotyledons of P. vulgaris cv. Red Mexican

	Treatment			
	1	2	3[a]	3´[a]
Cotyledon extract	1[b]	1	1	-
0.05M periodic acid	-	1	-	1
0.1M sodium acetate buffer pH 4.7	1	-	-	-
Glycerol	-[c]	-[c]	-	2 drops

[a] 3 and 3´ mixed after 2h incubation

[b] All volumes in cm^3 mixtures incubated in the dark at 20°C for 2h

[c] 2 drops added after 2h incubation

Further support for the hypothesis that the two agglutinating activities were caused by different substances was obtained from heat treatment of the sample. Incubation of the surface film from 100% $(NH_4)_2SO_4$ saturation of the pH 4.2 extract from 7-day cotyledons in a boiling water bath for 1 min, raised its temperature to c. 95°C and virtually destroyed its haemagglutinating activity, but hardly affected bacterial agglutination (Table 11). Indeed, bacterial agglutination was still largely unaffected after 30 min incubation at this temperature.

Table 10 Agglutination of cells of Race 1 and 2 isolates of P. phaseolicola and erythrocytes after periodate oxidation of the surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of 7-day old cotyledons of P. vulgaris cv. Red Mexican

	Race 1 R1(X) Treatment[a]			Race 2 882 Treatment			Erythrocytes Treatment		
Dilution	1	2	3+3´	1	2	3+3´	1	2	3+3´
Control	0[c]	0	0	0	0	0	0	0	0
2^1	4	0	4	3	0	4	4	L	L
2^2	4	0	4	3	0	2	4	L	L
2^3	4	0	3	0	0	0	4	L	L
2^4	4	0	2	0	0	0	4	L	L
2^5	4	0	0	0	0	0	4	3	3
2^6	0	0	0	0	0	0	4	0	3
2^7	0	0	0	0	0	0	2	0	2
2^8							0	0	0

a see Table 9 L lysis c agglutination index - see Plate 1

Table 11 Agglutination of cells of Races 1 and 2 of P. phaseolicola by unheated and heated surface film from 100% $(NH_4)_2SO_4$ saturated pH 4.2 extracts of 7-day old cotyledons of P. vulgaris cv. Red Mexican

	Race 1 isolate R1(X)		Race 2 isolate 882		Erythrocytes	
Dilution	U	H	U	H	U	H
Control	0[a]	0	0	0	0	0
2^1	4	4	2	3	4	2
2^2	4	4	0	2	4	0
2^3	2	0	0	0	4	0
2^4	0	0	0	0	4	0
2^5	0	0	0	0	4	0
2^6	0	0	0	0	0	0

U unheated sample H heated sample a agglutination index - see Plate 1

B. Identification of the substance causing bacterial agglutination

The suggestion that bacterial agglutination was due to carbohydrate in the sample leads to speculation as to its identity and location in the plant cells. If binding to plant cell walls *in vivo* is important, plant cell wall polysaccharides would be important candidates, since they present the surface available for binding in the intercellular spaces. It was decided to isolate cell wall polysaccharides from RM and CW and assess their ability to agglutinate bacterial cells. Since low pH conditions released the activity into the extract from RM cotyledons, and low pH also solubilizes pectic polysaccharide from cell walls, this was suspected as a likely candidate for the fuchsin staining material observed in the spacer gels after disc-PAGE. To test these hypotheses it was decided to extract pectic polysaccharide by various methods from cotyledons and leaves and measure its bacterial agglutinating activity. The effect on agglutinating activity of incubation of some of the extracts with enzymes was also investigated.

a) Extraction of pectic polysaccharides with dilute HCl (see Materials and Methods A a) ii), B a) and b))

i) 1-day cotyledons of RM and CW

The acetone and ethanol washing steps were used to remove lipid and low molecular weight phenolics respectively along with any other substances soluble in these solvents. The mean yield of 5 extractions of CW cotyledons was 1.23g of ethanol precipitate for each 100g fresh weight of cotyledons, whereas for RM cotyledons was 2.48g for each 100g cotyledons. The standard error of the difference between the two sample means = 0.54, and a statistical t-test indicated that the yield of ethanol precipitate from both cvs. was significantly different ($P = 0.05$). On dissolving in 0.02M sodium phosphate buffer pH 6.8 an opalescent solution was obtained,

which on centrifugation at 8,000g for 10 min gave a clear supernatant and a pellet which gave a blue-black colouration reaction with iodine dissolved in potassium iodide, indicating the presence of starch. The clear supernatant was used in agglutination assays. Table 12 shows the results when 10 mg cm^{-3} of buffer of each extract are present in the well of 2^1 dilution and suggest that the extract from RM is more active than the extract from CW, in terms of dilution end point, and relative degree of agglutination of the Race 2 isolate at the 2^1 dilution. However, the results for the extracts from the two cultivars are not strictly comparable since the relative proportions of starch in each was not known. However the much greater yield of extract (100% more) from equivalent fresh weights of RM cotyledons compared to CW cotyledons is perhaps indicative of why very little activity was obtained from CW cotyledons by the milder methods in Materials and Methods A a) i) and A b). In order to make the results more comparable, 20 mg cm^{-3} solutions of each extract in buffer were prepared and after centrifuging the pellets were dried and weighed. The differences gave the mass of substance in solution in each case and this was 14.8 mg cm^{-3} and 15.2 mg cm^{-3} for the extracts from CW and RM respectively. This suggests that the proportion of insoluble substance in the two extracts is similar and that the greater yield from RM is not due to the extraction of more buffer insoluble substance (presumably largely starch). When adjusted to contain 14 mg of dissolved material cm^{-3} the agglutinating activities of the extracts were similar (Table 13). However the dilution end points were greater for both races with the RM extract.

ii) RM leaves

Primary and secondary leaves of 2 week old RM seedlings were used to prepare an acetone powder which was subsequently extracted with dilute HCl at pH 3.0 (Materials and Methods 3 B a) and 3 B b)). 35 mg of extract in 2 cm^3 of 0.02M sodium phosphate buffer gave an opalescent solution which

Table 12 Agglutination of cells of Race 1 and 2 isolates of *P. phaseolicola* by pectic polysaccharide extracted from 1-day old cotyledons of *P. vulgaris* cvs. Red Mexican (RM) and Canadian Wonder (CW) by dilute HCl at pH 3.0

	RM		CW	
	Race 1	Race 2	Race 1	Race 2
Dilution	R1(X)	881	R1(X)	882
Control	0[a]	0	0	0
2^1	4	4	4	2
2^2	4	4	4	0
2^3	4	0	4	0
2^4	4	0	0	0
2^5	0	0	0	0
Dilution end-point	2^4	2^2	2^3	2^1

a agglutination index - see Plate 1

Table 13 Agglutination of cells of Race 1 and 2 isolates of *P. phaseolicola* by pectic polysaccharide extracted from 1-day old cotyledons of *P. vulgaris* cvs. Red Mexican (RM) and Canadian Wonder (CW) by dilute HCl at pH 3.0, after adjusting the test solution to contain the same concentrations of dissolved substances

	concen-tration of dissolved substance	RM		CW	
		Race 1	Race 2	Race 1	Race 2
Dilution	(mg cm^{-3})	R1(X)	882	R1(X)	882
Control	0	0[a]	0	0	0
2^1	7.00	4	3	4	3
2^2	3.50	4	2	4	0
2^3	1.75	4	0	4	0
2^4	0.88	2	0	0	0
2^5	0.44	0	0	0	0
Dilution end-point		2^4	2^2	2^3	2^1

a agglutination index - see Plate 1

on centrifugation gave a clear supernatant and a pellet which gave no colour reaction with iodine in potassium iodide solution. From the mass of the residue after centrifugation it was calculated that the supernatant contained 8 mg cm^{-3} of dissolved substance. No agglutinating activity could be detected with cells of Race 1 or 2 isolates of P. phaseolicola. Solutions of chelating agents are more efficient at extracting pectic polysaccharide than low pH, since they chelate the calcium associated with the carboxyl groups of the sugar acids and cross linking the chains of pectic polysaccharide, thus rendering them more soluble in aqueous solvents. Thus it was decided to use EDTA to extract pectic polysaccharide from cotyledons and leaves of RM and CW.

b) Extraction of pectic polysaccharides with EDTA (see Materials and Methods 3 A c) and 3 B b))

i) 1-day RM and CW cotyledons

The mean yield of 2 extractions of CW cotyledons was 4.95g ethanol precipitate for each 100g fresh weight of cotyledons, whereas the mean yield from 2 extractions of RM cotyledons was 7.63g for each 100g cotyledons. Opalescent solutions were obtained on dissolving the extracts in 0.02M sodium phosphate buffer pH 6.8, but on centrifuging and adjusting the supernatants to contain 20 mg of dissolved substance cm^{-3}, very similar agglutinating activity was shown by extracts from both cultivars (Table 14).

ii) Leaves

Primary and secondary leaves of 2 - 3 week old RM and CW seedlings were used to prepare acetone powders which were subsequently extracted with EDTA (Materials and Methods 3 B a) and 3 B c). The extracts dissolved completely in 0.02M sodium phosphate buffer pH 6.8 to give clear solutions,

Table 14 Agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by pectic polysaccharide extracted from 1-day old cotyledons of P. vulgaris cvs. Red Mexican (RM) and Canadian Wonder (CW) by EDTA

Dilution	concentration of dissolved substance (mg cm^{-3})	RM		CW	
		Race 1	Race 2	Race 1	Race 2
		RI(X)	882	RI(X)	882
Control	0	0[a]	0	0	0
2^1	10.00	4	4	4	4
2^2	5.00	4	3	4	4
2^3	2.50	4	1	3	2
2^4	1.25	3	0	3	0
2^5	0.63	0	0	0	0
Dilution end-point		2^4	2^3	2^4	2^3

A agglutination index - see Plate 1

so no adjustment for the quantity of dissolved substance for a given weight of extract was necessary. The mean yield of two extractions from RM and CW was 0.23 and 0.47g precipitate for each 10g of acetone powder respectively. The agglutinating activity of the extracts varied. The first extraction performed gave a very active preparation from RM leaves which differentially agglutinated cells of Race 1 and 2 isolates of P. phaseolicola, whereas the first extract from CW was virtually inactive. When the extractions were repeated however the preparation from RM leaves had much lower agglutinating activity and the preparation from CW leaves showed more activity than previously (Table 15). Since extraction of pectic polysaccharide with EDTA is somewhat degradative it would be expected to yield variable

Table 15 Agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by pectic polysaccharide extracted from leaves of P. vulgaris cvs. Red Mexican (RM) and Canadian Wonder (CW) by EDTA

		RM				CW			
		Race 1[a]	Race 2	Race 1[b]	Race 2	Race 1[a]	Race 2	Race 1[b]	Race 2
Dilution	concentration (mg cm^{-3})	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0	0[c]	0	0	0	0	0	0	0
2^1	10.00	4	4	4	3	2	0	4	3
2^2	5.00	4	2	2	1	2	0	2	1
2^3	2.50	4	2	0	0	0	0	0	0
2^4	1.25	4	1	0	0	0	0	0	0
2^5	0.63	0	0	0	0	0	0	0	0
Dilution end-point		2^4	2^4	2^2	2^2	2^2	-	2^2	2^2

a,b assays performed with preparations from different EDTA extractions

c agglutination index - see Plate 1

polysaccharide fractions. Therefore, rather than perform repeated extractions to try and obtain extracts of consistent agglutinating activity it was decided to use a milder, less degradative extraction procedure.

c) Extraction of pectic polysaccharides with citrate-oxalate buffer and sodium carbonate solution (Materials and Methods 3 B d) and 3 B e))

Primary and secondary leaves of 2 - 3 week old seedlings of RM and CW were used to prepare acetone powders which were extracted initially with 0.1M citrate-oxalate buffer pH 4.0 and the residue subsequently extracted with 0.1M Na_2CO_3. The citrate-oxalate buffer extract was fractionally precipitated with ethanol to give the acidic polysaccharide, which was very gelatinous, and on further addition of ethanol the neutral polysaccharide fraction, which formed a powdery precipitate. Agglutinating activity was associated only with the acidic polysaccharide, which showed similar differential agglutination of cells of Race 1 and 2 isolates of P. phaseolicola for RM and CW, both in degree of agglutination in corresponding wells and in dilution end points for the two races (Table 16). Table 17 shows the differential agglutination of 5 isolates of P. phaseolicola by the acidic pectic polysaccharides from RM and CW leaves. Comparing the results from Table 17 with those in Tables 1 and 2 for the agglutination of the same isolates by the crude pH 4.2 extract from cotyledons shows a large degree of similarity, suggesting that it may be pectic material in the cotyledon extracts which is causing agglutination.

The mean yield of 7 extractions from RM and 5 from CW leaves was 0.52g and 0.48g respectively. The standard errors of the difference between the two sample means = 0.12 and a statistical t-test indicated that the yield of acidic pectic polysaccharide from both cvs. was not different ($P = 0.05$).

Table 16 Agglutination of cells of Race 1 and 2 isolates of *P. phaseolicola* by pectic polysaccharides extracted from leaves of *P. vulgaris* cvs. Red Mexican (RM) and Canadian Wonder (CW) by 0.1M citrate-oxalate buffer pH 4.0

		RM				CW			
	concentration	acidic polysaccharide		neutral polysaccharide		acidic polysaccharide		neutral polysaccharide	
Dilution	(mg cm^{-3})	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0	0[a]	0	0	0	0	0	0	0
2^1	5.00	4	4	0	0	4	4	0	0
2^2	2.50	4	3	0	0	4	4	0	0
2^3	1.25	4	0	0	0	4	0	0	0
2^4	0.63	4	0	0	0	4	0	0	0
2^5	0.32	0	0	0	0	0	0	0	0
Dilution-end point		2^4	2^2	-	-	2^4	2^2	-	-

a agglutination index - see Plate 1

Table 17 Differential agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by acidic pectic polysaccharide extracted from leaves of P. vulgaris cvs. Red Mexican (RM) and Canadian Wonder (CW) by 0.1M citrate-oxalate buffer pH 4.0

		RM					CW				
		Race 1 isolates			Race 2 isolates		Race 1 isolates			Race 2 isolates	
Dilution	concentration (mg cm^{-3})	R1(X)	31A	710A	777A	882	R1(X)	31A	710A	777A	882
Control	0	0[a]	0	0	0	0	0	0	0	0	0
2^1	5.00	4	4	4	4	4	4	4	4	3	4
2^2	2.50	4	4	4	4	3	4	4	4	3	4
2^3	1.25	4	3	3	0	0	4	3	3	1	0
2^4	0.63	4	2	2	0	0	4	3	0	0	0
2^5	0.32	0	0	0	0	0	0	0	0	0	0
Dilution end-point		2^4	2^4	2^4	2^2	2^2	2^4	2^4	2^3	2^3	2^2

a agglutination index - see Plate 1

The fraction released by mild alkali treatment(0.1M Na_2CO_3)of the residue from the citrate-oxalate buffer extraction had no detectable agglutinating activity with cells of either race of P. phaseolicola.

The relative yields of active extracts from RM and CW cotyledons and leaves by the different extraction procedures (Table 18) suggest that using more efficient and less degradative extraction procedures for pectic material yields correspondingly more material. It also appears that significantly more active polysaccharide can be obtained from cotyledons of RM than CW, but that leaves of both cultivars show no significant difference. If there is more active material available for extraction in RM cotyledons than in CW cotyledons this would agree with the results in section A where little activity was released by treatment of CW cotyledons at pH 4.2, which would be less efficient than treatment at pH 3.0 for extracting pectic polysaccharides.

d) Protein content of pectic extracts from leaves

Some degree of contamination of the extracts of pectic polysaccharides with protein was probable and covalent linkage of protein to the pectic polysaccharide was possible.

Gels from disc-PAGE of 0.025 cm^3 aliquots of 20 mg cm^{-3} solutions of the EDTA extracts from RM and CW leaves showed very little staining with Kenacid blue (Figure 18)but appeared to have some peaks in common with those from pH 4.2 extracts of cotyledons (Figure 15 i)). PAS staining revealed very densely fuchsin stained regions in the spacer gels for both RM and CW extracts. In the CW extract gels the fuchsin staining region extended a little way into the resolving gel, suggesting the polysaccharide was slightly more degraded than in the RM extract. This would agree with the differences in agglutinating activity observed for the two extracts (Table 15). The results suggest there was little protein in the extracts,

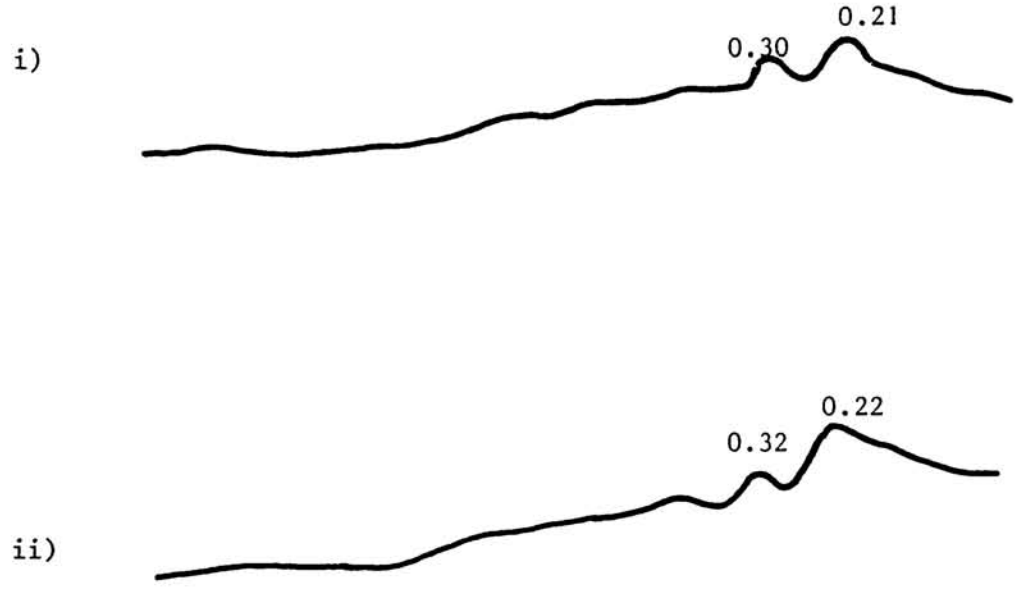


Figure 18. Gel scan at 550 nm of Kenacid blue stained gels after disc-PAGE of ethanol precipitates from EDTA extracts of leaves of :

i) P. vulgaris cv. Red Mexican

ii) P. vulgaris cv. Canadian Wonder

Relative mobilities were calculated with reference to a bromophenol blue marker

Table 18 Relative yields of active extracts from leaves and cotyledons of P. vulgaris cvs. Red Mexican (RM) and Canadian Wonder (CW) for the different extraction procedures used to obtain pectic polysaccharides

	Tissue				
	Cotyledons		Leaves		
Extraction method	RM	CW	RM	CW	
HCl pH 3.0	2.48^{a}_{g}	1.23^{a}_{g}	No activity detected		yields significantly different (P = 0.05)
EDTA	7.63^{b}_{g}	4.95^{b}_{g}	0.47^{b}_{g}	0.23^{b}_{g}	statistical analysis not performed
citrate-oxalate buffer	-	-	0.52^{c}_{g}	0.48^{a}_{g}	yields not significantly different (P = 0.05)

a mean of 5 values b mean of 2 values c mean of 7 values

and since the fuchsin staining regions did not stain with Kenacid blue, little, if any, protein was covalently linked to the polysaccharide.

Folin Lowry estimation of the protein content of 5 mg cm^{-3} solutions of the acidic pectic polysaccharide fractions from citrate-oxalate extraction of RM and CW leaves showed them to contain c. 0.30 mg cm^{-3} protein.

e) Periodate oxidation

Periodate oxidation of the acidic pectic polysaccharide fraction from citrate-oxalate extract of RM leaves was carried out and the effect on agglutination of cells of Race 1 and 2 isolates of P. phaseolicola studied. The treatments used are given in Table 19. Similarly to the results for the pH 4.2 extract from cotyledons bacterial agglutination was destroyed by the mild periodate oxidation regime employed (Table 20). This strongly suggests that carbohydrate in the fraction is the substance causing agglutination, and supports the hypothesis that the acidic pectic polysaccharide from bean cell walls is the carbohydrate in question.

