

**recent advances in phytochemistry**  
**volume 15**

# **The Phytochemistry of Cell Recognition and Cell Surface Interactions**

**Edited by**  
**Frank A. Loewus and**  
**Clarence A. Ryan**  
*Washington State University*  
*Pullman, Washington*

This volume examines the role of glycoproteins and other glycoconjugates as encountered in plants and other biological systems that interact with plants. Three general areas of this field of study are considered — an overview of the structures and properties of glycoconjugates, a detailed look at specific systems in terms of biological function, and selected examples of cell recognition and cell surface interactions as encountered in biology. Of special interest are chapters covering the structure and biosynthesis of certain glycoconjugates and the biochemical basis of adherence between bacteria and eukaryotic cells; the roles of several biologically active complex carbohydrates in plant-related host-pest relationships; extraction and properties of characteristic glycoproteins from plant tissues; the process of bacterial attachment to plant cell walls; and molecular events associated with pollen-stigma interactions and their immunochemistry.

A timely addition to the literature, *The Phytochemistry of Cell Recognition and Cell Surface Interactions* will be an important acquisition for researchers and workers in phytochemistry, biochemistry, botany, cell biology, and plant physiology.

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Cover photograph: Sprays of sweet cherry (*Prunus avium* L.) shortly after anthesis.

Photograph by Arden Literal, WSU, 1981

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Cell Recognition and  
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## **RECENT ADVANCES IN PHYTOCHEMISTRY**

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Institute of Biological Chemistry  
Washington State University  
Pullman, Washington

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Sweet cherry (Prunus avium L.) Blossoms

Here, the process of recognition and interaction between pollen and pistil is expressed as growth of pollen tubes. Self-pollinations are incompatible, as are cross pollinations between varieties within the same S-genotype. In both incompatible and compatible pollinations, the initial events are presumably identical but only pollen tubes from compatible crosses continue to grow through the style until the ovary is reached. Incompatible crosses result in arrested growth of pollen tubes within the style. See Chapter 8 by Clarke and Gleeson.

Photograph by Arden Literal, WSU, 1981

## PREFACE

The biological significance of carbohydrates in glycosylated biopolymers emerged from studies on viruses, microbial cells and animal tissues. Plant-related processes, a relative newcomer to this area of research, now offer challenging questions as regards the roles of glycosyl-conjugates and carbohydrate-binding proteins in such broadly based topics as pollination, fertilization, symbiosis (including nitrogen fixation), the chemical basis of morphogenesis, and the broad area of plant protection. While the impressive accomplishments on model systems, membrane-bound processes, receptor site biochemistry, and cell surface interactions fill numerous reports, reviews, and books, most of these involve biological systems other than plants. A real need exists for the present volume in which cell recognition and cell surface interactions as related to plants are examined.

Contributions to this volume may be sorted into three categories: first an overview of the structures and properties of glycoconjugates, then a closer look at specific systems in terms of biological function, and finally, selected examples of cell recognition and cell surface interactions as encountered in biology. To introduce the general subject, Alan Elbein reviews the structure and biosynthesis of certain glycoconjugates and examines the biochemical basis of adherence between bacteria and eucaryotic cells. Irwin Goldstein examines the properties of plant-derived lectins, in particular a group of lectins from Bandeiraea simplicifolia. The roles of several biologically-active complex carbohydrates in plant-related host-pest relationships are examined by Peter Albersheim and his colleagues. An in depth analysis of structural features found in exocellular and membrane-bound glycoconjugates of Penicillium charlesii is provided by John Gander and Cynthia Laybourne. Glycosidase activity accompanying phytohemagglutinin properties of plant-derived carbohydrate-binding proteins is described by Leland Shannon and Charles Hankins. Robert Brown and W. C. Kimmins review their work on extraction and properties of two characteristic glycoproteins from Phaseolus vulgaris, the so-called hydroxyproline-rich and hydroxyproline-poor glycoproteins.

Examples of cell recognition and cell surface interactions are drawn from five biological systems. Philip Larkin discusses plant protoplast agglutination and immobilization. Mariamne Whatley and Luis Sequeira review the process of bacterial attachment to plant cell walls, specifically the Agrobacterium tumefaciens, Pseudomonas spp., Xanthomonas and Erwinia, and Rhizobia interactions. Molecular events associated with pollen-stigma interactions, including the immunochemistry of these events, are presented by Adrienne Clarke and Paul Gleeson. Daniel Janzen explores the role of lectins in plant-herbivore interactions. In the final chapter, Daniel McMahon looks at lectins as determinants for cell surface glycoconjugates of slime mold.

The occasion of this Symposium was the first joint meeting of the Phytochemical Society of North America and the American Society of Plant Physiologists (in conjunction with the Western Section of the latter Society). It was held August 4-7, 1980 at Washington State University, Pullman, Washington. Symposium organizers included Tsune Kosuge (Univ. Calif., Davis) and Rodeny Croteau (Washington State Univ., Pullman), as well as the editors of this volume. The moderators of the Symposium were Tsune Kosuge and Leonard Beevers (Univ. Okla., Norman).

The meeting came only 10 weeks after a cataclysmic eruption of Mount St. Helens in western Washington. Volcanic ash was spread over a third of the state including Pullman. Subsequent eruptions, though less devastating, threatened the very existence of the meeting but plant scientists, hardy souls that they are, challenged Vulcan at his very doorstep. Over 1,200 attended the meeting and were rewarded with fair skies, balmy weather and a chance to listen to the eleven outstanding papers found in this volume.

The organizers and editors wish to thank all contributors for their efforts and prompt submission of manuscripts. Particular thanks goes to the National Science Foundation for a grant in support of travel for our participants and to the Graduate School, Washington State University and the Western Section, ASPP for generous funds. It was these sources that brought us to our goal.

Finally, acknowledgement must be made to Jane Bower in the Word Processing Center, College of Agriculture, Washington State University, under the direction of Doris Birch who provided camera-ready copy of excellent quality.

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## Chapter One

### THE STRUCTURE AND BIOSYNTHESIS OF LIPOPOLYSACCHARIDES AND GLYCOPROTEINS

ALAN D. ELBEIN

Department of Biochemistry  
University of Texas Health Science Center  
San Antonio, Texas 78284

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#### INTRODUCTION

The term recognition is defined by Webster as perceiving something clearly or perceiving something previously known. In that context, living cells are able to recognize various things in their environment, and this recognition must be one of the initial steps of many different cellular events.

Cell recognition can be visualized at several levels of complexity as shown schematically in Figure 1. At what might be considered the simplest level, cells recognize and interact with many types of molecules in their surroundings as depicted by the first example. These molecules, which are called chemical signals or mediators, may be as simple in structure as an amino acid or as complex as a protein. Examples of some chemical signals are hormones such as insulin, steroid hormones, auxins, gibberellins and so on. Or neurotransmitters, such as acetylcholine, or even compounds such as cholera toxin may be chemical signals.

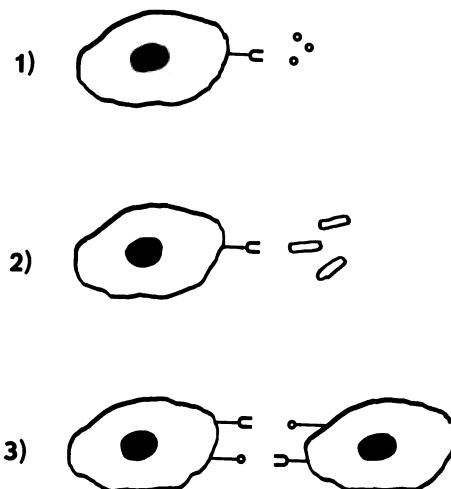


Figure 1. Model showing various types of recognition. In 1, cells recognize and interact with various small molecules of their environment such as hormones. In 2, eucaryotic (host) cells interact with bacteria or other microorganisms. In 3, eucaryotic cells adhere to each other.

These chemical signals interact with cellular structures called receptors, and this interaction must be initiated by some sort of recognition by the receptor for its specific mediator. Several of these systems have been studied in depth in animal cells and a number of receptor molecules have been isolated.

The highest level of complexity is probably the interaction between two different eucaryotic cells as is shown in example 3. Cell adhesion and cell communication result from this kind of interaction, and these are obviously important events in development, fertilization as well as other cell functions. Thus, it is clear that cells have the capacity to recognize like and unlike.<sup>1,2</sup> An excellent example of this level of recognition in plant systems is the pollen grain-stigma interaction which will be discussed later in this symposium by Dr. Clarke (See Chapter 9).

At an intermediate level of complexity, at least in terms of the cells involved, microorganisms interact with

Table 1. Some examples of plant-bacterial interactions

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I. Host-Parasite Interactions  
Pseudomonas solanacearum - potato, tobacco  
Agrobacterium tumefaciens - moss, beans

II. Host-Symbiont Interactions  
Rhizobium species - legumes

---

plant and animal cells as shown in example 2 of Figure 1. There have been numerous reports on the adherence of bacteria to eucaryotic cells, and these kinds of interactions have important implications in a variety of diseases<sup>3,4</sup> as well as in symbiotic associations. In this discussion, I would like to concentrate on the interaction between bacteria and eucaryotic cells.

Perhaps a good starting place is a quotation from Burnett:<sup>5</sup>

"All those positive recognitions between cells are readily interpreted as arising from specific union, reversible or irreversible, between chemical groupings on the surfaces of interacting cells."

As pointed out here, the interaction between cells and various ligands must involve the union between various chemical groups at the surfaces of the interacting species. So the question to be answered is what kinds of macromolecules are present on the surfaces of bacterial cells and what sorts of chemical groups do they interact with on host cell surfaces.

In plants there are several types of bacterial interactions that have been studied in some depth as indicated in Table 1. For example, there are host-parasite interactions as exemplified by Pseudomonas solanacearum, a pathogen of tobacco and potato<sup>6</sup> or by Agrobacterium tumefaciens, an organism that causes crown gall tumor in various plants.<sup>4-7</sup> Some of these systems will be discussed later in this symposium by Drs. Whatley and Sequeira. In addition, there are host-symbiotic associations as shown by the interaction

of various Rhizobium species with a number of leguminous plants.<sup>8,9,10</sup>

All the associations just mentioned involve gram-negative bacteria, and in all of these cases the bacterial cell wall lipopolysaccharide has been implicated in the adherence process. Thus, the first part of this discussion will involve the structure and biosynthesis of bacterial lipopolysaccharide. The other component of the recognition system, that is the host component, has been suggested to involve lectins, which are carbohydrate-binding proteins. Since many lectins are glycoproteins, the second part of this discussion will consider the structure and mechanism of biosynthesis of the asparagine-linked glycoproteins. And finally, in the last part of this discussion, I will briefly present some data on one system of bacterial adherence to show one example where a glycoprotein has definitely been implicated as a receptor molecule.

#### STRUCTURE AND BIOSYNTHESIS OF BACTERIAL LIPOPOLYSACCHARIDES

In terms of the bacterial lipopolysaccharides, Figure 2 shows the orientation of these molecules in the bacterial cell wall.<sup>11</sup> In gram-negative bacteria, the cell-envelope is composed of the cytoplasmic membrane, a layer of peptidoglycan and an outer membrane. The cytoplasmic membrane, like

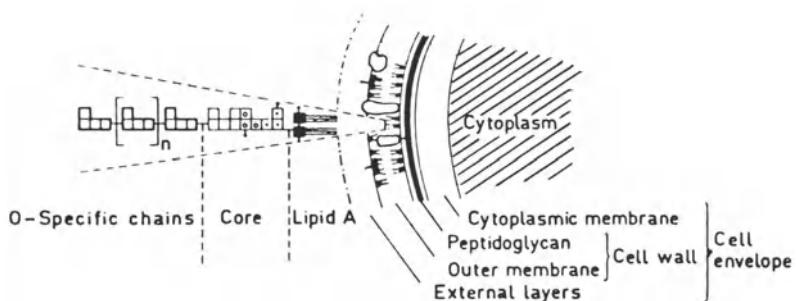
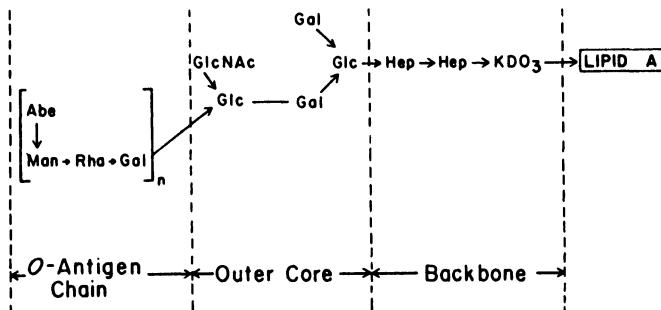


Figure 2. The organization of the gram-negative bacterial cell envelope. The location and organization of bacterial lipopolysaccharide is shown as an enlarged segment of the cell wall.

membranes from other types of cells, contains phospholipid and protein and these components are arranged as a phospholipid bilayer interspersed with integral proteins. On the outside of this cytoplasmic layer is a rigid layer of peptidoglycan. The peptidoglycan is a continuous network of carbohydrate chains that are crosslinked by peptide bridges, and this layer covers the whole surface of the cell in one giant macromolecule. This layer may be thought of as being analogous to a fishnet surrounding a balloon and is thought to give strength and rigidity to the cell.

External to the peptidoglycan is the outer membrane, which contains phospholipid, lipoprotein and lipopolysaccharide. The lipoprotein portion of this membrane is presumably covalently attached to the peptidoglycan and holds this outer membrane in place. The lipopolysaccharides are oriented at the external surface of the outer membrane in the arrangement shown in Figure 2. The lipid A portion of the molecule, which is quite hydrophobic, is oriented in the membrane and is probably associated with the phospholipids and the hydrophobic lipoprotein. On the other hand, the polysaccharide portion of the molecule composed of core polysaccharide and O-antigen side chains is at the surface and interfaces with the environment. In fact, it has been shown that the O-antigen portion is the most exposed to external agents and is the most antigenic portion of the molecule. Lipopolysaccharides have also been shown to be the receptor sites for a number of bacterial viruses. Thus, the lipopolysaccharides are likely candidates to be involved in recognition and attachment of bacteria to plant or animal cells. However, I should point out that many gram-negative bacteria, notably the Rhizobia, produce extracellular polysaccharides or capsules and these polymers could cover or mask the lipopolysaccharide. Space restrictions do not allow us to go into the many diverse structures of bacterial capsules, but they should be considered as a possible factor in bacterial adherence.

Figure 3 shows a more detailed structure of a lipopolysaccharide molecule. This particular structure is the lipopolysaccharide of Salmonella typhimurium which has been extensively studied.<sup>12</sup> Lipopolysaccharides from other bacteria probably have the same general structure, but may differ in terms of the specific sugar residues, the linkages and so on. Some of the differences will become evident as we discuss these structures in more detail.



**Figure 3.** The structure of the lipopolysaccharide of Salmonella typhimurium. Detailed structure of the backbone polysaccharide, outer core, and O-antigenic side chains is shown.

The lipopolysaccharides contain a hydrophobic core called lipid A, which is composed of glucosamine or some other amino hexose to which fatty acids are attached in ester bonds. Other sugars have also been found associated with lipid A in photosynthetic bacteria. Attached to lipid A is a backbone region of 2-keto-3-deoxyoctonic acid (KDO) and heptose (Hep). The configuration of the heptose apparently varies in different bacteria. Linked to the backbone is a pentasaccharide region called the outer core which is composed of the sugars glucose, galactose and N-acetylglucosamine (GlcNAc).

From a physiological standpoint, the most important region of the lipopolysaccharide may be the O-antigen side chain, which is involved in many reactions of these molecules. This is also the region that shows the greatest variation, both within closely related species and in diverse groups of bacteria. The O-antigen is attached to the glucose moiety of the outer core and is usually a long polymer made up of a number of repeating units. In Salmonella typhimurium, the repeating unit is the tetrasaccharide shown in Figure 3, i.e., a repeating trisaccharide of mannose-rhamnose-galactose with side branches of the deoxyhexose, abequose. In many Salmonella species, this same trisaccharide repeating unit is found, but the sugar abequose may be replaced by another sugar, or may be completely absent. Also, in some of these

Salmonellas, the linkages between the sugars in the trisaccharide and the anomeric configurations may vary.<sup>13</sup> Other organisms may have quite different O-antigenic side chains. For example, Escherichia species are usually much simpler than those shown here for Salmonella and contain fewer different kinds of sugars. The important point to stress from all of these data is that because of the enormous diversity that is possible, the O-antigens are prime candidates for a role in recognition. Unfortunately, there is not enough information available about the structures of the lipopolysaccharides of Agrobacteria or Rhizobia to tell us what their O-antigens are like, or to be able to point to differences among species. We hope such data will be forthcoming.

In terms of biosynthesis of these lipopolysaccharides, a great deal of research has been done in the laboratories of Osborn, Rothfield, Robbins, Heath and others (see several reviews listed on lipopolysaccharides). All of these studies have shown that the biosynthesis involves two rather different mechanisms; in the case of the core sugars, there is a direct transfer of sugars from their nucleotide sugar derivatives, whereas the O-antigenic side chains are synthesized via lipid intermediates. As an example of the first case, the core sugars glucose, galactose and GlcNAc are transferred from their nucleotide sugars, UDP-glucose, UDP-galactose and UDP-GlcNAc, to the lipid A backbone structure by individual glycosyl transferases which apparently reside in the cell envelope. Some of these glycosyl transferases have been highly purified by Rothfield and coworkers<sup>14,15</sup> and their properties have been studied.

Biosynthesis of the O-antigenic side chain, on the other hand, occurs in a rather interesting and unique manner that involves the participation of lipid carriers. In this mechanism, the sugars are transferred from nucleotide sugars to the lipid carrier to form a lipid-linked saccharide intermediate. The general structure of the lipid carriers in these reactions is shown in Figure 4. The upper structure shows that the carrier is a polyisoprenol made up of repeating isoprene units. In the bacterial systems, the polyisoprenol contains 50 to 55 carbons, or 10 to 11 isoprene units, all of which are unsaturated. A little later in this discussion, we will consider the biosynthesis of glycoproteins which involve similar types of lipid carriers that differ somewhat in

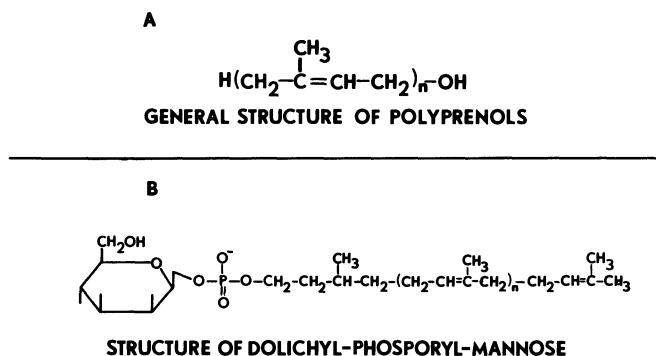


Figure 4. Structures of carrier lipids that participate in glycosylation reactions. A shows the general structures of the polyisoprenols which are composed of repeating isoprene units. The bacterial lipids are composed of unsaturated isoprene units whereas in eucaryotic cells, the dolichols have an  $\alpha$ -saturated isoprene unit. B shows the structure of one of the lipid-linked saccharides that participate in glycoprotein synthesis.

terms of chain length and number of unsaturations. The lower structure in Figure 4 shows one lipid-linked saccharide, dolichyl-phosphoryl-mannose, that is involved in glycoprotein biosynthesis.

Figure 5 shows the mechanism of biosynthesis of the O-antigen of *Salmonella*. The lipid carrier, called ACL (for antigen carrier lipid), is located in the cytoplasmic membrane of these bacteria. The individual sugars, mannose, rhamnose, and galactose, as well as any other sugars in the O-antigen, are transferred from their sugar nucleotides to the ACL to form the trisaccharide-lipid, which then becomes further elongated by the sequential addition of trisaccharide repeating units. At some undetermined stage of polymerization, this polysaccharide is transferred to the core region of the lipopolysaccharide to give a completed lipopolysaccharide molecule. Exactly how the lipopolysaccharide moves from the cytoplasmic membrane to the outer membrane is not clear at this time, nor is it clear how the degree of polymerization is determined.

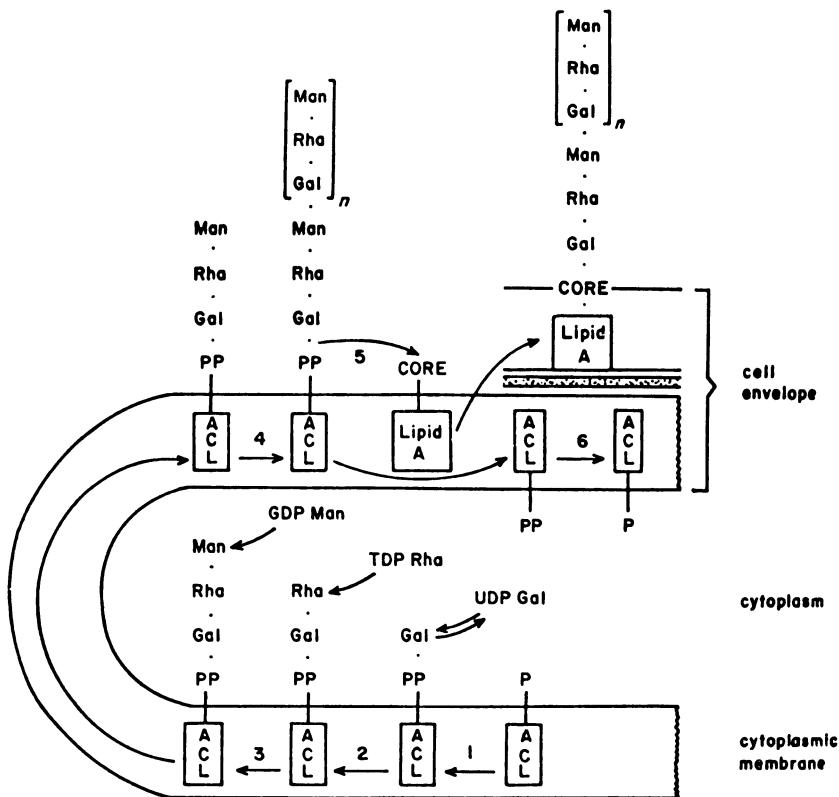


Figure 5. Mechanism of assembly of the O-antigenic side chain is shown. The antigen carrier lipid (ACL), which is located in the cytoplasmic membrane serves as the carrier for sugars which are donated from their nucleotide sugar derivatives.

#### STRUCTURE AND BIOSYNTHESIS OF ASPARAGINE-LINKED GLYCOPROTEINS

As indicated earlier, various workers have suggested that the plant components of these recognition systems are lectins. Many plant lectins are glycoproteins, and Figure 6 shows the structure of the oligosaccharide portion of soybean

lectin, one such glycoprotein that has been well studied.<sup>16</sup> This is one of the glycoproteins that are referred to as asparagine-linked, referring to the fact that the oligosaccharide is attached to the amide nitrogen of asparagine in a GlcNAc-asparagine bond. These kinds of oligosaccharides, which are called "high-mannose", because they contain large amounts of mannose, generally contain a common core structure. This core region is a pentasaccharide composed of 2 GlcNAc residues linked in a (1 $\rightarrow$ 4)  $\beta$ -bond to which are attached 3 mannose residues. The first mannose is linked to the GlcNAc in a (1 $\rightarrow$ 4)  $\beta$ -bond while the next 2 mannoses are attached in an (1 $\rightarrow$ 3) and (1 $\rightarrow$ 6)  $\alpha$ -linkage to give a branched structure.

From this point on, there may be considerable variation in the structure of these high-mannose oligosaccharides depending on the glycoprotein being considered. For soybean agglutinin, shown here, there are 7 or more additional mannose residues; all of them are in (1 $\rightarrow$ 6), (1 $\rightarrow$ 3) or (1 $\rightarrow$ 2)  $\alpha$ -linkages. This gives a highly branched structure. However, other glycoproteins may differ in terms of the number of mannoses, the extent of branching or the linkages involved. In the case of animal cells, there are additional complexities since the asparagine-linked oligosaccharides may contain other sugars such as galactose, sialic acid or fucose. However, in plants, so far as we know at this time, other sugars are not present in these oligosaccharides.<sup>17, 18</sup> I should point out that there is no evidence at this time to show that the carbohydrate portion of these molecules is involved in the receptor or recognition activity. In fact, the carbohydrate portion may be necessary in order to have the protein transported to the proper site in the cell, or for the protein to be inserted into the membrane, or simply for the protein to assume the proper configuration.

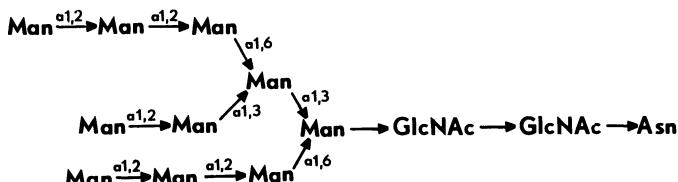


Figure 6. Structure of the oligosaccharide portion of soybean agglutinin. The oligosaccharide is linked to protein in a GlcNAc-asparagine bond.

As far as the formation of the oligosaccharide portion of these glycoproteins is concerned, this biosynthesis also involves the participation of lipid carriers in an analogous way to that previously discussed for lipopolysaccharides. As previously indicated in Figure 4, the lipid carrier is also a polyisoprenol, but in these glycoprotein systems, the polyisoprenol is usually a  $C_{100}$  to  $C_{110}$  lipid with the  $\alpha$ -isoprene unit being saturated. These saturated isoprene compounds are called dolichols, and the pathway of synthesis is referred to as the dolichol pathway. Figure 5 shows the structure of dolichyl-phosphoryl-mannose, one of the lipid-linked saccharides in this pathway.

The dolichol pathway of oligosaccharide biosynthesis is presented in Figure 7.<sup>19,20</sup> This pathway is initiated by the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P to form the first lipid-intermediate, dolichyl-pyrophosphoryl-GlcNAc. A second GlcNAc is added to form the N,N'-diacetylchitobiose-lipid, and this disaccharide is lengthened by the addition of a number of mannose residues. Some of these mannoses come directly from GDP-mannose while others come from the lipid, dolichyl-phosphoryl-mannose. This gives rise to a heptasaccharide-lipid containing 5 mannose and 2 GlcNAc residues. From this point on the further reactions of these lipids in the plant systems is not absolutely clear. In animal systems which form similar types of high-mannose oligosaccharides, there is good evidence to show that the lipid-linked oligosaccharide is further lengthened by the addition of 3 glucose and 4 mannose residues to form an oligosaccharide-lipid with the composition  $Glc_3Man_9GlcNAc_2$ . In the final step of this lipid carrier system, the oligosaccharide is transferred to protein while the protein is being synthesized on membrane-bound polysomes.

Following the transfer of oligosaccharide to the protein and the completion of the protein chain, the glycoprotein undergoes a number of processing or trimming reactions as outlined in Figure 8. Thus, within a short time after transfer of oligosaccharide, there is a rapid removal of all of the glucose residues by fairly specific membrane-bound glucosidases. The first one to two glucoses are apparently removed while the protein is still located in the endoplasmic reticulum, but the last glucose may be removed after the protein has been translocated to the Golgi apparatus. In the Golgi apparatus, a number of mannose residues may also be

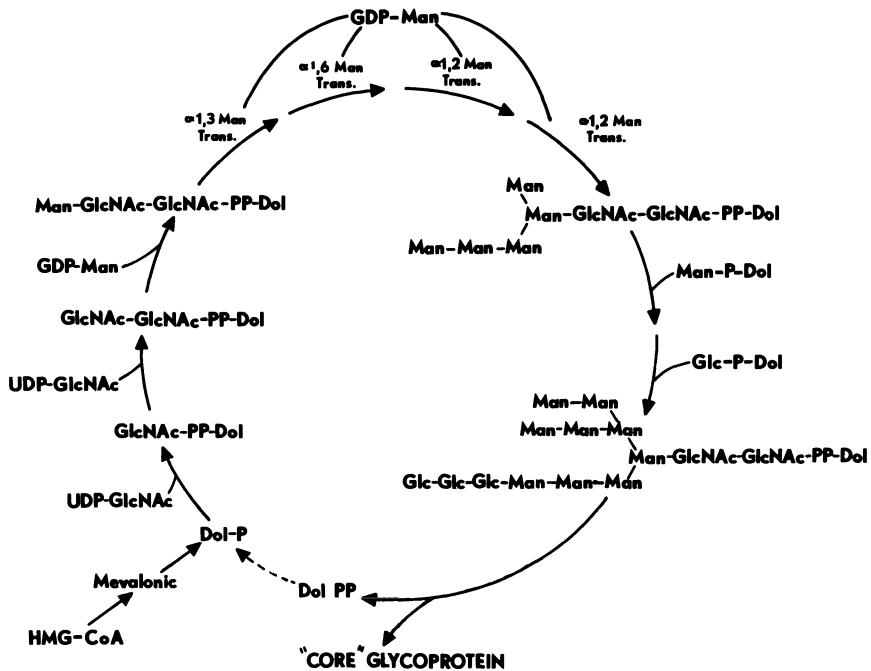


Figure 7. Pathway of biosynthesis of the oligosaccharide chains of asparagine-linked glycoproteins as demonstrated in animal cells. GlcNAc, mannose and glucose are added to the lipid carrier to give an oligosaccharide ( $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ )-lipid. The oligosaccharide is then transferred to protein.

removed depending on the protein in question. In the Golgi, a membrane-bound  $\alpha$ -mannosidase had been identified. In the case of animal systems, after trimming of a number of mannoses, the other sugars such as galactose and sialic acid may be added. But those types of reactions are not known in plant systems. So far there is nothing known about what controls the removal of mannose residues from these proteins. Nor is it clear what mechanism allows mannose residues to be removed from some glycoproteins, but not others.

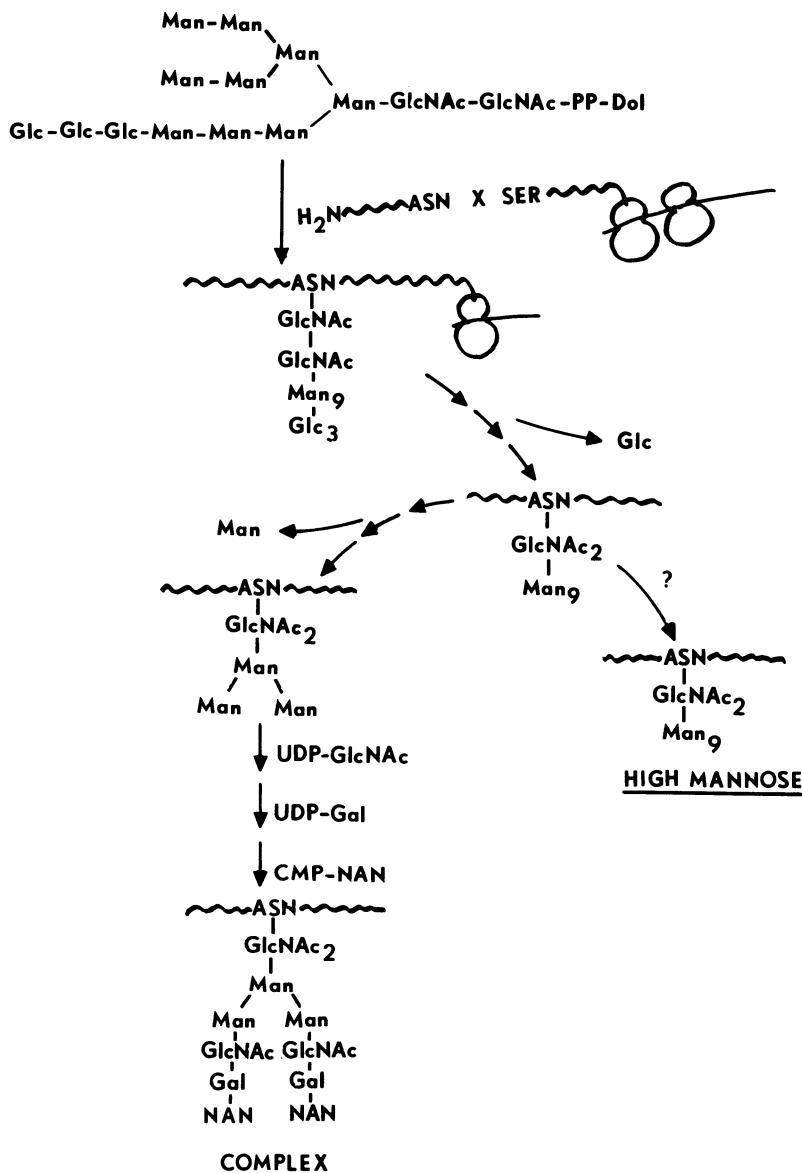


Figure 8. Reactions involved in the processing of the oligosaccharide chains of asparagine-linked glycoproteins. After transfer of oligosaccharide to protein, all of the glucoses and a number of mannose residues are removed by membrane-bound glycosidases. In animal cells, the protein may become a "high mannose" or a "complex" type of oligosaccharide.

## ROLE OF GLYCOPROTEIN IN RECOGNITION AND ADHERENCE OF BACTERIA

Now in terms of the functional role of these cell surface macromolecules, I would like to discuss one system of bacterial adhesion which we have been studying where a glycoprotein has definitely been implicated in the recognition and adherence of bacteria. Although this is not a system involving plants, the techniques used in these studies and the information obtained are certainly applicable to recognition systems in plants. This system is outlined in Figure 9. Several years ago, Dr. Barbara Sanford observed that upon infection of a canine kidney cell line with influenza virus, these infected cells contained receptors to which group B streptococci would adhere. However, these bacteria would not bind to normal uninfected kidney cells.<sup>21</sup> Influenza virus is a budding virus and the mature virus particle has two coat glycoproteins; one of these glycoproteins is a neuraminidase while the other is a hemagglutinin. When the host cell is infected with virus, the cell machinery is converted into the production of viral components. These two viral glycoproteins are synthesized and inserted into the host cell membrane. Thus when the virus particle leaves the cell, it buds off a piece of membrane taking the viral glycoproteins. So the preliminary results in this system suggested that the viral coat glycoproteins were serving as the receptors of adherence of the group B streptococci.

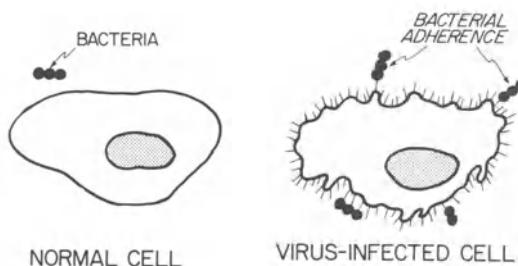


Figure 9. Schematic model showing the binding of group B streptococci to influenza virus-infected kidney cells, but not to uninfected kidney cells. In the infected cells, viral glycoproteins are inserted into kidney cell membrane.

In order to study this system in more detail, we developed a quantitative assay for examining bacterial adhesion. For this purpose we labeled the group B streptococci with a radioactive tag, either by growing them in  $^{14}\text{C}$ -fructose or by reacting them with a  $^3\text{H}$ -thiocyanate compound which forms covalent bonds with free amino groups at the cell surface.<sup>22</sup> The binding of these labeled bacteria to kidney cells was then followed as shown in Figure 10. In these experiments the kidney cells were grown in plastic dishes to confluence. In some cases the confluent monolayers were infected with influenza virus and all of the cultures were allowed to incubate for an additional 24 hours. At the end of this time, the medium was removed by aspiration and the monolayers were washed well with phosphate-buffered saline. The kidney cell monolayer was then incubated for 1 hour with various amounts of  $^3\text{H}$ -group B streptococci. After 1 hour, the unattached bacteria were removed by aspiration, the monolayers were washed 3 or 4 times with saline to remove free bacteria, and the cell monolayer was detached from the plates with trypsin. The detached cells were then placed in scintillation vials

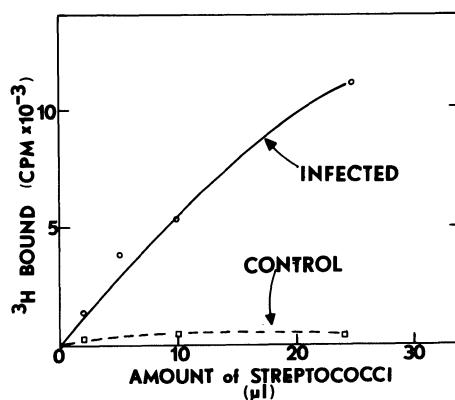


Figure 10. Radioactive assay of bacterial adhesion.  $^3\text{H}$ -labeled group B streptococci were mixed in increasing amounts with either virus-infected or uninfected (control) kidney cells. After thorough washing to remove unbound bacteria, the amount  $^3\text{H}$ -associated with the cell monolayer was measured.

for the determination of the amount of labeled bacteria bound to these cells. It can be seen from Figure 10 that the group B streptococci bound well to influenza virus-infected kidney cells, and this binding showed saturation at high levels of bacteria. However, no binding of group B streptococci to uninfected cells occurred.<sup>22</sup>

The binding to the virus-infected kidney cells was quite specific for group B streptococci as shown by inhibition studies. Thus the adherence of  $^{3}\text{H}$ -labeled group B streptococci could be inhibited if the labeled bacteria were mixed with increasing amounts of unlabeled group B streptococci. However, unlabeled streptococci from a number of other serological groups, besides group B, had very little effect on the adherence. These data indicate that the receptor on the kidney cell surface is specific for some recognition site in the group B streptococci. That this recognition site in the group B streptococci resides in the cell wall is shown by the experiment presented in Figure 11. As shown here, when cell walls prepared from group B streptococci are mixed with the kidney cell monolayers, they block the attachment of  $^{3}\text{H}$ -group B streptococci. Our interpretation of these results is that the cell walls bind to the kidney cell receptors and tie them

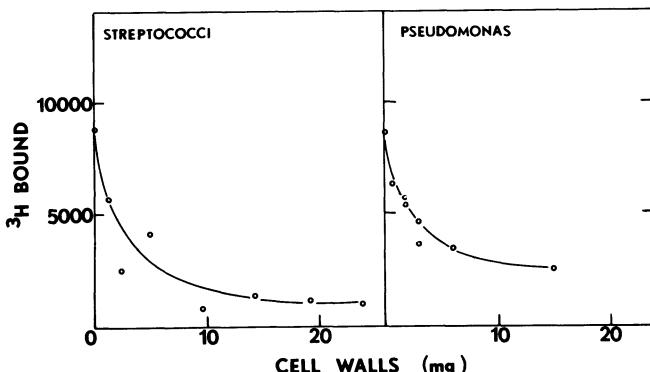


Figure 11. Inhibition of binding of  $[^{3}\text{H}]$  group B streptococci to virus-infected kidney cells by increasing amounts of group B streptococcal cell walls. Various amounts of cell walls were mixed with kidney cell monolayers and after an incubation of 30 min, the monolayers were challenged with  $^{3}\text{H}$ -bacteria.

up so that they are not available for binding the intact bacteria. Interestingly enough, as shown on the right hand side of this figure, *Pseudomonas* cell walls also block the adherence of group B streptococci to these cells. In other experiments it was found that *Pseudomonas aeruginosa* also binds to the kidney cells, but in this case the *Pseudomonas* adhere equally well to the infected and normal cells. Apparently the receptor for these bacteria is not the viral glycoproteins, but is another receptor normally present in these cells.

Evidence that the receptor for group B streptococci is indeed the viral glycoproteins is shown in Figure 12. In this experiment, various amounts of group B streptococci were mixed with free influenza virus and this mixture was tested for its ability to bind as compared to group B streptococci not mixed with virus. It can be seen from the figure that in the presence of virus, fewer group B streptococci bound to the kidney cells. We assume that the reason for this observation is that the group B streptococci bind to influenza virus and therefore are not available to bind to kidney cells. These studies also implicate the viral coat glycoproteins as the streptococcal receptor.

The influenza virus coat glycoproteins have been partially characterized in terms of their carbohydrate composition and they are asparagine-linked oligosaccharides of both the "high mannose" and the "complex type" as shown in Figure 13. Apparently both of these types of oligosaccharides are found in the viral hemagglutinin and the neuraminidase.<sup>23</sup> As indicated earlier in this discussion, these asparagine-linked oligosaccharides are synthesized by means of the lipid-linked saccharide pathway discussed earlier. There is a very useful antibiotic called tunicamycin which has been shown to block the formation of some of the lipid-linked saccharides. More specifically, tunicamycin blocks the reaction shown in Figure 14, that is, it prevents the formation of dolichyl-pyrophosphoryl-GlcNAc.<sup>24,26</sup> Since this lipid is the first lipid intermediate in the synthetic pathway for glycoprotein synthesis if the formation of this compound is blocked, then the lipid-linked oligosaccharides cannot be formed and the protein cannot be glycosylated. The tunicamycin is very useful for studying the role of the carbohydrate in the function of various glycoproteins. Since the influenza viral glycoproteins involve the lipid-linked

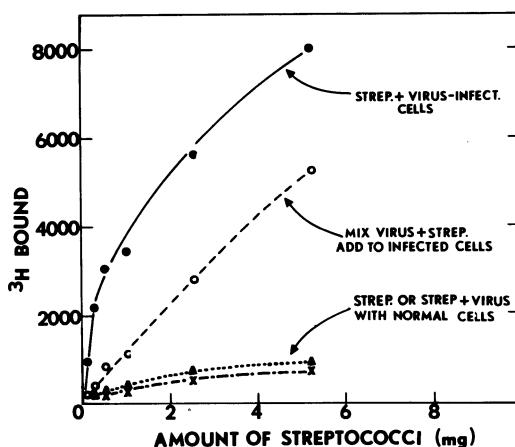


Figure 12. Effect of mixing free virus with group B streptococci. Increasing amounts of [<sup>3</sup>H] streptococci were mixed with free virus and this mixture was compared to group B streptococci alone for its ability to bind to virus-infected kidney cells.

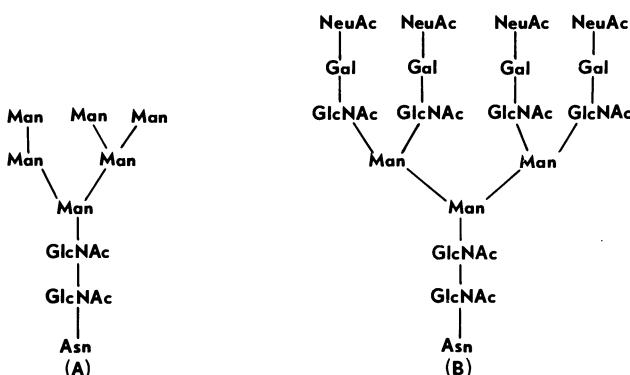


Figure 13. The "high mannose" (A) and "complex" (B) types of oligosaccharides found in the influenza virus glycoproteins. Although the detailed structures of the viral glycoproteins are not known, they have a general structure like those shown here.

saccharide pathway,<sup>27</sup> it was of interest to determine the effect of tunicamycin on the synthesis of these molecules and on bacterial adhesion.

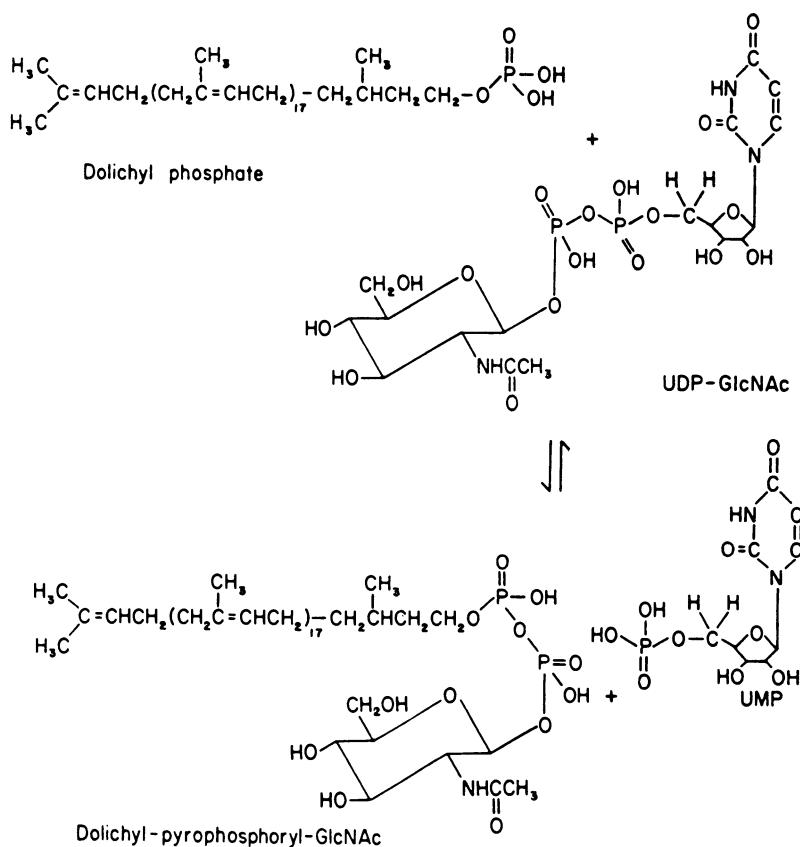


Figure 14. Tunicamycin sensitive reaction in the lipid-linked saccharide pathway. The transfer of GlcNAc-1-P from UDP-GlcNAc to form dolichyl-pyrophosphoryl-GlcNAc is the first step in the dolichol pathway.

Figure 15 shows the effect of tunicamycin on the synthesis of protein and on protein glycosylation in the virus-infected kidney cells. In this experiment, kidney cell monolayers were infected with influenza virus and after one hour, 0.9  $\mu\text{g}/\text{ml}$  of tunicamycin was added. The cells were incubated with antibiotic for another hour in order to allow the antibiotic to take effect and then various radioactive precursors were added. [ $^3\text{H}$ ]Mannose was added to these cells as a measure of the incorporation of mannose into protein (i.e., protein glycosylation), and [ $^3\text{H}$ ]leucine to measure leucine incorporation into protein (i.e., protein synthesis). After incubation of these cells with the labeled precursors for 3 hours, the cells were harvested and the incorporation of isotope into protein was measured. It can be seen from the curves on the left that mannose was rapidly incorporated into protein in control cultures (i.e., minus antibiotic), but tunicamycin almost completely inhibited protein glycosylation at about 1  $\mu\text{g}/\text{ml}$ . However, as shown by the curves on the right, this antibiotic had relatively little effect on the incorporation of leucine into protein.

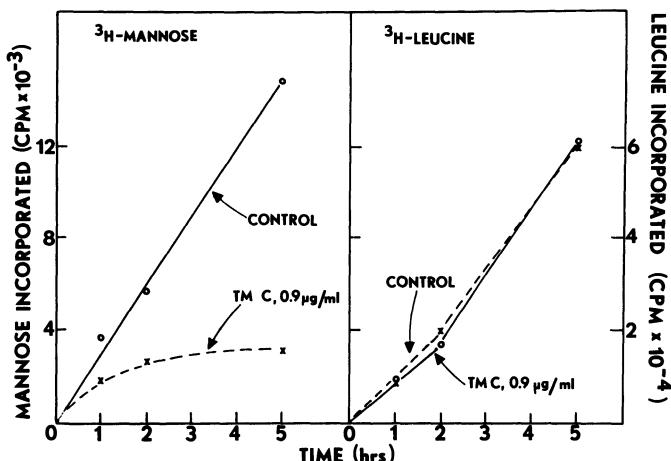


Figure 15. Incorporation of [ $^3\text{H}$ ]mannose and [ $^3\text{H}$ ]leucine into protein in MDCK cells. Virus-infected cells were incubated for 1 hour with tunicamycin and then labeled precursors were added for 3 hours. Control cells did not have tunicamycin.

Since this antibiotic prevented the glycosylation of proteins, it was of interest to examine its effect on the adherence of streptococci in these virus infected kidney cell monolayers. The results of such an experiment are shown in Table 2. In this experiment, kidney cells were

Table 2. Effect of tunicamycin on influenza virus:  
Infection and binding of group B streptococci

| Tunicamycin<br>( $\mu\text{g}/\text{ml}$ ) | Hemadsorption<br>plaque assay <sup>a</sup><br>(% of control) | Hemag-<br>glutinin<br>titer <sup>b</sup><br>(super-<br>natant) | Binding<br>of group<br>B streptococci <sup>c</sup><br>(% of control) |
|--|--|--|--|
| 0  | 100  | 1:128  | 100  |
| 0.078                                      | 100  | 1:16   | 71   |
| 0.156                                      | 50   | 1:11 <sup>d</sup>  | 6  |
| 0.32                                       | 7.7  | --   | 2  |
| 0.625                                      | 0  | --   | 0  |
| 1.25                                       | 0  | --   | 0  |
| 2.5  | 0  | --   | 0  |
| 5.0  | 0  | --   | 0  |
| 10.0                                       | 0  | --   | 0  |

<sup>a</sup>Hemadsorption by kidney cells. The control (100%) is equal to the mean number of hemadsorption plaques on virus-infected monolayers which have not been pretreated with tunicamycin.

<sup>b</sup>Hemagglutinin released from cells as measured by agglutination of erythrocytes.

<sup>c</sup>Binding of streptococci as measured by microscopic observation. The control (100%) is equal to the mean number of bacterial adherent plaques on virus-infected monolayers which have not been pretreated with tunicamycin. This assay correlated well with the  $^3\text{H}$ -binding assay done in other experiments.

<sup>d</sup>--, Undiluted supernatant was hemagglutination negative.

infected with virus and, after one hour, various concentrations of antibiotic were added. The cells were allowed to incubate for 24 hours in order to allow the virus to replicate. At the end of this time, the monolayers were tested for the presence of virus particles and for their ability to bind group B streptococci. The hemadsorption assay is a measure of the presence of influenza-viral glycoproteins in the kidney cell membrane while hemagglutination is a measure of the release of mature virus particles into the medium. The table shows that at about 0.3 µg/ml of tunicamycin, the viral glycoproteins are no longer found in the kidney cell surface, and there is no detectable virus in the medium. At this level of antibiotic, the kidney cells also lack the receptor to which the group B streptococci adhere. Thus, these experiments in conjunction with those already discussed indicate that the viral glycoproteins that have been synthesized in these infected cells and inserted into the membrane are able to serve as receptor sites for the attachment of certain bacteria. It is not known at this time whether the carbohydrate portion of these molecules is involved in bacterial adhesion. That is, the carbohydrate portion of the glycoproteins may be necessary in order for the protein to be inserted into the membrane and, in the absence of glycosylation, the viral proteins may remain in the cytoplasm of the cells.

#### SUMMARY

Cell surface macromolecules from bacteria and from the eucaryotic host cells undoubtedly interact in specific ways to allow binding of these cells to each other. In some plant-microbe interactions, bacterial lipopolysaccharides and plant lectins have been implicated in these processes. However, bacterial cells have many different macromolecules at their surface including various extracellular polysaccharides. These molecules could also be involved in recognition and adherence. In order to demonstrate which components are involved, they must be isolated from the respective organism, purified to homogeneity, and tested both in vivo and in vitro for their effect on adhesion.

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## Chapter Two

### PLANT DERIVED LECTINS

IRWIN J. GOLDSTEIN

Department of Biological Chemistry  
University of Michigan  
Ann Arbor, Michigan 48109

The term lectin [Latin, legere - to pick out, choose] was coined by W. C. Boyd<sup>1</sup> in order to call attention to a group of plant seed proteins which could distinguish among human blood groups. Boyd had discovered that extracts of the lima bean (Phaseolus lunatus) specifically agglutinated type A erythrocytes (Fig. 1).

| Reaction of extract with cells of group: |      |      |       |
|--|------|------|-------|
|  | A    | B    | O     |
| EW                                       | ++++ | LH ± | BD 0  |
| MF                                       | ++++ |      | BR 0  |
| DA                                       | ++++ |      | SJ 0  |
| JB                                       | ++++ |      | ON 0  |
| AL                                       | ++++ |      | BA 0  |
| WCB                                      | ++++ |      | CTS 0 |

Figure 1. Test of lima bean extract (December 10, 1945)<sup>2</sup>

Table 1. Examples of enzymes that act like lectins

- 
1. Crystalline  $\alpha$ -amylase forms an insoluble complex with glycogen at 4°C.<sup>4</sup>
  2. Muscle phosphorylase a and b precipitate glycogen at 0°C.<sup>5</sup>
  3. Galactose oxidase agglutinates sialidase-treated human erythrocytes at 0°C. At higher temperatures, the agglutinate disperses.<sup>6</sup>
  4. Mung bean  $\alpha$ -galactosidase agglutinates trypsinized rabbit erythrocytes at pH 8.5 and 0 - 5°C (pH optimum of enzymatic activity is 6.5; at pH 8.5 activity is only 10%). At 6.5 and higher temperatures the clot rapidly dissolves.<sup>7</sup>
  5. Lysozyme cross-linked by treatment with glutaraldehyde agglutinates human erythrocytes.<sup>8</sup>
- 

Lectins are naturally occurring, carbohydrate binding (glyco) proteins of non-immune origin which agglutinate cells and/or precipitate complex carbohydrates (polysaccharides, glycoproteins, glycolipids).<sup>3</sup> Although no known lectin has been shown to exhibit enzymatic activity, under certain defined conditions some enzymes may act like lectins, i.e. display lectin-like properties. Examples of some enzymes which act like lectins are listed in Table 1.

A major concern of many plant physiologists and biochemists is the biological role which lectins may serve. Although many hypotheses have been advanced, none has been proved. Possible functions of plant seed lectins are tabulated in Table 2.

Although the function of lectins has not yet been clarified, these fascinating substances display a host of remarkable biological properties (Table 3) which find wide application in many biomedical investigations (Table 4).

Table 2. Possible functions of plant lectins

---

|   |
|---|
| Sugar transport, storage and immobilization                               |
| Carbohydrases or procarbohydrases   |
| Plant antibodies  |
| Binding nitrogen-fixing bacteria to legumes                               |
| Involvement in germination, differentiation, maturation and cell division |
| Protection against insect and fungal predators                            |
| Involvement in cell wall metabolism                                       |

---

Table 3. Biological properties of lectins

---

|   |
|---|
| Agglutination of cells and particles: erythrocytes, lymphocytes, tumor cells, microorganisms, viruses, vesicles |
| Mitogenic stimulation of lymphocytes  |
| Inhibition of fungal growth   |
| Inhibition of activity of glycoprotein enzymes  |
| Degranulation of mast cells   |
| Insulin-like activity on fat cells  |
| Cytotoxic activity toward mammalian cells   |
| Modulation of the immune response   |

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Plant seed lectins may be classified into a limited number of carbohydrate binding specificity groups. Six carbohydrate binding specificity groups together with examples and some biological properties of each group are tabulated in Table 5.

Table 4. Some uses of lectins

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|  |
|--|
| Blood typing, the detection of "secretors" and polyagglutination   |
| Detection, preliminary characterization and structural studies on complex carbohydrates                          |
| Isolation and purification of carbohydrate-containing molecules  |
| Fractionation of cells and particles (e.g., viruses, cellular organelles, vesicles)                              |
| Models for carbohydrate-protein and antibody-antigen interactions  |
| Identification of carbohydrate-containing, cell surface markers (antigens)                                       |
| Mitogenic stimulation of lymphocytes in clinical and immunological studies                                       |
| Generation of lectin-resistant variants of eukaryotic cells for studies of glycoprotein structure and metabolism |

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Closer examination of these lectin classes allows us to discern some common structural and phylogenetic features (Table 6). For example, all plant lectins are multimeric (glyco) proteins, i.e. they consist of 2 or more polypeptide chains. With the notable exception of concanavalin A, all seed lectins from the family Leguminosae are glycoproteins and most are metalloproteins which lose all biological activity when deprived of their metal ions. Furthermore, most leguminous lectins are deficient in sulfur-containing amino acids. Concanavalin A contains methionine but no cysteine residues, the lima bean lectin (*Phaseolus lunatus*) contains cysteine but no methionine residues and the lectin from the red kidney bean (*Phaseolus vulgaris*) contains neither cysteine nor methionine residues.

A structural basis for some lectin classes which display the same sugar binding specificity is suggested by recent

Table 5. Classification of lectins into a limited number of carbohydrate-binding specificity groups.

1. D-Mannose (D-Glucose)-binding lectins

jack bean, pea, lentil, broad bean lectins  
(blood group non-specific; mitogenic)

2. N-Acetyl-D-glucosamine-binding lectins

B. simplicifolia II, jimson weed, potato, wheat germ  
lectins  
(bind to chitin; very weak or non-mitogenic;  
blood group non-specific)

3. N-Acetyl-D-galactosamine-binding lectins

Dolichos biflorus, soy bean, lima bean, edible snail  
lectins  
(blood group A - specific; lima bean and  
polymerized soy bean lectins are mitogenic).

4. D-Galactose-binding lectins

A. precatorius agglutinin, peanut, B. simplicifolia I,  
castor bean agglutinin  
(BS I - B<sub>4</sub> is blood group B-specific; A. precatorius is mitogenic).

