



Alpha-galactoside-binding proteins from plant membranes : distribution, function, and relation to helminthosporoside-binding proteins of sugarcane
by Douglas Sherwood Kenfield

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Plant Pathology
Montana State University
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Abstract:

The critical, specific reaction during the molecular interplay between *Helminthosporium sacchari* and *Saccharum of-ficinarum*[^] is the binding of helminthosporoside by a toxin receptor in the plasmalemmas of susceptible sugarcane. Because the toxin is an alpha-galactoside and other alpha-ga-lactosides compete with the toxin for receptor sites, the normal role of toxin receptors in plants may be as alpha-galactoside transporters. If so, similar proteins should exist elsewhere in the plant kingdom. A survey of nine plants revealed alpha-galactoside binding in trichloroace-tate extracts of cellular membranes from each. Raffinose, galactinol, and helminthosporoside binding occurred in extracts from susceptible sugarcane, mint, tobacco, wheat, barley, and beet. Extracts from resistant sugarcane, corn, and potato displayed raffinose and galactinol binding only.

Binding proteins from susceptible and resistant sugarcane, mint, and tobacco were purified via melibiose-Sepha-rose-6B affinity chromatography. All four proteins had a molecular weight of about 100,000 and displayed subunit species of about 50,000 and 14,000-20,000. Amino acid compositions were similar, but not identical. The protein from resistant sugarcane had less mobility than the other three during polyacrylamide gel electrophoresis,. At high protein concentrations (100 ug/ml), resistant cane and mint proteins did not bind helminthosporoside: susceptible sugarcane and tobacco proteins did, At low protein concentrations (4 ug/ml), all four proteins yielded Kd's of about 10-5M for raffinose, galactinol, and helminthosporoside.

The inverse correlation of binding activity with protein concentration was a logarithmic relationship for each protein-toxin combination. Presence of high molecular weight polymers was observed and the oligomer/polymer ratio was also found to be an inverse logarithmic function of protein concentration. In aqueous solutions, then, self-association of binding proteins is dependent on protein concentration and presence of ligand. Polymers are favored by high protein concentrations: oligomers are stabilized by ligand.

Differential toxin binding by receptors from susceptible and resistant sugarcane required the presence of integral membrane components or exogenous galactolipids. Also, sensitivity to helminthosporoside increased in older portions of susceptible leaves. In vivo concentrations of receptors and composition of the membrane milieu likely modulate expression of the potential for toxin binding inherent in proteins from both susceptible and resistant sugarcane.

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ALPHA-GALACTOSIDE-BINDING PROTEINS FROM PLANT MEMBRANES:
DISTRIBUTION, FUNCTION, AND RELATION TO HELMINTHOSPOROSIDE-
BINDING PROTEINS OF SUGARCANE

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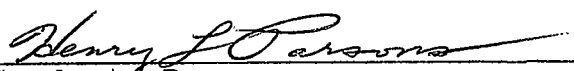
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ABSTRACT

The critical, specific reaction during the molecular interplay between Helminthosporium sacchari and Saccharium officinarum is the binding of helminthosporoside by a toxin receptor in the plasmalemmas of susceptible sugarcane. Because the toxin is an alpha-galactoside and other alpha-galactosides compete with the toxin for receptor sites, the normal role of toxin receptors in plants may be as alpha-galactoside transporters. If so, similar proteins should exist elsewhere in the plant kingdom. A survey of nine plants revealed alpha-galactoside binding in trichloroacetate extracts of cellular membranes from each. Raffinose, galactinol, and helminthosporoside binding occurred in extracts from susceptible sugarcane, mint, tobacco, wheat, barley, and beet. Extracts from resistant sugarcane, corn, and potato displayed raffinose and galactinol binding only.

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Differential toxin binding by receptors from susceptible and resistant sugarcane required the presence of integral membrane components or exogenous galactolipids. Also, sensitivity to helminthosporoside increased in older portions of susceptible leaves. In vivo concentrations of receptors and composition of the membrane milieu likely modulate expression of the potential for toxin binding inherent in proteins from both susceptible and resistant sugarcane.

INTRODUCTION

Nearly every carbon atom in heterotrophs first exists as part of a sugar molecule in a plant. This fact marks the importance of expanding our knowledge of carbohydrate synthesis, translocation, and utilization if, in a melioristic sense, we, as plant scientists, hope to enhance the efficiency of food production.

Sucrose is by far the most common sugar in most plants. The alpha-galactosyl derivatives of sucrose, the raffinose family of carbohydrates, comprise the second-most prevalent group of sugars (40). Major enzymes which accomplish the biosynthesis of the most common alpha-galactosides have been identified (37). In certain plants (squash, white ash, and members of the Labiatae) galactinol, raffinose, and stachyose are known to be among the first products of photosynthesis where raffinose and stachyose predominate among translocated carbohydrates (8, 25, 53, 77). More recently, chloroplasts from peas have been shown to contain the enzymes necessary for the synthesis of myo-inositol, galactinol, and raffinose (28, 29).

Of interest now is an elaboration of the mechanisms by which these sugars are delivered out of the synthesizing cells and into the translocation system of the phloem. Few types of carbohydrates are translocated, the major ones

being sucrose, the raffinose family, and the alditols mannitol and sorbitol (77). Geiger has discussed in detail certain mechanisms which may account for this relative selectivity during phloem loading (19). One postulated mechanism entails mediation of solute transport by selective carrier systems in the membranes of phloem cells. Isolation and characterization of such selective carriers are the next logical projects, but before pursuing this course, consideration of a related mechanism of pathogenesis will be enlightening.

The eyespot disease of sugarcane is caused by Helminthosporium sacchari (van Breda de Haan) Butl. (= Drechslera sacchari (Butl.) Subram. & Jain). The pathogenicity of this fungus is dependent upon its ability to produce helminthosporoside, an alpha-galactoside with the proposed structure 2-hydroxycyclopropyl-alpha-D-galactopyranoside (57, Figure 1). Sensitivity to the toxin and susceptibility to the fungus are strongly correlated with the presence of a functional toxin-binding protein in the plasma membranes of host cells (61). After extraction with Triton X-100 from membranes of a susceptible clone of sugarcane (51NG97), the binding protein appears to be an oligomeric protein with a molecular weight of 49,000, containing four

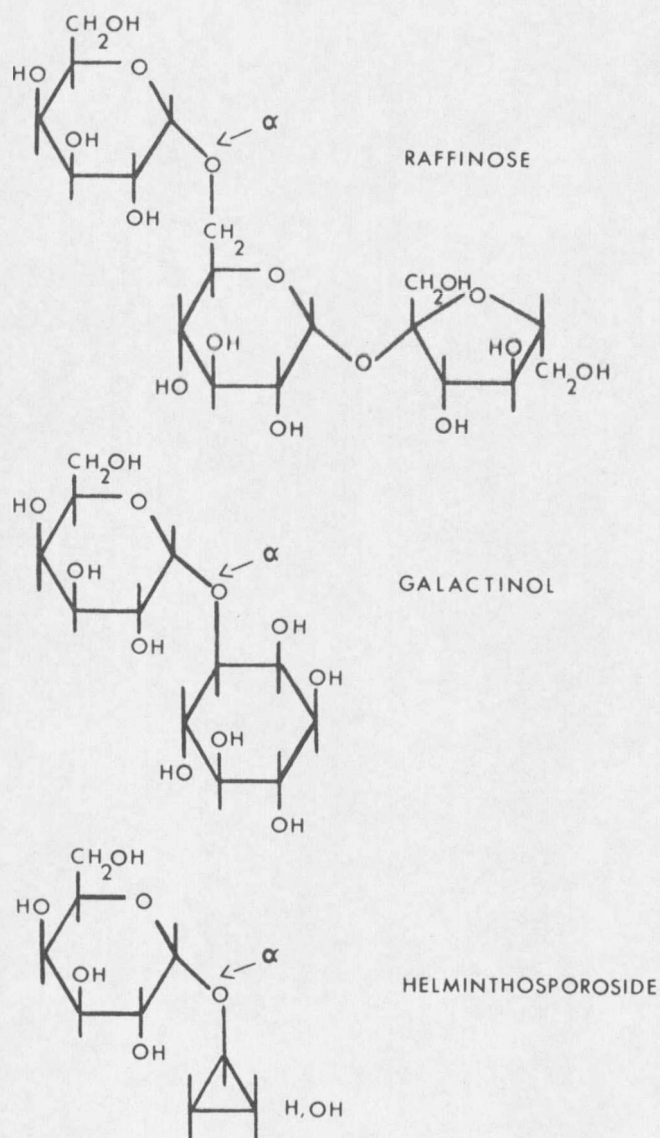


Figure 1. Illustration of raffinose and galactinol, common alpha-galactosides from higher plants, and helminthosporoside, a host-specific toxin produced by *Helminthosporium sacchari* (57). The aglycone of helminthosporoside is best represented as $\alpha\text{-C}_3\text{H}_5\text{O}$ at this time (Strobel-personal communication).

subunits and at least two binding sites (60). A resistant clone, H50-7209, contains a protein of the same molecular weight and subunit composition yet different from the 51NG97 protein in antigenicity, amino acid composition, and electrophoretic mobility. Further, freshly isolated H50-7209 protein lacks detectable toxin-binding activity just as protoplasts from resistant sugarcane do not exhibit a net uptake of helminthosporoside or raffinose (62). After three weeks in Triton X-100 or after treatment with sodium dodecyl sulfate (SDS), the detergent-solubilized protein from H50-7209 acquires the ability to bind toxin in vitro (61).

Based on these and other extensive observations, a molecular model of pathogenesis for the eyespot disease has been proposed (65). Briefly, the fungus infects susceptible hosts and produces at least one toxin, helminthosporoside. Helminthosporoside binds to sensitive proteins in plasmalemmas causing a perturbation of membrane constituents. Subsequently, ionic imbalance in the cells occurs; the cells swell and eventually burst. These events culminate in the leaves as water-soaked streaks which develop into reddish runners, characteristic symptoms of eyespot.

Many modes of resistance to H. sacchari are possible. The fungus's ability to produce helminthosporoside is controlled by the availability of either serinol or a "high-molecular-weight inducer," the syntheses of which occur in the host (5, 46). Potential hosts may prevent pathogenesis passively through a lack of or subminimal production of either or both of these inducers. In contrast to such passive resistance, sugarcane may elaborate inhibitors of helminthosporoside synthesis. To date, experimental evidence for this type of resistance is lacking. Alternatively, alpha-galactosides are known to protect leaves from the detrimental effects of the toxin (60), thus it is possible that sufficiently high levels of indigenous alpha-galactosides confer resistance. Another mode of resistance arises from the presence of toxin receptors which are modified such that, in situ, they express a diminished affinity for the toxin, even to the point of lacking toxin-binding ability altogether. This last example appears to be represented by the proteins from 51NG97 and H50-7209. Any, some, or all of these mechanisms of resistance could be operative in particular clones and confer to them high, intermediate, or low phenotypes relative to susceptibility to H. sacchari.

In the presence of helminthosporoside, however, the crucial step, the reaction which determines the possibility of subsequent processes leading to cellular death, is the binding of helminthosporoside to a toxin receptor in the plasma membrane. If binding is blocked, pathogenesis is precluded.

Certain ideas in the foregoing discussion are important in terms of relating this type of pathogenesis to carbohydrate transport. First, alpha-galactosides effectively inhibit activity of the toxin in leaves. Further, Triton X-100-solubilized proteins from 51NG97 exhibit similar K_d 's for helminthosporoside, raffinose, and melibiose. Comparable kinetics are obtained for raffinose binding by isolated proteins and raffinose uptake by sugarcane protoplasts. Furthermore, pretreatment of protoplasts from susceptible sugarcane with helminthosporoside blocks uptake of raffinose. These results support the hypothesis that the helminthosporoside-binding protein is part of an alpha-galactoside transport system normally present in sugarcane cells (63). As noted in the original presentation of this hypothesis, an anomaly occurs in H50-7209, resistant sugarcane, whose receptor lacks detectable raffinose- and toxin-binding activities. If alpha-galactoside transport is a

normal process in the translocation of carbon skeletons in sugarcane, how does H50-7209 compensate for the apparent lack of this process? Abstractly related to this question, consideration of the probability of alpha-galactoside transport in sugarcane and the prevalence of alpha-galactosides in the plant kingdom leads to the expectation that toxin-binding (= alpha-galactoside-binding) proteins exist elsewhere among plants. If such proteins do exist, what additional information could be gleaned from them in terms of both the toxicity of helminthosporoside and the mechanisms of alpha-galactoside transport? Answers to the first two of these questions are apparent upon evaluation of the results of a survey of the plant kingdom for alpha-galactoside-binding activity (33).

Membrane preparations from numerous, diverse plants were assayed for abilities to bind galactinol, raffinose, and helminthosporoside (Figure 1). Crude membrane proteins were prepared as outlined by Strobel (60) with one exception. Trichloroacetate, a chaotrope, was used instead of Triton X-100 to release the proteins from the membranes. All of the plants possessed galactinol- and raffinose-binding activities. All but three displayed a detectable toxin-binding activity.

Apparently, alpha-galactoside-binding activity in general and toxin-binding activity in particular are prevalent among plants. Also, resistant sugarcane does contain alpha-galactoside-binding proteins, the activity of which is somehow masked in detergent extracts and protoplasts. Ligand specificity in crude extracts is exemplified by the analogous activities of corn, potato, and H50-7209 proteins: they bind raffinose and galactinol but not helminthosporoside. This apparent specificity looms in view of the binding data reported in Chapter 3.

Intriguing also is the knowledge that toxin-binding activity exists in most of the plants tested, yet only susceptible sugarcane develop symptoms upon exposure to the toxin. Further, tobacco protoplasts are not sensitive to the toxin even though membrane extracts from this plant possess toxin-binding activity. Upon treatment with purified toxin-binding protein from 51NG97, however, tobacco as well as H50-7209 protoplasts acquire sensitivity to helminthosporoside and burst in its presence (64). Two possible explanations exist for the situation in tobacco and the other plants which bind yet are not sensitive to the toxin. The binding sites may be unavailable to extracellular toxin

because of the inaccessibility of the protein or the presence in the membrane of effectors which inhibit binding. Alternatively, binding may occur but subsequent events essential for cellular demise may be precluded. Once toxin binding has occurred in susceptible sugarcane, a heat-sensitive reaction(s) must follow or the cell remains intact. Sugarcane clones which initially are susceptible to H. sacchari and sensitive to helminthosporoside become resistant and insensitive after exposure to warm temperatures (30-35C), treatment with hot water (30-50C), preincubation in an N₂ atmosphere, or preincubation with protein-synthesis inhibitors. Although hot-water-treated leaves could revert to a sensitive condition, cold temperatures (5-15C), N₂, or protein-synthesis inhibitors prevent recovery of sensitivity (12). Concomitant with the temporary, heat-induced resistance in usually susceptible sugarcane is a 50% reduction in the activity of membrane ATPase but no loss of helminthosporoside-binding activity. Recovery of sensitivity and restoration of ATPase activity occur within 24 h. Treatment of plasma-membrane-enriched suspensions with helminthosporoside causes a 30% activation of ATPase activity in susceptible sugarcane preparations. No activation is detected in analogous preparations from normally resistant

sugarcane, heat-induced resistant sugarcane, or mint. Arrhenius plots of toxin-induced symptoms and membrane ATPase activity reveal a sharp change in slope at 32°C for each. Finally, inhibitors of membrane ATPase activity protect susceptible leaves from the effects of helminthosporoside. Furthermore, protoplasts of susceptible sugarcane are insensitive to toxin in a K^+ , Mg^{++} -deficient medium. Sensitivity is restored upon addition of these ions (66). Thus, one heat-sensitive reaction subsequent to toxin binding and essential for pathogenesis appears to be the activation of a plasma-membrane-bound (K^+ , Mg^{++})-ATPase. Whether this activation is directly or indirectly associated with toxin binding or whether stimulation of ATPase activity is the only reaction pursuant to toxin binding is unknown. Regardless of the exact nature of the reactions following binding, they are mediated via the initial interaction of helminthosporoside with the toxin receptor. The receptor from susceptible sugarcane must possess certain unique properties which cause it somehow to perturb the plasma membrane upon binding helminthosporoside. Such a perturbation occurs even in heterologous protoplasts, further evidence that the binding protein from susceptible sugarcane is the potentiator of this mechanism of pathogenesis.

This dissertation presents the isolation, purification, and characterization of the alpha-galactoside-binding proteins from susceptible sugarcane, resistant sugarcane, mint, and tobacco. One aim of this comparative biochemical analysis is to illustrate the uniqueness of the 51NG97 protein. An additional goal is to make feasible the study of these and similar carbohydrate-binding proteins in plants.

The dissertation is divided into three major topics: the comparative biochemistry of the four proteins, analysis of the in vitro binding activity, and an appraisal of the role of sugarcane proteins in the eyespot disease. Following these chapters will be a discussion of the involvement of binding proteins in such phenomena as disease resistance, carbohydrate transport, and cellular ageing.

MATERIALS AND METHODS

Isolation

Unless otherwise specified, the following isolation scheme was employed to obtain purified binding proteins. Crude membrane preparations were obtained as described by Strobel (60). These preparations were either lyophilized or homogenized immediately in Tris-HCl, 50 mM, pH 7.0 containing 1.0 M sodium trichloroacetate. A ratio of 20 ml of buffer per 100 g fresh weight of leaves was used. After one hour at 4C, the homogenate was centrifuged at 48,000g for 15 minutes at 4C. The supernatant was passed over a Sephadex LH-20 column (0.9 x 20 cm), eluting with Tris buffer which lacked the trichloroacetate. Five A_{265} -absorbing peaks were observed plus a large amount of colored material which passed from the column in the 70% ethanol rinse following each run. Only the material which eluted near the void volume contained measurable binding activity.

Fractions at or near the void volume were collected and diluted with three volumes of buffer then passed over a melibiose-Sepharose-6B affinity column having a bed volume of 160 ml (17). The column was washed with Tris buffer until no uv-absorbing material was detectable. Ten ml of 1.0 M melibiose was then added followed by Tris buffer. One column volume was collected, concentrated in vacuo at 30C to

approximately 10 ml, then desalted on a Sephadex G-25 column (2.5 x 50 cm). The fractions near the void volume were collected, concentrated in vacuo at 30C to 20 ml, then dialyzed under vacuum against either water or Tris buffer. The dialysate was collected, then the dialysis bag was rinsed with Tris-HCl, 1.0 M, pH 7.0. The dialysate and rinse material were combined, then desalted twice on the G-25 column. The excluded material was again concentrated in vacuo at 30C to 1 ml at which time it was either assayed directly or frozen and lyophilized (Figure 2).

Protein quantitation

Purified protein was quantitated based on the difference in absorption at 215 and 225 nm using a Beckman Model 25 Spectrophotometer (71). A standard curve was developed with bovine serum albumin. Determinations made by this nondestructive method of quantitation compared well with values obtained using the methods of Lowry, et al. (38), Schaffner and Weismann (51), or Bio-Rad (9). Because residual trichloroacetate strongly interfered with the differential absorption method, crude protein preparations were quantitated by the Lowry or Bio-Rad procedures.