Table 19 Treatments used in the periodate oxidation of the acidic pectic polysaccharide fraction from citrate-oxalate extraction of RM leaves

	Treatment			
	1	2	3[a]	3´[a]
acidic pectic[b] polysaccharide	1[c]	1	1	-
0.05M periodic acid	-	1	-	1
0.1M sodium acetate buffer pH 4.7	1	-	-	-
Glycerol	d	d	-	2 drops

a 3 and 3´ mixed after incubation b 20 mg cm^{-3} solution in water
c all volumes in cm^3, mixtures incubated in the dark at 20°C for 2h
d 2 drops added after 2h incubation

Table 20 Effect on agglutination of cells of Race 1 and 2 isolates of P. phaseolicola of periodate oxidation of the acidic pectic polysaccharide fraction from citrate-oxalate extraction of leaves of P. vulgaris or Red Mexican

	Race 1 R1(X) Treatment[a]			Race 2 882 Treatment[a]		
Dilution	1	2	3+3´	1	2	3+3´
Control	0[b]	0	0	0	0	0
2^1	4	0	4	1	0	1
2^2	4	0	4	0	0	0
2^3	3	0	3	0	0	0
2^4	0	0	0	0	0	0

a see Table 19 b agglutination index - see Plate 1

Table 21 Treatments used in incubation of acidic pectic polysaccharide from citrate-oxalate extracts of leaves of P. vulgaris cv. Red Mexican with polygalacturonase enzymes

	Treatment			
	1	2	3	4
pectic polysaccharide[a]	1[b]	1	1	-
polygalacturonase[c]	-	1	-	1
autoclaved polygalacturonase[d]	-	-	1	-
buffer[e]	1	-	-	1

a 10 mg cm^{-3} acidic polysaccharide from citrate-oxalate extraction of RM leaves. Dissolved in the appropriate buffer

b all volumes in cm^3

c Pectinase (Sigma), PG II, III or PG IV at c. 1 mg cm^{-3} in the appropriate buffer

d 121°C for 15 min

e 0.05M sodium acetate buffer pH 4.5 for Pectinase (Sigma)
0.02M sodium phosphate buffer pH 5.8 for PG II, III and PG IV

f) Enzyme digestions

i) Pectic enzymes

10 mg cm^{-3} solutions of the acidic pectic polysaccharide from citrate-oxalate extractions of RM and CW leaves were incubated with polygalacturonase enzymes at 30°C for 1.5h. The treatments used are given in Table 21. All the polygalacturonase enzymes destroyed the agglutinating activity of the extracts (Table 22, 23), confirming that pectic polysaccharide was the substance causing agglutination. The incubation with autoclaved

Table 22 Effect on agglutination of cells of P. phaseolicola isolates R1(X) (Race 1)and 882 (Race 2) of incubation with polygalacturonase enzymes of the acidic pectic polysaccharide from citrate-oxalate extraction of leaves of P. vulgaris cv. Red Mexican

	Pectinase (Sigma)								PG II, III								PG IV							
Treatment[a]	1		2		3		4		1		2		3		4		1		2		3		4	
Dilution	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2^1	4	3	1	0	4	2	0	0	4	4	0	0	4	4	0	0	4	4	0	0	4	3	0	0
2^2	4	2	0	0	4	2	0	0	4	1	0	0	4	1	0	0	4	3	0	0	4	1	0	0
2^3	4	1	0	0	4	1	0	0	3	0	0	0	3	0	0	0	3	1	0	0	3	0	0	0
2^4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilution end-point	2^3	2^3	2^1	-	2^3	2^3	-	-	2^3	2^2	-	-	2^3	2^2	-	-	2^3	2^3	-	-	2^3	2^2	-	-

a see Table 21 b agglutination index - see Plate 1

Table 23 Agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) after incubation of the acidic pectic polysaccharide from citrate-oxalate extraction of leaves of P. vulgaris cv. Canadian Wonder with PG IV

	Treatment[a]							
	1		2		3		4	
Dilution	R1(X)	882	R1(X)	822	R1(X)	882	R1(X)	882
Control	0[b]	0	0	0	0	0	0	0
2^1	4	4	0	0	4	3	0	0
2^2	4	1	0	0	4	0	0	0
2^3	3	0	0	0	3	0	0	0
2^4	0	0	0	0	0	0	0	0
Dilution end-point	2^3	2^2	-	-	2^3	2^1	-	-

a see Table 21 b agglutination index - see Plate 1

Table 24 Treatments used in incubation of acidic pectic polysaccharide from citrate-oxalate extracts of leaves of P. vulgaris cv. Red Mexican with pectin methyl esterase (PME) enzymes from apple or orange

	Treatment				
	1	2	3	4	5
pectic polysaccharide[a]	0.5[b]	0.5	0.5	-	0.5
PME[c]	-	0.5	-	0.5	-
autoclaved PME[d]	-	-	0.5	-	-
buffer[e]	0.5	-	-	0.5	0.5
toluene	-	1 drop	1 drop	1 drop	1 drop

a 10 mg cm^{-3} acidic pectic fraction from citrate-oxalate extraction of RM leaves. Dissolved in buffer

b all volumes in cm^3 c apple or orange PME c. 1 mg protein cm^{-3}

d 121°C for 15 min e 0.02M sodium phosphate buffer pH 6.8

polygalacturonase preparations was similar to the control in agglutination, suggesting that the reduction in agglutination observed with non-autoclaved preparations was due to enzymic activity. Polygalacturonase enzymes alone were tested and found to cause no agglutination of bacterial cells (Treatment 4).

The pectinase (Sigma) contained exo- and endo-polygalacturonase activity and PME activity. PG II, III had endopolygalacturonase activity and on investigation was shown to have a slight degree of PME activity. PG IV had endopolygalacturonase activity and no detectable PME activity, and thus by endo-hydrolytic activity alone was able to destroy the agglutinating properties of the acidic pectic polysaccharide. This suggests that linear sequences of sugar and/or uronic acid residues in the main chain or side chains, or their 3-dimensional arrangements are important for agglutination of bacterial cells. If agglutinating activity had simply been due to the presence of certain monomers within the chains endo-cleavage would not be expected to destroy it.

Incubation of the acidic pectic polysaccharide from citrate-oxalate extracts of RM leaves with apple PME at 30°C for 18h had no detectable effect on agglutination of cells of Race 1 and 2 isolates of P. phaseolicola (Tables 24 and 25). However, incubation with more active freshly prepared orange PME caused a slight reduction in the degree of agglutination of cells of both Races (Table 26). Since the effect of demethylation was only slight this suggests that the number of free carboxyl groups and therefore the overall change on the molecule is relatively unimportant for agglutination of bacterial cells, which is therefore not caused by non-specific charge effects. This lends further support to the hypothesis that it is the structural integrity of the polysaccharide which is important for agglutination. The toluene was added to the incubation mixtures to prevent microbial contamination during the 18h incubation. However, the results for treatments 1 and 5, one with and the other without toluene were identical. This suggests

Table 25 Effect on agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) of incubation with apple PME of the acidic pectic polysaccharide from citrate-oxalate extraction of leaves of P. vulgaris cv. Red Mexican

	Treatment[a]									
	1		2		3		4		5	
Dilution	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0[b]	0	0	0	0	0	0	0	0	0
2^1	4	3	4	3	4	3	0	0	4	3
2^2	4	2	4	2	4	2	0	0	4	2
2^3	4	1	4	1	4	1	0	0	4	1
2^4	0	0	0	0	0	0	0	0	0	0
Dilution end-point	2^3	2^3	2^3	2^3	2^3	2^3	-	-	2^3	2^3

a see Table 24 b agglutination index - see Plate 1

Table 26 Effect on agglutination of cells of *P. phaseolicola* isolates R1(X) (Race 1) and 882 (Race 2) of incubation with orange PME of the acidic pectic polysaccharide from citrate-oxalate extracts of leaves of *P. vulgaris* cv. Red Mexican

	Treatment[a]									
	1		2		3		4		5	
Dilution	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0[b]	0	0	0	0	0	0	0	0	0
2^1	4	2	4	1	4	2	0	0	4	2
2^2	4	2	4	1	4	2	0	0	4	2
2^3	3	1	2	0	3	1	0	0	3	1
2^4	0	0	0	0	0	0	0	0	0	0
Dilution end-point	2^3	2^3	2^3	2^2	2^3	2^3	-	-	2^3	2^3

a see Table 24 b agglutination index - see Plate 1

that microbial contamination during the 18h incubation was minimal and also that the presence of toluene did not affect the agglutination assay.

Time course studies of the digestion by PG IV of the acidic pectic polysaccharide from citrate-oxalate extracts of RM leaves were performed. PG IV was incubated with 20 mg cm^{-3} acidic pectic polysaccharide in 0.02M sodium phosphate buffer pH 5.8 at 30°C, and the reduction in viscosity was measured (see Materials and Methods section 10).

PG IV incubations set up as shown in Table 21, had the digestion stopped at various time intervals by placing the tubes in a boiling water bath for 5 min, and the reduction in agglutination of bacterial cells studied (Table 27). The reduction in viscosity which is proportional to the chain length of the pectic polysaccharide molecules is closely followed by the reductions in agglutination of cells of the Race 1 isolate R1(X) of P. phaseolicola (Figure 19), suggesting that agglutination of bacterial cells is also proportional to the chain length of the pectic polysaccharide.

Incubation of the pH 4.2 extract from 7-day RM cotyledons with pectinase (Sigma) for 1.5h at 30°C caused no detectable reduction in agglutination of bacterial cells. This is puzzling and suggests that another substance may be present in the extract which was able to differentially agglutinate cells of Race 1 and 2 isolates of P. phaseolicola. However the results for periodate oxidation of the extract (Table 10) are in agreement with the active substance being carbohydrate. It is possible that enzyme activity was interfered with due to a naturally occurring polygalacturonase inhibitor present in the cotyledons and carried through the stages of the extraction procedure. The incubation of relatively pure acidic pectic polysaccharide from cvs. RM and CW with highly purified pectic enzymes show that pectic polysaccharide from French bean can differentially agglutinate cells of the two races of P. phaseolicola. Since the acidic pectic fractions also contained small quantities of protein, incubations with protease were carried out to investigate possible effects on agglutination.

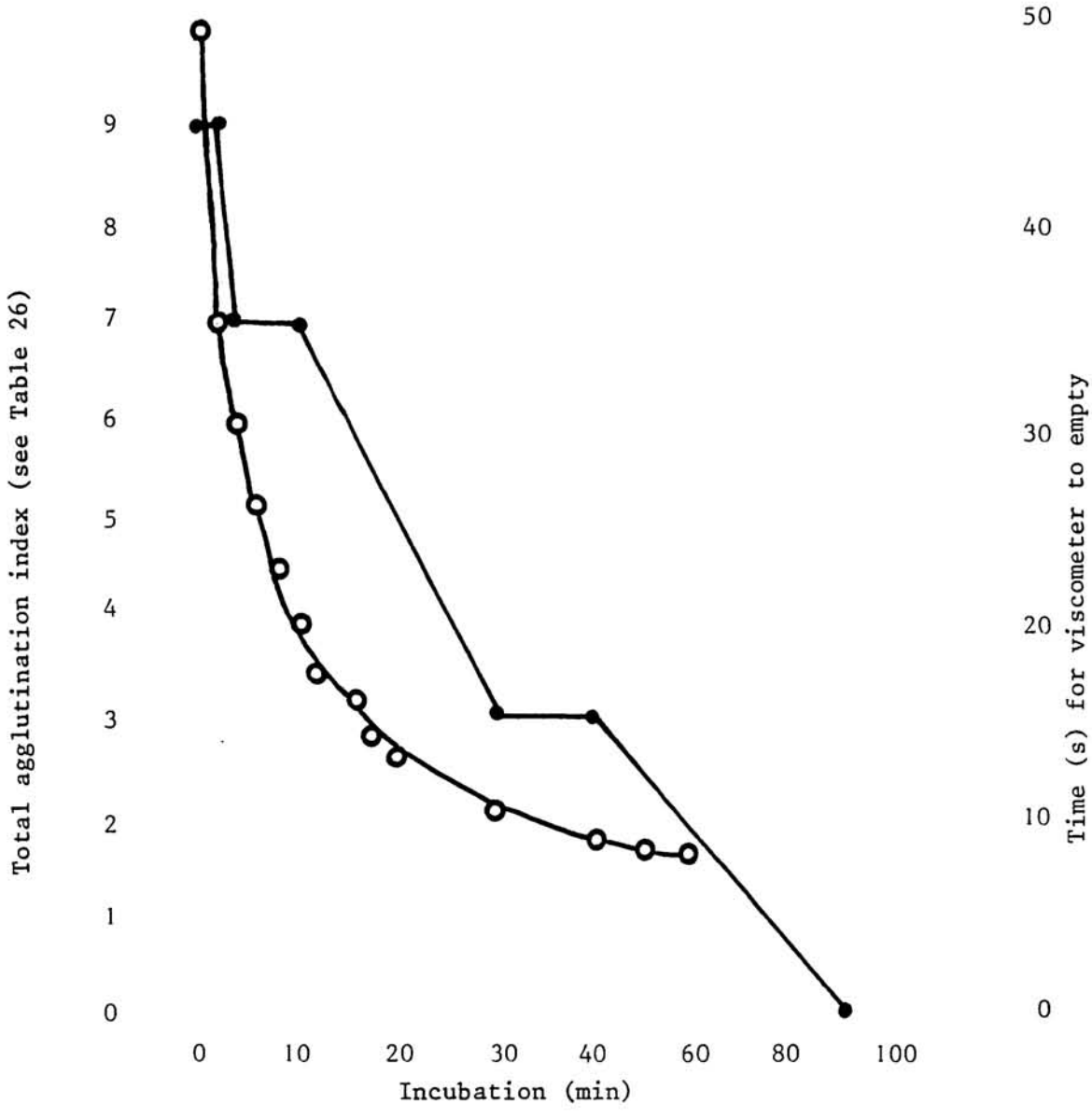


Figure 19. Reduction of agglutination (●-●-●) and time taken for viscometer to empty (o-o-o) with time of incubation of acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican with PG IV

Table 27 Time course of effect on agglutination of cells of *P. phaseolicola* isolates R1(X) (Race 1) and 882 (Race 2) by incubation of the acidic pectic polysaccharide from leaves of *P. vulgaris* cv. Red Mexican with PG IV

	Incubation time (min)																	
	0		1		2		4		5		10		30		40		90	
Dilution	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2^1	4	2	4	2	4	2	4	2	4	2	4	1	3	0	3	0	0	0
2^2	4	0	4	0	4	0	3	0	3	0	3	0	0	0	0	0	0	0
2^3	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2^4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total agglutination index[b]	9	2	9	2	9	2	7	2	7	2	7	1	3	0	3	0	0	0

a agglutination index - see Plate 1

b total agglutination index - sum of the agglutination indexes for each treatment

ii) Protease

1 mg cm^{-3} Protease V (Pronase AS) a protease from Streptomyces griseus with a very broad spectrum of activity was incubated for 1.5h at 30°C with 5 mg cm^{-3} acidic pectic fraction from citrate oxalate extraction of RM leaves. The treatments used are given in Table 28. The results (Table 29) show a slight reduction in the degree of agglutination of the P. phaseolicola Race 2 isolate 882. Compared with the total destruction of activity caused by PG IV this effect is marginal, and may be due to an effect on the bacterial cell surface.

Table 28 Treatments used in incubation of acidic pectic polysaccharide from citrate-oxalate extracts of leaves of P. vulgaris cv. Red Mexican with Protease V

	Treatment			
	1	2	3	4
pectic fraction[a]	1[b]	1	1	-
Protease V[c]	-	1	-	1
autoclaved[d] Protease V	-	-	-	-
buffer[e]	1	-	-	1

a 10 mg cm^{-3} acidic pectic polysaccharide from citrate-oxalate extraction of RM leaves. Dissolved in buffer

b all volumes in cm^3

c 1 mg cm^{-3} dissolved in buffer

d 121°C for 15 min

e 0.02M sodium phosphate buffer pH 6.8

Table 29 Effect on agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) of incubation of the acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican with Protease V

	Treatment[a]							
	1		2		3		4	
Dilution	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0[b]	0	0	0	0	0	0	0
2^1	4	4	4	2	4	4	0	0
2^2	4	1	4	0	4	1	0	0
2^3	3	0	3	0	3	0	0	0
2^4	0	0	0	0	0	0	0	0
Dilution end-point	2^3	2^2	2^3	2^1	2^3	2^2	-	-

a see Table 28 b agglutination index - see Plate 1

C. Characterization of the active pectic polysaccharide and the agglutination process

Characterization of the agglutination process was attempted by incorporating different substances into the agglutination assay mixtures to see if there was any interference. The substances used were generally those which are found in pectic polysaccharide, and the experiments were similar in concept to those used to determine the specificity of a lectin by hapten inhibition studies. Synthetic polysaccharides related to pectic material were also assessed for ability to agglutinate bacterial cells.

a) Neutral sugar interactions

10 mg cm^{-3} acidic pectic polysaccharide from RM in 0.02M sodium phosphate buffer pH 6.8 was used to prepare a dilution series in the usual way for an agglutination assay. The bacteria were suspended in 0.02M sodium phosphate buffer pH 6.8 containing the following test sugars at 0.2M concentration: rhamnose, xylose, galactose, mannose, mellibiose and arabinose. Thus on mixing the bacteria into the wells containing the dilution series the final concentration of sugar in each well became 0.1M. None of the neutral sugars tested appeared to have any effect on agglutination of cells of Race 1 and 2 isolates of P. phaseolicola (Table 30).

b) Interactions of sugar acids and their derivatives

The agglutination assays were set up as in the previous section with the final concentration of test substance in each well 0.1M. The uronic acids galacturonic acid and glucuronic acid caused agglutination of bacteria in their own right, as there was a high degree of agglutination in the controls. However, an interaction with agglutination of bacterial cells by

Table 30 Effect of various neutral sugars on agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

		Control		Rhamnose[a]		Xylose[a]		Galactose[a]		Mannose[a]		Mellibiose[a]		Arabinose[a]	
Dilution	Concentration acidic pectic polysaccharide (mg cm^{-3})	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0	0[b]	0	0	0	0	0	0	0	0	0	0	0	0	0
2^1	5.00	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2^2	2.50	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2^3	1.25	4	3	4	2	3	3	4	3	4	3	4	3	4	2
2^4	0.63	4	2	4	2	3	2	4	2	4	2	4	2	3	2
2^5	0.32	1	0	1	0	1	0	1	0	1	0	1	0	0	0
2^6	0.16	0	0	0	0	0	0	0	0	0	0	0	0	0	0

a 0.1M in each well

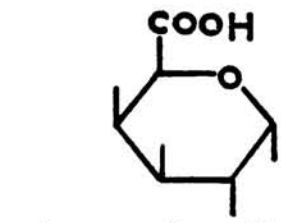
b agglutination index - see Plate 1

pectic polysaccharide was observed. The agglutination caused by the uronic acids in the control gave rise to a very even, smooth "button" of bacteria in the wells (see Plate 1 at the 2^5 dilution for the R1(X) isolate). The interaction was detected because in the wells with higher concentrations of pectic polysaccharide the consistency of the "button" of bacteria was much more granular than in the control, in fact approximating to the appearance of agglutination index 3 (Plate 1). This effect was gradually diluted out, returning to the agglutination pattern of the control by the 2^7 dilution well.

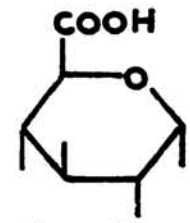
The interaction suggests more that pectic polysaccharide interferes with agglutination of the bacterial cells by uronic acids than vice versa. However it indicates that uronic acid groups in the pectic polysaccharide may be important for agglutination.

The agglutination of bacterial cells by uronic acids is intriguing, since to cause agglutination in the usual manner a substance has to have at least two binding sites, i.e. be "divalent". It is difficult to imagine how small molecules such as galacturonic and glucuronic acids can be divalent in this way. It seems more likley that the agglutination is brought about by an effect of the uronic acid molecules on the bacterial cell surface, causing autoflocculation. A series of uronic acid derivatives and gluconic acid were studied for their ability to agglutinate cells of P. phaseolicola. The structures are given in Figure 20.

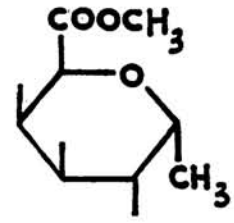
Dilution series were prepared using solutions of the test substances from 0.1M in the maximum concentration. Neither of the methylated galacturonic acid derivatives caused any agglutination of bacterial cells nor did D-gluconic acid (sodium salt). Since methyl-D-galacturonate did not cause agglutination of bacteria and neither did gluconic acid which has the carboxyl group on carbon 1 not carbon 6, this strongly suggests the presence of a free carboxyl group at carbon 6, as in galacturonic and glucuronic acids, is a requisite for this type of agglutination. The pH of 0.1M galacturonic



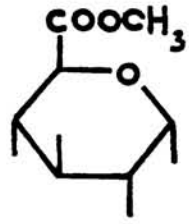
galacturonic acid



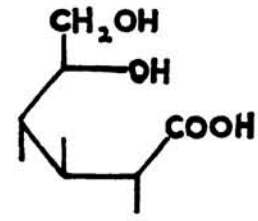
glucuronic acid



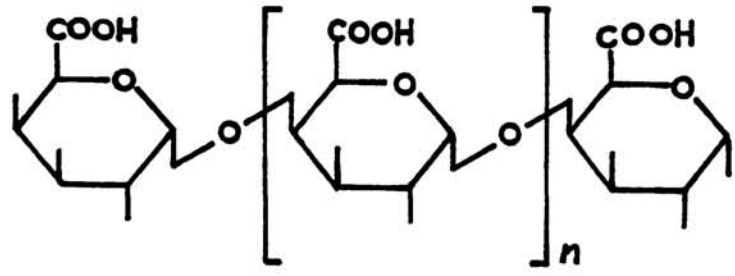
methyl(methyl-α-D-galactopyranoside)uronate



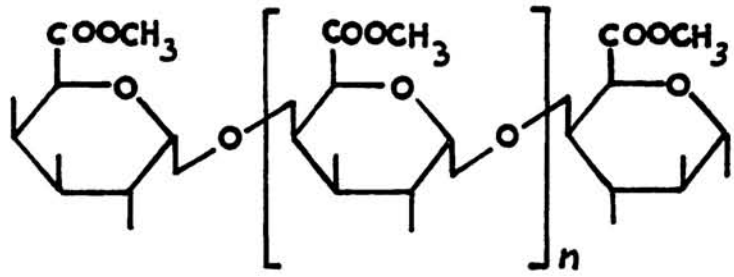
methyl-D-galacturonate



D-gluconic acid



polygalacturonic acid



polymethylpoly-galacturonic acid

Figure 20. Structure of gluconic acid and the uronic acids and their derivatives used in section C b). Vertical bars represent hydroxyl (OH) groups

acid is 2.5, but suspension of bacteria in 0.1M HCl/KCl buffer pH 2.5 caused no agglutination, ruling out that agglutination might be due to a pH effect on the bacterial cell surface. Galacturonic acid differentially agglutinated cells of the Race 1 and 2 isolates of P. phaseolicola used, with cells of the Race 1 isolate being agglutinated to a slightly greater degree than cells of the Race 2 isolate (Table 31).