5. L-Fucose-binding lectins

Eel, L. tetragonolobus, Ulex europeus I lectins  
(blood group O-specific; weak or non-mitogenic)

6. Complex carbohydrate-binding lectins

Red kidney bean (PHA) - a potent mitogen  
Meadow mushroom - a potent mitogen  
Horse-shoe crab agglutinin - binds sialic acid

reports on the structure and amino acid sequence of several of these carbohydrate-binding (glyco) proteins. For example, there are only two differences in the amino acid sequence of the first 25 amino acids in the  $\beta$ -chain of the D-mannose-binding pea and lentil lectins, and the N-terminal sequence of amino acids of the  $\alpha$ -chain of these agglutinins is nearly identical.<sup>9,10</sup> The soybean and peanut agglutinins are identical in 11 of the first 25 N-terminal amino acids and the N-terminal sequence of amino acids of the soybean agglutinin is similarly identical at 11 positions with that of the  $\beta$ -chain of the lentil lectin. Many of the amino acid differences observed in these cases could have resulted from a single nucleotide substitution.

Even more remarkable, the primary structure of the entire  $\alpha$  subunit from the lentil lectin was found to be homologous with the region between positions 72 and 121 from con A and the amino-terminal sequence of the  $\beta$ -chain is homologous to another portion of the con A molecule between position 123 and 165.<sup>11</sup> Similarly, Cunningham and his colleagues<sup>12</sup> found that the  $\alpha$ -chain of the lectin from the broad bean (Vicia faba) is homologous to a region in the middle of the con A sequence (residues 70-119), and that the  $\beta$ -chain is homologous to two discrete segments of con A comprising a circular permutation of amino acid sequences.

A further striking structural feature observed for several lectins has been the almost identical sequence of N-terminal amino acids in the two distinctive subunits present in a single lectin or isolectin thereof. Thus, the first 10 N-terminal amino acids of the two soybean lectin subunits<sup>13</sup> and the first 30 amino terminal amino acids of the two subunits of the Dolichos biflorus lectin<sup>14</sup>, are identical, and there are considerable homologies evident between the sequences of N-terminal amino acids of the two subunits of the lectin (PHA) from Phaseolus vulgaris seeds.<sup>15</sup> The discovery of extensive homologies between different lectins strongly suggests a common genetic origin. Phylogenetic trees have been constructed which reveal these relationships and support the notion that lectins may have an important physiological role in plants.

As an example of how lectins are isolated, purified and characterized and used in biological studies, I would like to briefly discuss the lectins present in Bandeiraea

Table 6. Lectins: Some physical-chemical properties.

|   | Subunits<br>No./M <sub>r</sub> | Glyco-<br>protein | Metalo-<br>protein |
|---|--------------------------------|-------------------|--------------------|
| 1. <u>D</u> -Mannose ( <u>D</u> -Glucose)-binding lectins |                                |                   |                    |
| Jack bean ( <u>Canavalia ensiformis</u> )                 | 4/26,000                       | -                 | +                  |
| Lentil ( <u>Lens culinaris</u> )                          | 2a, 2B/5,900, 17,000           | +                 | +                  |
| Pea ( <u>Pisum sativum</u> )                              | 2a, 2B/5,900, 17,000           | +                 | +                  |
| Broad bean ( <u>Vicia faba</u> )                          | 2a, 2B/5,000, 20,000           | +                 | +                  |
| 2. N-Acetyl- <u>D</u> -glucosamine-binding lectins        |                                |                   |                    |
| Bandeiraea <u>simplicifolia</u> II                        | 4/30,000                       | +                 | +                  |
| Jimson weed ( <u>Datura stramonium</u> )                  | 2, 4/40,000                    | +                 | -                  |
| Potato ( <u>Solanum tuberosum</u> )                       | 2/50,000                       | +                 | -                  |
| Wheat germ ( <u>Triticum vulgaris</u> )                   | 2/18,000                       | -                 | -                  |
| Gorse seed ( <u>Ulex europeus</u> II)                     | 4/30,000                       | +                 | +                  |
| Poke weed mitogen ( <u>Phytolacca americana</u> )         | 1-5/19,000-31,000              | +                 | ?                  |
| 3. N-Acetyl- <u>D</u> -galactosamine-binding lectins      |                                |                   |                    |
| Horse gram ( <u>Dolichos biflorus</u> )                   | 4/26,000                       | +                 | +                  |
| Soy bean ( <u>Glycine max</u> )                           | 4/30,000                       | +                 | +                  |
| Edible snail ( <u>Helix pomatia</u> )                     | 6/13,000                       | +                 | -                  |
| Lima bean ( <u>Phaseolus lunatus</u> )                    | 4, 8/31,000                    | +                 | +                  |
| 4. <u>D</u> -Galactose-binding lectins                    |                                |                   |                    |
| Jequitity bean ( <u>Abrus precatorius</u> )               | 4/33,000                       | +                 | +                  |
| Peanut ( <u>Arachis hypogaea</u> )                        | 4/27,500                       | -                 | ?                  |
| Bandeiraea <u>simplicifolia</u> I                         | 4/28,500                       | +                 | +                  |
| Castor bean ( <u>Ricinus communis</u> )                   | 4/31,000, 33,000               | +                 | -                  |
| 5. <u>L</u> -Fucose-binding lectins                       |                                |                   |                    |
| Eel ( <u>Anguilla anguilla</u> )                          | 3/40,000                       | <0.4%             | -                  |
| Asparagus pea ( <u>Lotus tetragonolobus</u> )             | 2, 4/29,000                    | +                 | ?                  |
| Gorse seed ( <u>Ulex europeus</u> I)                      | 2/28,000                       | +                 | -                  |
| 6. Complex carbohydrate-binding lectins                   |                                |                   |                    |
| Red kidney bean ( <u>Phaseolus vulgaris</u> )             | 4/31,000                       | +                 | +                  |
| Vicia graminea  | 4/25,000                       | +                 | ?                  |
| Meadow mushroom ( <u>Agaricus campestris</u> )            | 4/16,000                       | +                 | ?                  |
| Horse-shoe crab ( <u>Limulus polyphemus</u> )             | 18-20/20,000                   | +                 | +                  |

simplicifolia (more properly termed Griffonia simplicifolia) seeds. The B. simplicifolia plant is a shrub which grows in the tropical rain forests of West Africa, particularly in Ghana. It reaches a height of 3 to 4 meters.

Thus far we have isolated 4 distinctly different lectins from B. simplicifolia seeds (Table 7). These lectins differ in their physical-chemical properties, carbohydrate binding specificity, and immunochemical cross-reactivity. The fourth lectin, BS IV, has recently been isolated and shown to be active against  $Le^b$  blood group substance which contains L-fucose as its immunodominant sugar.<sup>16</sup>

Bandeiraea simplicifolia I plant seed isolectins comprise a family of tetrameric  $\alpha$ -D-galactopyranosyl-binding glycoproteins composed of various combinations of two different kinds of subunits designated A and B.<sup>17 18</sup> The A subunits exhibit a primary specificity for N-acetyl- $\alpha$ -D-galactosaminyl groups but also cross-react with  $\alpha$ -D-galactosyl groups whereas the B subunit shows a sharp specificity for  $\alpha$ -D-galactosyl units. Subtypes of the A and B subunits have been demonstrated by isoelectric focusing.

The five isolectins have been purified by use of two affinity columns. All five BS I isolectins bind to a melibionate-Bio Gel column. The  $A_4$  and  $A_3B$  isolectins were displaced as purified components by increasing quantities of N-acetyl-D-galactosamine. The  $B_4$ ,  $B_3A$  and  $B_2A_2$  components,

Table 7. Bandeiraea simplicifolia seeds contain 4 lectins

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BS I - A system of 5  $\alpha$ -D-galactosyl-binding isolectins

BS II - An N-acetyl-D-glucosamine-binding lectin

BS III - A system of 5 N-acetyl- $\alpha$ -D-galactosaminyl-binding isolectins

BS IV - A lectin with  $Le^b$ -specificity

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after elution from the melibionate column with methyl  $\alpha$ -D-galactopyranoside, were resolved on a column of insolubilized blood group A substance according to the procedure of Murphy and Goldstein.<sup>17</sup> The latter affinity column has N-acetyl- $\alpha$ -D-galactosaminyl determinant groups which interact with the subunit A binding sites.

Physical-chemical characterizaton of the A<sub>4</sub> and B<sub>4</sub> isolectins revealed the A and B subunits to be homologous structures with closely similar amino acid compositions although they differ markedly in one respect: the B subunit has one methionine residue whereas the A subunit contains no methionine. Ouchterlony analysis indicated that in addition to common structural features, each subunit contains its own distinct antigenic determinants. The B subunit has a molecular weight ( $M_r$  33,000) approximately 1000 daltons greater than the molecular weight of the A subunit ( $M_r$  32,000). These data combined with our observation that the isolectins present in individual seeds are never present in equivalent amounts but rather exhibit a skewed distribution have led us to postulate a precursor-product relationship between the B and A subunit.<sup>19</sup>

The BS I-B<sub>4</sub> isolectin has been labeled with fluorescein, ferritin, colloidal gold and tritium and used as a probe for the detection of  $\alpha$ -D-galactosyl groups in plant and animal cells. The immobilized form of the lectin has also been used for a one step purification of the galactomannan from Cassia alata seeds.<sup>20</sup> Fluorescein-labeled BS I-B<sub>4</sub> (FITC-B<sub>4</sub>) was shown to bind to and agglutinate Ehrlich ascites tumor cells, an observation confirmed by the detection on the electron microscopy of ferritin-labeled B<sub>4</sub> bound to Ehrlich ascites cell membranes.<sup>21</sup> Using immobilized BS I-B<sub>4</sub> as an affinity matrix we have been able to isolate and characterize a family of  $\alpha$ -D-galactosyl containing glycoproteins from an Ehrlich cell membrane fraction. FITC-B<sub>4</sub> was also shown to bind to murine kidney sections. Specific inhibition by methyl  $\alpha$ -D-galactoside and abolition of binding by  $\alpha$ -galactosidase in addition to morphological evidence indicates the lectin binds to  $\alpha$ -D-galactosyl groups of basement membrane.<sup>22</sup>

BS I-B<sub>4</sub> has also been shown to be cytotoxic to a variety of animal cells and used to generate a variant clone of B<sub>4</sub>-resistant 3T3 murine cells.<sup>23</sup>

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## Chapter Three

### STRUCTURE AND FUNCTION OF COMPLEX CARBOHYDRATES ACTIVE IN REGULATING THE INTERACTIONS OF PLANTS AND THEIR PESTS

PETER ALBERSHEIM, MICHAEL McNEIL, ALAN G. DARVILL,  
BARBARA S. VALENT, MICHAEL G. HAHN, BORRE K.  
ROBERTSEN, AND PER ÅMAN

Department of Chemistry  
University of Colorado  
Boulder, Colorado 80309

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### INTRODUCTION

Our laboratory has recently come to realize that complex carbohydrates of higher plants, fungi, and bacteria can act as regulatory molecules, that is, as molecules which in

minute quantities alter the metabolism of receptive cells by causing the synthesis of specific proteins. It is not surprising that these structurally complex and exquisitely specific molecules can possess regulatory properties, as many diverse classes of molecules including glycoproteins, proteins, peptides, steroids and a variety of smaller molecules such as epinephrine, indoleacetic acid, gibberellic acid, cytokinins, and even ethylene, are known to possess regulatory properties.

The carbohydrate portions of glycoconjugates are critically involved in recognition phenomena in biology (see, for example, references<sup>1-3</sup>). One of the earliest known functions of such carbohydrates in recognition processes is defining the blood group substances of mammals. The carbohydrate portions of glycoconjugates act on the cell surface of bacteria as serological determinants and as receptors for phage and bacteriocins. More recently, the carbohydrate portions of glycoconjugates have been recognized as cell surface specific antigens of at least some fungi, and the receptors of hormones and toxins in eucaryotic cells. The carbohydrate portions of glycoconjugates participate critically in determining the movement of glycoproteins within and between cells by acting as signals for directed transport of these molecules. Cell surface glycoconjugates are also important in differentiation of invertebrates, and they are the receptors for mitogenic lectins. Thus, the complex carbohydrates of glycoconjugates participate in a wide range of critical recognition phenomena. Evidence has even been obtained that the carbohydrate portion of a low molecular weight glycoconjugate has the ability to induce cell differentiation in a slime mold,<sup>4</sup> a function related to those which will be described in this paper.

In spite of knowing this wide range of recognition functions of the carbohydrate portion of glycoconjugates, there has been no suggestion until recently that carbohydrates themselves can act as regulatory molecules that alter protein synthesis in receptive cells, but recent evidence, accumulated with several experimental systems, establishes that carbohydrates can do just that. We describe below several plant and plant-microbe systems in which carbohydrates act as regulatory molecules.

PLANTS, WHEN EXPOSED TO CERTAIN  $\beta$ -GLUCAN FRAGMENTS OF FUNGAL ORIGIN, DEFEND THEMSELVES BY SYNTHESIZING AND ACCUMULATING PHYTOALEXINS

Many plants respond to invasion by a pathogenic or a nonpathogenic microorganism, whether a fungus, a bacterium, or a virus, by accumulating phytoalexins. Phytoalexins are defined as low molecular weight antimicrobial compounds that are both synthesized by and accumulate in plants after exposure to microorganisms. Many plants attempt to defend themselves against microbes and, perhaps, against other pests<sup>5-13</sup> by producing phytoalexins. Molecules which trigger phytoalexin production in plants have been called elicitors.<sup>14</sup>

The best characterized and most effective known elicitor of biological origin is composed of fragments of  $\beta$ -glucans present in the mycelial walls of many fungi.<sup>15 16</sup> This " $\beta$ -glucan elicitor" can be obtained by partial acid hydrolysis of purified mycelial walls of the fungal pathogen of soybeans, Phytophthora megasperma f.sp. glycinea which causes root stem rot. The " $\beta$ -glucan elicitor" is very active in stimulating phytoalexin accumulation in soybean tissues. The smallest  $\beta$ -glucan fragments which have elicitor activity contain approximately nine  $\beta$ -glucosyl residues interconnected by 3-, 6-, and 3,6-glucosidic linkages.

The " $\beta$ -glucan elicitors" isolated from different races of Phytophthora megasperma f.sp. glycinea<sup>5</sup> and from the yeast Saccharomyces cerevisiae<sup>17</sup> do not differ significantly in their elicitation of phytoalexin accumulation in several soybean cultivars,<sup>5,17</sup> in French beans,<sup>18</sup> and in potatoes.<sup>18</sup> Thus, as is common for regulatory molecules, elicitors are not species specific with regard to their source nor with regard to the cells whose metabolism they regulate. Also like other regulatory molecules, the " $\beta$ -glucan elicitor" is effective in very small concentrations; approximately ten nanograms of the " $\beta$ -glucan elicitor" stimulates accumulation in a single soybean cotyledon of more than sufficient amounts of phytoalexins to stop the growth of a variety of microorganisms in vitro.

Evidence has been obtained that the " $\beta$ -glucan elicitor" like many other regulatory molecules, stimulates de novo enzyme synthesis in receptive plant cells. The " $\beta$ -glucan elicitor" causes soybean cells to accumulate at least five

chemically and metabolically related pterocarpan phytoalexins. Ebel, Hahlbrock, and Grisebach and their coworkers<sup>19-21</sup> have studied the biosynthesis of these soybean phytoalexins. Apparently, synthesis of these phenylpropanoid compounds is a result of de novo synthesis of the necessary enzymes. Dixon and his coworkers have shown that enzymes responsible for the biosynthesis of the phytoalexin phaseollin in French beans (*Phaseolus vulgaris*) are also synthesized de novo as a result of elicitation by the " $\beta$ -glucan elicitor".<sup>18, 22, 23</sup> Thus, the " $\beta$ -glucan elicitor" causes receptive cells to synthesize new proteins.

#### POLYSACCHARIDE FRAGMENTS FROM THE WALLS OF PLANT CELLS ELICIT PHYTOALEXIN ACCUMULATION IN PLANT CELLS

Realization that the polysaccharides of the walls of growing plant cells are extremely complex structures<sup>24-26</sup> has made us wonder about the function of these molecules. Until recently, these complex molecules had been thought to have only a structural function, but it is difficult to believe that such complex molecules have evolved for only structural requirements. This skepticism proved well founded for we have recently demonstrated that at least two plant cell wall polysaccharides, or fragments thereof, serve as regulatory molecules.

We have shown that one of these cell-wall derived regulatory molecules, which elicits phytoalexins in soybean cotyledons, is a component of isolated cell walls of soybean stems and of the walls of suspension-cultured cells of tobacco, sycamore, and wheat. This elicitor can be released from the isolated walls by partial acid hydrolysis, and purified by ion exchange and gel filtration chromatography. The elicitor-active fragments thus obtained are heterogeneous in size. Their elution volume by gel chromatography suggests that many of the elicitor-active fragments consist of 10 to 15 glycosyl residues. These elicitor-active fragments are called the "endogenous elicitor".

The "endogenous elicitor" originates from a galacturonic acid-rich cell wall polysaccharide; treatment of the "endogenous elicitor" with a endopolygalacturonase destroys its elicitor activity. The "endogenous elicitor" of soybean cell walls does not appear to originate from either rhamnogalacturonan I or rhamnogalacturonan II, the two pectic

polysaccharides which have been partially characterized in this laboratory.<sup>24,26</sup> This is not surprising as more than half of the pectic polysaccharides of the walls of growing cells have yet to be characterized.

We were not the first to discover that plants have an "endogenous elicitor". Bailey, Hargreaves and Selby<sup>27-29</sup> found a heat stable, dialyzable component which is released from damaged pea or bean tissues and which elicits phytoalexin accumulation in these tissues. It is not known whether the "constitutive" elicitor discovered by Bailey, Hargreaves and Selby is the same as the "endogenous elicitor" present in cell walls, but it seems likely that both elicitors are the same.

The realization that the "endogenous elicitor" is a fragment of a cell wall pectic polysaccharide is made more intriguing by observations that two enzymes which degrade pectic polysaccharides are elicitors. Stekoll and West<sup>30</sup> have studied an elicitor of casbene, a castor bean phytoalexin. The elicitor, present in culture filtrates of the pathogenic fungus *Rhizopus stolonifer*, is a pectic-degrading enzyme, an endopolygalacturonase. More recently, G. Lyon and P. Albersheim (unpublished results) obtained evidence that an elicitor secreted by the bacterial pathogen, *Erwinia carotovora* which causes soft rot, is a polygalacturonic acid lyase, another pectic-degrading enzyme. Partially purified preparations of this enzyme are effective elicitors of phytoalexin accumulation in soybean cotyledons.

The ability of pectic-degrading enzymes secreted by *Rhizopus stolonifer* and *Erwinia carotovora* to stimulate phytoalexin accumulation suggests that these enzymes could release the "endogenous elicitor" present in the cell walls of plants. However, we have not successfully released the "endogenous elicitor" of soybean cell walls by treatment of the walls with the *E. carotovora* polygalacturonic acid lyase. An alternative mechanism by which the pectic-degrading enzymes may elicit phytoalexin accumulation is indirectly by damaging plant cells.<sup>31-34</sup> The damaged cells might release or activate a plant enzyme which liberates the "endogenous elicitor". We have experimental support for this alternative explanation (G. Lyon and P. Albersheim, unpublished results). We have solubilized and partially purified an enzyme from soybean stems that elicits phytoalexin accumulation in soybean

cotyledons. This enzyme has only been isolated from stems whose cells had been damaged by a freeze-thaw procedure. Experiments have not yet been carried out to determine whether this enzyme works by releasing the "endogenous elicitor". The activation of an elicitor-releasing enzyme in damaged cells could explain the manner by which phytoalexin accumulation is stimulated by a variety of abiotic elicitors such as ultraviolet light, freeze-thawing, heavy metals, and antibiotics, and perhaps even by the " $\beta$ -glucan elicitor".

The "endogenous elicitor" is likely to be distributed throughout the plant. The enzyme, putatively responsible for releasing the "endogenous elicitor", must be regulated in some manner. The enzyme might be compartmentalized, such as in lysosomes, or bound to the cell membrane, or stored in an inactive form, perhaps as a zymogen. If this putative enzyme is released or activated by cell damage and if this enzyme is also distributed throughout the plant, all parts of the plant would, as observed, be capable of localized phytoalexin accumulation in response to any stimulus which causes cell damage.

#### THE PROTEINASE INHIBITOR INDUCING FACTOR (PIIF) IS A FRAGMENT OF A CELL WALL POLYSACCHARIDE

A third complex carbohydrate found to be a regulatory molecule is the plant hormone known as "PIIF" - the proteinase inhibitor inducing factor - which, like the "endogenous elicitor", is a fragment of a polysaccharide present in the walls of growing plant cells. Ryan and his coworkers<sup>35</sup> discovered 10 years ago that the leaves of the potato and tomato plants that had been attacked by the Colorado potato beetle rapidly accumulate two proteinase inhibitors. The proteinase inhibitors accumulate even in unattacked leaves distant from the site of attack. The proteinase inhibitors are proteins which have been purified to homogeneity and well-characterized.<sup>35</sup>

Ryan and his coworkers found that insects are not necessary for stimulation of inhibitors. Virtually any type of extensive crushing or tearing of the vegetative tissues of tomato, potato, and other dicotyledonous plants releases PIIF into the vascular system of the plant where it is transported to other tissues of the plant and initiates accumulation of proteinase inhibitors.<sup>35</sup>

Ryan and his colleagues found that PIIF was heat stable, but they were unable to purify PIIF to homogeneity. Nevertheless, the properties of their partially purified preparations suggested that PIIF might be a carbohydrate. Our laboratory formed a collaboration with Ryan's group and analyzed their PIIF-active fractions for carbohydrate constituents.

The first of Ryan's preparations of PIIF-active material that our laboratory examined was impure and contained a variety of different glycosyl residues connected by a still larger variety of glycosyl linkages. However, this mixture contained those characteristically-linked glycosyl residues present in rhamnogalacturonan I,<sup>26</sup> a pectic polysaccharide accounting for approximately 7% of the walls of suspension-cultured sycamore cells. Assay of several different highly purified plant cell wall components for PIIF activity showed that rhamnogalacturonan I was the only component tested in tomatoes which possessed PIIF activity. Studies of more purified preparations of PIIF-active material extracted from tomato plants, and of other rhamnogalacturonan I preparations from sycamore have demonstrated that PIIF is, in fact, a fragment of rhamnogalacturonan I. Thus, just as with the "endogenous elicitor", it is evident that damage of plant cells releases fragments of a cell wall polysaccharide, in this case rhamnogalacturonan I or fragments thereof, which induces the synthesis in plant cells of proteins involved in defense of the plant (C. A. Ryan, P. Bishop, G. Pearce, A. Darvill, M. McNeil, and P. Albersheim, manuscript submitted).

PIIF-active rhamnogalacturonan I can be released from isolated cell walls by the action of a highly purified fungal endopolygalacturonase. The PIIF-active rhamnogalacturonan I has been purified by ion exchange and gel filtration chromatography. Purified rhamnogalacturonan I has a molecular weight of approximately 200,000 and is composed of L-rhamnosyl, D-galacturonosyl, L-arabinosyl, and D-galactosyl residues in the ratio of approximately 2:5:3:3. The backbone of rhamnogalacturonan is composed predominantly, if not entirely, of D-galacturonosyl and L-rhamnosyl residues. There are about 500 glycosyl residues in the backbone, but it is not known whether the backbone is a single contiguous glycan or whether each molecule contains a number of interconnected backbone chains. About half of the rhamnosyl residues of rhamnogalacturonan I are 2-linked, have a galacturonosyl residue attached to C-2, and are glycosidically

attached to C-4 of a galacturonosyl residue. The other half of the rhamnosyl residues are 2,4-linked, have a galacturonosyl residue glycosidically attached at C-2, and are glycosidically attached to C-4 of a galacturonosyl residue. Side chains averaging six glycosyl residues in length are attached to C-4 of the 2,4-linked rhamnosyl residues. There are many different side chains containing variously linked L-arabinosyl and/or D-galactosyl residues. The size or even the composition of the smallest rhamnogalacturonan I fragment which possesses PIIF activity is not known.

#### GLYCOPROTEINS SECRETED BY INCOMPATIBLE RACES (RACES THAT CAN NOT INFECT THE PLANT) OF A FUNGAL PATHOGEN OF SOYBEANS ACT AS REGULATORY MOLECULES AND PROTECT THE PLANT FROM ATTACK BY COMPATIBLE RACES

Almost all the microorganisms and other pests with which a plant comes in contact cannot successfully pathogenize the plant. The few microorganisms which are plant pathogens are often highly specialized and are pathogenic on only one or a few species of plants. Most "host-specific" pathogen species have a number of races, each of which is distinct from the others in its ability to attack various varieties (cultivars) of its host plant species. In other words, race 1 of a pathogen of a particular crop may attack variety A but not variety B, while race 2 of the pathogen may attack variety B but not variety A. Both races might be able to attack variety C and neither variety D, and so on. In this type of host-pathogen system, for each gene that governs resistance in the host plant there is a corresponding gene in the fungal pathogen that governs avirulence. This type of relationship is referred to in the plant pathology literature as a gene-for-gene host-pathogen system.<sup>36-38</sup>

Gene-for-gene resistance in plants is determined by dominant Mendelian genes.<sup>36-39</sup> Each such resistance gene that a plant possesses can make the plant totally resistant to one or more races of at least one of its pathogens. However, a resistance gene is effective in protecting a plant against only those pathogen races which produce molecules capable of a specific interaction with the product of the resistance gene. Since these molecules of the pathogen cause the pathogen to be avirulent, the genes responsible for the synthesis of these molecules are called avirulence genes rather than virulence genes.

The interdependence of resistance and avirulence genes leads to the conclusion that the products of specific resistance genes of the host must recognize (interact with) the products of specific avirulence genes of the pathogen. This recognition reaction is the key to whether a race of gene-for-gene pathogens will be compatible with (virulent on) a variety of its host.<sup>1, 36</sup> A positive interaction of a product of a resistance gene with the product of an avirulence gene initiates a resistance or incompatible response in the plant.

We have hypothesized that the avirulence genes of a gene-for-gene pathogen are manifest as cell surface or extracellular structures.<sup>1</sup> The only fungi whose surface structures have been extensively studied are the yeasts. Ballou et al.<sup>40, 41</sup> have demonstrated that in yeast the immunodominant species-specific cell surface structures are portions of mannan-containing glycoproteins. The species-specific differences in the glycoproteins reside in small differences in the structures of the carbohydrate portion of these glycoproteins.

At least some of the enzymes secreted by yeast are themselves mannan-containing glycoproteins, and the structures of the mannan portions include the same antigenically active carbohydrate structures as the species-specific cell surface mannan-containing glycoproteins.<sup>42, 43</sup> The carbohydrate portions of the cell surface and extracellular glycoproteins are synthesized by the same glycosyl transferases.<sup>40</sup> Thus, each species of yeast has a unique set of glycosyl transferases that is responsible for the synthesis of these species-specific antigens.

We have suggested that the products of the avirulence genes of fungal pathogens are glycosyl transferases, enzymes which function in the synthesis of complex carbohydrates which are present both on fungal cell surface and on at least some secreted glycoproteins. We think of the products of a plant's resistance genes as receptors for the glycoproteins synthesized by the avirulence gene-encoded glycosyl transferases of the pathogen. Thus, we propose that complex carbohydrates, present on the cell surface and/or extracellular glycoproteins of the pathogen, are recognized by receptors in resistant varieties of the pathogen's host and that this interaction activates the host's defenses. If the

hypothesis is correct and if the plant pathogenic fungi are similar in this respect to yeast, at least some of the glycoproteins secreted by a pathogen will contain race-specific complex carbohydrates.

We have been studying the race- and cultivar-specific interaction of soybeans and Phytophthora megasperma f.sp. glycinea, the causal agent of root and stem rot. This host-pathogen system appears to be a gene-for-gene system, since there exist at least 16 fungal races and many differently susceptible cultivars of the host plant.<sup>44</sup> Invertase, which is one of the many proteins secreted by this pathogen, was chosen for study as a typical extracellular protein of this pathogen. As with yeast, the invertases secreted by races 1, 2, and 3 of Phytophthora megasperma f.sp. glycinea are mannan-containing glycoproteins.<sup>45</sup> The glycosyl linkage compositions of the carbohydrate portions of the invertases produced by three different Phytophthora races are clearly different.<sup>45</sup> The demonstration of race-specific carbohydrate structures in differentially virulent Phytophthora races provides support for the hypothesis that such complex carbohydrates are involved in determining specificity in gene-for-gene host-pathogen systems, for the only known way to discriminate between the races and the only known selection pressure to cause differences in the races is by their differing abilities to infect the various soybean cultivars.

We reasoned that if the race-specific carbohydrates of the extracellular glycoprotein population determine host-pathogen specificity, the biological activity of these molecules should be demonstrable. In other words, the extracellular glycoproteins from incompatible (avirulent) races of Phytophthora megasperma f.sp. glycinea, but not those from compatible (virulent) races, should be capable of activating a defense reaction in seedlings of a soybean cultivar which would thereby protect the seedlings from attack by compatible races of the fungal pathogen.

Our approach to demonstrating the biological activity of the extracellular glycoproteins was first to partially purify the glycoprotein fraction from the extracellular culture medium of three races of Phytophthora megasperma f.sp. glycinea. The macromolecules obtained were composed on the average of 81.5% protein and 18.5% carbohydrate. Analysis of the carbohydrate fractions showed quantitative

but not qualitative differences in their composition.<sup>46</sup> This result is similar to that obtained for the carbohydrate fractions of the extracellular invertases of these three races.<sup>45</sup>

The important question was whether the extracellular glycoproteins of incompatible Phytophthora races can protect soybean cultivars from compatible races of this fungal pathogen. This would be expected if these glycoproteins are the race-specific determinants, that is, the biochemical expression of the avirulence genes. Experiments to answer this important question were carried out with three races of Phytophthora megasperma f.sp. glycinea and four soybean cultivars that are differentially susceptible or resistant to the races of Phytophthora. In the combinations tested, the extracellular glycoproteins from incompatible, but not from compatible, races of Phytophthora megasperma f.sp. glycinea protect seedlings from infection by compatible races of the pathogen. For example, the extracellular glycoproteins from races 1 or 2 protect the cultivar Harosoy 63, with which races 1 and 2 are incompatible, from infection by race 3, while the extracellular glycoproteins from compatible race 3 do not protect the Harosoy 63 seedlings from race 3 fungus.<sup>46</sup> On the other hand, the extracellular glycoproteins from races 1 or 3 protect the cultivar Sanga, with which races 1 and 3 are incompatible, from the compatible race 2 fungus, although the extracellular glycoproteins from race 2, which do protect Harosoy 63 from race 3, do not protect Sanga from race 2.<sup>46</sup>

These positive results encouraged us. We are even more encouraged by results of our first protection experiments with the alkali-released carbohydrate portion of the race-specific glycoproteins. These experiments tentatively indicate that the carbohydrate portions, by themselves, are more effective race-specific protection factors than the intact glycoproteins.

A long term goal is to look for receptors in the soybean seedlings for the specificity factors. If our hypothesis is correct, the receptors should be present in those plants which interact with the race-specific glycoproteins, that is, in resistant cultivars, but should not be present in plants which do not interact with the race-specific glycoproteins, that is, in susceptible cultivars. The receptors are likely

to be the products of the resistance genes of the soybean cultivars.

#### ACIDIC POLYSACCHARIDES SECRETED BY THE SYMBIOTIC NITROGEN-FIXING RHIZOBIA APPEAR TO REGULATE THE ENTRY OF THESE BACTERIA INTO THE ROOTS OF LEGUMES

A great many publications have demonstrated the essential function of the cell surface and extracellular polysaccharides of Gram-negative bacteria in the interaction of these bacteria with other cells, including the cells of both plants and animals.<sup>1, 47, 48</sup> The nitrogen-fixing Rhizobium are Gram-negative bacteria, therefore their surface and extracellular polysaccharides are likely to be active as regulatory molecules by determining with which higher plants the Rhizobium can form symbiotic nitrogen-fixing relationships. This hypothesis has been supported by the results described in this section.

Numerous extracellular polysaccharides of Gram-negative bacteria have been structurally characterized, and these polysaccharides are, in general, serotype or species specific. This also appears to be generally true for Rhizobium species for, with the possible exception of R. leguminosarum and R. trifolii, the acidic polysaccharides secreted by the various Rhizobium species appear to be nodulation group specific, that is, Rhizobium which nodulate different legumes secrete different extracellular polysaccharides. For example, R. japonicum<sup>49</sup> which nodulates and fixes nitrogen in soybeans secretes a markedly different acidic polysaccharide than does R. meliloti<sup>50</sup> which nodulates alfalfa. R. leguminosarum, the pea symbiont, R. trifolii, the clover symbiont, and R. phaseoli, the true bean symbiont, are the most closely related Rhizobium species.<sup>51, 52</sup> We have found that R. phaseoli secretes an acidic polysaccharide with a different structure than that secreted by R. leguminosarum and R. trifolii (P. Åman, L.-E. Franzén, M. McNeil, A. Darvill and P. Albersheim, unpublished results). However, we have also shown that the acidic polysaccharides secreted by R. leguminosarum and R. trifolii have basically the same structures.<sup>53</sup> The structure of the acidic extracellular polysaccharides produced by R. leguminosarum and R. trifolii, both fast-growing rhizobia, has some similarities to the acidic polysaccharides secreted by R. meliloti and R. phaseoli, which are also fast-growing species. However, there is no relationship between the

structures of the acidic polysaccharides secreted by the fast-growing *Rhizobium* species and the acidic polysaccharide secreted by slow-growing *R. japonicum*.

We have established that the glycosyl residue sequence and the anomeric configurations of the glycosyl linkages of the acidic polysaccharides secreted by two *R. trifolii* and two *R. leguminosarum* strains are identical. We have not, however, investigated the possibility of differently substituted acetyl, succinyl, or other alkali-labile residues in these polysaccharides. Therefore, it is not yet established that the acidic extracellular polysaccharides from *R. leguminosarum* and *R. trifolii* have identical structures. Analyses for alkali-labile substituents are important, for Jansson et al.<sup>50</sup> have determined that the acidic polysaccharide secreted by *R. trifolii* U226 possesses at least one O-acetyl residue per repeating unit; the O-acetyl residue(s) is attached to C-2 and/or C-3 of a 4-linked glucosyl residue(s). It remains to be ascertained whether the *R. leguminosarum* polysaccharide has the same acetyl substitution.

*R. leguminosarum* is a symbiont of pea (*Pisum sativum*) and *R. trifolii* a symbiont of clover (*Trifolium pratense*). In some instances these two species cross nodulate their legume hosts. Some strains of *R. leguminosarum* nodulate *Trifolium* species and some strains of *R. trifolii* nodulate *Pisum* species,<sup>54-57</sup> although the nodules formed in each case are unable to fix nitrogen. Both *R. leguminosarum* and *R. trifolii* cause curling and branching of root hairs in a host of *R. trifolii*, *Trifolium glomeratum*, phenomena generally induced only by *Rhizobium* capable of forming a symbiosis with that legume.<sup>57, 58</sup> *R. leguminosarum* and *R. trifolii* have also been reported to have a high degree of homology between their DNA molecules.<sup>51</sup> Thus, it is not very surprising that the polysaccharides secreted by *R. leguminosarum* and *R. trifolii* are very similar if not identical. The facts that *R. leguminosarum* and *R. trifolii* can in some instances cross nodulate legumes and that these two *Rhizobium* species secrete identical or nearly identical acidic polysaccharides support the hypothesis that the secreted acidic polysaccharides participate as regulatory molecules in the recognition processes which permit rhizobia to enter their host legumes.

Strong support for a regulatory function of acidic polysaccharides secreted by *Rhizobium* species is provided by

findings of W. D. Bauer and his coworkers at the Charles Kettering Institute. They have obtained evidence that the acidic polysaccharides are required for the development of legume root hairs capable of being infected by symbiont rhizobia; root hairs developed in the absence of these polysaccharides can not be infected (ref. 59 and personal communication). Therefore, these polysaccharides constitute a good example of complex carbohydrates with regulatory properties, in this case an ability to cause a specific differentiation of the epidermal cells of legume roots.

#### RHAMNOGALACTURONAN II - AN EXTRAORDINARILY COMPLEX POLYSACCHARIDE PRESENT IN THE WALLS OF GROWING PLANT CELLS

We have recently isolated, from the walls of suspension-cultured sycamore cells, a previously unknown pectic polysaccharide called rhamnogalacturonan II.<sup>24</sup> Rhamnogalacturonan II, which accounts for about 4% of the cell wall, is completely solubilized from the walls of suspension-cultured sycamore cells by the action of an endo- $\alpha$ -1,4-polygalacturonase and separated from the other solubilized pectic polysaccharides by anion exchange and gel permeation chromatography.

A brief description of rhamnogalacturonan II is included here because the extreme structural complexity of this molecule suggests that it too will be found to function as a regulatory molecule. This polysaccharide, which has been purified to apparent homogeneity, possesses a well-defined structure and molecular size. As isolated, rhamnogalacturonan II contains a total of about 50 glycosyl residues. It contains nine different glycosyl constituents including the rarely observed sugars 2-O-methyl fucose, 2-O-methyl xylose, which have nevertheless previously been recognized to be trace components of pectic polymers,<sup>60,63</sup> and apiose, a branched pentose, which has also been recognized as a component of the pectic polysaccharides of *Lemna* species. The *Lemna*-type apiose-containing pectic polysaccharide has not been found to be widespread in nature and is not structurally related to rhamnogalacturonan II. Apiose and the 2-O-methyl derivatives of fucose and xylose have never previously been recognized to be associated in a single polysaccharide, although all three sugars have been isolated from leaves of deciduous trees.<sup>64</sup>

Rhamnogalacturonan II is characterized by many different terminal glycosyl residues including terminal galacturonosyl, terminal galactosyl, terminal arabinosyl, terminal 2-O-methyl xylosyl, terminal 2-O-methyl fucosyl, and terminal rhamnosyl residues. The large content of terminal glycosyl residues and of a variety of branched glycosyl residues indicates a highly branched structure. Rhamnogalacturonan II also contains a number of unusually linked glycosyl residues including 2-linked glucuronosyl, 3'-linked apiosyl, 3-linked rhamnosyl, 2,4-linked galactosyl, and 3,4-linked fucosyl residues. The glycosyl composition of rhamnogalacturonan II remains constant throughout the lag, log, and stationary phases of growth of suspension-cultured sycamore cells. We also have evidence that a molecule very similar or identical to rhamnogalacturonan II is present in the primary cell walls of the four other dicots examined; namely, pea, French bean, and tomato seedlings, and suspension-cultured tobacco cells.

It is interesting to consider how a polysaccharide as complex as rhamnogalacturonan II is synthesized; synthesis by any of the known pathways would require on the order of 100 enzymes. This is an enormous investment by the cell to achieve structural complexity in a polymer that represents only 4% of the wall. Why is there such an investment? We can't help but think that the reason has been to evolve a molecule with regulatory functions. We are very curious to learn the function of this molecule.

#### CONCLUDING REMARKS

It has been an exciting experience for us to realize that the plant cell wall polysaccharides, whose structures we have been struggling to decipher, are functioning not only as structural polymers but also in a regulatory capacity. At least two of the complex polysaccharides which are present in the walls of growing plant cells contain fragments which possess the remarkable properties of hormones, that is, molecules formed by one cell which in minute amounts, stimulate receptive cells to synthesize specific proteins. Two different pectic polysaccharides contain within themselves the glycosyl sequences which constitute either the plant hormone known as PIIIF or the "endogenous elicitor". PIIIF and the "endogenous elicitor" are apparently released from the cell walls surrounding injured cells and then stimulate receptive cells to synthesize proteins involved in defense of the plant.

Two of the other complex carbohydrates described in this paper, the " $\beta$ -glucan elicitor" and the acidic polysaccharides secreted by Rhizobium species, also appear to possess the attributes of hormones, except that these regulatory carbohydrates are produced by one organism and affect receptive cells in another organism.

PIIF, the "endogenous elicitor", and the " $\beta$ -glucan elicitor" have, in addition to being carbohydrates with regulatory properties, two other characteristics in common. They are "stored" as insoluble cell wall polysaccharides; and they are released or "activated" by cleavage of the wall polysaccharides, presumably by specific enzymes.

The fact that the walls of growing plant cells contain these oligosaccharide "hormones" means that the walls function as a "pseudogland" containing regulatory molecules which can be released as needed. We can envision the cell wall containing a variety of messages capable of controlling physiological processes of a developing plant.

The fact that these oligosaccharide hormones originate as portions of larger polymers is strikingly similar to the origin of a number of animal peptide hormones. For example, several polypeptide hormones synthesized in the pituitary gland originate in a common precursor polypeptide.<sup>65</sup> A 16,000 dalton fragment of the precursor polypeptide is removed and the remaining polypeptide cleaved to produce the hormones corticotropin and  $\beta$ -lipotropin. The corticotropin can be further cleaved to yield  $\alpha$ -melanotropin, and the  $\beta$ -lipotropin can be cleaved to yield  $\alpha$ -lipotropin and  $\beta$ -endorphin. This process is analogous to cleavage of a wall polysaccharide to yield biologically active fragments.

We hope that the knowledge that plant cell wall polysaccharides possess a number of interesting biological functions will stimulate other laboratories to focus on unraveling their complex structures. Certainly, the increasing realization that complex carbohydrates play key roles in biological recognition processes will stimulate efforts to develop rapid and efficient methods for the structural characterization and synthesis of these molecules.

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## Chapter Four

### GALACTOFURANOSYL-CONTAINING LIPOGLYCOPEPTIDE IN PENICILLIUM

J. E. GANDER AND CYNTHIA J. LAYBOURN

Department of Biochemistry  
College of Biological Sciences  
University of Minnesota  
St. Paul, Minnesota 55108

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#### INTRODUCTION

Fungi secrete numerous macromolecules which are derived from a) walls, b) enzymes, and c) subcellular organelles. These macromolecules are subject to the action of lytic enzymes located in the nutrient medium. Therefore, many polymers found in media supporting fungal growth are degradation products of more complex substances. For instance, galactocarolose, a 5-O- $\beta$ -D-galactofuranosyl-containing decasaccharide, and mannocarolose, an  $\alpha$ -D-mannopyranosyl-containing nonasaccharide, first isolated from 28-day culture filtrates of Penicillium charlesii and partially characterized in W. N. Haworth's laboratory<sup>1, 2</sup> have been shown to be derived from a more complex glycopeptide<sup>3-5</sup> which first appears in the growth medium soon after formation of conidia.<sup>6</sup> Because of the composition of the complex glycopeptide we have referred to it as a peptidophosphogalactomannan.<sup>3</sup> The glycopeptide may be the major polysaccharide-containing substance secreted

prior to general lysis of the fungus.<sup>6,7</sup> Peptidophosphogalactomannans and/or peptidogalactomannans have been obtained from Cladosporium werneckii,<sup>8,9</sup> species of Aspergillus,<sup>10-12</sup> several species of dermatophytes from the genera of Trichophyton and Microsporum,<sup>13,14</sup> Fulvia fulva (Cooke) Ciferri<sup>15</sup> and several species of Penicillium<sup>10,16,17</sup> and may be common constituents of many genera of fungi.

It was initially assumed that polysaccharides found in media supporting fungal growth were derived from cell walls. This assumption may be correct for those polysaccharides which accumulate in large quantities in culture media over a period of two to four weeks. However, the peptidophosphogalactomannans and peptidogalactomannans derived from two to four grams (dry weight) of mycelia constitute less than one percent of the total mass. These glycopeptides are not major cell wall mannans or galactomannans. Cell walls of 14-day stationary cultures of P. charlesii contain no galactofuranosyl residues<sup>18</sup> and no phosphogalactomannan or galactomannan was released from cell walls obtained from 3-day cultures of P. charlesii when these walls were treated with alkali.<sup>19</sup> Bartnicki-Garcia<sup>20</sup> divided 13 taxonomic groups of fungi and yeasts into 8 different categories on the basis of the composition of the major polysaccharides of cell walls. Cell walls of Ascomycetes, the class to which Penicillium, Aspergillus, Neurospora, Trichophyton, Alternaria, Fusarium and Hansenula belong, contain an inner layer of chitin surrounded by an outer layer of  $\beta$ -glucan.<sup>21,22</sup> Hunsley and Burnett<sup>23</sup> suggested that a layer of protein or glycoprotein resided between the layers of chitin and  $\beta$ -glucan. The composition of the protein or glycoprotein has not been established.

Because the glycopeptides are somewhat unique in that they contain 5-O- $\beta$ -D-galactofuranosyl residues and at least those from P. charlesii contain 2-aminoethanol<sup>4</sup> and 2-dimethylaminoethanol<sup>24</sup> residues which are attached to the mannan through phosphodiester linkage,<sup>5</sup> we initiated a search for intracellular galactofuranosyl-containing polymers. The objective is to determine a) the parent polymer from which exocellular peptidophosphogalactomannan is derived, and b) the location of the parent polymer and its function in the fungus. This chapter reviews the work from this and other laboratories resulting in the partial description of the primary structure of this class of exocellular

glycopeptides, work from this laboratory on the location, isolation, and partial characterization of a membrane-bound galactofuranosyl-containing polymer, and a suggested function for the extracellular glycopeptide.

#### PARTIAL CHARACTERIZATION OF EXOCELLULAR FUNGAL GLYCOPEPTIDES

Investigations on structure and function of fungal glycopeptides originated in laboratories interested in the causative agent(s) of the immediate and delayed immunological responses observed when extracts of Trichophyton mentagrophytes or the polymers obtained from media supporting Trichophyton mentagrophytes growth were tested in patients suffering from tinea caused by Trichophyton species.<sup>25,27</sup> The immediate response of the glycopeptides, collectively named "trichophytin", was shown to be caused by the polysaccharide portion and the delayed response was due to the polypeptide(s).<sup>27</sup> Trichophytin obtained from submerged cultures contained a galactomannan, galactose:mannose ratio from 1:3 to 1:8, attached to a polypeptide which constituted about 10 percent of the total mass. In contrast, the polysaccharide of the glycopeptide obtained from surface cultures was shown to be a glucomannan.<sup>25</sup> Galactomannans contained numerous nonreducing terminal galactofuranosyl residues and the mannopyranosyl residues were attached predominantly by 1-2 and 1-4 linkages. They resolved trichophytin on DEAE-cellulose into two glycopeptides which had differing amino acid composition. Related galactomannans have been obtained from other species of Trichophyton and Microsporum.<sup>14,28,29</sup> Alkali effected the release of galactomannan and mannobiosyl residues from the polypeptide of Trichophyton granulosum.<sup>14</sup> The galactomannan contained 16 percent galactose which occurred as nonreducing terminal galactofuranosyl residues, and 84 percent mannopyranosyl residues which were attached by 1-2 and 1-6 linkages. Galactomannans were also obtained from Trichophyton schoenleinii, Trichophyton rubrum, Trichophyton interdigitale and Microsporum quinckaeum.<sup>29</sup> Galactofuranosyl residues occurred as nonreducing termini. The galactomannans were fractionated into galactomannans I and II. In general, galactomannan I contained less galactose than galactomannan II.

Aspergillus fumigatus, the organism responsible for aspergillosis, produces a peptidogalactomannan which contains galactofuranosyl residues.<sup>30,31</sup> The polypeptide region

is rich in seryl, threonyl, and alanyl residues and contains only traces of tyrosine, cysteine, and methionine.<sup>10,30</sup> The mannopyranosyl residues are attached to one another by (1 $\rightarrow$ 2) and (1 $\rightarrow$ 6)  $\alpha$ -linkages. Galactomannans obtained from either the growth medium or mycelia had a mass of 20,000 to 30,000 daltons and were homogenous by ultracentrifugal or electrophoretic analysis. A ratio of galactose:mannose of 1:1 was obtained. Galactomannans were also obtained Aspergillus flavus, Aspergillus effsus, Penicillium charlesii, Penicillium notatum, Penicillium frequentans, and Penicillium expansum by Azuma et al.<sup>10</sup> They conclude that galactomannans are common constituents of Aspergillus and Penicillium species. Galactomannan from culture filtrates of Penicillium chrysogenum contain galactofuranosyl residues and phosphorus.<sup>16</sup>

Lloyd<sup>8,9</sup> examined the "peptido-polysaccharides" from Cladosporium werneckii and found polymers of mass 150,000 to 200,000 daltons, composed of 11 percent protein, mannosyl-containing oligosaccharides and phosphogalactomannan attached to the polypeptide through seryl and threonyl linkages. The peptide is rich in seryl and threonyl residues and it contains only traces of tyrosine, phenylalanine, methionine and cysteine. Methylation analyses showed that mannopyranosyl residues are attached primarily by (1 $\rightarrow$ 2) links. Smaller quantities of (1 $\rightarrow$ 6) and (1 $\rightarrow$ 3) links also occur. The phosphogalactomannan contains phosphodiester groups which bridge a side chain of seven mannopyranosyl and one galactofuranosyl residues to the main core of polysaccharide. The main core also contains 7 phosphodiesters which bridge about 33 hexosyl residues. Phosphogalactomannan contained both galactofuranosyl and galactopyranosyl residues.

Dow and Callow<sup>15</sup> recently reported the occurrence of glycopeptides in culture filtrates of Fulvia fulva (Cooke) Ciferri (syn. Cladosporium fulvum) which contained major quantities of D-galactofuranosyl, D-glucosyl, and D-mannosyl residues and smaller amounts of D-glucuronosyl, D-galactosaminyl and D-glucosaminyl residues. Phosphodiesters bridged glucosyl-, galactosyl- andmannosyl-containing side chains to the main core of polysaccharide. Bridging of side chain oligosaccharides to main polysaccharide chain through a phosphodiester which is attached to C-1 of the reducing terminal sugar of the oligosaccharide and to C-6 of a hexosyl residue in the main chain occurs

frequently in polysaccharides (mannans and galactomannans) obtained from fungi and yeasts.

It was suggested that the saccharides were responsible for determining the specificity in gene-for-gene interaction between Fulvia fulva (pathogen) and the host tomato plant.<sup>32,33</sup> However, Dow and Callow reported<sup>34</sup> that the leakage of electrolytes that occurred when the glycopeptide was administered to tomato leaf surfaces showed no race or cultivar specificity. The glycopeptides appeared to bind to the surface of the leaf, possibly to the plasmalemma surface.

Work on the biosynthesis of "galactocarlose" in our laboratory soon established that galactocarlose and manno-carose in 28-day stationary cultures of Penicillium charlesii<sup>2</sup> were degradation products of more complex polymers which contained galactofuranosyl, mannopyranosyl, and glucopyranosyl residues.<sup>7, 35, 36</sup> These polysaccharide-containing polymers, secreted into the growth medium, were shown to contain phosphorus also.<sup>35</sup> The major polymer was released from DEAE-Sephadex-borate with 0.1 N HCl-0.06 M LiCl and was shown to be heterogenous with respect to mass, with respect to ratio of galactose:mannose:glucose, and with respect to ratio of P:galactose but not with respect to P:mannose.<sup>36</sup> Treatment of this polymer with 70 percent formic acid at 100°C for 90 minutes resulted in the release of phosphorus-containing substances that chromatographed like glucose 6-phosphate, mannose 6-phosphate and inorganic orthophosphate.<sup>35</sup> Later it was established that this exo-cellular polymer was a glycopeptide<sup>4</sup> and that the maximum quantity of polymer occurred in the medium within 8 to 10 days when the culture of Penicillium was grown with vigorous aeration.<sup>6</sup> Under these conditions little or no glucose was found in the polymer.<sup>3</sup> Treatment with alkali effected β-elimination of the saccharides from the polypeptide(s).<sup>4</sup> This treatment revealed that a phosphogalactomannan and 10 to 12 mannosyl-containing low molecular weight saccharides are attached through seryl and threonyl residues to the polypeptide(s). The mannosyl-containing saccharides released were primarily mannose, mannobiose, and mannotriose. Methylation analyses of the glycopeptide and phosphogalactomannan showed that the mannopyranosyl residues were attached by (1→2) and (1→6) α-linkages.<sup>3</sup> Acetolysis of the glyco-

peptide and phosphogalactomannan released mannotetraose, mannotriose, mannobiose, and mannose in ratios of 11:5:13:16 and 11:4:5:13, respectively. These data show that 1:8:3 residues of mannotriose, mannobiose, and mannose, respectively, were released from the polypeptide during  $\beta$ -elimination. We also noted a phosphorus-containing saccharide that eluted in the position of a mannopentaose.<sup>24</sup> This substance has not been characterized further, but it most likely is not a phosphomannopentaose as the phosphate would modify the elution position of saccharides.

Phosphogalactomannan from Penicillium charlesii glycopeptide is comprised of a phosphomannan backbone to which approximately 10 galactan chains are attached, each by (1 $\rightarrow$ 3)  $\beta$ -linkage.<sup>3,5,37</sup> The galactan chains contain an average of 22 5-O- $\beta$ -D-galactofuranosyl residues when the glycopeptide is obtained from cultures prior to depletion of glucose.<sup>37</sup> The exocellular glycopeptide from these cultures has a mass of about 70,000 daltons. Glycopeptide isolated from cultures which are depleted of glucose contain an average of two galactofuranosyl residues per galactan chain and have a mass of about 22,000 daltons. Partial degradation of the galactan chains results from an exo- $\beta$ -D-galactofuranosidase which is secreted into the growth medium as the medium becomes depleted of glucose,<sup>37</sup> (Pletcher, Lomar and Gander, in press). This enzyme degrades the galactan rapidly until two galactofuranosyl residues remain per chain. These two galactofuranosyl residues are removed at least two orders of magnitude more slowly than those from the longer chain galactans. Carbon-13 nmr spectroscopy shows a major resonance signal at chemical shifts of 110.5 ppm, the chemical shift of the anomeric carbon of nonreducing terminal  $\beta$ -D-galactofuranosyl residue and no signal at 104 ppm which would be indicative of a nonreducing terminal  $\alpha$ -D-galactofuranosyl residue.<sup>5</sup> Thus, we conclude that the slow removal of the final two galactofuranosyl residues from the mannan must be due to steric restraints. However, an exocellular "polysaccharide" from 28-day cultures of Penicillium varians contains both  $\alpha$ - and  $\beta$ -linked D-galactofuranosyl residues.<sup>38</sup> We have obtained a glycopeptide from Penicillium varians which has a similar hexose composition (galactose, glucose and mannose) and have shown that the polysaccharide is attached to polypeptide as occurs in Penicillium charlesii (Gander and Unkefer, unpublished).

During the course of studies to determine the N-terminal amino acyl residue(s) of the glycopeptide, 2-aminoethanol was found as a constituent and shown to occur in a molar ratio of 2-aminoethanol:glycopeptide of about 1:1.<sup>4</sup> This work was extended to show that the glycopeptide also contained 2-dimethylaminoethanol<sup>24</sup> and that 2-aminoethanol and 2-dimethylaminoethanol were probably attached to the mannan through phosphodiester bridges.<sup>5</sup> <sup>31</sup>P nmr spectra are consistent with this interpretation of the data and suggest that some 2-methyl aminoethanol and choline also occur

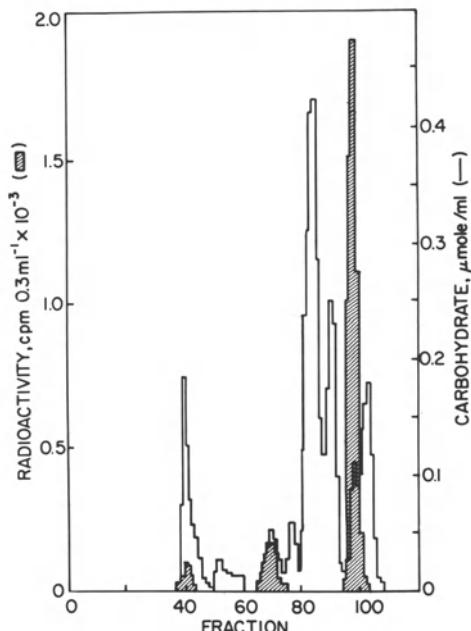


Figure 1. Distribution of <sup>14</sup>C and carbohydrate following chromatography of the low molecular weight substances from (<sup>14</sup>C)ethanolamine-labeled glycopeptide on Bio-Gel P2. Glycopeptide labeled with <sup>14</sup>C from [2-<sup>14</sup>C]ethanolamine was treated with alkali and fractionated on Sephadex G-50 as fractions 20 to 25 were combined, their volume was reduced to about 1 ml, and they were chromatographed on Bio-Gel P2 column. Fractions 35 to 110 were analyzed for <sup>14</sup>C (■) and carbohydrate (□).<sup>24</sup>

in the glycopeptide.<sup>39</sup> There is some evidence for the attachment of a neutral substance, possibly a saccharide, to the mannan through a phosphodiester bridge (Unkefer and Gander, unpublished). Treatment of <sup>14</sup>C-2-aminoethanol- or <sup>3</sup>H-methyl-labeled glycopeptide with alkali released about 40 percent of the <sup>14</sup>C or <sup>3</sup>H with low molecular weight substances and the remainder was nondialyzable and was reisolated with phosphogalactomannan.<sup>24</sup> In contrast, 95 percent of the phosphorus remained with phosphogalactomannan following treatment of glycopeptide with alkali. The phosphate which was released with low molecular weight substances eluted with "mannopentaose" fraction from BioGel P-2 column as did a small fraction of the <sup>14</sup>C-2-aminoethanol and <sup>3</sup>H-methyl-labeled substances. The remainder of the <sup>14</sup>C- and <sup>3</sup>H-labeled substances eluted near the monosaccharides (Fig. 1). Initially we concluded that the phosphodiesters were in two environments; the alkali stable phosphodiesters being attached to C-6 hydroxymethyl groups of the mannan and the alkali unstable phosphodiesters being attached to alkali unstable C-2 position of mannopyranosyl residues.<sup>24</sup> Preliminary evidence shows that alkali releases choline, probably because of its neutral charge even in 0.4 N alkali which is in contrast to 2-aminoethanol and its mono- and dimethylamino derivatives (Unkefer and Gander, unpublished).