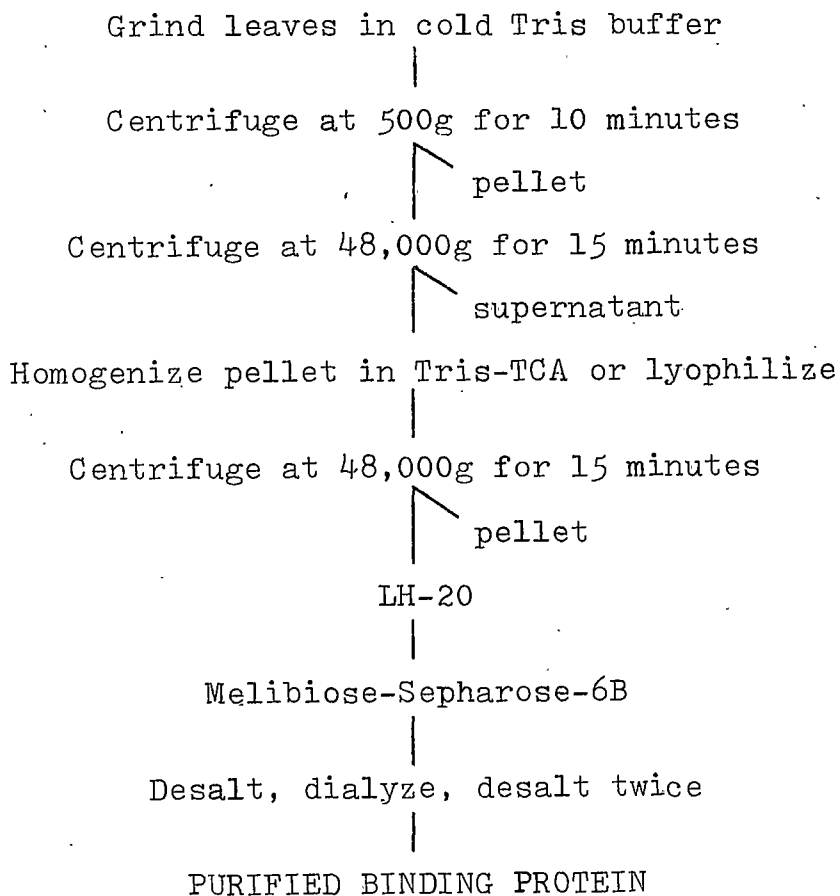


Figure 2. Flow chart illustrating the isolation of alpha-galactoside-binding proteins from plant leaves.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) on 7.5% gels was performed according to Ornstein and Davis (15). Coomassie blue stained the proteins poorly, but attempts to stain with Amido Schwarz, Stains-all (Sigma), or oil red O were unsuccessful.

Fluorescamine staining

Fluorescamine staining of the proteins was accomplished by dissolving 15-20 ug of lyophilized binding protein in 50 ul of sodium borate, 50 mM, pH 8.1, containing glycerol at a final concentration of 50% (v/v). Fluorescamine (Sigma) was then diluted with distilled acetone to a concentration of 3 mg/ml and added quickly to the protein preparation while mixing vigorously. The mixture was then allowed to stand at room temperature for 10 minutes prior to electrophoresis (49).

Molecular weight estimations

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to estimate molecular weights of the purified binding proteins (75). Also, molecular weight estimates via gel filtration were obtained using columns of Bio-Gel P-100 and P-200, Sephadex A1.5, and

Sepharose 6B. The elution buffer was Tris-HCl, 10 mM, pH 7.0. Standard proteins used were those in the Combithek kit (Boehringer-Mannheim, Figure 4) and Corynebacterium insidiosum toxin which has a molecular weight of 5×10^6 (48). Column effluents were monitored with a Gilson UV-RP flowmeter equipped with an A₂₆₅ interference filter.

Amino acid analyses

Amino acid analyses of 50 ug aliquots of purified binding proteins were performed by the aaa Laboratory, Seattle, WA. Proteins were hydrolyzed for 24 h at 110°C in 6N HCl, then subjected to ion exchange chromatography on a Durrum Analyzer, Model D-500. Yields of threonine and serine were augmented 5% and 10%, respectively, to account for loss during hydrolysis. Tryptophan and cystine were not quantitated.

Ligand affinities

Ligand affinities were measured via equilibrium dialysis using a Kontron Diapak dialysis unit (60). The dialysis membrane, Spectra/Por 1, had a molecular weight exclusion limit of 8000 (Spectrum Inc.). Equilibration of raffinose required 4 h. Dialysis time for measuring the ligand affinities was 8 h. Dialysis chambers were immersed

in a 30C water bath and rotated at 24 rpm. At the end of the dialysis period, the contents of each half cell (0.5 ml) was placed in 5 ml of Aquasol (Nuclear Chicago) and counted for 100 minutes in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320. The total counts over this time period ranged from 10,000 to 30,000 cpm. At the 90% confidence level (1.6 standard deviations), the % deviation was 1.6 to 0.9 (3). Counts were corrected to dpm using the channel ratios method for quench correction.

Preparation of radio-labeled ligands

^{14}C -Labeled ligands were variously prepared. Galactinol was labeled using a galactosyl transferase isolated from pea seeds to combine myo-inositol with ^{14}C -UDP-galactose (18). Radioactive raffinose was prepared by a transgalactosidation between ^{14}C -sucrose and raffinose (41). Galactinol and raffinose were also radio-labeled by incubating mint plants for 2 hours with $^{14}\text{CO}_2$ and isolating the sugars (53). Authentic galactinol was generously supplied by C. E. Ballou.

^{14}C -Helminthosporoside was purified from stationary phase cultures of Helminthosporium sacchari incubated with ^{14}C -sucrose 4 days at 24C (57).

The specific radioactivities of the alpha-galactosides were:

<u>Ligand</u>	<u>uCi/umole</u>	<u>kBq/umole</u>
raffinose	3.87×10^{-1}	1.43×10^1
galactinol	2.58×10^{-3}	9.55×10^{-2}
helminthosporoside	4.59×10^{-3}	1.70×10^{-1}

Plant material

Plants used in this study include sugarcane clones 51NG97 and H50-7209 (susceptible and resistant to H. sacchari respectively), mint (Mentha spicata), tobacco (Nicotiana tabacum 'White Burley'), and napier grass (Penisetum purpureum). These plants were maintained in the university greenhouse with no auxiliary lighting. They were potted in coarse, sandy loam and watered with a fertilizer solution consisting of 20:20:20 NPK, 0.5 lb/gal, diluted 1:100 with tap water. Temperature was maintained between 18-24C.

Cultures of H. sacchari were originally obtained from Dr. Gary Steiner, Hawaiian Sugar Planters Association. The fungus was maintained on cane agar slants prepared according to Pinkerton (47).

Testing susceptibility and sensitivity of plants

Susceptibility of the study plants was tested by

spraying the leaves with aqueous suspensions of spores and mycelia of H. sacchari. The misted plants were enclosed in plastic bags for two days after which the bags were removed. Plants were then maintained in a growth chamber for six weeks with the following daily regimen:

<u>Period</u>	<u>Duration</u>	<u>Temperature</u>	<u>Relative Humidity</u>
interim	2 h	70C	80%
day	8 h	80C	60%
interim	2 h	70C	80%
night	12 h	60C	90%

During the day and interim periods, the light intensity was 3×10^4 ergs cm^{-2} sec^{-1} .

Plants were assayed for sensitivity to helminthosporoside by the technique of Steiner and Strobel (57). Droplets of aqueous toxin (2 μl , 4.5 mM) were placed over puncture sites in the leaves. The leaves were placed in a humidity chamber at room temperature and visually monitored each day for six days.

Chemicals

2-Chloroethanol (Aldrich) was distilled before use. Monogalactosyl diglyceride and digalactosyl diglyceride (Applied Sciences, Inc.) were originally isolated from soybean. The remaining chemicals were obtained from Sigma Chemical Company.

COMPARATIVE BIOCHEMISTRY OF ALPHA-GALACTOSIDE-BINDING PROTEINS FROM MEMBRANES OF DIVERSE PLANTS

One way to evaluate the importance of a particular molecule in a biological process is to remove that entity from the biological system and examine its properties under controlled circumstances. This rationale perfuses the present approach to the study of membrane-bound, carbohydrate-binding proteins from plants. The isolation of these proteins is augmented by affinity chromatography whereby relatively easy separations can be effected under the mildest of conditions. Further, because of the specificity inherent in the affinity technique, active proteins may be obtained in a highly purified state.

Of course, removal of a protein from its normal milieu imparts an artificiality to subsequent observations. This artificial environment can be turned to the plant scientist's advantage, however, as long as some measure of biological activity is retained by the isolated proteins. Selective alterations in experimental conditions may then divulge the potential activity of these proteins and offer insight into the expression, or lack of expression, of activity in vivo.

Preparatory to such selective experiments, the isolated proteins must be characterized in terms of purity, chemical composition, and kinetic activity. These criteria were

obtained on proteins isolated from resistant and susceptible sugarcane, mint, and tobacco, an assemblage of plants representative of both monocots and dicots. Mint, a member of the Labiatae, was included because of the prevalence and importance of alpha-galactosides in the photosynthates of this family (54). Tobacco was chosen because it exhibited a low level of alpha-galactoside-binding activity in crude preparations (33). Also, tobacco is amenable to transport studies with protoplasts. The sugarcanes, of course, supplied the impetus for this entire study because of involvement of helminthosporoside, a host-specific alpha-galactoside, in the eyespot disease of this plant.

Following is a comparison of the chemical and kinetic characterizations of the binding proteins from these plants including a discussion of their relevance to sugar metabolism and disease.

Yields

The yield of purified binding protein from each of the plants averaged 70 to 80 ug per 100 g fresh weight of leaves. No apparent difference was noted in the relative amount of binding protein from different plants. Addition of dithiothreitol to the grinding buffer (0.1 g/100 ml

buffer) did not alter the yields or activity of purified protein. If the LH-20 chromatography was omitted, a 15 to 20% reduction in yield occurred. Diluting the protein preparation with 3 volumes of buffer just prior to affinity chromatography enhanced yields approximately 10%. Solubilization with 1.0 M trichloroacetate for 1 h was optimal for releasing the proteins from the membranes (Table 1, Table 2).

Once isolated, the protein preparations maintained activity at least 12 months if lyophilized. However, binding activity disappeared 5 days after reconstituting the proteins with water or buffer and storing at 4C. The binding activity of crude protein preparations was stable for at least 6 weeks at 4C.

Electrophoresis

PAGE was performed on three separate isolations of each protein. Single bands were obtained for each (Figure 3). H50-7209 protein migrated more slowly than did the other three proteins (Figure 3, Table 3). After pretreatment of the proteins with fluorescamine, each of the proteins migrated with the tracking dye during electrophoresis and showed no remarkable differences in relative mobilities

Table 1. Efficacy of different concentrations of trichloroacetate during solubilization of crude membranes. Lyophilized membrane pellet (0.1 g) from 51NG97 was taken up in 5 ml Tris-HCl, 10 mM, pH 7.0, containing different amounts of trichloroacetate then processed as outlined in Figure 2.

Trichloroacetate (M)	Yield of Binding Protein ^a (ug)
0.50	10
0.75	23
1.00	73
1.25	65
1.50	27

^aAverage of 2 replicates. Variability was less than $\pm 10\%$ of the average.

Table 2. Yield of binding protein at various times during solubilization of membranes. Lyophilized membrane pellet (0.1 g) from 51NG97 was taken up in 5 ml Tris-HCl, 10 mM, pH 7.0, containing 1.0 M trichloroacetate. The suspension was incubated at 4°C for varying intervals then processed as outlined in Figure 2.

Incubation Time (h)	Yield of Binding Protein ^a (ug)
0.5	47
1.0	73
2.5	33
4.0	35
8.0	27

^a Average of 2 replicates. Variability was less than $\pm 10\%$ of the average.

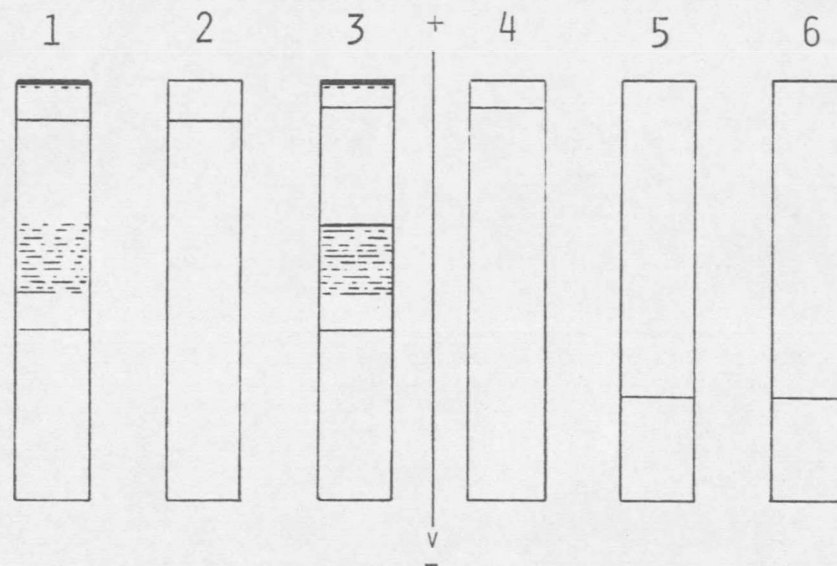


Figure 3. Electrophoretic patterns of binding proteins. PAGE performed according to Davis (15).

- 1 = crude membranes proteins from 51NG97
- 2 = purified binding protein from 51NG97
- 3 = crude membrane proteins from H50-7209
- 4 = purified binding protein from H50-7209
- 5 = purified binding protein from 51NG97
after pretreatment with fluorescamine
- 6 = purified binding protein from H50-7209
after pretreatment with fluorescamine

Arrow indicates direction of migration during electrophoresis.

Table 3. Electrophoretic mobilities of binding proteins on 7.5% polyacrylamide gels. Mobilities were calculated as described by Weber and Osborn (75).

Plant	Mobility	
	Before Fluorescamine ^a	After Fluorescamine ^b
51NG97	0.37	0.87
H50-7209	0.33	0.86
Mint	0.36	0.86
Tobacco	0.37	0.87

^aMeans of mobilities obtained for three separately isolated proteins from each plant. Variability was less than $\pm 5\%$ of the mean.

^bFluorescamine treatment was performed once on each protein.

(Figure 3, Table 3). Fluorescamine is noted for its ability to modify the free amino groups of the N-terminal amino acid and lysine causing oligomeric proteins to dissociate into subunits (49). The 7.5% gels did not resolve these proteins sufficiently to calculate subunit molecular weights, however.

uv Absorption

The A_{280}/A_{260} ratios of the proteins given in Table 4 reveal a consistent value less than one, indicative that nonproteinaceous material was present in the final preparations. When the carbohydrate content of the protein preparation was determined using the anthrone method (56), the total weight of carbohydrate + protein constituted only 65 to 85% of the gravimetric weight obtained using a Cahn Electrobalance, Model G. Further evidence that additional material was present came from the appearance of 10 spots after chromatography of purified proteins on silica gel plates in chloroform:methanol:water (65:25:4). This additional material was not extractable with chloroform. Further characterization was not attempted.

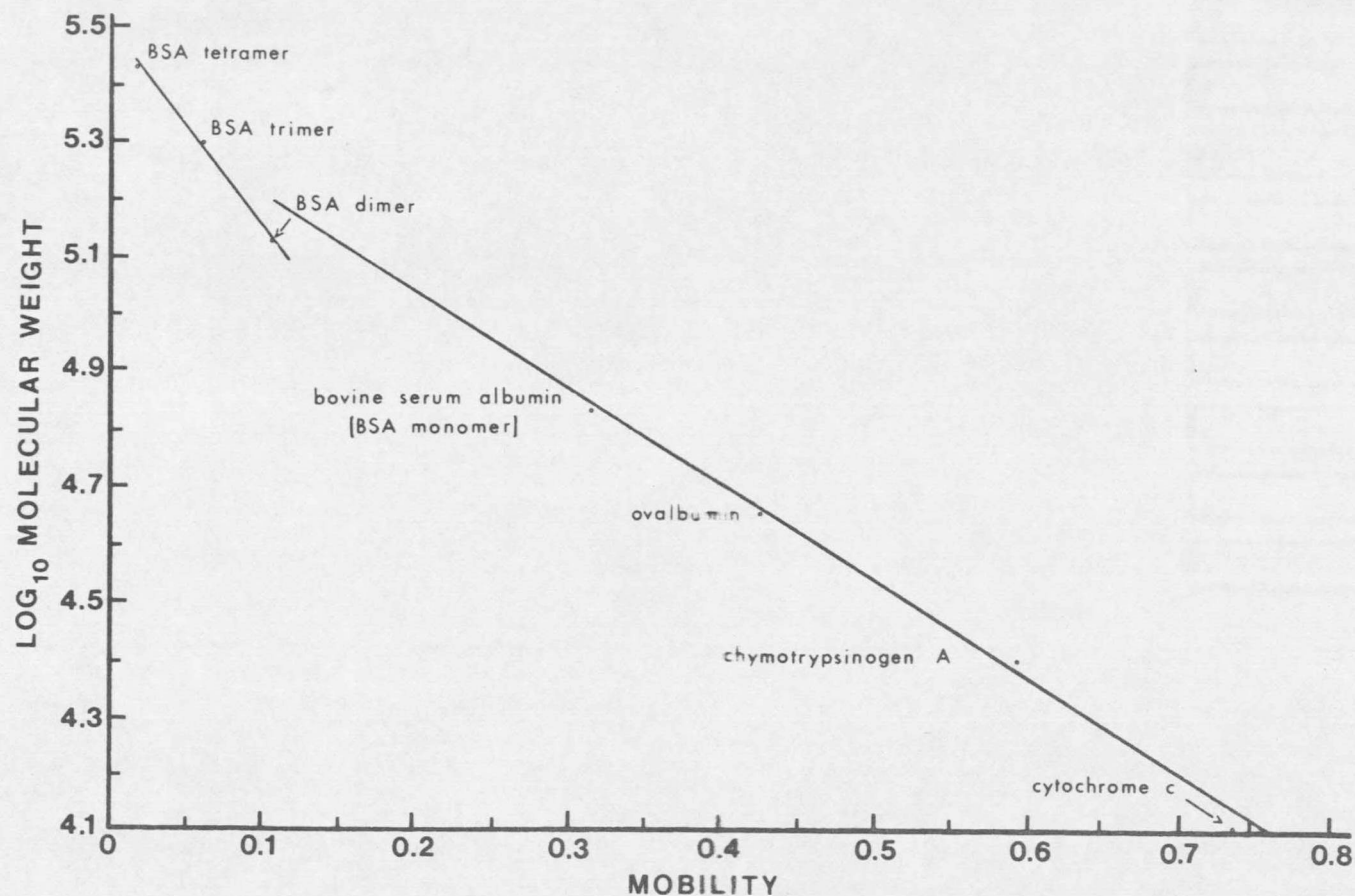
Molecular weight estimates

Figure 4 depicts the standard curves used to estimate

Table 4. A_{280}/A_{260} ratios of purified binding proteins in water.

Plant	A_{280}/A_{260}^a
51NG97	0.88 ± 0.03
H50-7209	0.82 ± 0.04
Mint	0.84 ± 0.06
Tobacco	0.85 ± 0.04
Napier Grass	0.86 ± 0.03

^aMeans of 6 to 8 protein preparations $\pm$ standard deviation.