Table 31 Agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) by galacturonic acid

Concentration galacturonic acid (M)	Race 1 R1(X)	Race 2 882
0.100	4[a]	4
0.050	4	4
0.025	4	4
0.013	4	0
0.006	0	0

a agglutination index - see Plate 1

A series of polymethylpolygalacturonic acid polymers, ranging in methylation from 96%, 79%, 68%, 57%, 32%, 19%, 6% and non methylated polygalacturonic acid were tested for their abilities to agglutinate bacteria. 10 mg cm^{-3} solutions of each in 0.02M sodium phosphate buffer pH 6.8 were used to prepare dilution series and cells of isolates R1(X) and 882 of P. phaseolicola added in the usual manner. No agglutination was caused by any of the methylated series or the non-methylated polymer. This suggests that it is not just the presence of a free carboxyl group at carbon 6 which

is necessary for agglutination. Possibly the hydroxyl groups at carbons 1 and/or 4 are important, since these are present in the monomer but not in the internal residues of the polymer. Alternatively the small size of the monomer may allow it access to parts of the outer layers of the bacteria necessary for it to cause agglutination, which the polymeric material (MW greater than 6000) cannot reach. That polygalacturonic acid derivatives do not cause agglutination of bacterial cells further suggests that agglutination caused by the acidic pectic polysaccharide from RM and CW leaves is due to a specific structural component of these molecules. Possibly there is steric compatibility with a substance on the bacterial cell surface, which brings together groups between which hydrogen bonding can occur. The surface of bacterial cells in most natural environments has an overall negative charge, although positively charged groups are present (e.g. N-acetyl groups). Thus it is possible that complementary structural interaction brings together negatively charged carboxyl groups of the uronic acid residues in the pectic polysaccharide and positive groups in the bacterial surface, giving localized areas of high ionic attraction which can overcome the general negative repulsive forces (see literature review p. 32). High ionic concentrations in solution tend to reduce the electrostatic attraction in ionic bonds due to dielectric effects, but have little effect on hydrogen bonding.

An agglutination assay was carried out with the dilution series prepared in the usual way, but the bacteria were suspended in 0.02M sodium phosphate buffer pH 6.8 containing 1M $(NH_4)_2SO_4$. Thus after mixing bacterial suspension into the wells, giving a final concentration of 0.5M NH_4SO_4 in each well. Only slight reduction in agglutination of cells of the Race 1 isolate R1(X) was observed (Table 32), which suggests that ionic bonds are probably not important in the agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by the acidic pectic fraction from leaves of RM.

Table 32 Effect of 0.5M $(NH_4)_2SO_4$ on the agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by the acidic pectic polysaccharide from citrate-oxalate extraction of leaves of P. vulgaris cv. Red Mexican

Dilution	Concentration acidic pectic polysaccharide (mg cm^{-3})	Control		0.5M$(NH_4)_2SO_4$ in each well	
		Race 1 R1(X)	Race 2 882	Race 1 R1(X)	Race 2 882
Control	0	0[a]	0	0	0
2^1	5.00	4	4	4	4
2^2	2.50	4	4	4	3
2^3	1.25	4	3	2	3
2^4	0.63	3	0	1	0
2^5	0.32	0	0	0	0
Dilution end-point		2^4	2^3	2^4	2^3

a agglutination index - see Plate 1

c) Electrophoresis

Due to the large size of the polysaccharide molecules support matrices for electrophoresis must have large "pore" size so as not to impede migration. Thus glass fibre paper and electrophoresis in solution over a urea gradient were employed (Materials and Methods 7 B, C). Usually the polysaccharide preparation is pre-treated with dilute Na_2CO_3 solution to demethylate the carboxyl groups and thus give better separation of the components. However, treatment of the acidic pectic polysaccharide from RM leaves with 0.1M Na_2CO_3 at room temperature for 1.5h virtually destroyed its agglutinating activity. This was not due to the effect of high pH on the agglutination process itself, or simply the presence of Na_2CO_3, since dialysis of the Na_2CO_3 treated sample

overnight at 4°C against two changes of 0.02M sodium phosphate buffer, with the agglutination assay carried out as usual at pH 6.8 still showed the loss of activity (Table 33). It is also unlikely to be due solely to demethylation, since treatment with PME enzymes did not destroy agglutination (Tables 25, 26). Thus an effect on the structure of the pectic polysaccharide is indicated. In view of this the Na_2CO_3 pre-treatment was not carried out when active fractions were required on recovery after electrophoresis.

Table 33 Effect of 0.1M Na_2CO_3 treatment on agglutination of cells of Race 1 and 2 isolates of P. phaseolicola by the acidic pectic fractions from leaves of P. vulgaris cv. Red Mexican

Dilution	Concentration acidic pectic polysaccharide (mg cm^{-3})	Control		Treated with 0.1M Na_2CO_3[a]	
		Race 1	Race 2	Race 1	Race 2
		R1(X)	882	R1(X)	882
Control	0	0[b]	0	0	0
2^1	2.50	4	3	2	0
2^2	1.25	4	0	0	0
2^3	0.63	4	0	0	0
2^4	0.32	0	0	0	0
Dilution end-point		2^3	2^1	2^1	-

a dialysed after incubation against 0.02M sodium phosphate buffer pH 6.8

b agglutination index - see Plate 1

i) Glass fibre paper

0.02 cm^3 3 mg cm^{-3} solutions of the acidic pectic polysaccharide from Red Mexican, citrus "pectin"(Sigma), polygalacturonic acid and galactose

were used. The procedure was as in Materials and Methods, section 7 B. After a 1h run the carbohydrates were immobilized with absolute alcohol and visualized using α-napthol reagent. The neutral sugar galactose had not moved from the origin, showing that no detectable endo-osmotic flow had occurred. Polygalacturonic acid, RM pectic polysaccharide and citrus "pectin" (Sigma) migrated 43, 16.5 and 18 mm respectively (means of 4 runs). The RM pectic polysaccharide migrated as a single spot and left no trace of any neutral polysaccharide at the origin. Since the α-napthol reagent is very sensitive and can show up as little as 0.2 μg of carbohydrate this suggests that the preparation was substantially free of a neutral component. In view of this it was decided to carry out some preliminary analysis of the active extract without further purification.

The distance migrated for a substance of given MW is directly proportional to its charge. Completely demethylated pectic polysaccharide would migrate the maximum possible distance in a given time, whereas native extract (i.e. without modification) would migrate some fraction of this distance. The ratio of the two distances migrated indicates the degree of esterification of the carboxyl groups in the native pectic polysaccharide, assuming that the reduction in MW due to the loss of the methyl groups is negligible.

The samples were de-esterified by mixing 6 mg cm^{-3} solutions of carbohydrate with equal volumes of 0.1M Na_2CO_3 followed by incubation at $4^{o}C$ for 12h. In view of the postulated structural effects due to Na_2CO_3 treatment (see previous section), the method was tested using citrus "pectin" (Sigma) which was quoted by the manufacturers as c. 7.5% methylated, i.e. 7.5% of the carboxyl groups were present as methyl esters. As a mean of 4 runs the ratio of distance migrated by native to de-esterified citrus "pectin" was 0.93, suggesting that the native citrus "pectin" had 93% of the charge on the de-esterified material, i.e. c. 7.0% of the carboxyl groups were methylated. This is in good agreement with the manufacturer's figure.

Electrophoresis of Na_2CO_3 treated acidic pectic polysaccharide from RM gave a single spot, suggesting that the postulated structural effects of Na_2CO_3 treatment do not involve fragmentation into smaller units. As a mean of 4 runs the ratio of distance migrated by native to de-esterified pectic polysaccharide was 0.73. This suggests that the native acidic pectic polysaccharide had 73% of the charge carried by the de-esterified materials, i.e. 27% of the carboxyl groups were methylated.

ii) Column electrophoresis

10 mg samples of the acidic pectic polysaccharide fractions from citrate-oxalate extracts of RM and CW leaves were applied separately to a 12 - 50% (w/v) urea gradient and electrophoresis carried out (see Materials and Methods section 7 C a)). 5 cm^3 fractions were collected and assayed for carbohydrate by the phenol-sulphuric and carbazole-sulphuric acid procedures (Materials and Methods 7 C b)), and for protein by ninhydrin. Fractions which were ninhydrin positive were subsequently assayed quantitatively for protein by the Folin-Lowry method (Materials and Methods 5 A and B).

Figures 21 and 22 show the absorbancies at the respective wavelength for each assay plotted against the fraction number for RM and CW respectively. The plots are similar for both RM and CW pectic polysaccharides, one major and an adjacent minor peak was present in both cases. The polysaccharide in the major peak had a greater proportion of uronic acid to neutral sugar residues than the polysaccharide in the minor peak. This can be seen directly from the plots. Carbazole and phenol-sulphuric acid procedures give greater colour production with uronic acid and neutral sugar residues respectively, and the relative positions of the two plots are reversed in each peak. Using the procedures outlined in Materials and Methods section 7 C b) iii) the quantities of carbohydrate in each fraction as uronic acid and galactose equivalents were calculated. The data are shown in Tables 34 and 35. As a mean of all the assays for the separations performed

Figure 21. •-•-•- Absorbance at 485 nm o-o-o- Absorbance at 525 nm after phenol-sulphuric and carbazole-sulphuric acid procedures with the fractions from columns electrophoresis of the acidic pectic fraction from RM (A.S. = anode solution)

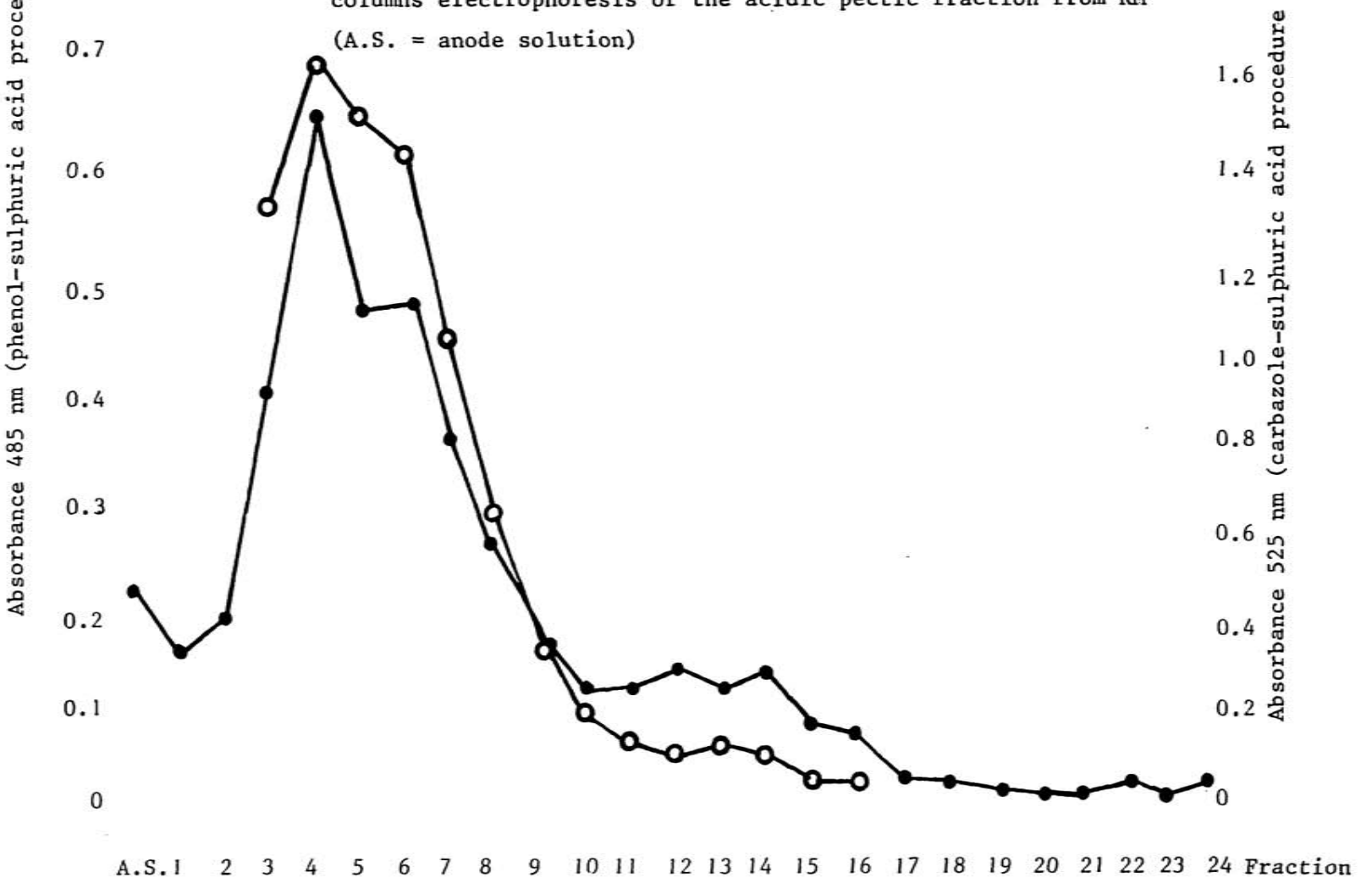


Figure 22. ●-●-●- Absorbance at 485 nm o-o-o- Absorbance at 625 nm
after phenol-sulphuric and carbazole-sulphuric acid procedures with the fractions from column electrophoresis of the acidic pectic polysaccharide fraction from CW
(A.S. = anode solution)

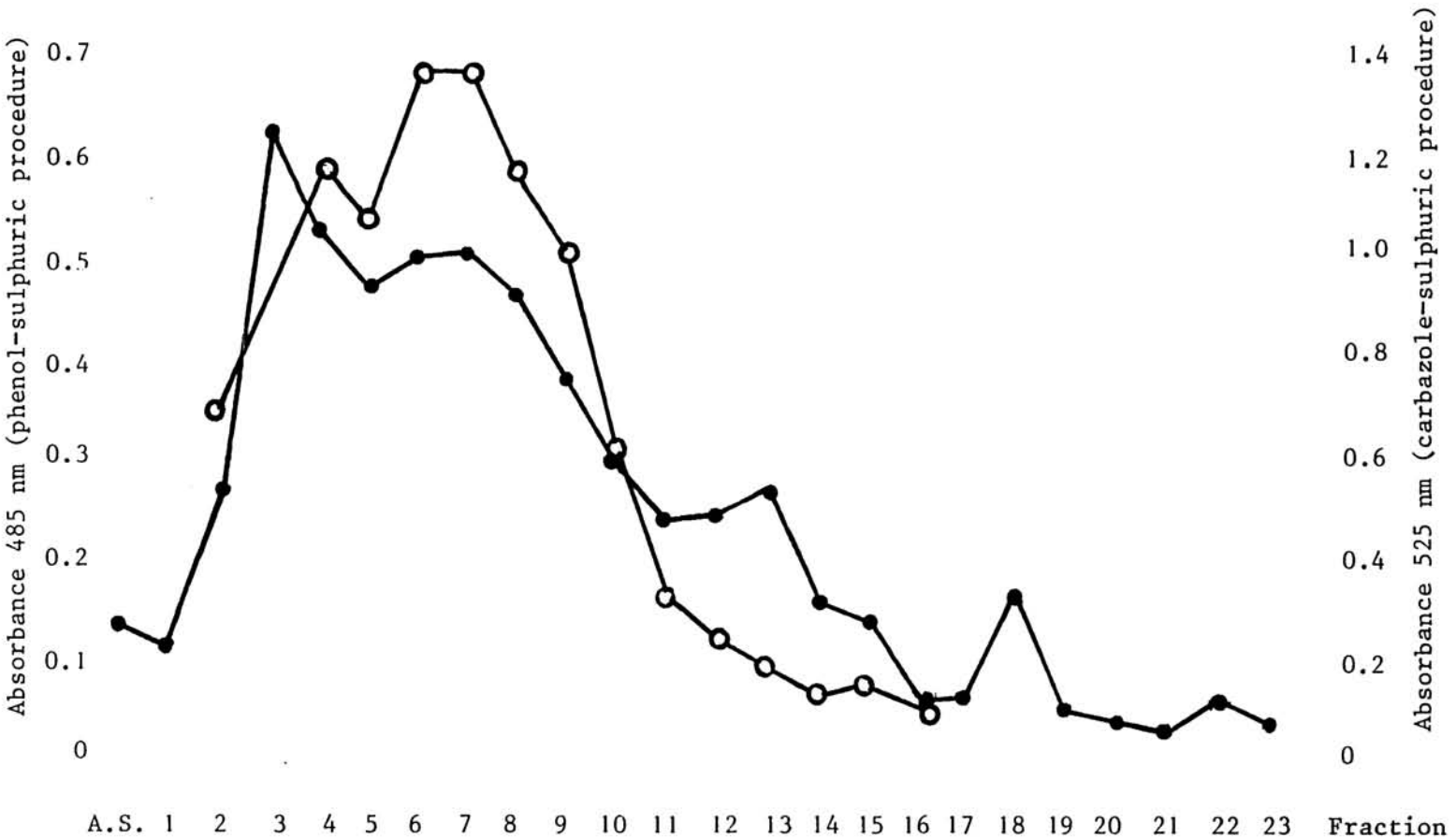


Table 34 Estimation of the quantities of uronic acid and neutral sugar present in the fractions after column electrophoresis of the acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

Fraction	Phenol sulphuric acid procedure A 485nm	Carbazole-sulphuric acid procedure A 525nm	Galacturonic acid equivalent (mg cm^{-3})	Galactose equivalent (mg cm^{-3})	% galacturonic acid
anode solution	0.224	1.255[a]	-	-	-
1	0.154	1.350[a]	-	-	-
2	0.198	1.525[a]	-	-	-
3	0.447	1.305[a]	-	-	-
4	0.744	1.633	0.17	0.06	74
5	0.535	1.500	0.16	0.07	70
6	0.543	1.419	0.15	0.03	83
7	0.387	1.059	0.11	0.02	85
8	0.255	0.614	0.06	0.02	75
9	0.162	0.325	0.03	0.01	75
10	0.111	0.175	0.02	0.01	67
11	0.116	0.111	0.01	0.02	33
12	0.136	0.081	0.007	0.02	26
13	0.116	0.107	0.01	0.02	33
14	0.134	0.087	0.007	0.02	26
15	0.078	0.027	0.002	0.01	17
16	0.066	0.024	0.002	0.01	17
17	0.018	ND[b]	-	-	-
18	0.016	ND	-	-	-
19	0.008	ND	-	-	-
20	0.002	ND	-	-	-
21	0.000	ND	-	-	-
22	0.016	ND	-	-	-
23	0.000	ND	-	-	-
24	0.017	ND	-	-	-

a a reaction was present which had an absorption maximum at 585 nm therefore the results were not used

b not determined

Table 35 Estimation of the quantities of uronic acid and neutral sugar present in the fractions after column electrophoresis of the acidic pectic polysaccharide from leaves of P. vulgaris cv. Canadian Wonder

Fraction	Phenol sulphuric acid procedure A 485 nm	Carbazole-sulphuric acid procedure A 525 nm	Galacturonic acid equivalent (mg cm^{-3})	Galactose equivalent (mg cm^{-3})	% galacturonic acid
anode solution	0.127	0.614[a]	-	-	-
1	0.106	0.255[a]	-	-	-
2	0.265	0.684[a]	-	-	-
3	0.623	0.925	0.09	0.08	53
4	0.520	1.163	0.12	0.04	75
5	0.462	1.064	0.11	0.03	79
6	0.498	1.373	0.14	0.02	88
7	0.499	1.361	0.14	0.02	88
8	0.453	1.144	0.12	0.03	80
9	0.367	0.994	0.10	0.02	83
10	0.273	0.591	0.06	0.02	75
11	0.229	0.311	0.03	0.03	50
12	0.233	0.217	0.02	0.04	33
13	0.260	0.162	0.01	0.05	17
14	0.142	0.108	0.01	0.02	33
15	0.128	0.129	0.01	0.02	33
16	0.046	0.092[b]	0.01	-	-
17	0.051	ND	-	-	-
18	0.153	ND	-	-	-
19	0.034	ND	-	-	-
20	0.026	ND	-	-	-
21	0.017	ND	-	-	-
22	0.095	ND	-	-	-
23	0.048	ND	-	-	-

a a reaction product was present which had an absorption maximum at 585 nm, therefore the results were not used

b ND - not determined

the percentage of uronic acid in the carbohydrate of the major and minor peaks was 80% and 31% for RM and 81% and 33% (w/w) for CW respectively.

All the fractions except those at the cathode end of the column gave no colouration with ninhydrin. Fraction 22 from the electrophoresis of the RM extract gave a yellow colouration while fractions 23 and 24 gave purple colourations. Fraction 22 from the electrophoresis of the CW extract also gave a yellow colouration while fraction 23 gave purple. The yellow colouration suggested the presence of the amino acid hydroxyproline which is commonly found in proteinaceous material from plant cell walls. Folin-Lowry estimation of these fractions gave readings not significantly different from the controls, suggesting that the concentration of protein was very low. No protein was associated with either carbohydrate peak.

The acidic pectic polysaccharide extracts from RM and CW behaved very similarly on electrophoresis, giving rise to similar peaks with similar proportions of uronic acid to neutral sugar residues. This, coupled with the similar differential agglutinating activity towards Race 1 and 2 isolates of P. phaseolicola suggested that the extracts from the two cultivars were very similar in composition.