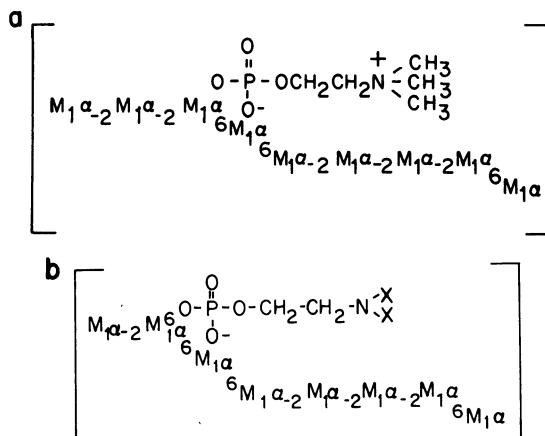


Figure 2. Proposed repeating units found in 2-aminoethanol-containing phosphomannan region of peptidophospho-galactomannan.

There was no evidence, based on  $^{31}\text{P}$  NMR spectra, for either phosphomonoester or cyclic phosphate occurring in the mannan after treatment with 0.4 N alkali. A model which is consistent with the data obtained is shown in Figure 2. This is but one of several feasible models and it assumes that phosphogalactomannan is composed of two types of repeating units; one with phospho-2-aminoethanol, or its mono- or dimethylamino derivative, bridged to C-6 of the reducing terminal residue of a mannooctaose (Fig. 2b) and the other with phospho-choline bridged to the C-6 of a mannononaose region of the phosphogalactomannan. As yet, we do not know to what region of phosphogalactomannan the neutral phosphodiester is attached. The single phospho-2-dimethyl-aminoethanol- and mannosyl-containing oligosaccharide which is released by treatment with alkali<sup>24</sup> may serve as a "stop" signal during biosynthesis of phosphomannan or phosphogalactomannan. Drewes and Gander<sup>40</sup> observed that a mutant of Penicillium charlesii which was selected because of its inability to grow on D-galactose, produced exocellular glycopeptide of about 23,000 daltons which contained only one mole of phosphorus and two to three moles of galactose per mole of glycopeptide. This suggests that phosphoryl residues are attached only after galactofuranosyl residues are attached. The occurrence of the one phosphoryl residue may represent that incorporated as the "stop" signal during synthesis of the mannan. The data also show that in the mutant the mannan can be synthesized without attachment of the galactan or phospho-2-aminoethanol derivative. It is not known whether the two or three galactofuranosyl residues are attached to the region of the mannan proposed as the stop signal or to some other region.

In vitro studies on the biosynthesis of the mannose-containing low molecular weight saccharides have established that GDP-mannose serves as the mannosyl donor in formation of mannobiosyl-peptidophosphogalactomannan from mannosyl-peptidophosphogalactomannan.<sup>41</sup> However, there was no evidence for formation of mannotriosyl or mannotetraosyl residues. A system which incorporates mannosyl residue(s) into phosphogalactomannan region was separated from that which incorporates mannosyl residues into the low molecular weight saccharides.<sup>42</sup> This system resulted in adding (1 $\rightarrow$ 6)-linked and (1 $\rightarrow$ 2)-linked mannosyl residues to phosphogalactomannan.

A peptidophosphogalactomannan-dependent incorporation of 2-aminoethanol from CDP-2-aminoethanol is catalyzed by membrane-bound enzymes.<sup>43</sup> Treatment of the isolated peptidophosphogalactomannan containing <sup>14</sup>C-2-aminoethanol with alkali resulted in the release of about 40 percent of the 2-aminoethanol with the low molecular weight substances and the remainder of the 2-aminoethanol was associated with phosphogalactomannan.

Investigations on the composition and sequence of amino acyl residues in the polypeptide region of the glycopeptide have shown that it contains 8 seryl, 7 threonyl, 4 alanyl, 2 valyl, 3 glycyl, 2 prolyl, 1-2 glutamyl-glutaminyl, and 1 aspartyl-asparaginyl residues and less than one residue each of histidine, leucine, isoleucine, and lysine.<sup>4</sup> The polypeptide has negligible quantities of S-containing and aromatic amino acyl residues. Treatment of the glycopeptide with alkali released carbohydrate from 6 of the seryl residues and 7 of the threonyl residues. The molecular weight of the polypeptide is about 3,000 and was shown to be nearly homogenous by gel filtration chromatography.<sup>44</sup> Analyses for N-terminal amino acyl residues showed that the glycopeptide contained N-terminal seryl, aspartyl, glycyl and glutamyl-glutaminyl residues.<sup>4</sup> Although treatment of the glycopeptide with pronase released one to three amino acyl residues<sup>44</sup> this treatment did not eliminate the heterogeneity at either C-terminal or N-terminal ends of the polypeptide (Tonn and Gander, unpublished). Treatment of the glycopeptide with anhydrous HF<sup>45</sup> removes the carbohydrate without cleavage of the polypeptide (Tonn and Gander, unpublished). The polypeptide was fractionated into four polypeptides containing N-terminal seryl, glycyl, and aspartyl-asparaginyl residues and part of the heterogeneity with respect to the number of leucyl, isoleucyl and lysyl residues was resolved by this treatment. However, we have not been able to obtain a unique sequence for any of these polypeptides. From these studies we conclude that the exocellular glycopeptide is derived from several different glycoproteins. The role of the phosphogalactomannan may be to provide the proper recognition for transport and exocytosis of these proteins.

Exocellular glycopeptides were isolated and purified from the following species of *Penicillium*: notatum, chrysogenum, patulum, claviforme, raistrickii and

roqueforti. Each glycopeptide was shown to have an amino acyl composition similar but not identical to that of Penicillium charlesii. Carbon-13 nmr spectroscopy of glycopeptides containing natural abundance  $^{13}\text{C}$  showed that each glycopeptide had large quantities of 5-O- $\beta$ -D-galactofuranosyl residues (Gander and Rees, unpublished). Thus it is apparent that 5-O- $\beta$ -D-galactofuranosyl-containing glycopeptides are common among the Ascomycetes.

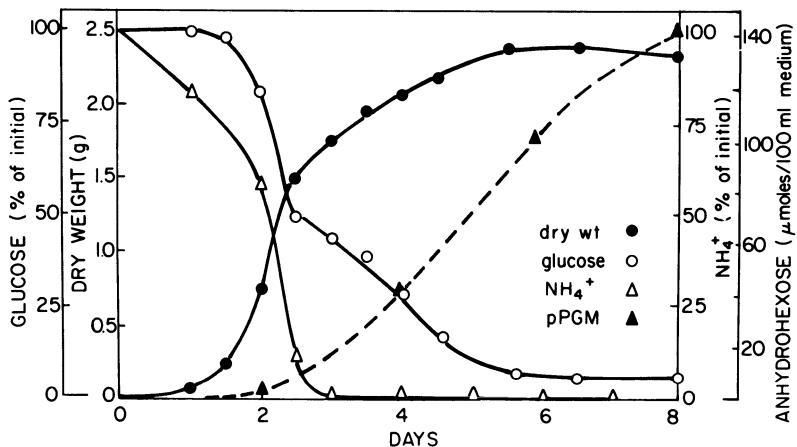


Figure 3. Time course of D-glucose and  $\text{NH}_4^+$  uptake from the growth medium and increase in dry weight of mycelia of P. charlesii cultures, and the release of peptidophosphogalactomannan, pPGM, into the medium. The organism was cultured in a modified Raulin-Thom medium (Jordan, J. M. and J. E. Gander. 1966. *Biochem. J.* 100:694-701). The contents of the flasks were removed at the indicated intervals, filtered, and the filtrate was assayed for the total carbohydrate (o-o),  $\text{NH}_4^+$  ( $\Delta-\Delta$ ), dry weight ( $\bullet-\bullet$ ) and pPGM ( $\blacktriangle-\blacktriangle$ ). The mycelia were dried for 24 hours at  $80^\circ\text{C}$  and weighed. The initial concentrations of D-glucose and  $\text{NH}_4^+$  were 278 and 36.3 mM, respectively.

### PARTIAL CHARACTERIZATION OF CELLULAR PEPTIDOPHOSPHO-GALACTOMANNAN

No galactofuranosyl-containing substances were found in the growth medium before 2.5-3 days (Fig. 3).<sup>6</sup> However, radioactive exocellular glycopeptide was isolated from 9-day cultures when Penicillium charlesii was cultured for 2.5 days in <sup>32</sup>P inorganic phosphate, D-[<sup>14</sup>C]glucose, or L-[<sup>14</sup>C]-threonine and the culture transferred into nonradioactive medium which had supported Penicillium charlesii for 2.5 days. The quantity of radioactivity incorporated suggested that glycopeptide synthesis started at the time, or soon after, germination.

Three day cultures of Penicillium charlesii were washed, the mycelia were broken with Al<sub>2</sub>O<sub>3</sub>, and the cell walls and unbroken cells were separated from membranes and soluble-cytoplasmic fraction by ultracentrifugation. Both the membranes and the cytoplasmic fraction contained galactofuranosyl residues which were released by exo-β-D-galactofuranosidase.<sup>46</sup> Glycopeptides in the soluble-cytoplasmic fraction were isolated by procedures routinely used in this laboratory<sup>3</sup> (Fig. 4). A galactofuranosyl-containing glycopeptide was obtained. The glycopeptide was somewhat larger in that it contained approximately 60 amino acyl residues and a mass of 80,000 to 90,000 daltons as determined by gel filtration chromatography and SDS disc polyacrylamide gel electrophoresis.<sup>46</sup> Treatment of the glycopeptide with alkali released mannosyl, mannobiosyl, and a galactofuranosyl-containing phosphogalactomannan. The glycopeptide contained N-terminal seryl and glycyl residues and dansyl-2-aminoethanol was found following treatment of the glycopeptide with 1-dimethylamino naphthalene 5-sulfochloride (dansyl chloride) and hydrolysis in acid. The polypeptide contained approximately 16 and 11 seryl and threonyl residues, respectively, and only traces of S-containing or aromatic amino acids were found. Polypeptide accounts for only 14 percent of the polymer. Some of the chemical properties of the glycopeptides obtained in this experiment are shown in Table 1. The small, but finite, quantity of the glycopeptide in the growth medium at 3 days is consistent with the data obtained previously.<sup>6</sup>

The ratio of galactofuranosyl:mannosyl residues is 2:1 which is typical of that observed previously<sup>37</sup> in glycopeptide

from culture filtrates prior to the release of large quantities of exo- $\beta$ -D-galactofuranosidase. Based on our previous studies<sup>3,7</sup> we anticipated a molar ratio of hexosyl residues:P of 30:1. The observed ratio of 15:1 suggests that each mole of glycopeptide contains 20 moles of P. We note that the sum of moles of mannotetraosyl, mannotriosyl, and mannobiosyl residues per mole of P derived from phosphogalactomannan by actolysis is 1:1. This suggests that each

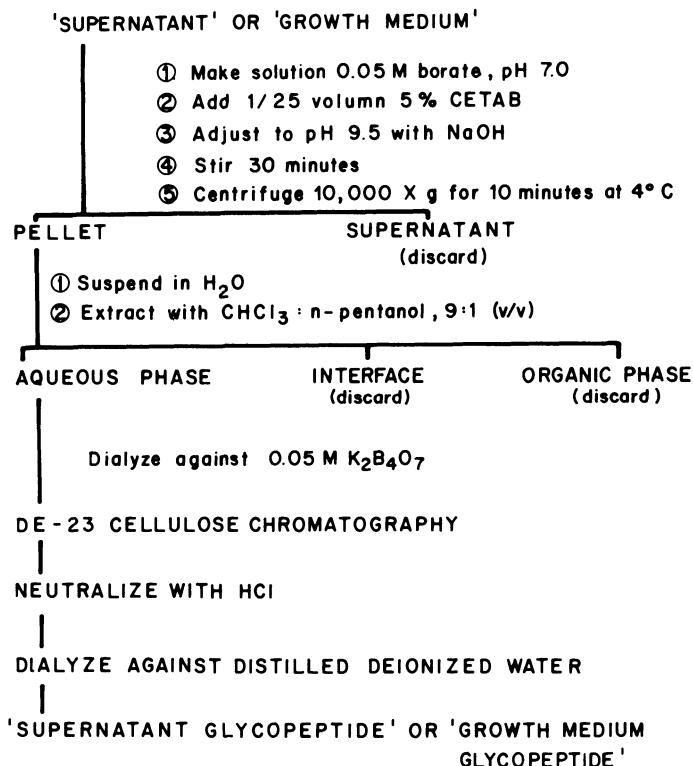


Figure 4. Flow diagram for isolation of soluble glycopeptide obtained from the soluble cytoplasmic fraction.

Table 1. Chemical characterization of glycopeptides

| Source of Glycopeptide | $\mu$ moles An-hex <sup>a</sup><br>Flask | An-hex <sup>a</sup><br>Phosphate <sup>b</sup> | An-hex <sup>a</sup><br>Protein | Galactose<br>An-hex <sup>a</sup> |
|------------------------|--|---|--------------------------------|----------------------------------|
| Growth Medium          | 2.5                                      | 16  | 6.7                            | 0.66                             |
| Supernatant            | 10.4                                     | 15  | 7.0                            | 0.64                             |
| DOC-soluble            | 4.0                                      | 12  | 3.8                            | 0.60                             |

<sup>a</sup>Anhydrohexose.

<sup>b</sup>Polymer treated with 0.05 N HCl for 90 minutes at 110°C. Galactose released measured by the coupled galactose oxidase-horseradish peroxidase assay.

region containing  $\alpha$ -1-2-linked mannopyranosyl residues may contain a phosphodiester attached to it. Recent studies using  $^{31}$ P nmr spectroscopy to characterize the glycopeptide and phosphogalactomannan suggest that glycopeptides isolated from culture filtrates of 6-day Penicillium charlesii may contain P in an environment not represented by those reported previously (Unkefer and Gander, unpublished). Using data from Table 1, molar ratios of P:glycopeptide of 20:1, 20:1 and 22:1 were obtained for exocellular glycopeptide, soluble cytoplasmic glycopeptide and membrane-bound glycopeptide, respectively.

By all of the criteria which we have used, the glycopeptide obtained in the fraction containing soluble cytoplasmic substances appears to be a precursor to the exocellular polymer. Its major difference is in having about twice the number of amino acyl residues.

#### PARTIAL CHARACTERIZATION OF MEMBRANE-BOUND LIPO-PEPTIDO-PHOPHOSGALACTOMANNAN

The membranes obtained by centrifuging the cell-free, wall-free homogenate described above were fractionated by isopycnic sucrose gradient ultracentrifugation. Six bands of membranes were observed and the occurrence of galactofuranosyl residues and GDP- $\underline{\text{D}}$ -mannose mannosyltransferase in

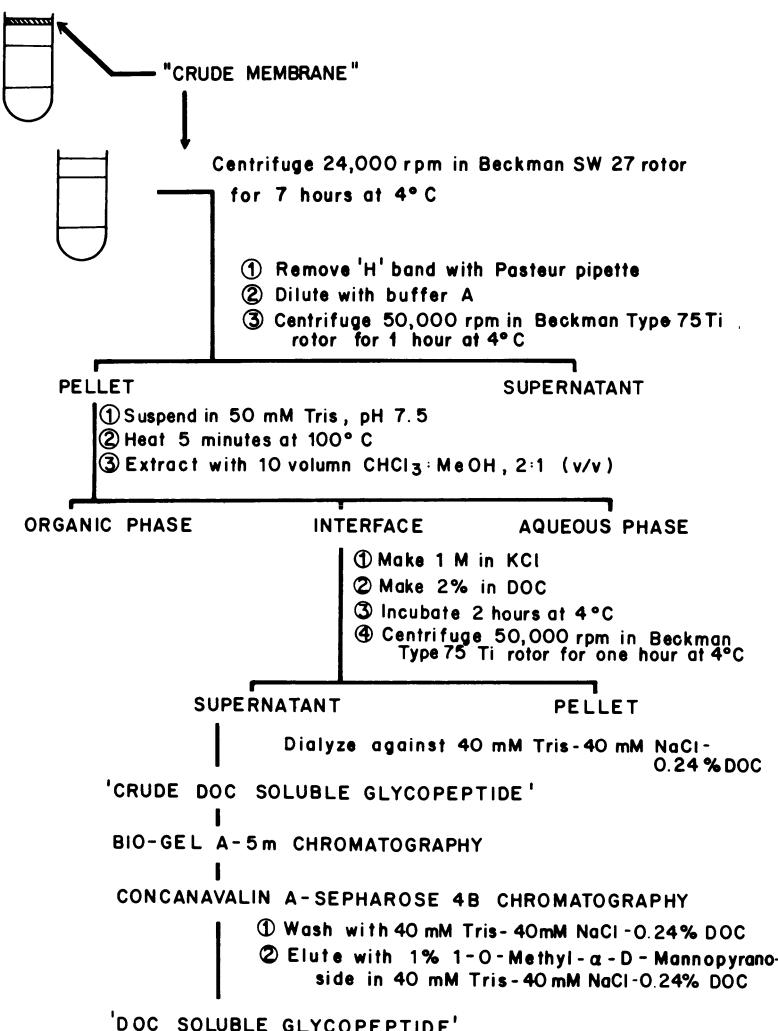


Figure 5. Flow diagram for isolation of lipo-glycopeptide obtained from membrane fraction V,  $\rho = 1.18$  g/cc.

each was determined.<sup>46</sup> Mannosyltransferase activity was located primarily in the low density membranes and galactofuranosyl residues were located in membranes of density 1.18 g/cc. The galactofuranosyl-containing substances were solubilized with KC1:deoxycholate and purified by chromatography on BioGel A 5m followed by chromatography on concanavalin A-Sepharose 4B<sup>46</sup> (Fig. 5). The galactofuranosyl-containing polymer was amphipathic.

The composition of this polymer was determined and it was found to contain 26 percent protein, a galactofuranosyl:mannopyranosyl ratio of 3:2, an estimated mass of about 105,000 daltons, an amino acyl composition similar to that of exocellular and soluble glycopeptide except seryl and threonyl residues, 24 and 19, respectively, account for only 38 percent of the total amino acyl residues. Treatment with alkali resulted in the loss of 11 seryl and 9 threonyl residues with the release of primarily mannobiose. No phosphogalactomannan was obtained. This suggests that the hydrophobic portion of the molecule was attached to this region of the polymer. Only 49 percent of the carbohydrate applied to BioGel P-2 column was recovered.

Evidence that the membrane-bound glycopeptide(s) contained a hydrophobic substance not present in the soluble glycopeptide was obtained by adding [1-<sup>14</sup>C]acetate to the growth medium and the glycopeptides from the medium, soluble cytoplasmic fraction and membranes from 3-day cultures of Penicillium charlesii were isolated (Table 2). Glycopeptide obtained from the growth medium and that in the soluble-cytoplasmic fraction contained 2,000-3,000 cpm/ $\mu$ mole of hexosyl residues.<sup>46</sup> In contrast, the membrane-bound glycopeptide contained 35,000 cpm/ $\mu$ mole of hexosyl residues. The <sup>14</sup>C was not released from the glycopeptide by alkaline conditions used to saponify fatty acyl esters or steryl esters. The <sup>14</sup>C was released by conditions required for releasing sphingosine bases, or their derivatives from sphingolipids. Although the <sup>14</sup>C migrated like sphingosine in two solvent systems when the CHCl<sub>3</sub>:methanol extract obtained following treatment of the <sup>14</sup>C-labeled glycopeptide with 4 N NaOH at 110°C for 5 hours, was subjected to thin layer chromatography in four solvent systems, the <sup>14</sup>C migrated unlike sphingosine in the other two solvent systems. Treatment of the <sup>14</sup>C-containing substances with IO<sub>4</sub><sup>-</sup> resulted in the formation of a product which, based on

Table 2.  $^{14}\text{C}$  from  $[1-^{14}\text{C}]$ acetate incorporated into deoxycholate-soluble glycopeptide

| Source of glycopeptide <sup>a</sup> | $^{14}\text{C}/\mu\text{mole Anhydrohexose}$ |
|-------------------------------------|--|
| Growth medium                       | $\text{cpm} \times 10^{-3}$<br>1.9           |
| Supernatant                         | 2.7  |
| Deoxycholate-soluble                | 35.3   |

<sup>a</sup>Glycopeptides were isolated and purified from cultures that were grown in four flasks under standard conditions. Twenty-four hours after inoculation 1 mCi  $[1-^{14}\text{C}]$ acetic acid (58 mCi/mmole) was added to each of two flasks.

preliminary analyses by gas chromatography-mass spectrometry, suggests that the base is phytosphingosine or dehydrophingosine. Dehydrophingosine occurs to the extent of 3 percent of the sphingolipid bases in yeasts.<sup>47</sup>

The position of attachment of the hydrophobic moiety or moieties to the phosphogalactomannan is unknown, nor do we know what signal serves to release the peptidophosphogalactomannan from lipo-peptidophosphogalactomannan. The occurrence of lipo-peptidophosphogalactomannan seems to be restricted to membranes with a density of about 1.2 g/cc.<sup>46</sup> The organelles from which these membranes are derived are unknown, but it seems unlikely that they are either endoplasmic reticulum or Golgi as they have little or no GDP-D-mannose mannosyl transferase activity. Schibeci *et al.*<sup>47</sup> fractionated plasma membranes from yeast protoplasts into two major bands with buoyant densities of 1.15-1.17 and 1.17-1.19 g/cc. Thus, the membranes obtained from Penicillium charlesii<sup>46</sup> may be derived from plasma membrane.

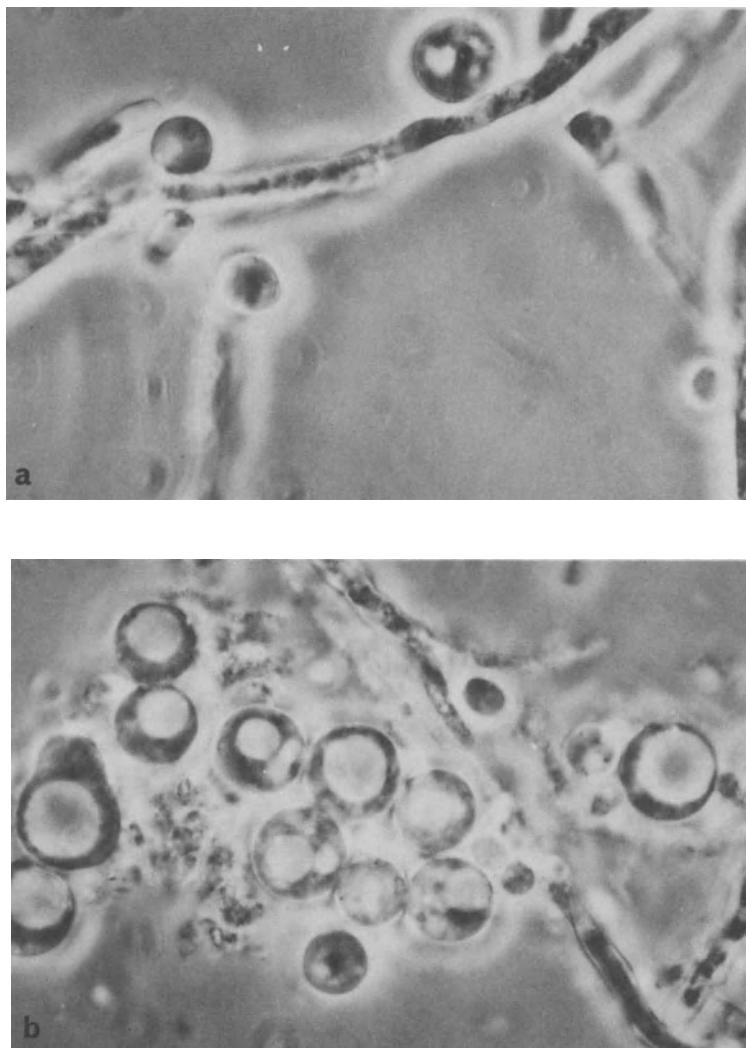


Figure 6. *Penicillium charlesii* hyphae and protoplasts a) 0.5 hour and b) 2.5 hours after initiation of treatment with  $\beta$ -D-glucuronidase (3 mg/ml) and cellulysin (20 mg/ml) in 50 mM maleate-0.5 M KC1, pH 6.0 buffer. 980x magnification.

## LOCATION OF LIPO-PEPTIDOPHOSPHOGALACTOMANNAN

Experiments were conducted to locate lipo-glycopeptide in Penicillium charlesii. Protoplasts were prepared by treating hyphae which were obtained after culturing the fungus for 42 hours, with a mixture of cellulysin (20 mg/ml) and  $\beta$ -D-glucuronidase (3 mg/ml) in 50 mM maleate-0.5 M KC1 buffer, pH 6.0 for 2.5 hours (Laybourn and Gander, unpublished). A typical protoplast preparation obtained after treatment with cellulysin and  $\beta$ -D-glucuronidase for 0.5 and 2.5 hours is shown in Figure 6a and 6b, respectively. The protoplasts were washed with maleate-KC1 buffer and filtered through Pellon to remove the debris (Fig. 7). A preparation was fixed with glutaraldehyde, sectioned and stained with OsO<sub>4</sub>. The preparation contained protoplasts which were essentially free of cell walls and protoplasts which had started resynthesis of cell wall material, (spheroplasts) (Fig. 8a and 8b, respectively).

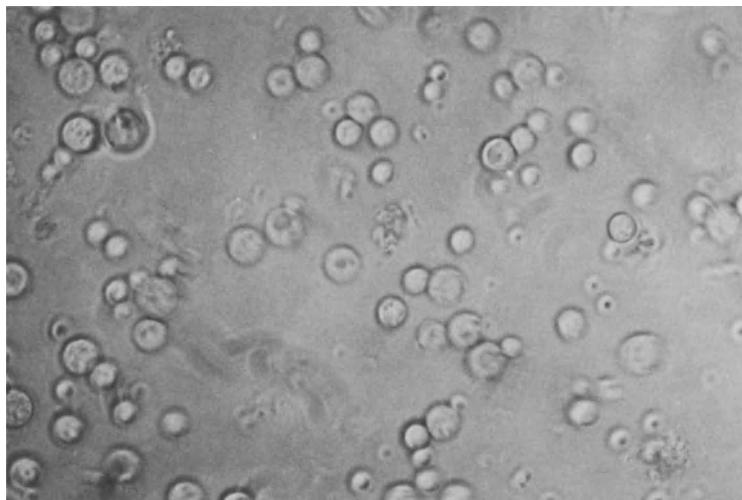


Figure 7. Protoplasts obtained from P. charlesii hyphae after removal of cell debris by filtration through medium porosity Pellon. 980x magnification.

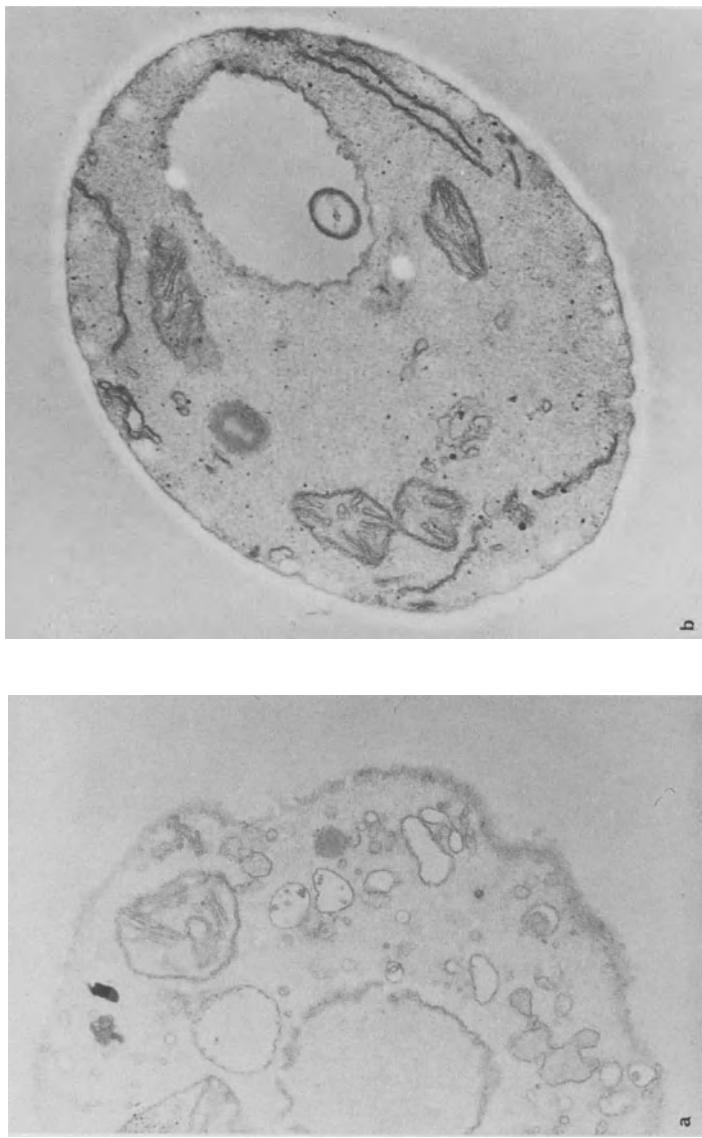


Figure 8. Electron micrograph of a) protoplast and b) spheroplast from Penicillium charlesii hyphae; 55,000 $\times$  magnification. The protoplast/spheroplast preparation was fixed with 2.5% (v/v) glutaraldehyde and 2.3% (w/v) formaldehyde in the 50 mM maleate-0.5 M KCl, pH 6.0 buffer. The protoplasts were rinsed in this buffer and post fixed in 1% (w/v)  $OsO_4$  and buffer. The preparation was embedded in Spurr's (Fullam), cut with a diamond knife on a Sorvall NT2B, stained in alcoholic uranyl acetate and post-stained with lead citrate. Sections were observed with a Hitachi model HU11C electron microscope.

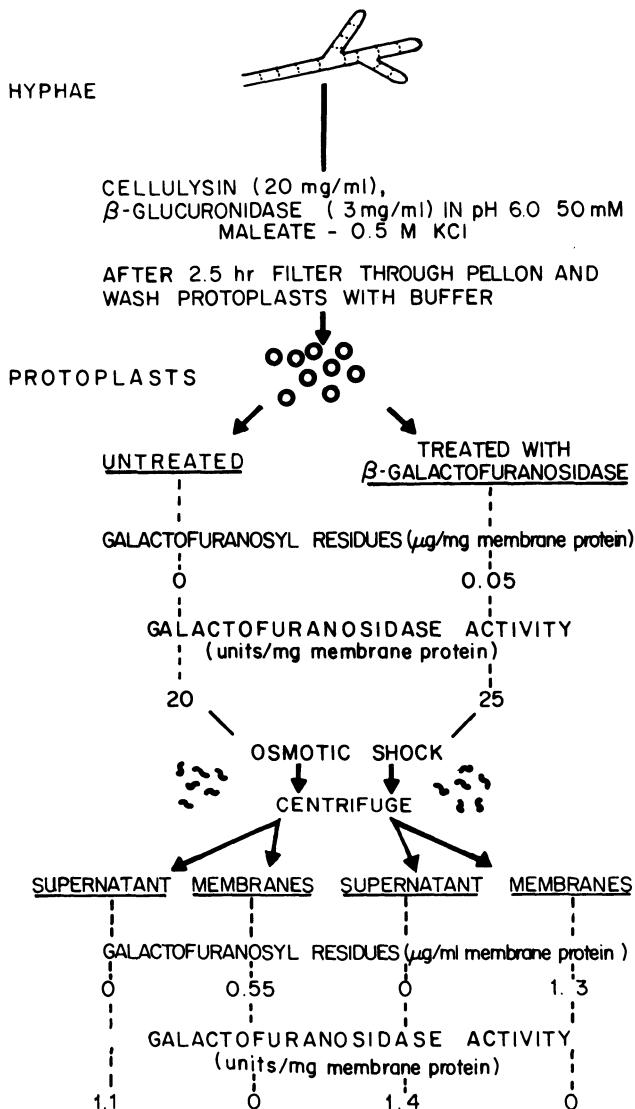


Figure 9. Distribution of lipo-glycopeptide and exo- $\beta$ -D galactofuranosidase in *Penicillium charlesii* protoplasts.

Investigations were undertaken to locate the galactofuranosyl-containing substances and exo- $\beta$ -D-galactofuranosidase in the protoplast/spheroplast preparations. Protoplasts were either pretreated with added exo- $\beta$ -D-galactofuranosidase at pH 4 for 24 hours or held for 24 hours at pH 4 without added exo- $\beta$ -D-galactofuranosidase. This treatment released 0.05  $\mu$ g of galactofuranosyl residues from the protoplasts treated with galactofuranosidase (Fig. 9). The protoplast preparation was washed with acetate-KCl buffer and exo- $\beta$ -D-galactofuranosidase activity was measured. Both preparations contained about 20 units of enzyme activity (Fig. 9). Separate samples of galactofuranosidase treated and untreated protoplasts were lysed by transferring them to distilled, deionized H<sub>2</sub>O and the membranes were separated from the soluble cytoplasmic material by ultracentrifugation. Membrane and soluble cytoplasmic fractions were analyzed for galactofuranosyl residues and for galactofuranosidase activity. Galactofuranosyl residues were found in the membranes but not in the soluble cytoplasmic fraction. The membranes derived from protoplasts which were pretreated with galactofuranosidase contained 1.3  $\mu$ g of galactofuranosyl residues per mg of protein in the membranes as compared with only 0.55  $\mu$ g in the membranes which were untreated. Pretreatment with galactofuranosidase may have made the membranes more susceptible to the action of proteases so that the relative quantity of membrane protein per unit of membrane was less in those protoplasts which were pretreated with galactofuranosidase. In contrast to the location of galactofuranosyl residues, galactofuranosidase activity was found only in the soluble cytoplasmic fraction (supernatant), and the activity per unit of membrane protein was decreased considerably from that observed in the intact membranes. This suggests that activity was destroyed during lysis and separation of the membranes from the soluble substances. This destruction may have been the result of proteolysis.

The membranes obtained following lysis were fractionated by isopycnic sucrose gradient ultracentrifugation. Galactofuranosyl residues were found primarily in fractions with densities of 1.21 to 1.24 g/cc and negligible quantity was found in the lighter membranes (Fig. 10). These data are consistent with those obtained with membranes isolated from homogenates of mycelia.<sup>46</sup> We conclude that the galactofuranosyl-containing lipoglycopeptide is located at least in

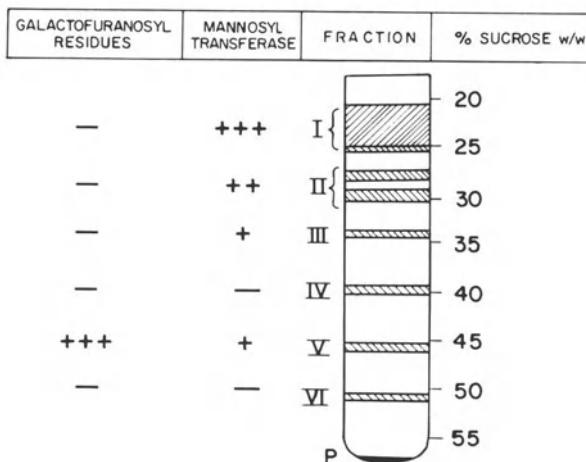


Figure 10. Separation of membranes of Penicillium charlesii protoplasts and location of galactofuranosyl residues and mannosyltransferase activity.

part on the outer surface of the plasma membrane, but that the larger portion of lipo-glycopeptide may be attached to an organelle which fuses with the plasma membrane.

#### PROPOSED FUNCTION OF EXOCELLULAR PEPTIDOPHOSPHOGALACTOMANNAN

These results when coupled with those presented previously<sup>46</sup> show that at least two types of galactofuranosyl-containing substances occur in Penicillium charlesii: a) a membrane-bound lipo-glycopeptide and b) soluble glycopeptides. The data suggest that lipoglycopeptide is the precursor of the soluble glycopeptides; that is, the polypeptide constitutes a greater portion of the total mass in the lipo-glycopeptide than in the soluble forms. The lack of galactofuranosyl-containing substances in the soluble-cytoplasmic fraction of protoplasts suggest that the "soluble cytoplasmic" glycopeptide obtained from mycelia<sup>46</sup> is not intracellular but instead represents glycopeptide released from the plasma membrane. This glycopeptide is probably that which is in transit from the surface of the membrane to the outside of the cell wall and is only loosely associated with the cell wall. During the transition of the glycopeptide from its membrane-bound form to exocellular

glycopeptide the polypeptide becomes degraded by proteases located on the outside surface of the plasma membrane, in the cell wall, and in the growth medium. This conclusion is consistent with the observation that the number of amino acyl residues in the polypeptide region decrease from about 100 in lipo-glycopeptide to 30 in exocellular glycopeptide. The data suggest that this serves as a mechanism for degrading fungal glycoproteins in regions of hyphae containing older cells, when the culture becomes deficient in N-containing nutrients. The degradation process may occur primarily on the outer surface of the plasma membrane, a process which serves to release amino acids out into the medium. The released amino acids are then available to the growing hyphal tips. The heterogeneity observed in the polypeptide region of exocellular glycopeptide<sup>44</sup> suggests that the lipo-glycopeptide is derived from many intracellular glycoproteins. This process may represent the mechanism by which Ascomycetes couple turnover of glycoproteins to conservation of N-containing substances until these substances are needed by growing and new members of the fungal colony. This conclusion is also consistent with the onset of appearance of proteases in the medium.

The role of phosphogalactomannan in this process may be of prime importance. It may contain the necessary recognition sites required for endocytosis by appropriate organelle(s) of the glycoproteins to be turned over. These organelles may fuse with the plasma membrane; a process which would place the glycoprotein on the outside surface of the plasma membrane.

Currently we do not know whether the phosphogalactomannan is put onto the glycoprotein as one unit or if it is derived from mannoproteins which are modified by the addition of galactofuranosyl and the appropriate phospho-2-aminoethanol derivative(s). Experiments are in progress to resolve this question.

The exocellular glycopeptide may have an important role in maintaining adequate minimal quantities of key nutrients so that the Penicillium can survive when the growth medium becomes nearly depleted of these nutrients. The sequence of events, as they are currently known, following formation of lipo-glycopeptide and release of exocellular glycopeptide and exo- $\beta$ -D-galactofuranosidase are

summarized in Figure 11. Following depletion of  $\text{NH}_4^+$  exo-cellular glycopeptide is released from the organism and diffuses away. Proteases, phosphatases, and some glycosidases are also released. Following depletion of glucose, the pH increases and organic acids are utilized by the fungus. These conditions result in the release of exo- $\beta$ -D-galactofuranosidase (Pletcher, Lomar and Gander, in press). Degradation of the galactan region of exocellular glycopeptide provides sufficient carbohydrate for survival of the Penicillium. The glycopeptide also contains other substances that may be critical for survival of the organism; that is, substances like phosphorus, and 2-aminoethanol and its methyl derivatives. We believe that "survivalin" is an

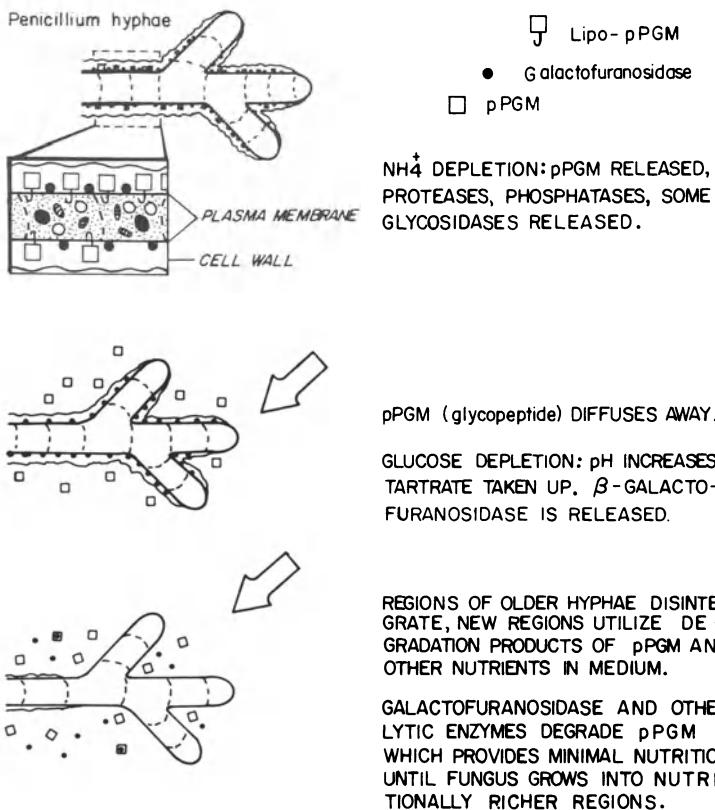


Figure 11. Model of proposed lipo-glycopeptide metabolism in Penicillium charlesii.

appropriate descriptive name for this group of glycopeptides as they undoubtedly provide quantities of carbohydrate, N-containing substances and phosphate sufficient to allow the organism to survive in a nutritionally deficient environment.

The exocellular glycopeptide may also have a role in directing growth of Penicillium toward a new nutrient source. The proposed roles of exo- $\beta$ -D-galactofuranosidase and exocellular glycopeptide in directing growth on a solid surface is summarized in Figure 12. This figure shows a sequence of events in which the glycopeptide is released and diffuses on the surface away from the fungal colony. As the colony depletes available carbohydrate in its growth region, exo- $\beta$ -D-galactofuranosidase is released and also diffuses over the surface. If a polymer-containing nutrient source is located on the surface then exo- $\beta$ -D-galactofuranosidase will be concentrated on the polymer if the enzyme binds tightly to any of the polymers. Increased enzyme activity will result in increased rate of galactose release from exocellular glycopeptide which, in turn, will establish a gradient of galactose concentration back to the fungal colony. The fungus will grow along with the gradient of galactose toward the nutrient source.

This model suggests that a portion of the specificity between host plant and pathogenic fungus may reside with the ability of the glycohydrolase released when the nutrient source of carbohydrate becomes depleted, to bind to polymers which are secreted by the plant. Dow and Callow suggest that exocellular fungal glycopeptide from Fulvia fulva binds to the tomato leaf and increases permeability of phosphate which results in its release from the cell.<sup>34</sup> However, binding of glycopeptide to the leaf plasma membrane was not specific for race of pathogen or cultivar of host.

#### CONCLUDING REMARKS

This chapter reviews the status of work in our laboratory on the structure and function of the galactofuranosyl- and phospho-2-aminoethanol-containing fungal glycopeptides. We suggest that the glycopeptides are derived from a series of glycoproteins and that these glycoproteins are, at least in part, degraded on the outside of the plasma membrane but only after the medium becomes essentially depleted of its

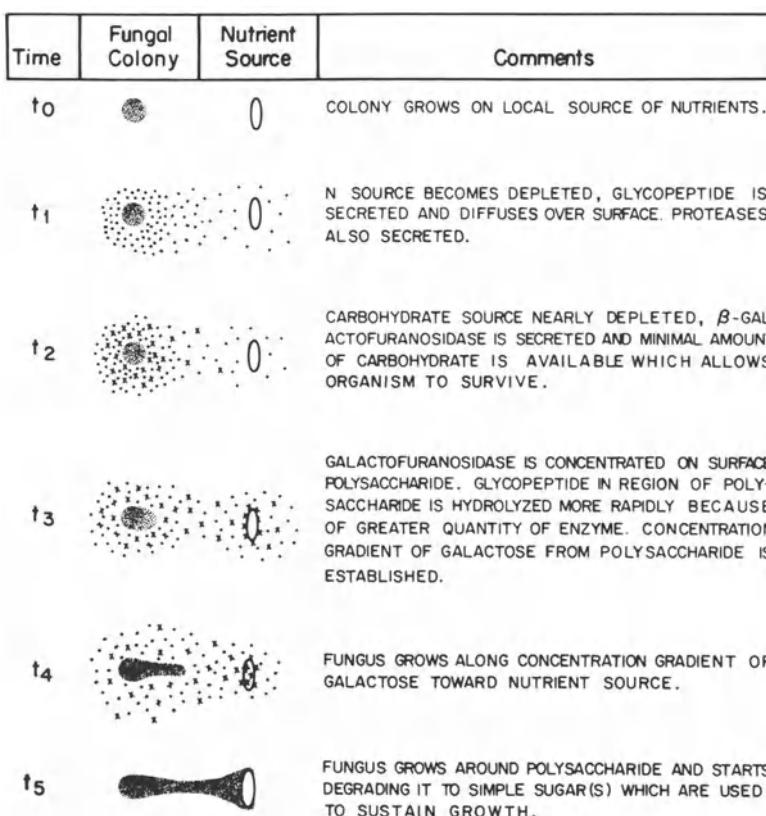


Figure 12. Proposed role for exocellular glycopeptide in directing growth of Penicillium.

N-containing nutrient(s). Thus the process of protein turnover is coupled to conservation of nitrogen, particularly amino acids, to allow the growing regions of the fungal colony to survive even under nutritionally stressful conditions. We have suggested that this class of glycopeptides might be called "survivalins" because of their role in the survival of the fungus when it is subjected to nutritionally stressful conditions. We also suggest three functions for the phosphogalactomannan region of the glycopeptides: a) it contains the necessary recognition sites

for the endocytosis and exocytosis process which must occur in transporting protein into specific organelles within the cell and exporting these lipo-glycoproteins out of the cell, respectively, b) it provides a minimal source of amino acids, carbohydrates and other nutrients as the medium in the region of the colony becomes depleted of these substances, and c) it aids in directing the growth of the organism toward a new source of nutrients. The model will undoubtedly need to be refined and the details of the various processes need to be defined in terms of the molecular events that occur and the regulation of these events. However, the general model presented is currently serving as a working hypothesis in our studies on the structure and function of lipopeptidophosphogalactomannans in Ascomycetes.

#### ACKNOWLEDGEMENT

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## Chapter Five

### ENZYMATIC PROPERTIES OF PHYTOHEMAGGLUTININS

LELAND M. SHANNON AND CHARLES N. HANKINS

Department of Biochemistry  
University of California  
Riverside, CA 92521

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### INTRODUCTION

Carbohydrate binding proteins that interact with animal cells and agglutinate them (hemagglutinins, lectins) have been under study for almost 100 years.<sup>1</sup> Best characterized are those hemagglutinins which have been isolated from plant species, particularly those isolated from species of the Leguminosae. Although hundreds of legume species contain hemagglutinins<sup>2</sup> and many have been purified and characterized,<sup>3</sup> the physiological function(s) of these proteins is unknown. Since the relationships among hemagglutinins from different legume species have only recently come under careful scrutiny, functional classification of these proteins is not possible at this time.

The recent discovery<sup>4, 5</sup> that certain legume enzymes possess hemagglutinin properties opened an exciting area of

research with new avenues of approach to the general study of plant hemagglutinins. Here we summarize what is currently known about enzymatic-hemagglutinins, how they are related to the "classic" legume hemagglutinins and, importantly, how the study of these proteins has led to new, useful approaches for the study of hemagglutinins. The literature of hemagglutinins has been reviewed in several<sup>3,6,7</sup> excellent, recent articles. This information is useful in the development of generalizations and provides an overview of the prospective from which we approach our studies.

#### $\alpha$ -GALACTOSIDASE-HEMAGGLUTININS

Crude seed extracts of mung bean (*Vigna radiata*) contained a potent agglutinin of trypsinized<sup>8</sup> rabbit erythrocytes. These extracts showed no activity with normal or trypsinized human (A, B or O) erythrocytes or with untrypsinized rabbit cells. They did exhibit two unique characteristics. First, hemagglutinin activity (MBH) was inhibited by xylose and myo-inositol, as well as galactose, a specificity unlike that encountered in previously described hemagglutinins. Secondly, these extracts possessed an interesting property, referred to here as "clot-dissolving" activity.<sup>4</sup>

Since observation of clot-dissolving activity led directly to the discovery of  $\alpha$ -galactosidase-hemagglutinins and also provided a diagnostic test for the presence of this type of hemagglutinin, this phenomenon is described in some detail. When a concentrated crude extract of mung bean seeds was mixed with rabbit erythrocytes, the cells were totally agglutinated within one minute, but within ten minutes the cells disaggregated and all evidence of hemagglutinin disappeared. Upon addition of a fresh sample of extract to the disaggregated cells they failed to re-aggregate. If instead, fresh erythrocytes were added to the disaggregated cell mixture (or a dilution of same) agglutination occurred. These observations suggested that the dissolution of cell aggregates was due to an alteration of rabbit erythrocytes rather than an inactivation of the hemagglutinin.

When normal titer assays (serial two-fold dilutions) were performed, the first dilution (most concentrated) gave rise to cell aggregates which disappeared (disaggregated)

after about ten minutes. In the second serial dilution agglutination persisted for about twenty minutes while in the third dilution series aggregates were no longer evident at the end of one hour. Agglutination was observed in all subsequent dilutions to a final dilution of 1:2000 (titer = 2000). For twenty-four hours, the final titer remained unchanged (2000), but cell aggregates in the fourth, fifth and sixth serial dilutions had successively disappeared. The final result of a twenty-four hour titer assay, then, was agglutination in titer wells corresponding to dilutions of about 1:128 to about 1:2000, but no agglutination at dilutions of less than about 1:128.

The observations described above were made at room temperature, but similar studies were done at 5°C and 37°C. These studies revealed that the time required for a clot (agglutinated cells) to disappear was extended at lower temperatures (3 to 4 times longer at 5°C than at 22°C), but considerably shortened at higher temperatures (about 1/2 as long at 37°C as at 22°C).

The temperature and concentration dependence of clot-dissolving activity suggested that it might be the result of an enzymatic process. Therefore, the next step was to examine mung bean extracts for glycosidase activities in an attempt to determine if one or more of these enzymes were responsible for clot dissolution. Glycosidases were assayed using nitrophenylglycosides as substrates. Carbohydrates were then tested for their ability to specifically inhibit each activity that was found. If a specific glycosidase were involved in clot dissolution, such inhibitors might be used to block clot-dissolving activity during the hemagglutinin titer assay.

When the  $\alpha$ -galactosidase activity in mung bean extracts was examined it was found to be inhibited by galactose, xylose and myo-inositol, a specificity identical to that displayed by the hemagglutinin activity. Further, the substrate used for the assay of  $\alpha$ -galactosidase, p-nitrophenyl  $\alpha$ -galactoside was a very potent inhibitor of the hemagglutinin activity. Other carbohydrates were tested but none were found which inhibited clot-dissolving activity or  $\alpha$ -galactosidase activity without also inhibiting the hemagglutinin.

Since the hemagglutinin and  $\alpha$ -galactosidase activities displayed such similar specificities it appeared possible that the enzyme might be recognizing and enzymatically altering these cellular sites recognized by the hemagglutinin. Further studies along these lines, however, required that the activities be purified.

#### Mung Bean $\alpha$ -Galactosidase-Hemagglutinin

Attempts to separate mung bean  $\alpha$ -galactosidase from hemagglutinin activity were unsuccessful. Both activities exactly co-purified by every separation method tested. A combination of ion exchange chromatography and gel filtration produced a preparation that contained a single protein species with very high specific enzyme and hemagglutinin activities. A summary of the overall purification is given in Table 1.

The native protein had a molecular weight of about 160,000 and appeared to be composed of a single type of subunit (45,000 mol. wt). The mung bean enzymatic hemagglutinin (MBH) was, therefore, assumed to be a tetramer of identically sized subunits.<sup>4</sup>

Table 1. Summary of the purification of mung bean enzymatic-hemagglutinin

| Step                         | Volume | ?    | Protein Hemagglutinin $\alpha$ -Galactosidase Ratio |                         | A/B    |
|------------------------------|--------|------|---|-------------------------|--------|
|                              |        |      | ml  | mg/ml                   |        |
| Crude                        | 2200   | 17.7 |   | 4.5<br>$\times 10^{-3}$ | 3.84   |
| $(\text{NH}_4)_2\text{SO}_4$ | 300    | 57.0 |   | 6.3                     | 5.15   |
| CM pool                      | 93     | 0.16 |   | 4,500                   | 4,750  |
| S-200 pool                   | 18.5   | 0.13 |   | 18,600                  | 14,400 |

Attempts to selectively inactivate one of the activities of the MBH by the use of heat or p-chloromercuribenzoate were unsuccessful. In fact, both activities were equally sensitive to these treatments and equal protection from inactivation was provided by galactose, xylose and myo-inositol.<sup>4</sup>

MBH displayed linear Michaelis-Menten kinetics with galactose, xylose or myo-inositol acting as competitive inhibitor of p-nitrophenyl  $\alpha$ -galactoside utilization. A summary of the kinetic parameters obtained is given in Table 2. The carbohydrate specificity of the hemagglutinin activity (Table 3) was virtually identical both qualitatively and quantitatively to that of the enzyme.

Attempts to separate the enzymatic and hemagglutinin activities by adsorption on rabbit erythrocytes were unsuccessful.<sup>4</sup> Both of these activities were adsorbed by cells under conditions favorable to hemagglutinin activity and both were released from cells under conditions favoring enzymatic activity. Further, both activities were released from rabbit cells following the addition of galactose, xylose or myo-inositol.

Recent studies revealed that MBH can be reversibly converted by pH shifts, from a tetrameric, enzymatically active hemagglutinin to an enzymatically active monomeric protein that is devoid of hemagglutinin activity.<sup>9</sup> This  $\alpha$ -galactosidase therefore, also exists in two different molecular weight forms as has been shown to be the case with the enzymes from Vicia faba<sup>10</sup> and soybeans.<sup>11</sup> Although both monomeric and tetrameric forms of MBH are enzymically active, they display quite different pH optima, kinetic properties and carbohydrate specificities.<sup>9</sup>

#### Soybean $\alpha$ -Galactosidase Hemagglutinin

Soybean (Glycine max.) seeds and leaves were found to contain a clot-dissolving hemagglutinin activity.<sup>12</sup> In seeds, this activity cannot be visualized without first inhibiting or removing (by affinity chromatography) SBA, the well characterized N-acetylgalactosamine specific hemagglutinin of soybeans. The soybean clot-dissolving hemagglutinin has been purified to homogeneity by both conventional procedures and by affinity chromatography on melibiose

Table 2. Kinetic parameters of the  $\alpha$ -galactosidases in several legumes<sup>1</sup>

| Non-Hemagglutinin<br>Forms | $K_m$               |               | $K_i$           |              |                |
|----------------------------|---------------------|---------------|-----------------|--------------|----------------|
|                            | p-Nitrophenol<br>mM | $\alpha$ -Gal | Galactose<br>mM | Xylose<br>mM | Inositol<br>mM |
| Amorpha                    | 1.25                |               | 0.53            | 2.95         | 12.6           |
| Bandeiraea s.              | 1.11                |               | 0.62            | 4.45         | 14.7           |
| Bauhinia p.a.              | 1.00                |               | 0.50            | 3.10         | 15.8           |
| Caragana a.                | 0.80                |               | 0.61            | 3.85         | 9.5            |
| Cercis s.                  | 1.82                |               | 0.47            | 3.40         | 9.0            |
| Colutea a.                 | 0.44                |               | 0.37            | 3.20         | 17.3           |
| Conavalia e.               | 1.18                |               | 0.22            | 1.75         | 9.5            |
| Cytisus m.                 | 1.00                |               | 0.30            | 1.85         | 6.1            |
| Dolichos b.                | 1.18                |               | 0.51            | 3.60         | --             |
| Genista m.                 | 0.58                |               | 0.35            | 1.60         | 11.0           |
| Laburnum a.                | 1.43                |               | 0.53            | 2.75         | 14.7           |
| Lathyrus l.                | 1.33                |               | 0.61            | 3.50         | 8.7            |
| Lens c.                    | 1.21                |               | 1.19            | 3.30         | 6.9            |
| Lespedeza b.               | 0.47                |               | 0.35            | 2.70         | 11.7           |
| Mimosa p.                  | 0.65                |               | 0.38            | 2.2          | 11.6           |
| Phaseolus v.               | 0.71                |               | 0.50            | 3.15         | 12.8           |
| Sophora j.                 | 0.57                |               | 0.39            | 2.25         | 9.0            |
| Spartium j.                | 1.66                |               | 0.45            | 3.55         | 11.4           |
| Ulex e.                    | 0.67                |               | 0.37            | 1.75         | 10.0           |
| Wistaria s.                | 0.65                |               | 0.58            | 3.65         | 17.8           |
| <u>Hemagglutinin forms</u> |                     |               |                 |              |                |
| Mung bean                  | 0.20                |               | 0.75            | 5.2          | 20.0           |
| Pueraria                   | 0.42                |               | 0.90            | 2.9          | 8.8            |
| Thermopsis                 | 0.20                |               | 0.40            | 1.6          | 8.8            |
| Lupine                     | 0.39                |               | 0.30            | 1.5          | 7.5            |
| Lima bean                  | 0.38                |               | 0.33            | 2.0          | 15.0           |
| Soybean                    | 0.33                |               | 1.06            | 3.80         | 12.9           |

<sup>1</sup>Data collected from references 4, 9, 12 and 13.

substituted sepharose.<sup>13</sup> As with the mung bean protein, this hemagglutinin was found to exactly co-purify with an  $\alpha$ -galactosidase activity.