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Figure 4. Plot of the mobilities of protein standards after SDS-PAGE.
 For the lower five points (BSA dimer - cytochrome c):
 $\log_{10}(\text{molecular weight}) = 5.38 - 1.71(\text{mobility}), r^2 = 0.99.$
 For the upper three points (BSA dimer - BSA tetramer):
 $\log_{10}(\text{molecular weight}) = 5.50 - 3.40(\text{mobility}), r^2 = 0.99.$

the molecular weights of the proteins via SDS-PAGE. SDS-PAGE was performed on purified proteins after one of the following treatments with the buffer of Weber and Osborn: 5 min, 24C; 2 h, 37C; 3 min, 100C (Table 5, Figure 5).

Because the molecular weight estimates for the 100,000 molecular weight species (Table 5) were about twice that reported for 51NG97 protein (60), the following experiments were performed. Crude membranes from 51NG97 were solubilized using 1% Triton X-100 instead of trichloroacetate. A molecular weight of 50,800 was obtained for the protein isolated from the affinity column. Also, when 30 ug of purified binding protein from the trichloroacetate procedure was incubated for 2 h at 24C in 0.1 ml Tris-HCl, 50 mM, pH 7.0, containing 1% Triton X-100, subsequent SDS-PAGE revealed a faint band corresponding to a molecular weight of 52,000 in addition to a strong band corresponding to 104,400 (Figure 6).

Estimates of molecular weights by gel filtration were complicated by self-association of the proteins at concentrations greater than 1 ug/ml. A more complete discussion of this phenomenon appears in Chapter 4. For the moment, it is important to note that the bulk of the protein on P-100, P-200, A1.5, and 6B columns eluted within

Table 5. Molecular weight estimates via SDS-PAGE for alpha-galactoside-binding proteins from plants.

Plant	Molecular Weight ^a		
	5 min, 24C	2 h, 37C	3 min, 100C
51NG97	104,400	104,400 50,300	104,400 50,300 14,400
H50-7209	101,600	ND ^b	101,600 48,700 14,500
Mint	107,500	107,500 44,800	107,500 43,900 17,800
Tobacco	108,400	ND ^b	108,400 49,000

^aValues represent means of four determinations for each treatment. All determinations were made using different protein preparations for each plant. Variation was less than $\pm 7\%$ of the mean.

^bND = not determined.

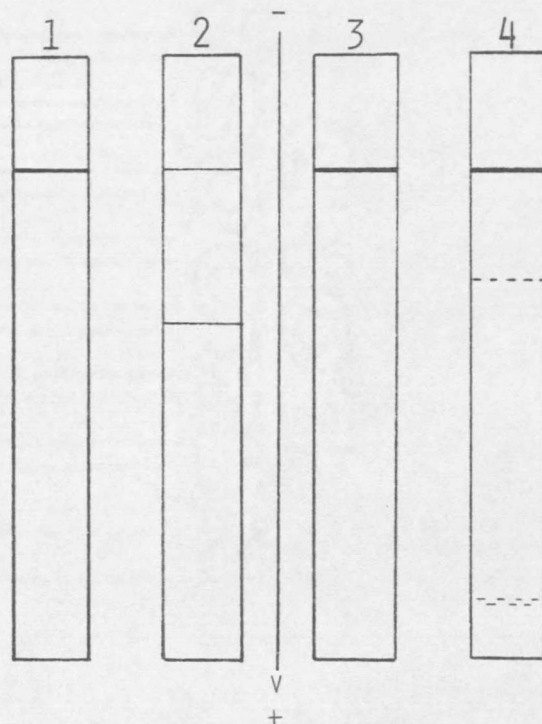
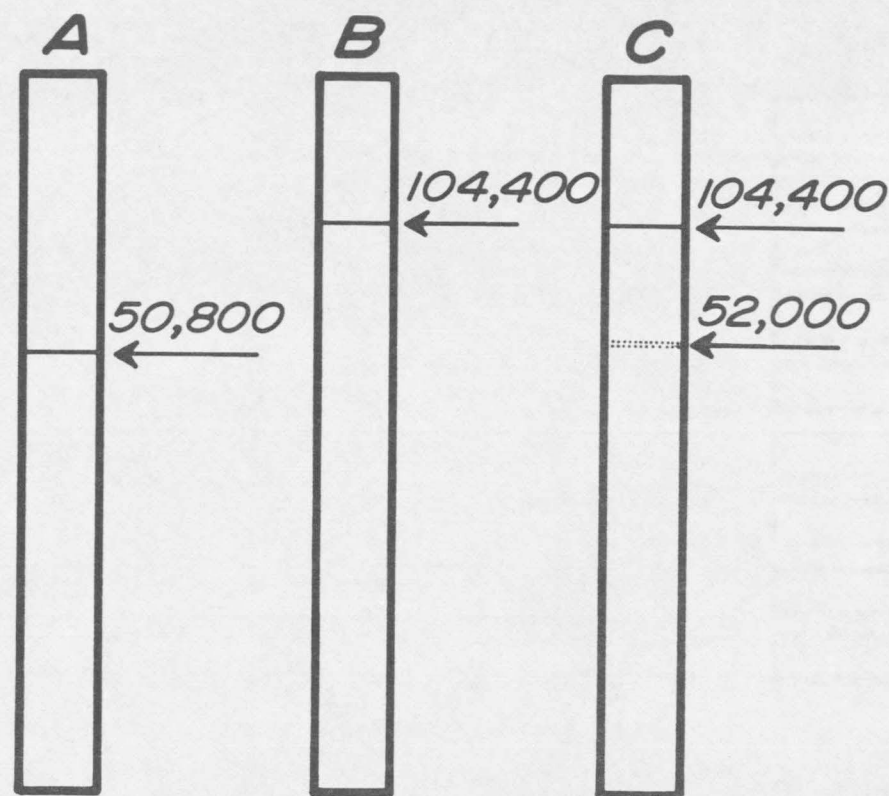


Figure 5. Mobilities of binding proteins after SDS-PAGE. The following treatments with the buffer of Weber and Osborn (75) were performed:

- 1 = mint protein - 5 min, 24C
- 2 = mint protein - 2 h, 37C
- 3 = 51NG97 protein - 5 min, 24C
- 4 = 51NG97 protein - 3 min, 100C

The arrow indicates the direction of migration during electrophoresis. Table 5 contains the estimates of molecular weights.



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Figure 6. Schematic diagram of the effect of Triton X-100 on purified 51NG97 protein.

A = 30 ug of alpha-galactoside-binding protein obtained by solubilizing the membrane pellet with 1% Triton X-100.

B = 30 ug of alpha-galactoside-binding protein obtained by solubilizing the membrane with 1.0 M trichloroacetate.

C = 30 ug of B protein which had been incubated with 1% Triton X-100 for 2 h at 24C. SDS-PAGE performed according to Weber and Osborn (75).

the void volume of each column: additional peaks representing approximately 10 to 16% of the total protein eluted in the fractions corresponding to 130,000 and 20,000 on P-200 and 133,000; 67,000; and less than 45,000 on 6B and A1.5 columns.

Amino acid compositions

Purified protein preparations which yielded a single band after gel electrophoresis were analyzed for their amino acid composition. Histograms representing those data are shown in Figure 7. The minimum molecular weights per monomer were calculated to be:

<u>Plant</u>	<u>Minimum molecular weight</u>
51NG97	16,300
H50-7209	13,300
Mint	20,900
Tobacco	25,600

Relative affinities for alpha-galactosides

Quantitating the ligand affinities of the various proteins was initially complicated by the apparent loss of helminthosporoside binding by the mint protein after purification (Table 6) as well as a general decrease in the specific binding activity of each of the proteins when measured at concentrations of 100 ug/ml. This decrease in

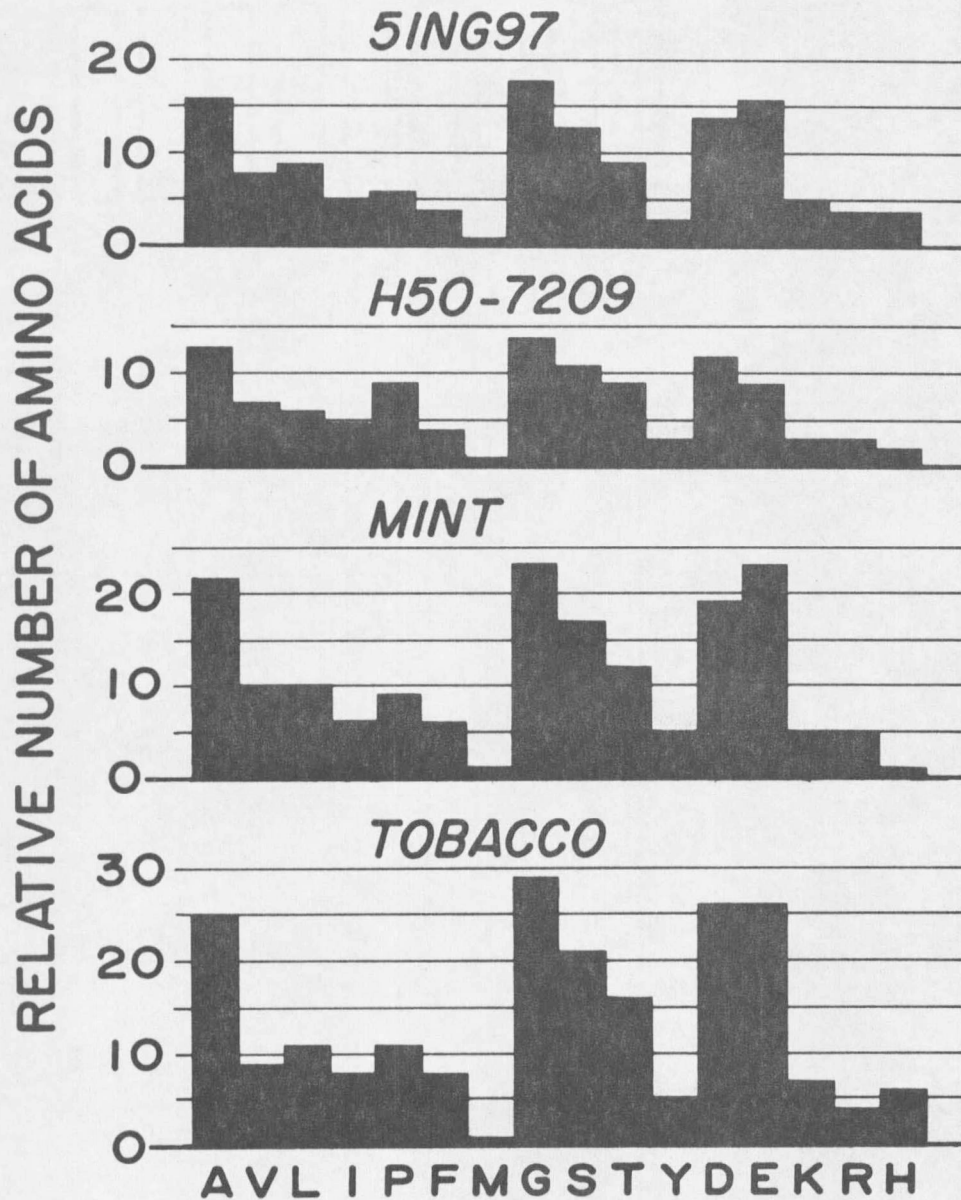


Figure 7. Histograms of amino acid compositions of binding proteins. The relative number of amino acid residues per subunit were calculated by assuming the presence of at least one residue of methionine per subunit. Values represent the average of analyses on two separately isolated protein preparations from each plant. Variability was less than ± 1 residue per subunit.

Table 6. Helminthosporoside-binding activity at different steps during purification of binding proteins.

Toxin concentration = 2.2×10^{-4} M.

Fractionation Step	Toxin-Binding Activity ^a			
	<u>mmole toxin</u> <u>g protein</u>			
	51NG97	H50-7209	Mint	Tobacco
Membranes ^b	<0.005	<0.005	ND ^c	ND ^c
Crude membrane proteins ^d	2.7	<0.005	4.7	0.2
Binding protein, 100 ug/ml	0.8	<0.001	<0.001	0.1
Binding protein, 4 ug/ml	1.7	1.2	0.8	0.9

^aValues represent an average of at least two determinations on different protein preparations. Variability was less than $\pm 8\%$ of the average.

^bMembranes = crude membrane pellet from 4 g leaf material solubilized in 0.5 ml Tris-HCl, 50 mM, pH 7.0.

^cND = not determined.

^dValues taken from Kenfield and Strobel (33). Protein concentration = 3 to 5 mg/ml.

activity at higher protein concentrations was noted for each protein-ligand interaction (unpublished data). Among the many possible reasons for an apparent loss of activity, the first to be characterized was the effect of protein concentration on binding activity. It was necessary to optimize the protein concentration during binding studies since there was an indication from gel-filtration data that some type of protein-protein interaction was occurring and may have been interfering with binding. Over a protein concentration range of 4 to 100 ug/ml, it was noted that the highest specific binding activity occurred at the lowest concentration. The table of affinities (Table 7) was then developed using low protein concentrations (4 ug/ml).

Figure 8 depicts the Michaelis-Menten and Lineweaver-Burke plots for the raffinose binding by tobacco protein. These plots are representative of all the protein-ligand kinetics, in particular the decrease in specific binding activity at the second lowest substrate concentration, which was repeatable for each protein-ligand interaction. The K_d 's of the various ligands were calculated using the specific binding activities at the six higher concentrations only. Scatchard analysis of the data was meaningless because of the anomalous kinetic behavior at penultimate

Table 7. Relative affinities of purified binding proteins for various alpha-galactosides.

Ligand concentration range: 1×10^{-6} to 3.5×10^{-4} M.
 Protein concentration: 3.6 to 4.0 ug/ml.
 Buffer: Tris-HCl, 10 mM, pH 7.0.

Plant	K_d ($\times 10^{-5}$ M) ^a		
	Raffinose	Galactinol	Helminthosporoside
51NG97 (susceptible cane)	1.0	2.7	2.8
H50-7209 (resistant cane)	1.8	3.4	8.2
Mint	6.0	1.2	1.3
Tobacco	1.8	6.0	6.6
Napier Grass	ND ^b	ND ^b	2.1

^aAverage of at least two determinations for each protein-ligand interaction. Each determination was performed on a different protein preparation. Variability was less than $\pm 6\%$ of the average.

^bND = not determined.

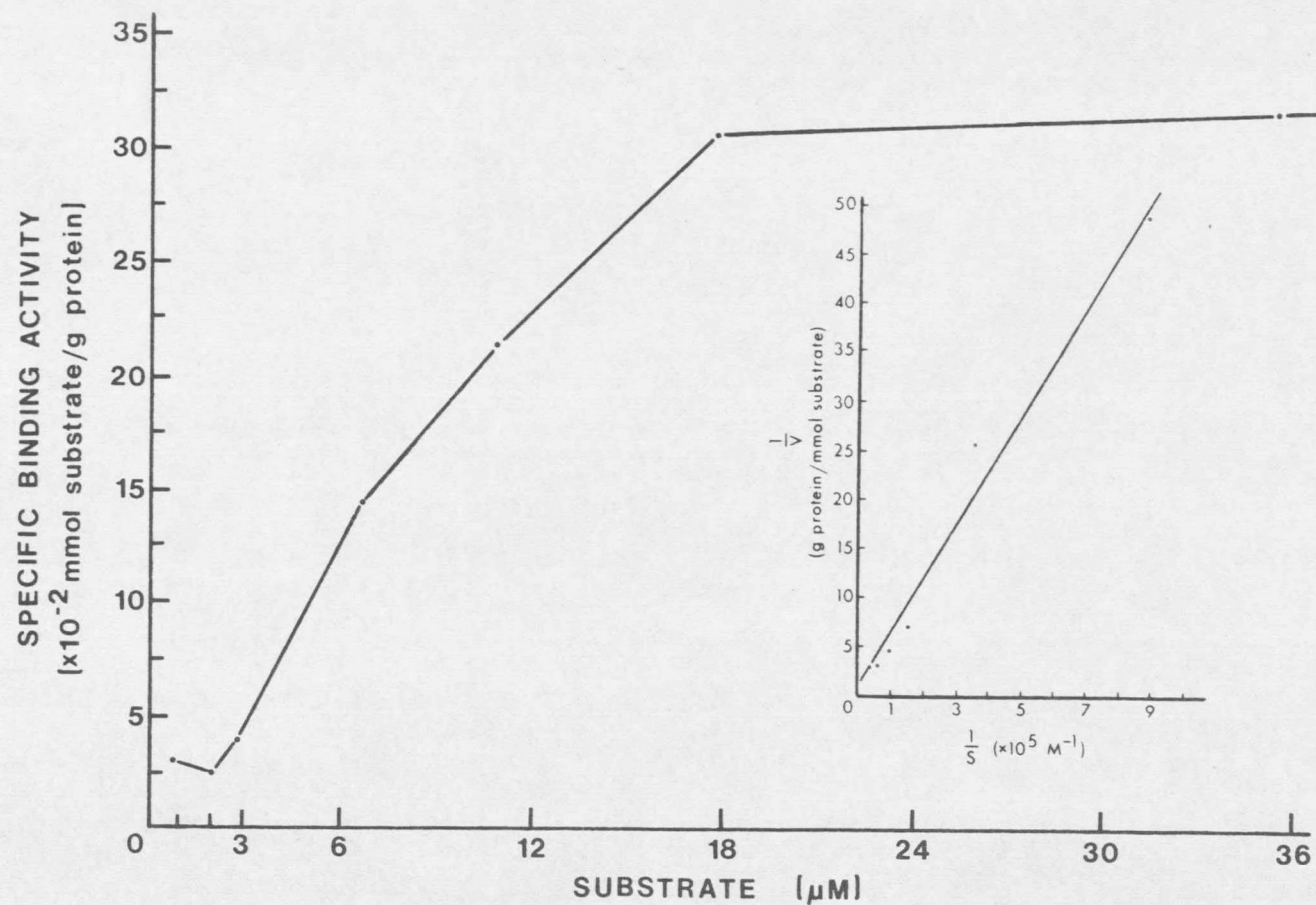


Figure 8. Plot of the binding activity of tobacco protein at different ligand concentrations. Insert: Reciprocal plot of the binding activity versus ligand concentration.

ligand concentrations. Also, ligand concentrations were not sufficiently low to permit a credible estimate of high and low affinity binding sites.

Discussion and summary

If the alpha-galactoside-binding proteins are involved in membrane transport and toxin recognition, it is important to establish their location. Evidence has been published that the helminthosporoside-binding protein from 51NG97 is detectable on the outer surface of the plasma-lemma (62). The ability to extract alpha-galactoside-binding proteins from plant cell membranes using either a mild chaotrope or low-ionic-strength buffer (see Chapter 5, Table 19) is indicative that these proteins are only peripherally associated with membranes (21). In contrast, solubilization by a non-ionic detergent (Triton X-100) and exhibition of certain in vitro qualities (self-association, lipid sensitivity) signify that such proteins are hydrophobic and feasibly could be expected to be intimately associated with membrane constituents in vivo. Further, the increase in yield upon implementation of the LH-20 column during isolation portends the presence of lipophilic factors in the solubilized membranes which adversely affect

binding (76). It seems likely, then, that these proteins are integrated at least partially into the membrane yet extend into the peripheral spaces. Whether they traverse the entire membrane as do five well-characterized transport proteins in mammalian cells is not known (20).

The tacit assumption in the above discussion is that, even though the membrane pellet contains material other than plasma membranes, the isolated proteins are present in the plasmalemma. Their occurrence in other cellular membranes, particularly the chloroplast membrane, is a possibility, even a probability since the chloroplast is a site of synthesis of alpha-galactosides (28). In view of the localization of 51NG97 protein on the plasmalemma, the marked physical and kinetic similarities of the binding proteins and the prevalence of alpha-galactosides in the translocation stream of many plants (54, 77), it seems likely that the mint and tobacco proteins are also located in, but not confined to, the plasma membrane.