To investigate which components of the acidic pectic polysaccharide fraction was binding to bacterial cells the following method was tried. 10 mg of acidic pectic polysaccharide from RM leaves was dissolved in 10 cm^3 sodium acetate buffer pH 5.0. 25 cm^3 of a 24h King's B broth shake culture of P. phaseolicola isolate R1(X) grown at 20°C, was washed as in Materials and Methods section 1 B b), and the pellet of bacterial cells resuspended in the acidic pectic polysaccharide solution. After 5 min incubation at room temperature the suspension was centrifuged (8000g for 10 min) and the pellet discarded. A fresh pellet of washed bacterial cells was resuspended in the supernatant and the incubation and centrifugation steps

repeated. The pellet was discarded and the supernatant brought to 18% w/v solution with urea. It was then loaded on to a column and electrophoreis carried out. It was hoped that one or more of the components in the extract would have bound to the bacterial cells and be removed on centrifugation. However, analysis of the fractions from the column for carbohydrate and protein showed no difference to any of the other electrophoretic separations which had not had the bacterial incubation step. Thus it can be concluded either that 5 min is too short a time for binding to occur or that the molecules were loosely bound and were sloughed off in the centrifugation steps as the bacteria sedimented.

It was decided to bulk the fractions making up the major and minor peaks and ninhydrin staining regions from three runs of RM acidic pectic polysaccharide, and after dialysis, assay each fraction for ability to agglutinate cells of P. phaseolicola. The fractions were dialysed for 3 days at $4^{o}C$ against several changes of distilled water, then concentrated by rotary evaporation at $45^{o}C$. Agglutination assays were then carried out. The ninhydrin positive fraction was used undiluted, but the polysaccharide fractions were adjusted to contain c. 5 mg cm^{-3} carbohydrate, determined by the phenol-sulphuric and carbazole-sulphuric acid procedures.

The results of the agglutination assays are shown in Table 36. The ninhydrin positive fraction had no detectable agglutinating activity. Pectic polysaccharide comprising the major peak differentially agglutinated cells of the two races in the same manner as the whole extract, i.e. Race 1 to a greater degree than Race 2. The pectic polysaccharide comprising the minor peak agglutinated cells of the Race 2 isolate 882, but not the Race 1 isolate R1(X). This differential agglutination is in the opposite sense to that caused by the pectic polysaccharide of the major peak. The effect of the minor polysaccharide component appears to be overshadowed by the effect of

the major polysaccharide component in the whole extract, presumably due to the relative amounts present. However, some of the agglutination of cells of Race 2 isolates observed with the whole extract is likely to be due to the minor polysaccharide component, which has a greater activity at the same concentration than the major polysaccharide component (Table 36). The absence of protein in the carbohydrate fractions is further evidence that agglutination is due to pectic polysaccharide rather than to protein with lectin-like activity.

Table 36 Agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) by components separated by column electrophoresis of the acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

	Ninhydrin positive region		Concentration polysaccharide (mg cm^{-3})	Major peak		Minor peak	
Dilution	R1(X)	882		R1(X)	882	R1(X)	882
Control	0[a]	0	0	0	0	0	0
2^1	0	0	2.5	4	1	0	2
2^2	0	0	1.25	4	0	0	0
2^3	0	0	0.63	3	0	0	0
2^4	0	0	0.32	0	0	0	0

a agglutination index - see Plate 1

d) GLC analysis

Gas-liquid chromatography (GLC) was used to analyse the neutral sugar composition of the major polysaccharide peaks obtained from column electrophoresis of the acidic pectic polysaccharide from leaves of P. vulgaris cvs. Red Mexican and Canadian Wonder. The pectic polysaccharide was first hydrolysed with trifluoroacetic acid (TFA) then reduced with $NaBH_4$ to form alditols. The alditols were then derivatized to give penta-acetates which were separated by GLC. The procedures used are given in Materials and Methods sections 11 a), b) and c). This technique does not detect uronic acids.

The galacturonic acid content of the samples was calculated from carbazole-sulphuric and phenol-sulphuric acid procedures (see Materials and Methods sections 8 C b) i), ii) and iii).

The results are shown in Table 37. The composition of the major component of the acidic pectic polysaccharide was very similar for both Red Mexican and Canadian Wonder. The major neutral sugar components were arabinose, galactose and rhamnose, with small amounts of fucose, mannose and xylose present. Glucose was absent from both samples.

Table 37. Composition of the major peaks obtained from column electrophoresis of acidic pectic polysaccharide from leaves of P. vulgaris cvs. Red Mexican (RM) and Canadian Wonder (CW)

	RM	CW
Galacturonic acid[a]	79.5[c]	81.2
Neutral monosaccharides[b]		
Rhamnose	3.1	2.8
Fucose	1.0	1.1
Arabinose	7.6	7.0
Xylose	0.6	0.6
Mannose	0.6	0.6
Galactose	7.6	6.7
Glucose	0	0

a determined by carbazole-sulphuric and phenol-sulphuric acid procedures

b determined by GLC

c all values quoted as mg 100 mg^{-1} polysaccharide

D. Investigation of the bacterial surface component involved in binding with acidic pectic polysaccharide

Purified preparations of various bacterial cell surface components were investigated to see if they interacted with the acidic pectic polysaccharide from leaves of Red Mexican.

a) EPS and LPS

EPS was extracted from the culture filtrate and LPS from the bacterial cells of 24h shake cultures of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) as described in Materials and Methods section 12 a), b).

i) Ouchterlony double diffusion assays

1% w/v Bacto agar of low ion content (Difco) or 0.8% w/v agarose gels were used as an inert support for Ouchterlony double diffusion assays. The supports were prepared as in Materials and Methods section 13. Agarose has advantages over agar for this purpose since it gives a high gel strength for a lower concentration of gel material and hence a larger pore size can be obtained in a gel not so weak as to be impracticable to use. A 0.8% w/v agarose gel has an upper molecular size exclusion limit of 1×10^8 Daltons for spherical molecules (Anon, 1977), which is well above the upper estimate of c. 4.0×10^5 Daltons required for pectic polysaccharide (Jirgensons, 1962).

Visible lines of precipitation form when the reactants diffuse to the point of equivalent concentration, and if the product formed is insoluble.

$0.02\ cm^3$ solution of acidic pectic polysaccharide from RM leaves was placed in the central well of the test matrix and LPS and EPS solutions in the surrounding wells. The concentrations used are given in Table 38.

After incubation for 5 days at room temperature, checking at 24h intervals, no precipitation lines were observed with either agar or agarose matrices for any of the concentrations of test substances used. This suggests that the pectic material does not interact with LPS or EPS on the surface of cells of either race of P. phaseolicola, or that the product(s) of the interaction are soluble, and therefore not detected. It is also possible that the ranges of concentration used for the test substances did not allow equivalent concentrations necessary for precipitation to occur.

Table 38. Concentrations of substances used in Ouchterlony double diffusion assay to investigate interaction of bacterial cell surface components with acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

Concentration of acidic pectic polysaccharide solution in central well (mg cm^{-3})	Concentration (mg cm^{-3}) in surrounding wells		
	LPS[a]	EPS1[a,b]	EPS2[a,c]
2	2, 4	2, 4	2, 4
3	2	4	4
4	2, 4	2, 4	2, 4

a From P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2)

b 2:1 absolute alcohol precipitate from concentrated, dialysed culture filtrate

c 4:1 absolute alcohol precipitate from concentrated, dialysed culture filtrate

It is also possible that since pectic polysaccharide molecules are linear rather than spherical in final configuration they could have been excluded even from the large pores of the agarose gel.

Thus the results are equivocal, but suggest that a substance other than LPS or EPS on the bacterial surface may interact with the acidic pectic polysaccharide.

Bacteria were treated in various ways before agglutination was assayed to provide information about the nature of the surface components interacting with the pectic polysaccharide.

b) Autoclaved cells

24h King's B broth shake cultures of P. phaseolicola isolates R1(X) and 882 grown at 25°C were washed by centrifugation. The cells were resuspended in distilled water. The cell suspension was divided into two samples, one of which was autoclaved at 121°C for 15 min. Both samples were then washed by centrifugation to remove any substance released by autoclaving which might interfere with agglutination. The cells were then resuspended in 0.02M sodium phosphate buffer pH 6.8 and adjusted by nephelometry to contain c. 1.5×10^{10} cells cm^{-3}. Agglutination was then assayed to compare the agglutination of autoclaved and viable cells by acidic pectic polysaccharide from leaves of Red Mexican.

The results are shown in Table 39. Autoclaving the cells significantly reduced agglutination caused by pectic polysaccharide. Both Race 1 and 2 isolates were affected similarly. This suggests that the active substance on the bacterial cell surface is heat labile. This indicates that LPS (which makes up the O-antigens of the bacterial surface) is not involved in the interaction, since O-antigens are heat stable (Meadow, 1975).

The composition of the bacterial cell surface varies with age of

Tabl 39. Agglutination of viable and autoclaved cells of P. phaseolicola isolates R1(X) and 882 by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

Acidic pectic polysaccharide concentration (mg cm^{-3})	Viable cells		Autoclaved cells	
	R1(X)	882	R1(X)	882
0	0[a]	0	0	0
5.00	4	4	3	3
2.50	4	3	0	0
1.25	4	2	0	0
0.63	4	0	0	0
0.32	4	0	0	0
0.16	0	0	0	0

a agglutination index - see Plate 1

culture and nutrient source. P. phaseolicola cultures of various ages grown on various media were used in agglutination assays to investigate any variation in agglutination which might occur.

c) Variation of agglutination with age of culture

King's B agar slope cultures of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) were grown at 25°C for 24, 48, 72, 96 and 120 h. The cultures were washed by centrifugation, resuspended in 0.02M sodium phosphate buffer pH 6.8 then adjusted to contain 1.5×10^{10} cells cm^{-3} by nephelometry. Agglutination was then assayed to investigate variations with age of culture. The results are shown in Table 40.

Agglutination of the Race 1 isolate R1(X) was consistent for the age ranges of cultures tested. Cultures of the Race 2 isolate 882 up to 72h behaved as expected - i.e. showed some agglutination with the acidic polysaccharide, but less than that observed with the Race 1 isolate. 96 and 120h Race 2 cultures showed spontaneous agglutination. This suggests that there is not only a "static" difference in surface composition between the two isolates which allows differential agglutination to occur, but also that composition of the surface varies differently with time for the two isolates. As with the agglutination caused by galacturonic acid (see Experimental and Results section C b), the presence of the acidic pectic polysaccharide interferes with spontaneous agglutination, the effect being diluted out on descending the dilution series.

d) Varied culture conditions

The surface composition of bacterial cells varies with culture conditions. Race 1 and 2 isolates of P. phaseolicola were cultured on a range of solid and liquid media and agglutination assays were done with

Table 40. Variation with age of culture of agglutination of cells of *P. phaseolicola* isolates R1(X) (Race 1) and 882 (Race 2) by acidic pectic polysaccharide from leaves of *P. vulgaris* cv. Red Mexican

Acidic pectic polysaccharide concentration (mg cm^{-3})	Age of culture									
	24h		48h		72h		96h		120h	
	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
0	0[a]	0	0	0	0	0	0	4	0	4
5.00	4	4	4	3	4	3	4	4	4	4
2.50	4	3	4	2	4	2	4	3	4	3
1.25	4	1	4	0	4	1	4	2	4	3
0.63	0	0	0	0	0	0	0	4	0	4
0.32	0	0	0	0	0	0	0	4	0	4

a agglutination index - see Plate 1

acidic pectic polysaccharide from Red Mexican leaves to investigate any variation in agglutination. The following media were used :- King's B agar slopes and broth, nutrient agar slopes and broth, M9 minimal agar slopes and defined Pseudomonas liquid medium; the recipes are given in Materials and Methods section 1 B b). All cultures were grown for 24h at 25°C and bacteria were washed by centrifugation before suspending in 0.02M sodium phosphate buffer pH 6.8 in the usual manner for agglutination assays.

The results are shown in Table 41. Differential agglutination of the Race 1 and 2 isolates of P. phaseolicola by acidic pectic polysaccharide from leaves of Red Mexican was fairly consistent irrespective of the culture medium or conditions of growth. This suggests that the surface difference between the races is independent of culture on a given medium. The range of media used was small, and of course, the bacteria were grown in artificial environments. It would be better to do the assays with bacteria recovered directly from diseased plants in the field, but this would be difficult. At present, therefore all that can be said is that virulent cultures, actively growing in a variety of conditions, showed differential agglutination by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican.

e) Periodate oxidation of bacterial surface

If the component on the bacterial cell surface which interacts with the pectic polysaccharide is carbohydrate in nature, then periodate oxidation of the bacterial cells might be expected to affect agglutination.

Distilled water suspensions of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) prepared from washed bacteria from 24h KB broth cultures and containing c. 3×10^{10} cells cm^{-3} were mixed with 0.05M periodate in 0.1M sodium acetate buffer pH 4.7 and kept at 20°C for 2h in the dark. The treatments used are given in Table 42.

Table 41. Agglutination of isolates R1(X) and 882 of P. phaseolicola grown on different media by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

Dilution	Acidic pectic polysaccharide concentration (mg cm^{-3})	Culture Medium: King's B broth		King's B slope		Nutrient broth		Nutrient agar slope		M9 minimal medium		Defined Pseudomonas liquid medium	
		R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
	0	0[a]	0	0	0	0	0	0	0	0	0	0	0
2^1	5.00	4	4	4	4	4	4	4	4	4	4	4	4
2^2	2.50	4	2	4	3	4	3	4	2	4	3	4	3
2^3	1.25	4	0	4	1	4	1	4	2	4	1	4	1
2^4	0.63	4	0	3	0	4	0	3	0	3	0	3	0
2^5	0.32	3	0	2	0	4	0	3	0	2	0	3	0
2^6	0.16	0	0	0	0	0	0	0	0	0	0	0	0
Dilution end point		2^5	2^2	2^5	2^3	2^5	2^3	2^5	2^3	2^5	2^3	2^5	2^3

a agglutination index - see Plate I

Table 42. Treatments used in the periodate oxidation of bacterial cells

	Treatment			
	1	2	3[a]	3´[a]
Bacterial suspension c. 3.0×10^{10} cells cm^{-3}	1[b]	1	1	-
0.05M periodate	-	1	-	1
0.1M sodium acetate buffer pH 4.7	1	-	-	-
Glycerol	-[c]	-[c]	-	2 drops

a 3 and 3´ mixed after incubation

b all volumes in cm^3, mixtures incubated in the dark at 20°C for 2h

c 2 drops added after 2h incubation

The dilution series for the agglutination assays were prepared with aqueous solutions of the acidic pectic polysaccharide from Red Mexican leaves, thus the final concentration of buffer in each well was 0.025M.

The results are shown in Table 43. The wells all showed a great deal of cloudiness and the values given for the agglutination indexes are only the best approximation to those in the key shown in Plate 1. However the wells should be comparable to each other within the limitations of the experiment. Periodate oxidation did not affect agglutination of the Race 1 isolate R1(X), but appeared to cause a reduction in agglutination of the Race 2 isolate 882. However, the reduction in agglutination was also observed with the 3 + 3´ treatment, which should serve as a control by reducing the periodate before mixing with the cells. This suggests that some product of

Table 43. Effect of periodate oxidation of the bacterial cell surface on agglutination by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

Pectic polysaccharide concentration (mg cm^{-3})	Dilution	Race 1 isolate R1(X) Treatment[a] 1	2	3 + 3′	Race 2 isolate 882 Treatment[a] 1	2	3 + 3′
0		0[b]	0	0	0	0	0
5.00	2^1	4	4	4	2	1	1
2.50	2^2	4	4	4	2	0	0
1.25	2^3	3	2	2	1	0	0
0.63	2^4	2	2	2	0	0	0
0.32	2^5	0	0	0	0	0	0
Dilution end point		2^4	2^4	2^4	2^3	2^1	2^1

a See Table 42 b agglutination index - see Plate 1

the reaction of periodate with glycerol may be interfering with agglutination. Were this the case one would have expected a similar effect in the other periodate oxidation experiments (see Experimental and Results sections A f), B e)). However, in these cases the 3 + 3´ treatments behaved similarly to the controls which were without periodate. This anomaly is puzzling. But, if the reduction in agglutination observed for the Race 2 isolate is not an artifact this would suggest that the surface components involved in interaction with pectic polysaccharide may be chemically different in each race. Since Race 1 cells were unaffected by periodate oxidation the active surface component cannot be carbohydrate, whereas for Race 2 carbohydrate is indicated. This accords with the results from agglutination assays with polysaccharides obtained from column electrophoresis of the acidic pectic polysaccharide from Red Mexican leaves (Experimental and Results section C c) ii)). Here the minor pectic polysaccharide component caused agglutination of cells of Race 2 isolate 882 but not of Race 1 isolate R1(X). This implies that the differential agglutination observed between the two races may not depend on quantitative differences in a common surface component, but on the presence or absence of different specific substances. However, since the major pectic polysaccharide component caused agglutination of both races there may also be a common surface substance in both races which can interact with pectic material.

f) Protease V digestion of the bacterial cell surface

None of the bacterial cell surface carbohydrates tested interacted with the acidic pectic polysaccharide from P. vulgaris cv. Red Mexican and the results from periodate oxidation (see previous section - d)) suggests that the relevant bacterial surface component may not be carbohydrate in nature, at least for the R1(X) (Race 1) isolate of P. phaseolicola. Since proteinaceous components of the bacterial cell surface have been associated with bacterial attachment (see Review of the Literature, p. 36) it was

decided to incubate bacteria with protease V to see if this affected agglutination.

Cells from 24h King's B broth shake cultures of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) grown at 25°C were washed by centrifugation and resuspended in 0.02M sodium phosphate buffer pH 6.8 to give suspensions containing c. 3.0×10^{10} cells cm^{-3}. Aliquots of the suspension were mixed with equal volumes of various test solutions and incubated at 30°C for 1.5h before use in agglutination assays with acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican. The treatments are given in Table 44. No attempt was made to stop enzyme activity after the 1.5h incubation since it has already been shown (Experimental and Results section B f) ii)) that incubation of the acidic pectic polysaccharide from P. vulgaris cv. Red Mexican with protease V had little effect on agglutination.

Table 44. Treatments used in protease V incubation experiment. Incubation was at 30°C for 1.5h

	Treatment		
	1	2	3
Bacterial suspension c. 3.0×10^{10} cells cm^{-3}	1[a]	1	1
Protease V[b]	-	-	1
Autoclaved protease V[c]	-	1	-
Buffer[d]	1	-	-

a all volumes in cm^3

b 1 mg cm^{-3} in buffer

c 121°C for 15 min

d 0.02M sodium phosphate buffer pH 6.8

A 10 mg cm^{-3} solution of acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican in buffer was used to prepare the dilution series. The results are shown in Table 45. Protease V digestion of the bacterial cell surface caused no alteration in agglutination of the cells compared with controls which were incubated with autoclaved enzyme or with no enzyme. This suggests that the bacterial cell surface component involved in agglutination with acidic pectic polysaccharide from P. vulgaris cv. Red Mexican is not proteinaceous, or if it is proteinaceous it is not digested by protease V. The latter conclusion is unlikely since protease V has an unusually wide spectrum of activity.

g) Streptomycin treated cells

To investigate whether de novo protein synthesis by the bacterium was necessary for agglutination an assay was performed with streptopmycin treated cells. Streptomycin inhibits bacterial protein synthesis via an effect on the 30S ribosome. Suspensions of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) containing c. 3.0×10^{10} cells cm^{-3} 0.02M sodium phosphate buffer pH 6.8, prepared from 24h King's B broth cultures, were mixed with equal volumes of 240 µg cm^{-3} solution of streptomycin in buffer. After 30 min incubation at 25°C the bacteria were used in an agglutination assay with acidic pectic polysaccharide from leaves of Red Mexican. The final concentration of streptomycin in each well was 85 µg cm^{-3}.

The results are shown in Table 46. Agglutination was not affected by the presence of streptomycin. Thus it can be concluded that de novo protein synthesis by the bacterium is not required for agglutination.

h) Phytohaemagglutinin (PHA) treated cells

Phytohaemagglutinin (PHA) is a lectin obtained from seeds of P. vulgaris which binds specifically to N-acetyl glucosamine residues

Table 45. Effect on agglutination of cells of P. phaseolicola by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican of incubation of the cells with protease V

Acidic pectic polysaccharide concentration (mg cm^{-3})	Treatment[a] 1		2		3	
	Race 1 RI(X)	Race 2 882	Race 1 RI(X)	Race 2 882	Race 1 RI(X)	Race 2 882
0	0[b]	0	0	0	0	0
5.00	4	4	4	4	4	4
2.50	4	3	4	3	4	3
1.25	4	2	4	2	4	2
0.63	3	0	3	0	3	0
0.32	3	0	3	0	3	0
0.16	0	0	0	0	0	0

a see Table 44 b agglutination index - see Plate 1

Table 46. Agglutination of streptomycin treated cells of P. phaseolicola isolates R1(X) and 882 by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

Acidic pectic polysaccharide concentration (mg cm^{-3})	Control		+ streptomycin (85 µg cm^{-3})	
	Race 1 R1(X)	Race 2 882	Race 1 R1(X)	Race 2 882
0	0[a]	0	0	0
5.00	4	4	4	4
2.50	4	4	4	4
1.25	4	3	4	3
0.63	4	2	4	2
0.32	1	0	1	0
0.16	0	0	0	0

a agglutination index - see Plate 1

(Anon, 1981), which are present in bacterial LPS and some types of EPS. PHA also binds to N-acetylgalactosamine residues (Sharon, 1980). If these residues were involved in interaction with the acidic pectic polysaccharide from bean then pretreatment of the cells with PHA might be expected to have an effect on agglutination. Suspensions of P. phaseolicola isolates R1(X) and 882 containing c. 3.0×10^{10} cells cm^{-3} 0.02M sodium phosphate buffer pH 6.8, prepared from 24h King's B broth cultures, were mixed with equal volumes of PHA in buffer. After 30 min incubation at 25°C the bacteria were used in an agglutination assay. The final concentration of PHA in each well was 100 µg cm^{-3}.