Table 3. Inhibitor specificities of the hemagglutinin activities of several  $\alpha$ -galactosidase-hemagglutinins

| Inhibitor                                | Mung<br>beans | Soybean   | Pueraria  | Thermopsis | Lima<br>beans | Lupine<br>beans |
|--|---------------|-----------|-----------|------------|---------------|-----------------|
| p-Nitrophenyl<br>$\alpha$ -galactosidase | 0.34          | 0.1       | 1.0       | 1.0        | 3.1           | 0.6             |
| Galactose                                | 1.0           | 0.38      | 1.5       | 0.8        | 3.1           | 0.8             |
| Xylose                                   | 4.0           | 0.76      | 3.0       | 3.0        | 6.3           | 6.2             |
| Inositol                                 | 20.0          | 3.06      | 25.0      | 25         | 25            | 31              |
| Galactosamine                            | $\geq$ 50     | $\geq$ 50 | $\geq$ 50 | $\geq$ 50  | $\geq$ 50     | $\geq$ 50       |

<sup>a</sup>Minimum concentration required to totally inhibit four hemagglutinin units.

Detailed characterization of the soybean  $\alpha$ -galactosidase hemagglutinin revealed that it was nearly identical to the mung bean protein in most respects (see data in Tables 2, 3 and 4) and was immunologically indistinguishable from same.<sup>13</sup> One major difference between the two proteins was noted; the soybean  $\alpha$ -galactosidase was composed of two very similar but distinct subunits with molecular weights of about 38,000 and 40,000. The soybean protein displayed pH dependent association-disassociation behavior similar to that described for mung bean, and possesses the same aggregation dependent variations in enzymatic and hemagglutinin properties.

Soybean  $\alpha$ -galactosidase hemagglutinin is probably the same  $\alpha$ -galactosidase purified and characterized by Harpaz et al.<sup>11</sup> however, these authors did not describe any associated hemagglutinin activity, nor did they discuss the changes in enzymic properties that accompany changes in aggregation state.

Table 4. Summary of gel filtration molecular weight determinations for various legume  $\alpha$ -galactosidases

| Plant                          | $\alpha$ -Galactosidase |                                   |
|--------------------------------|-------------------------|-----------------------------------|
|                                | I                       | II <sup>1</sup>                   |
| <b>Non-hemagglutinin forms</b> |                         |                                   |
| Colutea                        | 135,000                 | 34,000                            |
| Cytisus                        | 190,000                 | 40,000                            |
| Genista                        | 160,000                 | 37,000                            |
| Laburnum                       | 155,000                 | 30,000                            |
| Lathyrus                       | 160,000                 | -- <sup>2</sup>                   |
| Lespedeza                      | 120,000                 | 31,000                            |
| Sophora                        | 150,000                 | -- <sup>2</sup>                   |
| Spartium                       | 160,000                 | 30,000                            |
| Ulex                           | 160,000                 | 42,000                            |
| <b>Hemagglutinin forms</b>     |                         |                                   |
| Mung bean                      | 160,000                 | 45,000 <sup>3</sup>               |
| Pueraria                       | 150,000                 | -- <sup>2</sup>                   |
| Thermopsis                     | 180,000                 | -- <sup>2</sup>                   |
| Lupine                         | 190,000                 | 50,000                            |
| Lima bean                      | 180,000                 | 39,000                            |
| Soybean                        | 160,000                 | 38,000 and<br>40,000 <sup>3</sup> |

<sup>1</sup> Galactosidase II may generally be representative of a monomeric (subunit) form of  $\alpha$ -galactosidase I. This appears to be the case in mung beans and soybeans.

<sup>2</sup> $\alpha$ -Galactosidase II not observed in crude extracts of these species.

<sup>3</sup>Determined by SDS gel electrophoresis of pure  $\alpha$ -galactosidase I.

Other  $\alpha$ -Galactosidase-Hemagglutinins

In a survey of 27 legume species from 26 genera four plants in addition to mung bean and soybean were found to contain clot-dissolving hemagglutinins (Table 5).<sup>12</sup> These clot-dissolving hemagglutinins exactly copurified by ion exchange and gel filtration chromatography with an  $\alpha$ -galactosidase activity.<sup>12</sup> From the data in Tables 2, 3, and 4, it can be seen that the physical, enzymatic and hemagglutinin properties of these proteins are very nearly identical to each other and to MBH.

The  $\alpha$ -galactosidase hemagglutinin in lima bean is a distinctly different protein from the well characterized N-acetylgalatosamine specific hemagglutinin that is present in lima bean.<sup>12</sup> We have examined three lima bean varieties (Burpee "Bush", "Fordhook", and "Sieva") and found the clot-dissolving, enzymic hemagglutinin in all three. The N-acetylgalactosamine specific lima bean hemagglutinin however, appears to be present in significant quantities only in the "Sieva" variety of lima bean.

Non-Agglutinating  $\alpha$ -Galactosidases

From Table 5 it can be seen that most legume species examined did not contain clot-dissolving hemagglutinin activity. In fact many plants appeared to be devoid of hemagglutinin activity altogether.<sup>14</sup> All species examined, however, contained  $\alpha$ -galactosidase activity. The kinetic and physical properties of the non-agglutinating  $\alpha$ -galactosidases were examined to determine if they were related to the hemagglutinating forms.

Kinetic studies (summarized in Table 2) showed that the enzyme in each extract displayed simple Michaelis-Menton substrate utilization with competitive inhibition by galactose, xylose and myo-inositol.<sup>14</sup> The striking feature of this data is the remarkable similarities, not only among the non-agglutinating  $\alpha$ -galactosidases, but also between these forms and the  $\alpha$ -galactosidase-hemagglutinins.

Gel filtration experiments revealed that all plants examined contained a large molecular weight enzyme activity and many also contained a small molecular weight form. From the data in Table 4 one can readily see the similarities in

Table 5.  $\alpha$ -Galactosidase and related activities in several legume species

| <u>Plant</u>                    | $\alpha$ -Galactosidase<br>units/g dry seed | Hemagglutinin<br>activity of<br>any kind | Clot-dissolving<br>hemagglutinin<br>activity | MBH-CRM         |
|---------------------------------|---|--|--|-----------------|
| <i>Amorpha fruticosa</i>        | 22  | No <sup>1</sup>                          | No   | No <sup>2</sup> |
| <i>Cercis siliquastrum</i>      | 39  | No                                       | No   | Yes             |
| <i>Colutea arborescens</i>      | 104   | No                                       | No   | Yes             |
| <i>Genista monosperma</i>       | 450   | No                                       | No   | Yes             |
| <i>Lespedeza bicolor</i>        | 300   | No                                       | No   | No              |
| <i>Mimosa pudica</i>            | 75  | No                                       | No   | Yes             |
| <i>Spartium junceum</i>         | 50  | No                                       | No   | Yes             |
| <i>Gymnocladus</i>              | 4   | No                                       | No   | No              |
| <br>                            |   |  |  |                 |
| <i>Vigna radiata</i>            | 111   | Yes                                      | Yes  | Yes             |
| <i>Glycine max</i>              | 62  | Yes                                      | Yes  | Yes             |
| <i>Pueraria thunbergiana</i>    | 110   | Yes                                      | Yes  | Yes             |
| <i>Thermopsis caroliniana</i>   | 116   | Yes                                      | Yes  | Yes             |
| <i>Lupinus arboreus</i>         | 43  | Yes                                      | Yes  | Yes             |
| <i>Phaseolus limensis</i>       | 26  | Yes                                      | Yes  | Yes             |
| <br>                            |   |  |  |                 |
| <i>Bandeiraea simplicifolia</i> | 20  | Yes                                      | No   | No              |
| <i>Bauhinia purpurea alba</i>   | 72  | Yes                                      | No   | Yes             |
| <i>Caragana arborescens</i>     | 200   | Yes                                      | No   | Yes             |
| <i>Conavalia ensiformis</i>     | 225   | Yes                                      | No   | No              |
| <i>Cytisus multiflorus</i>      | 36  | Yes                                      | No   | Yes             |
| <i>Dolichos biflorus</i>        | 60  | Yes                                      | No   | Yes             |
| <i>Lathyrus latifolia</i>       | 300   | Yes                                      | No   | Yes             |
| <i>Lens culinaris</i>           | 175   | Yes                                      | No   | No              |
| <i>Phaseolus vulgaris</i>       | 60  | Yes                                      | No   | Yes             |
| <i>Sophora japonica</i>         | 140   | Yes                                      | No   | Yes             |
| <i>Wistaria sinensis</i>        | 180   | Yes                                      | No   | Yes             |

<sup>1</sup> As determined by the visual agglutination of trypsinized rabbit or human (A, B and O) erythrocytes.

<sup>2</sup> As determined by Ouchterlony double diffusion of extracts with antisera raised against pure Vigna  $\alpha$ -galactosidase-hemagglutinin.

molecular weights, no only among the non-hemagglutinin forms, but between these enzymes and the  $\alpha$ -galactosidase hemagglutinins.

#### Immunochemical Studies

Immunological evidence has been presented which reveals that the various  $\alpha$ -galactosidase-hemagglutinins are evolutionarily closely related.<sup>12</sup> In fact, those from mung bean and soybean appear to be identical immunologically.<sup>13</sup> Similarities in physical and kinetic properties among these proteins as well as their related immunological properties suggest a strong homology.

A survey of several legume species (Table 5) revealed that seed extracts of most plants tested contained cross-reacting materials (CRM) which were immunologically related to MBH (MBH-CRM), even though most of the plants did not contain  $\alpha$ -galactosidase hemagglutinins. Immunoaffinity chromatography was used to determine if the non-agglutinating  $\alpha$ -galactosidases were responsible for the MBH-CRM seen in these plants. In this method IgG was purified from both anti-MBH sera and pre-immune sera. Each IgG was then coupled to sepharose 4B. Crude extracts from different legume species, all containing  $\alpha$ -galactosidase activity, were passed over columns of pre-immune IgG sepharose and anti-MBH IgG sepharose. The results (Table 6) show that  $\alpha$ -galactosidase activity is specifically absorbed from every extract by anti MBH IgG but not by pre-immune IgG sepharose. No specific binding was seen for several other glycosidases tested. By using a larger quantity of column adsorbant or by prolonged incubation of the extracts with adsorbant, virtually 100% of the  $\alpha$ -galactosidase activity could be adsorbed from each of the extracts. These studies<sup>14</sup> clearly indicate that non-agglutinating  $\alpha$ -galactosidases are closely related immunologically to the  $\alpha$ -galactosidase hemagglutinins. This result coupled with the great similarities in their physical and kinetic properties suggests that both types of  $\alpha$ -galactosidases are members of one specific functional class of protein (i.e., they are homologues).

The evidence collected to date indicates that the  $\alpha$ -galactosidase hemagglutinins and the "classic" legume hemagglutinins are members of distinct classes of proteins.<sup>12</sup> Immunological studies<sup>15</sup> reveal, however, that these two

Table 6. The binding of  $\alpha$ -galactosidase activities in crude legume extracts to anti Vigna IgG-Sepharose

| Plant        | Protein | % Specific Binding*     |                        |                      |                       |
|--------------|---------|-------------------------|------------------------|----------------------|-----------------------|
|              |         | $\alpha$ -Galactosidase | $\beta$ -Galactosidase | $\beta$ -Glucosidase | $\alpha$ -Mannosidase |
| 1. Sophora   | 0       | 95                      | 2                      | 0                    | 0                     |
| 2. Lespedeza | 0       | 70                      | 9                      | 0                    | 0                     |
| 3. Amorpha   | 0       | 68                      | 0                      | 0                    | 0                     |
| 4. Caragana  | 0       | 66                      | 0                      | 0                    | 0                     |
| 5. Ulex      | 0       | 71                      | 0                      | 0                    | 0                     |
| 6. Colulea   | 0       | 60                      | 0                      | 0                    | 0                     |
| 7. Lens      | 0       | 80                      | 0                      | 0                    | 0                     |
| 8. Conavalia | 0       | 40                      | 0                      | 0                    | 0                     |

\* Computed as the percentage of activity adsorbed to the specific IgG column minus the percentage bound to the preimmune IgG column.

classes of proteins are evolutionarily related (i.e., share at least some antigenic determinants). This fact derives from the observation that antisera to several "classic" lectins cross react with MBH and antiserum to MBH cross reacts with several classic lectins. It should be noted that relatively high concentrations of antigen and strong antisera are required in order to see these cross reactions.

**$\alpha$ -MANNOSIDASE-HEMAGGLUTININS**

Paus and Steen<sup>5</sup> have described an  $\alpha$ -mannosidase from Phaseolus vulgaris which displayed hemagglutinin properties. The hemagglutinin has a molecular weight of about 220,000 and apparently is a glycoprotein composed of two non-covalently bound subunits of 110,000 molecular weight. This protein has been studied in some detail<sup>16 17</sup> with respect to its enzymatic and physical properties, but very little has been reported about its hemagglutinin properties. Likewise its relationship (if any) to the classic hemagglutinin (PHA) in P. vulgaris or to other legume hemagglutinins is not known. The authors did, however, show that P. vulgaris  $\alpha$ -mannosidase was a mitogen with a potency comparable to other legume mitogenic hemagglutinins.<sup>5</sup> Evidence was also provided suggesting that similar enzymic-mitogenic hemagglutinins may exist in mammalian tissues.

**N-ACETYL GALACTOSAMINE-SPECIFIC HEMAGGLUTININS**Immunological Surveys

A great many of the legume hemagglutinins that have been studied show specificities for N-acetyl-galactosamine (GalNAc lectins).<sup>3</sup> Among those which have been characterized are the GalNAc lectins from species of Bauhinia, Dolichos, Glycine (soybean), Phaseolus (lima bean), Wisteria, Sophora and Caragana. The GalNAc lectins from most or all of these legume species are glycoproteins, bind metal ions, exist in tetrameric forms, are isolated from seeds and have similar sized subunits.<sup>17</sup> The great overall similarities in these proteins suggests that they may be homologues. This possibility is very strongly supported by the finding that these proteins are immunologically related<sup>15,19</sup> and may generally possess a high degree of amino acid sequence homology.<sup>20,21</sup> We have recently completed N-terminal amino acid sequence studies with GalNAc lectins from Bauhinia, Caragana, Sophora and the fucose specific lectin from Ulex and found that these proteins all show extensive sequence homology with one another and with previously analyzed lectins.<sup>20,21</sup>

In view of the above findings and the fact that GalNAc lectins often account for 1% or more of the seed protein, and are widely distributed among legume species,<sup>2</sup> we

wondered if they might be ubiquitous legume proteins. A review of the literature suggested that this possibility was unlikely, since many legume species (perhaps 50% or more of all species) appeared to be totally devoid of hemagglutinin activity of any kind. Furthermore, many of the species which contained hemagglutinins appeared to contain proteins with carbohydrate specificities distinct from the GalNAc lectins.<sup>2</sup> To confirm the validity of the earlier studies a survey of a number of legume species was performed and, indeed, seed extracts of many legume species displayed very little or no hemagglutinin activity (Table 7) or contained hemagglutinins which were not inhibited by GalNAc (unpublished data). One cannot rule out the presence of a hemagglutinin activity by the limited assay methods used. However, since all GalNAc hemagglutinins which have been described are very easily detected in crude extracts by the assay methods used, it is reasonable to conclude that none of these plants contain a detectable quantity of hemagglutinin activity comparable to the GalNAc hemagglutinins.

Table 7. Hemagglutinin content of crude legume seed extracts

| Plant Species                     | Hemagglutinin Titers<br>Blood Type <sup>1</sup> |       |   |
|-----------------------------------|---|-------|---|
|                                   | Rabbit  | Human |   |
|                                   | R   | A     | B |
| 1. <i>Acacia decurrens mollis</i> | 0   | 0     | 0 |
| 2. <i>Amorpha fruticosa</i>       | 0   | 0     | 0 |
| 3. <i>Cercis siliquastrum</i>     | 0   | 0     | 0 |
| 4. <i>Colutea arborescens</i>     | 0   | 2     | 2 |
| 5. <i>Delonix regia</i>           | 8   | 4     | 2 |
| 6. <i>Genista monosperma</i>      | 0   | 0     | 0 |
| 7. <i>Gleditsia triacanthos</i>   | 4   | 4     | 2 |
| 8. <i>Lupinus polyphyllus</i>     | 8000  | 0     | 0 |
| 9. <i>Lespedeza bicolor</i>       | 0   | 0     | 0 |
| 10. <i>Mimosa pudica</i>          | 4   | 0     | 2 |
| 11. <i>Parkinsonia aculeata</i>   | 16  | 4     | 4 |
| 12. <i>Poinciana gilliesii</i>    | 2   | 0     | 0 |
| 13. <i>Pueraria thunbergiana</i>  | 2000  | 0     | 0 |
| 14. <i>Spartium junceum</i>       | 0   | 0     | 8 |
| 15. <i>Thermopsis caroliniana</i> | 500   | 0     | 0 |

1. All assays were done using trypsinized<sup>8</sup> erythrocytes.

We decided to examine extracts of a number of GalNAc hemagglutinin "negative" species to determine if they contained proteins (CRM) immunologically related to any of the GalNAc hemagglutinins. Results (Table 8) reveal that every plant tested contained CRM to one or more of the GalNAc hemagglutinins tested. The antisera used in these studies were from rabbits immunized with very pure hemagglutinin preparations. Although many plants showed CRM with antisera raised against several different lectins, it appeared likely that one major protein was responsible since adsorption of the extracts by any one of the reactive sera results in a loss of reactivity with all the other sera (unpublished observations).

Since the GalNAc hemagglutinins are glycoproteins, the antisera raised against them might recognize glycoproteins in plant extracts which are totally different from the hemagglutinins, but which possess similar carbohydrate determinants. This possibility was ruled out in the following way: The quantity of hemagglutinin (antigen) required to totally inhibit the homologous antisera was determined. About 100 times this quantity of antigen was denatured by boiling and then subjected to several proteolytic treatments. The resulting material retained no hemagglutinin activity, showed no immunological reaction by Ouchterlony double diffusion and most importantly, showed no ability to block the reaction of antisera with CRM containing extracts. All the buffers and protease solutions were tested for the presence of several glycosidases and none were found. If carbohydrate determinants constituted a dominant part of the CRM reaction, then the glycopeptides remaining in the proteolyzed antigen preparation would be expected to have potent hapten inhibitors of the CRM reaction. Since no inhibition was seen, it was concluded that carbohydrate plays little or no role in determining the immunological similarities between hemagglutinins and their CRMs (unpublished observations).

#### Affinity Chromatographic Studies

Most plant hemagglutinins can be purified (or partially purified) by affinity chromatography.<sup>13</sup> Sepharose to which N-acetyl galactosamine has been attached provides an excellent adsorbant for the purification of most of the GalNAc specific hemagglutinins. There are now many examples of proteins which have carbohydrate binding properties, but

Table 8. Immunological cross reactions between crude seed extracts and antisera raised against purified Ga1NAc specific legume lectins

| Seed Extract       | Antisera* |          |         |
|--------------------|-----------|----------|---------|
|                    | Bauhinia  | Dolichos | Sophora |
| 1. Acacia          | +         | +        | +       |
| 2. Amorpha         | +         | -        | +       |
| 3. Cercis          | ±         | -        | +       |
| 4. Colutea         | +         | -        | +       |
| 5. Delonix         | +         | ±        | +       |
| 6. Genista         | +         | -        | +       |
| 7. Gleditsia       | +         | -        | +       |
| 8. Lupinus         | +         | -        | +       |
| 9. Lespedeza       | +         | -        | +       |
| 10. Mimosa         | +         | +        | +       |
| 11. Parkinsonia    | ±         | -        | +       |
| 12. Poinciana      | +         | -        | +       |
| 13. Pueraria       | +         | +        | +       |
| 14. Spartium       | +         | -        | +       |
| 15. Thermopsis     | +         | -        | +       |
| Total positive CRM | 13        | 3        | 15      |

\* + = precipitin line observed

- = no precipitin line observed

± = very weak reaction, questionable

which are not themselves hemagglutinins, that can be purified by carbohydrate affinity chromatography. It is also known that hemagglutinins may sometimes exist in forms (or can be converted to forms) which retain carbohydrate binding properties but are no longer hemagglutinins. This information leads one to question whether or not the CRMs, even though they do not display hemagglutinin activity, can be adsorbed by affinity matrices. Therefore, a number of CRM containing plant extracts were chromatographed on GalNAc sepharose columns with the result that very little, if any of the total protein and no CRM was retained by the columns (unpublished observations). These findings suggest that the CRMs, which do not display classic hemagglutinin activity, also do not possess the carbohydrate binding properties typical of GalNAc lectins.

#### Immunoadsorption Studies

The results obtained using the immunoadsorption methods developed for the study of  $\alpha$ -galactosidases suggested that similar techniques might be useful to further characterize GalNAc lectin CRMs. We therefore prepared IgG-sepharose columns using purified IgG from pre-immune sera and from several anti GalNAc lectin sera.

Columns containing pre-immune IgG did not bind CRMs (i.e., adsorb it from crude extracts). As expected, however, CRMs were very effectively adsorbed by columns containing IgG from cross reactive antilectin sera (unpublished observations). We could now determine whether any specific proteins (such as enzymes) were selectively adsorbed from extracts by anti-lectin IgG columns. Most of the work was done using IgG purified from antisera against the GalNAc lectins from Bauhinia and Sophora.

Very little of the total protein in extracts was adsorbed by either pre-immune or antilectin IgG sepharose. However, when glycosidase activities were followed, it appeared that in extracts from several legume species a  $\beta$ -hexosaminidase activity was specifically adsorbed by the antilectin IgG columns (unpublished observations). This very interesting result led us to ask if plants containing GalNAc lectins, such as Sophora and Bauhinia also contained  $\beta$ -hexosaminidases and where these enzymes were related to the lectins.

Our studies in this area are just beginning so to date are preliminary. Despite unsuccessful attempts to purify the  $\beta$ -hexosaminidases from Sophora japonica and Bauhinia purpurea alba, some observations were made as regards to these activities:

1. The enzymes from each plant have molecular weights (by gel filtration on Sephadryl S-200) which are practically identical to those of the respective lectins.
2. The enzyme and lectin in each plant have very similar (but not identical) ion exchange behavior.
3. The lectin is adsorbed from extracts by the affinity adsorbent, GalNAc Sepharose, but not the  $\beta$ -hexosaminidases.
4. Material not retained by GalNAc sepharose, i.e., the lectin-depleted fraction still contains material immunologically related to the lectin.
5.  $\beta$ -Hexosaminidase activity in Sophora extracts from which the lectin has been removed by affinity chromatography, adsorbs to anti Sophora IgG columns but not to pre-immune IgG sepharose. The same results are seen with the Bauhinia enzyme.

Our studies suggest the legume  $\beta$ -hexosaminidases and GalNAc specific lectins are evolutionarily related proteins. If these observations are substantiated by further investigation, then studies of the relationship between these two GalNAc binding proteins should provide a new and rewarding approach toward understanding legume hemagglutinins.

#### CONCLUDING REMARKS

The evidence at this time suggests that there are at least two evolutionarily related but functionally distinct classes of legume protein which possess hemagglutinin properties. These are the "classic" lectins and the  $\alpha$ -galactosidase hemagglutinins. Whether or not the  $\alpha$ -mannosidase hemagglutinin seen in Phaseolus constitutes a member of a third class remains to be seen. Also it is not yet clear whether the "classic" lectins are all homologues or

proteins from several distinct functional classes. However, the GalNAc specific lectins that have been described certainly appear to be homologues. This is an important conclusion because we can now refer to these proteins (GalNAc lectins) as members of a specific physiologically functional class even though we do not know their function.

In comparing the studies of the GalNAc lectins with findings obtained for the  $\alpha$ -galactosidase-hemagglutinins, a number of analogies are readily apparent. We know that all legumes contain a specific  $\alpha$ -galactosidase but that only rarely does this enzyme possess hemagglutinin activity. Likewise, it appears that all legumes contain a GalNAc-lectin-like protein (CRM) but only occasionally contain a GalNAc specific hemagglutinin activity. We suggest that all legumes probably contain a functional homologue of the GalNAc lectins and that it is this protein which gives rise to the majority of the GalNAc lectin CRM seen in many legumes. Thus, just as there may be hemagglutinin "active" and "inactive" forms of  $\alpha$ -galactosidase, there may be hemagglutinin "active" and "inactive" forms of the GalNAc lectins. We further suggest that the "hemagglutinin activity" of the GalNAc lectins, just as with the  $\alpha$ -galactosidase hemagglutinins, is probably not important with respect to the general functioning of this class of proteins.

Our studies with  $\beta$ -hexosaminidases strongly suggest that this enzyme is closely related to the GalNAc lectins. Just how these two kinds of GalNAc binding proteins are related remains to be seen. They might be functionally distinct but evolutionarily related proteins, as appears to be the case between the  $\alpha$ -galactosidases and the classic lectins or they could be more closely related, perhaps displaying a precursor-product relationship.

Independent of the ultimate validity of the foregoing speculations, we feel the current body of knowledge indicates that great care must be exercised with respect to the terms lectins and hemagglutinin. A variety of proposals have been advanced with regard to the function of plant lectins,<sup>7</sup> however, virtually all these theories rely on the assumption that hemagglutinin activity is a direct reflection of the in vivo activity of these proteins. This assumption is not necessarily valid and by discarding it, one can view lectins from a much broader perspective. For example,

one might view lectins as functionally inactive or altered forms of specific plant proteins, in which case hemagglutinin activity might represent little more than an artefactual property. The idea that hemagglutinin activity may not be a required manifestation of the physiological function (activity) of these proteins is not unreasonable since their in vivo role is totally unknown and thus no specific assay for this function exists. One cannot safely define a class of proteins with respect to a physiological function by a criterion (hemagglutinin activity) which has never been shown to be a direct reflection of that function. Thus, one should not conclude that a plant is devoid of a physiologically active "lectin" simply because it may lack a protein with hemagglutinin activity or may contain a protein with activities in addition to hemagglutinin activity.

The rapidly expanding area concerning immunological and amino acid sequence homologies is a good trend, we think, and may offer the best prospects for overcoming the difficulties associated with terminology. This is particularly relevant here because these methods are "blind" with respect to functions or activities and yet, can reveal much about the "relatedness" of proteins. Clearly a great many questions about these interesting and yet elusive proteins remain to be answered. We hope our ideas and observations will stimulate additional work from broader perspectives, which will provide the necessary foundation from which their physiological roles will ultimately emerge.

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## Chapter Six

### RECOVERY OF GLYCOPROTEINS FROM PLANT TISSUES

ROBERT G. BROWN AND W. C. KIMMINS

Department of Biology  
Dalhousie University  
Halifax, Nova Scotia, Canada

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#### INTRODUCTION

The study of plant glycoproteins received a major stimulus by the discovery of plant lectins and their commercial exploitation. Many lectins are glycoproteins and some have structural similarities with cell wall glycoproteins. Some glycoproteins have well-defined and important biological functions, whereas, the function of other glycoproteins remains elusive. This is particularly true of cell wall glycoproteins which are widely distributed among plants suggesting an important function which has yet to be discovered.

Recovery of glycoproteins from plant tissue poses some unique problems, particularly, if the glycoproteins are to be obtained intact. This is particularly true of cell wall glycoproteins which are firmly bound to the wall and generally must be degraded before extraction is possible. Cell walls of higher plants contain at least two glycoproteins, termed hydroxyproline-rich glycoprotein<sup>1</sup> or extensin<sup>2</sup> and hydroxyproline-poor glycoprotein.<sup>1</sup> The main glycoprotein of Chlamydomonas reinhardtii may be cleaved into hydroxyproline-rich and hydroxyproline-poor fragments suggesting that hydroxyproline-rich and hydroxyproline-poor glycoprotein may be parts of a larger complex.<sup>3</sup> Wounding leaves of Phaseolus vulgaris increases production of a hydroxyproline-poor

glycoprotein which binds to cellulose and therefore may have a cross-linking role in the cell wall.<sup>4-8</sup> Methods of extracting hydroxyproline-rich and hydroxyproline-poor glycoprotein will be examined in this article as a means of assessing techniques available for the extraction of glycoproteins involved in recognition phenomena which are associated with the cell wall. Although recovery of both hydroxyproline-rich and hydroxyproline-poor glycoproteins will be discussed, emphasis will be placed on the latter glycoprotein. In particular, the distribution of hydroxyproline-poor glycoprotein among land plants and within plant cells will be emphasized. An attempt will be made to explore the relationship between hydroxyproline-poor glycoprotein found in the cell wall and that produced in response to wounding.

#### EXTRACTION AND PROPERTIES OF GLYCOPROTEINS

Although many methods have been used to extract glycoproteins from plant tissue, not all are suitable for extracting hydroxyproline-rich or hydroxyproline-poor glycoprotein. Hydroxyproline-rich glycoprotein has been extracted from hypocotyl cell walls with alkali<sup>9,10</sup> and cell walls from other tissue with sodium chlorite.<sup>1,11,12</sup> In addition, sodium perchlorate has been used to extract hydroxyproline-rich glycoprotein from algal cell walls.<sup>3</sup> Hydroxyproline-rich glycoprotein extracted with sodium chlorite from cell walls of *P. vulgaris* has structural features summarized in Fig. 1. The glycoprotein is low molecular weight with hydroxyproline as the most abundant amino acid, twice as much arabinose as galactose and the glycosidic linkages expected for a tetraarabinoside-containing structure. The presence of (1→4)-linked glucose residues is unexpected but this has also been found in a similar preparation from *P. coccineus*.<sup>11</sup> Hydroxyproline-poor glycoprotein has been extracted from wounded leaf tissue with alkali<sup>4</sup> and from cell walls with sodium chlorite.<sup>1,12</sup> Following extraction, hydroxyproline-poor glycoprotein may be purified by gel filtration and isoelectric focusing (Fig. 2).<sup>4,13</sup> Hydroxyproline-poor glycoprotein from *P. vulgaris* has the structural features summarized in Fig. 3. This glycoprotein is high molecular weight, with lysine as the only N-terminal amino acid suggesting the presence of one polypeptide chain. Arabinofuranose residues are linked (1→2), (1→3) and (1→5), galactose is terminal and glucose occurs as (1→4)-linked units. Hydroxyproline-poor glycoprotein binds to cellulose,

| Amino acids (Mol. %) |    | Carbohydrate (Mol. %)                 |    |
|----------------------|----|---------------------------------------|----|
| Hyp                  | 15 | Ara                                   | 50 |
| Asx                  | 12 | Xyl                                   | 3  |
| Thr                  | 11 | Man                                   | 5  |
| Ser                  | 12 | Gal                                   | 27 |
| Glx                  | 6  | Glc                                   | 15 |
| Pro                  | 5  | <b>Protein-Carbohydrate linkage</b>   |    |
| Gly                  | 10 | Ser - gal ( $\alpha$ -linked)         |    |
| Ala                  | 5  | Hyp - ara, gal (ara, $\beta$ -linked) |    |
| Val                  | 4  | <b>Linkage (Mol. %)</b>               |    |
| Met                  | -  | Ara (terminal)                        | 16 |
| Ile                  | 2  | Ara (1 $\rightarrow$ 2)               | 24 |
| Leu                  | 2  | Ara (1 $\rightarrow$ 3)               | 12 |
| Tyr                  | 1  | Ara (1 $\rightarrow$ 5)               | 2  |
| Phe                  | 1  | Xyl (terminal)                        | 3  |
| Trp                  | 3  | Gal (terminal)                        | 5  |
| Lys (lys + AAA)      | 9  | Gal (1 $\rightarrow$ 4)               | 7  |
| His                  | 1  | Glc (terminal)                        | 2  |
| Arg                  | 2  | Glc (1 $\rightarrow$ 4)               | 27 |
| Cys (Cys A)          | 1  |                                       |    |

Figure 1. Structural features of hydroxyproline-rich glycoprotein from Phaseolus vulgaris (extracted with sodium chlorite<sup>1</sup>).

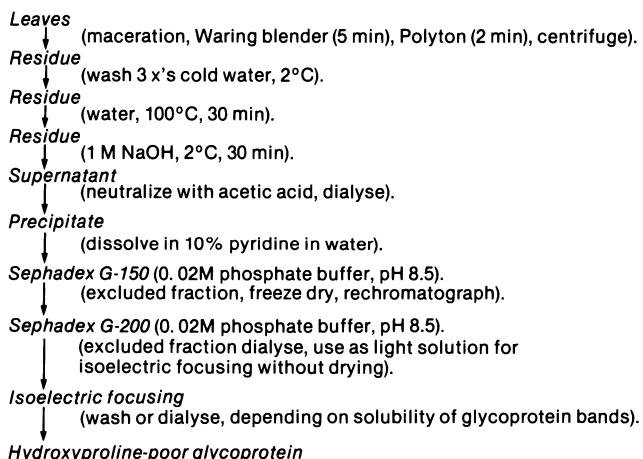


Figure 2. Extraction and purification of hydroxyproline-poor glycoprotein.

(Matteuccia struthiopteris) dextran. The latter may be conveniently purified using Sephadex G-150. The glycoprotein

agarose, and, in one case binds to the gel during chromatography; the gel containing the bound glycoprotein is removed from the column and eluted with 1%  $\alpha$ -methylglucoside or  $\beta$ -methylglucoside to yield the glycoprotein.

Most hydroxyproline-poor glycoproteins purified by isoelectric focusing were enriched with glucose following this purification procedure (Table 1). Loss of xylose and mannose following isoelectric focusing indicates that these sugars were probably impurities. The isoelectric points of hydroxyproline-poor glycoproteins from four sources were similar; all were acidic proteins. Isoelectric focusing (pH 3-10) of glycoproteins from M. struthiopteris, Rheum rhabonticum and P. vulgaris was terminated after 18 hours, because focusing for longer times resulted in precipitation of glycoproteins which settled to the bottom of the column. However, the glycoprotein from Nicotiana tabacum did not precipitate and it was possible to focus until equilibrium was achieved (48 h, pH 2.5 - 5.0). Additional unidentified compounds detected during carbohydrate analyses following isoelectric focusing indicated that ampholytes may bind to the glycoproteins. In addition, substantial increases in the apparent lysine and histidine content of glycoproteins form M.

| <b>Amino acids (Mol. %)</b> |    | <b>Carbohydrate (Mol. %)</b>   |    |
|-----------------------------|----|--|----|
| Hyp                         | 2  | Ara  | 28 |
| Asx                         | 7  | Xyl  | 5  |
| Thr                         | 5  | Gal  | 36 |
| Ser                         | 7  | Glc  | 31 |
| Glx                         | 10 | <b>Protein-Carbohydrate linkage</b>                                      |    |
| Pro                         | 7  | Ser - Ara, Gal, Glc  |    |
| Gly                         | 11 | Hyp - Ara, Gal, Glc  |    |
| Ala                         | 11 | <b>Molecular weight</b>  |    |
| Val                         | 6  | 520,000  |    |
| Met                         | 1  | <b>Isoelectric point 3.6</b>   |    |
| Ile                         | 5  | <b>N-terminus lysine</b>   |    |
| Leu                         | 7  | <b>Binding:</b> Cellulose, Agarose; Dextran ( <u>M. struthiopteris</u> ) |    |
| Tyr                         | 4  |  |    |
| Phe                         | 4  |  |    |
| Tryp                        | 1  |  |    |
| Lys                         | 7  |  |    |
| His                         | 1  |  |    |
| Arg                         | 4  |  |    |
| <b>Linkage (Mol. %)</b>     |    | <b>Linkage (Mol. %)</b>  |    |
|                             |    | Ara (terminal)   | 4  |
|                             |    | Ara (1 $\rightarrow$ 2)  | 9  |
|                             |    | Ara (1 $\rightarrow$ 3)  | 10 |
|                             |    | Ara (1 $\rightarrow$ 5)  | 9  |
|                             |    | Xyl (terminal)   |    |
|                             |    | Xyl (1 $\rightarrow$ 4)  | 5  |
|                             |    | Gal (terminal)   | 18 |
|                             |    | Glc (terminal)   | 2  |
|                             |    | Glc (1 $\rightarrow$ 4)  | 41 |

Figure 3. Structural features of hydroxyproline-poor glycoprotein from Phaseolus vulgaris (extracted with alkali<sup>4</sup>).

Table 1. Sugar composition of hydroxyproline-poor glycoproteins before and after isoelectric focusing.

| Sugars     | Source                   |        |                   |        |                       |        |           |                    |        |           |
|------------|--------------------------|--------|-------------------|--------|-----------------------|--------|-----------|--------------------|--------|-----------|
|            | <i>M. struphiopterus</i> |        | <i>N. tabacum</i> |        | <i>R. rhabonticum</i> |        |           | <i>P. vulgaris</i> |        |           |
|            | %                        | Before | pl<br>3.2         | Before | pl<br>3.7             | Before | pl<br>4.5 | pl<br>5.0          | Before | pl<br>3.6 |
| <i>Ara</i> | 4                        | 12     |                   | -      | -                     | 26     | 21        | 10                 | 11     | 6         |
| <i>Xyl</i> | 2                        | -      |                   | 4      | -                     | -      | -         | -                  | 13     | -         |
| <i>Man</i> | 4                        | -      |                   | 15     | -                     | -      | -         | -                  | -      | -         |
| <i>Gal</i> | 6                        | -      |                   | -      | -                     | 40     | 49        | 62                 | 21     | 6         |
| <i>Glc</i> | 81                       | 88     |                   | 81     | 100                   | 30     | 30        | 28                 | 55     | 88        |

pl; isoelectric point.

*struphiopterus* and *R. rhabonticum* and glycine in the glycoprotein from *N. tabacum* were detected following isoelectric focusing. For instance, the glycine content of *N. tabacum* glycoprotein was 57% after isoelectric focusing. Therefore, isoelectric focusing was employed, in conjunction with gel electrophoresis, to verify homogeneity, rather than as a preparative procedure.

#### OCCURRENCE OF GLYCOPROTEINS

Hydroxyproline-arabinosides are present in cell walls of plants from algae to angiosperms suggesting a wide distribution of hydroxyproline-rich glycoprotein among plants.<sup>14</sup> Likewise, a study of the distribution of hydroxyproline-poor glycoprotein has suggested a wide distribution of this glycoprotein among land plants.<sup>13</sup> Representatives of Pteridophyta, Gymnospermae and Angiospermae contain hydroxyproline-poor glycoproteins which have similar amino acid compositions (Table 2). Linkage analysis indicates that the glyco-mieties of glycoproteins are also similar in structure (Table 3). In addition, trifluoroacetylation,<sup>15</sup> a technique which cleaves peptide bonds but does not cleave glycosidic linkages except those to serine or threonine,<sup>16</sup> followed by deacetylation, fractionation on Sephadex G-25 or G-150 and sugar analysis indicates that the protein moiety was degraded to a molecular weight less than 500 and the carbohydrate side chains of glycoproteins from a variety of sources

Table 2. Amino acid composition of hydroxyproline-poor glycoprotein from representatives of Pteridophyta, Gymnospermae and Angiospermae.

| Amino acid<br>(Mol %) | Source                   |                  |                   |                |                   |                       |                |
|-----------------------|--------------------------|------------------|-------------------|----------------|-------------------|-----------------------|----------------|
|                       | <i>M. struthiopteris</i> | <i>P. stobus</i> | <i>N. odorata</i> | <i>V. faba</i> | <i>N. tabacum</i> | <i>R. rhiponticum</i> | <i>Z. mays</i> |
| Hyp                   | trace                    | trace            | trace             | 6              | 2                 | trace                 | trace          |
| Asx                   | 8                        | 8                | 12                | 6              | 9                 | 9                     | 11             |
| Thr                   | 5                        | 4                | 5                 | 5              | 5                 | 4                     | 6              |
| Ser                   | 7                        | 5                | 5                 | 7              | 10                | 5                     | 6              |
| Glx                   | 5                        | 7                | 10                | 9              | 14                | 8                     | 10             |
| Pro                   | 6                        | 5                | 6                 | 5              | 2                 | 6                     | 5              |
| Gly                   | 9                        | 16               | 10                | 8              | 11                | 22                    | 12             |
| Ala                   | 9                        | 7                | 10                | 10             | 8                 | 9                     | 10             |
| Val                   | 8                        | 5                | 10                | 7              | 7                 | 7                     | 7              |
| Met                   | -                        | -                | 1                 | 1              | -                 | 1                     | 1              |
| Ile                   | 6                        | 4                | 5                 | 4              | 5                 | 4                     | 5              |
| Leu                   | 10                       | 6                | 8                 | 10             | 7                 | 7                     | 8              |
| Tyr                   | 2                        | -                | 3                 | 2              | 6                 | 3                     | 3              |
| Phe                   | 4                        | -                | 3                 | 3              | 6                 | 3                     | 4              |
| Tryp                  | -                        | 4                | 2                 | 3              | trace             | -                     | -              |
| Lys                   | 11                       | 17               | 6                 | 8              | 5                 | 7                     | 6              |
| His                   | 2                        | 8                | -                 | 2              | 1                 | 1                     | 2              |
| Arg                   | 6                        | 4                | 4                 | 4              | 2                 | 4                     | 4              |

Table 3. Linkage analysis of hydroxyproline-poor glycoproteins from representatives of Pteridophyta, Gymnospermae and Angiospermae.

| Linkage<br>(%) | Source                   |                  |                   |                |                   |                       |                |
|----------------|--------------------------|------------------|-------------------|----------------|-------------------|-----------------------|----------------|
|                | <i>M. struthiopteris</i> | <i>P. stobus</i> | <i>N. odorata</i> | <i>V. faba</i> | <i>N. tabacum</i> | <i>R. rhiponticum</i> | <i>Z. mays</i> |
| Ara (terminal) | -                        | 8                | 1                 | 2              | 2                 | 6                     | -              |
| Ara (1 → 2)    | -                        | -                | -                 | -              | -                 | 11                    | -              |
| Ara (1 → 3)    | -                        | 2                | trace             | -              | trace             | -                     | trace          |
| Ara (1 → 5)    | 2                        | -                | 1                 | -              | -                 | 6                     | -              |
| Xyl (terminal) | -                        | 1                | 4                 | 1              | 1                 | 6                     | 2              |
| Xyl (1 → 4)    | 15                       | 8                | 17                | 2              | 2                 | 33                    | 6              |
| Gal (terminal) | 6                        | 4                | -                 | -              | -                 | 13                    | -              |
| Glc (terminal) | 3                        | 7                | 13                | 11             | 6                 | 8                     | 9              |
| Glc (1 → 4)    | 75                       | 70               | 63                | 84             | 89                | 17                    | 81             |

were similar in molecular weight (Table 4). The optical rotation of fractions containing predominately glucose indicate that this sugar is both  $\alpha$ - and  $\beta$ -linked. Side chains with a molecular weight greater than 2,000 were composed

Table 4. Molecular weight and sugar composition of oligosaccharides obtained by trifluoroacetolysis of glycoproteins.

| Fraction | Molecular Weight | Source | Percent (Weight) | Sugar Composition (%) |     |     |     |     | $[\alpha]_D^{20}$ |
|----------|------------------|--------|------------------|-----------------------|-----|-----|-----|-----|-------------------|
|          |                  |        |                  | Rha                   | Ara | Man | Gal | Glc |                   |
| 1        | > 2,000          | a      | 33               | 10                    | 28  | 8   | 27  | 28  | -                 |
|          |                  | b      | 49               | -                     | -   | 5   | 12  | 79  | -                 |
|          |                  | c      | 34               | -                     | 2   | -   | -   | 98  | -                 |
|          |                  | d      | 42               | -                     | -   | -   | -   | 100 | + 111°            |
|          |                  | e      | 34               | -                     | -   | -   | -   | 100 | + 96°             |
|          |                  | f      | 12               | 1                     | 14  | 3   | 24  | 58  | + 159°            |
| 2        | 900-2,000        | a      | 17               | 4                     | 55  | 12  | 15  | 14  | -                 |
|          |                  | b      | 18               | -                     | 30  | 9   | 16  | 45  | -                 |
|          |                  | c      | 16               | -                     | 17  | 15  | 67  | -   | -                 |
|          |                  | d      | 40               | -                     | -   | 5   | 13  | 82  | + 165°            |
|          |                  | e      | 66               | -                     | -   | -   | -   | 100 | + 96°             |
|          |                  | f      | 83               | 3                     | 10  | 4   | 16  | 67  | + 108°            |
| 3        | 500-900          | a      | 14               | -                     | 48  | 18  | 18  | 15  | -                 |
|          |                  | b      | 6                | -                     | 45  | 16  | 9   | 33  | -                 |
|          |                  | c      | 15               | -                     | 25  | 28  | 10  | 38  | -                 |
|          |                  | d      | 18               | -                     | -   | 5   | 20  | 75  | + 180°            |
|          |                  | e      | -                | -                     | -   | -   | -   | -   | -                 |
|          |                  | f      | 5                | 20                    | 26  | 13  | 6   | 35  | -                 |
| 4        | 342-500          | a      | 11               | -                     | 61  | 13  | 19  | 8   | -                 |
|          |                  | b      | 11               | -                     | 43  | 10  | 15  | 29  | -                 |
|          |                  | c      | 20               | -                     | 5   | 25  | 10  | 52  | -                 |
|          |                  | d      | -                | -                     | -   | -   | -   | -   | -                 |
|          |                  | e      | -                | -                     | -   | -   | -   | -   | -                 |
|          |                  | f      | -                | -                     | -   | -   | -   | -   | -                 |
| 5        | 180-342          | a      | 24               | -                     | 47  | 14  | 34  | 5   | -                 |
|          |                  | b      | 16               | -                     | 22  | 8   | 28  | 41  | -                 |
|          |                  | c      | 16               | -                     | 44  | -   | 56  | -   | -                 |
|          |                  | d      | -                | -                     | -   | -   | -   | -   | -                 |
|          |                  | e      | -                | -                     | -   | -   | -   | -   | -                 |
|          |                  | f      | -                | -                     | -   | -   | -   | -   | -                 |

- a. *P.vulgaris* hydroxyproline-rich glycoprotein.
- b. *P.vulgaris* hydroxyproline-poor glycoprotein.
- c. *M.struthiopteris* hydroxyproline-poor glycoprotein.
- d. *N.tabacum* hydroxyproline-poor glycoprotein.
- e. *Z.mays* hydroxyproline-poor glycoprotein.
- f. *R.raponticum* hydroxyproline-poor glycoprotein.

mainly of glucose. Gel chromatography on Sephadex G-150 indicated the molecular weight of this fraction was heterogeneous with the largest fragments having molecular weights as high as 100,000. Side chains with a molecular weight

from 342 to 900 contained arabinose, galactose, glucose and mannose, whereas, the fraction containing single sugar residues contained arabinose, galactose and, in the case of P. vulgaris, glucose. No uronic acids were detected in any of the fractions. Carbohydrate with a molecular weight greater than 500 was pooled and placed on Sephadex DEAE (A50, acetate form). Most of the carbohydrate was eluted with distilled water. For example, 80% of the carbohydrate from N. tabacum was eluted before the gradient of sodium acetate (0→2M) appeared in the effluent, providing further evidence that the carbohydrate moiety of hydroxyproline-poor glycoprotein does not contain uronic acids.

Hydroxyproline-poor glycoproteins from M. struthiopteris and P. vulgaris both have N-terminal lysine indicating further structural homology and suggesting that a hydroxyproline-poor glycoprotein with common structural features is widely distributed among land plants. The isolation of a proteoglycan from a red alga having an amino acid composition very similar to hydroxyproline-poor glycoprotein suggests that a glycoprotein with some structural similarities also occurs among algae.<sup>17</sup>

Trifluoroacetolysis and gel chromatography of hydroxyproline-rich glycoprotein indicates that a significant portion of the carbohydrate moiety of this glycoprotein has a molecular weight greater than 2,000 (Table 4). The high optical rotation of glucose-rich fraction indicated most glucose was  $\alpha$ -linked ( $[\alpha]_D^{20} + 180^\circ$ ; glucose, 65%; galactose, 13%; arabinose, 7% and mannose, 6%). Consequently, hydroxyproline-rich and hydroxyproline-poor glycoproteins both appear to have (1→4)-linked glucose residues most of which are  $\alpha$ -linked. Investigations to ascertain if this is a structural feature of these glycoproteins or merely indicates a tightly-bound glucose polymer are currently being initiated.

#### EXTRACTION OF $^{14}\text{C}$ -LABELLED GLYCOPROTEINS

The presence of hydroxyproline-poor glycoprotein in all plants examined suggests an important function for this glycoprotein in plant cells.<sup>13</sup> Knowledge of the cellular location of this glycoprotein would aid elucidation of its function. Addition of [ $^{14}\text{C}$ ]proline to the Celite abrasive used to wound leaf tissue of P. vulgaris,<sup>5</sup> labelled both

hydroxyproline-poor and hydroxyproline-rich glycoprotein within 36 hours. Leaves were harvested 36 hours and 96 hours after wounding and extracted by using one of three methods which did not differ significantly from each other in the extractants employed, but did differ in the order in which the extractions were made. In the first method (Fig. 4), cell walls were not treated with alkali before sodium chlorite extraction. In the second method, hydroxyproline-poor glycoprotein was extracted with cold alkali (2°C) before preparation of cell walls and extraction with sodium chlorite. In method three, cell walls were extracted with alkali (1M and 4M at 20°C) after cell wall preparation but before sodium chlorite extraction.

Radioactivity in each fraction was determined, after drying, using a Packard Tricarb oxidizer. Supernatant fluids were dialysed before freeze drying. Radioactivity in hydroxyproline and proline was determined after protein hydrolysis using a 10:1 flow splitter during amino acid analysis so that one-tenth of the column output went to the detector while the remainder was collected for radioactivity measurement. Radioactivity in proline and hydroxyproline accounted for at least 70% of the radioactivity present in the samples analysed. The ratio of hydroxyproline to proline is based on the amount of radioactivity in these two amino acids and is expressed as HP/P ratio.

Extraction by method 1 indicated that after 36 hours proline was incorporated mainly into hydroxyproline-poor protein that was extracted with sodium chlorite but was not precipitated by ethanol (Table 5). Some proline was incorporated into hydroxyproline-rich glycoprotein particularly if the specific activity of proline was low. Extending the post wounding time from 36 hours to 96 hours before harvesting resulted in incorporation of proline into protein which was associated with other cell wall fractions, mostly the hemicellulose I fractions and cellulose. Extraction of hydroxyproline-poor glycoprotein with cold alkali demonstrated that proline was incorporated into this glycoprotein; however, most proline was incorporated into material which occurred in the sodium chlorite supernatant fraction, but this was also hydroxyproline-poor (Table 6). Extraction by method 2 resulted in more incorporated proline being found in other cell wall fractions, particularly the hemicellulose I fractions, than method 1 (comparing results at 36 h).

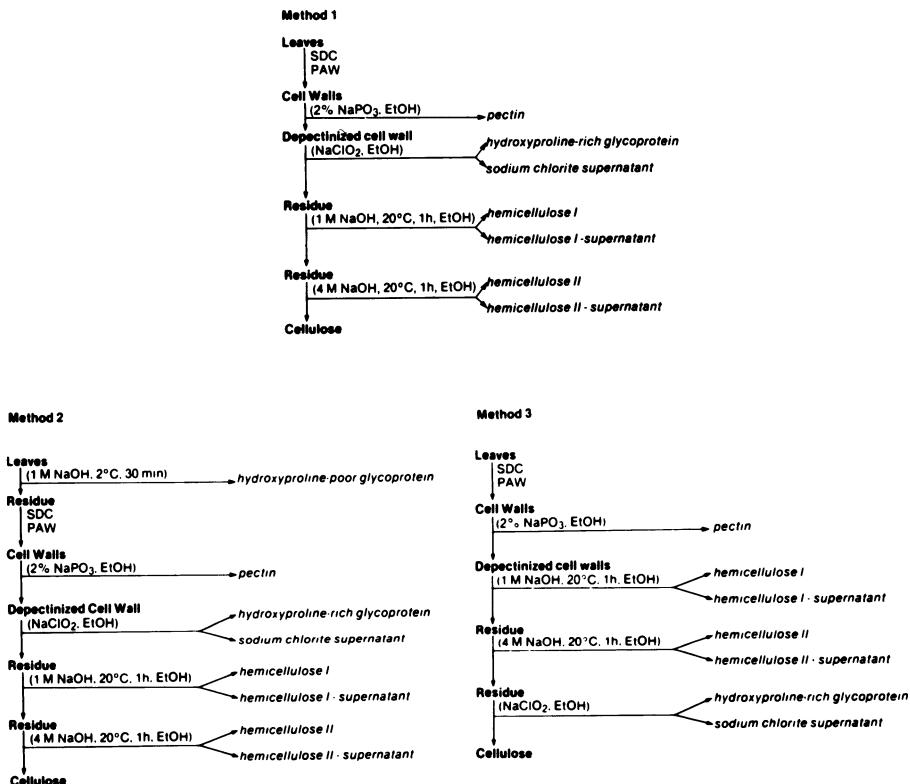


Figure 4. Extraction of leaves of *P. vulgaris* exposed to [<sup>14</sup>C]proline at wounding. Cell walls were prepared by a modification of the method of Ring and Selvendran,<sup>18</sup> whereby leaves were macerated in a Waring blender (10 min) followed by a Polytron treatment (5 min) while suspended in sodium deoxycholate (SDC, 1%). The insoluble material was collected on a sintered glass crucible and washed with SDC. Maceration and washing were repeated twice. The residue was suspended in phenol/acetic acid/water (PAW, 2:1:1), macerated for 5 minutes in a Polytron, then collected by centrifugation (5,000 g, 10 min). The PAW extraction was repeated twice to yield cell walls.

Table 5. Incorporation of [<sup>14</sup>C]proline into cell wall fractions of P. vulgaris (Method 1).

| Fractions                                     | Post Wounding Time |    |           |     |         |    |         |    |
|---|--------------------|----|-----------|-----|---------|----|---------|----|
|   | 36 h               |    |           |     | 96 h    |    |         |    |
|   | DPM                | %  | DPM       | %   | DPM     | %  | DPM     | %  |
| Hydroxyproline-rich glycoprotein <sup>b</sup> | 10,267             | 7  | 603       | -   | 2,252   | 18 | 4,035   | 12 |
| Sodium chlorite supernatant <sup>c</sup>      | 144,108            | 93 | 1,015,800 | 100 | 3,349   | 27 | 6,412   | 20 |
| Hemicellulose I                               | 627                | -  | 90        | -   | 1,769   | 14 | 5,046   | 16 |
| Hemicellulose I - supernatant                 | 647                | -  | ND        | -   | 761     | 6  | 5,000   | 15 |
| Hemicellulose II                              | 253                | -  | 4,025     | -   | 1,706   | 14 | 150     | -  |
| Hemicellulose II - supernatant                | 151                | -  | 2,899     | -   | 1,367   | 11 | 78      | -  |
| Cellulose                                     | ND                 | -  | 3,936     | -   | 1,432   | 11 | 11,785  | 36 |
| <b>Total</b>                                  | 156,053            |    | 1,027,353 |     | 12,636  |    | 32,506  |    |
| <b>Radioactivity used</b>                     | 0.8 μCi            |    | 8 μCi     |     | 0.8 μCi |    | 0.8 μCi |    |

<sup>a</sup> Expt. 1 Specific activity of Proline 10 μCi/mMol

<sup>b</sup> Expt. 2 Specific activity of Proline 283 mCi/mMol

<sup>c</sup> HP/P = 1.2; HP/P is the ratio Hyp/Pro

<sup>c</sup> HP/P = 0.2

Table 6. Incorporation of [<sup>14</sup>C]proline into cell walls fractions of P. vulgaris (Method 2).

| Fractions                        | Post Wounding Time |    |        |    |      |        |    |  |
|----------------------------------|--------------------|----|--------|----|------|--------|----|--|
|                                  | 36 h               |    |        |    | 96 h |        |    |  |
|                                  | DPM                | %  | DPM    | %  | HP/P | DPM    | %  |  |
| Hydroxyproline-poor glycoprotein | 5,252              | 17 | 1,998  | 3  | 0.3  | 7,155  | 14 |  |
| Hydroxyproline-rich glycoprotein | 5,692              | 19 | 8,576  | 15 | 1.2  | 5,765  | 11 |  |
| Sodium chlorite supernatant      | 12,725             | 42 | 28,000 | 48 | 0.3  | 13,590 | 26 |  |
| Hemicellulose I                  | 1,261              | 4  | 5,092  | 9  | 0.1  | 4,461  | 9  |  |
| Hemicellulose I - supernatant    | 3,650              | 12 | 12,100 | 21 | 0.3  | 14,398 | 28 |  |
| Hemicellulose II                 | 361                | 1  | 1,320  | 2  | -    | 213    | -  |  |
| Hemicellulose II - supernatant   | 100                | -  | 0      | -  | -    | 5,551  | 11 |  |
| Cellulose                        | 982                | 3  | 1,210  | 2  | -    | 1,038  | 2  |  |
| <b>Total</b>                     | 30,023             |    | 58,296 |    |      | 52,171 |    |  |
| <b>Recovery (%)</b>              | 90                 |    | 79     |    |      |        |    |  |

Specific activity of Proline: 10 μCi/mMol

Radioactivity used: 0.8 μCi

HP/P is the ratio Hyp/Pro.