The molecular weight differences between those reported here and the 49,000 earlier reported (60) may be caused by the difference in mechanism of extraction by trichloroacetate and Triton X-100. The chaotrope, because of its low charge density, releases membrane proteins by perturb-

ing the structure of water. When the boundary layers of water adjacent to membranes are disrupted, the membrane lipids also become disordered as they associate into new constructs of minimum free energy. This disorder releases macromolecular constituents. Detergents act by competing with the membrane lipids for the hydrophobic sites on the proteins, thus tending to competitively solvate membrane proteins (24). Triton X-100 can cause the detectable association of a 50,000 molecular-weight species (Figure 6). Because high degrees of self-association inhibit detergent binding, it is likely that an even stronger band would have been observed at the 50,000 mark if protein concentration had been lower during this experiment (43).

The high degree of self-association of purified binding proteins (Chapter 4) may be the reason for incomplete dissolution of the proteins in SDS. The predominance of high molecular weight species after boiling in 0.1% SDS implies that the hydrophobic forces causing the proteins to associate in water may have prevented the anionic detergent from penetrating. Lowering the protein concentration during incubation with SDS could overcome this problem.

The calculation of minimal molecular weight from amino acid composition (p 34), appearance of low molecular weight

species after treatment with SDS (Table 5), and rapid migration of the proteins after modification with fluorescamine (Table 3) substantiate the hypothesis that these proteins are oligomeric (49). If the molecular weight estimate of approximately 100,000 is accurate for the sugarcane proteins, it seems likely that they exist in the membranes as octomeric receptors comprised of 12,000 to 15,000 molecular-weight subunits. Still, it is possible that an equilibrium among octomeric, tetrameric, and monomeric forms occurs in the membrane. Results in the next chapter indicate that an even larger form may participate in this flux.

The subunit estimates for tobacco and mint proteins are higher than those for the sugarcane proteins (p 34). For tobacco, a subunit molecular weight of 25,000 points to the 100,000 molecular weight species being a tetramer. An interesting thought is that the tobacco subunit may represent a doubling of the sugarcane subunit: the molecular weight estimates are nearly a factor of 2 (p 34); 11 of the 16 amino acids in the tobacco protein are approximately twice their counterparts in the H50-7209 protein (Figure 7). Sequence data on these proteins would be desirable.

The comparative amino acid compositions are remarkable for their similarity. The most striking variation among

the proteins occurs in the ratios of aspartate and glutamate. The strong resemblance in the pattern of nonpolar (alanine through glycine) and polar, non-charged (serine, threonine, and tyrosine) amino acids support the idea that a strong evolutionary conservation in the hydrophobic portions of these membrane proteins has occurred. If these proteins are related, the variation in composition has taken place in the charged amino acids, those most likely to extrude into the spaces peripheral to the membrane and participate in the binding of alpha-galactosides.

Based on the total abundance of glutamate, aspartate, or glycine, the sugarcane proteins comprise a group distinct from mint and tobacco proteins (Figure 7). This grouping holds up in terms of subunit molecular weights (p 34). Evidence presented in Chapter 4 on the kinetics of protein-protein interaction agrees with the separation of the monocot proteins from the dicot (Table 8). Drawing such distinctions is tenuous at this point, however. An important contrast to the alignments above is the electrophoretic identity of 5LNG97, mint, and tobacco proteins. H50-7209 protein differs from these three, having a slower rate of migration (Table 3, Figure 3). One explanation for the slower rate may reside in the marked decrease in the

level of glutamate in resistant cane protein. If this value represents only or mostly glutamate, rather than glutamine (both appear as glutamate in amino acid analyses), the decreased level will confer a less negative charge to the protein at pH 8.5, the electrophoretic pH. The protein will then migrate more slowly towards the anode.

Physically and chemically, then, certain differences among the four proteins are apparent, but these differences are not phylogenetically consistent and are splayed against a background of general similarity. Kinetically, a patchwork comparison also prevails: differential toxin binding under certain conditions, no differences under other conditions. In the crude trichloroacetate extracts, differential toxin-binding activity agrees with the original report (61), i.e., 51NG97 extracts bind the toxin, H50-7209 extracts do not. Toxin-binding activity in mint extracts is nearly double that of 51NG97 while tobacco shows a low but detectable activity. Upon testing the purified proteins at a concentration of 100 ug/ml for toxin binding, the specific activity of 51NG97 and tobacco proteins have decreased while that of mint protein is no longer detectable. Reducing the protein concentration to 4 ug/ml causes a recovery of toxin-binding activity of the mint protein, in-

creases the specific activity of 51NG97 and tobacco, and reveals toxin-binding activity in H50-7209, resistant sugar-cane protein (Table 6).

One inference is that the protein preparations are self-inhibitory. This aspect is dealt with in Chapter 4. A second inference is that these proteins all have the potential to bind, yet this potential is not always expressible. An elaboration of this "potential and expression" inference appears in Chapter 5.

At low protein concentration, all four proteins yield similar K_d 's for the three ligands (Table 7). These K_d 's compare well with the affinities for helminthosporoside and raffinose obtained with Triton-solubilized 51NG97 protein and are sufficiently low to approximate ligand affinities necessary for transport phenomena (63). The Michaelis-Menten plots for alpha-galactoside binding appear to be sigmoidal (Figure 8) and stir speculations of cooperativity. Analysis of the binding data via the logarithmic form of the Hill equation (52) yields Hill coefficients of 0.93 to 0.98 for all the protein-ligand interactions. A Hill coefficient of 1 implies that all the binding sites are equivalent and commonly is taken to mean that no allosteric activity is evident. Yet, because the data do conform to the

Hill equation, a different form of cooperativity could be occurring. It is possible that the ligand stabilizes the association of the most active binding form of receptor. This type of stabilization has been documented (45). However, if it is occurring in the present situation, the interaction of ligand and receptor must be complex because of the logarithmic relationship. Unitary Hill coefficients are the first hint that alpha-galactosides influence the physical form of their receptors. This thought will be pursued after discussing pertinent experiments in Chapter 5.

The kinetic behavior at low ligand concentrations is perplexing. Near concentrations of 10^{-6} M, ligands appear to exert a negative effect on the binding activity. Resolution of this behavior will require more data points in the range of 10^{-7} to 10^{-6} M with ligands of high specific radioactivities.

While much more characterization is required for the isolated proteins, it is apparent that the use of carbohydrate affinity chromatography and mild chaotropic agents offers a facile method for purifying proteins involved in sugar transport and toxin binding.

The alpha-galactoside-binding proteins from plant cell membranes are prevalent throughout the plant kingdom (33).

They comprise a family of compositionally and kinetically similar, but not identical, macromolecules. Perhaps an analogy with cytochrome c is appropriate (16). The alpha-galactoside activities may be crucial to plants and the proteins have thus been largely conserved throughout evolution. Inclusion of gymnosperms and lower plants in a broader study range would be of interest in this regard.

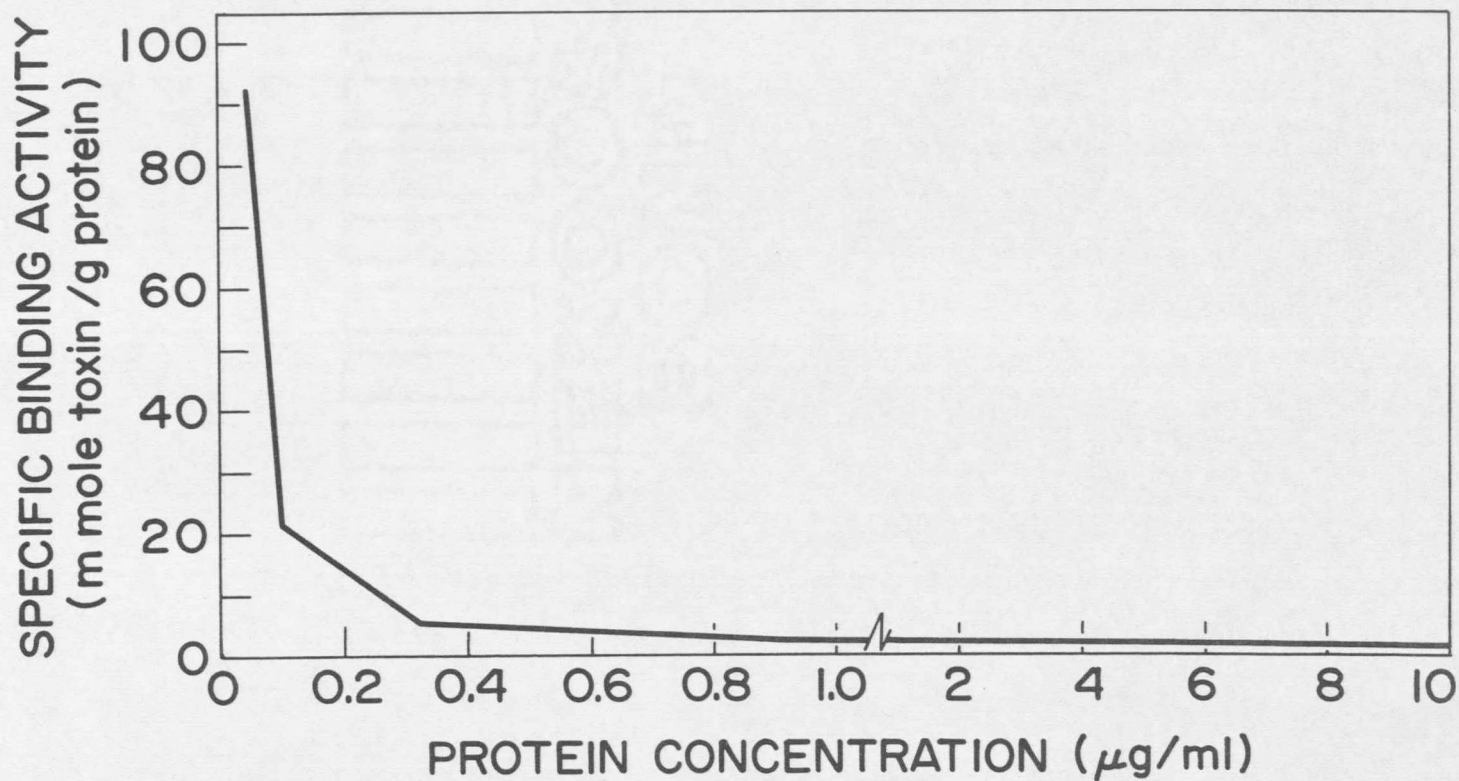
The differential toxin-binding activity that plays an important part in the molecular model of host specificity in the eyespot disease has been shown to be lacking under certain in vitro conditions. The remaining portions of this dissertation deal with the ability to stimulate or block this potential for binding.

LIGAND BINDING VERSUS SELF-ASSOCIATION OF ALPHA-GALACTOSIDE- BINDING PROTEINS

The kinetic experiments reported in Chapter 3 present a paradoxical situation. For each of the four proteins at high concentrations (100 ug/ml), binding activity is either non-detectable or greatly diminished compared to the binding activity at low concentration (4 ug/ml). Such behavior may well be an artifact of studying membrane proteins in buffered water. Nonetheless, the retention of even a remnant of activity in aqueous media permits a rational or empirical approach to reconstituting full activity. The remainder of this chapter contains the results and discussion of attempts to evaluate the inverse relationship between binding activity and the concentration of alpha-galactoside-binding proteins.

Specific binding activity versus protein concentration

Purified binding proteins from 51NG97, H50-7209, mint, tobacco, and napier grass were assayed for toxin-binding activity over a protein concentration range of 0.04 to 24 ug/ml. The concentration of helminthosporoside was maintained at 2.2×10^{-5} M, the approximate K_d concentration at 4 ug/ml protein (Table 7). Figure 9 depicts the results of testing variable concentrations of 51NG97 protein versus



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Figure 9. Toxin-binding activity of 5LNG97 protein at various concentrations. After five determinations utilizing three separately isolated protein preparations, the specific binding activity at 0.1 ug protein/ml was 21.5 ± 0.4 mmole toxin/g protein. Toxin concentration: 2.2×10^{-5} M.

constant concentrations of toxin. Analogous plots for proteins from napier grass and H50-7209 were nearly superimposable on Figure 9. Plots for tobacco and mint proteins depicted a similar inverse relationship of activity and protein concentration. Reciprocal plots and semilog plots failed to yield straight lines. When the $\log_{10}$ of the specific binding activity was plotted against the $\log_{10}$ of the protein concentration, however, straight lines were obtained (Figure 10, Figure 11). Correlation coefficients for the lines obtained in log-log plots were: 51NG97, 0.90; H50-7209, 0.99; mint, 0.96; tobacco, 0.85; and napier grass, 0.97. Linearity in a log-log plot signifies that the interaction is highly complex and can be described by the general formula $y = kx^a$ where y = specific binding activity and x = protein concentration. Equations of this form were developed for each of the proteins (Table 8).

A similar experiment in which raffinose-binding activity was monitored at varying concentrations of 51NG97 protein (Figure 12) produced a plot similar to Figure 9 and the equation: raffinose binding activity = $0.8(\text{protein concentration})^{-1.06}$. The correlation coefficient for this line, 0.75, was not as high as that for toxin binding, but reciprocal and semilog plots were much less fit ($r^2 = 0.39$ and

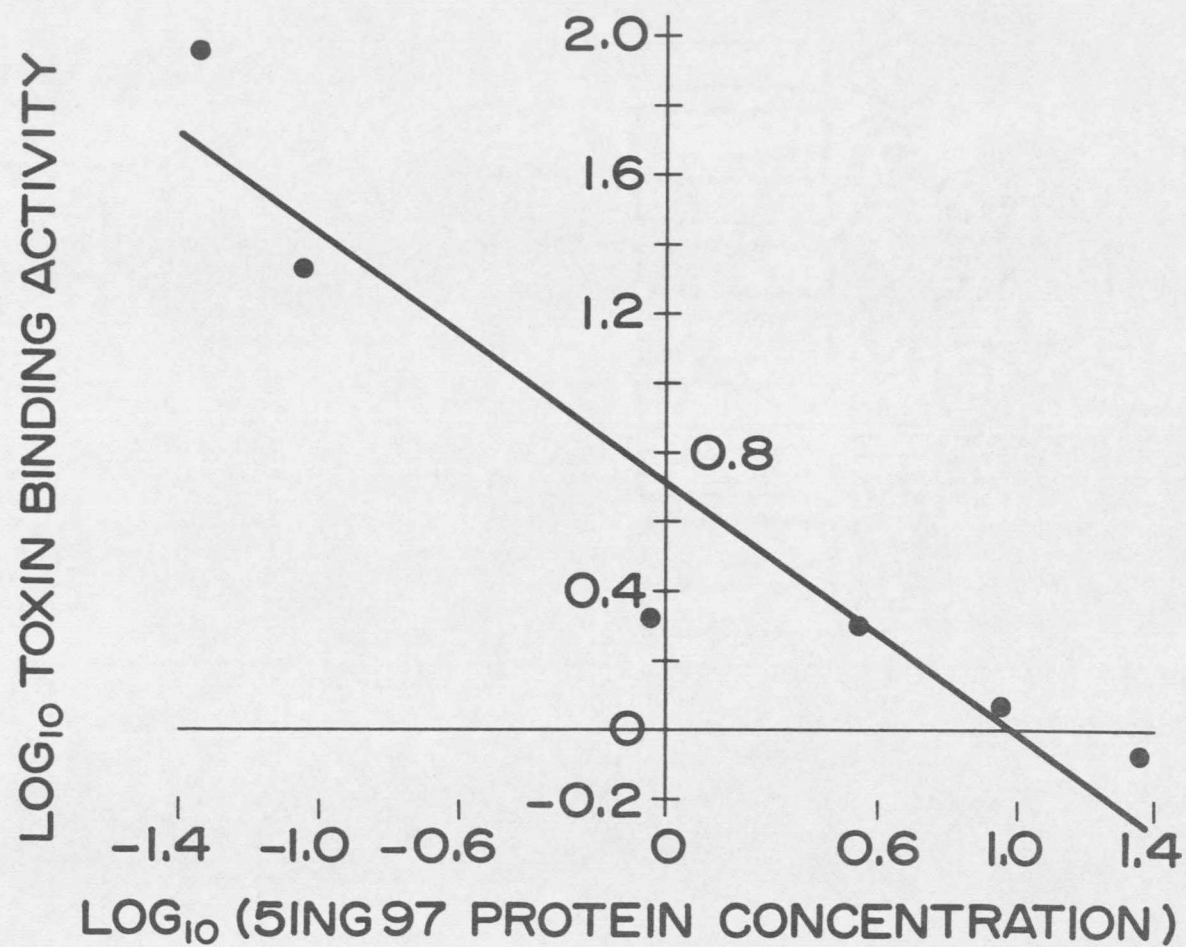


Figure 10. Log-log plot of specific binding activity versus concentration of 51NG97 protein. For the line of best fit, $r^2 = 0.90$. Toxin concentration: 2.2×10^{-5} M.

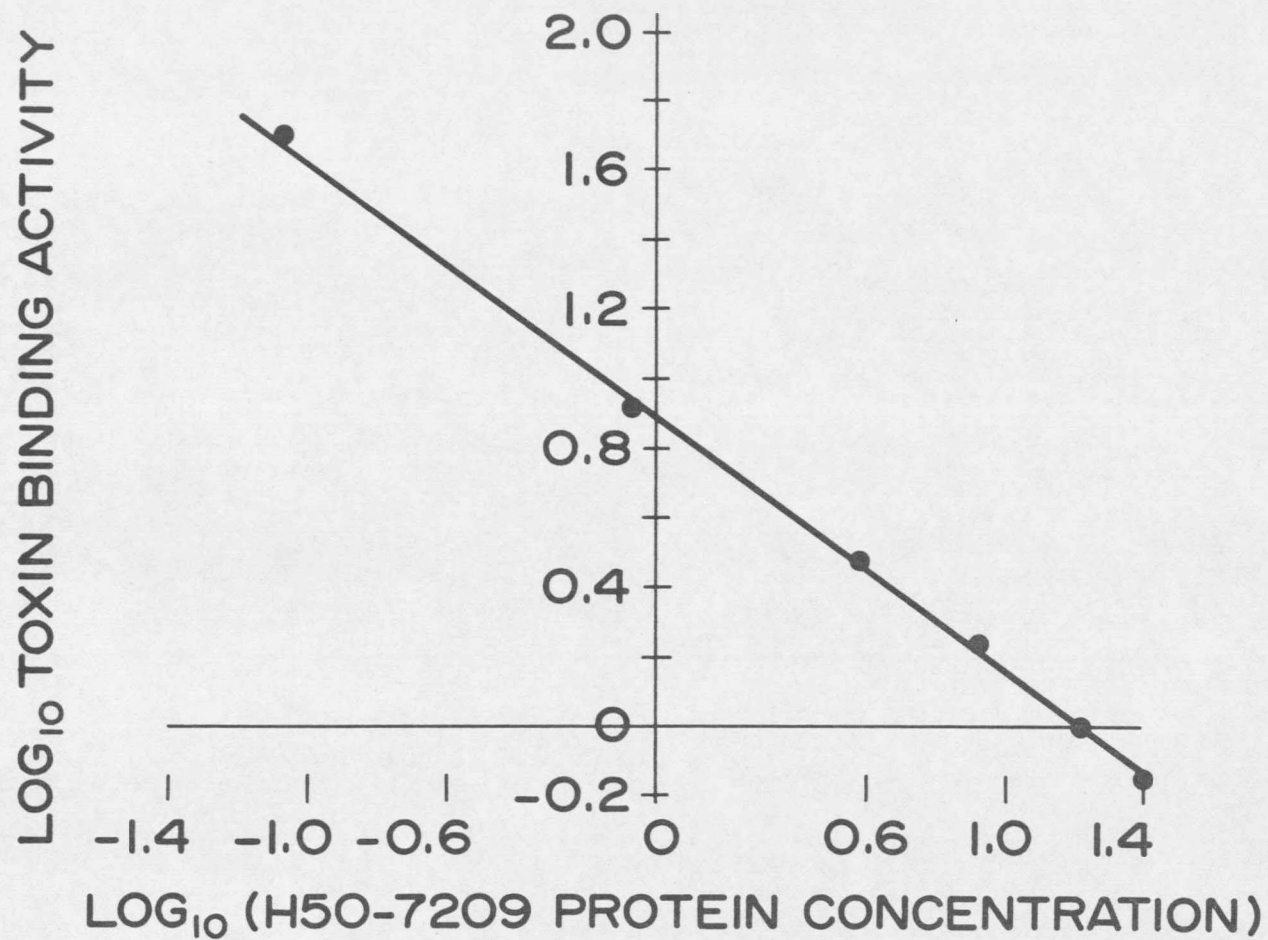


Figure 11. Log-log plot of specific binding activity versus concentration of H50-7209 protein. For the line of best fit, $r^2 = 0.99$. Toxin concentration: 2.2×10^{-5} M.