The results are shown in Table 47. Agglutination was not affected by pretreatment of the cells with PHA. This suggests that N-acetylglucosamine residues on the bacterial cell surface are not involved in the interaction with acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican.

Table 47. Agglutination of phytohaemagglutinin (PHA) treated cells of P. phaseolicola isolates R1(X) and 882 by acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican

Acidic pectic polysaccharide concentration (mg cm^{-3})	Control		+ PHA	
	Race 1	Race 2	Race 1	Race 2
0	0[a]	0	0	0
5.00	4	4	4	4
2.50	4	4	4	4
1.25	4	3	4	3
0.63	4	2	4	2
0.32	1	0	1	0
0.16	0	0	0	0

a agglutination index - see Plate 1

E. In vivo interactions

a) Effects on virulence

After the discovery that a crude extract from cotyledons of P. vulgaris cv. Red Mexican could differentially agglutinate cells of the two races of P. phaseolicola (Experimental and Results section A a)), it was decided to investigate whether virulence of the races was affected by incubation

with cotyledon tissue.

24h King's B agar slope cultures of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) grown at 25°C were prepared as for an agglutination assay (Materials and Methods section 1, B, b). Suspensions of cells in sterile distilled water were adjusted by nephelometery to contain c. 2 x 10^8 cells cm^{-3}, and were mixed with equal volumes of either sterile distilled water or a comminuted preparation of Red Mexican cotyledons, thus giving inoculum concentrations of c. 1 x 10^8 cells cm^{-3}. The comminutate was prepared from seeds which had been soaked for 24h on moist paper at 25°C in the dark. The testas were removed and 40g of tissue comminuted for 2 min at top speed in a homogenizer with 100 cm^3 sterile distilled water. Seeds of P. vulgaris cv. Canadian Wonder were soaked for 24h on moist paper at 25°C in the dark and the testas were then removed. The cotyledons were then immersed for 30 min in 50 cm^3 of the respective inocula in petri dishes, then potted individually into 3" pots containing John Innes No. 2 potting compost and grown in the glasshouse. 15 seeds of CW were used for each treatment. A control set of 5 cotyledons were soaked in sterile distilled water before potting.

The seedlings were scored for symptom development at 10 and 20 days after potting. The results are shown in Table 48. The Race 2 isolate 882 was more virulent than the Race 1 isolate R1(X) on Canadian Wonder. There was no significant difference between the severity of infection caused by a given race inoculated in water or in a suspension of comminuted Red Mexican cotyledons. Thus it can be concluded that if the bacteria do bind to particulate material in the Red Mexican cotyledon preparation this does not reduce their capacity to cause disease in Canadian Wonder.

b) Alteration of the hypersensitive reaction

If the hypothesis that bacteria need to attach to the plant cell wall in order to induce the HR in leaves is correct, then one might expect to see

Table 48. Symptom development on seedlings of P. vulgaris cv. Canadian Wonder after inoculation with P. phaseolicola suspended in sterile distilled water or a comminutate of Red Mexican cotyledons

	Number of seedlings not emerging	Symptom development[a] 10 days			20 days		
		-	+	++	-	+	++
P. phaseolicola inoculum (1×10^8 cells cm^{-3})							
R1(X) (Race 1) water suspension	2	1[b]	5	7	0	5	8
R1(X) (Race 1) in RM cotyledon comminutate	1	1	4	9	0	3	11
882 (Race 2) water suspension	4	0	0	11	0	0	11
882 (Race 2) in RM cotyledon comminutate	5	0	1	9	0	0	10

a - no visible symptoms + plant stunted, mild interveinal chlorosis
++ plants stunted, severe interveinal chlorosis, emerging leaves distorted

b number of seedlings

an effect on HR if binding sites on bacterial or host surfaces were occupied prior to inoculation of avirulent cells. Before doing experiments to investigate this, the effects of agglutination caused by pectic polysaccharide on bacterial metabolism were studied.

i) Effect of agglutination on bacterial metabolism

P. phaseolicola produces a non-selective antimetabolite toxin which inhibits the enzyme ornithine transcarbamylase. If agglutination of cells by pectic polysaccharide resulted in cell death or general suppression of bacterial metabolism, toxin production would be impaired. Toxin production by agglutinated and non-agglutinated cells was compared using a quantitative bioassay modified after Gasson (1977, 1980).

24h King's B agar slope cultures of P. phaseolicola isolate R1(X) (Race 1) grown at 25°C were washed by centrifugation, resuspended in sterile distilled water and adjusted by nephelometry to c. 1×10^9 cells cm^{-3}. Suspensions were mixed with 2 volumes of either sterile distilled water or an aqueous solution of pectic polysaccharide. The solution made from pectic polysaccharide extracted from cotyledons of Red Mexican (Materials and Methods section 3, A, b), ii)) was prepared by suspending 20 mg cm^{-3} in water and centrifuging at 8000g for 10 min to remove undissolved starch. This gave a solution containing c. 9 mg pectic polysaccharide cm^{-3}. 0.01 cm^3 of inoculum containing c. 3×10^8 cells cm^{-3} were inoculated as discrete droplets on to M9 minimal agar in 9.0 cm diameter petri dishes. After incubation at 20°C for 48h the plates were overlayed with 3.5 cm^3 of 0.7% w/v water agar suspension of E. coli containing c. 1.5×10^9 cells cm^{-3}, kept molten at 45°C. This suspension was prepared from a prototrophic strain of E. coli grown at 37°C for 24h on M9 minimal medium and washed by centrifugation in the usual manner. The plates were then incubated overnight at 37°C which allowed growth of E. coli, but prevented further growth of

P. phaseolicola. The experiment was also performed using acidic pectic polysaccharide from leaves of Red Mexican. 0.01 cm^3 of c. 3 x 10^9 cells cm^{-3} were inoculated as discrete droplets on to the plates suspended either in water or 4 mg cm^{-3} solution of acidic pectic polysaccharide. The E. coli overlay contained 9 x 10^8 cells cm^{-3}. The diameter of zones of inhibition where growth of E. coli was inhibited by phaseolotoxin which had diffused into the medium were recorded.

The results are shown in Table 49. The plates inoculated with the solution of pectic polysaccharide alone showed no inhibition of E. coli growth. The inhibition zones due to phaseolotoxin produced by P. phaseolicola were similar for inocula of agglutinated and non-agglutinated cells. This suggests agglutination of cells by pectic polysaccharide from bean does not result in death of the cells or suppression of metabolism. Rather there was slightly greater phaseolotoxin production by the cells agglutinated by acidic pectic polysaccharide from Red Mexican leaves, since the mean inhibition zone of E. coli growth was slightly larger than for the non-agglutinated cells. Thus any effect on the HR which may be caused by treatment of bacterial cells with pectic polysaccharide are not due to suppression of bacterial metabolism.

ii) Effect on symptoms of HR

Bacteria were incubated with pectic polysaccharide in an attempt to occupy binding sites on their surfaces prior to inoculation into the incompatible host. The subsequent development of HR was then monitored.

24h King's B agar slope cultures of P. phaseolicola isolate R1(X) (Race 1) grown at 25°C were washed by centrifugation, the cells resuspended in sterile distilled water and adjusted to contain c. 2 x 10^8 cells cm^{-3} by nephelometry. The suspension was mixed with an equal volume of sterile distilled water or a solution containing 10 mg cm^{-3} acidic pectic

Table 49. Effect on metabolism of P. phaseolicola isolate R1(X) (Race 1) of agglutination by pectic polysaccharide from P. vulgaris cv. Red Mexican cotyledons and leaves, measured by effect on production of phaseolotoxin

Inoculum	Diameter of inhibition zone of E. coli growth (mm)								Mean	Standard error
c. 3 x 10^8 cells cm^{-3} water suspension	30	36	34	29	30	31	32		32	0.94
c. 3 x 10^8 cells cm^{-3} solution of pectic polysaccharide from RM cotyledons	31	31	33	31	27	32	31		31	0.70
c. 3 x 10^9 cells cm^{-3} water suspension	38	42	38	40	34	39	37	41	39	0.89
c. 3 x 10^9 cells cm^{-3} solution of pectic polysaccharide from RM leaves	42	46	45	45	42	43	41	43	43	0.63

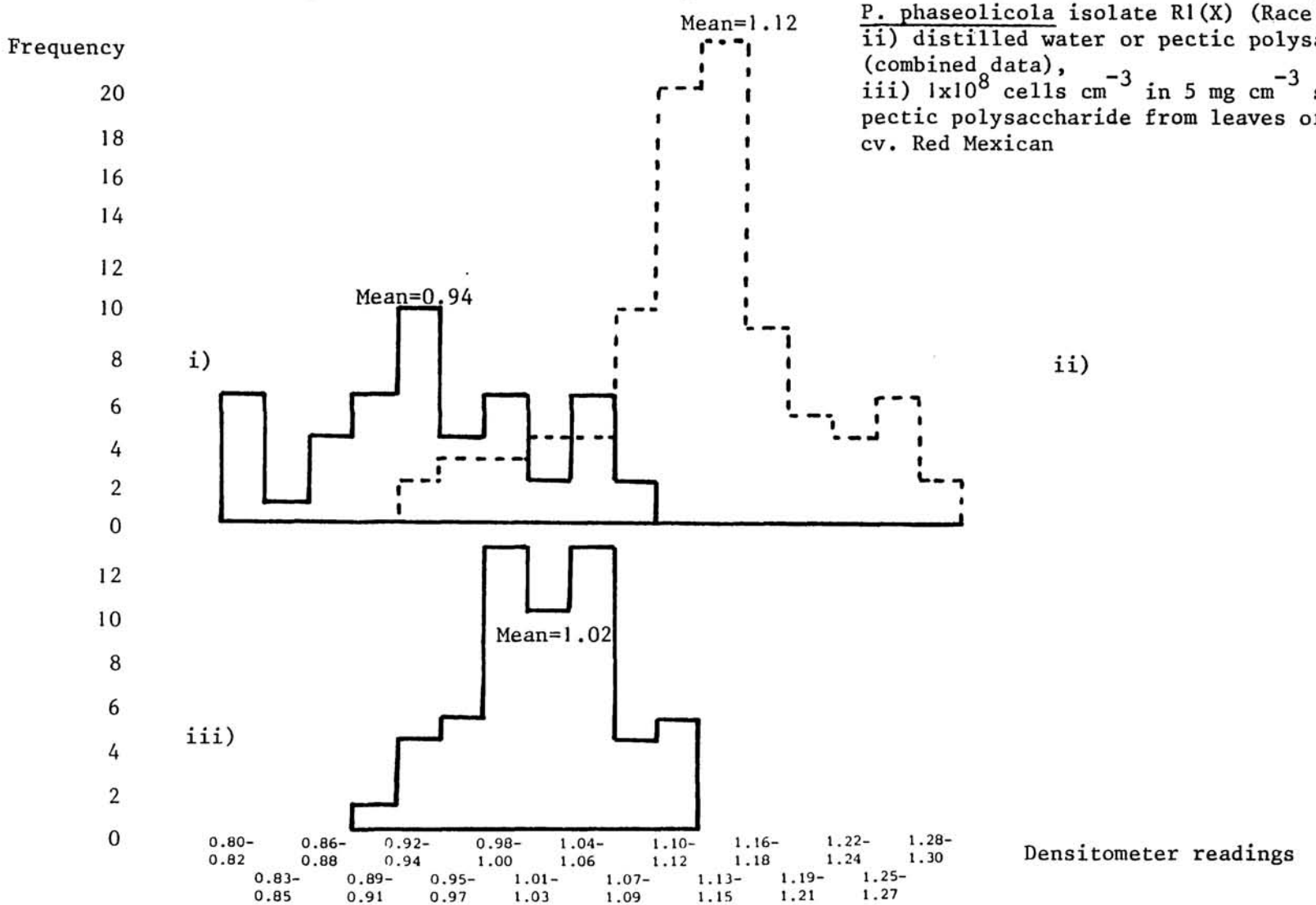
polysaccharide from leaves of P. vulgaris cv. Red Mexican. The suspensions were inoculated by syringe into primary leaves of 10 - 14 day old Red Mexican seedlings, grown individually in 3" pots in the glasshouse. Plants were also injected with sterile distilled water and 5 mg cm^{-3} solution of pectic polysaccharide to serve as controls. After inoculation the plants were returned to the glasshouse and the time of appearance of the initial symptoms of HR recorded. After 5 days to allow for full collapse and desiccation of the tissues the leaves were detached from the plants and transmission of light through the inoculated regions measured using a densitometer (Melico/Photolog B/W Transmission Densitometer, Model TDX).

The results are shown in Figure 23, which shows the distribution of densitometer readings for each treatment. The means and distributions of readings for the control treatments of water and acidic pectic polysaccharide solution were not significantly different so the data were combined. It can be seen that leaves inoculated with bacteria suspended in pectic polysaccharide solution gave a distribution of readings intermediate between those for healthy leaves and those for hypersensitively responding leaves inoculated with bacteria in water. Analysis of variance of the data indicated that the results were significantly different ($P = 0.001$). Macroscopically the lesion also appeared intermediate between healthy and fully collapsed leaf tissue, being mottled green/brown in appearance compared to bronze in the full HR.

The experiment was performed four times. The above result was achieved twice. However, the other two times the results were equivocal, analysis of variance showed that the results for the two different bacterial inoculations did not differ significantly from each other.

The early symptoms of HR appeared after c. 24h for both treatments. Because of the variability conclusions must be tentative. But the very significant results in two of the experiments ($P = 0.001$, by analysis of variance) do suggest that occupation of binding sites on the

Figure 23. Densitometer readings of Red Mexican leaves after inoculation with:i) $1x10^8$ cells cm^{-3} P. phaseolicola isolate R1(X) (Race 1) in water, ii) distilled water or pectic polysaccharide solution (combined data), iii) $1x10^8$ cells cm^{-3} in 5 mg cm^{-3} solution of acidic pectic polysaccharide from leaves of P. vulgaris cv. Red Mexican



surface of the bacterium before inoculation interferes in some way with presentation of the HR. This may depend on reduction in numbers of bacteria binding to plant cells, so that fewer cells per unit area would respond hypersensitively, resulting in a mosaic of healthy and collapsed cells.

iii) Effect on the HR induction period

The HR induction period is the time after inoculation when living bacteria are required for the HR to occur. It is measured by inj ecting solutions of antibiotics specific for procaryotic systems into leaves at various times after inoculation until further injection does not prevent the HR.

Suspensions of c. 2×10^8 cells cm^{-3} in sterile distilled water was prepared from 24h King's B agar slope cultures of P. phaseolicola isolated R1(X) (Race 1) grown at 20°C and washed by centrifugation. The suspensions were mixed with an equal volume of sterile distilled water or 10 mg cm^{-3} aqueous solution of acidic pectic polysaccharide from leaves of Red Mexican. Primary leaves of 12 day old RM seedlings were inoculated by syringe and the plants in a growth cabinet at c. 20°C (Materials and Methods section 1, A, b)). At various times groups of plants were injected with a 250 ppm aqueous solution of streptomycin sulphate in the previously inoculated regions. Four inoculation sites were used per plant, and 4 plants were used for each treatment at each time interval. Three plants were injected with streptomycin and the 4th with distilled water. The leaves were examined after 48h for HR development. In an earlier experiment (Experimental and Results section D, e)) it was shown that treatment of cells with streptomycin did not affect in vitro agglutination caused by acidic pectic polysaccharide from leaves of Red Mexican.

The results are shown in Table 50. The induction period was the same for both treatments and lay between 7 and 9h after inoculation. Thus it was concluded that treatment of bacterial cells with acidic pectic polysaccharide

Table 50. Measurement of the HR induction period in primary leaves of Red Mexican seedlings inoculated with P. phaseolicola isolate R1(X) (Race 1) suspended either in water or an aqueous solution of acidic pectic polysaccharide from leaves of Red Mexican

Time (h)	Plants inoculated with aqueous bacterial suspension. Subsequently injected with				Plants inoculated with bacteria suspended in pectic polysaccharide solution. Subsequently injected with			
	250 ppm streptomycin			water	250 ppm streptomycin			water
2	+[a]	−	−	++++	−	−	−	++++
	+	−	+	++++	−	−	−	++
	+	−	+	++++	−	+	−	+++
	+	−	+	++++	−	+	−	++++
4	−	−	−	++++	+	+	+	++++
	−	−	−	++++	+	−	+	++++
	+	−	−	++++	−	−	+	++++
	+	−	+	++++	−	−	−	+++
7	−	−	−	++++	++	−	−	+++
	−	−	−	++++	−	−	−	++
	−	+	+	++++	+	+	+	++++
	−	−	+	++++	−	−	+	+++
9	−	++++	++++	++++	++	++++	++++	++++
	−	++++	+	++++	+++	+++	++	++++
	−	+++	++	++++	+	++	++	+++
	−	+++	++	++++	++++	++++	+++	++++
12	+++	++++	++++	++++	++	+++	++++	++
	++++	+++	++++	++++	++++	++	+++	+
	+++	++	+	++++	++++	++++	++++	++++
	++	++	+	++++	++++	+++	+++	++++

a HR measured on scale from −, no visible effect on leaf through intermediate stages of mottling +, ++, +++ to fully collapsed and desiccated bronzed lesions ++++

before injection into an incompatible host does not affect the induction period of the hypersensitive reaction .

iv) Effect on the HR of pre-inoculation with bacterial LPS and EPS

Leaves were pre-inoculated with LPS or EPS in order to investigate further the possibility that these components of the bacterial cell surface carried the receptor site(s) which interacted with acidic pectic polysaccharide from bean. If binding sites on the plant cell walls were occupied this would reduce or prevent attachment of bacteria and thus reduce the intensity of the HR.

LPS and the two EPS fractions were isolated from P. phaseolicola isolates R1(X) (Race 1) as in Materials and Methods sections 12, a), b). 2 mg cm^{-3} solution of LPS and 5 mg cm^{-3} solution of the two EPS fractions were prepared in sterile distilled water by heating in a water bath at $50^{o}C$ for 1h. The solutions were injected into primary leaves of 10 - 14 day old Red Mexican seedlings and the plants returned to the glasshouse. After 24h the pre-inoculated regions were injected with an aqueous suspension containing 1×10^{8} cells cm^{-3} of P. phaseolicola isolate R1(X) (Race 1) prepared from a 24 King's B broth shake culture grown at $25^{o}C$ and washed by centrifugation. Each plant was inoculated with sterile distilled water, an aqueous solution of the test extract, an aqueous suspension of bacteria and a suspension of bacteria in test extract. The plants were returned to the glasshouse and after 5 days to allow full collapse and desiccation of the tissues the leaves were detached and transmission of light through the inoculated regions measured with a densitometer.

Analysis of variance of the data showed that the means for the densitometer readings of hypersensitively reacting leaves did not differ significantly ($P = 0.05$) when leaves were pretreated with LPS, EPS1 or EPS2

from P. phaseolicola isolate R1(X) (Race 1) or distilled water. Since none of the bacterial surface components tested interfered with the hypersensitive reaction this suggests that they may not be involved in attachment of bacteria to plant cell walls in this interaction.

c) In vivo attachment and recovery of bacteria

If differential attachment of cells of the two Races of P. phaseolicola to cell walls of bean occurred in vivo then using gentle methods to recover the intercellular fluid from inoculated leaves should give different recovery efficiencies for cells of the two Races.

Cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) were infiltrated into primary leaves of P. vulgaris cvs. Red Mexican and Canadian Wonder, time was allowed for bacteria to attach to plant cell walls, and the intercellular fluid was recovered by centrifugation. It was hoped that this method was gentle enough not to remove bacteria which had attached to plant cell walls. The intercellular volume of the leaves was estimated. Dilution plating of the intercellular fluid on King's B agar plates with subsequent counting of the colonies of P. phaseolicola was carried out. From these data the proportion of bacteria which attached in any given inoculation could be calculated (see Materials and Methods section 14, b)).

Thus, it was possible to compare attachment of cells of the two Races of P. phaseolicola to plant cell walls in a given cultivar and compare attachment of cells of a given Race of P. phaseolicola in the two cultivars. The procedures used are given in Materials and Methods section 14.