Treatment with alkali at 20°C before sodium chlorite extraction altered the distribution of incorporated proline in the cell wall fractions (Table 7). Hydroxyproline-rich glycoprotein was no longer precipitated by ethanol and consequently it was present in the sodium chlorite supernatant fluid. This suggests that carbohydrate attached to serine may be required for ethanol to precipitate sodium chlorite extracted hydroxyproline-rich glycoprotein. Hydroxyproline-poor protein was associated with cellulose. These two fractions accounted for most of the proline incorporated into the cell wall. The recoveries of radioactivity given in Tables 6 and 7 are based on the amount of radioactivity present following hot water extraction in the hydroxyproline-poor glycoprotein extraction procedure (Fig. 2) for Table 6 and the radioactivity present in cell walls for Table 7. In each case, a sample was freeze-dried and combusted to CO<sub>2</sub> for radioactivity determination. A comparison of HP/P ratios at 36 hours and 96 hours post-wounding, indicates an increase in hydroxyproline content of all fractions with time.

A comparison of the distribution of incorporated [<sup>14</sup>C]-proline in the fractions obtained by the three extraction methods (Table 8), indicated the following. Firstly, treatment with alkali increased the proportion of incorporated

Table 7. Incorporation of [<sup>14</sup>C]proline into cell wall fractions *P. vulgaris* (Method 3).

| Fractions                        | Post Wounding Time |    |      |        |    |      |
|----------------------------------|--------------------|----|------|--------|----|------|
|                                  | 36 h               |    |      | 96 h   |    |      |
|                                  | DPM                | %  | HP/P | DPM    | %  | HP/P |
| Hemicellulose I                  | 1,180              | 2  |      | 2,880  | 4  |      |
| Hemicellulose I supernatant      | 4,807              | 8  | 0.2  | 4,576  | 6  |      |
| Hemicellulose II                 | 5,226              | 9  | 1.2  | 360    | -  |      |
| Hemicellulose II - supernatant   | 8,436              | 14 |      | 693    | 1  | 0.8  |
| Hydroxyproline-rich glycoprotein | 544                | 1  | 0.9  | 2,049  | 3  | 1.5  |
| Sodium chlorite supernatant      | 20,775             | 36 | 2.5  | 30,905 | 41 | 3.4  |
| Cellulose                        | 17,231             | 30 | 0.4  | 33,899 | 45 | 0.6  |
| Total                            | 58,199             |    |      | 75,362 |    |      |
| Recovery (%)                     | 92                 |    |      | 64     |    |      |

Specific activity of Proline: 283 mCi/mMol  
Radioactivity used: 0.8  $\mu$ Cl

proline found in hydroxyproline-rich fractions. This was particularly apparent when cell walls were treated with alkali at 20°C before sodium chlorite extraction (method 3 vs. method 2). The explanation of this observation may be related to differences in the extraction methods or to the observation that HP/P ratios were higher in all material obtained by method 3 than methods 2 or 1. This may indicate that, although all leaves were harvested at either 36 or 96 hours, biosynthesis of glycoproteins in leaves used for extraction by method 3 was more complete, consequently, more hydroxylation of proline residues had occurred. Secondly, treatment with alkali rendered hydroxyproline-poor protein more difficult to extract with sodium chlorite. Hydroxyproline-poor glycoprotein was associated with hemicellulose after cold alkali treatment and with cellulose following treatment with alkali at 20°C. Again, this may be related to the extraction methods or differences in hydroxylation.

The designation of fractions as either hydroxyproline-poor or hydroxyproline-rich is arbitrary, that is, those fractions with a hydroxyproline/proline ratio less than 0.6 are termed hydroxyproline-poor; whereas, those fractions with a hydroxyproline/proline ratio greater than 0.6 are

Table 8. Distribution of [<sup>14</sup>C]proline in hydroxyproline-poor and hydroxyproline-rich glycoproteins extracted by three methods.

| Fractions                                | Percent    |            |            |            |            |          |
|--|------------|------------|------------|------------|------------|----------|
|  | 36 h       |            |            | 96 h       |            |          |
|  | Method 1   | Method 2   | Method 3   | Method 1   | Method 2   | Method 3 |
| Hemicellulose I (Hyp-poor)               | -          | 4          | 2          | 16         | 9          | 4        |
| Hemicellulose I - supernatant (Hyp-poor) | -          | 12         | 8          | 15         | 28         | 6        |
| Hemicellulose II - (Hyp-poor)            | -          | 1          | 9          | -          | -          | -        |
| Hemicellulose II - supernatant           | -          | -          | 14         | -          | 11         | 1        |
| Hydroxyproline-rich glycoprotein         | -          | 19         | 1          | 12         | 11         | 3        |
| Sodium chlorite supernatant              | 100        | 42         | 36         | 20         | 26         | 41       |
| Hydroxyproline-poor glycoprotein         | (Hyp-poor) | (Hyp-poor) | (Hyp-rich) | (Hyp-poor) | (Hyp-poor) | Hyp-rich |
| Cellulose (Hyp-poor)                     | -          | 17         | -          | -          | 14         | -        |
|  | -          | 3          | 30         | 36         | 2          | 45       |

Method 1 - no alkali before sodium chlorite.

Method 2 - alkali (2°C) before sodium chlorite.

Method 3 - alkali (20°C) before sodium chlorite.

HP/P<0.6 designated (Hyp-poor).

HP/P>0.6 designated (Hyp-rich).

termed hydroxyproline-rich. The choice of 0.6 as the determining ratio is based on values obtained for a variety of preparations of hydroxyproline-poor and hydroxyproline-rich glycoprotein (Table 9).

Amino acid, sugar and linkage analysis verified the fraction termed hydroxyproline-rich glycoprotein was indeed this material. The results of linkage analysis was compared to a similar analysis of material from *P. coccineus* (Table 10). The glycoproteins were either prepared by chromatography on Sephadex G-200 (*P. vulgaris*) or DEAE-Sephadex (*P. coccineus*). The similarity of the results obtained is apparent despite the difference in preparative methods. The results are compatible with a structure containing tetra-arabinoside and terminal galactose which are characteristic of hydroxyproline-rich glycoprotein. One unexpected finding is the presence of (1 $\rightarrow$ 4)-linked glucose residues in both preparations.

#### SPECULATION

This chapter concludes with some speculation concerning the following questions. Firstly, what is the relationship between the hydroxyproline-poor glycoprotein extracted from wounded leaf tissue<sup>4-8</sup> and the hydroxyproline-poor protein found in cell walls?<sup>1, 11, 12</sup> Secondly, what relationship, if any, exists between hydroxyproline-poor and hydroxyproline-rich glycoprotein?

Table 9. Ratio of hydroxyproline to proline in preparations of hydroxyproline-rich and hydroxyproline-poor glycoproteins from *Phaseolus*.

| Glycoprotein                            | HP/P | Source              | Reference                    |
|---|------|---------------------|------------------------------|
| <i>Hydroxyproline-rich glycoprotein</i> | 0.7  | <i>P. vulgaris</i>  | Lamport, 1965                |
|   | 1.1  | <i>P. vulgaris</i>  | Brown and Kimmins, in press  |
|   | 3.0  | <i>P. vulgaris</i>  | Brown and Kimmins, in press  |
|   | 7.0  | <i>P. coccineus</i> | Selvendran, 1975             |
|   | 7.3  | <i>P. coccineus</i> | O'Neill and Selvendran, 1980 |
| <i>Hydroxyproline-poor glycoprotein</i> | 0.3  | <i>P. vulgaris</i>  | Brown and Kimmins, 1978      |
|   | 0.2  | <i>P. vulgaris</i>  | Brown and Kimmins, 1978      |
|   | 1.0  | <i>P. vulgaris</i>  | Brown and Kimmins, 1978      |
|   | 0.3  | <i>P. vulgaris</i>  | Brown and Kimmins, in press  |
|   | 0.5  | <i>P. coccineus</i> | Selvendran, 1975             |

Table 10. Linkage analysis of hydroxyproline-rich glycoprotein from P. vulgaris and P. coccineus.

| Linkage        | Mol (%)            |                      |
|----------------|--------------------|----------------------|
|                | <u>P. vulgaris</u> | <u>P. coccineusb</u> |
| Rham (1 → 2)   | -                  | 1                    |
| Ara (terminal) | 16                 | 23                   |
| Ara (1 → 2)    | 24                 | 35                   |
| Ara (1 → 3)    | 12                 | 19                   |
| Ara (1 → 5)    | 2                  | 3                    |
| Xyl (terminal) | 3                  | 1                    |
| Xyl (1 → 4)    | -                  | 2                    |
| Gal (terminal) | 5                  | 9                    |
| Gal (1 → 4)    | 7                  | 1                    |
| Glc (terminal) | 2                  | -                    |
| Glc (1 → 4)    | 27                 | 5                    |

<sup>a</sup>Brown and Kimmins (in press): Extraction; Selvendran, 1975: purified by chromatography on Sephadex G-200.

<sup>b</sup>O'Neill and Selvendran, 1980: Extraction; modified Selvendran, 1975: purified by chromatography on DEAE-Sephadex.

Investigation of the first question depends on the presence of a characteristic structural feature which can be demonstrated to be common in hydroxyproline-poor glycoprotein from wounded leaf tissue and cell walls. Unlike hydroxyproline-rich glycoprotein which has a readily detectable alkali-stable hydroxyproline-O-arabinosyl linkage, no characteristic structural feature of hydroxyproline-poor glycoprotein is known at the present time. However, alkali treatment (0.2 M NaOH, 50°C, 5 h) produces at least three peptide fragments having proline and glycine as N-terminal units, in addition to lysine, which is the N-terminus of hydroxyproline-poor glycoprotein.<sup>8,13</sup> These polypeptide fragments have molecular weights of 143,000, 138,000, and 11,000, determined by PAGE electrophoresis and column chromatography. Addition of sodium [<sup>35</sup>S]sulphite during alkali treatment labels only the smallest peptide fragments (11,000) indicating that most carbohydrate attached to serine is associated with these fragments.<sup>8</sup> Investigations to determine if these structural features were present in cell wall hydroxyproline-poor glycoprotein were complicated by the finding that sodium chlorite-extracted hydroxyproline-

rich glycoprotein yielded  $^{35}\text{S}$ -containing material having a molecular weight of 17,000 following alkali treatment.

Alkali treatment in the presence of sodium borohydride (1 M) produced fragments from M. struphiopterus and R. rhabonticum hydroxyproline-poor glycoproteins which isoelectric focusing (48 h, pH 3-10) indicated had the following isoelectric points; 3.85, 4.15, and 4.3 (M. struphiopterus), 3.0 and 3.4 (R. rhabonticum). Gel electrophoresis indicated molecular weights in the range 650 to 1300. Material not soluble in the light solution of amphophytes was removed by centrifugation before isoelectric focusing. Present investigations seek to compare peptide fragments produced from leaf and cell-wall hydroxyproline-poor glycoproteins following treatment with alkali to determine if any homology exists between the two proteins.

Linkage analysis of hydroxyproline-rich glycoprotein after alkali treatment indicated that this procedure resulted in loss of (1 $\rightarrow$ 4)- $\alpha$ -linked glucose units and an increase in terminal, (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 5)-linked arabinose units as well as terminal galactose (Table 11). The latter result was unexpected as hydroxyproline-rich glycoprotein has single galactose residues attached to serine,<sup>19</sup> however, similar treatment of potato lectin released galactose residues from serine very slowly.<sup>20</sup> This was attributed to an inhibitory effect of the arabinofuranosidic residues on the  $\beta$ -elimination reaction caused by a negative charge on the hydroxy groups of adjacent arabinofuranosidic residues. If this explanation is correct, some (1 $\rightarrow$ 2)-linked arabinose units and (1 $\rightarrow$ 4)-linked glucose units attached to serine must be sufficiently removed from hydroxyproline-tetraarabinoside units so that this inhibition does not occur.

The occurrence of hydroxyproline-poor and hydroxyproline-rich glycoprotein in cell walls poses the question of what relationship, if any, exists between hydroxyproline-poor and hydroxyproline-rich glycoprotein? The possibilities include the following; (1) hydroxyproline-rich glycoprotein is a part of hydroxyproline-poor glycoprotein which is separated by the extractants employed in its isolation or as a normal development process during cell maturation, (2) hydroxyproline-poor glycoprotein is a precursor of hydroxyproline-rich glycoprotein and (3) the two glycoproteins are synthesized independently. Although there is little, if

Table 11. Linkage analysis of hydroxyproline-rich glycoprotein before and after  $\beta$ -elimination.

| Linkage                        | <u>Mol (%)</u>              |                            |
|--------------------------------|-----------------------------|----------------------------|
|                                | Before $\beta$ -elimination | After $\beta$ -elimination |
| <i>Ara</i> (terminal)          | 16                          | 23                         |
| <i>Ara</i> (1 $\rightarrow$ 2) | 24                          | 27                         |
| <i>Ara</i> (1 $\rightarrow$ 3) | 12                          | 23                         |
| <i>Ara</i> (1 $\rightarrow$ 5) | 2                           | 11                         |
| <i>Xyl</i> (terminal)          | 3                           | 5                          |
| <i>Gal</i> (terminal)          | 5                           | 11                         |
| <i>Gal</i> (1 $\rightarrow$ 4) | 7                           | .                          |
| <i>Glc</i> (terminal)          | 2                           | .                          |
| <i>Glc</i> (1 $\rightarrow$ 4) | 27                          | .                          |

any, information which supports any of these possibilities, an attractive hypothesis results from a combination of the first two, whereby, some proline residues of hydroxyproline-poor glycoprotein would be oxidized and glycosylated to yield regions high in hydroxyproline. Cleavage of these regions (enzymic or chemical) could release a hydroxyproline-rich glycoprotein. The two glycoproteins have common structural features including (1 $\rightarrow$ 2)-, (1 $\rightarrow$ 3)-, and (1 $\rightarrow$ 5)-linked arabinose units, (1 $\rightarrow$ 4)-linked glucose residues, and terminal galactose.

Hydroxyproline-poor glycoprotein binds to cellulose, agarose and in one case, dextran. These polysaccharides have no obvious structural features in common, therefore, binding to the polysaccharides may involve different regions of hydroxyproline-poor glycoprotein. Lamport has proposed that hydroxyproline-rich glycoprotein has a hydrophobic tail and hydrophilic head.<sup>21</sup> Thus, the larger complex, (hydroxyproline-poor glycoprotein) may have many regions which bind to a variety of material. Treatment of agarose-bound hydroxyproline-poor glycoprotein with alkali-sodium sulphite releases a fragment high in cysteic acid,<sup>7</sup> suggesting that carbohydrate attached to serine is responsible for this binding. Labelled hydroxyproline-poor glycoprotein is associated with hemicellulose if treated with cold alkali and with cellulose if treated with alkali at room temperature (Table 8) suggesting that this glycoprotein is associated with several cell wall components. The hypothesis proposed

is that hydroxyproline-poor glycoprotein associates with many cell wall components and selective cleavage of this glycoprotein could release cell wall moieties from the matrix in a prescribed manner. Several studies have demonstrated that an increase of cell wall hydroxyproline relative to cell wall proline is correlated with cessation of cell elongation.<sup>22,23</sup> In the model we propose (Fig. 5), hydroxyproline-poor glycoprotein would bind to a cell wall component, probably cellulose; then, hydroxylation of proline residues would occur as the cell matures. Ultimately, a hydroxyproline-rich glycoprotein moiety would be generated which may remain with the parent molecule or be cleaved enzymically to release a cell wall component whose role at the present time is not understood.

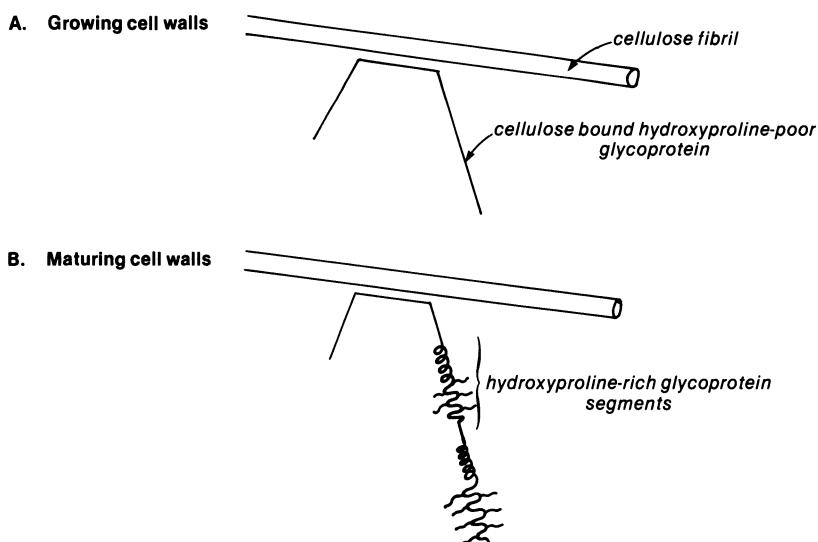


Figure 5. Postulated structure of hydroxyproline-poor glycoprotein complex in growing and non-growing cell walls.

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## Chapter Seven

### PLANT PROTOPLAST AGGLUTINATION AND IMMOBILIZATION

PHILIP J. LARKIN

Division of Plant Industry, CSIRO  
Canberra City, A.C.T. 2601, Australia

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### INTRODUCTION

It is now an almost ubiquitous notion amongst plant cell biologists that plant cells do possess recognition faculties. We have in mind models which envisage plant cells regulating some of their functions in response to external stimuli. These external stimuli may also be thought of in terms of communicated information such as in pollen/stigma interactions or pathogen elicitor induction of host phytoalexins.

The models for recognition processes generally include lock and key (antibody/antigen or enzyme/substrate) type interactions between the recognition binder and the information determinant. Many of the models also assume that the information for communication is encoded by carbohydrate moieties. This is so partly because lectins are currently so newsworthy.<sup>1-3</sup> Additionally, however, one may argue that carbohydrate moieties have a far greater information carrying capacity than a peptide with the same number of residues. This results from the fact that the polysaccharide may be branched, the glycosides linked through different positions and by either  $\alpha$ - or  $\beta$ -linkages. Also the resulting moiety is spatially rigid allowing a 3-dimensional "key" to be constructed to fit a given "lock". This trend to model

information determinants as carbohydrates has a conceptual drawback: polysaccharides are at least two steps removed from the genetic code. The central dogma requires us to see all heritable information as coded in nucleic acid. We do not yet fully understand how 3-dimensional polysaccharide information is faithfully translated from the genetic code. Nevertheless cells can achieve this as illustrated by the simple inheritance of the ABO blood group determinants.

Now a conceptual stumbling-block for such models is the location of the binders (recognition molecules) and determinants. The plant cell wall is an attractive location because complementary binders or determinants from the external milieu have unimpaired access to the wall. However it is difficult to conceive how a recognition interaction occurring on the external extremities of the wall can have an effect on the more active machinery of the cell. A plasmalemma location for the critical interaction is much more attractive in terms of switching on or off cell functions. However one can not help but be a little concerned that the wall may be an obstacle for some determinants arriving at the critical site. This conceptual difficulty would seem most acute where the recognition phenomenon is between two plant cells where the determinant and binder are both anchored in the plasmalemma.

These difficulties of location are sometimes solved for us by the details of a recognition system. For example in many fungal pathogenic systems the hyphae penetrate the wall and sometimes can even be seen to bind to the host plasmalemma.<sup>4,5</sup> Often a fungal haustorium develops between the host wall and plasmalemma.<sup>6,7</sup> In powdery mildew of barley the compatible or incompatible relationship seems to be determined when penetrating hyphae contact the host plasmalemma.<sup>8</sup> Tomiyama *et al.*<sup>9</sup> have observed that Phytophthora infestans hyphae initiate potato hypersensitive reactions only upon contact with the host plasmalemma. These authors also showed that fungal extracts increase potato protoplast death only in compatible combinations.

Another fascinating example where the location problem seems to be solved for us is in graft compatibility phenomena. Where the dividing cells of scion and stock come together the opposing cell walls are dissolved and the plasmalemmas of the two cell types come into direct contact.<sup>10</sup>

These examples not only implicate the plasmalemma as the primary location of the critical recognition interaction but also illustrate some ways in which the "obstruction" of the wall is overcome. It must also be said that our impulsive conception of the membrane and wall being discontinuous and divorced from each other is probably quite wrong. Roland<sup>11</sup> has reviewed the evidence for the close relationship and interdependence of the plant cell wall and membrane. Even ultrastructurally it is apparent that fibrils anchored in the membrane extend deep into the wall. There are also protoplasmic outgrowths and plasmalemma extensions that can be visualized in the wall. It is quite likely that some polysaccharides or glycoproteins anchored in the membrane may extend right through the wall to expose determinants beyond the wall.

The likelihood that membranes are the primary sites of recognition events is enhanced by the observation that many plant lectins appear to be localized to membranes.<sup>12-17</sup> However some have also been localized in the cytoplasm,<sup>14,19</sup> and some to cell walls.<sup>17,18</sup>

Isolated plant protoplasts are cells lacking a cell wall with the plasmalemma directly exposed to the external milieu. Protoplasts may offer advantages as an experimental approach to the study of recognition interactions which occur at the plasmalemma. The preparation of protoplasts is perhaps the only reliable means of obtaining a suspension of truly isolated single cells from a given plant tissue. As a consequence of these characteristics, it is proposed that protoplast agglutination or immobilization may be used as indicators of certain recognition events. Our consideration of these possibilities will be categorized on the basis of whether the determinant and binder are fixed (e.g. anchored to the plasmalemma) or free (e.g. solubilized from the cell).

#### PROTOPLAST AGGLUTINATION

##### Determinant fixed/ binder free

The naked plasmalemma of a protoplast will have its determinants entirely exposed. If a multivalent binder for one or more of these determinants is added exogenously to the protoplast suspension the interaction can manifest itself by protoplast agglutination. This has been demonstrated for

a number of isolated plant lectins with many species of protoplasts (Table 1, Fig. 1).

Table 1. Exogenous lectin-mediated agglutination

| Lectin source                                  | Lectin     | Agglutination | No. of protoplast species | Reference     |
|--|------------|---------------|---------------------------|---------------|
| <u>Arachis hypogaea</u><br>(peanut)            | PNA        | +             | 8                         | 23, 25        |
| <u>Glycine max</u><br>(soybean)                | SBA VII    | +             | 11                        | 23, 25, 26    |
| <u>Canavalia ensiformis</u>                    | ConA       | +             | 13                        | 20-24, 26, 27 |
| <u>Ricinus communis</u><br>(castor bean)       | RCA II     | +             | 8                         | 23, 26        |
| <u>Triticum aestivum</u><br>(wheat germ)       | WGA        | +             | 4                         | 24            |
|  |            | -             | 7                         | 23            |
| <u>Phaseolus vulgaris</u><br>(red kidney bean) | PHA-M or-P | +             | 4                         | 24            |
|  |            | -             | 7                         | 23, 26        |
| <u>Ulex europaeus</u>                          | UEA        | -             | 7                         | 23, 26        |
| <u>Bandeiraea simplicifolia</u>                |            | -             | 5                         | 23            |
| <u>Dolichos biflorus</u>                       | DBA        | -             | 4                         | 23, 26        |
| <u>Phytolacca americana</u><br>(pokeweed)      | PWM        | -             | 1                         | 26            |

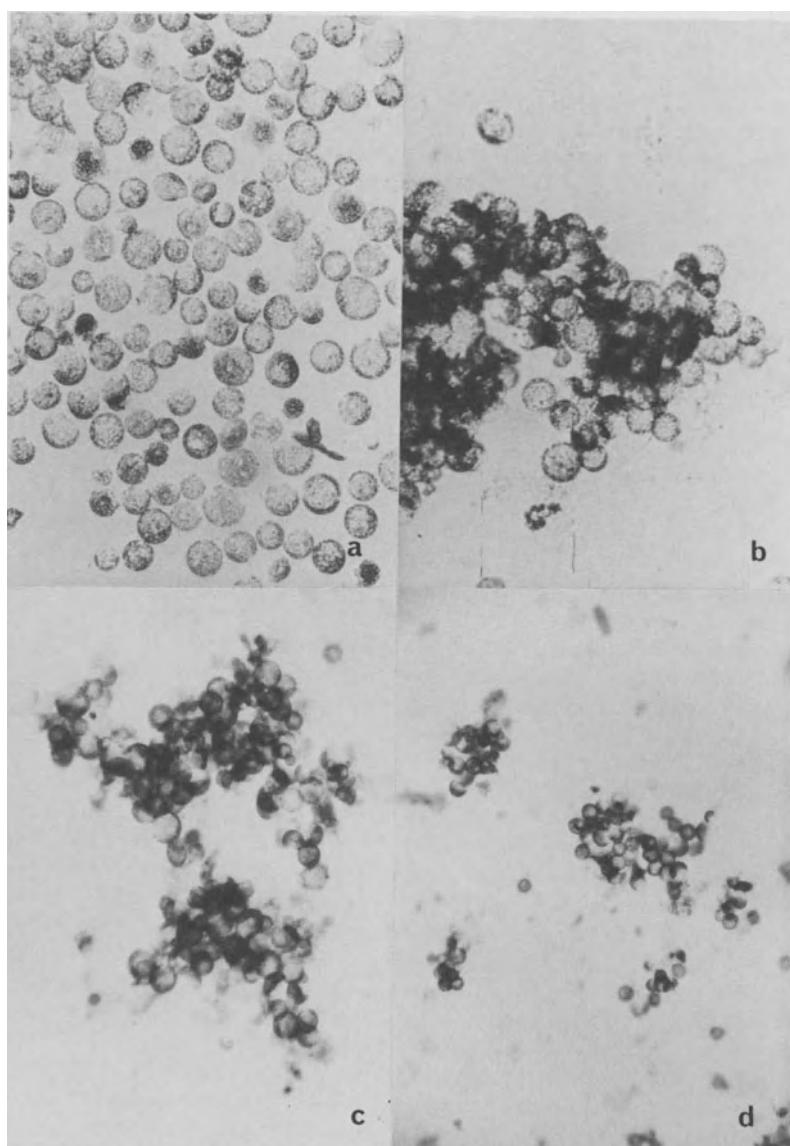


Figure 1. Agglutination induced by exogenous soluble lectins.  
Petunia hybrida leaf protoplasts. a) Control (x100).  
b) Agglutination with PNA (x100).  
Triticum aestivum leaf protoplasts. c) Agglutination  
with SBA VII (x80). d) Agglutination with RCA II (x50).

Multivalency (more than one binding site per molecule) is a critical factor in allowing the crosslinking between cells which leads to agglutination. Some of the well-characterized lectins require pH to be raised to about 7 before they form the multimers which are most efficient at cell agglutination.<sup>2,28</sup> The ability to agglutinate can be enhanced even further by cross-linking the lectin molecules with agents such as glutaraldehyde.<sup>29</sup> When attempting to use protoplast agglutination to investigate undefined receptors one needs to satisfy the requirement for multivalency either by varying parameters such as pH or crosslinking or covalently attaching the binders to a matrix (see later under protoplast immobilization).

The ability to agglutinate will also probably be dependent upon the density or mobility of the determinants on the protoplast surface. Effective bridging between cells may require many lectins (recognition molecules) to bind at any one contact site. This in turn may require the determinants to be mobile and "cap" at the site to give the required local density. Conceivably some determinants may be relatively immobilized due to functional relationships with certain structures. This inability to cap may prevent their use as receptors mediating protoplast agglutination.

The lectins reported to cause agglutination of plant protoplasts represent different glycoside specificities. The agglutination is inhibited by the appropriate simple sugars or glycosides.<sup>20,23,24</sup> This confirms that it is a function of the lectin activity. The lectins do not differentiate between plant species in the ability to bind to their protoplasts. This is perhaps no surprise. Legume lectin-mediated agglutination may be an entirely artificial phenomenon and the protoplast agglutination per se may be unrelated to any in vivo function of these seed lectins.

There is now a widespread consensus that legume lectins play a determining role in the specificity of Rhizobium-legume symbiosis,<sup>30-41</sup> although not all reports have concurred.<sup>42,43</sup> Most of this work has concerned the interaction of the plant lectin with the rhizobial cells. Dazzo and Hubbell<sup>33</sup> proposed a model for attachment based upon a cross-reactive antigen on both the Rhizobium and plant cell. The recognition lectin is proposed to bind to this antigen and, due to its multivalency, form a bridge attachment between the

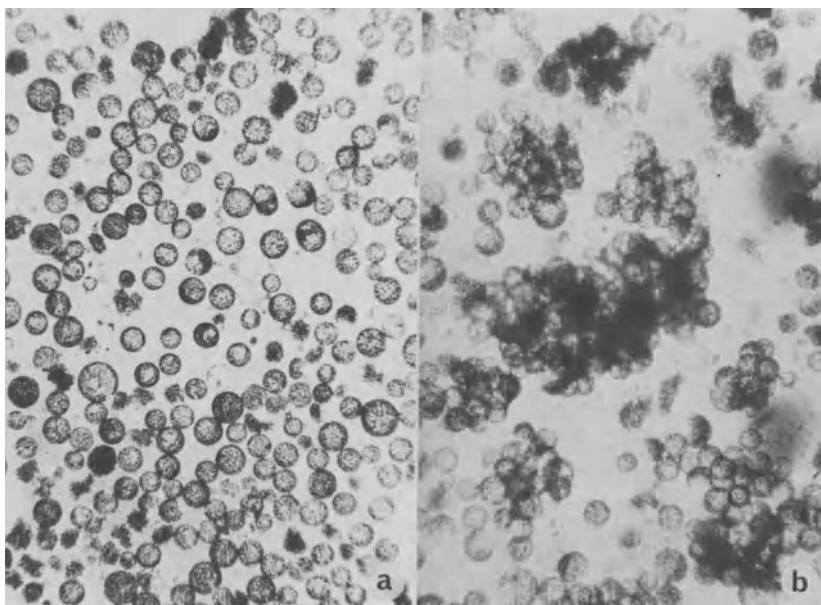
rhizobial cells and plant root hairs. There has however been very little work to confirm the existence of cross-reactive antigens. If they are anchored in the host plasmalemma then protoplast preparations may be agglutinated by the appropriate lectin.

Some toxins are known to have subunits which have lectin activity. Cholera toxin,<sup>44</sup> tetanus toxin,<sup>45</sup> and diphtheria toxin<sup>46</sup> are able to specifically bind to cell surface gangliosides or oligosaccharides. There may be toxins from plant pathogens which similarly have lectin-type activities. If these are multivalent, or can be made multivalent, with respect to that activity (e.g. by glutaraldehyde cross-linking) this surface interaction may be manifested as an ability to agglutinate protoplasts. Protoplast agglutination would then become a system for investigating the host-specificity and chemistry of the interaction.

Yeoman *et al.*<sup>10</sup> have reported agglutination of the protoplasts of one species caused by saline leaf extracts of another species when the two species are graft compatible. No agglutination was observed in graft incompatible combinations. These preliminary observations need extension and clarification. As they are they seem to indicate that the protoplast agglutination system has uncovered the recognition components involved with graft compatibility.

Mention should also be made of the use of antisera to agglutinate protoplasts. This is of course not a plant recognition system. However antibody-mediated agglutination is a potentially useful tool for confirming the plasmalemma-location of a given component. A number of reports have confirmed that antiserum causes protoplasts to aggregate.<sup>47-51</sup> However even control sera were found to cause high levels of agglutination apparently due to the interaction of a non-specific component of the serum of 9 animal species with protoplast surface arabinogalactan proteins<sup>50</sup> (Fig. 2). Partial purification of the immunoglobulin by ammonium sulphate fractionation did not remove the non-specific agglutinins.

Recently Raff *et al.*<sup>51</sup> confirmed that control (pre-immune) rabbit sera cause plant protoplast agglutination. Indeed they were using the IgG fraction prepared by Protein A-Sepharose affinity chromatography. At low IgG concentrations there appeared to be a greater ability to agglutinate



**Figure 2. Agglutination with pre-immune immunoglobulins. *Nicotiana tabacum* leaf protoplasts. a) Supernatant of 45% saturation  $(\text{NH}_4)_2\text{SO}_4$  fractionation of cat serum. b) Precipitate (immunoglobulins) of 45% saturation  $(\text{NH}_4)_2\text{SO}_4$  fractionation of cat serum.**

Both fractions were dialyzed to protoplast osmoticum and used at a concentration of 1/10 relative to original volume.

with the immune preparations. Nevertheless there was 75% agglutination when the control preparation was used at 5  $\mu\text{g}/\text{ml}$ . Hanke<sup>25</sup> observed agglutination triggered by submaxillary asialomucins. Similar components in serum may also bind to Protein A. There is a need to clarify which serum component is involved in the non-specific agglutination. If it proves possible to prepare highly purified immunoglobulin specific for surface antigens they will be useful for confirming the observations of Strobel and Hess<sup>49</sup> that the recognition protein of sugarcane for the *Helminthosporium sacchari* toxin exists in the protoplast plasmalemma.

Determinant free/binder fixed

The naked surface of a protoplast will have any plasmalemma-bound recognition binding molecules exposed for easy access of exogenous determinants. If the determinants are multiliganded the interaction may manifest itself as protoplast agglutination. This category of interaction may be illustrated with the agglutination induced by Yariv antigens. These are synthetic phenylazo-glycosides which are trivalent with respect to the glycoside moiety<sup>52,53</sup> (Fig. 3). These synthetic determinants, when bearing suitable  $\beta$ -linked glycosides, are bound by arabinogalactan proteins (sometimes referred to as  $\beta$ -lectins) on the protoplasts and agglutination results<sup>50,54</sup> (Fig. 4). It is not known what function the arabinogalactan proteins serve in plants. Gleeson and Clarke<sup>55</sup> found them as major components of stylar canals and suggested a recognition and/or nutritive role for the growing pollen tube. It has also been suggested that they may be pollen catching molecules in the stigma.<sup>56</sup> The structure and function of arabinogalactan proteins has recently been reviewed by Clarke *et al.*<sup>57</sup>

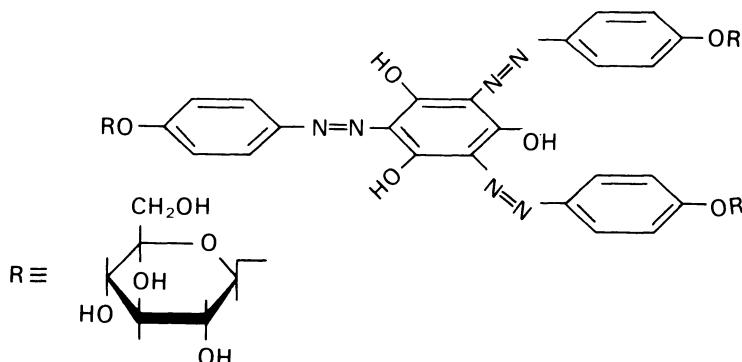


Figure 3. Structure of Yariv antigens. Shown is the  $\beta$ -glucosyl Yariv ( $\beta$ -D-Glu) which is 1,3,5-tri-(*p*- $\beta$ -D-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene.

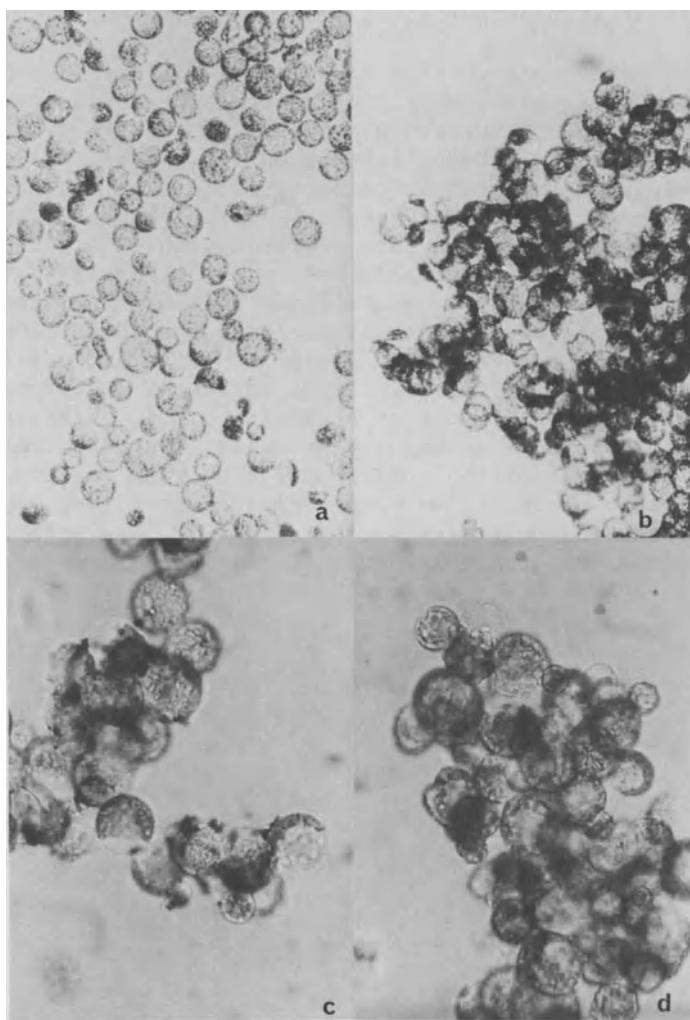


Figure 4. Agglutination of protoplasts induced by  $\beta$ -glycosyl Yariv antigens.

Petunia hybrida leaf protoplasts. a) Control (x100).  
b) Agglutination with 50  $\mu\text{g}/\text{ml}$   $\beta$ -lactosyl Yariv antigen (x100).

Triticum aestivum leaf protoplasts. c) Agglutination with 50  $\mu\text{g}/\text{ml}$   $\beta$ -galactosyl Yariv antigen (x250). d) Agglutination with 50  $\mu\text{g}/\text{ml}$   $\beta$ -cellobiosyl Yariv antigen (x250).

It is also interesting that arabinogalactans themselves are good determinants for other lectins, possibly due to their brush-like branching structure.<sup>58</sup> They have been used in cross-linked form as affinity matrices for the purification of castor bean and peanut lectins.<sup>59,60</sup> It seems quite likely that surface arabinogalactans are at least some of the determinants acting as receptors during RCA II, PNA- and SBA VII-induced protoplast agglutination.

It is a possibility that phenolic glycosides such as flavonoids are the natural analogues of Yariv antigens. There is evidence that O-hydroxymethylphenyl- $\beta$ -D-glucoside (salicin), p-nitrophenyl- $\beta$ -D-glucoside and indoxylyl- $\beta$ -D-glucoside are inhibitory to Yariv antigen-mediated agglutination.<sup>54</sup> Some partially purified flavonoid glycosides extracted from Ceratonia siliqua (carob), Hypericum perforatum, Echeveria gibbiflora and Pseudotsuga menziesii (Douglas-fir) leaves and Allium cepa (onion) bulbs also showed inhibitory activity.<sup>61</sup> Jermyn<sup>62</sup> has found that  $\beta$ -lectins are retained on affinity columns of myricetin-3- $\beta$ -D-galactoside or quercetin-3- $\beta$ -D-galactoside coupled to the bromoacetyl derivative of 6-amino-hexyl Sepharose. These flavanol glycosides also competitively inhibited the precipitation reaction between  $\beta$ -lectins and Yariv antigens.

A tantalising example of protoplast agglutination which has bearing on the theme of recognition is that reported by Professor Stelzig and his colleagues.<sup>63,64</sup> They find that the elicitor from Phytophthora infestans which induces phytoalexin production in potato discs also causes extensive agglutination of potato leaf protoplasts. The elicitor is a  $\beta$ -1,3-glucan with extensive 1,6-branching. The agglutination is taken as corroborative evidence that there is a recognition receptor on the host plasmalemma.

There are of course many other elicitor/phytoalexin systems. In most of these the elicitor is reported to be non-host-specific.<sup>65-70</sup> Keen<sup>71,72</sup> however has obtained a mannose-containing, wall-associated glycoprotein elicitor from Phytophthora megasperma var. sojae which appears to be host-specific.

Similarly there are race-specific glycoproteins from Cladosporium fulvum which induce hypersensitive cell death only in resistant tomato hosts.<sup>73,74</sup> It may prove valuable

to investigate the ability of these elicitors to agglutinate protoplast preparations. One could vary the genotype of the protoplasts to determine the host range for receptors (binders) of the elicitors.

It is interesting that a terpenoid glycoside with elicitor activity appears to have been recovered as a sub-fraction of P. infestans wall elicitors.<sup>66</sup> In potato these elicit phytoalexins which are themselves terpenoids. This observation requires further clarification. It is possible that arabinogalactan proteins ( $\beta$ -lectins) are the binding receptors for terpenoid elicitor moieties.

More recently hypersensitivity-inhibiting-factors (HIF) have been described in the potato/Phytophthora infestans system.<sup>75</sup> These suppressors, like the elicitors themselves, are glucans containing  $\beta(1\rightarrow 3)$  linkages. They are isolated from the fungal mycelia, zoospores and cytospores as small molecular weight water-soluble molecules. The glucans from compatible races have greater suppressor activity than those from incompatible races and it is suggested that these factors are responsible for the specificity. It is likely they act by competitive inhibition of the elicitor interaction with the plant cell receptor. If this mode of action is correct they should also inhibit potato protoplast agglutination in the presence of elicitor.

There are other examples of pathogenic toxins which appear to induce susceptibility rather than resistance (as in the case of hypersensitivity elicitors). El-Banoby and Rudolph<sup>76</sup> recently described water-soaking extracellular polysaccharides from Pseudomonas and Xanthomonas pathogens which appear to be specific for their respective hosts. The mal-secco disease of Citrus sp. is caused by Phoma tracheiphila and the pathogen produces a glycoprotein toxin (malseccin) which plays a role in the disease process.<sup>77</sup> Such toxins may also have specific binding receptors on host plasmalemma which may be indicated by protoplast agglutination in their presence. The host-specific toxin, victorin, from Helminthosporium victoriae causes susceptible oat protoplast lysis.<sup>78</sup> Cross-linking of the toxin into multimers may allow oat protoplast agglutination and possibly suppression of the lysis effect.

Determinant fixed/binder fixed

Many or most recognition phenomena will involve both determinant and binder fixed to separate cells. When one of these components can be solubilized the experimental possibilities discussed above apply. We will consider here also systems where neither determinant nor binder can be or need be solubilized. The phase-, sex-, and species-specific mating type reaction in Chlamydomonas is a naturally-occurring agglutination system.<sup>79</sup> The initial step in the copulation process is the adhesion of the flagella of opposite mating types of gametes of the same species. The adhesion appears to involve two independent binding phenomena: a binder on the + gamete with a determinant on the - gamete, and a binder on the - gamete with a determinant on the + gamete. All components in the interaction are fixed to the cell surfaces. It is possible to solubilize agglutinin activities with the predicted specificities. However the mating interaction can be observed as agglutination of + and - gametes under experimental conditions with all components in situ.

It may be possible to employ this approach with mixtures of plant protoplasts and pathogenic cells. If there is a binding-type recognition interaction between the two cell types, then a coagglutination matrix will form. There is considerable evidence that, as a prerequisite for plant tumor induction, the bacterium Agrobacterium tumefaciens attaches to specific sites on the plant cell wall.<sup>80-84</sup> All cell wall materials are synthesized and extruded through the plasmalemma. It is therefore possible that the protoplast will also bear the receptors for agrobacterial adhesion. A few hours preincubation in culture medium allowing synthetic activity may enhance this possibility.

Similar experimental possibilities exist with the hypersensitivity-inducing bacterium, Pseudomonas solanacearum. The hypersensitive response of the plant tissue is triggered by incompatible bacterial strains which are "rough" in lipopolysaccharide type. The initial event which triggers the response seems to be attachment of the bacteria via the lipopolysaccharide determinants to a plant cell surface lectin.<sup>85-88</sup> Solubilized potato lectin appears to have the required selectivity between virulent and avirulent strains.<sup>83</sup> Bacterial immobilization by plant cell surface components

has been postulated as a defence mechanism in a number of other systems as well.<sup>89-92</sup>

In many fungal pathogen interactions, the critical recognition event appears to occur when the penetrating hyphae contacts the plant plasmalemma.<sup>6-8</sup> Lectin-like activities have been implicated.<sup>67</sup> Hyphal fragments may bridge between appropriate plant protoplasts causing agglutination.

Reference has already been made to the cell contact between stock and scion in a plant graft. The cell walls of the dividing cells appear to be digested at the point of contact to allow direct contact between the plasmalemmas of the stock- and scion-derived cells.<sup>10</sup> This plasmalemma exposure and contact occurs both in compatible and incompatible grafts and it is only after this phenomenon that incompatibility becomes apparent. Protoplasts may be able to make a valuable contribution to the study of graft compatibility. Do protoplasts prepared from the stock callus and scion callus autoagglutinate when mixed? Preliminary observations with seed extracted agglutinins and leaf protoplasts showed selective agglutination with graft-compatible partners.<sup>10</sup> This work needs extension and clarification. There may be specific recognition binders and determinants produced at the time of graft contact.

#### Protoplast immobilization

An alternative experimental possibility for using protoplasts to investigate surface recognition events is to artificially fix one of the components (binder or determinant) to a solid support. If a solubilized determinant is unliganded, or a solubilized binder is univalent, they will be unable to initiate protoplast agglutination. If such components are first covalently linked to a solid matrix, the recognition interaction will be visualized as protoplast immobilization. We have been able to demonstrate this approach using Con A, RCA II, SBA VII, and PNA covalently bound with glutaraldehyde to collagen membranes or serum albumin sponges (themselves formed with glutaraldehyde cross-linking). Plant protoplasts adhere tenaciously to such surfaces so that they are stable to thorough rinsing and viable at least in the short term (Fig. 5).

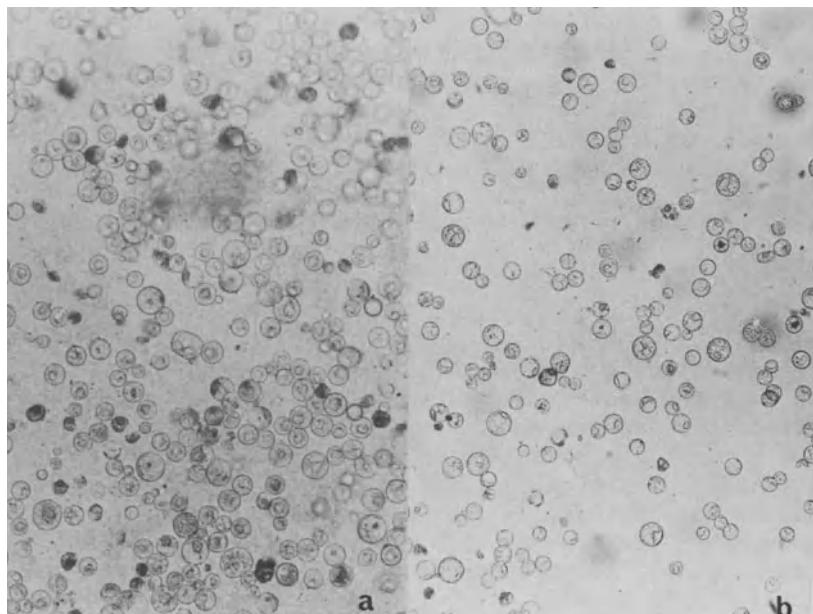


Figure 5. Protoplast immobilization. a) Tobacco "nia 63" culture protoplasts adhering to a glutaraldehyde cross-linked protein sponge containing bovine serum albumin and Con A. The non-adhering protoplasts have been removed by 3 consecutive rinses by immersion. b) Corn culture protoplasts immobilized similarly to above.

This approach, while technically more involved, has the advantage that components of unknown valency may be used. It may also be possible to differentiate between biological effects requiring uptake of the effector and effects requiring only surface binding. For example pathotoxins or phytohormones may be bound to a supporting matrix and plant protoplasts immobilized by interaction with the toxins or phytohormones. If the biological effects can still be observed it may be concluded that uptake of the effector is unnecessary.

A modification of this approach is to covalently cross-link effectors (determinants or binders) to erythrocytes or beads. The recognition interaction is then visualized as a rosetting of the erythrocytes or beads around the plant protoplasts. Raff *et al.*<sup>51</sup> have shown that the rosetting technique can be used to confirm the location of an antigen on plant protoplasts. The protoplasts were reacted with the rabbit antiserum. Erythrocytes bearing cross-linked sheep anti-rabbit immunoglobulin formed rosettes around the protoplasts.

#### CAUTION AND CONCLUSION

Anyone contemplating the use of protoplast agglutination to investigate some recognition phenomenon needs to be aware of the dangers. Firstly one must be aware that there are a number of macromolecules which cause non-specific agglutination of protoplasts. Those reported include gelatin proteins and peptides,<sup>93</sup> non-immune immunoglobulin preparations,<sup>50, 51</sup> IgA myeloma J539 protein,<sup>94</sup> submaxillary asialomucins,<sup>25</sup> polyethylene glycol,<sup>95, 96</sup> and polyvinyl-alcohol.<sup>97</sup> It is also noteworthy than some  $\alpha$ -galactosidases extracted from legume seeds acted as haemagglutinins.<sup>98</sup> Some species of protoplasts we have observed to be particularly prone to autoagglutination particularly after a few hours in certain culture media e.g. *Brassica* protoplasts in B5 salt<sup>99</sup> media. This autoagglutination is not necessarily damaging and divisions often initiate from such clumps. It remains to be seen whether developmentally-regulated lectins are responsible or perhaps surface charge effects.

Secondly some caution must be exercised with regard to the time between protoplast isolation and use, and the medium in which they have been stored. We have observed a dramatic effect of time in culture on agglutinability. The agglutinability with peanut lectin (PNA) or  $\beta$ -lactosyl Yariy antigen ( $\beta$ -LAC) drops dramatically in the first day or two of culture in normal medium. Coumarin<sup>101</sup> and 2,6-dichlorobenzonitrile (2,6-DB)<sup>101</sup> are both inhibitors of cell wall synthesis. In the presence of these compounds the drop in agglutinability is delayed (Fig. 6). This suggests that wall formation is responsible for the reduced agglutinability. In the case of ConA the first 20 to 30 hours in culture actually enhance agglutinability but thereafter wall formation interferes. Hanke<sup>25</sup> reported a drop with time in SBA-induced

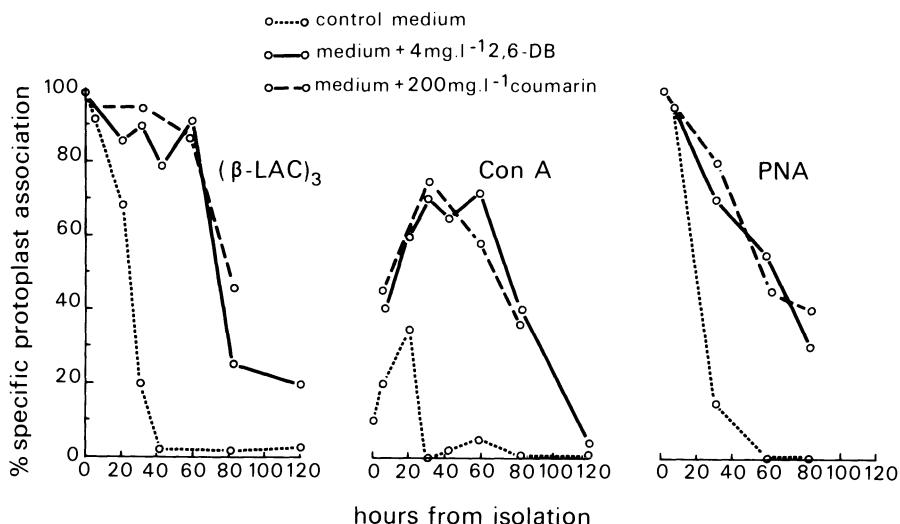


Figure 6. The effect of culture of *Petunia hybrida* protoplasts on their agglutinability with  $\beta$ -lactosyl Yariv antigen ( $\beta\text{-LAC}$ ), Con A and PNA. Culture was both with and without the cell wall synthesis inhibitors coumarin or 2,6-DB, as indicated. The percent specific protoplast association was determined as the difference between the controls (no agglutinin) and the tests each time and for each medium.

agglutination of soybean protoplasts but a rise with time in ConA-induced agglutination.

These results with ConA introduce another issue for caution. It is possible that the isolation procedures for protoplasts, which involve crude enzyme preparations, remove important recognition components or even expose artifactual determinants. The preincubation of petunia and *Nicotiana debneyi* protoplasts for 1 or 2 days in the presence of coumarin or 2,6-DB greatly enhanced their agglutinability with ConA (Fig. 6). This suggests that synthetic metabolism enhances the receptors for ConA. Those receptors are unlikely to be wall polymers.

The fact that wall formation inhibits agglutination does not necessarily negate the *in vivo* significance of the interaction being investigated. Cell deformability is a

very critical factor in agglutination.<sup>102 103</sup> Naked protoplasts are highly deformable. The reformation of wall will drastically reduce deformability and consequently agglutinability even if the binder/determinant interactions are occurring in the presence of the wall as they must in nature.

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## Chapter Eight

### MOLECULAR ASPECTS OF RECOGNITION AND RESPONSE IN THE POLLEN-STIGMA INTERACTION

ADRIENNE E. CLARKE AND PAUL A. GLEESON

School of Botany  
University of Melbourne  
Parkville 3052, Australia

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### INTRODUCTION

Although fertilization in plants is fundamental to life, our understanding of the process at the molecular level is restricted to a few facets of a few systems; predictably, the best information has come from the simplest systems. For example, the first event of fertilization in some algal species is direct membrane contact of gametes which do not

have cell walls, and the recognition of compatible gametes is apparently mediated by essentially the same sort of reaction which occurs during the mutual recognition of animal cells. The cellular events leading to fertilization in higher plants are more complex: in most cases not only are there a series of interactions between the haploid pollen housing the male gametes, and the diploid female tissues of the pistil, prior to fertilization, but the interactions involve contact of cells which have walls over-laying the plasma membrane. For this reason, we will consider the possible mechanisms for cell-cell recognition between plant cells. We will then outline the biology of fertilization in flowering plants with particular reference to the stages where cell-cell recognition interactions are evident. The information available concerning the nature of the surface components of cells which come into contact during pollination will then be considered, and finally we will present some experimental data on the structure and function of three classes of macromolecules implicated in the events of pollination.

#### MOLECULAR BASIS OF CELL-CELL RECOGNITION

Cell recognition is the initial event of cell-cell communication which elicits a defined biochemical, physiological or morphological response.<sup>1</sup> For animal cells, the receipt and translation of intercellular signals is believed to be largely a cell surface phenomenon; furthermore, it depends on formation of a complex between the signalling molecule and a membrane bound receptor. The reaction is specific both for receipt of a particular signal and for a particular cellular response. There are two general mechanisms envisaged for cell recognition: In the first, complex formation is at the surfaces of cells in contact, and is mediated by one or more pairs of complementary molecules (Fig. 1a) of which at least one is protein in nature. In many instances the partners in this interaction are saccharide components of glycoproteins or glycolipids and complementary proteins or glycoproteins (for reviews see references 2-5). If these saccharide-binding components are multivalent, they can be classified as lectins.<sup>6</sup> For the second major mechanism, it is assumed that cells may have specific membrane receptors for extracellular macromolecules (Fig. 1c); if the macromolecule is multivalent, it may effectively cross link cells (Fig. 1b). Implicit

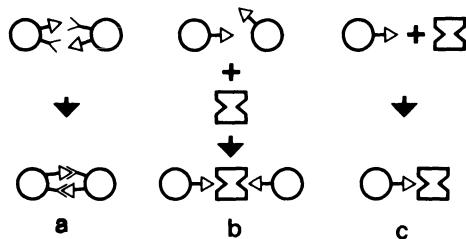


Figure 1. Possible mechanisms by which recognition in animal cells may be mediated. (a) Interaction of one or more pairs of sterically complementary molecules; (b) Cell attachment via multivalent extracellular molecule; (c) Attachment of extracellular molecule to plasma membrane receptor.

in both mechanisms is the idea that binding of a macromolecule to the surface receptor\*, whether it is itself part of another cell surface or is extracellular, initiates some change in the membrane which is then transmitted to the cytoplasm, eventually to give the observed response. Various possibilities are, that movement of the membrane receptors induced by the binding may cause permeability changes in the membrane, activation of peripheral enzymes, alterations in the underlying cytoskeleton or internalization of the bound receptors which then move through the cytoplasm to the target organelle, to initiate the response (Fig. 2).<sup>10</sup>

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\*In this discussion, we use the term "receptor" in the sense defined by Greaves<sup>7</sup> and Goldstein and Hayes<sup>8</sup> to describe a membrane structure which binds external molecules in a highly specific way, thereby transmitting signals from the environment to the cell. An alternative nomenclature has recently been suggested by Ballou and coworkers<sup>9</sup> in which "cognor" signifies the active, recognizer partner of the interaction, and "cognon" denotes the passive partner, which is the site recognized.