Table 8. Equations for toxin-binding activity of different proteins at variable protein concentrations.

Toxin concentration: 2.2×10^{-5} M.

Plant	Specific Binding Activity =
	$\frac{\text{mmole toxin}}{\text{g protein}}$
51NG97	$5.14(P)^{-0.71^a}$
H50-7209	$7.89(P)^{-0.74}$
Mint	$2.65(P)^{-0.96}$
Tobacco	$1.98(P)^{-0.64}$
Napier Grass	$4.14(P)^{-0.77}$

$^a(P)$ = protein concentration in ug/ml.

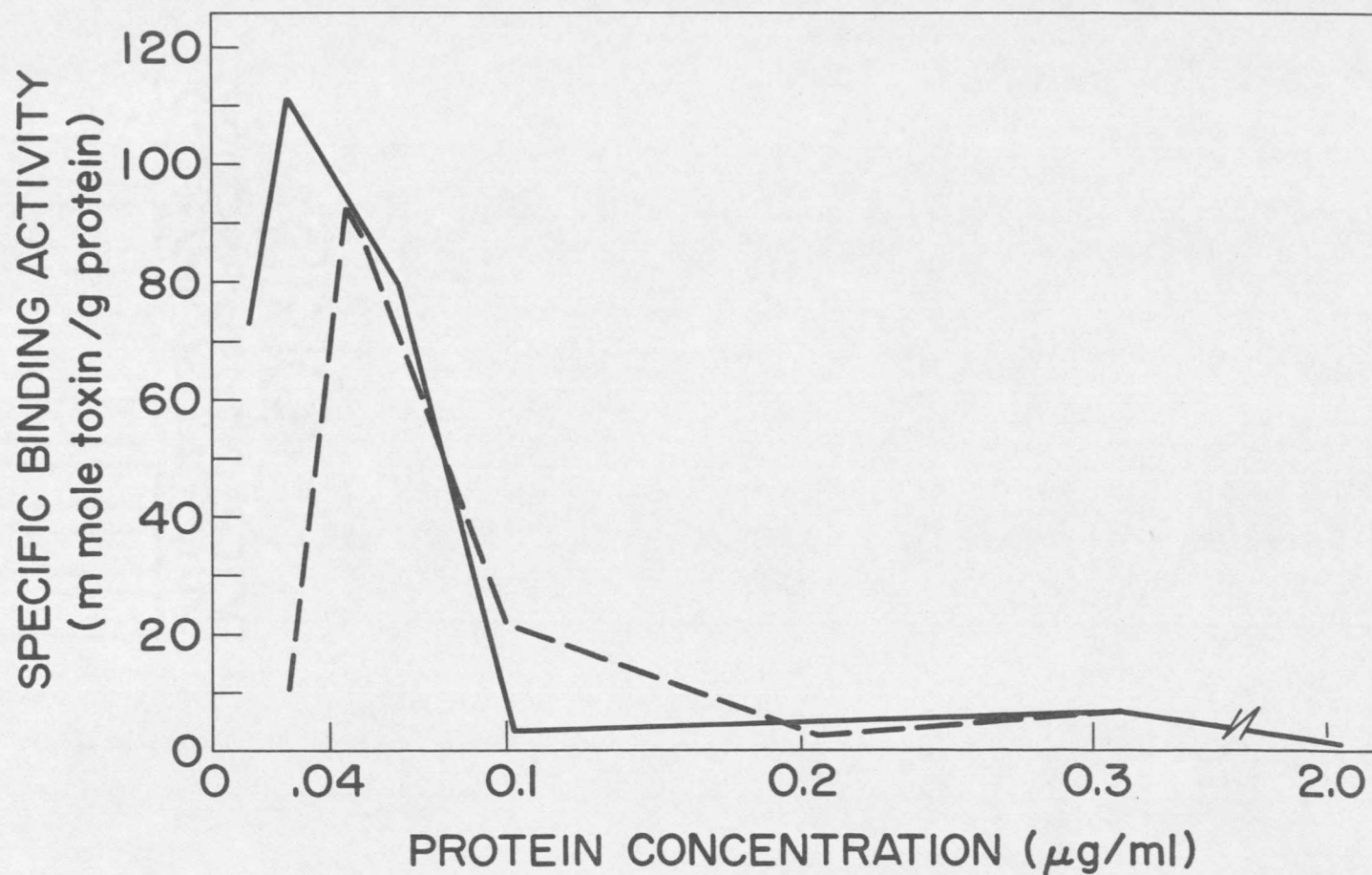


Figure 12. Comparison of raffinose binding (—) and toxin binding (----) at variable concentrations of 51NG97 protein. Toxin concentration: 2.2×10^{-5} M. Raffinose concentration: 3.6×10^{-5} M.

0.61, respectively).

Substrate binding activity peaked near protein concentrations of 0.04 to 0.05 ug/ml below which the specific binding activity decreased. Table 8 and Figures 10 and 11 were developed from the binding data for protein concentrations greater than and equal to that which produced the highest specific binding activity.

Slopes of the log-log plots (superscripts in the equations in Table 8) were different for each protein-toxin combination, an indication that the proteins differ kinetically in their response to ligand binding. An extension of this thought is suggested by a different interpretation of the data presented for the 5LNG97 protein-toxin interaction. The log-log plot (Figure 10) could be depicted as two lines, one for the lower three concentrations and one for the higher three concentrations. Respective slopes for these lines are -1.28 and -0.48, $r^2 = 0.98$ for each. Log-log plots of the tobacco-toxin combination revealed a similar breakpoint. A line derived from binding activity at the lowest three protein concentrations yielded a slope of -2.5, $r^2 = 0.99$. For the three highest concentrations, the slope was -0.24, $r^2 = 0.87$ (data not shown). For at least two of the five proteins, then, the

kinetics of toxin-binding activity at low protein concentrations (less than 1 ug/ml) may differ from that at higher concentrations. One reason for this difference could be the effect of the ligand on the active form of the protein. This thought will be discussed after clarifying certain physical aspects of these proteins in solution.

Polydispersity versus protein concentration

As noted in Chapter 3, purified binding proteins exhibited a polydisperse pattern during gel filtration in contrast to appearing as single bands during PAGE and SDS-PAGE. Observations of heterogeneity on sizing columns which is nondetectable by electrophoretic techniques are not uncommon for membrane proteins (43). The extreme dilutability of binding activity (detectable at 17 ng/ml) argues against the presence of contaminating proteins in the relative abundance apparent on sizing columns (Figure 13). It is possible, however, that these particular proteins, being amphiphilic, could be behaving much like amphiphilic lipids in water, i.e., a dynamic distribution of monomeric, oligomeric, and polymeric forms may be occurring. The heterogeneity of the protein preparations could then be one of size rather than composition. If a size distribution is

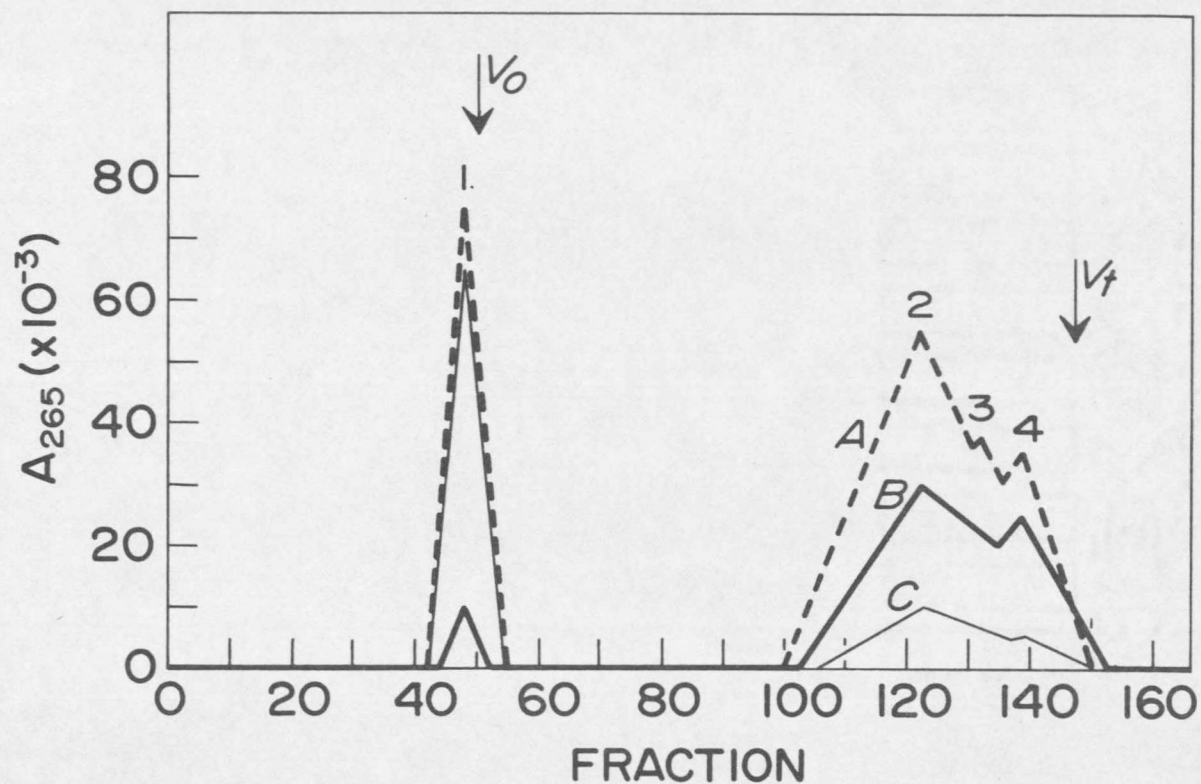


Figure 13. Elution profile of 51NG97 protein (54 ug/ml) on Sephadex A1.5.

A (----): first pass of protein preparation over column.

B (—): repass of the combined fractions under peaks 2,3, and 4.

C (—): repass of fractions under peak 1.

present and is dynamic, collecting the appropriate fractions from a sizing column and rechromatographing them should reproduce a qualitatively similar elution pattern to the original one.

Figure 13 illustrates the elution profile of 51NG97 protein on Sephadex A1.5. Initially, 54 ug of protein in 1 ml of water were passed over the column and four peaks were observed. The fractions represented by peak 1 were collected, desalted, and lyophilized. Fractions inclusive of peaks 2, 3, and 4 were treated identically. The two portions were resolubilized separately in 1.0 ml water and rechromatographed on the A1.5 column. The material which initially was excluded exhibited lower molecular weight peaks in addition to a peak at the void volume. The low molecular weight material from the first pass also showed a peak at the void volume as well as the later peaks (Figure 13).

Peaks 1, 2, 3, and 4 correspond, respectively, to molecular weights of greater than 1.5×10^6 ; 133,000; 67,000; and less than 45,000 based on a standard curve of known proteins (Table 9).

The specific toxin-binding activities of the initial protein preparation, the excluded material, and the bulked

Table 9. V_e/V_o of standard proteins on Sephadex A1.5 (95 x 0.5 cm).

Flow rate: 0.3 ml/h

Fraction volume: 0.45 ml

Buffer: Tris-HCl, 10 mM, pH 7.0.

Standards	Molecular Weight ^a	V_e (fraction)	V_e/V_o
<u>Corynebacterium insidiosum</u>			
Toxin	5×10^6	50	1
Ferritin	5.4×10^5	74	1.48
Aldolase	1.6×10^5	118	2.36
Bovine Serum Albumin	6.7×10^4	130	2.60
Picric Acid	2.3×10^2	147	2.94

^aTo calculate molecular weight of unknown: molecular weight = $1.17 - 4.25(V_e/V_o)$, $r^2 = 0.98$.

material representing peaks 2-3-4 were measured at protein concentrations of 4 ug/ml each. The binding activities for each fraction were nearly identical (Table 10).

In conjunction with binding activity, the distribution of high and low molecular weight forms may also have been influenced by the protein concentration. If so, passing different concentrations of protein over the sizing column should reflect a quantitative difference between peak 1 and peaks 2-3-4. 51NG97 binding protein was passed over the column at two initial concentrations of 70 ug/0.2 ml and 0.8 ug/0.05 ml. The elution profiles for these concentrations appear in Figures 14 and 15, respectively.

At the highest concentration, the ratio of polymer:oligomer was 1:0.19 by triangulation of the areas under the peaks and 1:0.17 by protein quantitation. Quantitation was not done for the experiments shown in Figures 12 and 14, however triangulation gave the data presented in Table 11.

Plotting the data in Table 11 as the $\log_{10}$ (protein concentration) versus $\log_{10}$ (oligomer/polymer) gave a straight line described by:

$$\text{oligomer/polymer} = 708(\text{protein concentration})^{-1.38}, r^2 = 0.99.$$
 The dissociation of polymers thus appeared to be an inverse, complex function of protein concentration in

Table 10. Toxin-binding activity of different fractions of 51NG97 protein after chromatography on Sephadex A1.5.

Toxin concentration: 2.2×10^{-5} M.

Protein ^a (4 ug/ml)	Toxin-Binding Activity
	$\frac{\text{mmole toxin}}{\text{g protein}}$
Pre A1.5	2.0
Peak 1	2.2
Peak 2-3-4	2.2

^aRefer to Figure 12 for identity of the fractions.

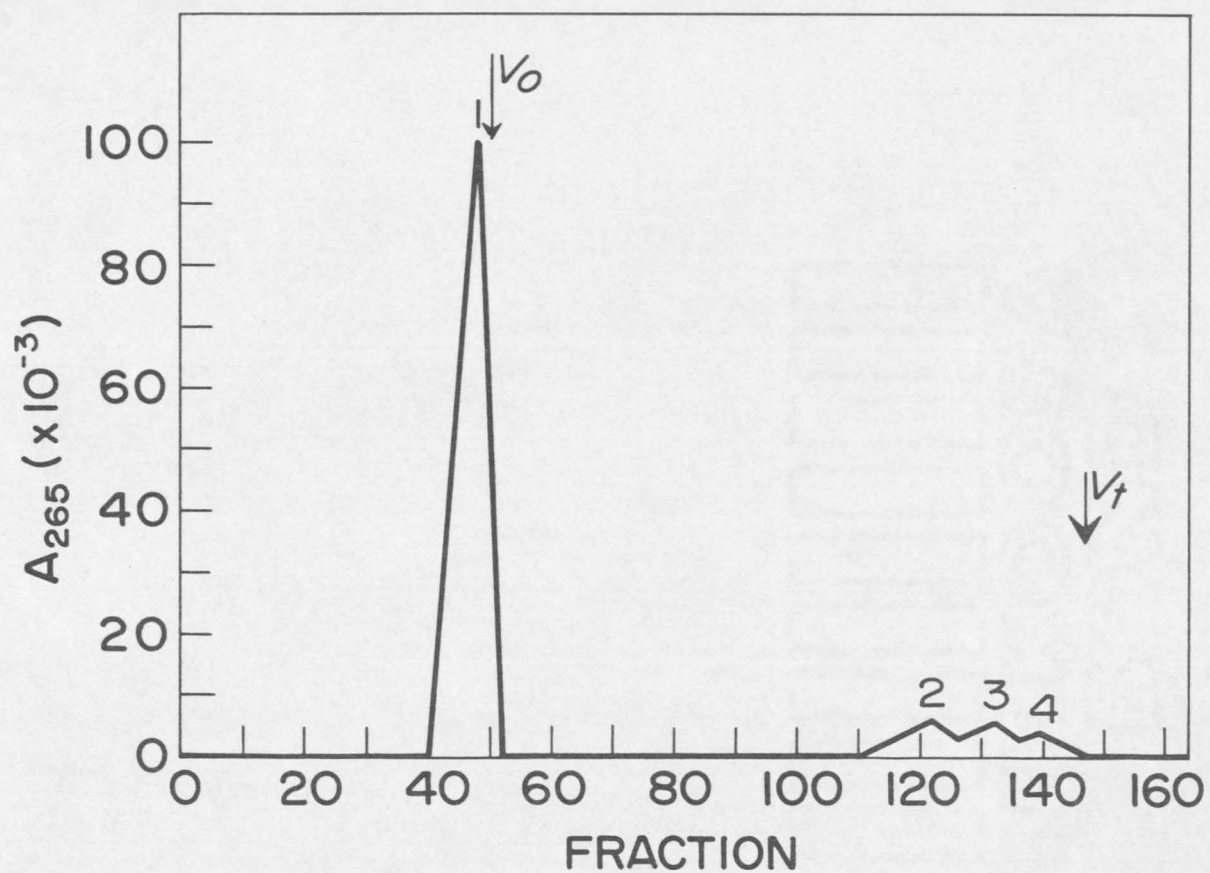
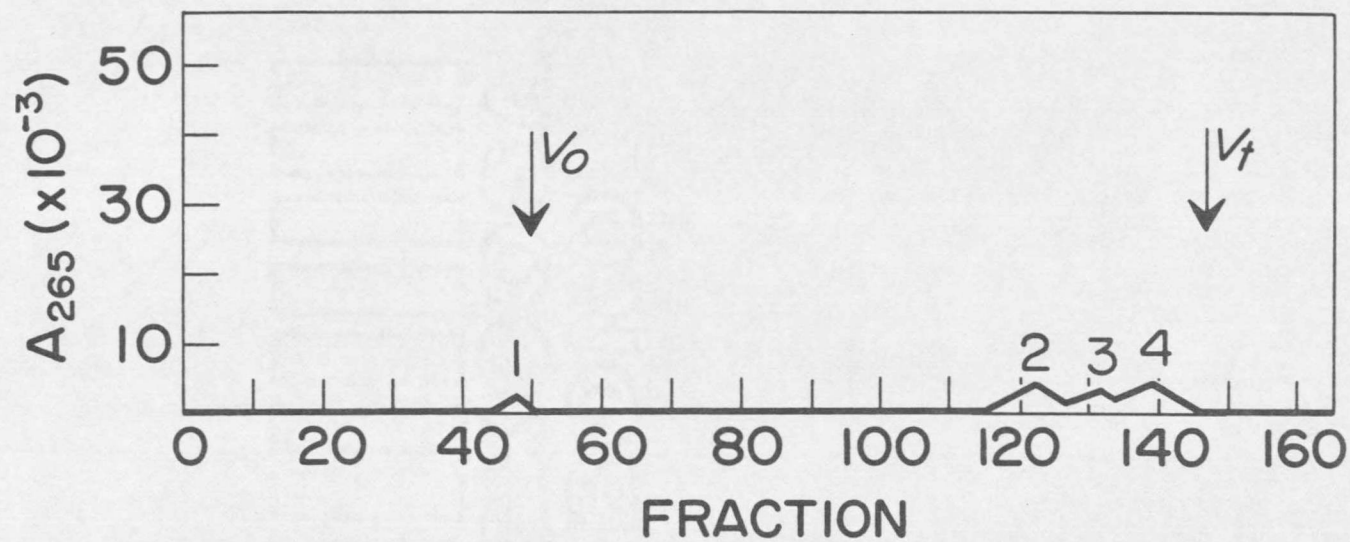


Figure 14. Elution profile of 51NG97 protein (70 ug/0.2 ml) on Sephadex A1.5.