The relative recoveries of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) from leaves of P. vulgaris cvs. Red Mexican and Canadian Wonder are given in Tables 51 and 52. With inoculum concentrations of 0.1, 0.5 and 1.0 x 10^8 cells cm^{-3} in both cultivars consistently more cells of the Race 2 isolate were recovered with the intercellular fluid than cells

Table 51. Number of colonies of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) counted on plates of King's B agar after recovery by centrifugation of the intercellular fluid from inoculated primary leaves of P. vulgaris cv. Red Mexican

Inoculum concentration x 10^8 cells cm^{-3}	Number of colonies counted per plate from 0.05 cm^3 of intercellular fluid recovered by centrifugation								Number of Race 1 colonies expressed as a percentage of the number of Race 2 colonies[a]
	Undiluted		Mean		10^{-2} dilutions		Mean		
	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	
0.1	448,474,202,148	1600,[b]	318	1600	11,7,5,3	45,40,26,20	7	33	21%
0.5	1140,1040,1620,1216	-[c]	1254	-	18,34,30,35	457,600,539,517	27	529	5%
0.5	1704[b]	-[c]	-	-	25,28,31,18	151,137,149,139	26	144	18%
1.0	-[c]	-[c]	-	-	21,23,58,71	52,170,167,108	43	134	35%

a calculated from averages for the 10^{-2} dilution

b only one plate counted, due to large numbers involved a segment was counted and the number multiplied up to give the total

c colonies too numerous to count

Table 52. Number of colonies of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) counted on plates of King's B agar after recovery by centrifugation of the intercellular fluid from inoculated primary leaves of P. vulgaris cv. Candian Wonder

Inoculum concentration x 10^8 cells cm^{-3}	Number of colonies counted per plate from 0.05 cm^3 of intercellular fluid recovered by centrifugation								Number of Race 1 colonies expressed as a percentage of the number of Race 2 colonies[a]
	Undiluted		Mean		10^{-2} dilutions		Mean		
	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	
0.1	b	32,39,199,240	-	125	b	3,3,0,1	-	2	-
0.5	13,25,23,27	154,164,103,136	25	139	0,1,0,0	5,2,1,21[c]	0.25	2.5	10%
1.0	d	d	-	-	201,190,96,91	572,516,546,460	145	524	28%

a calculated from averages for the 10^{-2} dilution

b no colonies recovered

c value ignored when mean calculated

d colonies too numerous to count

of the Race 1 isolate. This suggests that cells of the Race 1 isolate attach to bean cell walls more readily than do cells of the Race 2 isolate. The number of colonies from Race 1 cells recovered as a percentage of colonies from Race 2 cells recovered varied between 5 - 35% for Red Mexican (mean = 20%) and 10 - 28% for Canadian Wonder (mean = 19%). Table 53 shows the percentage of inoculum not recovered, presumably due to attachment of the bacteria to plant cell walls, for both P. phaseolicola isolates with both French bean cultivars. The mean values for R1(X) (Race 1) and 882 (Race 2) with cv. Red Mexican were 98.7 and 95.6% and with cv. Canadian Wonder were 98.9 and 96.1%. Most of the inoculum of both Races in both cultivars was not recovered. Thus although there may be differential attachment of cells of the two Races the quantitive difference in numbers of attached cells of each Race is small. Differential attachment occurred to similar degrees in both bean cultivars.

P. vulgaris cv. Canadian Wonder is susceptible to both Races of P. phaseolicola whereas cv. Red Mexican gives a hypersensitive reaction and is resistant to cells of Race 1 isolates. Since contact of bacteria with the plant cell wall is necessary for induction of the HR, it was postulated that differential attachment of the two Races might occur in Red Mexican, with Race 1 cells attaching to a greater degree than Race 2 cells, allowing a HR to be induced by Race 1 isolates only. In Canadian Wonder it was postulated that neither Race attached to any significant extent, thereby not being able to induce the plant cells to respond hypersensitively. These results suggest that differential attachment of bacterial cells in the two bean cultivars is not the critical factor which allows for their different responses to cells of the two Races of P. phaseolicola.

The binding coefficient (K) for the Race 1 isolate R1(X) of P. phaseolicola in Red Mexican (calculated as in Materials and Methods section 14, b)) was constant for the range of inoculum concentrations used (see Table 53). Similarly for the Race 2 isolate 882 in Red Mexican. This

Table 53. Percentage of inocula of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) not recovered by centrifugation from leaf discs of P. vulgaris cvs. Red Mexican and Canadian Wonder, and values of the binding coefficients for isolates of both Races in the two cultivars

Inoculum concentration x 10^8 cells cm^{-2}	RM percentage of inoculum not recovered		RM binding coefficient K		CW percentage of inoculum not recovered		CW binding coefficient K	
	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
0.1	98.96%	95.11%	0.010	0.051	-	-	-	-
0.5	97.51%	60.98%[a]	0.026	0.664[a]	-	-	-	-
0.5	99.03%	94.62%	0.010	0.057	99.98%	99.83%	0.0002	0.002
1.0	99.13%	97.30%	0.009	0.028	97.87%	92.32%	0.022	0.08
Mean values	98.66%	95.59%	0.014[b]	0.045[b]	98.93%	96.08%	-	-
Standard error			0.004	0.009			-	-

a value not used in calculation of mean

b values significantly different (P = 0.02)

suggests that initial attachment of bacterial cells to bean cell walls is a reversible process. Subsequent envelopment of bacterial cells by plant material may bring about irreversible attachment.

The greater the value of K the lower the affinity of the cell wall for the bacterium. The mean value of K for cells of R1(X) (Race 1) in Red Mexican was 0.014 and for cells of 882 (Race 2) was 0.045, suggesting that there was greater affinity between the cell walls and bacteria for R1(X) than for 882. The standard error of the difference between the two sample means was 0.009 and a statisitcal t-test indicated that the means were significantly different ($P = 0.02$).

F. Agglutination of bacterial cells by pectic polysaccharides from different plant species

a) Agglutination of phytopathogenic and non-phytopathogenic bacteria by acidic pectic polysaccharide from P. vulgaris cv. Red Mexican

Acidic pectic polysaccharide from leaves of P.vulgaris cv. Red Mexican (extracted as in Materials and Methods section 3, B a) and c) iii)) was dissolved in 0.02M sodium phosphate buffer pH 6.8 to give a 10 mg cm^{-3} solution which was used to prepare dilution series for agglutination assays. Various species of phytopathogenic and non-phytopathogenic bacteria were used in the assays. Bacteria were grown in shake culture for 24h at $25^{o}C$ in 25 cm^3 King's B broth in 250 cm^3 conical flasks. After washing by centrifugation the cells were resuspended in buffer and after adjusting the concentration by nephelometry, were used in agglutination assays. Concentrations were adjusted using the calibration curve prepared with cells of P. phaseolicola to contain c. 1.5×10^{10} cells cm^{-3}. Nephelometer calibration curves were not prepared for each bacterial species. Suspensions of bacteria

of different sizes would scatter light differently, thus using the nephelometer readings with the calibration curve for P. phaseolicola would in actual fact give suspensions containing various concentrations of cells. Due to the different sizes of cells of the various species the total surface area over which pectic polysaccharide can attach would be different in each suspension. Thus the results are not comparable from species to species to estimate relative degrees of agglutination, but are suitable to determine whether agglutination of a particular species does or does not occur.

The results are shown in Tables 54 and 55. Cells of all phytopathogenic species tested were agglutinated by acidic pectic polysaccharide from P. vulgaris cv. Red Mexican. Cells of E. carotovora showed spontaneous agglutination. However an interaction with pectic polysaccharide was still observed, the effect being diluted out as the dilution series was descended. Cells of E. atroseptica did not spontaneously agglutinate, but were agglutinated by the pectic polysaccharide. The relative degree of agglutination of the pseudomonads can be compared since the nephelometer calibration should be accurate for cells of the same genus. Cells of P. mors-prunorum and P. phaseolicola isolate R1(X) (Race 1) were agglutinated similarly. Cells of the saprophyte P. fluorescens showed a degree of agglutination intermediate between P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2). The degree of agglutination of cells of the two species of Agrobacterium tested should be comparable since both suspensions should contain similar concentrations of cells. A.radiobacter and A. tumefaciens were agglutinated to similar degrees.

Cells of the non-phytopathogenic species P. fluorescens and Sarcina subflava were agglutinated by the acidic pectic polysaccharide. Cells of Serratia marcescens and E. coli showed spontaneous agglutination, however there was evidence of an interaction with the acidic pectic polysaccharide.

The results show that bacteria from several phytopathogenic and non-phytopathogenic genera can interact with acidic pectic polysaccharide from

Table 54. Agglutination of cells of phytopathogenic bacteria by acidic pectic polysaccharide from leaves of *P. vulgaris* cv. Red Mexican

Acidic pectic polysaccharide concentration (mg cm^{-3})	*P.phaseolicola* Race 1 R1(X)	*P.phaseolicola* Race 2 882	*P.morsprunorum*	*Corynebacterium fascians*	*Erwinia carotovora*	*E.atroseptica*	*Agrobacterium radiobacter*	*A.tumifaciens*
0	0[a]	0	0	0	3	0	0	0
5.00	4	4	4	4	4	4	4	4
2.50	4	1	4	1	2	4	4	4
1.25	4	0	4	1	2	4	4	4
0.63	3	0	3	0	2	1	4	4
0.32	1	0	1	0	3	0	3	4
0.16	0	0	0	0	3	0	1	0
0.08	0	0	0	0	3	0	0	0

a agglutination index - see Plate 1

Table 55. Agglutination of cells of non-phytopathogenic bacteria by acidic pectic polysaccharide from leaves of *P. vulgaris* cv. Red Mexican

Acidic pectic polysaccharide concentration (mg cm^{-3})	*P. fluorescens*	*Sarcina subflava*	*Serratia marcescens*	*Escherichia coli*
0	0[a]	0	4	3
5.00	4	4	2	4
2.50	4	2	4	4
1.25	3	1	4	4
0.63	2	0	4	4
0.32	0	0	4	4

a agglutination index - see Plate 1

leaves of P. vulgaris cv. Red Mexican.

b) Agglutination of cells of P. phaseolicola by pectic polysaccharides from various higher plant species

It was decided to extract pectic polysaccharide from a range of plant species and assess their abilities to cause agglutination of cells of P. phaseolicola. The reactions of the plants used to cells of Race 1 and 2 isolates of P. phaseolicola are given in Table 56.

Table 56. Reactions of plant species and cultivars used as sources of pectic polysaccharide to Race 1 and 2 isolates of P. phaseolicola

Plant species and cultivars	Reaction to cells of P. phaseolicola	
	Race 1	Race 2
P. vulgaris		
cv. Red Mexican[a]	R	S
cv. Canadian Wonder[a]	S	S
cv. PI 150414[b]	R	R
cv. GN Nebraska 27[b]	R	R
P. mungo[a]	R	S
Vicia faba cv. The Sutton[a]	R	R
Pisum sativum cv. Onward[a]	R	R

R = resistant reaction S = susceptible reaction

a data from Lalyett (1977) b data from Taylor (1978)

Pectic polysaccharide was extracted from leaves of 2 - 3 week old seedlings as in Materials and Methods sections 3, B, a) and c) iii). Fractional precipitation with absolute alcohol to give an acidic pectic

polysaccharide fraction was carried out. Further addition of absolute alcohol to give a precipitate corresponding to the neutral pectic polysaccharide fraction of Barrett and Northcote (1965) was carried out with buffer extracts from P. mungo and Tulipa sp. The second ethanol precipitate obtained from the extract from Tulipa sp. was atypical. It was gelatinous and identical in appearance to the first ethanol precipitate, usually the neutral pectic polysaccharide fraction was a powdery precipitate different in consistency from the first precipitate. This suggests that with the extract from Tulipa sp. further addition of ethanol had precipitated more of the acidic pectic polysaccharide from solution. Tulips are members of the Liliaceae and there are few reports of pectic polysaccharide from monocots.

The pectic polysaccharides were dissolved in 0.02M sodium phosphate buffer pH 6.8 to make 10 mg cm^{-3} solutions which were used to prepare dilution series for agglutination assays. Bacteria were grown in shake culture for 24h at $25^{o}C$ in 25 cm^{-3} King's B broth in 250 cm^{3} conical flasks. After washing by centrifugation the cells were resuspended in buffer and the suspensions adjusted by nephelometry to contain c. 1.5×10^{10} cells cm^{-3}.

The results are shown in Tables 57 and 58. The acidic pectic polysaccharide fractions from all the higher plant species and cultivars tested differentially agglutinated cells of the two Races of P. phaseolicola. Cells of the Race 1 isolate R1(X) were agglutinated to a greater degree than were cells of the Race 2 isolate 882. This can be seen from Table 57 by comparing the corresponding agglutination indexes and dilution end points. Agglutination of cells of the Race 1 isolate R1(X) showed little variation for acidic pectic polysaccharide from all the plant species; the dilution end points were 2^{4} or 2^{5} in all cases. However agglutination of cells of the Race 2 isolate 882 was more variable, the dilution end points ranging from 2^{1} to 2^{3}. No correlation was apparent between the pattern of

Table 57. Agglutination of cells of P. phaseolicola isolates R1(X) (Race 1) and 882 (Race 2) by acidic pectic polysaccharides from leaves of 2-3 week old seedlings of various higher plant species and cultivars

Dilution	Acidic pectic polysaccharide concentration (mg cm^{-3})	Phaseolus vulgaris cv. Red Mexican		Phaseolus vulgaris cv. Canadian Wonder		Phaseolus vulgaris cv. PI 150414		Phaseolus vulgaris cv. GN Nebraska 27		P. mungo		Vicia faba cv. The Sutton		Pisum sativum cv. Onward		Tulipa sp.[a]		Citrus sp.[a,b]	
		R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882	R1(X)	882
Control	0	0[c]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2^1	5.0	4	4	4	1	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2^2	2.50	4	1	4	1	4	4	4	3	4	1	4	3	4	0	3	3	4	4
2^3	1.25	4	0	4	0	4	1	4	0	3	1	4	0	4	0	1	0	4	0
2^4	0.63	2	0	1	0	1	0	3	0	1	0	1	0	3	0	1	0	2	0
2^5	0.32	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
2^6	0.16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilution end point		2^5	2^2	2^4	2^2	2^4	2^3	2^5	2^2	2^5	2^3	2^4	2^2	2^4	2^1	2^4	2^2	2^4	2^2

a age of plants from which extract made unknown

b obtained from Sigma (London)

c agglutination index - see Plate 1

Table 58. Agglutination of cells of *P. phaseolicola* isolates R1(X) (Race 1) and 882 (Race 2) by first and second ethanol precipitates of citrate-oxalate buffer extracts of leaves of *P. mungo* and *Tulipa* sp., and agglutination of cells of *Agrobacterium radiobacter* by the 1st ethanol precipitate from *Tulipa* sp.

Dilution	Pectic polysaccharide concentration (mg cm^{-3})	*P. mungo* 2nd ethanol precipitate[b]		*Tulipa* sp. 2nd ethanol precipitate[b]		*Tulipa* sp. 1st ethanol precipitate[a]		
		R1(X)	882	R1(X)	882	R1(X)	882	*Agrobacterium radiobacter*
Control	0	0[c]	0	0	0	0	0	0
2^1	5.00	0	0	4	4	4	4	4
2^2	2.50	0	0	3	1	3	3	4
2^3	1.25	0	0	2	0	1	0	4
2^4	0.63	0	0	0	0	1	0	4
2^5	0.32	0	0	0	0	0	0	3
2^6	0.16	0	0	0	0	0	0	0
Dilution end point		-	-	2^3	2^2	2^4	2^2	2^5

a corresponding to acidic pectic polysaccharide of Barrett and Northcote (1965)
b corresponding to neutral pectic polysaccharide of Barrett and Northcote (1965)
c agglutination index - see Plate 1

agglutination by extracts from the different species and cultivars and their reaction of resistance or susceptibility to cells of the two Races of P. phaseolicola.

The neutral pectic polysaccharide fraction obtained from leaves of P. mungo caused no detectable agglutination of cells of either Race of P. phaseolicola. In this respect it behaved similarly to the neutral pectic polysaccharide fractions obtained from P. vulgaris cvs. Red Mexican and Canadian Wonder (see Experimental and Results section B, c)). However, with Tulipa sp. the fraction corresponding to the neutral pectic polysaccharide fraction from other species did cause agglutination of cells of both Races of P. phaseolicola. Cells of the Race 1 isolate R1(X) were agglutinated to a greater degree than were cells of the Race 2 isolate 882 (Table 58). The fraction probably contained a large proportion of acidic pectic polysaccharide, not all of which appeared to have been precipitated by the first addition of ethanol (see earlier).

The acidic pectic polysaccharide fraction from Tulipa sp. caused agglutination of cells of Agrobacterium radiobacter (Table 58).

From the results it can be concluded that agglutination of bacteria by acidic pectic polysaccharide is a widespread phenomenon.

DISCUSSION

There have been many recent reports of specific recognition between higher plants and micro-organisms. In particular there have been many reports of specific attachment of bacteria to plant cell walls in the intercellular spaces of leaves. Stall and Cook (1979) reported that contact of bacteria with plant cell walls was necessary for induction of the hypersensitive reaction (HR) but not the susceptible reactions (SR). The hypothesis was advanced that specific attachment of cells of avirulent but not virulent bacteria to plant cell walls enabled induction of the hypersensitive reaction in those cells (Sequeira, 1976; Klement, 1977). Many of the bacteria/higher plant interactions reported in the Review of the Literature appear to support this view.

In halo blight of French bean caused by Pseudomonas phaseolicola two races of the pathogen can be distinguished on P. vulgaris cv. Red Mexican, which is resistant to Race 1 but susceptible to Race 2 isolates. P. vulgaris cv. Canadian Wonder is susceptible to both races.

It was thought possible that the different reactions of the two cultivars of bean to the two races of P. phaseolicola might result from differential attachment of bacteria to a substance in the plant cell walls. If this supposition were correct, cells of Race 1 isolates of P. phaseolicola would be expected to attach to cell walls of cv. Red Mexican more readily than would cells of Race 2 isolates. Bacteria of both races would not be expected to attach readily to cell walls of cv. Canadian Wonder, in which the active substance might be absent or present in significantly lower concentrations

An electronmicrograph showing cells of an avirulent Race 1 isolate of P. phaseolicola attached to cell walls in the intercellular spaces of leaves of P. vulgaris cv. Red Mexican was published by Roebuck et al. (1978).

A large volume of literature suggests involvement of lectins in specific recognition between plants and micro-organisms (Callow, 1977; Sequeira, 1978).

It was decided to extract tissues of the two cultivars for lectins and assess the ability of the extracts to interact with cells of isolates of the two races of P. phaseolicola. Many lectins if they are di- or multivalent (i.e. have more than one binding site) will cause agglutination of the cells whose surfaces carry the sugar residues with which they interact. Agglutination of cells of P. solanacearum by a lectin from potato had been reported (Sequeira and Graham, 1977) and while this work was in progress Anderson and Jassalavich (1979) reported agglutination of Pseudomonad cells by extracts from P. vulgaris cv. Dark Red Kidney. However, the slide agglutination assay used by these and other authors working with phytopathogenic bacteria was found to be rather subjective in comparing the agglutination of different bacterial isolates. The assay used in the work reported in this thesis was a modification of a standard method used for haemagglutination by serum (Hudson and Hay, 1976; Roitt, 1973).

Using this assay comparison of the agglutination of cells of different bacterial isolates was facilitated by :

a) A readily observable dilution end-point beyond which no agglutination occurs,

b) The appearance of the agglutinated clumps of cells of different isolates in wells containing equivalent concentrations of agglutinin (see Plate 1).

Using a standard key prepared from a photograph of an assay, an "agglutination index" can be based on the appearance of the button of cells in the test well (see Plate 1). The standard key has been published (Slusarenko and Wood, 1981). Comparison of an agglutination index quoted in a table of results with the standard key defines with some precision what occurred in the experiment. This is much less subjective than the usual way of reporting the results of a slide agglutination test, with agglutination being rated on a scale from 0 to ++++, without an adequate definition of what

each symbol represents. What is classified by one worker as ++++ may be classed by another as +++ or even ++. Whereas this is adequate for a single experiment, the modified haemagglutination assay has obvious advantages for wider comparison.

Using dilute HCl to extract lectins (after Moreira and Perrone, 1977) a fraction was isolated from cotyledons of cv. Red Mexican which agglutinated cells of Race 1 isolates of P. phaseolicola more actively than cells of Race 2 isolates. A fraction with similar properties was not obtained from cotyledons of cv. Canadian Wonder. This supported the hypothesis outlined on page 182. Extracts from cotyledons of both cultivars caused agglutination of erythrocytes. It was thought that agglutination of bacteria and erythrocytes might be caused by the same substance which was probably a lectin. However, no bacterial agglutinating activity was detected in HCl extracts of leaves of either cv. Red Mexican or Canadian Wonder. This was important for the hypothesis (page 182); a bacterial agglutinin would have to be present in leaves of cv. Red Mexican. Extracts of cotyledons made with dilute saline caused agglutination of erythrocytes, but not bacteria. Subsequent experiments showed that erythrocyte and bacterial agglutination were caused by different substances; bacterial agglutination was caused by carbohydrate in the extracts.

Resistance of P. vulgaris cv. Red Mexican to Race 1 isolates of P. phaseolicola is monogenic and dominant (Taylor, 1978). It was suggested (Slusarenko and Wood, 1981) that the gene might specify a glycosyl transferase which would give a cell wall polysaccharide structurally different from the corresponding polysaccharide in susceptible cultivars, and which was able to cause agglutination of avirulent Race 1 isolates of P. phaseolicola.

The active carbohydrate was identified as part of the pectic polysaccharide fraction of bean cell walls which had been fortuitously extracted by the dilute HCl used to extract lectins. More efficient methods for extraction of pectic polysaccharides gave fractions active in bacterial

agglutination from cotyledons and leaves of cvs. Red Mexican and Canadian Wonder. Agglutination of bacteria was caused by part of the acidic pectic polysaccharide fraction from both cultivars. Purifcation and analysis showed the extracts to be very similar in composition. This, and the similar abilities of the acidic pectic polysaccharide from the two cultivars to agglutinate cells of the two races of P. phaseolicola, suggest that the hypothesis of a unique cell wall polysaccharide in cv. Red Mexican was incorrect. Similar amounts of active pectic polysaccharide were obtained from leaves of both cultivars. However, significantly more pectic polysaccharide could be extracted from a given fresh weight of cotyledons of cv. Red Mexican than from those of cv. Canadian Wonder. This is probably why the initial dilute HCl "lectin" extracts of Canadian Wonder cotyledons did not cause agglutination. The mild low pH conditions could not solubilize sufficient of the smaller amount of pectic polysaccharide present in this cultivar.

In order for the specific response of cv. Red Mexican to Race 1 isolates of P. phaseolicola to depend on recognition by specific attachment of avirulent bacteria to plant cell walls, the active substance, although present in leaves of cv. Canadian Wonder would have to be unavailable at the surface of plant cell walls in the intercellular spaces, and therefore be unable to interact with bacterial cells.