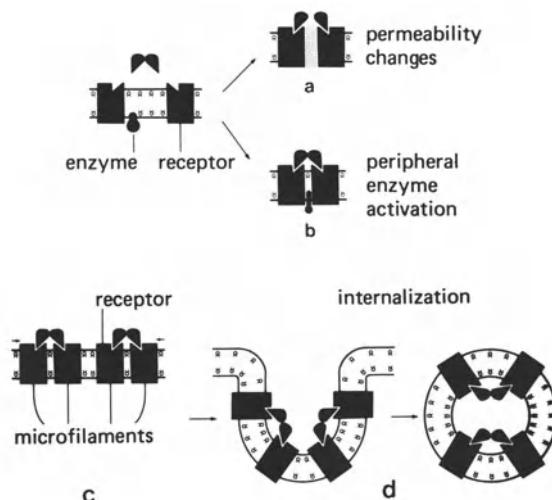


Figure 2. Some possible mechanisms for response of a cell to receipt of an extracellular molecular signal: Binding of a multivalent molecule to membrane receptors may cause (a) Permeability changes by clustering of receptors; (b) Activation of enzymes bound to the membrane; (c) Alterations in the cytoskeletal elements; (d) Internalization of bound macromolecules (adapted from Reference 10).

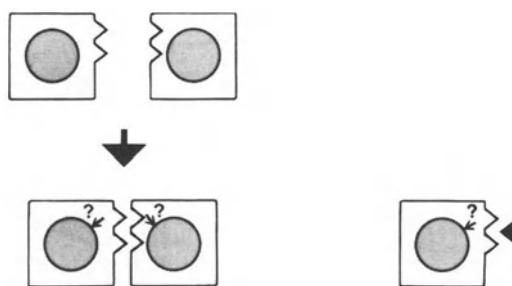
These types of complementary interaction, (Fig. 1) involving surface saccharides and complementary lectins, are known for a number of animal cell systems. These are also operative in some plant cell interactions which involve direct membrane-membrane contact. Thus, contact of naked gametes during mating of certain algal species depends on binding between surface saccharides and specific surface saccharide-binding components. For example, mating in the unicellular, biflagellate, fresh water alga Chlamydomonas proceeds by adhesion at the flagella tips of gametes of the opposite mating type, followed by cell fusion to form a zygote. In some species this initial adhesion possibly involves interaction between  $\alpha$ -mannosyl groups on the flagellar surfaces of the plus gametes and a complementary

receptor (lectin) on the flagellar surface of the minus gametes.<sup>11</sup> Mating in *Fucus*, a member of the brown algae Fucales apparently depends on a similar saccharide-saccharide binding interaction. In this system, fertilization of the large non-motile eggs is highly species-specific and is mediated by attachment of the tip of the anterior flagellum of the sperm to the egg cell membrane. The available evidence indicates that egg cell surface saccharides containing both fucosyl and mannosyl residues are involved in the specific initiating events of fertilization.<sup>12</sup>

Direct communication between higher plant cells, by contact between the extracellular faces of the plasma membranes is precluded by the presence of the cell walls; however, there are two major mechanisms by which recognition of extracellular signals can be envisaged.

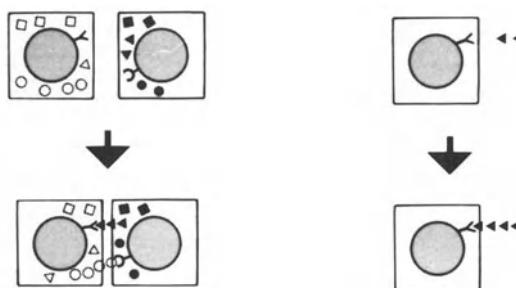
#### 1. Complementary interaction of components at the wall surfaces of cells in contact

For cells which come into physical contact such as pollen on stigma surfaces, pollen tubes and the cells of the style canal, fungal zoospores at root surfaces, or fungal hyphae with epidermal cells, it is possible that direct contact between walls of cells could initiate some sort of reorganization or deformation of wall component(s), which in turn may transmit a signal to the plasma membrane and thence to the cytoplasm (Fig. 3a). One class of likely candidates for this type of interaction would be the polysaccharide matrix components, which in contrast to the fibrillar components have considerable informational potential in the type and arrangement of their constituent monosaccharides. These polysaccharides may interact with complementary sequences of polysaccharides in the interacting cell wall, with subsequent changes in the gel properties of the walls. Certainly, interactions between complementary regions of polysaccharides in solution are known,<sup>13</sup> and they do affect the gel properties. Alternatively, the matrix polysaccharides may be modified by interaction with proteins or glycoproteins such as lectins or enzymes. The current information regarding the molecular architecture of the cell wall is not sufficiently detailed to allow assessment of the likelihood of this possibility: we know a great deal about the structure and form of some of the wall polysaccharides and glycoproteins of a few cell types, but very



**a** Direct cell wall-cell wall contact. Interaction via complementary macromolecules at the surface

**b** Extracellular molecule interacts with complementary wall molecules at the surface



**c** Direct cell wall-cell wall contact. Interaction via wall macromolecules which diffuse through the wall of the interacting cell to a plasma membrane receptor

**d** Extracellular molecule diffuses through cell wall to a plasma membrane receptor

**Figure 3.** Possible mechanisms by which recognition in plant cells may be mediated. In each case a signal ultimately reaches the plasma membrane through the cell wall. In (a) and (b) the wall itself is the site of initial receipt of information - that is, the wall plays an active part in the recognition reaction. In (c) and (d) the wall is regarded as a passive barrier through which the signal molecule may pass to a membrane receptor. The square outline represents the outer boundary of the cell wall. The circle represents the plasma membrane.

little about their precise intermolecular associations and organization within the wall.<sup>14</sup> For instance, there may be concentration gradients of particular components, some, for example being localized at the outer wall surface rather than the region adjoining the plasma membrane. In spite of the lack of knowledge in this area, there is evidence that wall-wall contact does operate in one situation - the sex-specific agglutination of yeasts. For several species, including Hansenula wingei, specific agglutination is apparently mediated by complex formation between complementary surface molecules which are evenly distributed over the whole cell wall: furthermore the interaction involves a glycoprotein and saccharide residues (Fig. 3a).<sup>9</sup> A variant of this mechanism is one in which an extra-cellular macromolecular signal may be received at the outer surface of the wall and be transmitted to the plasma membrane by some mechanism involving a similar perturbation of the wall structure or secondary messenger in the wall (Fig. 3b). In this type of interaction the wall components would play an active role.

## 2. Transmission of an extracellular signal through the cell wall to a plasma membrane receptor

An alternative mechanism in which the wall components play a passive role can also be envisaged. It may be that a molecular signal originating from one cell, could diffuse to the cell surface and then directly through the walls of the interacting cell to be received at the plasma membrane by a complementary receptor (Fig. 3c). For cells within the same tissue this would also involve passage through the middle lamella. There is evidence that molecules of up to 5nm in diameter (equivalent to approx. MW 17,000 for a globular protein or 6,500 for a dextran) can pass through the gel matrix of the primary wall of some cells,<sup>15</sup> so that information transmission by this mechanism is possible. The variant of the mechanism corresponding to that shown in Fig. 3b, is that in which an extracellular macromolecular signal diffuses through the cell wall of the target cell to the plasma membrane receptor (Fig. 3d).

In spite of the lack of information regarding details of the mechanism, there is evidence that this type of interaction may be the basis of at least one example of cell-cell recognition. This is the interaction between Phytophthora

megasperma var. sojae and soybean in which an extracellular 3,6  $\beta$ -glucan, related to the fungal cell wall matrix components, will initiate phytoalexin production in both tissue slices and cultured callus cells.<sup>16</sup> There is also indirect evidence for the presence of a specific receptor for these and related (1 $\rightarrow$ 3)-linked  $\beta$ -glucans at protoplast surfaces.<sup>17</sup> In vivo the interaction could be envisaged in terms of diffusion of a low MW fungal wall polysaccharide, through the host plant cell wall to a plasma membrane receptor. Binding at the membrane receptor then somehow triggers phytoalexin biosynthesis (Fig. 2).

There are also a number of interactions between higher plants and microorganisms in which recognition, either in a symbiotic or pathogenic relationship probably involves surface saccharides and saccharide receptors (for reviews see References 1, 16, 18-23).

Apart from contact via the walls, cells in somatic tissues may also be in contact via plasmodesmata, which are continuities of the plasma membrane through the cell wall. Movement within these intercellular channels is apparently restricted to molecules of up to 1,000 daltons. Communication of this kind is important in maintaining a state of differentiation in a tissue; it allows some exchange of intercellular information, within the symplast, and is separate from the extracellular recognition systems discussed above, which operate within the apoplast.<sup>24</sup>

We will now outline the biology of fertilization in flowering plants as a basis for considering how these views of cell-cell recognition apply to pollination.

#### BIOLOGY OF FERTILIZATION IN FLOWERING PLANTS

The interacting partners are the pollen and the pistil. Pollen grains containing the male gametes are transported, usually by wind currents or by animal vectors to the female organ, the pistil. If the mating is compatible, the pollen hydrates at the stigma surface of the pistil, produces a pollen tube which penetrates the stigma surface and grows intercellularly through the style to the embryo sac, where fertilization occurs (Fig. 4). This involves release of two sperm cells, one fertilizes the egg and the other fuses with primary endosperm nucleus. Breeding is controlled by

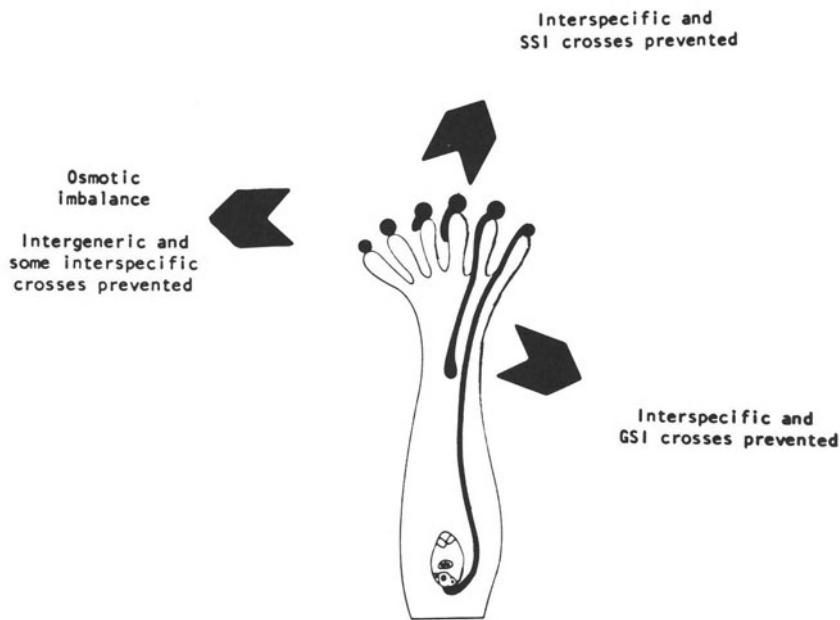


Figure 4. Diagrammatic representation of the events of pollination and the various stages at which growth of incompatible pollen tubes may be arrested. The hand-like structure represents the pistil, and the finger-like processes represent individual papillar cells. The solid black structures represent the pollen grains and tubes. (Adapted from Reference 1). (SSI = sporophytic self-incompatibility; GSI = gametophytic self-incompatibility).

selection between the different types of pollen which may be received by the stigma: intergeneric crosses are usually prevented; interspecific crosses are occasionally successful, but usually intraspecific crosses are successful. The exceptions to this are in the 70 or so plant families in which specific self-incompatibility genes operate to prevent inbreeding. There are usually no morphological differences between the breeding groups, and the system favours out-crossing by preventing self-pollination.

By defining the points at which these different types of crosses are prevented, we can start to ask questions regarding the nature of the interacting surfaces involved in the recognition. It is not easy to categorize these interactions, because of the enormous variation in behavior of foreign pollen on a pistil. However, for many inter-generic pollinations and some interspecific pollinations, it is believed that some type of osmotic imbalance prevents the pollen from germinating, as although the pollen may not germinate on a particular stigma, it can be induced to germinate in artificial media of appropriate concentration. This barrier to germination is not necessarily based on interaction between a cell surface receptor and an external molecular signal, and will not be considered further in this paper.

For some interspecific crosses and for one of the two types of self-incompatibility (sporophytic incompatibility) the foreign pollen may germinate, but fail to penetrate the stigma surface. There is some information regarding the nature of the interaction in sporophytic self-incompatibility systems of the Cruciferae and Compositae. In these systems, germination of the pollen is controlled not by its own genotype, but by that of the plant which produced it. There is a single gene locus with multiple alleles; where there is a match between an allele of the pollen parent and the female tissues of the stigma and style, the pollen tubes are inhibited on the stigma surface (Fig. 5a). The interaction can be viewed as a cell-cell recognition event in which components of the stigma surface and the surface of either the pollen grain or rudimentary pollen tube surface interact to signal either continued or arrested growth of the pollen tube. A most useful observation has been that arrest of tube growth in incompatible matings is commonly accompanied by rapid deposition of material which gives a brilliant yellow fluorescence with aniline blue, both at the stigma surface adjacent to the contact point with the rudimentary pollen tube and also in the tip of the tube.<sup>25,26,27</sup> Because of this characteristic staining with aniline blue the material is referred to as "callose" (see section beginning on page 194). In compatible pollinations callose is also detected: in this situation it is laid down in pads which apparently "block off" older sections of the tube, restricting the cytoplasm containing the sperm cells to the growing tip.

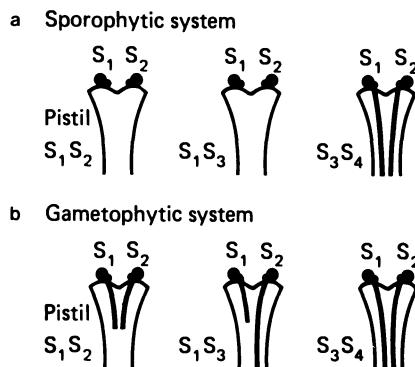


Figure 5. Behaviour of pollen in the two major self-incompatibility systems.

(a) Sporophytic incompatibility. The pollen parent genotype is  $S_1S_2$ . When an allele in the pollen parent is matched with that of the pistil (e.g.  $S_1S_2$  or  $S_1S_3$ ) pollen germination is arrested at the stigma surface. Where there is no match ( $S_3S_4$ ) the pollen may germinate and grow through the style to the embryo sac. The central diagram of Fig. 5a only applies if the allele  $S_1$  is dominant to or codominant with  $S_2$  in the pollen and  $S_1$  is dominant to or codominant with  $S_3$  in the style. If  $S_3$  is dominant to  $S_1$  in the style, or  $S_2$  dominant to  $S_1$  in the pollen, then pollen from  $S_2S_2$  parent will be compatible. (b) Gametophytic incompatibility. The pollen parent genotype is  $S_1S_2$ . When an allele in the individual haploid pollen grain is matched with that of the diploid stylar tissues, growth of the pollen tube will be arrested, usually in the style. For example, both  $S_1$  and  $S_2$  pollen will be inhibited in an  $S_1S_2$  style, but  $S_2$  pollen will grow successfully through an  $S_1S_3$  style. Where there is no match the pollen tube will grow through the style to the embryo sac. For example,  $S_1S_2$  pollen grains, on an  $S_3S_4$  pistil.

With regard to the interacting surfaces, integrity of the stigma surface is important, because by disrupting the stigma chemically (organic solvents, e.g. hexane), physically (heat, mechanical or electrical damage) or enzymically (with trypsin), pollen tubes which would normally be arrested

at the stigma surface, maybe allowed to grow into the style (for reviews see References 1, 28, 29). There is no information available regarding the structure of the stigma surface components which are involved in this reaction. However, some information regarding the nature of the pollen components has been derived from experiments in which observations of "callose" production have been used as a qualitative bioassay. For several systems, "callose" deposition can be induced at the stigma surface by application of a protein-containing fraction derived from the outer wall (exine) of ungerminated incompatible pollen.<sup>30,31</sup> This implies that exine-derived pollen proteins originating from the anther tapetum of the pollen parent, contain information related to the S-genotype, which is perceived at the stigma surface. Presumably this "callose"-eliciting component is carried onto the growing pollen tube surface, and recognition at the stigma surface results in "callose" deposition. The finding that stigma extracts of an incompatible genotype, but not of a compatible genotype, will inhibit pollen tube growth in vitro, indicates that the pollen tube has some means of perceiving material of the stigma surface and responding to this by arrest of growth (Fig. 6).

The nature of these interacting components is not known. We would expect to see products of the S-alleles present at the stigma surface and in the parentally derived exine components of the pollen; attempts to demonstrate this by immunological methods for Brassica oleracea have shown that an antigen which correlates with the S-genotype is indeed present in stigmas,<sup>32,33</sup> although no corresponding antigen has been reported for pollen extracts.

The next point where mismatch between pollen and stigma leads to a cut-off in the events of fertilization, is within the style canal (Fig. 4). After some interspecific pollinations and self-pollinations in the gametophytic incompatibility system growth of pollen tubes is arrested in the style. In gametophytic incompatibility pollen behaviour is controlled by its own genotype. Usually there is a single gene locus - the S locus with multiple alleles. A pollen tube carrying a single given allele is inhibited if the same allele is present in the style (Fig. 5b). If the S-allele of the pollen tube is not present in the style, then pollen tube growth proceeds through style to the embryo sac.

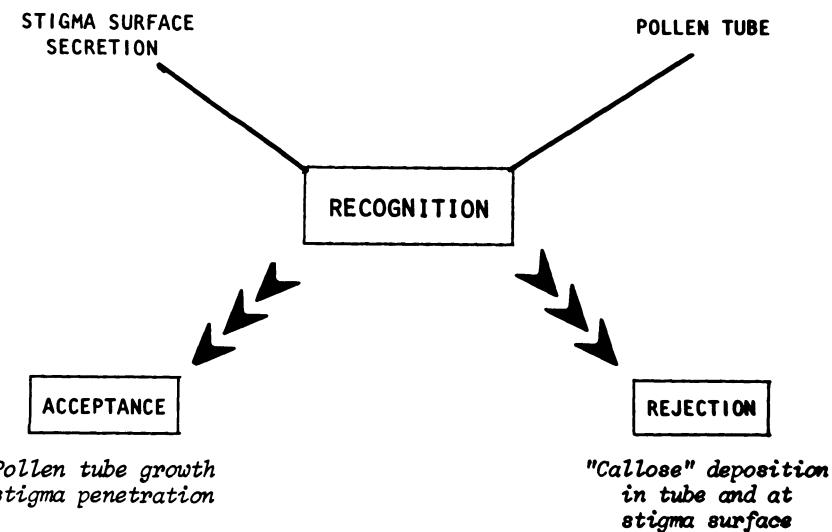


Figure 6. Diagrammatic representation of recognition and response during pollination in a sporophytic self-incompatible system.

In most gametophytic incompatibility systems, incompatible and compatible tubes appear identical initially, but at some stage during their growth through the style, incompatible tube tips appear abnormal; in some cases the tube bursts and in other cases growth ceases and the tip becomes occluded with aniline blue-staining "callose".<sup>34,35</sup> In these systems, the pollen tubes grown through the style, either intercellularly through the transmitting tissue or through a hollow style canal which is filled with a mucilage at maturity. In both cases, the contact is with extracellular materials of the style. Thus again, the difference in genotype of the pollen tube and style canal is somehow perceived and the observable results are deposition of "callose" and cessation of tube growth (Fig. 7). In these cases, the interacting surfaces are that of the pollen tube and the secretions of the style canal; a pollen tube receptor for an extracellular component of the style is implied; on the basis of the previous discussion we might expect this receptor to be located either at the pollen tube surface (Fig. 3b) or at the plasma membrane (Fig. 3d). The possible

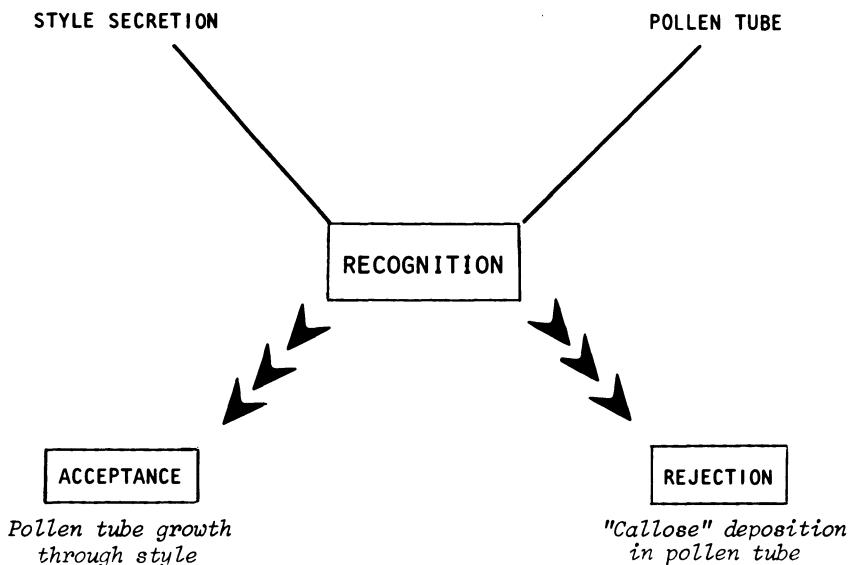


Figure 7. Diagrammatic representation of recognition and response during pollination in a gametophytic self-incompatible system.

nature of the style component involved in gametophytic self-incompatibility in Prunus avium is discussed in the section beginning on page 188. More detailed descriptions of the biology of pollination are given in references.<sup>36,38</sup>

#### NATURE OF CELL SURFACES INVOLVED IN POLLEN-STIGMA RECOGNITION

The nature of the molecules involved in the specific interactions between pollen tube and stigma or style has not been defined for any pollination system. However, many stigmas have been examined for particular components; often the examination has been cytochemical so that the presence of certain classes of components is inferred from their staining reactions. These studies are fragmentary and it is difficult to piece together the analyses to get an overview of the composition. For example, low molecular weight carbohydrates such as glucose, fructose and sucrose,

phenolic compounds such as flavone glycosides and sterols have been detected. In many genera, stigma exudates have lipid esters of phenolic compounds as the major component; also many stigma surface secretions are enzymically active and hydrolyze artificial substrates such as  $\alpha$ -naphthyl acetate and  $\alpha$ -naphthyl phosphate in cytochemical tests for esterase and phosphatase activity. There is even less information available regarding the composition of the style mucilage although in a pioneering study, Loewus and Labarca published a complete monosaccharide analysis of the stigmatic exudate of Lilium longiflorum<sup>39</sup> (see the next section).

For pollen, again, most information has been obtained cytochemically; usually the object has been either to examine the pollen grains with specific stains or to examine the wall layers for specific components. Briefly, these studies have shown that most types of pollen grains have a lipid surface coat containing carotenoid or flavanoid pigments. Some wall components diffuse into the medium when pollen is moistened; these components are derived from both the exine (outer-wall) and the intine (inner-wall). Pollen diffusates contain enzymes such as glycosidases, esterases, and glycosyl transferases. The "esterase" activity detected cytochemically has been equated with "cutinase" which is the enzyme(s) responsible for the degradation of the stigmatic cuticle by pollen.<sup>36</sup> Allergenic components have also been detected in grass pollen and ragweed pollen. This information is reviewed in References 1, 18, 37, 40.

Another approach to examining the nature of both pollen and stigma components has been to raise antisera to extracts and to examine the number and identity of the antigenic components. This approach was introduced by Lewis in 1952<sup>41</sup> and has most often been used to study S-gene products in self-incompatible pollination systems, and also to study pollen allergens. The value and limitations of this approach are discussed in the section beginning on page 188.

As to the pollen tubes there is very little analytical data or studies to indicate whether pollen tubes do secrete material, although pollen tubes have been used most successfully as model systems for studying cell wall growth and development.<sup>42</sup>

## DEFINED COMPONENTS INVOLVED IN POLLINATION.

An analysis of Gladiolus stigma surface secretion and style canal mucilage. Arabinogalactans are major components.

Because of fragmentary information available on composition of stigma surface secretions and style mucilages and because of their apparent role in the pollination process, we have undertaken a detailed analysis of these secretions. Preliminary experiments were with Gladiolus; this monocot has large flowers with large accessible pistils and anthers, and has the great advantage of being available commercially throughout the year. Ideally we would have worked with defined genotypes in a self-incompatible system. However, there are problems in collecting sufficient material for analysis in all the available defined self-incompatible systems - either the flowers can be grown in glasshouse conditions, but have tiny stigmas, such as Brassica, and the grasses, or they can be grown in the field but have a very short flowering time, for example Prunus avium, the sweet cherry.

Gladiolus is self-compatible, but inter-specific crosses arrested either at the stigma surface and/or within the style canal, so that the pistils have capacity for recognition.<sup>28,43</sup> As the stigmas of these flowers become receptive to compatible pollen, they develop and adhesive outer layer<sup>44,45</sup> which is the first point of contact with pollen.

Detection of arabinogalactans. This surface secretion contains protein, carbohydrate and lipid in the ratio 20:23:0.1. Examination by SDS-polyacrylamide gel electrophoresis showed more than 15 bands which stained with Coomassie blue, 9 of which also stained for carbohydrate with periodate-Schiff reagent.<sup>43</sup> By far the most dominant component is a high molecular weight polymer which is detected by staining the polyacrylamide gel with the  $\beta$ -glucosyl artificial carbohydrate antigen.<sup>43</sup>

The use of this artificial carbohydrate antigen as a stain is important to the eventual finding that arabinogalactans are components of the stigma surface and style canal of many plants, and in some they are major components. The background is that in 1962, Yariv, during attempts to

detect low concentrations of antibodies to glycosides; prepared artificial carbohydrate antigens by coupling diazotized 4-aminophenyl glycosides to phloroglucinol.

These artificial antigens gave a precipitin reaction with the corresponding anti-glycosyl antibodies.<sup>46</sup> Later, in 1967, Yariv and his co-workers at the Weizmann Institute in Israel, made the chance observation that the  $\beta$ -glucosyl artificial carbohydrate antigen precipitated an arabinose- and galactose-containing polymer from soybean, jackbean and maize extracts.<sup>47</sup> This observation remained a curiosity for the next 8 years until Jermyn extended Yariv's observation and showed that the  $\beta$ -glucosyl artificial carbohydrate antigen specifically precipitated arabinogalactan-proteins from a remarkably wide range of plant seed and tissue extracts.<sup>48,49</sup> These artificial carbohydrate antigens are a brilliant red color which has made them valuable cytochemical stains. Usually, the  $\beta$ -glucosyl artificial antigen, being the most water soluble is used in the test; control experiments are performed with the  $\alpha$ -galactosyl artificial antigen, which does not precipitate arabinogalactans.

The precise nature of the interaction between the  $\beta$ -glucosyl artificial carbohydrate antigen and the arabinogalactan-protein is still unresolved. However, there is some information available relating to the requirements of both the artificial carbohydrate antigen and the arabinogalactan-protein for effective interaction. In summary: analysis of the stereochemical requirements of the artificial carbohydrate antigens show that they must bear a glyco-pyranose residue with a  $\beta$ -D-configuration at C(0)1 and the D-gluco configuration at C(0)2.<sup>50</sup> A further requirement is the 1:4 orientation of the azo and glycosyloxy groups to the phenyl ring (Fig. 8). The nature of the surfaces of the arabinogalactan-protein which bind the artificial antigen is not clear, although the interaction appears likely to depend on the overall physical and chemical properties of the arabinogalactan-protein, rather than a specific binding site. The arabinogalactan-proteins which interact have similar physical and chemical properties. They are very high molecular weight polymers containing a highly branched (1 $\rightarrow$ 3):(1 $\rightarrow$ 6)  $\beta$ -galactan with arabinose residues in terminal positions.<sup>51</sup> The protein component usually represents between 5 to 15% of the molecule and is very resistant to proteolysis, indicating that the protein is

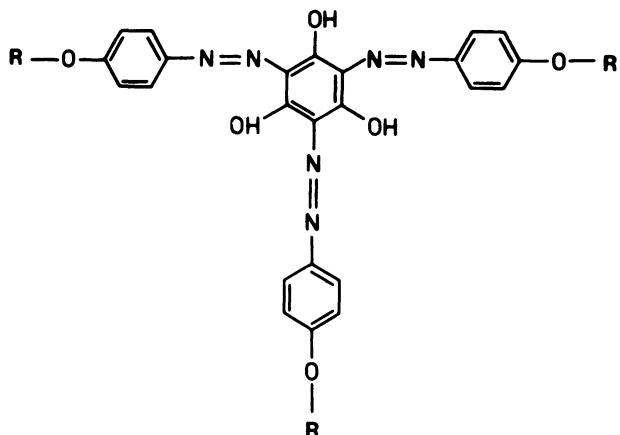


Figure 8. Structure of the  $\beta$ -glucosyl artificial carbohydrate antigen ( $R = \text{glucosyl residue}$ ).

buried within the molecule.<sup>52</sup> Enzymic removal of the terminal arabinosyl residues has no apparent effect on the capacity of the molecule to interact with the artificial carbohydrate antigen (Gleeson and Clarke, unpublished observation). However, the integrity of the arabinogalactan-protein is required for effective interaction, as mild acid hydrolysis with 12.5 mM oxalic acid abolishes its capacity for interaction. Analysis of the arabinogalactan-protein after this hydrolysis indicates that the galactan chains are extensively fragmented.<sup>53</sup> Moreover, recent experiments have shown that the ability of the arabinogalactan-protein to interact with the artificial antigens is destroyed by treatment with a crude galactanase preparation (Jermyn, unpublished observations) indicating that the branched galactan portion of the molecule is somehow involved in interaction; a more precise definition of the interacting surface is not available at present. The most cogent evidence for the specificity is, that of all the polysaccharides present in plant extracts, it is always the arabinogalactans which are precipitated on addition of a solution of a  $\beta$ -glucosyl artificial carbohydrate antigen.

This  $\beta$ -glucosyl artificial carbohydrate antigen was used to identify the major high molecular weight component of Gladiolus stigma extracts, as an arabinogalactan. The nature stigma of Gladiolus<sup>49</sup> stained with this reagent and conditions for washing this material off the stigma surface while leaving the underlying cell intact were established. Sufficient surface material for analysis was obtained from 3,500 stigmas. The contents of the style canal also stained intensely with the  $\beta$ -glucosyl artificial carbohydrate antigen<sup>53</sup> (Fig. 9a). It is worth noting that the success of this stain as a cytochemical reagent, apart from its brilliant red color, also depends on its ability to precipitate the material with which it interacts; that is, it partially fixes the soluble polysaccharide during staining. This is important as it is often difficult to detect soluble cell surface carbohydrates microscopically as they are not fixed by the usual aldehyde fixatives and are often lost in the tissue preparation.<sup>54</sup>

Isolation of arabinogalactans. The material precipitated from style extracts by the artificial antigen contained galactose and arabinose as the major monosaccharides.<sup>53</sup> One drawback of isolating the polymer in this way is the difficulty of dissociation of dye from arabinogalactan; direct analysis of the insoluble complex is not satisfactory as glucose, originating from the precipitating artificial carbohydrate antigen, is always detected. An alternative procedure for isolation of the arabinogalactan which obviates this difficulty is based on affinity chromatography using an insolubilized galactosyl-binding lectin from the giant clam Tridacna maxima.<sup>55</sup> The binding of saccharides to this lectin depends on the presence of  $\text{Ca}^{++}$  ions, so that material bound in the presence of  $\text{Ca}^{++}$  can be simply eluted with a  $\text{Ca}^{++}$ -free buffer. In this way, the major component from both the stigma surface and style of Gladiolus was isolated.<sup>53,56</sup> The recovered material represented 30 to 50% of the total dry weight of the material washed from the stigma surface, (less than 1  $\mu\text{g}$  per flower) and 40% of the total dry weight of the soluble style material (greater than 100  $\mu\text{g}$  per flower). The material can be visualized at the stigma surface by its binding to FITC-tridacnin (Figure 9b).

Analysis of arabinogalactans. Both the stigma and style material isolated in this way behaved as single

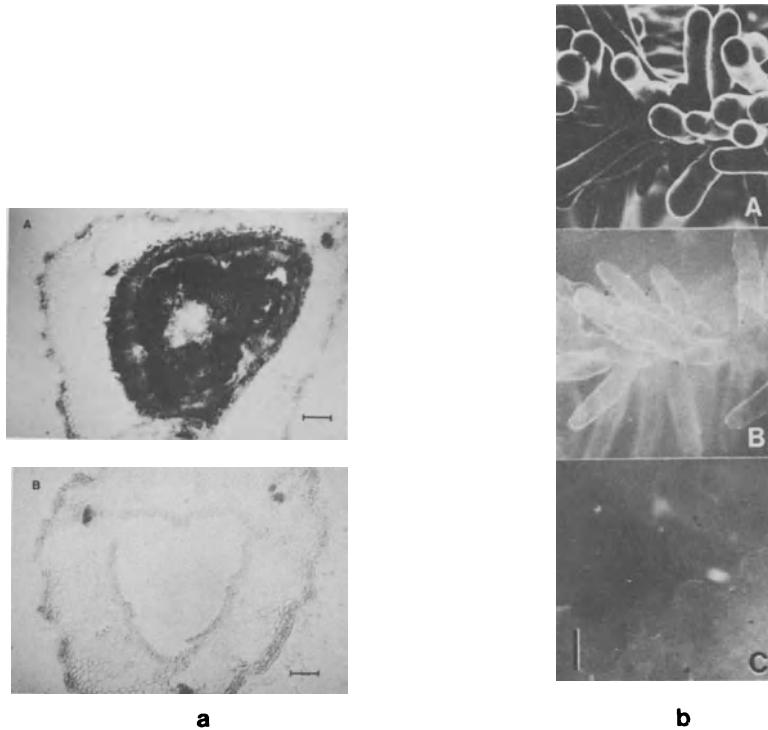


Figure 9. (a) A transverse section of the Gladiolus style stained for the arabinogalactan-protein with  $\beta$ -glucosyl artificial carbohydrate antigen (A). As a control of the staining specificity a section was treated with  $\alpha$ -galactosyl artificial carbohydrate antigen (B). The bar represents 150  $\mu\text{M}$ . (Adapted from Reference 48).

(b) Binding of fluorescein isothiocyanate-tridacnin to surface of stigmatic papillae of Gladiolus. A. Appearance of untreated papillae by scanning electron microscopy. B. Fluorescence of surface after treatment with labelled tridacnin. C. Absence of fluorescence after treatment with labelled tridacnin in presence of 0.1 M lactose. Bar = 100  $\mu\text{M}$ . (From Reference 44).

components on cellulose acetate electrophoresis at pH 8.8; for each sample only one positively charged diffuse band was detected by staining the strip with the  $\beta$ -glucosyl artificial carbohydrate antigen. However, ultracentrifugal analysis of the style material indicated that it was polydisperse in the molecular weight range 150,000 to 400,000. Nevertheless, methylation analysis of fractions collected across this molecular weight range showed that the arabino-galactan was homogeneous with respect to both monosaccharide composition and linkage type.

Monosaccharide and methylation analyses of the stigma and style arabinogalactans are shown in Tables 1 and 2. The analyses are similar: both contain galactose and arabinose as the major monosaccharides in similar proportions. The linkage composition of the two samples is also similar: all the arabinose is present as terminal arabinofuranosyl residues; glucose is also present solely in terminal positions. The galactose is also present solely in terminal positions. The galactose is mainly 1,3,6-linked with smaller amounts of (1 $\rightarrow$ 3)-linked, (1 $\rightarrow$ 6)-linked and terminal residues. Thus, the molecules are highly

Table 1. Monosaccharide composition of stigma and style arabinogalactans from Gladiolus and Lilium.

| MONOSACCHARIDE       | COMPOSITION (% by weight) |                        |                                  |
|----------------------|---------------------------|------------------------|----------------------------------|
|                      | <i>Gladiolus</i> Stigma   | <i>Gladiolus</i> Style | <i>Lilium</i> Stigma*<br>exudate |
| Galactose            | 76.1                      | 85.8                   | 64.1                             |
| Arabinose            | 20.0                      | 14.2                   | 30.1                             |
| Glucose              | 4.0                       | —                      | —                                |
| Rhamnose             | —                         | —                      | 5.8                              |
| Galactose: arabinose | 3.8                       | 6.0                    | 2.1                              |

\*No analysis for uronic acids was undertaken.

Table 2. Methylation analysis of stigma and style arabino-galactans from Gladiolus ad Lilium.

| LINKAGE TYPE                   | LINKAGE COMPOSITION (moles %) |                        |                               |
|--------------------------------|-------------------------------|------------------------|-------------------------------|
|                                | <i>Gladiolus Stigma</i>       | <i>Gladiolus Style</i> | <i>Lilium Stigma exudate*</i> |
| <i>Terminal rhamnosyl</i>      | 0                             | 0                      | 7                             |
| <i>Terminal arabinosyl</i>     | 17                            | 13                     | 32                            |
| <i>Terminal glucosyl</i>       | 7                             | trace                  | 0                             |
| <i>Terminal galactosyl</i>     | 16                            | 29                     | 11                            |
| <i>1,3 linked galactosyl</i>   | 13                            | 14                     | 10                            |
| <i>1,6 linked galactosyl</i>   | 6                             | 6                      | 5                             |
| <i>1,3,6 linked galactosyl</i> | 41                            | 39                     | 30                            |

\* Data of Aspinall and Rosell, recalculated, excluding glucuronic acid

branched. The two molecules differ in the terminal residues, the stigma arabinogalactan having a higher proportion of terminal arabinose and glucose than the style arabino-galactan, which has a higher proportion of terminal galactose.

Enzymic hydrolysis of the style arabinogalactan-protein with  $\alpha$ -L-arabinofuranosidase confirmed the exclusive terminal position of the arabinose residues and established the  $\alpha$ -configuration of these linkages. Lectin binding and optical rotation studies indicate that the galactosyl residues are most likely linked in the  $\beta$ -configuration. These data are consistent with a structure having a (1 $\rightarrow$ 3)-linked  $\beta$ -galactan backbone with side branches of 1,6-linked  $\beta$ -galactosyl residues, some of which carry the terminal L-arabinofuranosyl residues (Fig. 10).

Also included in Table 2 are figures calculated from the data of Aspinall and Rosell<sup>57</sup> on the composition of a fraction of the surface exudate of stigmas of another monocotyledon Lilium longiflorum (var. Ace) from a different

family (Liliaceae). The number of molecular species in this fraction was not established, although the methylation analyses indicate that a major component is an arabino-3,6-galactan. We have confirmed this finding; freshly collected stigma exudate from Lilium longiflorum formed a single precipitin band with the  $\beta$ -glucosyl artificial carbohydrate antigen, and the precipitated material represented about 35% dry weight of the total stigma exudate. It contained galactose, arabinose, and rhamnose in the proportions 11:5:1 (Table 1) - a neutral sugar analysis similar to that given by Aspinall and Rosell for the stigma exudate fraction which they examined. Methylation analysis has shown that although the total content of non-terminal galactosyl residues is somewhat lower in the Lilium than in the Gladiolus arabinogalactans, the molar ratios are approximately the same (2:1:6.6) for (1 $\rightarrow$ 3)-, (1 $\rightarrow$ 6)- and 1,3,6-linked residues. The Lilium arabinogalactan has a higher proportion of arabinose (32%) and a significant content of terminal rhamnose residues (7%), a monosaccharide which was not detected in the Gladiolus arabinogalactans. It also contains a lower proportion of terminal galactosyl residues, no terminal glucose and glucuronic acid (11%) both as terminal units and as (1 $\rightarrow$ 4)-linked residues. In contrast, the uronic acid content of the arabinogalactan of the Gladiolus style is low (0.9%). A total monosaccharide analysis of the high molecular weight fraction of the stigmatic exudate of Aptenia cordifolia (Aizoaceae) shows that it contains high proportions of both galacturonic and glucuronic acids as well as a range of neutral monosaccharides (Glu, Fru, Gal, Ara, Man, Xyl and Rha).<sup>58</sup> In this respect it differs from the Gladiolus and Lilium stigmatic exudates.

Because the Gladiolus style arabinogalactan was available in larger amounts, we have been able to characterize it in more detail.<sup>53</sup> This arabinogalactan is associated with 3% protein; as the protein remains associated with the carbohydrate after chromatography in 8 M urea, it is likely to be covalently bound, although the carbohydrate-protein linkage point(s) has not been identified. Whether protein is associated with the stigma arabinogalactans is not known.

Characterization of the Gladiolus style arabinogalactan-protein also provides evidence in favour of the backbone type structure shown in Fig. 10, rather than other possible models such as a branch-on-branch type structure or one

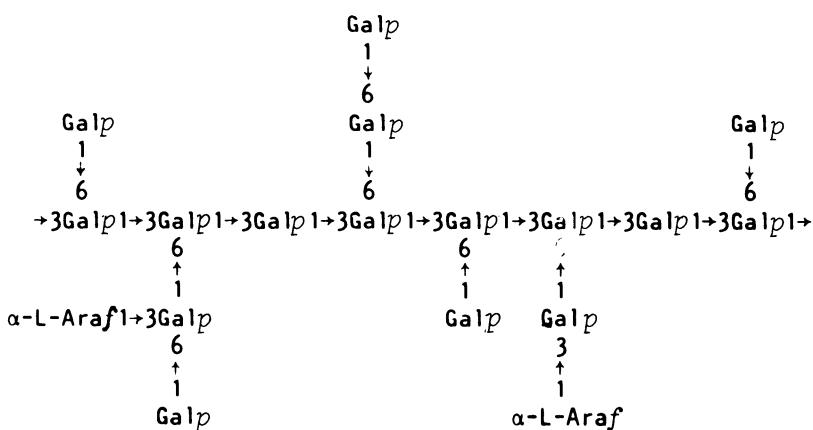


Figure 10. A proposed structure for a portion of the Gladiolus style arabinogalactan-protein.

involving mixed (1→3);(1→6) linkage galactan chains. This additional evidence comes, first of all, from observations of the solubility of the molecule, before and after modification. The arabinogalactan-protein is extremely water soluble (> 5 mg/ml); this solubility may reflect the highly branched nature of the molecule. Linear homopolymers containing (1→3) β-linkages (e.g. paramylon) on the other hand, are often insoluble as individual polysaccharide chains can aggregate in a highly ordered fashion.<sup>59</sup> Therefore, it might be predicted that modification of the side chain character of this molecule to facilitate interaction between (1→3) β-galactan backbones would result in a decrease in solubility. Indeed, enzymic removal of terminal arabinosyl residues does reduce the solubility of the molecule from more than 5 mg/ml to 1 mg/ml.<sup>53</sup> Another modification of the side branches which decreases the solubility is periodate oxidation. On the basis of the structure shown in Fig. 10, periodate oxidation would be expected to cause extensive degradation of the side branches containing (1→6)-linked galactosyl residues as well as the terminal

galactosyl and arabinosyl residues, but no oxidation of the (1 $\rightarrow$ 3)-linked galactan backbone. The oxidized arabino-galactan-protein remained in solution when stored at 4°C; however, after freeze-drying or freezing the solution at -20°C, it became quite insoluble.<sup>60</sup> These marked changes in solubility are compatible with a backbone-type structure, but are incompatible with a branch-on-branch type structure.

Immunological analysis of arabinogalactans. Supporting evidence for the backbone model was also obtained from immunochemical studies.<sup>60</sup> These studies were undertaken primarily to provide a characterized specific antiserum as an additional structural and cytochemical probe for the investigation of these polymers in other tissues. An antiserum was raised in rabbits to the isolated style arabino-galactan-protein. The specificity of the antiserum was investigated by immunoprecipitation using [<sup>3</sup>H]arabinogalactan-protein. The [<sup>3</sup>H]-label was introduced into the arabinogalactan-protein by oxidation of the terminal galactosyl residues with galactose oxidase followed by reduction with sodium [<sup>3</sup>H]borohydride.

The hapten concentrations required for 50% inhibition of the binding between [<sup>3</sup>H]-arabinogalactan-protein and antiserum are shown in Table 3. The specificity is directed primarily to the monosaccharide components, D-galactose and L-arabinose. The disaccharide 6-O- $\beta$ -D-galactopyranosylgalactopyranose was the most potent inhibitor and the antiserum showed preference for galactosides in the  $\beta$ -configuration.

The antigenic features of the arabinogalactan-protein were investigated by examining the interaction of the antiserum with chemically and enzymically modified arabino-galactan-protein.<sup>60</sup> The most significant finding was that periodate oxidation destroyed all the antigenic determinants as the oxidized material was a poor inhibitor of the homologous interaction.

In summary, the antiserum to the Gladiolus style arabinogalactan-protein is directed to the monosaccharides arabinose and galactose present in the accessible side chains while the inaccessible (1 $\rightarrow$ 3)-galactosyl residues of the backbone are not apparently involved.

Table 3. Comparison of saccharides as haptens inhibitors of the [<sup>3</sup>H]arabinogalactan-protein-specific anti-serum binding.

| Inhibitor                            | Concentration required for 50% inhibition (mM) |
|--------------------------------------|--|
| 6-0- $\beta$ -D-Galactopyranosyl-    |  |
| D-galactopyranose                    | 0.08   |
| Methyl $\beta$ -D-galactopyranoside  | 6.6  |
| 3-0- $\beta$ -D-Galactopyranosyl-    |  |
| D-arabinofuranose                    | 7.6  |
| 4-0- $\beta$ -D-Galactopyranosyl-    |  |
| D-glucopyranose (lactose)            | 12.5   |
| D-Galactose                          | 23   |
| L-Arabinose                          | 30   |
| Methyl $\alpha$ -D-galactopyranoside | 100  |

The cross-reactivity of various polysaccharides and glycoproteins with this antiserum was examined using an assay based on coupling the antiserum to red blood cells and examining the ability of the polysaccharides to agglutinate these antibody-coated red cells. Haemagglutination with the native Gladiolus style arabinogalactan-protein was detectable at 31 µg/ml. The Gladiolus stigma arabinogalactan-protein also cross-reacted strongly as did the preparation from Lilium longiflorum stigma exudate. In general, the more distant the structural relationship, the less cross-reactivity with the specific antiserum.<sup>60</sup>

Overall, these investigations of the Gladiolus and Lilium arabinogalactans show that they are major components of the stigma and style secretions and that they have

similar core structures with differences being expressed in the side branch monosaccharides.

The next question is, whether these arabinogalactans are present in the female sexual tissues of all plants. To answer this question, a number of style extracts have been screened for their capacity to interact with the  $\beta$ -glucosyl artificial carbohydrate antigen. Positive reactions were found for Prunus avium, Primula spp., Petunia spp. and Secale cereale, indicating the presence of arabinogalactans. We have now started to use the defined antiserum to Gladiolus style arabinogalactan-protein to confirm the presence of arabinogalactans in female sexual tissues of these and other species.

Structurally, these arabinogalactans are part of a widely distributed group of macromolecules - the arabino-galactans and arabinogalactan proteins, which all have similar structural features; macromolecules of this group are secreted into the medium of many callus cultures, are a major component of many plant gums and are found in extracts of a wide range of plant tissues (for review see Reference 51). In addition, they are apparently present at the surface of protoplasts in a number of different species.<sup>61</sup> It is also of interest that the arabinogalactans of the Gladiolus sexual tissues are apparently tissue specific as they differ from the arabinogalactans of the somatic tissues of Gladiolus in their monosaccharide composition, electrophoretic behaviour and reactivity towards various galactose-binding macromolecules.<sup>62</sup>

The question of the role of these arabinogalactans in the pollination process remains unresolved: a number of functions consistent with their physical and chemical properties are possible. Firstly, they may hold the key to specificity in the pollen-stigma interaction; this seems unlikely in view of the wide distribution of this class of macromolecules, although variations in the terminal saccharide sequences could contain specific information. Indeed, the terminal sequences in the arabinogalactans of the genus Acacia have been considered as a possible taxonomic marker.<sup>63</sup> Secondly, arabinogalactans at the stigma surface could act as adhesive gums, binding pollen grains to the surface. The Gladiolus stigma surface is adhesive and effectively binds a number of macromolecules.<sup>43</sup> Also,

the highly branched nature of the arabinogalactan fulfils the requirements of an adhesive base; but there is no experimental evidence showing that the isolated arabinogalactans are effective adhesives. Thirdly, the arabinogalactans may play a nutritional role, acting as a source of carbohydrate to nurture pollen tube growth. In Lilium longiflorum, Labarca and Loewus<sup>64, 65</sup> showed that growing pollen tubes incorporate exogenous substrate into their walls: when high molecular weight labelled stigma exudate was injected directly into the style canal, at least 25% of the carbohydrate of the excised pollen tubes was shown to be derived from the labelled exudate. As an arabinogalactan is a major component of the Lilium exudate, it seems likely that it may play a part in nutrition of the pollen tubes. Finally, the arabinogalactans may play a passive role in forming a matrix which supports pollen germination and pollen tube growth.

These studies have given us detailed information regarding the structure of the major component of the stigma and style secretions of Gladiolus: however, the structure and function of the range of other components detected in these secretions<sup>43</sup> have not been examined.

An immunological analysis of the style mucilage of Prunus avium, an antigenic component which correlates with S-genotype can be identified.

The problem of which component(s) is involved in the expression of specificity in pollination has been approached by examining a gametophytic self-incompatibility system - Prunus avium - the sweet cherry, by both immunological and traditional analytical methods.

As macromolecules from plant cells, like those from animal cells are often antigenic; antibodies to these molecules can be raised and are useful tools in identification of their structure and function. In 1952 Lewis<sup>41</sup> pioneered the application of immunological methods to detect the S-gene products in the evening primrose (Oenothera organensis), which has a sporophytic self-incompatibility system. He raised antisera to pollen extracts of different genotypes, and showed a precise correlation between S-genotype and particular antigens.

The success of this approach has encouraged other groups who have consistently been able to show a correlation between S-genotype and a specific antigen. For example, Nasrallah, Wallace and coworkers<sup>32</sup> raised antisera to homogenates of pistils of Brassica oleracea; the S-genotype specific antigen was partially characterized as a high iso-electric point glycoprotein. The antigen was extremely soluble and appeared at the same time as incompatibility developed, about three days prior to anthesis. In a more detailed study Nishio and Hinata<sup>33</sup> have shown that the antigen corresponding to genotype S<sub>7</sub> of Brassica campestris is a glycoprotein, isolectric point pH 5.7, MW 57,000, with a carbohydrate to protein ratio of 1.2:1. The principal amino acids were serine, glutamic acid and glycine.

We have examined the style mucilage of Prunus avium in an attempt to define the components involved in expression of specificity and their relationship to the arabino-galactans detected in style mucilages of this and other plants.

In Prunus avium, all self-pollinations are incompatible, as are cross pollinations between varieties with the same S-genotype. In both incompatible and compatible pollinations, the initial events are apparently identical. The pollen tubes germinate and grow into the style. The cells of the transmitting tract of the style are joined by plasmodesmata into vertical files.<sup>75</sup> At maturity, the extracellular gel matrix becomes progressively less viscous, so that pollen tubes grow through an amorphous fluid matrix between quite separated files of cells. At some point within the style, growth of incompatible tubes is arrested. The precise zone of the style at which incompatible tube growth ceases, varies with ambient temperature (Lewis, 1942). Incompatible tubes swell at the tip and often burst; usually a deposit of aniline-blue staining material ("callose") is observed just behind the tip of the arrested tube. The behavior of pollen tubes during the growth, suggests that the reaction might involve contact between the growing pollen tube and the style secretions. We examined style secretions from two S-allele groups, S<sub>1</sub>S<sub>2</sub> and S<sub>3</sub>S<sub>4</sub> (Table 4).

Immunological approaches. By raising antisera to a pistil extract of styles of the S<sub>3</sub>S<sub>4</sub> genotype (Lambert) we were able to demonstrate antigenic components which correlate

precisely with the S-allele group. In the homologous reaction between antiserum and the diffusible material of mature Lambert pistils, two distinct antigenic components are separated\* (Fig. 11). On immunodiffusion, there is an outer band which correlates with the S-genotype (S-antigen) and an inner band which is apparently specific to pistil tissues (P-antigen) of members of the Rosaceae. On immuno-electrophoresis at pH 8.9 the S-antigen moves toward the cathode and the P-antigen moves toward the anode.

When antiserum is tested with pistil diffusates from other Prunus avium varieties of the same S-allele group (Napoleon, Bing), an identical pattern is produced. However, when the antiserum is tested with pistil diffusates from varieties of a different S-genotype group ( $S_1S_2$ ) the P-antigen is present but a band corresponding to the S-antigen is barely detectable. When the antiserum is absorbed with the eliciting antigen preparation or with the corresponding style diffusates from other varieties of the same S-genotype group ( $S_3S_4$ ), and then tested, both S- and P-antigen are removed. However, if the antiserum is absorbed with style diffusates from the  $S_1S_2$  group and tested, the P-antigen is completely removed but the S-antigen remains (Table 4).

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\*These antigens, which correlate with the S-allele, can also be detected using antisera raised to material secreted by callus cells of Prunus avium, grown in liquid suspension culture. Callus cells secrete a range of antigens into their culture media: not only antigens specific to the parental organ, but also antigens typical of other organs of the parent plant.<sup>66</sup> In this study callus was raised from leaf tissue of cv Napoleon ( $S_3S_4$ ); the culture filtrate was taken to 80% saturation with ammonium sulphate and antisera was raised in rabbits to this protein fraction. A number of antigens were detected, including the major antigen which is specific to leaf tissues of the genus Prunus, and an antigen apparently identical with the antigen found in pistil extracts which correlates with the S-allele group. This extraordinary finding that an antigen as specific in function as the S-allele antigen of the pistil is secreted by callus cells derived from tissues other than the pistil, dramatically illustrates the potential of callus cells for wide expression of the plant genome.<sup>67</sup>

The S-antigen is also developmentally regulated. It is absent in early stages of style development - at mid balloon and petal show, but is present at late balloon and in mature pistils (24 hrs after flower opening). Also, it is present in both the upper and lower portions of the style but is absent from the ovary and receptacle. Thus, it is present at the right time (flower maturity), in the right place (style) to function in control of sterility. However, although there is a precise correlation of this antigen with S-genotype group, a direct function for this material has not been demonstrated.

Another interesting feature of this S-antigen is its precise tissue specificity. It could not be detected in pollen diffusates, ovary or receptacle of mature flowers or in leaf tissue of the  $S_3S_4$  genotype. Nor could it be detected in a number of diffusates prepared from pistils of other members of the Rosaceae such as peach (Prunus persica) or rose (Rosa domestica).

Chemical approaches. These components have been isolated and partially characterized by traditional methods.

An extract of style collected from mature flowers of genotype ( $S_3S_4$ ) was fractionated by ammonium sulphate precipitation. The individual fractions were examined for their capacity to interact with antiserum raised to the whole pistil extract.

Both the S and P antigens were concentrated in the 30 to 45% ammonium sulphate fraction and have been isolated on the basis of their differing charge properties, by DEAE-cellulose chromatography. They are quantitatively minor components, each accounting for less than 5% of the soluble material of the style (yield < 2 mg from 6,000 pistils). Both antigens are glycoproteins (S antigen 16.3%, P antigen 17.2% carbohydrate as glucose). There is no apparent immunological cross-reactivity, in that [ $^{125}I$ ]-P antigen gave a single peak corresponding to molecular weight 32,000 when precipitated by its homologous antiserum, but no precipitation in the heterologous antiserum. [ $^{125}I$ ]-S antigen gave two closely related peaks, apparent molecular weights, 37,000 and 39,000, when precipitated with its homologous

Table 4. Antigens detected in style extracts of Prunus avium by immunodiffusion and immunoelectrophoresis. Cultivars in S-allele group  $S_1S_2$  were Bedford and Early Rivers; in group  $S_3S_4$  were Bing, Lambert and Napoleon.

| S-allele group of<br>Pistil extract  | $S_3S_4$ | $S_1S_2$    |
|--|----------|-------------|
| Antiserum to extract<br>of Lambert pistils ( $S_3S_4$ )  | P S      | P (S faint) |
| Antiserum to extract<br>of Lambert pistils ( $S_3S_4$ )<br>absorbed with pistils<br>of S-allele group ( $S_3S_4$ ) | - -      | - -         |
| Antiserum to extract<br>of Lambert pistils<br>absorbed with pistils<br>of S-allele group ( $S_1S_2$ )              | - S      | - -         |

antisera, but no precipitation in the heterologous interactions (Mau, S-L, Raff, J and Clarke, A. E., unpublished observations).