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Figure 15. Elution profile of 51NG97 protein (0.84 ug/0.05 ml) on Sephadex A1.5.

Table 11. Ratio of oligomers to polymers of 51NG97 protein as a function of protein concentration.

Protein concentration (ug/ml)	Oligomer/Polymer
350	0.19
54	3.33
16.8	12.50

aqueous buffers. The slope, -1.38 was markedly different from the slope of either toxin (-0.71) or raffinose (-1.06) binding as a function of protein concentration.

Toxin-binding activity versus pH

The effects of pH and buffer ion on toxin-binding activity of purified 5LNG97 protein reveal an extreme sensitivity of this protein to electrostatic alterations (Table 12). No binding activity was observed in MES or at pH 6 in phosphate. Binding activity peaked at pH 7 in Tris and pH 8 in phosphate.

Toxin-binding activity versus ionic strength

The dependence on ionic strength of the toxin-binding activity of purified 5LNG97 protein was measured at low and high concentrations of protein (Table 13). The general trend showed an inverse relationship of binding activity to buffer concentration at pH 7.0. At higher ionic strengths, however, it was possible to totally negate binding at the low protein concentration. Conversely, the activity at the higher protein concentration decreased by 6-fold yet was still detectable at the highest ionic strength tested.

Table 12. Toxin-binding activity of 51NG97 protein as a function of pH and buffer ion.

Toxin concentration: 2.2×10^{-5} M.
 Protein concentration: 0.1 ug/ml.
 Buffer concentration: 10 mM.

pH	Specific Binding Activity ^a $\frac{\text{mmole toxin}}{\text{g protein}}$		
	MES	Tris	Phosphate
5	<0.001	ND ^b	23.3
6	<0.001	ND ^b	<0.001
7	<0.001	21.5	15.1
8	ND ^b	8.2	63.1
9	ND ^b	8.2	38.4

^aValues are an average from 2 replicates.
 Variability was less than $\pm 5\%$.

^bND = not determined.

Table 13. Toxin-binding activity of 51NG97 protein as a function of ionic strength.

Toxin concentration: 2.2×10^{-5} M.

Buffer: Tris-HCl, 1 to 100 mM, pH 7.0.

Ionic Strength (mM)	Specific Binding Activity ^a <u>mmole toxin</u> <u>g protein</u>	
	Protein Concentration	
	0.10 ug/ml	3.4 ug/ml
1	65.9	4.1
5	48.1	3.1
10	21.5	2.1
25	9.6	0.7
50	<0.001	0.7
100	<0.001	0.7

^aValues represent an average of 2 replicates. Variability was less than $\pm 5\%$.

Effects of heterologous proteins on toxin-binding activity

Alpha-galactoside-binding protein from 5LNG97 appeared to self-associate under given conditions of protein concentration and electrostatic parameters. Nonspecific hydrophobic forces affected the polymer-oligomer distribution, but was the protein-protein interaction dependent only on hydrophobic forces and protein concentration or was it necessary to have specific proteins present to cause the behavior? An answer to this question came from the following four experiments.

First, aliquots of purified binding proteins from 5LNG97 were heated to 50C for 1 h, a treatment which destroyed binding activity. Equal amounts of the heated and non-heated proteins were mixed and assayed for binding activity at three concentrations (Table 14). Again, the binding activity decreased with increasing protein concentration. The specific binding activities of the mixtures, however, were higher than those expected for analogous concentrations of nontreated proteins. It is possible that certain of the molecules which co-purify with the binding proteins (see p 27) were released during heat treatment. Such molecules could have associated with the non-treated proteins and acted as positive effectors. Further exper-

Table 14. Toxin-binding activity of 51NG97 protein amended with heat-inactivated 51NG97 protein.

Toxin concentration: 2.2×10^{-5} M.

Buffer: Tris-HCl, 10 mM, pH 7.0.

Heat inactivated 51NG97 Protein	Non-treated 51NG97 Protein	Total Protein	Specific Binding Activity ^a	
ug/ml	ug/ml	ug/ml	$\frac{\text{mmole toxin}}{\text{g protein}}$	
0.068	0	0.068	<0.001	70
0	0.068	0.068	33.2	
0.034	0.034	0.068	99.8	
0.068	0.068	0.126	70.7	
0.102	0.102	0.204	11.1	

^aSpecific binding activity calculated using amount of non-treated protein only. Values represent an average of 2 replicates. Variability was less than $\pm 5\%$.

imentation was not performed to test this speculation, but the observation does warrant some attention because of the possibility of identifying specific factors which stimulate toxin-binding activity.

A second experiment involved mixing purified 51NG97 protein with bovine serum albumin such that the concentration of binding protein remained constant while total protein increased from 0.04 to 150 ug/ml. A 5-fold decrease in binding activity was observed over this 3750-fold increase in protein concentration (Table 15).

In the third experiment, equal amounts of purified binding proteins from 51NG97 and H50-7209 clones of sugarcane were combined to produce a concentration range of 0.052 to 0.8 ug/ml. It would have been desirable to have had concentrations below 0.05 ug/ml, but the loss of binding activity of extremely dilute proteins interfered with these attempts (Figure 12). In the concentration range tested, a profile of decreasing binding activity with increasing protein concentration did occur (Table 16, Figure 16).

The fourth experiment was identical to the third except that mint binding protein was used instead of the H50-7209 protein. Again, an inverse profile was obtained which ap-

Table 15. Toxin-binding activity of 51NG97 protein amended with increasing amounts of bovine serum albumin.

Toxin concentration: 2.2×10^{-5} M.

Buffer: Tris-HCl, 10 mM, pH 7.0.

51NG97 Protein	Bovine Serum Albumin	Specific Binding Activity ^a
ug/ml	ug/ml	<u>mmole toxin</u> <u>g protein</u>
0.046	0	92.2
0.046	0.06	89.0
0.046	1.2	87.0
0.046	12.0	24.6
0.046	32.0	24.6
0.046	150.0	18.6
0	150.0	<0.001

^aAverage of 2 replicates. Variability was less than $\pm 8\%$.

Table 16. Toxin-binding activity of combined 51NG97 and H50-7209 proteins at variable concentrations.

Toxin concentration: 2.2×10^{-5} M.

Buffer: Tris-HCl, 10 mM, pH 7.0.

Protein Concentration ug/ml	Specific Binding Activity ^a <u>mmole toxin</u> <u>g protein</u>		
	51NG97	H50-7209	Combined ^b
0.052	86.9	88.4	ND ^c
0.104	21.5	50.5	22.6
0.200	2.2	7.2	4.2
0.420	4.1	5.8	1.1
0.600	ND ^c	ND ^c	5.2
0.800	2.2	6.4	2.8

^aValues represent averages of 2 replicates. Variability was less than $\pm 7\%$.

^bCombined preparations consisted of 51NG97 and H50-7209 proteins mixed in equal amounts to comprise the final concentration.

^cND = not determined.

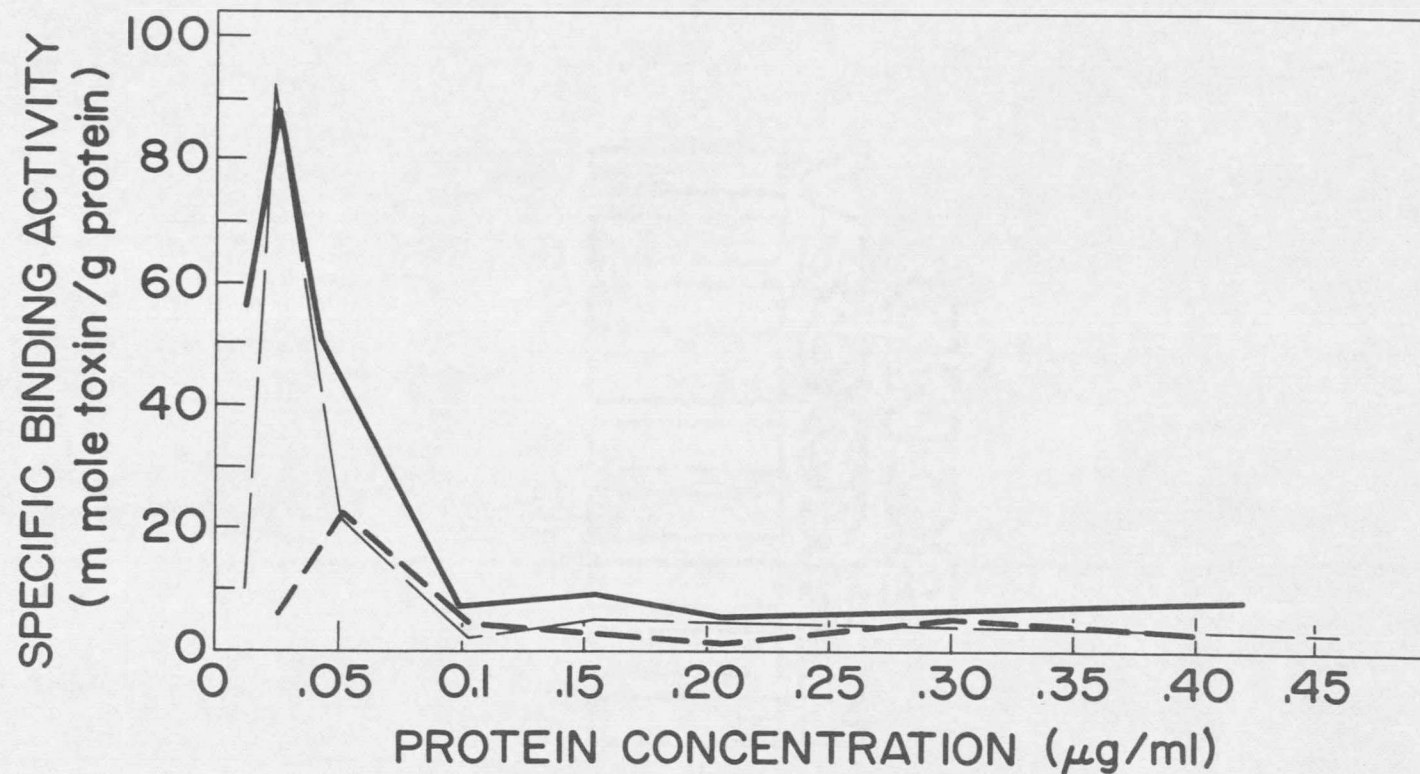


Figure 16. Comparison of toxin-binding activities of 51NG97 protein (—), H50-7209 protein (— —), and mixed 51NG97/H50-7209 proteins (----) at variable concentrations. Toxin concentration: 2.2×10^{-5} M.

proximated that of either protein by itself. Pertinent data from this and the other three experiments were compared (Table 17).

Discussion and summary

For a discussion of protein-protein interaction in solution, it is important to distinguish between self-association and aggregation. Here, self-association will be defined as the reversible formation of oligomeric and polymeric species of proteins from the protomeric form. The protomer is the lowest molecular weight species observable and in the present discussion will be equivalent to monomer. Aggregation is the irreversible formation of higher-molecular-weight species. Thus, aggregates fail to dissociate upon dilution. Self-association, on the other hand, is governed by the laws of mass action and is concentration dependent (43).

The elution profile of 51NG97 protein on the A1.5 column displays 4 peaks, the lower three of which may well correspond to the monomeric, tetrameric, and octomeric species (Figure 13). The octomeric molecular weight of 133,000 and tetrameric 67,000 are about 30% larger than the corresponding estimates obtained by SDS-PAGE. The low

Table 17. Toxin-binding activity of heterologous mixtures of proteins.

Toxin concentration: 2.2×10^{-5} M.

Buffer: Tris-HCl, 10 mM, pH 7.0.

Added Protein	Concentration of Additive ug/ml	51NG97 ug/ml	Specific Binding Activity	
			<u>mmole toxin</u> g protein	
51NG97	0	0.052	86.9	76
	0.052	0	86.9	
	0.052	0.052	21.5	
H50-7209	0.052	0	88.4	
	0.052	0.052	22.6	
Bovine Serum Albumin	0	0.046	92.2	
	0.060	0	<0.001	
	0.060	0.046	89.0	
Mint	0	0.068	33.2	
	0.068	0	20.8	
	0.068	0.068	20.8	

ionic strength of the elution buffer (10 mM) may be causing the protein to assume a more hydrated form with a larger Stokes radius thus contributing to a larger apparent molecular weight on sizing columns. Peak 4 (Figure 13) is beyond the resolving capacity of the A1.5 column, but estimates from P-200 and P-100 column chromatography show this peak to correspond to about 20,000. SDS-PAGE and amino acid analysis yield estimates of 14,400 and 13,300 for the monomer. The combined data from column chromatography, SDS-PAGE, and amino acid analysis are interpreted to mean that the polydispersity observed during gel filtration is caused by the presence of polymeric and oligomeric species of a single protein undergoing self-association. Although the evidence is not rigorous, the oligomeric series will be designated as monomer=protomer, tetramer, and octomer. The designation, even if incorrect, will bolster the discussion of the equilibrium between the polymer and oligomers.

Self-association as a function of protein concentration is receiving attention by those people interested in the lipoproteins of mammalian plasma (50). Utilizing density gradient centrifugation and circular dichroism, researchers have amassed much data on the physical aspects of association of apolipoproteins, delipidated lipoproteins.

Apo A-II, one of the major constituents of high density lipoproteins, displays a concentration-dependent monomer-dimer association; apo C-1, one of the minor proteins, exhibits a monomer-dimer-trimer equilibrium. For both proteins, the higher molecular weight species are favored by higher protein concentrations. In addition to protein concentration, self-association of plasma lipoproteins is extremely sensitive to ionic strength, pH, type of salt present, and temperature (43).

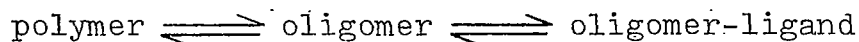
Because of the extremely dilute concentrations at which the 51NG97 protein is most active, it is difficult to compare the plant protein directly with the mammalian lipoproteins. The self-association of both types of proteins is similar, increasing protein concentration favors the formation of the high molecular weight species; however one notable difference is that a polymeric form of apolipoproteins is not apparent, even at protein concentrations up to 2.5 mg/ml (43). With 51NG97 protein, the polymeric form predominates at 350 ug/ml (Figure 14). A second difference, one of function rather than structure, is the capacity of 51NG97 protein to bind alpha-galactosides - an assayable function which can be utilized to monitor perturbing effects of amendments. This binding ability is also use-

ful for monitoring the self-association phenomena at concentrations far below the resolving ability of most physical techniques.

The polydispersity of purified 51NG97 protein is not due to the presence of discrete, stable molecular weight species in solution, i.e., an equilibrium exists. If discrete, stable associations were present, only polymers or oligomers would have been detectable upon rechromatographing the separate volumes of the first run. Such was not the case (Figure 13). Also, the near-equality of the specific binding activities of the peak 1 and peak 2-3-4 proteins, when measured at the same protein concentration, argues that a dynamic association - dissociation is occurring (Table 11). If the molecular-weight species were not equilibrating, the peak 2-3-4 proteins would have shown a higher specific binding activity because the binding activity is highest when the relative abundance of oligomers is highest, i.e., at low protein concentrations (Table 8, Table 11).

The slopes of the log-log plots for toxin-binding, raffinose binding, and oligomer/polymer-ratio for 51NG97 protein are -0.71, -1.06, and -1.38 (Table 8, p 61). The difference in the slopes signifies that ligands alter the

equilibrium among the physical forms of binding protein at a particular protein concentration. Because the magnitude of the negative slope is less for either raffinose or toxin binding than for the oligomer/polymer ratio, it is arguable that ligands increase the rate of dissociation of the polymer. At a particular protein concentration, then, the relative abundance of oligomers will be greater if ligand is present. A second idea can be gleaned from the difference in slopes between raffinose and toxin binding. These two ligands were tested at slightly different concentrations (raffinose = 3.6×10^{-5} M, toxin = 2.2×10^{-5} M), thus the different slopes signify that either or both ligand concentration or type of ligand are important parameters in the protein-protein vs protein-ligand activity. At this point, it is possible to present the following model:



The laws of mass action dictate that the polymer-oligomer equilibrium be dependent on protein concentration, i.e., high protein concentrations force the equilibrium to the left. This aspect of the model has been supported (Table 11). Another prediction from the model is that increasing the ligand concentration should drive the equili-

brium to the right. As noted above, negative slopes of lesser magnitude for ligand binding than for the purely physical oligomer/polymer ratio offer tentative support for the prediction. Experiments which would clarify the validity of the model include measuring the specific binding activity at variable protein concentrations in the presence of different, constant concentrations of ligand. If the slopes at different ligand concentrations differ, then the ligands must be modulating the dissociation of the polymer. Also, monitoring the oligomer/polymer ratio during gel filtration on Sephadex A1.5 in the presence of different concentrations of ligand in the elution buffer should give a direct estimate of the effect of ligand on the physical form of the binding protein. Testing the effect of different ligands at the same concentration should reveal if the type of ligand is important. All three types of experiments are in progress.

The response of 51NG97 protein to changing ionic strength is peculiar: the binding activity of high concentrations of protein is much less sensitive than binding activity of low concentrations (Table 13). At high protein concentrations, the hydrophobic forces governing self-association may be more effective than the salting-out

ability of Tris, thus the bulk of the protein in solution is in the polymeric form which consistently releases the more active oligomers. Certain substantiative evidence is available for these suggestions. When 51NG97 protein (80 ug/ml) is chromatographed on an A1.5 column and the elution buffer is 50 mM Tris rather than 10 mM, the low molecular weight peaks are nearly undetectable (unpublished observation). Secondly, the decrease in ligand-binding activity below a critical protein concentration (0.03 to 0.04 ug/ml, Figure 9) occurs when the monomeric species should be more abundant - if the laws of mass action are governing the association-dissociation activity. High ionic strengths should also favor formation of monomers at low protein concentrations. It seems likely, then, that the monomer lacks binding activity. The polymer may be active, but not nearly so active as the oligomers. Which of the oligomers is the most, or only, active species is not known.

51NG97 protein appears to act much like an amphiphilic lipid in water (67). A lower critical concentration is required to enable association of the molecules into active oligomers. A narrow range of concentrations exist in which the association-dissociation phenomena are extremely sensi-

tive to environmental parameters. An upper critical concentration exists above which micellar lipids and polymeric proteins form spontaneously.