Investigation of in vivo attachment of bacteria to plant cell walls showed that cells of the Race 1 isolate R1(X) attached more readily than did cells of the Race 2 isolate 882 in both cultivars. Similar proportions of a given inoculum attached in each cultivar. Thus it must be concluded that differential attachment of cells of the two races of P. phaseolicola to plant cell walls is not the crucial recognition event which enables cv. Red Mexican to respond specifically with a hypersensitive reaction to cells of the avirulent isolates. The "switching point" must be determined by a subsequent event, either in the host cells or those of the bacteria.

These results question the conclusions of Klement (1977) working with P. pisi in tobacco, that specific cell contact recognition "was connected or coincided with multiplication of bacterial cells and that the multiplication of bacteria was the essential factor of HR induction" and "toxic bacterial metabolites are not responsible for the induction of the HR." Although there is differential attachment of cells of the two races of P. phaseolicola to cell walls of P. vulgaris cv. Red Mexican, a significant proportion of a given inoculum of both races attaches. Omer and Wood (1969) showed that multiplication of cells of the two races of P. phaseolicola in cv. Red Mexican was very similar for the first two days after which time multiplication of cells of the Race 1 isolate slowed down. Ercolani and Crosse (1966) observed a similar effect with cells of P. phaseolicola and P. mors-prunorum in homologous and heterologous hosts. Klement (1977) does not demonstrate that actual multiplication of bacteria is required rather than cells which are simply metabolically active. At one point he states "multiplication of the bacterium or at least metabolically active bacterial cells are necessary", but later ignores this proviso in his final conclusions. It is difficult to conceive how attachment and multiplication of bacteria could pass the stimulus to the host cell and induce the HR without the participation of some chemical "messenger" as an intermediate. Klement (1977) does not speculate on this. His failure to demonstrate a discrete substance which acted as an inducer of HR in his experiments does not justify his conclusion that one does not exist.

In view of the work presented in this thesis alternative hypotheses are needed to explain the specific response of P. vulgaris cv. Red Mexican to cells of avirulent Race 1 isolates of P. phaseolicola. A possible sequence of events is :

a) Cells of both races of P. phaseolicola attach to cell walls of P. vulgaris cvs. Red Mexican and Canadian Wonder.

b) Cells of both races produce unstable inducers of HR which pass across the plant cell wall to the membrane and/or beyond into the host cytoplasm.

c) The product of the dominant resistance gene of cv. Red Mexican serves as a receptor for the inducer from Race 1 isolates of P. phaseolicola but not for the different inducer from Race 2 isolates. Interaction of inducer and receptor causes the sequence of events which lead to cell collapse. Cv. Canadian Wonder would lack receptors for the inducers from both races of P. phaseolicola.

O'Brien and Wood (1973) showed that treatment of P. vulgaris cv. Red Mexican with cycloheximide suppressed the leakage of electrolytes and tissue browning usually associated with infiltration of avirulent cells of P. morsprunorum into the leaves. Keen et al. (1981) showed that blasticidin S prevented cell collapse if infiltrated into soybean leaves up to 9h after inoculation with cells of avirulent Pseudomonads. Cycloheximide and blasticidin S inhibit protein synthesis in eukaryotic cells. Keen et al. (1981) concluded that the processes leading to hypersensitive cell death depend on protein synthesis in the host. However the HR inducer of the bacterium may be itself induced by some substance from the host. This would accord with the observations :

a) Cells of both races of P. phaseolicola attach to cell walls of Red Mexican and Canadian Wonder.

b) The product of the gene for resistance (sensitive to inhibitors of eucaryotic protein synthesis) passes across the plasma membrane and plant cell wall to the attached cells.

c) Cells of Race 1 but not Race 2 isolates respond by synthesising the unstable HR inducer (sensitive to inhibitors of procaryotic protein synthesis) which on entering the host cytoplasm causes

d) the sequence of events which lead to cell collapse (sensitive to eucaryotic protein synthesis inhibitors).

Stages a) to c) of the above scheme would occur during the induction period of HR whereas d) would occur afterwards. Thus, if a selective inhibitor of stage b) could be found it would be possible to test the hypothesis. If the hypothesis is correct the inhibitor for b) would only be effective for the length of the induction period (determined by infection of leaves with streptomycin); whereas an inhibitor of d) would be effective for a longer period.

Differential attachment of cells of the two races of P. phaseolicola to bean cell walls may be related to virulence (i.e. degree of pathogenicity (Anon, 1973). In the compatible host Canadian Wonder the Race 1 isolate R1(X) of P. phaseolicola was less virulent than the Race 2 isolate 882. It would therefore be interesting to investigate if there were correlations between virulence of other isolates of both races of P. phaseolicola, their degree of in vitro agglutination by acidic pectic polysaccharide from the host and in vivo attachment of bacteria to host cell walls, and to compare binding coefficients K for the different isolates to in vitro agglutination. High positive correlations would support the contention that it is acidic pectic polysaccharide to which these bacteria attach in vivo.

The differential recovery of cells of isolates of the two races from leaves is interesting in view of work published by Hildebrand et al. (1980), who reported no differences in attachment and entrapment in bean leaves 3h after inoculation with cells of compatible or incompatible isolates of P. syringae or the incompatible phytopathogens P. marginalis, P. pisi and P. tomato. They proposed an entirely non-discriminatory physical process for

attachment. However, their proposal was based only on observations by electronmicroscopy and were not quantitative.

An earlier report by Hildebrand and Schroth (1968) using quantitative techniques showed differences in recovery (and therefore by implication attachment to plant cell walls) of cells of saprophytic and phytopathogenic bacteria from inoculated leaves of cowpea.

Hildebrand et al. (1980) also reported that if inoculated tissues were kept watersoaked for 12h after inoculation and then examined, no bacterial entrapment was evident. The authors did not leave plants long enough to see if a HR developed to incompatible phytopathogens. Lyon (neé O'Brien) and Wood (1975) reported that French beans inoculated with avirulent bacteria and then maintained at high humidity still responded hypersensitvely. These observations suggest either :

a) attachment of avirulent bacteria to host cell walls is not necessary for induction of the HR in bean,

or

b) attachment is necessary, and is not prevented by keeping the tissues watersoaked, but Hildebrand et al. (1980) did not observe attachment in their electronmicrographs.

Evidence presented in this thesis shows a reduction in intensity of the HR to avirulent bacteria in leaves of bean when binding sites on the bacterial surface were occupied before inoculation. This suggests that attachment of bacteria to plant cell walls is important for induction of the HR in bean. Hildebrand et al. (1980) state "Bacterial attachment should not be prevented by the presence of water if this were an active process." There is no theoretical reason why active or passive attachment of bacteria to a surface should be prevented by a water film. Indeed free water would be considered the usual environment for surface interactions to occur in nature.

The initial attachment of cells of P. phaseolicola to cell walls of bean was shown to be a reversible process. In a water film bacteria can swim by flagellar action. However, when bacteria come into "contact" with the plant cell wall, attachment and detachment should occur freely. In intercellular spaces of leaves full with water proportionally more bacteria would be expected to be out of contact with the cell wall. Thus the equilibrium would be shifted but attachment should still occur. Hildebrand et al. (1980) may not have looked hard enough for attached bacteria.

Pretreatment of cells of avirulent P. phaseolicola isolate R1(X) with acidic pectic polysaccharide to occupy binding sites on the bacterial surface did not perceptibly alter the induction time of the HR in cv. Red Mexican. Presumably a reduction in number of bacteria binding to plant cells in a given leaf area results in fewer leaf cells which can respond hypersensitively, and in a mosaic of healthy and collapsed cells which gives rise to the apparent reduction in intensity of the HR mentioned earlier. Turner and Novacky (1974) showed that concentrations of inoculum of P. pisi below the threshold necessary to induce confluent necrosis in tobacco gave rise to dead leaf cells at a ratio of 1:1 to the number of bacteria introduced into the leaf. Thus although the number of plant cells within a given area which have bacteria adhering to their surface may be reduced by pretreatment, the HR is induced normally by those bacteria which are attached. An induction period of 7 - 9h was observed in the experiments reported in this thesis. Other values quoted for P. vulgaris cv. Red Mexican and avirulent Race 1 isolates range from 1 - 1.5h (Lyon (neé O'Brien) and Wood, 1975) to 4h (Roebuck et al., 1978). Lyon (neé O'Brien) and Wood (1975) reported that the HR developed most rapidly at 25°C, at which temperature the quoted induction periods were determined. However, the value of 7 - 9h for the induction period was determined using plants kept at 20°C. Possibly the induction period is longer at this lower temperature as is the presentation time.

Investigations to determine the nature of the component on the bacterial surface which serves as an attachment site for acidic pectic polysaccharide were not conclusive. Lipopolysaccharide (LPS) or extracellular polysaccharide (EPS) were obviously suspected because of their position at the cell surface and their implication in other bacteria/plant cell surface interactions (e.g. Sequeira and Graham, 1977; Dazzo and Hubbell, 1975; Wolpert and Albersheim, 1976; Whatley et al., 1976). However, no interactions with acidic pectic polysaccharide from P. vulgaris cv. Red Mexican could be demonstrated either in vitro in Ouchterlony double diffusion assays, or in vivo by pretreatment of leaves before infiltration with avirulent bacteria. If EPS or LPS were able to interact with host attachment sites for bacteria, pretreatment of leaves would be expected to reduce in intensity or prevent the HR. Such effects were found by Mazzucchi and Bazzi (1979) in tobacco, Graham et al., (1977) with LPS from P. solanacearum, E. coli and Serratia marcescens in tobacco. However, no such effects were found in cv. Red Mexican with P. phaseolicola, suggesting that LPS and EPS are not involved in the surface interactions. The non-involvement of LPS in attachment is further supported by the observation that in vitro agglutination of cells of P. phaseolicola by acidic pectic polysaccharide was reduced by autoclaving the cells. Bacterial "O" antigens, which comprise the LPS, are heat stable (Meadow, 1975). Lyon (1973) showed that pretreatment of leaves of P. vulgaris cv. Red Mexican with heat killed cells of Race 1 isolates of P. phaseolicola did not prevent the HR to living avirulent cells. Lozano and Sequeira (1970b) prevented the HR in tobacco leaves by pretreatment with heat killed bacteria. Bacterial LPS was later shown to be the bacterial surface component interacting with a host attachment site in this system (Sequeira and Graham, 1977).

Rough (R) colony forms of P. phaseolicola were reported by Adam and Pugsley (1934). R mutants lack the O side-chain and part of the core oligosaccharide of LPS. It would be interesting to carry out agglutination assays with R forms and acidic pectic polysaccharide, although Taylor (1970b)

reports that these R forms have a tendency to spontaneous agglutination. Taylor (1970b) also reported a heat labile antigen of P. phaseolicola which was present in 12 other species of phytopathogenic pseudomonads; other genera were not tested. In view of the ability of acidic pectic polysaccharide to cause agglutination of cells of a wide variety of bacterial species and genera it is possible that the widely distributed heat labile antigen described by Taylor and the heat labile binding site for acidic pectic polysaccharide are the same. However, P. fluorescens which was reported to lack this antigen still was agglutinated by acidic pectic polysaccharide from bean.

Adherence of Ruminococcus albus and R. flavefaciens to plant cell walls in the rumen of herbivores is prevented by treatment of bacteria with periodate or pepsin (Latham, 1978). It was concluded that the integrity of the well defined glycoprotein coat of these organisms was essential for adhesion. Periodate oxidation of cells of P. phaseolicola isolate R1(X) (Race 1) did not affect agglutination of the cells by acidic pectic polysaccharide from Red Mexican which implies that the surface receptor on the surface of cells of P. phaseolicola isolate R1(X) (Race 1) is not carbohydrate. There was a slight reduction in agglutination of cells of the race 2 isolate 882 after periodate oxidation of the cell surface. This is discussed on page 193.

The heat lability of the bacterial surface receptor for acidic pectic polysaccharide from bean suggests that it may be proteinaceous. A mannose specific lectin involved in attachment of bacteria to epithelial cells was shown to be present on the surface of E. coli (Eshdat et al., 1978). Group 1 pili have been implicated in adhesion of cells of E. coli, Salmonella sp. (including S. typhimurium), Klebsiella sp. and Proteus sp. to plant cells (Ward and Berkeley, 1980). Bacterial flagella must also be considered as possible carriers of binding sites involved in adhesion. Agglutination of bacterial cells by acidic pectic polysaccharides was shown to be a widespread phenomenon; possibly different bacterial surface receptors are important for

different genera and species. Treatment of cells of P. phaseolicola with a protease which had a broad spectrum of activity had no detectable effect on agglutination of cells of either race. This suggests either that protein is not important as a receptor, or that it was protected from enzyme action.

Column electrophoresis on a urea gradient resolved the acidic pectic polysaccharide from bean into two components. The major component contained c. 80% uronic acid residues and agglutinated cells of the Race 1 isolate R1(X) of P. phaseolicola more actively than it did cells of the Race 2 isolate 882. The minor component contained c. 30% uronic acid residues and caused agglutination of cells of the Race 2 isolate 882 but not the Race 1 isolate R1(X). This illustrates that although seemingly widespread,the interaction of bacteria with pectic polysaccharides can show some specificity. That surface differences exist between the two races is illustrated by their differential reactions with these acidic pectic polysaccharide fractions. However, the nature of the differences remains obscure.

Both the major (80% uronic acid) and minor (30% uronic acid) polysaccharide components of the acidic pectic polysaccharide fraction obtained from bean caused agglutination of cells of the Race 2 isolate 882. Thus, in agglutination assays with the whole acidic pectic fraction from bean, a small amount of agglutination of Race 2 cells would be caused by the minor (30% uronic acid) pectic polysaccharide component. Periodate oxidation caused a slight reduction in agglutination of cells of the Race 2 isolate 882 but not the Race 1 isolate R1(X).

It is possible that the bacterial surface receptor for the minor (30% uronic acid) component of the acidic pectic fraction is carbohydrate and that its oxidation by periodate caused the observed slight reduction in agglutination.

Serra Yoldi (1976) after serological and immuno-electrophoretical

investigation of isolates of the two races of P. phaseolicola concluded that the Race 2 isolate investigated had a series of substances at its cell surface which were not present on the surface of the Race 1 isolate. It was assumed that these substances were important in the early stages of infection, affecting either the establishment of a compatible relationship or the induction of a defense reaction by the host plant. Since they report that cells of the Race 2 isolate had surface substances lacking in the Race 1 isolate it is possible that the receptor for the major (80% uronic acid) acidic pectic polysaccharide component on cells of Race 1 isolates is obscured to some degree in Race 2 isolates and thus rendered less accessible for binding. However, the acidic pectic polysaccharides may interact with other substances on the bacterial cell surface not detected by the techniques used.

The contention that acidic pectic polysaccharides in the cell walls of dicotyledonous plants serve as attachment sites for Agrobacterium tumefaciens in tumour formation has been proposed (Lippincott et al., 1977; Lippincott and Lippincott, 1977, 1980; Pueppke and Benny, 1981). Wounding, which may expose more binding sites and allow transference of the tumour inducing principle to host cells is an essential pre-requisite for tumour formation (Lippincott and Lippincott, 1969), but is not essential for attachment. Cells of A. tumefaciens adhere to the protonema of the moss Phylaisiella selwynii without wounding (Whatley and Spiess, 1977), shown in an electronmicrograph by Lippincott and Lippincott (1980).

Preinoculation of compatible hosts with A. radiobacter inhibits tumour formation by virulent isolates of A. tumefaciens by competing for attachment sites on host cell walls. Work presented in this thesis showed that cells of both species were agglutinated in vitro to similar degrees by acidic pectic polysaccharide from bean, which is a compatible host. This suggests that they have similar affinities for the cell wall attachment sites. Cells of representative species of Pseudomonas and Corynebacterium did not

inhibit tumour formation. However, cells of bacteria from both genera were agglutinated *in vitro* by acidic pectic polysaccharide from bean. Cells of *C. fascians* were only weakly agglutinated, apparently less actively than were cells of *A. radiobacter* or *A. tumefaciens*, although caution must be exercised in such comparisons for reasons discussed on page 174. This suggests that cells of *C. fascians* would not compete successfully with cells of *A. tumefaciens* for attachment sites and hence would not be efficient inhibitors of tumour formation.

Lippincott *et al.* (1978) used plant cell walls from a selection of higher plants to study inhibition of tumour formation. Cell walls were inoculated with bacteria into leaves and inhibited tumour formation if they had binding sites to which the virulent bacteria attached. Monocots in general are resistant to *A. tumefaciens* infection (Lippincott and Lippincott, 1975). The authors found cell walls from 5 families of monocots, including asparagus and onion in the Liliaceae, did not inhibit the tumour formation. This is interesting in view of the *in vitro* agglutination of cells of *A. radiobacter* by acidic pectic polysaccharide extracted from leaves of *Tulipa* sp., a member of the Liliaceae. The cells were agglutinated as actively as by acidic pectic polysaccharide from bean which is a compatible host of *A. tumefaciens*. If *Tulipa* sp. is resistant to infection the resistance may be determined by means other than non-attachment of the bacterial cells to the host cell wall. Kaupi (1975) reported that cell walls of *Tulipa* sp. had thickened over a wide area by 48h wounding and that this was due principally to an increase in pectic substances. Spherical "warts" of pectic substances appeared in the walls of many cells bordering intercellular spaces. Since pectic polysaccharide can interact with Agrobacteria these warts should provide ideal sites for attachment. There has been a report of *A. tumefaciens* infection of *Gladiolus* sp. (Baker, 1972) which is a monocot in the family Iridaceae. It would be interesting to investigate the ability of pectic

polysaccharides from Gladiolus sp. to cause agglutination of cells of A. tumefaciens.

Cell walls from monocots do not inhibit tumour formation, but after pectinesterase treatment become inhibitory (Lippincott and Lippincott, 1977). Cell walls from dicots inhibit tumour formation but become non-inhibitory after pectinmethyltransferase treatment. This suggests that the degree of methylation of the uronic acid residues is important for the attachment of A. tumefaciens to cell walls. Removal of the methyl groups esterified to uronic acid residues of the acidic pectic polysaccharide from bean slightly reduced its ability to agglutinate cells of both races of P. phaseolicola. This implies that there may be different mechanisms of adhesion of cells of A. tumefaciens to its hosts, and cells of P. phaseolicola to bean. The O side-chain of A. tumefaciens LPS has been suggested as the bacterial surface component which interacts with pectic polysaccharide (Lippincott and Lippincott, 1980). LPS has an overall negative charge, but has groups which can bear a positive charge. A straightforward ionic attraction of positively charged groups in the LPS and negatively charged uronic acid residues in the pectic polysaccharide may be the prime cause of adhesion. Adhesion of P. phaseolicola to cell walls of bean is probably due to hydrogen bond formation between complementary specific portions of a bacterial surface component and a region of the acidic pectic polysaccharide molecule, since it was shown that ionic interactions were probably not important.

Results presented in this thesis show that at least a part of the acidic pectic polysaccharide fraction from a range of higher plant species can agglutinate cells of diverse genera of Gram negative and Gram positive bacteria, including phytopathogens and non-phytopathogens. The concentration of pectic substances in the primary plant cell wall increases towards the exterior of the cell (Northcote, 1958). Thus pectic material may be suitably positioned to interact with bacteria in intercellular spaces. There currently exists what may be called the "lectin paradigm" that when a micro-organism

attaches to a plant surface, especially with some degree of specificity, then a lectin is involved in the interaction. The attachment of bacteria to surfaces is ecologically important (Leben and Whitmoyer, 1979). Many bacteria strongly prefer the surface regions of soils, teeth, plant fibres, roots, etc., where nutrients may be concentrated by adsorption, or where concentration gradients resulting from metabolism may be found (Pethica, 1980). Bacteria in streams are typically found attached to rocks and plant surfaces, and bacteria may bridge clay particles in a form of heteroflocculation in soils (Marshall, 1976). For many micro-organisms attachment is the norm rather than the exception, and many of the adhesions observed in diseased plants may be of this general type. I suggest that the adhesion of bacterial cells to plant cell walls in intercellular spaces shown in electronmicrographs depends mainly on attachment of the bacteria to pectic substances. This does not rule out a later recognition event involving specific interactions between lectins and receptor molecules, but questions the involvement of lectins in attachment. The strongest evidence for the involvement of lectins in specific recognition/ attachment is with rhizobia and their hosts. However, even here Sequeira (1978) concluded that sufficient exceptions were known to question the validity of the models which proposed lectins as primary determinants of specificity. More recently Etzler (1981) concluded that "it has not yet been established that lectins have a physiological role in protecting plants against pathogenic fungi" and "more information on the distribution and properties of lectins in plants must be obtained before their physiological role(s) can be determined". Pectic polysaccharides provide attachment sites for A. tumefaciens and in a recent review Lippincott and Lippincott (1980) stated that no evidence for lectin participation in adherence of A. tumefaciens to its host had been found. Colvin and Leppard (1973) reported fibrillar, modified polygalacturonic acid extending from plant cell walls into intercellular spaces and suggested that it might serve as a"promoter of symbiosis".

Leben and Whitmoyer (1979) showed attachment of cells of P. lachrymans, P. glycinea, P. aeruginosa, P. putida, P. fluorescens, A. tumefaciens, Corynebacterium michaganense, Erwinia stewartii, Xanthomonas phaseoli, Serratia marcescens and Esherichia coli to ruthenium red staining polymeric material associated with cucumber cell walls. Ruthenium red stains negatively charged anions and is traditionally used as a stain for pectic macterial (Rougier, 1970), which the polymeric material might have been. Anderson and Jassalavich (1979) reported agglutination of cells of saprophytic and phytopathogenic pseudomonads by commercially obtained "pectin". El-Banoby and Rudolph,(1980) used dilute HCl to make extracts from leaves of P. vulgaris cvs. Red Kidney and "02". Agglutination of cells of P. phaseolicola was caused by concentrated extracts from the resistant breeding line "02" but not the susceptible cv. Red Kidney. The authors suggested that the activity might be due to lectin in the extracts. However, in view of the work reported in this thesis it is possible that the agglutination of cells of P. phaseolicola was caused by pectic polysaccharide.