The most remarkable feature of the analysis was the presence of a single major component: on the basis of the experience with Gladiolus and Lilium we expected to find an arabinogalactan, but found a high molecular weight (>70,000) macromolecule containing 95% protein, 5.4% carbohydrate (as glucose) with monosaccharides Glc:Man:Gal:Xyl in the ratio 5.3:1.4:2.2:1.0. The component was present in the 20 to 30% ammonium sulphate fraction. It was purified by G-200 Sephadex and Biogel P-6 chromatography and accounts for about 30% of the total soluble material of the whole style extract. As expected, from the monosaccharide analysis, there was no interaction with either the anti-arabinogalactan protein serum, or the  $\beta$ -glucosyl artificial carbohydrate antigen. However, whole homogenates of Prunus avium styles do interact with these probes, indicating the presence of arabinogalactans in the tissues. We located these arabinogalactans in the supernatant of the 85% ammonium sulphate fraction by their binding to both probes. In contrast to the arabinogalactans of Gladiolus and Lilium, they

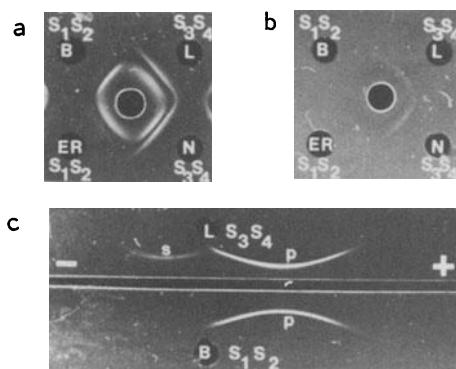


Figure 11. S-Allele associations of antigens from the pistil of the sweet cherry, *Prunus avium*. (a) Immunodiffusion: antiserum in the inner well was raised to a style extract from Lambert ( $S_3S_4$ ). The outer wells contained antigens prepared from mature pistils of different varieties, with known S-allele types: Lambert (L) and Napoleon (N) belong to the S-allele group  $S_3S_4$  and produce two bands with the antisera. Bedford (B) and Early Rivers (ER) of the S-allele group  $S_1S_2$  produce a single band continuous with the inner band of the two produced with the  $S_3S_4$  groups.

(b) Immunodiffusion: The same antiserum (to  $S_3S_4$ ) was adsorbed with pistil extract from Bedford ( $S_1S_2$  inner well). The outer wells contained antigens prepared from mature pistils of different varieties. The inner band, common to both  $S_3S_4$  and  $S_1S_2$  extracts was removed by the absorption. The outer band was formed between the  $S_3S_4$  varieties and the absorbed antiserum.

(c) Immunoelectrophoresis: The antiserum in the centre trough was raised to a style extract from Lambert ( $S_3S_4$ ). The upper wells contained antigen preparation from Lambert ( $S_3S_4$ ); two bands were produced. The P band, was common to pistils of the other S-allele group  $S_1S_2$  and the S band was only present in extracts of the  $S_3S_4$  group.

were minor components of the *Prunus* style, accounting for less than 2% of the total soluble material. Alerted by Larkins' observations<sup>61</sup> that protoplasts derived from many cell types are agglutinated by  $\beta$ -glucosyl artificial antigen, we also examined a membrane preparation from the style. The style homogenate was exhaustively extracted and washed with

buffer, and then extracted with 0.1% Nonidet-P40. This detergent extract gave a precipitin band with both the anti-arabinogalactan-protein serum and the  $\beta$ -glucosyl artificial antigen.

In summary, the style extract of Prunus avium contains a major single high molecular weight of glycoprotein component. There are two minor components which can be detected by their antigenic properties; one is characteristic of the S-genotype and is restricted to mature P. avium styles, the other is present in the styles of all Prunus species tested. The arabinogalactans, which are major components in Gladiolus and Lilium styles, are minor components of the soluble material in P. avium styles and are also present in a membrane preparation.

An analysis of the aniline blue-staining material produced as a result of self pollination of Secale cereale - "callose" contains both (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)-glucosidic linkages.

The third aspect of the pollen-stigma interaction which we will consider is the deposition of aniline blue-staining material as a response to incompatible matings in both the gametophytic and sporophytic systems. For the sporophytic system, these deposits are formed in the initiating pollen tube and in stigmatic cells at the stigma surface. In the gametophytic system they are formed in the tip of the pollen tube within the style. This characteristic deposition is such a reliable test that it is used horticulturally to establish relationships between potential breeding groups. Material which gives the characteristic brilliant yellow fluorescence with decolorized aniline blue, is generally referred to as callose although the precise nature of the material stained is not known. Because in one situation (sieve tube callose of grape vine), the staining material was shown to be a (1 $\rightarrow$ 3)  $\beta$ -glucan, it has been assumed that a polysaccharide with the same structure is always involved in this staining interaction. We have examined the nature of the aniline blue staining material produced as a response to incompatible self-mating in the grass Secale cereale.<sup>68</sup>

In this system, compatible pollen tubes penetrate the stigma surface, grow intercellularly along the multicellular papillae of the stigma, and enter the main stigma branch, where they continue to grow intercellularly towards the

base of the stigma and into the ovary (Fig. 12). In incompatible matings, the pollen tubes grow intercellularly for a distance of four or five cells in the stigmatic papillae and growth is arrested after 45 minutes. Aniline blue-staining droplets are detected 20 minutes after pollination and by 1 hour have coalesced into distinct plugs. Deposition continues until 3 hours after pollination when the material occludes about one third of the pollen grain surface and the basal part of the pollen tube, after which there is no further deposition.

Under the conditions used for pollination, no wound callose was produced. This was shown by the poor staining of compatible pollen and tubes with aniline blue. Also, when excised stigmas were treated with polystyrene beads (40  $\mu\text{m}$  diameter) or sunflower pollen under the same conditions, no wound callose was detected with aniline blue-staining.

To examine the nature of this incompatibility callose, 2,000 stigmas were self pollinated. Three hours later the stigmas were homogenized; the callose deposits were released as fragile solid plugs which fragmented on further homogenization. The deposits could not be separated by density gradient method and could only be solubilized under degradative conditions. The approach of removing other components, leaving the callose as a residue was adopted. Initially, the homogenate was extracted with chloroform and methanol to remove lipids from both the pollen tube and the outer layer of the pollen grains, the pollenkitt. This was followed by a hot-guffer extraction to remove soluble cell wall components and an acid chlorite treatment to oxidize sporopollenin and any lignin. The total yield from 2,000 pollinated stigmas (dry weight 600 mg) was 80 mg. This material was further fractionated on the basis of its solubility in dimethyl sulphoxide (DMSO). The whole "callose" preparation and both the DMSO soluble and insoluble fractions retained the capacity to stain with aniline blue, characteristic of the deposits in the whole tissue.

Monosaccharide analyses of the "callose preparation" and both the DMSO soluble and insoluble fractions are shown in Table 5. The whole preparation contained glucose as the major monosaccharide as well as arabinose, xylose, galactose and mannose. The DMSO insoluble fraction was extremely difficult to hydrolyze - only 25% of the total material was

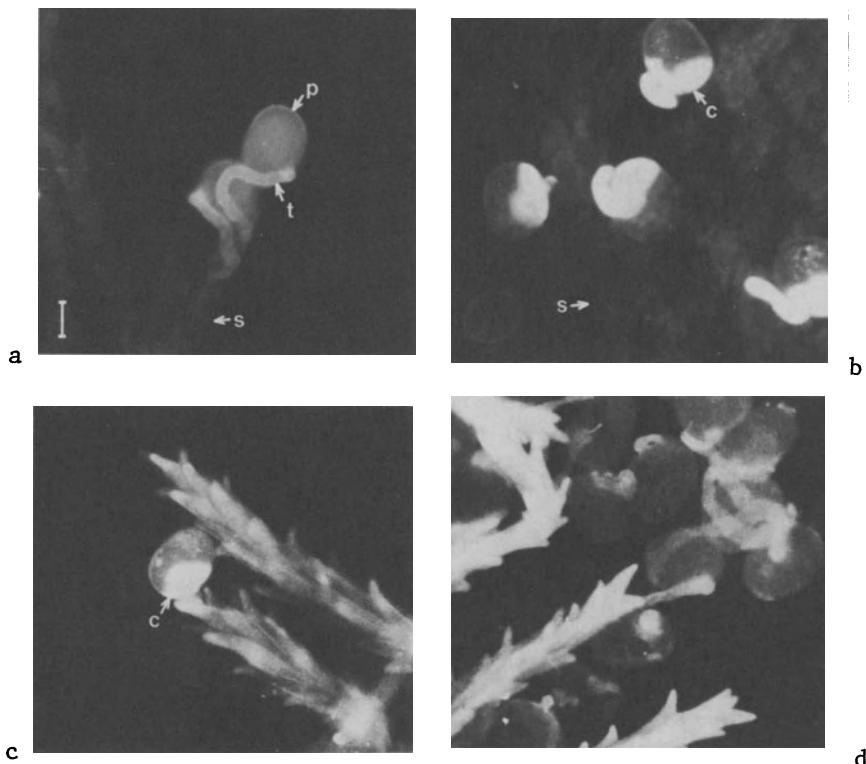


Figure 12. Deposition of "callose", as a response to self pollination in Secale cereale.

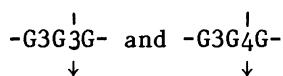
Excised mature stigmas were pollinated by dipping in fresh compatible or incompatible pollen. After 60 minutes in a moist chamber at room temperature, the samples were squashed, stained and examined by fluorescence microscopy (magnification  $\times 900$ ; internal marker  $25 \mu\text{m}$ ).

(a) Compatible pollen (p) showing the tube growth (t) through the multicellular stigmatic hair (s); stained with decolorized aniline blue. (b) Incompatible pollen on self stigma (s) showing the comma-shaped callose deposit (c) which occludes part of the pollen grain and the short pollen tube; stained with decolorized aniline blue. (c) and (d) as for (a) and (b) except the preparations were stained with Calcofluor M2R New and viewed by fluorescence microscopy. Stigma, pollen grains and tubes were all stained, however the area corresponding to depositon of "callose" (c) is intensely stained (Panel c).

recovered as reducing sugars and in the material, glucose was the major component with arabinose as a minor component.

Methylation analysis (Table 6) of this DMSO soluble fraction showed most of the glucose to be (1 $\rightarrow$ 4)-linked. However, as well as the expected terminal glycosyl residues, 9% of the material was recovered as a peak tentatively identified as 1,3,5 tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol corresponding to 1,3-linked glycosyl residues. All the arabinose, which is present as a minor component, was recovered as terminal residues. Whether this material represents a mixture of a (1 $\rightarrow$ 4)-glucan and a (1 $\rightarrow$ 3)-glucan or a macromolecule containing both linkage or a mixture of all three types of polymer cannot be stated from the data. X-ray diffraction of the isolated "callose" gave a pattern similar to that of cellulose indicating that the predominant organizational feature in the "callose" preparation is linear runs of (1 $\rightarrow$ 4)  $\beta$ -glucosidic linkages. The absence of bands corresponding to the (1 $\rightarrow$ 3)  $\beta$ -glucan paramylon, also indicates the absence of long runs of (1 $\rightarrow$ 3)-linkages. Again these data can be interpreted as the presence of both cellulose and a glucan with runs of (1 $\rightarrow$ 3)-linkages, or of a single mixed linkage glucan.

This question was partially resolved by enzymic hydrolysis with purified,  $\beta$ -glucan hydrolases with defined linkage specificity. The results and the specificity of the enzymes are given in Table 7. The exo-acting (1 $\rightarrow$ 3)-glucan hydrolase released glucose from the whole callose preparation as well as both the DMSO-soluble and insoluble fractions - indicating that there are at least some chains with terminal (1 $\rightarrow$ 3)- $\beta$ -glucosyl residues. The Rhizopus arrhizus endo-(1 $\rightarrow$ 3)- $\beta$ -glucan hydrolase will cleave either (1 $\rightarrow$ 3)- or (1 $\rightarrow$ 4)-glucosidic linkages, joining 3-substituted glycosyl residues in a linear chain. Thus linkages in the sequences



will be hydrolyzed as shown. The products of hydrolysis of the whole "callose" preparation as well as the DMSO-soluble and insoluble fractions included laminaribiose as well as the mixed-linkage trisaccharide G4G3G. A similar pattern of products was produced by enzymic hydrolysis with the

Table 5. Monosaccharide analyses of the "callose preparation" from rye pollen after self-pollination, and the fractions obtained from the "callose preparation" by DMSO fractionation. Results are expressed as % (by weight) of total carbohydrate.

| Monosaccharide | "Callose<br>preparation" | DMSO fractionation |          |
|----------------|--------------------------|--------------------|----------|
|                |                          | Insoluble          | Soluble  |
|                |                          | fraction           | fraction |
| Glucose        | 61                       | 26                 | 90       |
| Arabinose      | 18                       | 58                 | 10       |
| Xylose         | 11                       | 5                  | 0        |
| Galactose      | 6                        | 7                  | 0        |
| Mannose        | 4                        | 4                  | 0        |

Table 6. Methylation analysis of the "callose preparation" from rye pollen after self-pollination, and the fractions obtained from this preparation by DMSO fractionation.

| Linkage type                | Linkage composition (% peak area) |                       |                     |
|-----------------------------|-----------------------------------|-----------------------|---------------------|
|                             | "callose pre-<br>paration"        | DMSO fractionation    |                     |
|                             |                                   | Insoluble<br>fraction | Soluble<br>fraction |
| Terminal Ara <sup>f</sup>   | 7                                 | 7                     | 5                   |
| 1,3 linked Ara <sup>f</sup> | 1                                 | trace                 | 0                   |
| Terminal Glc                | 0                                 | 0                     | 8                   |
| 1,5 linked Ara <sup>f</sup> |                                   |                       |                     |
| 1,5 linked Ara <sup>f</sup> | 11                                | 11                    | 0                   |
| Terminal Gal                | 6                                 | 7                     | 0                   |
| 1,3 linked Glc              | 8                                 | 5                     | 9                   |
| 1,2 linked Glc              |                                   |                       |                     |
| 1,2 linked Man              | 17                                | 16                    | 0                   |
| Xyl (? linked)              |                                   |                       |                     |
| 1,4 linked Glc              | 50                                | 54                    | 77                  |

Bacillus subtilis (1 $\rightarrow$ 3):(1 $\rightarrow$ 4)  $\beta$ -glucan endo-hydrolase. This enzyme will hydrolyze glucans containing both (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)  $\beta$ -glucans linkages in a linear sequence but will not hydrolyze (1 $\rightarrow$ 3)-glucans or (1 $\rightarrow$ 4)-glucans which are homogeneous with respect to linkage type. This enzyme degraded the DMSO-soluble fraction most effectively, but also released trace amounts of oligosaccharides from the DMSO-insoluble and whole "callose" preparation. The major products were G4G3G and G4G4G3G.

The Streptomyces enzyme gave the same products with all three callose fractions: cellobiose (G4G), cellotriose (G4G4G), cellotetraose (G4G4G4G), and the mixed-linkage trisaccharide (G3G4G). The specificity of this enzyme is for (1 $\rightarrow$ 4)-glucosidic linkages where the glycosyl residue is 4-substituted. Thus, it will hydrolyze either (1 $\rightarrow$ 4)  $\beta$ -glucans such as cellulose or the linear heteropolymers such as barley glucan. The findings, that each of the three endoacting enzymes produced mixed linkage trisaccharides from the whole callose preparation and from the DMSO-soluble and insoluble fractions indicates that the (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)-linkages must be present in the same molecule.

Thus the "callose" produced in response to self pollination of Secale cereale contains linear glucose polymers which have (1 $\rightarrow$ 3)- $\beta$  and (1 $\rightarrow$ 4)- $\beta$ -linkages in the same molecule; the evidence available does not rule out the possibility that this preparation may also contain substantial amounts of the (1 $\rightarrow$ 4)- $\beta$ -glucan cellulose, and/or some homogeneous (1 $\rightarrow$ 3)- $\beta$ -glucan, and poses the question, 'Are all macromolecules which give positive fluorescence with aniline blue (1 $\rightarrow$ 3)- $\beta$ -glucans?'.

The question is difficult to resolve, because the material in the cell or tissue may react differently when it is isolated - that is, the secondary and tertiary structure and the hydration state as well as the primary sequence of monosaccharide, may influence the interactions. The available evidence indicates that whereas glucans containing high proportions of (1 $\rightarrow$ 3)-linkages are usually stained, glucans with other linkage types may also stain. Furthermore, other cell wall components such as cellulose and mixed (1 $\rightarrow$ 3):(1 $\rightarrow$ 4)-linkage glucans also bind the fluorochrome.<sup>69,70,71</sup> The fluorescent brightener, Calcofluor also stained the callose deposits in Secale stigmas more intensely than the other cell

Table 7. Tentative identification of the products from enzyme hydrolysis of "calllose preparation" and the DMSO insoluble and DMSO soluble fractions.

| Enzyme treatment   | Linkage specificity   | Products of enzyme treatment identified by paper chromatography    |  |   |  |
|--|---|--|--|---|--|
|  |   | Callose* preparation   | DMSO insoluble fraction  | DMSO soluble fraction   | G<br>(oligomers also)                                    |
| <i>Euglenes gracilis</i><br>(EC 3.2.1.58)<br>(exo 1,3 ase) | $\downarrow$<br>G <sub>3</sub> G -  | G (trace)  | G (trace)  |   |  |
| <i>Rhizopus arrhizus</i><br>(EC 3.2.1.6)<br>(endo 1,3 ase) | - G <sub>3</sub> G <sub>3</sub> G -<br><br>or - G <sub>3</sub> G <sub>4</sub> G - | G (trace)<br>G <sub>3</sub> G<br>G <sub>4</sub> G <sub>3</sub> G   | G (trace)<br>G <sub>3</sub> G<br>G <sub>4</sub> G <sub>3</sub> G   | G (strong)<br>G <sub>3</sub> G<br>G <sub>4</sub> G                                    |  |
| <i>Bacillus subtilis</i><br>(EC 3.2.1.73)<br>(1,3;1,4 ase) | - G <sub>4</sub> G <sub>3</sub> G <sub>4</sub> G -                                | G (trace)<br>G <sub>4</sub> G <sub>3</sub> G<br>(trace)            | G (trace)<br>G <sub>4</sub> G <sub>3</sub> G<br>(trace)            | G (strong)<br>G <sub>3</sub> G<br>G <sub>4</sub> G<br>G <sub>4</sub> G <sub>3</sub> G |  |
| <i>Streptomyces</i><br>(EC 3.2.1.4)<br>(1,4 ase)           | - 4G <sub>4</sub> G -   | G <sub>4</sub> G<br>G <sub>3</sub> G <sub>4</sub> G<br>?           | G <sub>4</sub> G<br>G <sub>3</sub> G <sub>4</sub> G<br>?           | G <sub>4</sub> G<br>G <sub>3</sub> G <sub>4</sub> G<br>?                              | G <sub>4</sub> G<br>G <sub>3</sub> G <sub>4</sub> G<br>? |
|  |   | G <sub>4</sub> G <sub>4</sub> G<br>G <sub>4</sub> G <sub>4</sub> G | G <sub>4</sub> G <sub>4</sub> G<br>G <sub>4</sub> G <sub>4</sub> G |   | G <sub>4</sub> G<br>G <sub>3</sub> G <sub>4</sub> G<br>? |

\*After incubation with the enzyme, there was an insoluble residue.

wall regions: this dye binds strongly to cellulose,<sup>72,73</sup> but will also bind mixed linkage (1 $\rightarrow$ 3):(1 $\rightarrow$ 4)-glucans.<sup>73,74</sup> Certainly these dyes, aniline blue and Calcofluor, as well as the  $\beta$ -glucosyl artificial carbohydrate antigen are useful cytochemical probes: for each dye we can define the major class of compounds stained, but not the precise physico-chemical requirements for staining.

Aniline blue-staining material is also found in pollen tube walls. Herth and coworkers<sup>76</sup> examined the nature of Lilium pollen tube wall material and found both (1 $\rightarrow$ 3) and (1 $\rightarrow$ 4)  $\beta$ -glucosidic linkages in an isolated wall fraction. They faced a similar dilemma as to whether these originated from a mixture of (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)- $\beta$ -glucans, or a mixed linkage (1 $\rightarrow$ 3):(1 $\rightarrow$ 4)- $\beta$ -glucan. The staining of pollen tube walls with resorcin blue, another "callose specific" stain, is abolished by treatment with 1,3  $\beta$ -glucan hydrolase indicating the presence of (1 $\rightarrow$ 3)-glucosidic linkages.<sup>77</sup>

Observations on "callose" formed in response to an incompatible fungal infection support the idea that aniline blue-staining indicates, but is not necessarily restricted to (1 $\rightarrow$ 3)- $\beta$ -glucans. Collars of aniline blue staining material "callose", are laid down on walls of Zea mays root epidermal cells as a response to contact with invading hyphae of Phytophthora cinnamomi. In sections of infected root, fluorescence with aniline blue is abolished by digestion of the section with (1 $\rightarrow$ 3)- $\beta$ -glucan exo-hydrolase (from Euglena gracilis) (1 $\rightarrow$ 3)- $\beta$ -glucan endo-hydrolase (from Rhizopus arrhizus) and (1 $\rightarrow$ 3):(1 $\rightarrow$ 4)- $\beta$ -glucan endo-hydrolase (from Bacillus subtilis) but not (1 $\rightarrow$ 4)- $\beta$ -glucan endo-hydrolase (from Streptomyces sp.). Also the "callose" collars give a positive stain with periodate-Schiff reagent indicating the presence of linkages other than (1 $\rightarrow$ 3)- $\beta$ -glucosidic linkages. However, although the capacity to stain with aniline blue is abolished by enzymic digestion, the "callose" collars retain their capacity to stain with the periodate-Schiff reagent.<sup>78</sup>

Other similarities can be seen between pollen tube growth through the style canal and fungal infection of roots (Fig. 13) (Table 8). In each case the host tissues are penetrated by a genetically foreign invader (pollen tube or fungal hypha). In both cases, the invader may be accepted and grow through the tissue, or the growth may be arrested.

In instances where there are highly specific interactions such as self-incompatible pollinations or race-specific pathogenesis, the control is through either a single gene or a few gene loci. This recognition seems to depend on an interaction between surface secretions and their complementary receptors. In both cases, there are a number of responses to the interaction, among which is the deposition of "callose". The precise nature of the molecules which elicit this and other responses and of the receptors for these signals, is just now beginning to be unravelled.

#### CONCLUSIONS

1. The study of the events of pollination at the molecular level is a new field of cell biology. The groundwork has been laid by the careful microscopic observations of pollination biologists. These studies have pointed to the stigma surface and style secretions as likely candidates for mediating recognition between the pollen-grain or tube and the female sexual tissues.
2. The nature of the components present at the interacting surfaces can be partially established by classic analytical techniques. Immunological techniques have unexploited potential for analysis of minor components of plant cell surfaces. The use of these techniques has allowed an insight into the nature and organization of animal cell surface components, and there is no doubt that immunological methods will be of great value in future studies of plant cell surfaces.
3. A major obstacle in studies of cell recognition during pollination is the development of a rapid quantitative assay for compatible and incompatible pollination. In vivo assays which depend on seed set are time-consuming and may be subject to environmental hazards. Assays for pollen tube growth usually depend on subjective observations of the numbers of tubes in a style canal. Assays which depend on the interaction between pollen tubes grown in vitro and particular components have some potential, although there are ultrastructural differences between pollen tubes growth in vivo and in vitro.
4. It seems likely that the recognition events of pollination will be based on the same mechanisms operative for

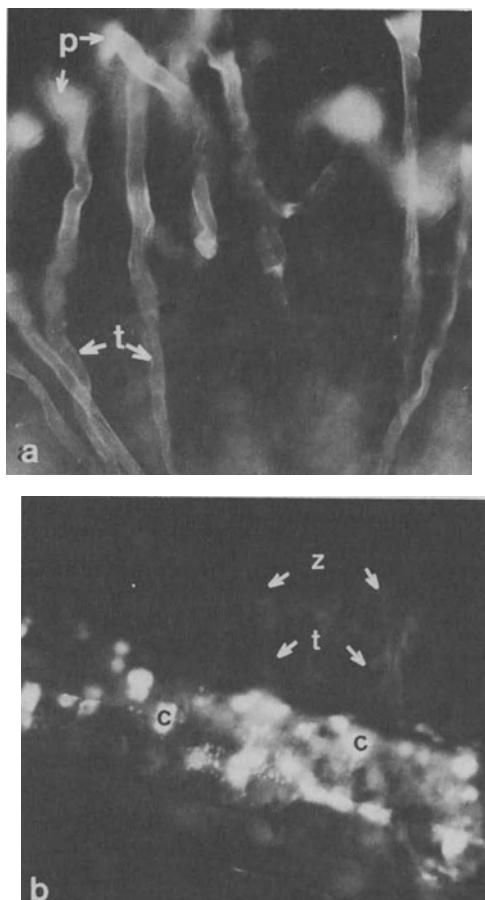


Figure 13. Fluorescent micorgraphs showing a comparison of pollination and fungal infection. (a) The pollen grains (p) at the surface of the stigma produce tubes (t) which grow through the style canal. (b) The zoospores (z) produce germ tubes (t) and hyphae which grow through the root tissue. The host responds by deposition of callose (c).

recognition between higher plant cells in other situations. That is, receipt of extracellular information, either at the cell wall surface or at the plasma membrane after diffusion through the wall. One response to recognition of

Table 8. Comparison of self-incompatible pollination and race specific pathogenesis in terms of the possible recognition events and response. The ultimate response is arrest of growth of the pollen tube or fungal hypha. One of many facets of the total response is deposition of callose in the pollen tube or cell walls of the infected tissue.

|  | SIGNAL                                   | RECEPTOR                                   | RESPONSE  |
|--|--|--|---|
| Gametophytic<br>self-incompatible<br>pollination | soluble<br>S-gene<br>product in<br>style | pollen-tube<br>(wall?<br>plasma membrane?) | Arrest of<br>pollen tube<br>growth<br>↓<br>↓<br>callose in<br>pollen tube |
| Race specific<br>pathogenesis                    | secretion of<br>fungal<br>hyphae         | host plant<br>(plasma membrane?)           | Arrest of<br>fungal growth<br>↓<br>↓<br>callose in<br>host wall           |

incompatible pollen is production of callose, identified by its fluorescent staining with aniline blue. The material is also produced as a response to many fungal infections: its formation is also part of the general wound response.

5. Analytical work has so far only been undertaken for a few components of a few systems, there is insufficient data available to draw any generalizations regarding the molecular basis of the recognition events.

6. There is no information available regarding the putative receptors at the pollen tube surface. The nature of the secreted components of the stigma and the pistil have been examined for the monocotyledons Gladiolus and Lilium. Both contain a variety of components, among which is an arabino-(1→3):(1→6)-galactan which accounts for about half the total non-dialyzable material of the secretion. Related

arabinogalactans are present in style extracts of other species. The role these components or indeed the other minor components play in pollination is not known.

7. For a number of systems, components which correlate with S-genotype have been demonstrated; these components have an implied, but not proven, role in recognition of compatible pollen.

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## Chapter Nine

### BACTERIAL ATTACHMENT TO PLANT CELL WALLS

MARIAMNE H. WHATLEY AND LUIS SEQUEIRA

Department of Plant Pathology  
University of Wisconsin-Madison  
Madison, WI 53706

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### INTRODUCTION

A plant encounters a large number of potential pathogens in its environment, but, because of the highly specific nature of most host-pathogen interactions, rarely does successful infection occur. This specificity apparently is dependent on the initial recognition between the plant and pathogen, which may be mediated by the interaction of complementary macromolecules on the surfaces of both organisms. Recognition can facilitate growth of both organisms, as is the case of symbiotic relationships. Recognition also could function as a defense mechanism. A plant can recognize and immobilize a potential pathogen, thus preventing its multiplication. Though the hypothesis of recognition as a specific defense mechanism is an attractive explanation of various resistance phenomena, it has not been demonstrated unequivocally. Most of the work in this area involves symbiotic or plant pathogenic bacteria. This paper will examine the evidence for attachment of bacteria to plant cell walls and then proceed to a discussion of the nature of the bacterial and plant components that may be involved.

## EVIDENCE FOR BACTERIAL ATTACHMENT TO PLANT CELL WALLS

When bacteria invade a plant, the plant often responds actively. For this response to occur, there must be close proximity between the bacterium and host cell wall.<sup>1</sup> Other than in the case of infection by soft rot bacteria, in which plant material is enzymatically disintegrated ahead of the pathogen, bacteria generally interact with the host. In the cases of crown gall tumor induction, in which plasmid transfer occurs between Agrobacterium and its host, and root nodule formation, in which nitrogen-fixing bacteria are encapsulated in the root hair, it is clear that close association between host and parasite is necessary. In these cases, the cell wall, though it is sometimes thought of as an inert barrier, is very much involved in the host response. Incompatible and saprophytic bacteria also stimulate a host response. Though the need for specific recognition and attachment is apparent in the case of infections by crown gall and nitrogen-fixing bacteria, there is still some controversy as to the role of specific attachment in other interactions. The following section reviews and discusses evidence for specific attachment in plant-bacterial interactions.

Agrobacterium tumefaciens

Infection of a susceptible host by Agrobacterium tumefaciens results in non-self limiting tumors. A wound is necessary for tumor induction. Once the induction process is complete, the presence of the bacteria is no longer required for the continued growth of the tumor. By means of a pinto bean leaf tumor bioassay, Lippincott and Lippincott<sup>2</sup> showed that bacterial attachment to a plant host wound site is a necessary first step in tumor induction. Tumor initiation by virulent strains of A. tumefaciens is inhibited by the presence of either heat-killed virulent Agrobacterium cells or certain living avirulent cells. Some avirulent strains of Agrobacterium show no inhibitory effect, however. Reduction in tumor number occurs if the inhibitory cells are added with or 15 minutes before the virulent inoculum, but not if added 15 minutes later. It appears, therefore, that the binding process is complete in 15 minutes.<sup>2</sup>

Binding of Agrobacterium has been shown to occur in other hosts besides pinto beans.<sup>3-6</sup> Electron microscopy has also provided evidence for Agrobacterium-plant attachment. Bogers<sup>7</sup> and Schilperoort<sup>8</sup> showed a close association between the virulent bacterium and the plant cell wall, with apparent envelopment of the pathogen by material that appeared to be of host origin. Agrobacterium also binds to carrot cells in suspension culture<sup>8</sup> and to Datura cells in suspension.<sup>9</sup>

The necessity of binding between Agrobacterium and its host has become more significant since the discovery that a portion of the Ti plasmid is actually transferred to and transcribed in the host cell.<sup>10,11</sup> For this transfer to occur, close contact probably is necessary since DNA would have to move through the bacterial envelope and plant cell wall during the course of tumor induction. The binding and transfer processes may involve several steps, of which initial recognition may be only one. Many aspects of cell binding, including changes in surfaces that would allow plasmid transfer, remain to be investigated.

Besides causing tumor induction, A. tumefaciens has also been shown to speed up normal plant development. In the moss Pylaisiella selwynii, the presence of virulent, viable A. tumefaciens speeds up gametophore induction and increases the number of gametophores.<sup>12</sup> This process requires physical contact between the moss protonema and the bacteria.<sup>13</sup> Scanning electron microscopy also has been used to document the close moss-bacterial cell association.<sup>14</sup> The germ tube appeared to be the site where effective attachment may occur. The different species of Agrobacterium tested adhered with different orientations, A. tumefaciens cells attaching lengthwise on the filament. Though a non-site binding strain of A. radiobacter also adhered, it had an end-on orientation and binding appeared to be mediated by the flagellae. This difference in adherence may be responsible for the ineffectiveness of this strain on gametophore development.

#### Rhizobium-legume interactions

As with the closely related Agrobacteria, it is clearly important to have a close association between Rhizobia and their hosts during the nodulation process. The initial

steps in this process involve deformation of the root hair, invagination of the root hair plasmalemma, and formation of an infection thread. There is a great deal of specificity in these processes, since most Rhizobia will nodulate only one host. Though specificity theoretically could be determined at many points in the long and complex process of nodulation, it appears that the initial recognition and binding may be the key determinant. It has been suggested that attachment is not the basis of host-symbiont specificity because some Rhizobia have been shown to attach strongly to non-hosts.<sup>15</sup> Although these data suggest that simple attachment is not necessarily sufficient to determine specificity, they do not disprove attachment as a prerequisite for nodulation.

When observed by electron microscopy, Rhizobia appeared to attach in an end-on, polar fashion; there was production of microfibrillar material, identified as cellulose, at the site of attachment.<sup>16</sup> Similar microfibrils are produced by the bacteria in culture and the authors suggested that continued production during the infection process may help mediate adsorption to the root hair surface. The possibility that the surface fibrillar material is of host origin is not entirely ruled out, however. The authors also concluded that the outer fibrillar layer of the infection thread is cellulose of host origin.

Dazzo et al.<sup>17</sup> compared attachment of non-nodulating Rhizobium and nodulating R. trifolii to clover root hairs quantitatively and found attachment was four to five times greater with the latter.

#### Pseudomonas spp.

When large populations of incompatible strains of Pseudomonas solanacearum are introduced into tobacco leaves, there is a rapid hypersensitive response (HR) which is characterized by collapse of plant cells and reduction in bacterial numbers.<sup>18</sup> Compatible bacteria, however, do not induce the HR, multiply rapidly, and spread from the area of infiltration.

Sequeira et al.<sup>19</sup> studied the ultrastructural changes associated with the introduction of virulent, avirulent or incompatible strains of P. solanacearum into tobacco leaves.

The avirulent or incompatible bacteria are attached to the cell walls and enveloped by fibrillar and granular host material. Also, there are changes in the host cell wall. A pellicle, probably of cuticular origin, separates from the and surrounds the bacteria. The plasmalemma separates from the cell wall and there is an accumulation of membrane-bound vesicles in the space between the plasmalemma and the cell wall. The virulent cells, however, are not attached and remain free to multiply in the intercellular fluid. There is some breakdown of cell wall structure, apparently caused by proteolytic and cellulolytic enzymes, but no extensive changes in organelle structure for 48 hours or longer. Heat-killed avirulent cells bind in the same manner as live ones, but do not induce the cell collapse associated with the HR. The HR can be prevented by pre-treatment with dead cells; live cells infiltrated 24 hours later will not attach and the HR is not induced.

Goodman et al.<sup>20</sup> obtained similar results with the inoculation of P. pisi and saprophytic species of *Pseudomonas* into tobacco leaves. They reported the separation of the wall cuticle from the cell wall at the point of attachment of the incompatible bacteria. The cuticle became progressively thicker and contained fibrillar material and large numbers of vesicles. The bacteria near the plant cell wall appeared to be enveloped and immobilized by the cuticle. Part of the enveloping material seemed to involve plant contents which had migrated out from the cell wall. The saprophytic bacteria also induced separation of the cuticle. Compatible bacteria, P. tabaci, induced aggregation of some of the loose fibrillar material, but none of the vesicles that were apparently involved in the formation of the thickened cuticle.

Sing and Schroth<sup>21</sup> reported initially that saprophytic P. putida cells infiltrated into "Red Kidney" bean leaves were enveloped by fibrillar material of apparently plant origin after the initial attachment between bacteria and host cell walls. The cuticular material was described as less granular than in tobacco. Pathogenic strains, either compatible or incompatible, did not attach and were not enveloped. Unlike the tobacco system, attachment did not appear necessary for induction of the HR since P. tomato did not attach, but caused the HR.

A more recent paper from the same laboratory suggested that the apparent attachment and envelopment is an artifact.<sup>22</sup> They suggested that when the intercellular spaces of bean leaves are infiltrated, the water dissolves materials from the cell surface. As the fluid recedes, due to evaporation or imbibition, the dissolved material forms a film at the water-air interfaces. Infiltrated bacteria are physically trapped by this film. Attachment of saprophytes was prevented by watersoaking the leaves continuously. They also observed that films were associated not necessarily with bacteria, but with the interstices between plant cells. In addition, they suggested the film may aid pathogenesis by maintaining a liquid phase around the bacterial cell.

Obviously, this paper raises several important criticisms of the electron microscopic evidence for bacterial attachment. If the attachment and envelopment observed in tobacco result from physical drying rather than an active host response, there must be a re-evaluation of the specificity of attachment and its role as a defense mechanism.

It should be remembered that the results obtained with bean are not necessarily applicable to those with tobacco or other hosts. The nature of the envelopment is visibly different in the two hosts.<sup>21, 23</sup> There are also differences in the nature of the host responses. When introduced at high concentrations into tobacco leaves, incompatible bacteria do not multiply. Daub and Hagedorn,<sup>23</sup> however, found that in both resistant and susceptible bean leaves, *P. syringae* multiplied in the intercellular spaces and usually was not enveloped. Only rarely was there a response that resembled envelopment and this occurred with all host-pathogen combinations. Other studies with susceptible and resistant bean leaves also did not show close attachment between *P. phaseolicola* and the cell wall.<sup>24</sup> However, in pods there was evidence in all cases of envelopment of bacteria by fibrillar material from host cell walls.<sup>23</sup> The response did not prevent bacterial multiplication, however. This apparent non-specificity of attachment is consistent with the other studies with bean previously described.<sup>22</sup>

It is important to examine the possibility that there is non-specific physical binding in tobacco. One of the

points made by Hildebrand et al.<sup>22</sup> is that films and deposits form very non-specifically, around available objects such as bacteria and in the interstices between plant cells. From this, it would be expected that any object infiltrated into the intercellular spaces of leaves would be expected to be enveloped. However, when infiltrated into tobacco leaves in the same manner as bacteria, both polystyrene balls and asbestos fibers settled on the wall surface upon drying of the water, but there was no apparent attachment or envelopment (de Zoeten and Sequeira, unpublished). Tobacco rattle virus, infiltrated by the same methods, will attach end-on to tobacco cell walls, but there is no envelopment.<sup>25</sup> If physical non-specific trapping is responsible for envelopment, this should be seen with virus particles, also. In tobacco, some bacteria do become trapped in the interstices between host cells. However, bacteria also are attached along surfaces of cell walls that do not offer this same potential for trapping. Huang and Van Dyke<sup>26</sup> provided evidence of immobilization of bacteria by callus cell walls. An incompatible bacterium, *P. pisi*, formed aggregates which were enveloped by a network of fibrillae of plant origin.

In tobacco, true envelopment, rather than physical entrapment, occurs only with avirulent and incompatible bacteria. It is argued by Hildebrand et al.<sup>22</sup> that a compatible bacterium, through a combination of extracellular polysaccharides (EPS) and the surrounding film, can maintain a moist environment and can continue to multiply, eventually displacing the film and spreading. This could provide an explanation for the binding of the non-fluidal avirulent *P. solanacearum* strains, but not of the fluidal virulent strains. However, this does not explain why incompatible Race 2 strains of *P. solanacearum*, which are attached and induce the HR, also produce large amounts of EPS.<sup>19</sup>

Hildebrand et al.<sup>22</sup> imply that in the initial stages of entrapment there is no active host response, but merely physical effects brought on by changes in the status of water in intercellular spaces. However, the evidence from tobacco-bacteria interactions supports the concept of an active host response. Politis and Goodman<sup>27</sup> followed the changes in the host cell wall very soon after inoculation with incompatible *P. pisi*. As seen in other systems, the

bacteria were in close association with the cell wall after 2 hours. At this time, the plasmalemma had separated from the cell wall and loose microfibrils began to accumulate. By 6 hours, organized structures, called appositions, invaginated the plasmalemma opposite the attached bacteria. Membrane-bound vesicles, that originated from the plasmalemma opposite the bacteria, seemed to carry and deposit the fibrillar material in the appositions. Their results, as do the other studies with tobacco, point to an active host response only in the area close to the point of bacterial attachment, resulting in the formation of specific structures. This kind of response would not be expected if the interaction was dependent on mere physical trapping.

In an electron microscopic study, it is obviously important to be aware of possible artifacts and not to interpret physical effects as physiological phenomena. However, the arguments put forth by Hildebrand et al.<sup>22</sup> do not invalidate conclusions that apply to the interactions of bacteria and tobacco. Though physical trapping of bacteria in the interstices between host cells may be responsible for immobilizing some bacteria, it cannot account for the degree of specificity seen in the attachment process.

#### Xanthomonas and Erwinia

Results similar to those found in tobacco-Pseudomonas interactions have been reported for the cotton-Xanthomonas malvacearum interaction. Cason et al.<sup>28</sup> found that inoculation of incompatible bacteria into cotyledons of resistant cotton plants caused a detachment of the surface cuticle and envelopment of adjacent bacteria. The enveloping structures contained fibrillar material. In contrast, when bacteria were inoculated into susceptible host tissue, none of these changes in the plant cell wall occurred and bacteria were not enveloped. Though the authors felt that the envelopment was too fragile to result in immobilization of bacteria, they also suggested that the disruption of the cell wall stimulated by the presence of the bacteria may be essential for the HR.

In the interaction of Xanthomonas phaseoli var. sojensis with soybean, a virulent strain remained free and multiplied in the intercellular fluid, but two avirulent strains, which did not induce the HR, were attached to the

mesophyll cell walls and enveloped by fibrous material. Another strain which elicits the HR was not enveloped, however.<sup>29</sup> In this case, the results with soybean were similar to those reached by Sing and Schroth with a bean system.<sup>21</sup>

The HR can be prevented by maintaining fluid continuously in the intercellular spaces or by introducing bacteria in agar.<sup>30,1</sup> Water-soaking may prevent the HR by preventing close contact of the bacterium with the host cell wall, as Stall and Cook have shown for Xanthomonas vesicatoria introduced into pepper leaves.<sup>1</sup> However, this may not be the reason for prevention of the HR, because in some systems the HR is induced without attachment.

When virulent strains of the soft rot bacterium Erwinia chrysanthemi are introduced into corn leaves, they remain free and actively dividing in the intercellular spaces. However, when incompatible E. carotovora strains are infiltrated, the bacteria become attached and enveloped by granular material by 4 hours.<sup>31</sup> The plasmalemma separates slightly from the cell walls and vesicles appear near the points of bacterial attachment. There is also noticeable disruption of the bacterial cytoplasm and, in some cells, apparent detachment of the bacterial envelope. Although very limited work on the ultrastructure of this interaction has been done, the available evidence suggests that it follows the pattern described for tobacco and cotton.

#### THE BACTERIAL COMPONENT OF RECOGNITION

When bacteria invade a potential host, components on the outer surface of the bacterial cell are the first to interact with the host cell surface. The high specificity in these initial interactions suggests that a bacterial cell surface component is likely to be an important determinant of specificity. Strong candidates for this role are the extracellular polysaccharide (EPS) and the lipopolysaccharide (LPS) of the outer membrane. In bacteria with a true capsule, the oligosaccharide constituents would make first contact with the plant cell wall and may have sufficient structural stability to bind to specific receptors. In bacteria with either soluble or no EPS, the LPS would be a more likely candidate for recognition. In animal pathogens, the O-antigen of the LPS is highly variable and

determines the antigenic specificity. The lipid A and core regions of LPS are quite constant and would not be expected to vary much within a species.

#### Agrobacterium tumefaciens

By means of inhibition of tumor formation in the pinto bean bioassay, Whatley et al.<sup>32</sup> studied the nature of the *A. tumefaciens* component involved in host recognition. Lipopolysaccharides (LPS), extracted from virulent and binding avirulent strains of *A. tumefaciens* by the standard phenol water method, were highly inhibitory in this assay. A concentration of 1.0 ng/ml gave 35% inhibition of tumor formation. As would be expected of the binding component, inhibition only occurred when LPS was added before or along with the virulent inoculum, but not when added 15 minutes later. The LPS from non-binding strains of *A. radiobacter* did not show any inhibitory effect. Removal of LPS from bacteria by EDTA treatment reduced tumor-inducing ability of the bacteria.<sup>32</sup>

When *Agrobacterium* LPS is hydrolyzed by mild acetic acid treatment, the lipid A and the polysaccharide components separate, but only the polysaccharide inhibits tumor formation.<sup>5</sup> Lipid A, when complexed with bovine serum albumin to increase solubility, has no effect on tumor formation. The polysaccharide, which contains the core and O-antigen, showed approximately the same level of biological activity as whole LPS. It is not known what part of the polysaccharide is responsible for recognition, though the O-antigen is a likely candidate because of its greater variability.

The Ti or tumor-inducing plasmid of *A. tumefaciens* carries sufficient genetic information to transform an avirulent, non-binding strain of *A. radiobacter* into a virulent, binding strain.<sup>33</sup> When any of several Ti plasmids is introduced into these non-binding strains, their LPS becomes inhibitory in the binding assay; similarly, loss of the plasmid results in loss of binding ability. However, when other virulent strains are cured of their plasmids, they retain site-binding ability, indicating that genetic information for binding may also be located on the chromosome.

Inhibition of gametophore induction in the moss assay was used to determine the bacterial component involved in recognition. Whatley and Spiess<sup>34</sup> demonstrated that LPS of *A. tumefaciens* is the binding component involved in this interaction. The LPS showed the same binding specificity as whole cells and showed an order of addition effect; LPS was no longer inhibitory when added 24 hours after the virulent cells. As with tumor induction, it is the polysaccharide, not the lipid A, which is effective as an inhibitor.

#### Rhizobia-legume interactions

There has been a great deal of debate concerning the identity of the recognition component of Rhizobia. Both EPS and LPS have been proposed. Wolpert and Albersheim<sup>35</sup> examined the interaction between the LPS of four different rhizobia and four different lectins immobilized on columns. Only the LPS of the appropriate symbiont bound to the lectin of its host. Chemical modification of the LPS molecules indicated that the O-antigen portion was active in the binding process. Work by Maier and Brill<sup>36</sup> indicated that the O-antigen may play a role in nodulation of soybeans by *R. japonicum*. Using paper chromatography of LPS, they found three major differences in composition between the O-antigen of a non-nodulating mutant and that of the wild-type. In another study, it was reported that host plant lectins bound specifically to *R. japonicum* and *R. leguminosarum* and this interaction could be inhibited by LPS extracted from these cells.<sup>37</sup>

There is other evidence the LPS may not be involved in certain Rhizobial interactions. In a study of the LPS from *R. leguminosarum*, *R. phaseoli*, and *R. trifolii*, there was no obvious correlation between immunochemistry or chemical composition of the LPS and the nodulating group.<sup>38</sup> Though the authors did not rule out involvement of LPS, they felt that the data did not lend support to the LPS hypothesis.

Calvert et al.<sup>39</sup> found that ferritin-labeled soybean lectin binds specifically to the capsular (EPS) material of *R. japonicum* cells and not to the outer membrane (LPS) layer. The ability of *R. japonicum* to bind soybean lectin changes with different stages in the growth cycle<sup>40</sup> and

these changes are associated with specific changes in the sugar composition of the capsule.<sup>41</sup> A mutant of *R. leguminosarum* which secretes less than normal amounts of extracellular polysaccharide was incapable of nodulating host roots, though the parental and mutant types both had the same LPS structure.<sup>42</sup> This suggests that EPS rather than LPS may play a role in Rhizobial specificity, though there may be other explanations for the failure to nodulate host roots.

In the *R. trifolii*-clover system, Dazzo and Hubbell<sup>43</sup> reported that the apparent binding component is an acidic heteropolysaccharide. Serologically, it is similar to an antigen present on the surface of clover roots. Since 2-keto-3-deoxyoctonic acid was absent and there was no endotoxin activity, the authors assumed their preparation contained no LPS. However, in a more recent paper, Dazzo and Brill<sup>44</sup> found that the active EPS is serologically identical to the O-antigen of *R. trifolii* LPS. *R. leguminosarum* produces a polysaccharide that is different from either EPS or LPS.<sup>45</sup> It was suggested that this polysaccharide might help explain the apparent involvement of both EPS and LPS in recognition. This polysaccharide does not appear to be part of the LPS.<sup>46</sup>

Other researchers, in trying to resolve this controversy, have attempted to find a role for both LPS and EPS. One suggestion is that the binding process may actually be in two steps: an initial recognition involving EPS and a tighter binding, necessary for further infection, involving LPS. Kamberger<sup>47</sup> suggests that EPS is responsible for absorption of *Rhizobium* to pea root hairs, but that EPS-mediated binding is not sufficient for nodulation. A more specific recognition involving LPS may be necessary for bacterial invasiveness. In the binding of labeled peanut lectin to *Rizobium*, it was reported that there are major binding sites in EPS, but that there are other binding sites in LPS.<sup>48</sup>

#### Pseudomonas solanacearum

All virulent strains of *Pseudomonas solanacearum* produce large amounts of slime, a soluble, non-capsular form of EPS. Avirulent, cultural variants of this bacterium can induce the HR in tobacco or potato leaves and lack EPS.

Sequeira and Graham<sup>49</sup> found that a low concentration of potato lectin agglutinated avirulent, but not virulent cells of the bacterium. If virulent cells were washed to remove EPS, they were agglutinated to a certain degree, but when EPS was added back, agglutination was reduced. The LPS from either HR or non-HR inducing bacteria was precipitated by the lectin, though the latter not as extensively. It appeared from these data that LPS is the binding component in both HR and non-HR inducing strains, but that in virulent strains, EPS prevents binding and allows bacterial multiplication.<sup>49</sup> Whether the effect of EPS was due to competitive binding or a general blocking effect was not established.

Further studies with a series of virulent and avirulent strains, the latter including HR and non-HR inducers, indicated a strong correlation between LPS structure and ability to induce the HR.<sup>50</sup> Based on sugar composition of LPS, particularly on the relative amounts of xylose and glucose, and on mobility by SDS polyacrylamide gel electrophoresis, it was shown that all the HR-inducing strains had rough (R) LPS, that is, they lacked part or all of the O-antigen.<sup>50</sup> All but one of nine non-HR inducing strains had smooth (S) or complete LPS. The one exception (strain Q) which had R-LPS based on gel separation and was intermediate in sugar composition, may have been unable to induce the HR for other reasons. Strain Q is an intermediate strain that will be useful to define the precise configuration of LPS that is necessary for binding to host cell wall. A bacteriophage that lyses both virulent and avirulent non-HR inducers, but not the HR inducers, is also being used to establish LPS structure in these strains. Pretreatment of the phage with S-LPS inhibited lysis of a virulent strain of the bacterium, whereas R-LPS had no effect.<sup>51</sup>

These studies suggested that the differences in LPS structure determine the ability of *P. solanacearum* to cause the HR. Also, they suggest that the binding component resides in either the core or lipid A portion, since O-antigen is lacking in the rough strains. In the smooth LPS, the highly branched O-antigen side-chain may mask the recognition site.

Lipopolysaccharide also plays a role in induced resistance in tobacco leaves. If heat-killed avirulent or

virulent Pseudomonas cells are introduced into tobacco leaves, the leaves will be protected against subsequent challenge by a variety of pathogens.<sup>52,53</sup> In protected leaves, induction of the HR by avirulent P. solanacearum is prevented. Heat-killed cells attach and are enveloped in a manner similar to live HR-inducing cells,<sup>19</sup> but no HR results. Graham et al.<sup>54</sup> found that protection was induced by LPS alone, whether from rough or smooth strains, and determined that the active portion was located in the core-lipid A region. When LPS was injected into leaves, ultrastructural changes occurred in host cells similar to those obtained with heat-killed cells. The role of LPS in HR induction and in induced resistance evidently is not the same, since the former requires rough LPS, while the latter is obtained with either R- or S-LPS. Similarly, Mazzuchi et al.<sup>55,56</sup> found that both the HR and susceptible response in tobacco could be prevented by a protein-LPS complex from phytopathogenic pseudomonads.

#### Xanthomonas and Erwinia

In the interaction of Xanthomonas phaseoli var. sojensis with soybean, it was shown that virulent strains do not bind to host walls, while avirulent do bind and are enveloped.<sup>29</sup> All five strains examined had equal amounts of EPS of identical sugar composition. The virulent strains, however, all had S-LPS, as determined by sugar composition and SDS polyacrylamide gel electrophoresis. In contrast, the avirulent all had predominantly R-LPS. LPS from virulent strains had approximately 30% fucose and less than 10% glucose, while the avirulent contained no detectable fucose and over 30% glucose (Whatley, unpublished). The difference in binding between virulent and avirulent would appear, therefore, to reside in the LPS. In other Xanthomonas systems, however, there is some evidence that the EPS plays a role in determining specificity.<sup>57</sup>

It is possible that the specificity of other bacterial-plant interactions depends on the rough-smooth difference in LPS structure. The O-antigen may provide a way to mask the recognition component and allow the pathogen to remain undetected. It is also possible that an indirect effect may be involved. There can be major changes in cell membrane protein associated with smooth-rough transitions<sup>58</sup> which may be important. For example, Bruegger and Keen

found a specific elicitor of glyceollin production in soybean which could be extracted from the cell envelope of P. glycinea.<sup>59</sup> Neither LPS nor EPS were active as elicitors; the elicitor appears to be a glycoprotein.

Strains of Erwinia stewartii, which are virulent on corn, produce large amounts of EPS. These cells, as well as several avirulent, EPS-producers, were not agglutinated by a corn agglutinin. Those avirulent strains which lack EPS were readily agglutinated.<sup>60</sup> There were no differences between the LPS from agglutinable and non-agglutinable strains (Whatley, unpublished). It appears that LPS may be involved in binding, while EPS may prevent the interaction of the LPS with the plant receptor.

#### THE PLANT CELL WALL RECEPTOR

In most instances, the bacterial component recognized by the host appears to be a polysaccharide, either LPS or EPS. Therefore, the plant cell wall receptor must be able to recognize polysaccharides specifically. Because lectins are proteins or glycoproteins which are able to bind specific sugars, these proteins fit the requirements for receptors. Lectins can be located on host cell walls and there is some evidence that they play a role in plant-bacterial interactions, although there is still no definitive proof. To obtain proof, it is important to show that the lectin binds the bacterial polysaccharide specifically in vivo and in vitro, that this binding is hapten-reversible, and that the lectin is localized in the parts of the plant that normally bind the bacteria. Other possible receptors must be considered. For instance, a plant polysaccharide could interact specifically with the bacterial polysaccharide.<sup>57, 61, 62</sup> Evidence points in the direction of a carbohydrate-carbohydrate interaction in a few systems.

#### Agrobacterium tumefaciens

Since a wound is necessary for tumor induction, it is possible that the plant binding component could be on an exposed plasma membrane or on the cell wall. By means of the pinto bean bioassay, Lippincott et al.<sup>63</sup> concluded that the binding component resides on the cell wall. In the tumor induction assay, the inhibitory effect of the cell wall preparations could be neutralized by pretreatment of

the cell walls with certain whole heat-killed bacteria or their LPS. Non-binding cells or their LPS do not alter the binding ability of the cell walls, indicating that the same binding specificity is exhibited in vivo and in vitro.

Cell wall preparations from a number of different plants or tissue cultures were tested for their binding ability to inhibit tumor formation.<sup>64</sup> Cell walls from dicots, but not those from monocots were inhibitory.<sup>64</sup> Though this finding correlates with the host range of Agrobacterium, the difference in binding probably does not explain the specificity of the interactions. In the same study, it was indicated that after transformation, cell walls from tumors are no longer able to bind Agrobacterium, indicating that there are changes in cell wall structure associated with tumor induction.

When isolated cell wall components were tested, it was found that polygalacturonic acid was highly inhibitory and pectin was mildly inhibitory.<sup>62</sup> Removal of pectin and hemicelluloses by strong acid treatment reduced the binding ability of the cell walls. Differences in effectiveness of pectin and polygalacturonic acid appeared to be due to differences in degree of methylation. When non-binding cell walls, such as those from monocots, tumors, or embryonic tissue, were pretreated with pectin methyl esterase, they became highly inhibitory. Lippincott and Lippincott concluded that the binding site may involve the polygalacturonic acid in the middle lamella and primary cell wall.<sup>62</sup> In its highly methylated form (pectin), polygalacturonic acid loses this binding ability. In spite of this evidence, the possibility that lectins are involved in recognition has not been ruled out. Because A. tumefaciens has such a wide host range, it is likely that the binding component is similarly non-specific, such as polygalacturonic acid. Recognition in crown gall tumor induction may involve an LPS-polygalacturonic acid interaction.

#### Rhizobium-legume interactions

The role of lectins in Rhizobium-legume interactions has been reviewed recently<sup>65,66</sup> and will be mentioned here only briefly. Hamblin and Kent<sup>67</sup> first suggested a role for lectin in recognition with the demonstration that there was a specific binding of bean lectin to R. phaseoli.