The double peak of the binding activity in phosphate buffer is unusual (Table 12). A total inhibition of binding activity near pH 6 could mean that histidine is involved in the active site. Certainly, histidine modification experiments would be of interest in this regard. From the activities in Tris and phosphate buffer, 51NG97 protein is most active between pH 7 and 8.

The physical and kinetic evidence discussed so far lend credence to the hypothesis that the binding protein from susceptible sugarcane is a classical oligomeric, hydrophobic protein. If self-association of monomers and oligomers is occurring for all four binding proteins in solution, it should be possible to mix heterologous proteins at low concentrations and produce similar binding activity-protein concentration effects - providing the monomers of the different proteins are similar enough to interassociate. If monomers from one protein can associate only with themselves, the presence of two discrete populations of binding proteins in solution should cause an additive effect in terms of the specific binding activity. As

total protein concentration increases, the specific binding activity of either 51NG97/H50-7209 or 51NG97/mint mixtures decreases (Table 16, Table 17). Bovine serum albumin, which is not a subunit protein, does not appreciably affect the specific binding activity of 51NG97 protein (Table 15). Kinetically, the mixtures of alpha-galactoside-binding proteins from diverse plants act as though they interassociate.

The hypothesis that monomers of different alpha-galactoside-binding proteins can interassociate is testable. PAGE of 51NG97/H50-7209 should display a band of intermediate mobility (refer to Figure 3). Elution profiles on an A1.5 column should reflect a concentration dependence analogous to either protein alone. A most important experiment relating to the mechanism of disease resistance would be to treat resistant protoplasts with the chimera binding protein and observe the sensitivity of treated protoplasts to toxin. Also, the ability of chimera protein to stimulate ATPase activity of susceptible sugarcane warrants attention.

Elucidation of some aspects of the in vitro behavior of alpha-galactoside-binding proteins has opened certain doors of understanding. The inverse relationship of activ-

ity to protein concentration may be an isolated instance of artificial behavior or it may be an important property of membrane proteins. A recent review of the structural and functional properties of acetylcholine receptors notes that the activity of the preparation is lowest when high molecular weight species are present (22). Hepatic binding protein from plasma membranes of liver cells self-associates in aqueous solution and has a molecular weight of 500,000. In the presence of Triton X-100, however, the self-association is prevented and a molecular weight of 250,000 is obtained (4, 32). Perhaps careful protein concentration - activity studies with these important mammalian receptors will reveal inverse patterns similar to the activity of 5LNG97 protein.

At the least, an inverse concentration-activity relationship offers an additional concept to the model of resistance to eyespot disease. The in vivo concentrations of binding proteins may determine the sensitivity of sugarcane clones to helminthosporoside. It is possible to totally inhibit toxin binding by H50-7209 protein at high concentrations (Table 6). In addition, it is possible to severely inhibit toxin binding by 5LNG97 protein at high concentrations and even obliterate that activity by altering the

assay environment (Table 6, Table 12, Table 13). Extrapolation of these in vitro effects to the possible situation in the membrane milieu invites the hypothesis that susceptibility and resistance are temporal conditions in sugarcane.

Experiments reported in the next chapter expand upon this hypothesis.

ROLE OF ALPHA-GALACTOSIDE-BINDING PROTEINS IN THE SPECIFICITY OF PATHOGENESIS

In the previous chapter, experiments revealed that one parameter which governed both the specificity and kinetics of ligand binding in vitro was the protein concentration. This idea led to a hypothesis that carbohydrate transport in general and sensitivity to the toxin in sugarcane may be a function of the protein concentration in vivo. An extension of this hypothesis evolves from the rationale that protein concentration in the membranes of cells is not constant. A common feature during maturation of plant cells is enlargement. As the cell expands so must the membranes increase in surface area. One alteration associated with this expansion may be a change either in the absolute quantity of or lateral localization of membrane-bound alpha-galactoside-binding proteins. Either variation would effect a change in the protein concentration at a particular site in the membrane and alter the chances of either sugar transport or toxic reaction.

Other changes in the membrane upon ageing could involve the amount and type of lipids present at different stages. Lipid-protein interactions are known to affect the activity of many integral membrane proteins. Apo-D-(-)-beta-hydroxybutyric dehydrogenase, the delipidated form of the

enzyme, is inactive. Reinsertion of the apoprotein into micelles of phosphatidyl choline causes a reactivation of enzymatic activity (31). Alteration of the acyl chain length by two carbon atoms causes significant changes in the activity and conformations of two mammalian membrane diglycerol phosphotransferases in micellar suspensions (42). When the glycoprotein of thyroid thyrotropin receptor is embedded in a liposome of dipalmitoyl phosphatidyl choline, it binds both thyrotropin and tetanus toxin. Changing the liposomal matrix to dioleoyl phosphatidyl choline stimulates thyrotropin binding but does not affect tetanus toxin binding (36). A bacterial galactosyl transferase loses activity upon removal from the membranes. Activity is restored when the protein is incorporated into a matrix of phosphatidyl ethanolamine and lipopolysaccharide. Neither phosphatidyl inositol or phosphatidyl choline is as effective at restoring the transferase activity (7). Membrane proteins have also been shown to exhibit a species specificity in terms of lipid binding (35). Sheep red cell membranes are characterized by a low phosphatidyl choline and high sphingomyelin content: human erythrocyte membranes are just the opposite. Results of recombining delipidated sheep membrane proteins with either human or sheep membrane stroma demon-

strate a preferential binding of sphingomyelin by the sheep proteins.

The presence of specific types of lipids, then, do control the activity and specificity of membrane proteins. Simply placing membrane proteins into a hydrophobic environment is not always sufficient to enable those proteins to mimic biological activity.

It is fortunate the alpha-galactoside-binding proteins do retain measurable activity in a hydrophillic medium. Such activity permits experimental modulations of the assay environment which may reveal pertinent conditions required for isolated proteins to regain normal biological activity. Alterations in the assay medium were evaluated in vitro as they affect helminthosporoside binding. The hypothesis concerning the effect of ageing on toxin sensitivity was tested in vivo. Results of these experiments and a discussion of their relevance follow.

Reaction of plants to H. sacchari and helminthosporoside

The ability to bind helminthosporoside is a critical property of clones of sugarcane susceptible to H. sacchari (59, 60). However, toxin binding alone is not sufficient for pathogenesis (11, 33, 66). Because all the plants in

this study, including resistant sugarcane H50-7209, can bind the toxin under certain conditions (Table 7), it was necessary to evaluate the susceptibility of each plant to the pathogen as well as the sensitivity of whole leaves to helminthosporoside preparatory to a discussion of the effects of assay environment on the specificity of toxin binding.

Only 51NG97 sugarcane and napier grass were found to be susceptible to the fungus. Only 51NG97 was sensitive to the toxin (Table 18). In regard to the model of pathogenesis presented for the eyespot disease (65), the susceptibility yet lack of sensitivity demonstrated by napier grass make this plant a good subject for evaluation of the reactions subsequent to toxin binding (12, 66). Some plants of napier grass were reported to develop red runners when infected, but the majority developed only the reddish flecks noted in Table 18 (70).

Effect of extraction medium on differential toxin binding

In addition to the intrinsic differences in the binding proteins as depicted in Chapter 3, different plants and different varieties of the same plant may harbor innate differences in the composition of their plasmalemmas. Such differences, if they modify the in vitro activity of binding

Table 18. Reaction of different plants to H. sacchari and helmintholporoside.

Plant	Susceptible? ^a	Symptoms	Sensitive? ^b
51NG97	yes	eyespot, runners	yes
H50-7209	no	none	no
Mint	no	none	no
Tobacco	no	none	no
Napier Grass	yes	red flecks	no

^aSusceptible plants were those which developed symptoms upon inoculation with H. sacchari and from which the fungus was reisolated after 6 weeks.

^bSensitivity to the toxin was evaluated by the leaf puncture assay (57) using 2 ul helminthosporoside (4.5 mM). Reddish runners appeared on 51NG97 within 24 h. The other plants remained symptomless for 6 days after which they were discarded. Three leaves from each plant were tested on two different occasions.

proteins, should be detectable when assaying the toxin-binding activity of variously extracted preparations of membrane proteins. This expectation is based on the premise that different membrane constituents (glycolipids, proteins) should be selectively extracted by different solvents.

Crude membranes from 51NG97 (susceptible) and H50-7209 (resistant) sugarcane were obtained as outlined on Figure 2. Each membrane preparation was divided into three portions, then homogenized in Tris-HCl (10 mM, pH 7), Tris containing 1.0 M trichloroacetate, or Tris containing 1% Triton X-100. After 1 h at 4C, the suspensions were centrifuged at 48,000g for 15 min. The supernatant fluids (which varied from a turbid yellow-green in the Tris and Tris-trichloroacetate extracts to a clear dark green in the Tris-Triton extract) were then assayed for toxin-binding activity via equilibrium dialysis.

Toxin-binding activity was consistently demonstrable in preparations from susceptible sugarcane regardless of extracting agent (Table 19). However, H50-7209 showed toxin binding in Tris buffer extracts only. Extraction via either the chaotrope or non-ionic detergent inhibited the helminthosporoside binding in fresh preparations from resistant sugarcane. If the trichloroacetate and Triton X-100

Table 19. Effect of extraction medium on differential toxin-binding activity of 51NG97 and H50-7209 proteins.

Toxin concentration: 2.2×10^{-5} M.

Protein concentration: 100 ug/ml.

Extraction Buffer	Specific Binding Activity ^a $\times 10^{-2} \frac{\text{mmole toxin}}{\text{g protein}}$	
	51NG97	H50-7209
Tris	5.5	3.7
Tris + 1.0 M Trichloroacetate	2.8	<0.1
Tris + 1% Triton X-100	9.6	<0.1

^aValues represent the average of two determinations. A different membrane preparation was used for each determination. Variability was less than $\pm 15\%$.

extracts of resistant sugarcane were stored for three weeks at 4°C, the helminthosporoside binding activities were 0 and 5.2×10^{-2} mmole/g protein, respectively. Strobel also noted this long-term acquirement of toxin-binding ability in detergent solubilized membranes from H50-7209 (63).

Effect of detergent or chaotropic amendments on toxin-binding activity

One inference from Table 19 might be that the extracting agent is acting directly on the binding protein rather than effecting a selective extraction of associated membrane molecules. To test this inference, various amendments were added to the usual equilibrium dialysis assay medium (Tris-HCl, 10 mM, pH 7) containing purified binding protein. These amendments, present in both half-cells of the dialysis chamber, were 1% Triton X-100, 0.2 M trichloroacetate, and 80% 2-chloroethanol. The non-ionic detergent inhibited toxin-binding activity whereas the two chaotropes stimulated binding (Table 20).

2-Chloroethanol was an intriguing perturbant to use in this situation. A chaotrope, it has as yet inexplicable properties of promoting alpha-helical conformations of proteins in solution thereby preventing entanglement of polypeptide chains (72). It caused a larger increase in binding

Table 20. Effects of detergent and chaotropic amendments on toxin-binding activity of purified binding proteins.

Toxin concentration: 1.4×10^{-4} M.

Protein concentration: 3.8 to 4.0 ug/ml.

Buffer: Tris-HCl, 10 mM, pH 7.0.

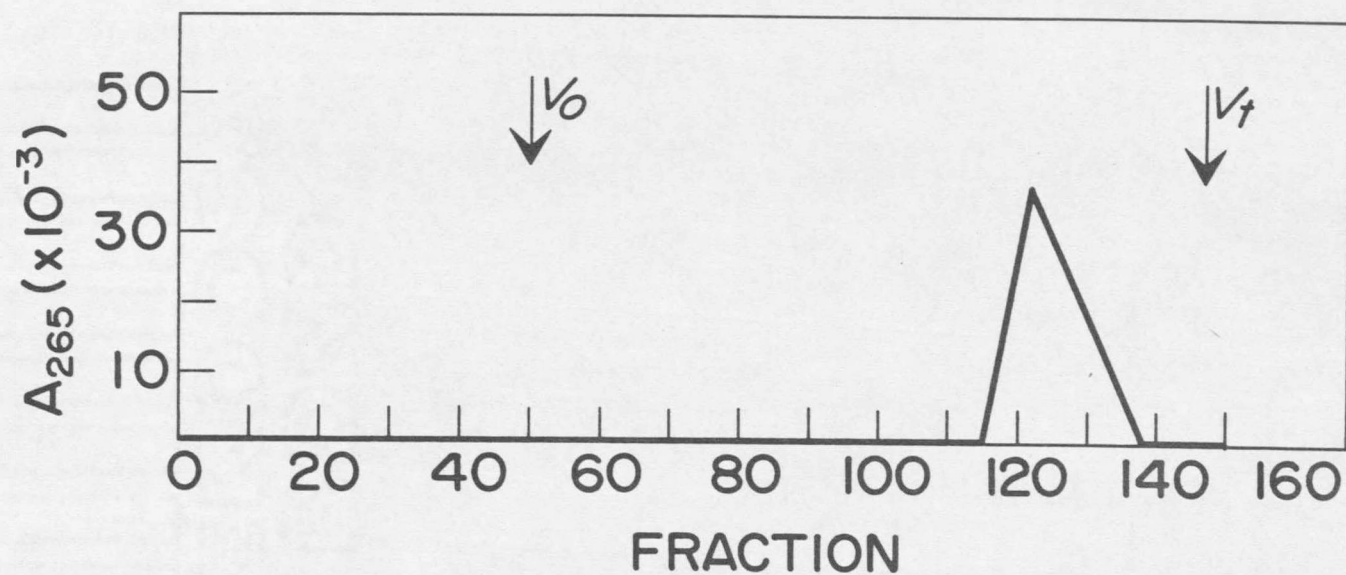
Amendment	Specific Binding Activity ^a		
	<u>mmole toxin</u> <u>g protein</u>		
	51NG97	H50-7209	Napier Grass
none	6.6	6.6	3.9
1% Triton	5.2	4.0	1.4
0.2 M trichloroacetate	7.3	7.1	4.5
80% 2-chloroethanol	16.3	20.8	18.6

^aValues represent the average of 2 replicates.
Variability was less than $\pm 8\%$.

activity than did trichloroacetate, the stimulations for 5LNG97, H50-7209, and napier grass being 2.5-fold, 3.2-fold, and 4.7-fold (Table 20). One insight into its promotive effects was revealed when purified 5LNG97 binding protein was incubated with 80% 2-chloroethanol:20% Tris-HCl, 10 mM, pH 7 (25 ug protein/0.1 ml) for 1 h at room temperature then chromatographed on a Sephadex A1.5 sizing column which had been equilibrated with the incubation buffer. The elution profile (Figure 17) showed a single peak corresponding to a molecular weight of 130,000. In Tris buffer lacking 2-chloroethanol, this concentration of protein yielded four peaks representing different species of molecular weights (Figure 13). 2-Chloroethanol, then, may be stimulating binding activity via its ability to prevent protein-protein interaction and the resultant polymer formation.

Effect of galactosyl glycerides on binding activity

To observe the effects of exogenous lipids on the binding activity of purified proteins, monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) (Figure 18) were added separately to the assay medium, then either toxin binding or raffinose binding was evaluated. Galactosyl



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Figure 17. Elution profile of 51NG97 binding protein on Sephadex A1.5 in the presence of 80% 2-chloroethanol. Protein concentration: 50 ug/0.2 ml.

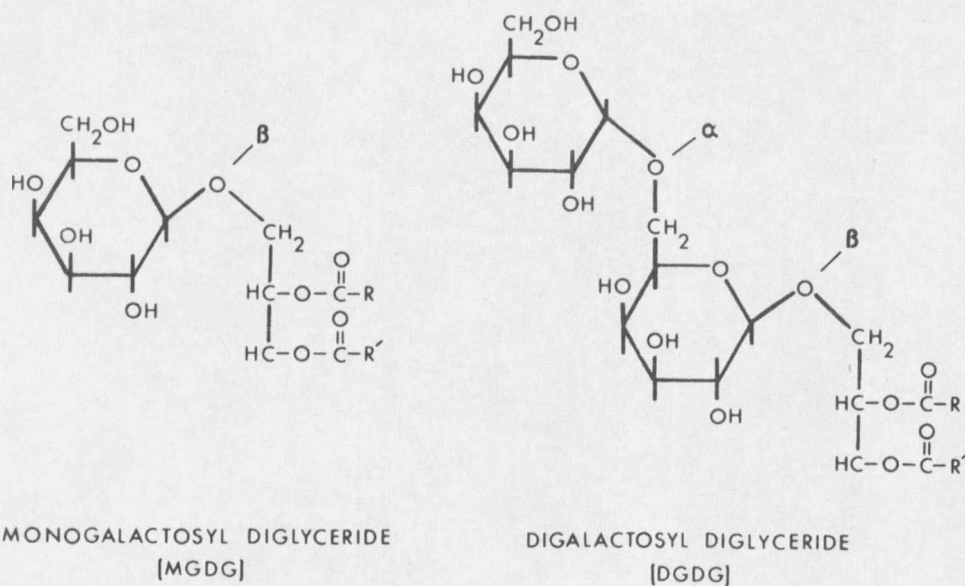


Figure 18. Structures of monogalactosyl diglyceride and digalactosyl diglyceride. Note the location of alpha and beta linkages.

glycerides were chosen because DGDG was found to comprise approximately 75% of the lipids in the plasmalemma of 51NG97 (55). DGDG contains two galactosyl units, one in a beta-linkage to the glycerol backbone and one in an alpha-linkage to the first galactose (Figure 18). MGDG, which is a beta-galactoside only, was included to aid in discerning inhibitory effects of the lipid versus a possible competitive inhibition with raffinose or helminthosporoside due to the alpha-galactosyl linkage present in DGDG. A cautionary note when interpreting the effects of these glycolipids is necessary because the fatty-acid side chains may not be identical for both.

Concentrations of the glycolipids were established at 2.4×10^{-11} M because the critical micelle concentration for biological lipids is in the range of 10^{-9} to 10^{-10} M (67). Assays in which the glycolipid concentration was 10^{-5} M revealed a total inhibition of binding activity of each protein for both raffinose and helminthosporoside.

At the lower concentration of MGDG and DGDG, the following observations were noteworthy: a stimulation of raffinose binding by MGDG; a 57% inhibition of toxin binding by H50-7209 protein in the presence of MGDG in contrast to no effect on 51NG97 protein; total inhibition of both

raffinose and toxin binding by H50-7209 protein in the presence of DGDG in contrast to no effect on raffinose binding and an 85% inhibition of toxin binding by 51NG97 protein (Table 21).

Effect of tissue age on response to helminthosporoside

The hypothesis has been presented that the physiological age of tissue, by virtue of the fluxes in protein concentrations in the plasmalemma during maturation, may be correlated to effective carbohydrate transport or to toxin sensitivity. One way to test this hypothesis is to compare the reaction to exogenous helminthosporoside in different parts of leaves from susceptible sugarcane, 51NG97. It is known that the length of the runner produced by toxin is concentration dependent (59). However, this and other reports indicate a large variability in response, even in different sections from the same leaf (11, 59). Perhaps this variability was due to the comparison of samples of different physiological age.

In monocots, leaves elongate acropetally from a residual intercalary meristem near the juncture with the stem. Thus, the youngest tissue in a leaf will be that nearest the stalk. It is not known if the chronological age of the tis-

Table 21. Effects of lipids on toxin-binding activity of 51NG97 and H50-7209 proteins.