One possible objection to the hypothesis that bacteria attach to acidic pectic polysaccharides in plant cell walls adjacent to intercellular spaces would be the presence of an internal cuticle. This covering if complete would prevent bacteria from coming into contact with pectic material in the plant cell wall. The waxy cuticle covering the outer surfaces of aerial plant parts is stated to extend as a much thinner layer into the substomatal space (Martin and Juniper, 1970; Frey-Wyssling, 1976). The extent to which internal cutinisation extends beyond the substomatal cavity is debateable. Frey-Wyssling (1976) suggests that an extremely thin layer of nonlpolar material which is not visible with the light microscope extends beyond the substomatal cavities, but evidence is contradictory. Scott (1949 - 50) reports internal suberization of leaf tissue to be widespread giving rise to a thin covering visible by light microscopy. However, Sargent

(personal communication) believes that except in certain xerophylls, cutinisation of internal cell walls is not widespread. Jones and Milne (1963) give circumstantial evidence for the absence of an internal cuticle in oat.

Goodman et al. (1976a) presumed that a thin osmiophilic layer lining mesophyll cell walls in leaves of tobacco was an internal cuticle, but did not attempt to test this assumption. Cuticular material does not take up osmium well whereas pectic material does and Sargent (personal communication) believes that the layer observed by Goodman et al., which was contiguous with the middle lamella, may have been pectic in nature. Were a thin internal cuticle present in a plant, it is still possible that pectic material is exposed to the intercellular spaces, for example the fibres of modified polygalacturonic acid reported by Colvin and Leppard (1973) projected into intercellular spaces. Sargent (1976) demonstrated fibrils of pectic material penetrating the thick external cuticles of some xerophytes.

Hildebrand et al. (1980) reported physical entrapment of cells of pseudomonads in bean leaves by amorphous and for fibrillar material dissolved from plant cell walls and condensing out of solution as the intercellular water film receded by transpiration. The material was osmiophilic and in view of the work presented in this thesis I suggest that it may have been pectic polysaccharide.

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APPENDIX I

Data used in calibration curves for phenol-sulphuric and carbazole-sulphuric procedures

Concentration (mg cm^{-3})	Absorbance	
Galacturonic acid	A_{485}[a]	A_{525}[b]
0.272	0.748[c]	-
0.181	0.523	-
0.136	0.376	1.149
0.091	0.227	0.749
0.068	-	0.683
0.045	0.107	0.465
0.023	-	0.225
Galactose		
0.300	1.394	-
0.200	1.009	-
0.150	0.757	0.140
0.100	0.475	0.075
0.075	-	0.067
0.050	0.271	0.042
0.010	-	0.015

a Phenol-sulphuric procedure

b Carbazole-sulphuric procedure

c Assays were performed in duplicate and individual values which gave the best line through the points are quoted here (Williams and Wilson, 1975)

APPENDIX II

Calculated and actual concentrations of galacturonic acid and galactose in mixtures of both

A_{484} [a]	A_{525} [b]	Concentration (mg cm^{-3})			
		Galacturonic acid		Galactose	
		Calculated[c]	Actual	Calculated[c]	Actual
0.35	1.02	0.11	0.11	0.01	0.01
0.39	0.93	0.10	0.10	0.025	0.02
0.51	0.64	0.06	0.06	0.07	0.06
0.71	0.23	0.01	0.01	0.14	0.11

a phenol-sulphuric procedure

b carbazole-sulphuric procedure

c see Materials and Methods section 8 C b) iii)

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Differential agglutination of races 1 and 2 of *Pseudomonas phaseolicola* by a fraction from cotyledons of *Phaseolus vulgaris* cv. Red Mexican

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A fraction obtained from cotyledons of *Phascolus vulgaris* cv. Red Mexican agglutinated cells of avirulent Race 1 isolates of *Pseudomonas phaseolicola* more actively than it agglutinated cells of virulent Race 2 isolates; it also agglutinated human erythrocytes. Carbohydrate in the fraction agglutinated the bacteria whereas haemagglutination was caused by protein. A fraction with similar properties could not be obtained from cotyledons of cv. Canadian Wonder, susceptible to Races 1 and 2. Agglutination of bacteria was assayed by a modification of a standard microhaemagglutination assay.

INTRODUCTION

There are many recent reports on specific recognition between higher plants and micro-organisms [*1*, *3*, *4*, *5*, *6*, *13*, *14*, *15*]. In particular, it has been shown that extracts from leaves of *Phaseolus vulgaris* cv. Dark Red Kidney differentially agglutinated cells of saprophytic and phytopathogenic pseudomonads [*2*]. Another report suggests that close contact between bacteria and plant cells inside leaves is necessary for the hypersensitive response but not the susceptible reaction [*12*, *17*]. Evidence from observations of ultrastructure indicates that bacteria in intercellular spaces bind to plant cell walls in resistant but not in susceptible reactions [*10*, *16*]. In halo blight of French beans (*Phaseolus vulgaris*) caused by *Pseudomonas phaseolicola* two races of the pathogen can be distinguished on cv. Red Mexican which is resistant to Race 1 but susceptible to Race 2 isolates [*9*]. Cv. Canadian Wonder is susceptible to both races. The possibility that the different responses of cv. Red Mexican to Races 1 and 2 were linked with differential binding was investigated in the work described below.

MATERIALS AND METHODS

Pseudomonas phaseolicola

Isolates 31A, 710A (Race 1), 777A and 882 (Race 2) were kindly supplied by Dr J. D. Taylor (National Vegetable Research Station, Wellesbourne). R1 (X) (Race 1) came from the Botany Department, Imperial College. Cultures were grown on King's B medium [*8*], subcultured every 3 weeks and stored at 4 °C. The virulence and identity of isolates were determined by inoculation of *Phaseolus vulgaris* cv. Red Mexican and Canadian Wonder.

The bacteria for use in agglutination assays were grown for 24 h on King's B agar in the dark at 25 °C. Cells were washed from the surface with sterile distilled

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water, collected by centrifuging at 8000 *g* for 10 min and re-suspended in sterile distilled water to $1{\cdot}5 \times 10^{10}$ viable cells cm^{-3}, after calibration of cultures by nephelometry and counts of viability on dilution plates.

Extraction of agglutinating fraction

Seed was soaked for 24 h on moist paper at 25 °C in the dark. The testas were removed and 100 cm^3 of distilled water added for every 40 g of cotyledons which were then comminuted for 2 min in a homogenizer. The mixture was adjusted to pH 4·2 with 0·5M HCl and left for 6 to 8 h at room temperature with occasional stirring; it was then centrifuged at 16 000 *g* for 15 min and the pellet discarded. The supernatant was brought to 50% saturation with solid $(NH_4)_2SO_4$ and left overnight at 4 °C before centrifuging at 16 000 *g* for 15 min. The pellet was discarded and the supernatant brought to 100% saturation with solid $(NH_4)_2SO_4$ and left for 4 h at 4 °C. The sample was centrifuged at 16 000 *g* for 15 min and the surface film was retained. The pellet at the bottom of the tube was found to be inactive in agglutination tests and was discarded. The surface material was taken up in a small volume of 5 mM sodium phosphate buffer pH 6·8 and dialysed against 1 dm^3 of buffer overnight at 4 °C with one change of buffer. The dialysate was divided into 3 cm^3 samples and stored at −20 °C until required. Activity of samples did not decrease during 8 months storage even after thawing and re-freezing a number of times. Cotyledons taken from 7-day-old seedlings were also used.

Agglutination assay

Agglutination of bacteria was assessed by modification of a standard method used for haemagglutination by serum [*7*, *11*]. It was found to be better than the slide and test tube agglutination methods usually used with phytopathogenic bacteria because quantitative differences in agglutination for different samples, strains and isolates of bacteria could be more accurately determined. The assay uses microhaemagglutination trays each with 96 wells in 12 rows of 8. Numbering successive wells 1 to 8, 0·05 cm^3 of 5 mM sodium phosphate buffer pH 6·8 is placed in each except well 2, and 0·05 cm^3 of the test sample is added to wells 2 and 3. The contents of well 3 are mixed and 0·05 cm^3 of the mixture pipetted into well 4 and so on to well 8 from which, after mixing, 0·05 cm^3 is removed leaving a final volume in each well of 0·05 cm^3 with successive 50% dilution from wells 2 to 8. Well 1 is the control without sample. 0·05 cm^3 of bacterial suspension containing *c.* $1{\cdot}5 \times 10^{10}$ cells cm^{-3} is then placed in wells 1 to 8 and thoroughly mixed before removing 0·05 cm^3 to give a final concentration of *c.* $0{\cdot}75 \times 10^{10}$ cells cm^{-3} in each well. A lid is placed on the tray which is then incubated at 25 °C overnight. In the absence of agglutination the bacteria settle freely and are channelled by the sloping walls into a tight button in the centre of the well. When agglutination occurs, settling is impeded and the diameter of the button is greater than in the control. The size and appearance of the button is a measure of the degree of agglutination. In tests with bacteria, similar results were obtained when buffer was replaced with distilled water.

In bacterial agglutination assays, after periodate oxidation of the sample, the dilution series was prepared with 0·1 M sodium acetate buffer pH 4·7. In haemagglutination assays the test samples were brought to 0·9% (w/v) saturation with

NaCl, and 0·9% (w/v) saline was used as a diluent with a 2% (v/v) suspension of human erythrocytes (group O rhesus positive) in 0·9% (w/v) saline.

Heat treatment of fractions

One cm^3 aliquots of the sample were heated in test tubes in a boiling water bath for 1 min. This raised their temperatures to *c.* 95 °C.

Periodate oxidation

One cm^3 of the fraction from 7-day-old cotyledons was mixed with 1 cm^3 of 0·05 M periodic acid in 0·1 M sodium acetate buffer pH 4·7 and incubated for 2 h in the dark at 20 °C. Two drops of glycerol were then added and the tubes left for 30 min to reduce unreacted periodic acid. This mixture was used as a test sample in an agglutination assay. (See Table 6).

RESULTS

Table 1 and Plate 1 show the differential agglutination of cells of avirulent and virulent races of *Pseudomonas phaseolicola* by fractions from cotyledons of *Phaseolus vulgaris* cv. Red Mexican. Avirulent Race 1 isolates were agglutinated to a greater degree than virulent Race 2 isolates. This is shown by the dilution end points for agglutination (Table 2) and from the relative degree of agglutination in corresponding wells. Table 3 shows that the equivalent fraction from 1-day-old cotyledons of *Phaseolus vulgaris* cv. Canadian Wonder is less active in agglutination of the bacterial cells and does not differentiate between avirulent and virulent races. The amount of surface film from 7-day-old Canadian Wonder cotyledons was too small for collection and assay. Tables 1 and 3 show that fractions from both cultivars have haemagglutinating activity. On heating the fraction from 7-day-old Red Mexican cotyledons for 1 min in boiling water, haemagglutinating activity is largely destroyed, whereas bacterial agglutination is hardly affected (Table 4). Indeed, it is still unaffected after 30 min. Conversely, periodate oxidation of a sample eliminated its capacity to agglutinate bacteria, but only slightly reduced haemagglutination

TABLE 1

Differential agglutination of avirulent and virulent races of Pseudomonas phaseolicola *and erythrocytes by fractions from 1- and 7-day-old cotyledons of* Phaseolus vulgaris *cv. Red Mexican*

	Avirulent Race 1 isolates						Virulent Race 2 isolates					
	R1(X)		31A		710A		777A		882		Erythrocytes	
Dilution	Day 1	7	1	7	1	7	1	7	1	7	1	7
Control	0	0	0	0	0	0	0	0	0	0	0	0
2^1	4	4	4	4	4	4	4	4	4	3	4	4
2^2	4	4	4	3	4	4	1	2	2	2	4	4
2^3	4	4	2	3	3	3	0	0	0	1	4	4
2^4	0	3	0	1	0	3	0	0	0	0	3	4
2^5	0	2	0	0	0	2	0	0	0	0	3	4
2^6	0	0	0	0	0	0	0	0	0	0	3	2
2^7	0	0	0	0	0	0	0	0	0	0	3	1

TABLE 2

Dilution end-points for agglutination of avirulent and virulent cells of Pseudomonas phaseolicola *by fractions from cotyledons of* Phaseolus vulgaris *cv. Red Mexican and Canadian Wonder*

Agglutinating sample	Dilution end-point[a] Avirulent Race 1 isolates R1(X)	31A	710A	Virulent Race 2 isolates 777A	882
Fraction from 1-day-old Red Mexican cotyledons	2^{3}[b]	2^{3}	2^{3}	2^{2}	2^{2}
Fraction from 7-day-old Red Mexican cotyledons	2^{5}	2^{4}	2^{5}	2^{2}	2^{3}
Fraction from 1-day-old Canadian Wonder cotyledons	2^{1}	—	—	—	2^{1}

[a]Greatest dilution showing activity.
[b]Data from Tables 3 and 4.

TABLE 3

Agglutination of cells of avirulent and virulent races of Pseudomonas phaseolicola and *erythrocytes by a fraction from 1-day-old cotyledons of* Phaseolus vulgaris *cv. Canadian Wonder*

Dilution	Avirulent Race 1 isolate R1(X)	Virulent Race 2 isolate 882	Erythrocytes
Control	0	0	0
2^{1}	3	3	4
2^{2}	0	0	4
2^{3}	0	0	4
2^{4}	0	0	4
2^{5}	0	0	4
2^{6}	0	0	4
2^{7}	0	0	4
2^{8}			4
2^{9}			4
2^{10}			2

TABLE 4

Agglutination of cells of avirulent and virulent races of Pseudomonas phaseolicola *and erythrocytes by unheated and heated fractions from 7-day-old cotyledons of* Phaseolus vulgaris *cv. Red Mexican*

Dilution	Avirulent Race 1 isolate R1(X) U[a]	H[b]	Virulent Race 2 isolate 882 U	H	Erythrocytes U	H
Control	0	0	0	0	0	0
2^{1}	4	4	2	3	4	2
2^{2}	4	4	0	2	4	0
2^{3}	2	0	0	0	4	0
2^{4}	0	0	0	0	4	0
2^{5}	0	0	0	0	4	0
2^{6}	0	0	0	0	0	0
2^{7}	0	0	0	0	0	0

[a]Unheated sample.
[b]Heated sample.

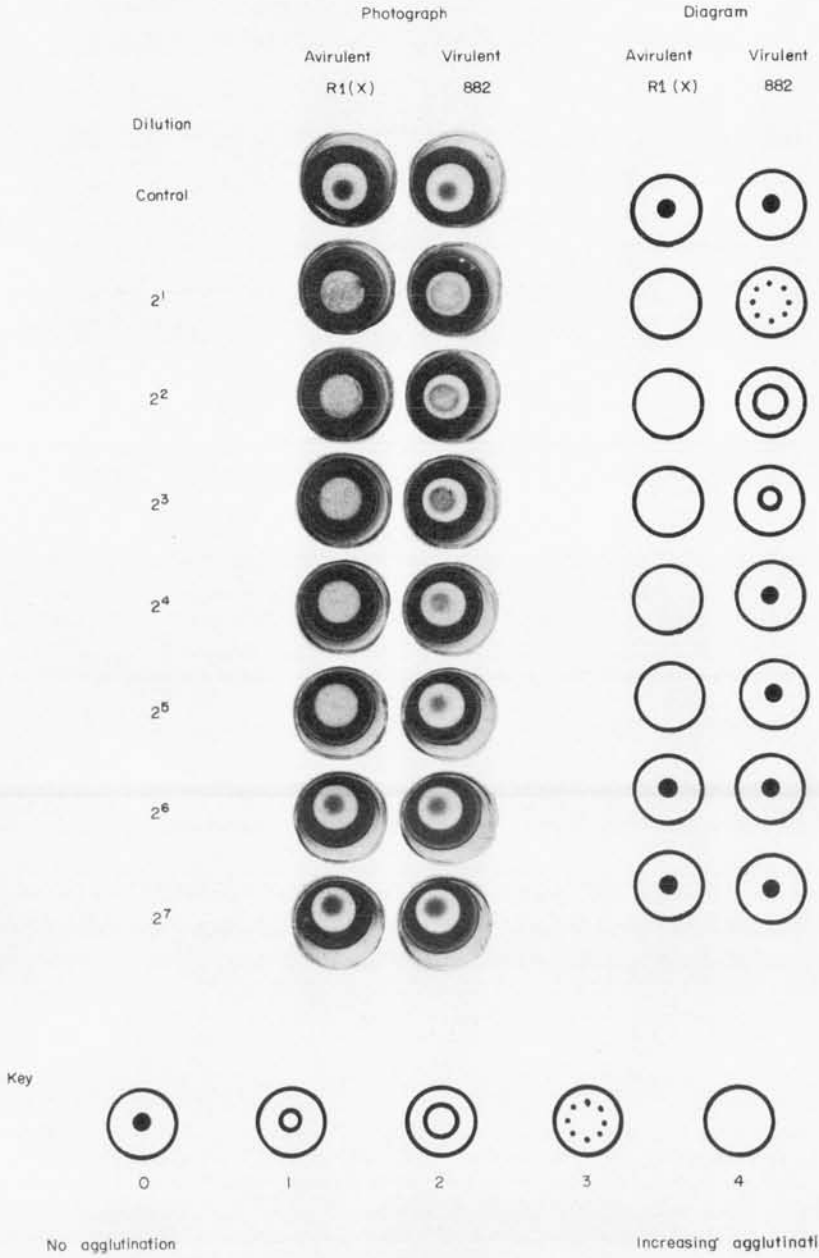


PLATE 1. Photograph and diagram showing differential agglutination of cells of avirulent and virulent races of *Pseudomonas phaseolicola* by an extract from 7-day-old cotyledons of *Phaseolus vulgaris* cv. Red Mexican.

[*facing page 190*]

TABLE 5

Effect on agglutination of cells of avirulent and virulent races of Pseudomonas phaseolicola *and erythrocytes after periodate oxidation of the fraction from 7-day-old cotyledons of* Phaseolus vulgaris *cv. Red Mexican*

	Avirulent Race 1 isolate R1(X) Treatment[a]			Virulent Race 2 isolate 882 Treatment			Erythrocyte Treatment		
Dilution	1	2	3+3′	1	2	3+3′	1	2	3+3′
Control	0	0	0	0	0	0	0	0	0
2^1	4	0	4	3	0	4	4	L[b]	L
2^2	4	0	4	3	0	2	4	L	L
2^3	4	0	3	0	0	0	4	L	L
2^4	4	0	2	0	0	0	4	L	L
2^5	4	0	0	0	0	0	4	3	3
2^6	0	0	0	0	0	0	4	0	3
2^7	0	0	0	0	0	0	2	0	2
2^8							0	0	0

[a]See Table 6.
[b]L, lysis.

TABLE 6

Treatments used in the periodate oxidation experiment

	Components in mixtures Treatment			
	1	2	3[a]	3′[a]
Cotyledon extract	1[b]	1	1	—
0·05 M periodic acid	—	1	—	1
0·1 M sodium acetate buffer, pH 4·7	1	—	—	—
Glycerol	—[c]	—[c]	—	2 drops

[a]3 and 3′ mixed after incubation.
[b]All volumes in cm^3, mixtures incubated in the dark at 20 °C for 2 h.
[c]2 drops added after 2 h incubation.

(Table 5). Table 6 shows the various treatments used in the periodate oxidation. The 3+3′ treatment was included to assess whether possible products of the reaction between periodate and glycerol affected agglutination. Periodate lyses erythrocytes, but after this effect had been removed by dilution, haemagglutination was still observed (Table 5).

DISCUSSION

Observation of differential agglutination of avirulent Race 1 and virulent Race 2 isolates of *Pseudomonas phaseolicola* was facilitated by the assay in which a dilution end point is apparent beyond which no agglutination occurs, and in which equivalent mixtures can be compared to assess the relative degree of agglutination of different isolates by a given concentration of test substances. This assay substantially decreases the subjectivity of the slide agglutination test usually used with phytopathogenic bacteria.

It was suspected that agglutination of the bacteria and of erythrocytes was caused by the same principle. However, heat and periodate treatments of extracts from 7-day-old cotyledons of *Phaseolus vulgaris* cv. Red Mexican separated the 2 properties, and suggested that haemagglutination is caused by protein in the fraction whereas bacterial agglutination is caused by carbohydrate (Tables 4 and 5).

Current views about hypersensitive resistance of plants to bacterial pathogens postulate close contact of bacteria with cell walls of resistant plants [*12*, *17*]. Most past work on resistance has been with leaves. In the current work the Red Mexican cotyledons that were used were resistant to Race 1 and susceptible to Race 2 isolates of *Pseudomonas phaseolicola*. The greater agglutination of avirulent Race 1 isolates compared with virulent Race 2 isolates by a fraction from these cotyledons may be significant in this resistance. *Phaseolus vulgaris* cv. Canadian Wonder cotyledons are susceptible to Races 1 and 2 of *Pseudomonas phaseolicola*. Compared with Red Mexican the fraction from Canadian Wonder cotyledons only slightly agglutinated isolates of *Pseudomonas phaseolicola* and did not differentiate the races (Table 3).

The resistance of Red Mexican to *Pseudomonas phaseolicola* Race 1 is monogenic and dominant [*18*]. It is possible that the gene specifies a glycosyl transferase which gives a wall polysaccharide, structurally different from the corresponding polysaccharide in susceptible cultivars, which is able to agglutinate avirulent Race 1 isolates of *Pseudomonas phaseolicola*.

Investigation of different wall polysaccharides for their abilities to differentially agglutinate avirulent and virulent races of *Pseudomonas phaseolicola* is in progress.

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