Bohlool and Schmidt<sup>68</sup> showed later that soybean lectin (SBL) combined specifically with 22 of 25 nodulating strains of *R. japonicum*. They did not find binding of the lectin to non-nodulating strains. The SBL binding sites are polar,<sup>69</sup> a fact which agrees with the end-on attachment reported by Dazzo et al.<sup>17</sup> These results of Bohlool and Schmidt were supported by extensive tests in Bauer's laboratory and the important criterion of hapten specificity was met, since N-acetyl-D-galactosamine, an SBL hapten, fully blocked bacterial-lectin binding specifically.<sup>40</sup>

There remained, however, the problems of nodulating strains that did not bind the lectin and of the non-nodulating ones that did.<sup>15</sup> The first problem was partly solved by the demonstration that many of these non-binding bacteria, when grown in soybean root exudates, developed receptors for SBL.<sup>70</sup> Discrepancies in results, therefore, may be due to differences in the culture conditions used. It also must be remembered that specific binding to the lectin need not always lead to nodulation, since there are many steps in the process at which it could be blocked.

There are many other unresolved questions. For example, a specific hapten for SBL is apparently not present in *R. japonicum* EPS,<sup>71</sup> though binding sites *in situ* do not necessarily have to involve a simple hapten. Also, SBL has not been detected in the roots of soybean at an age when the plant could still be nodulated.<sup>72</sup> An immunological study indicated that the roots of a number of soybean lines appeared to lack SBL totally, though SBL was found in a line thought to lack it.<sup>73</sup> There are a number of possible explanations for this. For example, lectin may be more tightly bound and not extractable by standard procedures.<sup>72</sup> It is necessary to settle these questions before a role for lectin can be demonstrated in the *R. japonicum*-soybean interaction.

The *R. trifolii*-clover system also has been extensively studied. Dazzo and Hubbell<sup>43</sup> reported that a carbohydrate antigen on the surface of clover roots was cross-reactive with a bacterial polysaccharide. A lectin named trifoliin was isolated from clover seed and was found to bind to both the bacteria and clover roots.<sup>74,75</sup> Binding of bacteria by trifoliin can be inhibited by the sugar 2-deoxy-D-glucose, a compound which can also elute lectin

from clover seedling roots. It was proposed that trifoliin forms a bridge between bacterium and host to establish binding.<sup>43,66</sup> The major problem with this hypothesis is that there is little evidence that trifoliin is on the roots.

#### Pseudomonas solanacearum

The bacterial component involved in the induction of the hypersensitive response (HR) in tobacco by Pseudomonas solanacearum seems to be LPS. Whether EPS plays a role in preventing binding of bacteria *in vivo* is still an open question. A lectin isolated from potato or tobacco agglutinated the HR-inducing strains of P. solanacearum much more strongly than virulent strains, and this was correlated with a stronger precipitation of R-LPS compared with S-LPS.<sup>49</sup> Fluorescein-labeled lectin bound to avirulent, but not to virulent cells of P. solanacearum and this binding was inhibited by chitin oligomers, which contain internal N-acetyl glucosamine moieties, the hapten for potato lectin.<sup>49</sup> Potato and tobacco lectins, therefore, seem to meet the requirement of specificity of binding of the bacterial recognition component. A nitrocellulose filter binding assay is currently being used to quantitate differences in binding between LPS and EPS of different strains.<sup>76</sup>

It is also important to show the lectin is present in tissues which bind bacteria. A lectin, with agglutinating specificity identical to that of the potato lectin, was isolated from tobacco leaves by infiltrating the leaves with saline and recovering the intercellular fluid by centrifugation.<sup>49</sup> That the lectin was located on the cell walls was demonstrated with the use of fluorescent anti-lectin antiserum.<sup>77</sup> Binding of the labeled antibodies to mesophyll cell walls of tobacco and potato was demonstrated by fluorescence microscopy. The potato and tobacco lectins are hydroxy-proline-rich proteins, as are other plant cell wall components, and also contain arabinogalactans, substances commonly found in the cell wall.<sup>49</sup> In further studies of localization, it will be necessary, therefore, to establish that the antiserum is specific for the lectin and not for other cell wall components.

Although most of the evidence points to the involvement of the lectin in the plant-bacterial interaction

resulting in the HR, further evidence is necessary in terms of localization of the lectin, hapten reversibility, and quantitation of binding of bacterial cell wall components.

#### Xanthomonas and Erwinia

A possible role for plant agglutinins in soybean-Xanthomonas phaseoli var. sojensis interactions was examined by Fett.<sup>29</sup> Standard SBL did not agglutinate the bacterium, but a new soybean agglutinin differentially agglutinated different strains of the bacterium. However, there was no apparent correlation with virulence, although these strains differed in LPS structure and in their ability to bind to cell walls. Both EPS and LPS inhibited agglutination by the agglutinin. It appears that either a lectin is not involved in this interaction or the correct one has not been identified as yet.

On the basis of in vitro interactions, Morris et al.<sup>57</sup> suggested that Xanthomonas-plant interactions may involve polysaccharides of both organisms. Xanthans, EPS isolated from Xanthomonas spp., gel when mixed with plant galactomannans, even at low concentrations. They suggest that this interaction may provide a "molecular holdfast" for the bacterium on the plant.<sup>57</sup> It is also reported that there is specificity in the gelling of both plant and bacterial components.<sup>78</sup> Though the in vitro studies show a specific interaction, there is as yet no in vivo demonstration of the role of this polysaccharide-polysaccharide interaction as a determinant of specificity.

An agglutinin extracted from corn seed agglutinated the avirulent strains of E. stewartii which did not produce EPS. Virulent strains, all of which produce EPS, were not agglutinated.<sup>60</sup> A role for this agglutinin in E. stewartii-corn interactions remains to be demonstrated, however.

#### CONCLUDING REMARKS

In the systems that have been studied, there is strong physical and chemical evidence for specific site-attachment between bacteria and plants. In two of these systems, root nodule formation and crown gall tumor induction, the binding

is necessary for the continued interaction of the bacteria with the host. In those interactions that result in an incompatible, hypersensitive response, attachment appears to be part of a host defense mechanism. There are, however, unexplained cases of HR induction without prior attachment. In either compatible or incompatible interactions, attachment results in a host response. How this response is linked to attachment is unknown, but it is one of the key questions in the entire area of recognition. There must be a system for transfer of information from the site of attachment on the cell wall to the target organelle in the host cytoplasm.

In all cases, the bacterial component appears to be either a cell surface polysaccharide (LPS or EPS) or a glycoprotein. However, the identity of the bacterial component in most interactions, including the Rhizobium-legume interaction, is still in doubt. There has been a great deal of conflicting data generated by researchers who have used different systems. An attempt must be made to study the same bacterial strains and the same host cultivars in different laboratories if the question is to be resolved.

The complementary plant component involved in recognition may be either a lectin or a polysaccharide. The interaction of bacteria and plant cell wall components must be sufficiently specific to explain the highly predictable nature of the host responses. However, the role of lectins in plant bacterial binding has not been adequately demonstrated and there is little evidence for polysaccharide-polysaccharide interactions. Future studies on lectins must center on the localization of these substances in the appropriate parts of the plant and on the specificity and hapten reversibility of their interaction. It is important to move past the stage of simple correlations into a detailed examination of chemical structure of both interacting components and the direct demonstration of their involvement in recognition. In particular, structural modifications which prevent the interactions should be examined.

These questions regarding the identity of the interacting components must be settled before we can begin to examine how changes in both host and parasite are stimulated after attachment.

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## Chapter Ten

### LECTINS AND PLANT-HERBIVORE INTERACTIONS

DANIEL H. JANZEN

Department of Biology  
University of Pennsylvania  
Philadelphia, Pennsylvania 19104

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### INTRODUCTION

When an animal takes a bite out of a plant, it is gustatorily and digestively treading on a battlefield implanted and strewn with traits generated by natural selection during millions of years of acts of herbivory. The glycoproteins called lectins are heterogeneous in kind, place and density in this battlefield. How animals respond to this pattern and its parts suggest that lectins may be more than simply one more of nature's many kinds of glue. They are quite unfortunately called "lectins" since it is their sticky nature that is the basis of their biological function; while they are specific in their attachment to certain sugars, the fact that the same sugars can occur on the surface of many different kinds of cells makes them in fact highly variable in the specificity of their stickiness at the cellular level of organization (which surely is the level of concern when one calls them "lectins").

- What are the relevant properties of a glue?
- How tightly does it adhere to two or more relevant surfaces?
- How specific is its adherence?
- How quickly and easily can it be dissolved or neutralized by the user?
- How expensive is it?

How can it be used for other purposes?  
What is its shelf life?  
How much is needed to do the job?  
How long does it retain its properties once in  
place?  
What is its setting time?

If we ask these sorts of questions of a child's paste pot, of a welder's rod, of electrician's tape, of a gardener's tanglefoot, of epoxy resins, of a spider's web, of a flower's stigma, to say nothing of what Fort Dix thought up during the Viet Nam war, we quickly note that lectins have a number of properties which should lead, through natural selection, to their becoming on occasion part of a plant's defense repertoire. Just as there are specific places in battle where boiling oil is an important direct defense (though hot oil is generally much more important as a motor lubricant), a moderate-sized protein that adheres tightly to very specific sorts of surfaces can on occasion be an effective defensive weapon.<sup>1</sup> Needless to say, direct defense against other organisms is certainly not the biological function of all lectins any more than providing a microbe binding site on roots is the biological function of the lectins in cecropia moth blood.<sup>2</sup>

Among the array of lectin kinds, concentrations and placements, where is it reasonable to search for a defense function against multicellular herbivores? Before embarking on this query, I must very briefly mention what lectins apparently do to a herbivore that consumes them. They apparently bind to gut surfaces, be they of the animal or of its essential microbial fauna, and alter the function of those surfaces.<sup>3</sup> In short, they fall in the category of digestion inhibitors, as do protease inhibitors,<sup>4,5</sup> polyphenols (tannin, lignin, etc.),<sup>5,6</sup> cellulose,<sup>7</sup> unassimilable starches, etc. Since a lectin molecule is small relative to the herbivore's gut surface area, and since it has only a few active sites,<sup>3</sup> for a lectin to be a significant digestion inhibitor it will have to occur as a relatively large proportion of the diet.

Without dipping further into the philosophical background of why study protein glues as defensive compounds as well as molecules useful to the plant in other ways, I would like to relate the early unfoldings of a study of an

exceptional little mouse, a study that bears strongly on lectins as defenses against seed predators.

#### LIOMYS SALVINI

Liomys salvini (Figure 1) is a hispid pocket mouse in the same family of professional seed-eaters, Heteromyidae, as is the more familiar desert kangaroo rat, Dipodomys. L. salvini, or 'guarda fiesta' as it is locally known, however is a forest-floor mouse. This tropical mouse is a common inhabitant of the deciduous forests of Guanacaste Province, on the lowland coastal plain of Costa Rica.<sup>8-10</sup> Its diet is almost entirely seeds of herbs, vines, shrubs and trees, which it finds in the litter and in animal dung. It also shells seeds out of certain species of newly fallen fruits. The specific area where I have studied this mouse, Santa Rosa National Park (about 35 km north of Liberia, Guanacaste Province, Costa Rica), has a flora of about 650 species of broad-leaved plants,<sup>11</sup> over half of which are woody perennials with seeds large enough for this mouse to bother with. Of this set of seeds, it is conspicuous that certain species



Figure 1. Adult female Liomys salvini (46 g) from Santa Rosa National Park, Guanacaste Province, Costa Rica (July, 1980).

are flatly rejected (Table 1). These seeds lie about on the forest floor, in or out of their fruits, for months without being eaten. They are never encountered in the pouches of trapped mice. They were rejected when presented to the mice as part of seed mixes on plates at which free-ranging mice nightly foraged in the forest.

It is possible that in some absolute sense some species of seeds are rejected because they are nutritionally a badly imbalanced food; however, this does not explain why they are not eaten as, for example, a carbohydrate source in combinations with other seed species. Some are rejected because they are in a container too hard or unwieldy for L. salvini to gnaw through (Acrocomia vinifera palm nuts are an example), but the great majority of rejected seeds are no harder than those that are eaten and are often bitten into in apparent testing behavior. I find the most reasonable working hypothesis to be that these seeds are rejected for the chemicals that they contain other than those that are normally thought of as dietary nutrients for small mammals. Since this essay is directed at the obnoxious protein portion of a seed's defenses, I will focus on that aspect rather than some of the other known (additional) potential defenses of these seeds (e.g., Mucuna pruriens seeds contain L-dopa,<sup>12</sup>

Table 1. Some species of native forest and forest-edge woody plant seeds and nuts eagerly eaten and thoroughly rejected by free-foraging Liomys salvini in Santa Rosa National Park.

| <u>Rejected</u>                   | <u>Accepted</u>                 |
|-----------------------------------|---------------------------------|
| <u>Acrocomia vinifera</u>         | <u>Phaseolus lunatus</u>        |
| <u>Spondias mombin</u>            | <u>Enterolobium cyclocarpum</u> |
| <u>Lonchocarpus acuminatus</u>    | <u>Cochlospermum vitifolium</u> |
| <u>Lonchocarpus costaricensis</u> | <u>Malvaviscus arboreus</u>     |
| <u>Pithecellobium platylobum</u>  | <u>Hymenaea courbaril</u>       |
| <u>Canavalia brasiliensis</u>     | <u>Forsteronia spicata</u>      |
| <u>Canavalia maritima</u>         | <u>Pithecellobium saman</u>     |
| <u>Dioclea megacarpa</u>          | <u>Cissus rhombifolia</u>       |
| <u>Mucuna pruriens</u>            | <u>Sesbania emerus</u>          |
| <u>Ateleia herbert-smithii</u>    |                                 |
| <u>Cassia grandis</u>             |                                 |

Dioclea and Canavalia seeds contain canavanine,<sup>13</sup> Ateleia seeds contain 2,4-methanoproline and 2,4-methanoglutamic acid,<sup>14</sup> etc).

With the current understanding of potentially toxic seed proteins, protease inhibitors and lectins are the two proteinaceous candidates to examine with respect to Liomys rejection of seeds as food. Turning briefly to protease inhibitors, in the accepted column of Table 1 there is Enterolobium cyclocarpum, a mimosaceous legume seed that contains a substantial amount of a protease inhibitor (C. A. Ryan, personal communication), but no lectins, at least as measured by agglutination of human, rabbit or hamster blood (I. Liener, personal communication). This seed, in the hard (dormant, ungerminated) or germinated state can serve as the sole diet of L. salvini for a month or more in the laboratory,<sup>10</sup> is avidly sought by L. salvini in the field, and is chosen over many other acceptable seed species in choice tests. This suggests that L. salvini may be sufficiently specialized as a seed predator that it carries gut enzymes or other digestive repertoires generally resistant to protease inhibitors (it is unlikely, however, to be like the bruchid beetle larvae that are resistant to protease inhibitors by having a digestive system that operates largely without proteases<sup>15</sup>). To test this, I fed 4 or 5 L. salvini on diets of pure rat chow, and on rat chow that was 1, 5 or 25% soybean trypsin inhibitor (Table 2). There was no reduction in weight (and even a hint of weight increase compared to animals on pure rat chow) or rejection of the adulterated food, nor was there any visible symptom of a toxic or otherwise debilitating effect of the adulterated food. As a working hypothesis, I will assume that L. salvini is highly resistant to protease inhibitors in legume seeds.

There are at least 4 species of seeds in the rejected column of Table 1 that show strong agglutinating activity towards human, rabbit and hamster red blood cells (I. Liener, personal communication): Canavalia brasiliensis, C. maritima, Dioclea megacarpa, and Cassia grandis. The most desirable test would be to extract the lectins from these seeds and feed them to L. salvini. This step is planned. However, black beans (Phaseolus vulgaris) are a rich source of a potent lectin that has been shown to be lethal when incorporated in the diet of the larvae of a bruchid beetle.<sup>1</sup>

**Table 2. Weight changes and survival of adult Liomys salvini (hispid pocket mice) fed a variety of diets containing potentially toxic proteins. The animals were wild-caught and maintained in individual cages with ad lib food and water at temperatures approximating those of their natural habitat (Santa Rosa National Park, Guanacaste Province, Costa Rica, May-July, 1980).**

| Treatment   | Mouse number | Initial <sup>1</sup> weight (g) | Weight change (g) | Final Health | Duration of feeding (days) | Weight change per day (g) |
|---|--------------|---------------------------------|-------------------|--------------|----------------------------|---------------------------|
| <u>Rat chow:</u>                                    |              |                                 |                   |              |                            |                           |
|   | 37 ♂         | 32                              | +1                | healthy      | 9                          | +0.11                     |
|   | 38 ♀         | 40                              | +1                | healthy      | 7                          | +0.14                     |
|   | 39 ♀         | 41                              | -4                | healthy      | 7                          | -0.57                     |
|   | 40 ♂         | 59                              | 0                 | healthy      | 7                          | 0                         |
|   | 41 ♀         | 36                              | +1                | eaten by boa | 4                          | +0.25                     |
|   | $\bar{x}$    | 41.6                            | -0.2              |              |                            | -0.02                     |
|   | s.d.         | 10.4                            | 2.2               |              |                            | 0.32                      |
| <u>Rat chow with 1% soybean trypsin inhibitor:</u>  |              |                                 |                   |              |                            |                           |
|   | 1 ♀          | 50                              | 0                 | healthy      | 6                          | 0                         |
|   | 5 ♀          | 40                              | 0                 | healthy      | 6                          | 0                         |
|   | 7 ♀          | 41                              | -1                | healthy      | 6                          | -0.17                     |
|   | 8 ♀          | 40                              | 0                 | healthy      | 6                          | 0                         |
|   | 10 ♀         | 47                              | +1                | healthy      | 6                          | +0.17                     |
|   | $\bar{x}$    | 43.6                            | 0.0               |              |                            | 0.00                      |
|   | s.d.         | 4.6                             | 0.7               |              |                            | 0.12                      |
| <u>Rat chow with 5% soybean trypsin inhibitor:</u>  |              |                                 |                   |              |                            |                           |
|   | 3 ♀          | 45                              | +1                | healthy      | 6                          | +0.17                     |
|   | 9 ♀          | 43                              | +2                | healthy      | 6                          | +0.17                     |
|   | 12 ♀         | 43                              | +2                | healthy      | 6                          | +0.33                     |
|   | 15 ♀         | 38                              | +1                | healthy      | 6                          | +0.17                     |
|   | $\bar{x}$    | 42.3                            | +1.3              |              |                            | +0.21                     |
|   | s.d.         | 3.0                             | 0.5               |              |                            | 0.08                      |
| <u>Rat chow with 25% soybean trypsin inhibitor:</u> |              |                                 |                   |              |                            |                           |
|   | 37 ♂         | 33                              | 0                 | healthy      | 5                          | 0                         |
|   | 38 ♀         | 41                              | +1                | healthy      | 5                          | +0.20                     |
|   | 39 ♀         | 37                              | +1                | healthy      | 5                          | +0.20                     |
|   | 40 ♂         | 59                              | +1                | healthy      | 5                          | +0.20                     |
|   | $\bar{x}$    | 42.5                            | +0.8              |              |                            | +0.15                     |
|   | s.d.         | 11.5                            | 0.5               |              |                            | +0.10                     |

Table 2 (continued)

| Treatment   | Mouse number | Initial <sup>1</sup><br>weight (g) | Weight<br>change (g) | Final<br>Health    | Duration of<br>feeding (days) | Weight change<br>per day (g) |
|---|--------------|------------------------------------|----------------------|--------------------|-------------------------------|------------------------------|
| <u>Cooked black beans:</u>                                  |              |                                    |                      |                    |                               |                              |
|   | 51 ♀         | 39                                 | +1                   | healthy            | 6                             | +0.17                        |
|   | 53 ♀         | 45                                 | -1                   | healthy            | 5                             | -0.20                        |
|   | 54 ♀         | 25                                 | +4                   | healthy            | 5                             | +0.80                        |
|   | 58 ♀         | 31                                 | +1                   | healthy            | 5                             | +0.20                        |
|   | 59 ♂         | 54                                 | -3                   | healthy            | 5                             | -0.60                        |
|   | ̄X           | 38.8                               | +0.4                 |                    |                               | +0.07                        |
|   | s.d.         | 11.4                               | 2.6                  |                    |                               | 0.52                         |
| <u>Uncooked black beans:</u>                                |              |                                    |                      |                    |                               |                              |
|   | 1 ♀          | 46                                 | -7                   | dying <sup>2</sup> | 3                             | -2.33                        |
|   | 3 ♀          | 36                                 | -7                   | dying <sup>2</sup> | 3                             | -2.33                        |
|   | 5 ♀          | 41                                 | -6                   | dying <sup>2</sup> | 3                             | -2.00                        |
|   | 7 ♀          | 35                                 | -4                   | dying <sup>2</sup> | 3                             | -1.33                        |
|   | 50 ♀         | 44                                 | -12                  | dead               | 10                            | -1.20                        |
|   | 52 ♀         | 38                                 | -6                   | dead               | 3                             | -2.00                        |
|   | 55 ♀         | 40                                 | -3                   | dead               | 3                             | -1.00                        |
|   | 56 ♀         | 34                                 | -5                   | dead               | 3                             | -1.67                        |
|   | 57 ♀         | 35                                 | -4                   | dead               | 3                             | -1.33                        |
|   | ̄X           | 38.8                               | -6.0                 |                    |                               | -1.69                        |
|   | s.d.         | 4.3                                | 2.6                  |                    |                               | 0.50                         |
| <u>Animals that refused to eat toxic seeds:<sup>3</sup></u> |              |                                    |                      |                    |                               |                              |
|   | 24 ♀         | 48                                 | -15                  | dead               | 7                             | -2.14                        |
|   | 21 ♂         | 36                                 | -9                   | dead               | 4                             | -2.25                        |
|   | 27 ♀         | 46                                 | -10                  | dead               | 6                             | -1.67                        |
|   | 28 ♂         | 38                                 | -10                  | dead               | 7                             | -1.43                        |
|   | 29 ♀         | 38                                 | -9                   | dead               | 4                             | -2.25                        |
|   | 30 ♀         | 32                                 | -6                   | dead               | 2                             | -3.00                        |
|   | ̄X           | 39.7                               | -9.8                 |                    |                               | -2.12                        |
|   | s.d.         | 6.1                                | 2.9                  |                    |                               | 0.55                         |

<sup>1</sup> When a mouse appears more than once in this table, the first time its "initial weight" is the weight at the time of capture. The second time its "initial weight" is the weight in the laboratory following at least 5 days in the laboratory feeding on high quality food.

<sup>2</sup> These 4 mice would not have lived another 12 hours and had the usual traits of starving mice in their last few hours of life (severe shakes, closing eyes, poor coordination, little response to stimulation).

<sup>3</sup> Given the data available, I will assume that mice that voluntarily starve themselves to death die at the same rate as those deprived of all food.

This result is particularly striking because bruchids as a group are seed predators.<sup>16</sup> Black beans are cheap and easily available in Costa Rica. They also suffer no depredation by rats when stored in rat infested habitations.

When L. salvini were offered a diet consisting solely of black beans that had been boiled for one hour, they maintained their body weight and otherwise appeared quite healthy (Table 2). However, when given a pure diet of only uncooked black beans, L. salvini lost weight at a rate no different from that of those eating no food at all (Table 2;  $t_{13 \text{ d.f.}} = 1.54$ , n.s.). The one animal out of 9 that still appeared healthy after the third day survived for 10 days and probably was in exceptionally good condition at the beginning of the experiment. During the course of the experiment, the mice did eat small but highly variable amounts of uncooked black bean seeds. The mouse that lived so long (number 50 ♀, Table 2) ate roughly half the weight of uncooked black bean seeds per day as it would have were it maintaining its body weight on laboratory rat chow. It lost 27% of its body weight before death, which is nearly twice the loss these mice usually tolerate.

As a working hypothesis, I conclude that it is the lectin in the black beans that is killing the mice. The mode of action is probably the combined effect of direct starvation caused by food rejection (averaged over 34 mouse days, the bean consumption per day per mouse was 0.16 gram, and these mice require 2 to 4 grams per day of laboratory rat chow to maintain their body weight) and reduced nutrient uptake through the intestinal wall, as suggested by there being no conspicuous relationship between rate of weight loss and amount of bean eaten. While there are protease inhibitors in black beans, the impressive ability of L. salvini to live on food rich in soybean trypsin inhibitor and live on protease inhibitor-rich Enterolobium cyclocarpum seeds suggests that protease inhibitors are not the likely cause of black bean seed rejection by L. salvini. There are no known alkaloids or uncommon amino acids in commercial black beans, nor are there any other known potentially toxic molecules besides the proteins. The ultimate test of this working hypothesis depends on the availability of purified black bean and other lectins in 10 to 30 gram amounts. L. salvini is a very abundant seed predator in the forests it occupies. If it can be definitively shown that it cannot eat seeds rich in lectins, it can be stated with certainty that these seed lectins serve as a defense role against rodents irrespective of their other uses to the seedling. Lectins in the diet at naturally occurring concentrations can kill the larvae of a seed predator.

bruchid.<sup>1</sup> This demonstrates that seed lectins are functional as are alkaloids, uncommon amino acids, cyanogenic glycosides, etc. in defending seeds against insect seed predators.

If a dietary chemical is lethal to *L. salvini*, the situation is more than the mere act of hitting a naive gut with just any potentially nasty compound. This seed-eating specialist is unaffected by the very protease inhibitors that are a major reason for humans to boil or otherwise process seeds before eating them. For example, the seeds of *E. cyclocarpum* are lethal if they are the sole diet of *Sigmodon hispidus*, another terrestrial rodent in the same habitat (though *S. hispidus* does quite well on them if boiled).<sup>17</sup> *L. salvini* can live on a pure diet of HCN-rich *Phaseolus lunatus* seeds. *E. cyclocarpum* seeds are also rich in pipecolic acid and albizzine (E. A. Bell, personal communication), two uncommon amino acids with conspicuous insecticidal properties at the concentrations found in seeds.<sup>18,19</sup> *Sesbania emerus* seeds are rich in canavanine (G. A. Rosenthal, personal communication) yet eaten readily by *L. salvini*. This animal has a versatile gut yet there appears to be at least one lectin that it cannot handle.

#### WHAT PROCESSES EVOLUTIONARILY PUT LECTINS IN SEED CHEMICAL REPERTOIRES?

There are five traits of the system that are relevant this question:

- 1) The forest has many kinds of seeds, and the seeds of each species contain a unique combination of potentially defensive compounds.
- 2) The forest has many kinds of seed predators, each with the ability to ignore or detoxify some of these compounds, but not all.
- 3) Lectins are just one of the many protective traits a seed contains.
- 4) There are two different seed-predator responses to a seed, each likely to generate different traits in the defense array.

a) The rodent-type animals, such as Liomys salvini, try a newly encountered seed, and if it has the appropriate defenses, reject it. Rejection will depend as well on hunger, gut conditioning, body weight of the animal, alternate available foods, health and fat condition of the animal, perceptability of the defense compound, etc. The selective pressure favoring better-defended mutants is essentially constant over the years. This is because each year there are new recruits in the habitat that have to learn about the seeds of that habitat and season, and because there is a rodent-specific rate of forgetting that leads to re-sampling and relearning as each species of plant comes into seed again year after year. Since small rodents are very common, an unprotected mutant will be quickly located and its seed crop probably eliminated by the local set of mice. On the other hand, as selection for resistance traits occurs, the seed will have to be a consistently important part of the diet of the mouse for there to be such a strong selection that the mouse evolutionarily increases or shifts its detoxification abilities to encompass the change. The more usual rodent response to a mutant seed that is better defended should be to eat less of it. This system does not proceed to total inedibility for all seeds because the mice are also dispersal agents, defenses have economic costs to both parent and off-spring, plants and rodent densities fluctuate, rodent detoxification abilities do change, etc.

b) The insect-type animals oviposit on a particular species of host seed (or on its fruit), usually the only species of seed in the habitat that their larvae can eat.<sup>16</sup> Their larvae are specialists on the chemistry of that seed.<sup>20-22</sup> In addition, all mature and immature stages are behaviorally as well as physiologically programmed to deal with the host plant's other traits (e.g., timing of fruiting, fruit chemistry, odor cues for location, susceptibility to parasitoid attack while in that species of seed, etc.). While these animals may be very regular and deadly in killing their host's seeds, they pose no threat in contemporary time to nearly all other plant species present in the habitat, even if the larvae could develop in their seeds. There are undoubtedly many

other seed species in the habitat, which, when only seed chemistry is considered, could serve as hosts for a particular species of insect that does not, in fact, feed on them.

However, probing of other hosts does occur in evolutionary time, but rarely. A probe occurs when an ovipositing female makes an oviposition error or owing to a shortage of its regular hosts, oviposits on almost anything that contains even a fraction of the oviposition stimuli emitted by the usual host. Furthermore, in the latter case (and possibly the former) a successful probe may lead to a shift in the host seed species used, rather than a broadening of the host list. This is because it is likely that the beetle will have a higher fitness as a specialist on either one or the other hosts than as a generalist on both, since thorough bypassing of a plant's defenses often requires very fine tuning at the behavioral, morphological, physiological, biochemical, etc. level. Fine tuning may well be impossible owing to differences in timing of seed production, seed chemistry, fruit morphology, etc. If *L. salvini* or small rodents cannot handle lectins in general, this will select for strong convergence in lectin traits in the seeds these rodents confront; convergence will be on that molecule that works the best at an optimal cost for the genetic lineage of the parent and offspring that bear it.

It is the insect-type seed predator that should be responsible for much of the fine tuning of a lectin's traits, when that lectin is serving primarily as a defense compound in a seed. If each kind of lectin requires a different chemistry of detoxification by a host-specific insect, then a seed's lectin traits will be occasionally evolutionarily modified because the mutant repels a host-specific insect seed predator, just as is the case with alkaloids, uncommon amino acids, cyanogenic glycosides, etc. In contrast to the case where a group of seed predators (e.g., rodents) can bypass a class of compounds (e.g., *Liomys* versus protease inhibitors) or is repelled by a class of compounds (e.g., rodents versus lectins), each time a lectin's properties are changed it is a novel defense as seen by the host-specific insect. Here, then, natural selection will not result in convergence of traits among lectins, but rather in the continual appearance of

new types. There should even be active selection that results in divergence in lectin types because a mutant, that is a change in a direction already occupied by other lectins, is likely to become susceptible to the host-specific seed predators that can bypass those other lectins. Both diversification and divergence in lectin types should, however, approach an equilibrium level. The level should be determined in part by all those other ecological processes besides physiological seed availability that determine the numbers, kinds and diversity of seed predators in the habitat, and in part by the other non-predator-related selective pressures on lectins.

5) Like other compounds found in the seedling's bag lunch, lectins should be under strong selection to be of multiple use in this weight-, volume-, resource quality-limited container. The ideal combination of compounds in a seed is that which maximizes the fitness of that genetic lineage. Surely this will require a complex balance of

- a) partitioning of parental resources among the seeds (seed size, weight, number, etc.),
- b) seed photosynthesis and therefore contribution to its own resources, and
- c) seed resources for seedling growth and protection against herbivores (probably no molecule is ideal for both functions).

Specifically, lectins will be the focus of selective pressures associated with

- a) their use as glue in development, such as in the attachment of symbiotic bacteria to roots or in the binding of different cells within the organism,
- b) their degradative destruction as an amino acid and small polypeptide source in seedling metabolism,
- c) their use as protective compounds in the cotyledons and in newly produced vegetative tissues (deterring contemporary herbivory by generalists and evolution of herbivory by specialists).

In some plants, past selection will have been such that lectins were never functional as anything more than one kind of glue, a glue of importance only in some very internal biochemical sense. Here, then, other compounds are the defenses and storage compounds in the seeds. But somewhere early in the dim history of legume seeds, there was a combination of herbivore susceptibility and plant lectin synthesis capacity that led to lectin-rich seeds becoming an integral part of the character of a 'successful' species of plant which then radiated in various ways to give us many species of lectin-rich legume seeds. Alternatively, one may hypothesize that this even occurred many times, owing to the general presence of the lectin-synthesis ability of legumes, which in turn pre-disposed them to selection by herbivores that got bad stomach aches from diets rich in protein glues. One cannot chose between these two historical scenarios with the data at hand, but it is obvious what sort of amino acid sequence studies are needed to distinguish among them. They are also not mutually incompatible. Owing to the chemical complexity of proteins, as contrasted with small defensive molecules like alkaloids and uncommon amino acids which can have absolutely convergent end products in their synthesis by different plants, the history of a protein molecule is to some more extreme degreee incorporated in its structure. The question has become not 'Is a lectin for defense?', but rather 'What are the various ways that sticky glyco-proteins are functional?' 'What selects for their detailed traits?' and 'What selects for deposition in certain plant parts in sufficient bulk to give a herbivore a gut ache?', bearing in mind that herbivores come in widely differing sizes, food consumption rates and intensity of desire to eat a particular plant part.

#### WHY ARE LECTINS SO PROMINENT IN SEEDS AND TUBERS?

Assuming that the relatively high concentrations of kinds and amounts of lectins in seeds and tubers is not a sampling artifact, there are several plausible ecological reasons why this severe distributional heterogeneity should occur.

Dilution. Many species of animals that commit severe seed predation on mature seeds are sufficiently specialized on this diet that they eat almost no other food for all or

much of their lives. This means that the contents of the seed are likely to be all or nearly all of their stomach and intestinal contents at a given time. That is to say, whatever is in the seed runs little risk of being diluted out in a larger bulk of different food (as occurs, for example, when a horse digests some of the hard lectin-rich legume seeds it swallows along with leafy feeds). On the other hand, seed contents are very concentrated nutrients, and compared to a foliage-eating animal, a seed-eater consumes a minuscule amount of food. For example, a bruchid beetle larva in a legume seed may only consume twice its last instar body volume in seed contents during its entire development, while a moth caterpillar eating leaves may consume its own volume of food during every 24 hours of active growth. In short, the amount of lectin required to maintain a 5% lectin titer in a seed-eater's gut is easily only 1 to 5% of that which would be required for the same effect in a foliage-eater's gut of the same body weight. That is to say, an expensive digestion inhibitor like a lectin or protease inhibitor may be economically most appropriate for a seed while the much cheaper (per gram) polyphenol digestion inhibitors are most appropriate for foliage. While both may be found in each type of plant part, here I am discussing the forces that keep the disproportionalities in the system.

Bag lunch. Seeds and tubers are storage devices, and in the case of the former, volume- and weight-conscious ones. A lectin (as well as a protease inhibitor) may double as a polypeptide and amino acid storage unit (and of course may even have its evolutionary roots in an innocuous storage protein). However, to the degree that only innocuous storage proteins are found in more vegetative plant parts, we are then again left with the disproportionality question. Furthermore, while it is all very well to beat your sword into a ploughshare, it may be best to put it into the closet and buy a plough when the war is over, because wars have a way of reappearing each generation. Finally, to give you a very high quality sword, your mother may well have used such high quality steel that it makes a lousy ploughshare.

Fitness. Finally, and most definitely not least, there is the simple answer that gram for gram, seeds and tubers have the highest fitness value of any plant part.

From the viewpoint of the juvenile in the seed, the seed is the essence of fitness. For the parent plant, the seeds are one of its throws of the dice to remain in a surviving lineage. Seeds are probably the most thoroughly protected of all plant parts and part of that thoroughness is achieved by containing quite physiologically active compounds of many kinds in high concentrations. Virtually all seeds contain at least 1 potent digestion inhibitor and 2 to 5 small molecules (cyanogenic glycosides, alkaloids, cardiac glycosides, uncommon amino acids, cyanolipids, etc.)<sup>23</sup> unless they are involved in some sort of population-level seed predator satiation (e.g., as in conifers, oaks, bamboos, niloo, chestnuts, dipterocarps, etc.),<sup>24-26</sup> physical protection (hard nuts), or very small size (many herbs). Our own village histories undoubtedly taught us this. Seeds and tubers are where the goodies are, but you cannot eat them unless either you process them (cook them, break them, dig them up, breed out their defenses) or they are so chemically defenseless as to be eaten by the bulk of the herbivorous animals in the habitat. The same ecological story applies to the presence of protease inhibitors and largely indigestible starches and other complex sugars in seeds and tubers, but I shall let that lie as it is not the subject of this symposium. On the other hand, it may be noted that the intensity of protection required is related not only to the value to the owner (mother and seed), but the value to the thief. Seeds and tubers contain the highest concentrations of animal nutrients in the plant world, and often occur at a density in time and space quite high enough to support many species of herbivores for much if not all of the year or generation. Such an array of barbarian hordes cannot be kept at bay with a few ditches and spears.

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## Chapter Eleven

### CELL INTERACTIONS AND PATTERN FORMATION IN *DICTYOSTELIUM DISCOIDEUM*

DANIEL McMAHON

Program in Genetics and Department of Zoology  
Washington State University  
Pullman, Washington 99164

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#### INTRODUCTION

The panoply of forms of plants and animals is as amazing as is the reproducibility with which each form is produced within a given species of plant or animal. The reproducibility is determined by a molecular mechanism through which cells in the organism determine their relative position. This process leads to the pattern of spots on the frog, stripes on a tiger, and the precise interconnections which occur between the ganglion cells of retina and their target neurons in the optic tectum of the frog.<sup>1</sup> In the cellular slime mold, *Dictyostelium discoideum*, the process of cellular position determination occurs in the pseudoplasmodium and leads to two types of cells: stalk cells and spore cells. Approximately 1/3 of the cells in the pseudoplasmodium become stalk cells; most of the remaining two-thirds of the cells become spores.<sup>2</sup> The proportioning is largely invariant over a wide variety of experimental conditions and over an approximately one hundred thousand fold difference in number of cells in the pseudoplasmodium.

While the developmental cycle of D. discoideum is rapidly becoming common knowledge among biologists, I can briefly summarize the major cellular events which occur during development. Unicellular amoebae reproduce asexually under favorable environmental conditions, including the presence of ample supplies of food. If the amoebae begin to starve, they become chemotactic to cyclic AMP and also begin to emit pulses of cyclic AMP. This behavior causes groups of amoebae to aggregate together. Their aggregation is facilitated by an increase in intracellular adhesivity which occurs at the same time. The resulting aggregate may be composed of up to one hundred thousand cells. The cells of the aggregate, under appropriate environmental conditions, form a migrating, slug shaped pseudoplasmodium. At this stage, the cells of the pseudoplasmodium determine their relative position and use this information to determine their direction of their differentiation. This fact was elegantly demonstrated by Kenneth Raper.<sup>3</sup> In his experiments, reciprocal grafts were made between pseudoplasmodia which had been produced from cells which had fed on colorless bacteria or on the red bacterium, Serratia marcescens. If a tip was taken from a red pseudoplasmodium and grafted onto a colorless pseudoplasmodium from which the tip had been removed, the stalk of the resulting sorocarp was colored red and the spores were uncolored. In the reciprocal graft, spores were colored red, and the stalk was uncolored. This experiment helped demonstrate that the cells of the pseudoplasmodium become predestined as a function of their position in the pseudoplasmodium.

A substantial number of experiments have shown that detectable position-dependent biochemical changes occur in the cells of the pseudoplasmodium. These include histochemical differences between the cells at the front and the cells at the rear. The content of periodic acid-Schiff's staining carbohydrates, alkaline phosphatase, and glycogen phosphorylase differ substantially between cells of the front and the back.<sup>4, 5, 6</sup>

#### THE CELL CONTACT MODEL

A number of molecular theories for the mechanism by which cells might determine their position in a morphogenetic field, such as the pseudoplasmodium, have been

advanced. These have been reviewed elsewhere.<sup>7</sup> I have proposed a theory which suggests that this mechanism is mediated by complimentary molecular contacts between molecules on the plasma membranes of adjacent cells.<sup>8</sup>

Propositions contained in this model are the following:

1. Cells have complimentary contact-sensing molecules on their cell surface.
2. These molecules, when activated by contact, have complimentary effects on the intracellular concentration of a morphogen, such as cyclic AMP, which regulates metabolism and gene action.
3. The effective "concentration" of these molecules on the cell surface is regulated by negative feedback (i.e., as the concentration of intracellular morphogen (e.g., cAMP) rises within the responding cell, the system which increases cyclic AMP is inactivated, whereas the system which degrades cyclic AMP is activated).
4. These molecules have a polarized distribution on the cell surface.

A schematic diagram which illustrates the operation of this model is presented in Figure 1. In Figure 1, the top row of six boxes represents six cells in a morphogenetic field. Each of the cells is identical both in concentration of intracellular morphogen as denoted by capital A and in their display of cell surface molecules as denoted by F and R. A simple physical fact is responsible for the initiation and propagation of changes in surface display. In each line of cells, the front cell has no cell in front of it and the rear cell has no cell behind it. Assuming that the contact-sensing molecules on the rear of the cell, designated by R in this figure, increase the intracellular concentration of cyclic AMP, and the contact-sensing molecules on the front of the cell, designated by F in this figure, decrease the concentration of cyclic AMP, a propagating change in intracellular cyclic AMP concentration occurs. The first cell in line has R molecules activated by contact with the second cell. Its F is not activated by contact since no cell precedes it. The system which

increases the concentration of cyclic AMP is activated. The system which degrades it is not. Therefore, the intracellular concentration of cyclic A increases within the cell. As a consequence, the number (or activity) of F's is increased, whereas the number (or activity) of R's is decreased. As the process proceeds, the second cell in line becomes functionally equivalent to the first cell in line (i.e., the R molecules on the cell preceding it disappear). Each row of cells in Figure 1 illustrates the progression of this process with time. The final result is a group of cells, as illustrated in the bottom row, in which the front cells have large intracellular concentrations of A, whereas the rear cells have small intracellular

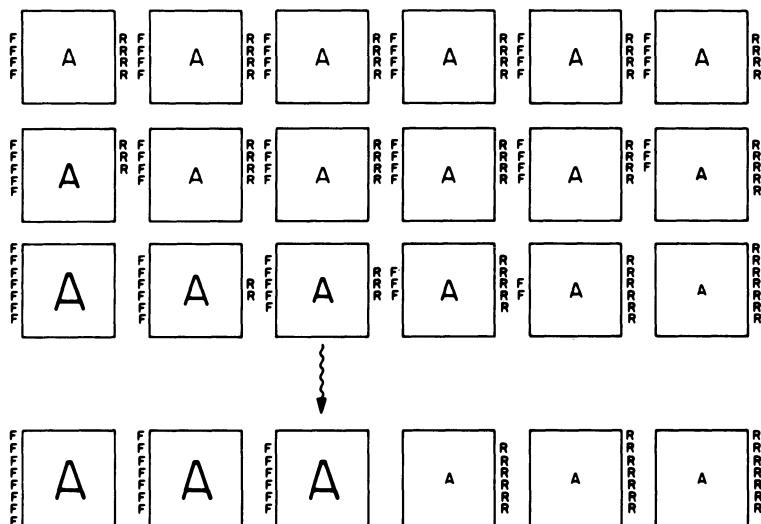


Figure 1. Schematic description of the cell contact model. Each row of boxes in this diagram represents a row of cells in a morphogenetic field. The letters represent: A, the concentration of intracellular morphogen; F, the contact-sensing molecules on the front of a cell; R, the contact-sensing molecules on the rear of a cell.

concentrations of A. In addition, the front cells have on their surface a substantial number of F molecules and lack R molecules. The reverse is true of the cells in the rear.

This model can be represented by simple differential equations and these equations were solved<sup>8</sup> on the digital computer, numerically, using the Runge-Kutta method. The result of this numerical simulation of this system led to results which agree with those illustrated in Figure 1. It predicted that a large intracellular concentration of cyclic AMP should be present in the cells which are destined to become stalk cells and that a sharp boundary should separate this from the cells destined to become spore cells, which would contain low intracellular concentrations of cyclic AMP.

#### TESTS OF THE MODEL

A number of predictions arise from the cell contact model. First, the model predicts that a discontinuous gradient of intracellular cyclic AMP should be produced as described above. Second, the model suggested that cyclic AMP was a mediator of the cytodifferentiation of the stalk cell. Third, the model indicates that a continuous chain of normally reacting cells is necessary for the propagation of information in a morphogenetic field. Fourth, the model suggests that intracellular signaling in the morphogenetic field is mediated by molecules found on the plasma membrane of the developing cells. Fifth, it suggested that these contact-sensing molecules should become regionally localized on cells of the pseudoplasmodium as a function of their position.

The first proposition of the model, i.e., that cyclic AMP would show an unusual distribution in cells of the pseudoplasmodium was tested by Pan et al.<sup>9</sup> They used an antiserum which had been raised against cyclic AMP. It was made fluorescent by conjugation with fluorescein isothiocyanate. Cells and sections of pseudoplasmodium were stained with the serum. These experiments demonstrated that a discontinuous gradient of cyclic AMP develops in the pseudoplasmodium. As predicted by the model, this histochemical method demonstrates that the cells of the front have high intracellular concentration of cyclic AMP and a sharp discontinuity separates these from the cells

of the rear with much lower concentrations of cyclic AMP. Experiments by others<sup>10,11</sup> indicate that there is a 50% difference in intracellular cyclic AMP that can be detected between the cells of the front and the rear of the pseudoplasmodium by radioimmunoassay. We (Goldberg and McMahon, unpublished results) have looked at the content of cyclic AMP in cells of the stalk and in spore cells at the time the pseudoplasmodium completes culmination, also by radioimmuno-assay. The stalk cells contain 10 times as much cyclic AMP as the spore cells.

Klaus and George<sup>12</sup> have examined the necessity for a continuous chain of viable cells for the normal propagation of information in the pseudoplasmodium of D. discoideum. They irradiated a pseudoplasmodium with a microbeam laser to kill small groups of cells within the pseudoplasmodium. When this was done, the pseudoplasmodium frequently fragmented into two pseudoplasmodia. They concluded that the results of their experiments agreed with the predictions of the model.

We have examined the ability of purified plasma membranes to interact with living cells during morphogenesis biochemical differentiation. Plasma membranes were purified by the method of McMahon et al.<sup>13</sup> from cells at various stages of development. Pseudoplasmodial cells were dissociated by trituration and plated on Millipore filters in the presence or absence of added plasma membranes. These experiments yielded a number of interesting results<sup>14</sup> (McMahon, unpublished results). Morphogenesis of the dissociated cells is prevented by added plasma membranes. Untreated cells are able to reform pseudoplasmodia within four hours after being placed on a Millipore filter and to culminate within six hours. Cells treated with plasma membrane were able to reaggregate but morphogenesis was disrupted at this point.

When dissociated pseudoplasmodial cells were plated on Millipore filters, the developmentally-regulated enzyme, alkaline phosphatase, increases in activity four hours after replating the cells; reaches a maximum of activity after approximately eight hours; then proceeds to decline in activity. If cells are plated on the filter in the presence of plasma membranes purified from pseudoplasmodial cells, this enzyme is superinduced. The superinduced level

of this activity is maintained at least throughout the next twelve hours.<sup>14</sup>

We investigated several characteristics of this superinduction of alkaline phosphatase activity by membranes. These experiments provided the following results: first, when extracts of membranes are mixed with extracts of cells *in vitro*, the enzyme is not activated; second, the effect required contact between the cells and the plasma membranes (it is not exhibited if the cells are separated from plasma membranes by a Millipore filter with a pore-size of 0.45 microns); third, the effect is developmentally specific. If vegetative amoebae are treated with pseudoplasmodial plasma membranes, the amoebae do not show an increase in alkaline phosphatase activity. On the other hand, plasma membranes prepared from vegetative amoebae are several times less active on a protein basis than pseudoplasmodial cell membranes in causing the superinduction of alkaline phosphatase when pseudoplasmodial cells are treated with them.

Treatment of the membranes with Pronase under conditions which degrade the major polypeptides of the plasma membrane but have little effect on the size of the glycoproteins do not destroy the activity of the membranes in this assay, whereas mild periodate oxidation of the plasma membrane does (McMahon, unpublished results). Since pure plasma membranes can be isolated which contain little contamination except the small percentage contamination with the inner membrane of the mitochondria,<sup>13,15</sup> these effects appeared to be caused by a component of the plasma membrane.

#### MACROMOLECULAR COMPOSITION OF THE PLASMA MEMBRANE

To examine the chemical and topographical changes which occur in the macromolecular components of the plasma membrane during development, we have examined the composition of the plasma membrane on SDS gels at various stages of development. Vegetative amoebae, aggregating amoebae, and pseudoplasmodial cells have been compared. The plasma membrane of the vegetative amoebae contained 55 major polypeptides. 50% of these change amount during development. In order to determine whether any of the polypeptides of the external surface of the plasma membrane change in surface exposure during development, cells were treated with

the proteolytic enzyme, Pronase, to degrade any exposed polypeptide chains. Plasma membranes were then isolated from these cells. These experiments indicated that approximately 10% of the plasma membrane polypeptides change in surface exposure during development.<sup>16,17</sup> When SDS gels were stained for carbohydrate with the periodic acid-Schiff's reagent, approximately 25 major glycoproteins were identified in the plasma membrane of vegetative amoebae and these were completely replaced in the course of development of vegetative cells to pseudoplasmodial cells. It is difficult to tell if any of these molecules change in surface exposure during development since the glycoproteins of Dictyostelium plasma membrane are quite resistant to Pronase treatment, either of the intact cell or the isolated membranes. If they are treated with Pronase in the presence of detergents which disrupt the structure of the plasma membrane, they can be digested by Pronase. The changes in membrane composition are under developmental control and do not result simply from starvation.<sup>18 19</sup>

Experiments described above suggested that carbohydrate containing components of the plasma membrane are important in development, and initial experiments analyzing the macromolecular composition of the plasma membrane indicated that the most dramatic changes in its macromolecular composition occurred in its glycoproteins during development.<sup>16</sup> We have developed procedures to examine the nature and topography of plasma membrane glycoproteins with more precision. In order to identify and analyze the glycoproteins, we have developed a technique<sup>15,19,20</sup> with which glycoproteins can be identified with great sensitivity after resolution by gel electrophoresis. Using this procedure, gels are fixed with glutaraldehyde to cross-link and immobilize bands of proteins. The glutaraldehyde is washed out, free carbonyl groups are reduced by treatment by sodium borohydride and finally fluorescent lectin is diffused into the gel in order to bind to the lectin receptor which has been immobilized in the gel. Finally, unbound fluorescent lectins is diffused from the gel. This procedure can be used on one-dimensional SDS or isoelectric focusing gels or on two-dimensional gels. Its analytical resolution is limited only by the resolution of the resolving gel system. Figure 2 represents an experiment using this technique in which purified plasma membranes from vegetative amoebae, aggregation phase cells and

pseudoplasmodium cells were analyzed by SDS gel electrophoresis. They were then stained for protein with Coomassie Blue or with fluorescent wheat germ agglutinin, N-acetyl-chitopentose. The three gels on the left of this figure have on them (from left to right) plasma membranes from vegetative amoebae, aggregating cells and preculmination cells stained for protein. The three gels in the middle present respectively vegetative amoebae, aggregating cells and preculmination cells stained with fluorescent wheat germ agglutinin. The three gels on the right of this figure represent equivalent gels stained with fluorescent wheat germ agglutinin in the presence of the hapten inhibitor. This panel illustrates that dramatic changes occur in the composition of wheat germ agglutinin receptors on the plasma membrane of *D. discoideum* during the development of the cell from a vegetative amoeba to a pseudoplasmodial cell. All of the wheat germ agglutinin receptors, except the diffuse band ranging in molecular weight from approximately 30 to 60 kilodaltons are glycoproteins. This is indicated by their sensitivity to digestion by Pronase in the presence of SDS.<sup>20</sup> When similar gels were stained with a variety of other lectins, only receptors for the lectin Concanavalin A could be demonstrated in Dictyoselium plasma membranes.

Lectins which bind to galactose, N-acetyl-galactosamine, and fucose bound receptors in plasma membranes that were purified from human erythrocytes. There were no receptors for these lectins in *D. discoideum* plasma membranes.<sup>20</sup> The results of these experiments correspond in general with what would be expected from analyses of the sugar composition of the plasma membranes from *D. discoideum*. Only mannose, glucose, glucosamine and fucose were detected in Dictyostelium discoideum plasma membrane. No galactose, galactosamine or sialic acid could be detected.<sup>19</sup> The absence of receptors for fucose-binding lectins may indicate that the fucose presence in *D. discoideum* plasma membrane is present in a steric conformation which does not allow interaction with fucose-binding lectins.

In summary, the fluorescent lectin-binding assay demonstrated that there are approximately 35 Concanavalin A receptors in the plasma membrane of vegetative amoebae and that two-thirds of these change in amount during differentiation, preculmination phase cells. All of the 25 wheat

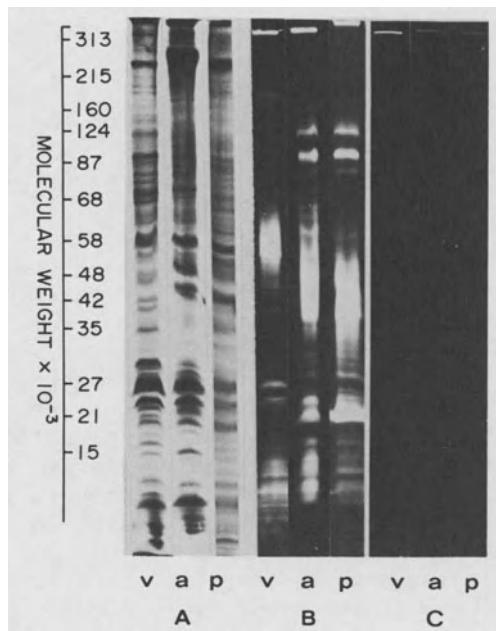


Figure 2. Wheat germ agglutinin receptors on plasma membranes of *Dictyostelium discoideum*. Plasma membrane proteins and glycoproteins were resolved by SDS gel electrophoresis and stained with Coomassie Blue (left 3 gels); fluorescent wheat germ agglutinin (central 3 gels); fluorescent wheat germ agglutinin plus N-acetyl-chitopentose (right 3 gels). Each group of 3 gels present from left to right plasma membranes from vegetative amoebae, aggregation phase cells, and pseudoplasmodium phase cells.

germ agglutinin receptors present in plasma membrane of vegetative amoebae disappear or are modified during the same period of development.

#### TOPOGRAPHY OF LECTIN RECEPTORS

Analysis of the topography of lectin receptors on the plasma membrane of intact cells using microspheres conjugated to lectins, provided the following results. All of the lectin receptors of the plasma membrane appear to be mobile in the plane of the plasma membrane. Both

wheat germ agglutinin receptors and Concanavalin A receptors appear to uniformly cover the surface of the cell at vegetative and aggregation stages of development. At pseudoplasmodial stage there appear to be patches of plasma membrane in which Concanavalin A receptors are either absent or inaccessible for binding to the Concanavalin A conjugated microspheres.<sup>21</sup>

Finally, we have used the sensitivity of the fluorescent lectin-binding assay to examine whether cells isolated from different positions along the longitudinal axis of the pseudoplasmodium differ in the macromolecular composition of their plasma membranes. In these experiments, pseudoplasmodia were dissected into four pieces and cells from the anterior piece, which are destined to become stalk cells, were compared with cells from a posterior piece, which are destined to become spore cells. These experiments showed that five lectin receptors for wheat germ agglutinin were present in distinctly different amounts on the plasma membrane from the two populations of cells, and that there were quantitative differences in the distribution of several Concanavalin A receptors. By using an antibody specific for pseudoplasmodial antigen, we could show that this antigen was found only in the cell from the anterior portion of the pseudoplasmodium, those cells destined to become stalk cells.<sup>22</sup>

#### SUMMARY

In summary, Dictyostelium discoideum provides in microscale an illustration of the process of cellular position determination which is so important in the development of both plants and animals. We have proposed a molecular model for the mechanism by which this process may operate. It has been tested in several laboratories and the results agree with the model's predictions. Finally, it has been possible to demonstrate that during development of such a simple organism as the cellular slime mold, unexpectedly substantial changes occur in the molecular composition of its plasma membrane. These changes are most obvious in the composition of the glycoproteins on the cell surface. These results are in general accord with the proposition that glycoproteins may be important determinants in cellular interaction and development.<sup>23</sup>

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**LIPID  
BIOCHEMISTRY  
of Fungi and Other Organisms**

**John D. Weete**

*Auburn University*

with contributions by

**Darrell J. Weber**

*Brigham Young University*

A comprehensive review of the chemistry, distribution, function, and metabolism of lipids in fungi, *Lipid Biochemistry of Fungi and Other Organisms* explores the current state of knowledge in lipid biochemistry, with emphasis on fungi. The book also examines relevant information on other living organisms. Differences between fungi and other organisms can thus be readily noted, and gaps in the current knowledge of lipids as they relate to fungi can be filled in.

The first chapter gives a definition of lipids, presents a scheme for lipid classification, and includes a brief history of lipid research. The second chapter focuses on the total amount of lipids in numerous species representing the major fungal taxa; in various vegetative and reproductive structures, such as spores; and in subcellular structures, such as membranes and cell walls. The various factors that influence the production of lipids are also described in this chapter. Subsequent chapters are concerned with specific types of lipids, including fatty acids, neutral glycerides, phospholipids, sphingolipids, aliphatic hydrocarbons, sterols, carotenoids, and polypropenols. Each chapter examines the nomenclature of the subject lipid, its distribution in fungi, and the pathways of its metabolism. The final chapters outline the metabolism of lipids during fungal spore germination and formation.

The areas covered are thoroughly reviewed, and students, teachers, and researchers in the fields of chemistry, biochemistry, mycology, physiology, pharmacy, and botany will find the book a useful reference.

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