Toxin concentration: 2.2×10^{-5} M.
 Protein concentration: 2.0 ug/ml.
 Raffinose concentration: 3.5×10^{-5} M.
 MGDG and DGDG concentrations: 1×10^{-11} M.
 Buffer: Tris-HCl, 10 mM, pH 7.0.

Protein	Ligand	Specific Binding Activity ^a <u>mmole toxin</u> <u>g protein</u>		
		Control	MGDG	DGDG
51NG97	Raffinose	1.0	2.5	1.1
H50-7209	Raffinose	0.2	0.3	<0.001
51NG97	Toxin	2.7	2.7	0.4
H50-7209	Toxin	3.0	1.3	<0.001

^aValues represent an average of 2 replicates.
 Variability was less than $\pm 10\%$.

sue varies transversely in the leaf, but the physiological age of the cells could be expected to increase towards the leaf margin. In any one section, then, the physiological age can vary tremendously.

To test the hypothesis on ageing, 5-day old to 3-month-old leaves were removed from 51NG97 sugarcane and cut into sections 10 cm long beginning at the base or end nearest the stalk. Sites midway between the midrib and margin, 2 cm from the base of the section were punctured then overlaid with 1 μ l of helminthosporoside (8.5×10^{-5} M). Runner lengths were measured 48 h later. Data were bulked into five groups representing increments of 20% of the total leaf length (Figure 19). A total of 199 observations were made on 24 leaves: the observations per group ranged from 39 to 43. Although the runner length varied from 0 to 8 cm in any age group, nearly all of the observations were within a relatively narrow range. For this reason, the highest value and lowest value for each age group were discarded when the bulked means were calculated. One source of this variation was the placement of the puncture site at the transverse midpoint. A subsequent study revealed that a site approximately two-thirds of the distance between midrib and margin, nearest the margin, yielded the maximal response per

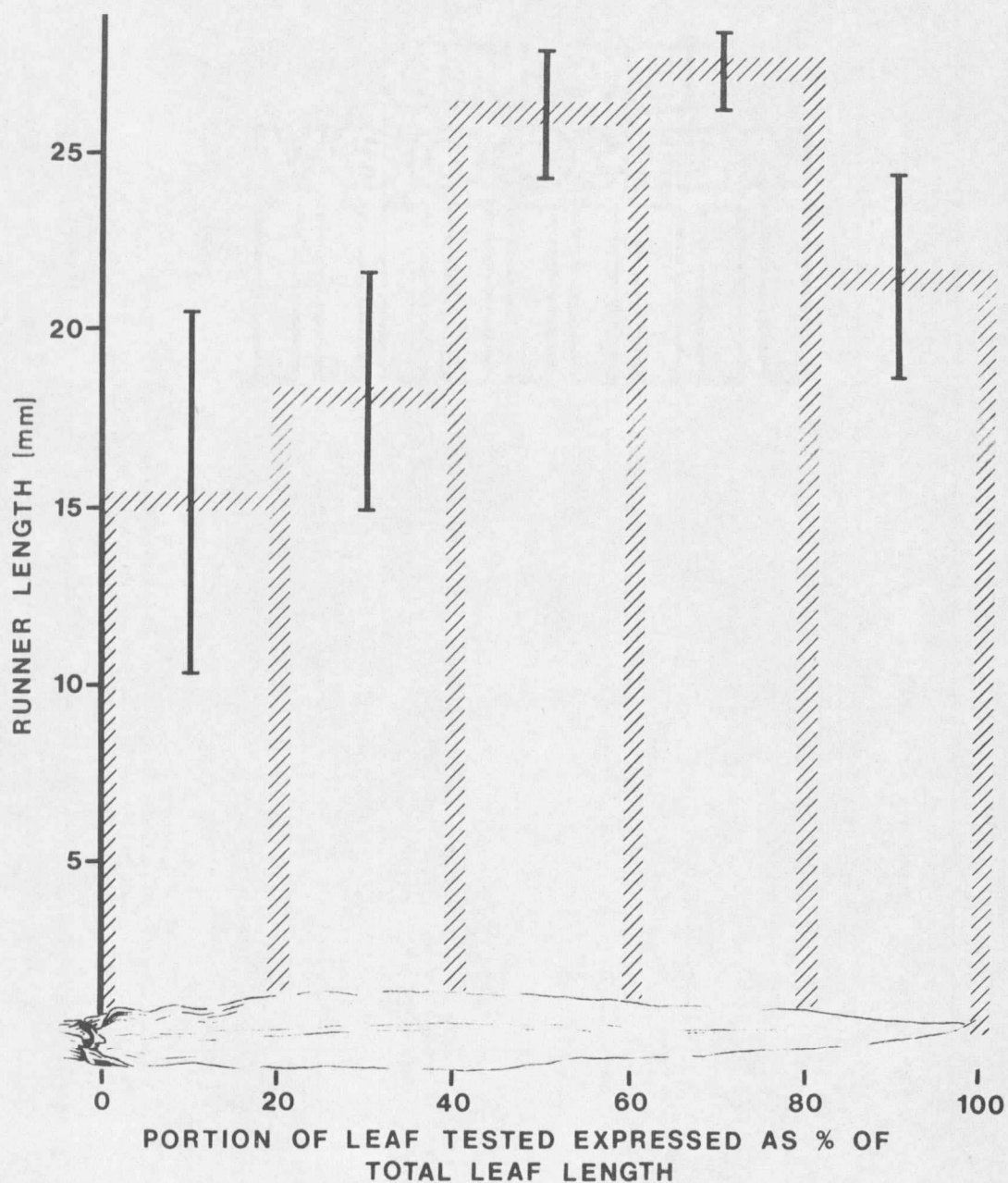


Figure 19. Response of incremental leaf sections to exogenous helminthosporoside (1 μ l, 8.5×10^{-5} M). The means of the runner lengths for each section are represented by the horizontal, slanted-line bars. Solid, vertical bars depict the standard deviation.

section. Because the physiological age increases both transversely and longitudinally, the midpoint of a section may not always be more mature than the midpoint of the section acropetal to it thus confounding age-related observations.

Regardless, the data reveal a maximal response to toxin occurring in the area comprising 40-80% of the total leaf length (Figure 19). When analyzed using Duncan's multiple range test the mean runner lengths of the per cent increments fell into three significantly different groups at the 95% confidence level: 0-20% = 20-40%; 40-60% = 60-80%; and 80-100%. The decrease in sensitivity in the oldest portion of the leaf was likely caused by the presence of dead and dying tissue in this region. Much of the leaf tip area appeared chlorotic and brown even when the rest of the leaf was healthy.

Discussion and summary

Two major conclusions are supported by the preceding experiments. First, the specificity and extent of binding by alpha-galactoside-binding proteins can be modified by exogenous effectors or by factors inherent in the cellular membranes. Second, sensitivity to exogenous helmintho-

sporoside is correlated to the physiological age of the tissue being tested.

To expand the first conclusion, it appears that, in addition to intrinsic differences in the binding proteins from resistant and susceptible sugarcane, qualitative differences which dictate toxin-binding abilities exist between the cellular membranes of these two varieties. That differential toxin-binding activity is not dictated solely by the binding protein is arguable by the following observation: treatment of purified binding protein from H50-7209 with trichloroacetate causes an 8% stimulation of toxin binding whereas toxin binding is not detectable in trichloroacetate-solubilized crude membranes (Table 19, Table 20). Triton-solubilized proteins from H50-7209 gradually acquire toxin-binding activity yet trichloroacetate-solubilized do not. This acquirement may be caused by a slow displacement by detergent molecules of lipids normally associated with the protein. Yet another argument supporting the contention that innate differences in membranous compositions are in part, responsible for differential sensitivity to helminthosporoside is that crude membranes homogenized in neat Tris buffer exhibit essentially no clonal differences in toxin binding (Table 19). This treatment of the membranes

likely precludes much, if any, extraction of integral membrane components.

The importance of the intrinsic properties of the binding must not be overlooked, however. Quantitative differences did occur in the response of purified proteins to different amendments (Table 20, Table 21). Most significantly, it was possible to severely inhibit the toxin-binding activity of purified H50-7209 protein with MGDG - a molecule which did not affect toxin binding by 51NG97 protein. Also, Strobel reported that transferral of 51NG97 protein to protoplasts of H50-7209 was concomitant with transferral of toxin sensitivity to these protoplasts. To express specificity, a protein must possess not only the potential to bind helminthosporoside, but also the potential to be responsive to a particular membranous milieu.

The second conclusion, that of the age-related sensitivity of plant tissue, is not new. Reports abound concerning changes in susceptibility as a plant ages. For the sugarcane-H. sacchari interaction, however, it may be possible to define this increased sensitivity upon maturation in molecular terms.

When the fungus invades the plant, the earliest symptoms appear as minute watery spots on the youngest leaves.

After four to six days, characteristic red runners emanate from these eyespot lesions (39). Our studies revealed no significant differences in the bulked means of runner lengths on 5-day-old leaves, the youngest tested, compared to runner lengths on older leaves. Perhaps in leaves less than five days old a significantly lesser response to toxin would occur similar to the intra-leaf variation depicted in Figure 19. G. W. Steiner, who helped pioneer the use of helminthosporoside as a screening agent for selection of resistant sugarcane stated that newer leaves were more resistant to the toxin than were middle-aged leaves. Spindle leaves, the youngest, rarely showed symptoms when sprayed unless the clone was highly susceptible (58).

Of the many types of maturation processes which could occur to increase the sensitivity of leaves, one which has experimental support (Chapter 4) entails the effective dilution of membrane-bound alpha-galactoside-binding proteins during elongation and expansion of individual cells. As the cells enlarge, the area of the plasmalemma will increase and membrane protein populations may approach optimal binding concentrations, at least on a statistical target site basis. Circumstantial evidence in further support of this hypothesis is that the yield of alpha-galactoside-binding

protein is 4-fold greater from the basal 10 cm section than from a similar section taken from 0.55-0.65 of the total leaf length (3.2 ug/g fresh weight versus 0.8 ug/g fresh weight).

Additional experimentation pertinent to the cellular ageing-protein dilution hypothesis involves treating the membranes in vivo with reagents which cross-link proteins (73). If the proteins in young cells are significantly more concentrated than receptors in old cells, it should be possible to cross-link them. Cross-linked proteins should not be sensitive to dilution effects so they should be detectable both kinetically and physically (Chapter 4).

The symptomology during pathogenesis may devolve because the younger portions of a susceptible leaf are more amenable to establishment of the fungus yet much less sensitive to the toxin produced by this pathogen because the binding protein is too concentrated. An eyespot sans runner is then an early symptom. After four to six days, enough cellular enlargement has occurred to effectively dilute the binding proteins to concentrations which permit sufficient toxin binding for macroscopic runners to be produced. Symptom expression in older portions of artificially inoculated leaves of 51NG97 were observed to vary from the

above pattern: runner development was concomitant with the appearance of the eyespot lesion - an observation consistent with the hypothesis that more effective concentrations of binding protein exist in the older parts of leaves.

PERSPECTIVES

"Nature, it seems, is the popular name for milliards and milliards and milliards of particles playing their infinite game of billiards and billiards and billiards." (23)

For an accurate observation of any shot in nature's game of billiards, a discerning probe is essential. The alpha-galactoside-binding activity of proteins isolated from plant cell membranes serves such a purpose. The receptors can be monitored both in solution and in the cell via observations on the kinetics and specificity of binding. From the observations presented in this dissertation, a perspective of pertinent biological mechanisms is possible.

A common theme throughout has been the applicability of these observations to carbohydrate transport in plants although no actual transport data were presented. In truth, until some physio-chemical method of identifying the isolated binding protein in the membrane transport receptor is developed, inferential evidence must suffice. The prevalence of alpha-galactoside-binding proteins among plants and their documented ability to bind naturally occurring alpha-galactosides at levels sufficient for transport phenomena offer strong corroborative support for the involvement of these proteins in membrane transport. If the binding pro-

teins do participate in alpha-galactoside transport, an elaboration of their potential would be fruitful in terms of learning how to control their activity in the plants.

An initial problem in understanding the mechanics of the transport process is deobfuscating the structure of the transporter as it resides in the membrane. The five best characterized transport proteins in mammalian cells are all transmembrane, oligomeric glycoproteins (20). They are asymmetric in the membrane and perform the transport process via some conformational change other than transmembrane migration. It is not known if the alpha-galactoside proteins are glycoproteins, but they are oligomeric. They may, indeed, be analogous to the transport proteins of other eukaryotic cells, but an arrangement like the histidine transport system in Salmonella typhimurium cannot be ruled out. The bacterial mechanism involves a two-part system comprised of an integral membrane component which is associated with a periplasmic histidine receptor. Genetic lesions in either protein can block transport (2). Perhaps the alpha-galactoside transport process in higher plants consists of a peripherally located receptor which is linked to an integral membrane component. An alteration in a membrane component which prevents the alpha-galacto-

side receptor from assuming an active binding conformation could be the reason for the lack of raffinose transport in resistant sugarcane protoplasts and inability of freshly isolated, detergent-solubilized H50-7209 protein to bind raffinose or toxin. The raffinose- and toxin-binding abilities of H50-7209 protein after preparation in a manner which minimizes the presence of integral membrane components is support for, but not restricted to, a two-component transport system.

An absolute structure of the transport system is desirable but not essential to understanding enough about the process to be able to control it. Kinetic studies loom in this regard. Many examples have been presented in which the binding activity, the first step of any transport mechanism, is dependent upon the environmental parameters. Even the specificity of binding, an immutable aspect in the biology of this phenomenon can be controlled by the presence of certain lipids or relative protein concentrations. Non-membrane molecules could also affect binding. Specificity in a bacterial melibiose transport system is controlled by the presence of Li^+ . Li^+ causes the transporter to bind both melibiose and beta-thiomethylgalactoside. In the absence of Li^+ only melibiose, an alpha-galactoside, is

bound (69). Thus, anomeric specificity could be a function of metal cofactors. Considering the current interest in plant productivity, methods which stimulate carbohydrate binding activity should be very useful for those wishing to increase the nutritive value of desirable crop organs — seeds, leaves, tubers. Perhaps useful levels of reagents like 2-chloroethanol could be found. Alternatively, plant breeders could use carbohydrate transport proteins for genetic markers and potentiate the plant for higher carbohydrate uptake and storage — a feasible way of increasing photosynthetic efficiency.

The dependence of binding activity on protein concentration has implications for a link between carbohydrate uptake and cellular differentiation. If a temporal flux in membrane protein concentration is occurring in vivo, a type of non-nuclear regulation of carbohydrate transport is possible. It makes sense that physiologically immature cells in the leaf likely do not contribute much to the carbohydrate flow out of the leaf. Most of their energy would be utilized in the construction of the cell itself. As growth slows, however, the cell would be capable of producing more than it required and could then participate in the source-sink relationship of translocation. Highly con-

centrated, kinetically dampened binding proteins in the immature cell membrane would limit carbohydrate efflux. Diluted, active proteins in more mature cells would allow the efflux and automatically establish a disequilibrium in the carbohydrate flow within the cell without the need for specific inducers or activators. The system is hypothetical and certainly not the only way of controlling sugar metabolism. The hormonally-induced glycogenolysis in animal tissues could be occurring in plants. Here again, immature cells less capable of participating in sugar release would benefit if the activity of membrane-bound hormone receptors is concentration-dependent. Target tissues would then be temporal matters rather than specific tissues as in animals. An increased activity upon dilution offers an energy-efficient method for regulation of carbohydrate transport in leaf cells.

The converse situation is possible in "sink" cells. Here, it would be more efficient if sugar uptake were more active under conditions of highly concentrated transport proteins, a likely situation in tissues of densely compacted cells as in the embryo and endosperm. Evaluation of seeds or storage organs would be very interesting in this regard.

In sugarcane, the alpha-galactoside transport system

is linked to a varietal susceptibility to the eyespot organism through the activity of the toxin, helminthosporoside. The same protein appears to mediate alpha-galactoside uptake and sensitivity to toxin (63). The binding of toxin by receptor in susceptible sugarcane is the specific, first step in the molecular events of pathogenesis, but is not sufficient of itself to produce symptoms (33). The potential for toxin binding is an intrinsic property of all the alpha-galactoside binding proteins studied, even those from resistant sugarcane (Table 7). Expression of this toxin-binding potential in vivo is subject to control by additional membrane constituents, glycolipids or proteins. Thus, an ability to respond to natural effectors is an additional intrinsic property of the binding proteins which governs both the specificity and kinetics of toxin binding.

The proteins from 51NG97 and H50-7209 differ physically and chemically. They differ kinetically in their response to self-association and presence of lipids. The exact nature of the difference which confers the varietal sensitivity to helminthosporoside in vivo has not been resolved in this study, but many nuances in mechanisms of resistance have been revealed.

Sensitivity is seen to be temporal, even in suscepti-

ble plants. One hypothesis for the increased sensitivity is the dilution of membrane receptors to a more active form. This phenomenon may not be restricted to plants. Digitalis, a plant-produced glucoside effective in stimulating ionotropic responses in heart muscle, binds with a membrane receptor that is either identical to or closely associated with a mammalian ATPase. Digitalis has a narrow dose range above which it is extremely toxic. With increasing age of the patient, the toxicity of digitalis increases - an observation analogous to the toxicity of helminthosporoside in sugarcane (6, Figure 19). Perhaps, ageing in mammalian cells is also manifested in part as fluctuating receptor concentrations - an as yet unexplored aspect of differentiation and senescence.

From the results of the lipid-amendment experiments, these membrane molecules also participate in the specificity of binding. Using a deuterated lipid as a probe, researchers have shown that membrane proteins do interact with membrane lipids and restrict the motion of those lipids. This perturbation effect is apparent both adjacent to and distant from the protein (14). In sugarcane, varietal differences in the type or amount of pertinent lipids could confer specificity.

Clones intermediate in their sensitivity to toxin will be interesting subjects. The intermediate reactions could arise from a mosaic of binding and nonbinding proteins present in the membranes. At the same level of toxin, then, the number of toxicity-inducing hits could be less than in highly susceptible varieties. A further possibility is inherent in the oligomeric structure of binding proteins. Given that equilibrium monomer $\rightleftharpoons$ tetramer $\rightleftharpoons$ octomer occurs in the membrane, mosaic binding proteins could be produced having less of an affinity for the toxin.

All of these hypothetical situations are testable and likely exist somewhere in the world's sugarcane population. They deal only with the binding step, however, and the ensuing reactions of pathogenesis must be considered for a more complete understanding of this disease. Just as many factors influence the ability of a protein to bind the toxin, many conditions must control the mediation of the heat-sensitive reactions by the protein-toxin complex. Evidence that activation of plasmalemmar (K⁺, Mg⁺⁺)-ATPase is one of the reactions pursuant to binding is credible (66). The ability to obtain highly purified, detergent-free binding proteins offers another test of the involvement of ATPase. The toxin is known to stimulate ATPase activity in

plasma membranes prepared from susceptible sugarcane (63). Similar treatment of plasmalemmas from resistant sugarcane or mint do not cause this stimulation (63, Strobel-personal communication). Addition of purified 51NG97 protein to mint and H50-7209 plasma membranes should cause increased ATPase activity if the only difference in sensitivity resides in the binding protein. Purified mint protein added to H50-7209 plasma membranes should not cause the ATPase stimulation under this premise.

Full resolution of the role of alpha-galactoside-binding proteins in pathogenesis and carbohydrate transport will come only when the membranes can be synthesized in vitro with specific constituents. Purification and characterization of the binding proteins is a step along the way, but quantitating the additional components essential for biological activity remains.

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