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Structural Requirements for the Binding of Phenylglycosides to the Surface of Protoplasts[1]

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EUGENE A. NOTHNAGEL* AND J. L. LYON
Department of Botany and Plant Sciences, University of California, Riverside, California 92521

ABSTRACT

A variety of phenylglycosides have been synthesized and tested for binding to the surface of protoplasts from suspension-cultured cells of "Paul's Scarlet" rose (*Rosa* sp.). Multivalent phenylglycosides in the form of Yariv antigens (1,3,5,-tri-[*p*-glycosyloxyphenylazo]-2,4,6,-trihydroxybenzene) agglutinated the protoplasts. Fluorescence-labeled derivatives of other monovalent and polyvalent phenyl-β-glycosides did not bind to the protoplast surface. Agglutination was induced by Yariv antigens only if these probes contained β-anomeric, O-glycosidic linkages. Yariv antigens containing α-anomeric or thio-glycosidic linkages did not agglutinate protoplasts. These same structural features of Yariv antigens were also required for the precipitation of gum arabic-Yariv antigen complexes. The results suggest that plasma membranes of "Paul's Scarlet" rose protoplasts contain arabinogalactan-proteins that interact with phenyl-β-glycosides. The results further show that binding at these plasma membrane sites is not solely dependent upon the carbohydrate portion of single phenylglycosides, but may also require specific spatial orientations of adjacent phenylglycosides.

AGPs[2] are water-soluble proteoglycans found in most flowering plants (10) as well as in liverworts and other lower plants (4). At the cellular level, AGPs are found predominantly in the extracellular space (4, 5, 10, 16) with lesser amounts present in the cytoplasm (1, 19) and at the protoplast surface (4, 5, 17). Functional roles in cell-cell adhesion, pollen-stigma recognition (3), and water retention have been suggested (2, 7) for these macromolecules, although none of these proposed functions have been established.

A property of AGPs that may be related to their biological function is their affinity for certain phenylglycosides. Investigations of this property (4, 8–10, 12, 23) have generally relied on use of synthetic, multivalent probes of the general structure (1,3,5,-tri-[*p*-glycosyloxyphenylazo]-2,4,6-trihydroxybenzene). These probes are commonly referred to as Yariv antigens (24), and the structure of one such Yariv antigen is shown in Figure 1. This particular Yariv antigen, (β-D-Glc)$_3$, contains three β-D-glucoside arms, but similar molecules containing other sugars in either α- or β-anomeric linkage can be synthesized (24). Because Yariv antigens self-associate in aqueous solutions to form complexes of 10 to 50 molecules (22), the effective valency of these molecules is probably much greater than the trivalency suggested by their chemical formula.

Certain Yariv antigens form tight complexes with AGPs, as evidenced by the facts that these Yariv antigens can precipitate AGPs from aqueous solution (1, 10) and can specifically stain AGPs in isoelectrofocusing gels (17). In a pioneering study, Jermyn and Yeow (10) found that Yariv antigens could precipitate macromolecules from buffer extracts of a wide variety of plants if the Yariv antigens contained β-linked D-gluco-, D-galacto-, D-xylo-, malto-, lacto-, or cellobiopyranosyl residues. Since Jermyn and Yeow (10) found that precipitation did not occur with Yariv antigens containing any of these sugars in α-linkage, they gave the name 'all β-lectins' to the precipitated macromolecules. These β-lectins found by Jermyn and Yeow are now known as AGPs (2, 7).

The presence of some form of AGP on the plasma membrane was first suggested by observation that Yariv antigens, when used as histochemical reagents, stained the membrane-cell wall interface (4, 5). Larkin (11, 12) subsequently showed that protoplasts from a wide variety of plants could be agglutinated by many of the same Yariv antigens that precipitated AGPs. Recently, Samson *et al.* (17) have shown that a plasma membrane fraction from *Phaseolus* hypocotyls contains AGP, as judged by the presence of hydroxyproline and the staining of a low pI component by a Yariv antigen. Thus it seems likely that the plasma membrane component mediating the protoplast agglutination observed by Larkin (11, 12) is closely related to the water-soluble AGPs, although the details of this relationship have not been established.

The present study was undertaken to characterize further the binding of phenyl-β-glycosides to the protoplast surface. Specifically, we have tested the binding of some modified Yariv antigens in protoplast agglutination experiments and have compared the agglutination potencies with the abilities of these Yariv antigens to precipitate gum arabic. We have also synthesized some phenyl-β-glycosides that are not Yariv antigens and have tested these phenyl-β-glycosides for binding at the protoplast surface. A preliminary report of some of these results has appeared as an abstract of a poster presentation (15).

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[2] Abbreviations: AGP, arabinogalactan-protein. The nomenclature used here for Yariv antigens is similar to that used by Larkin (12). Thus, (β-D-Glc)$_3$ represents the Yariv antigen containing three β-linked D-glucopyranosyl arms. Similarly, (Thio-β-D-Gal)$_3$ represents the Yariv antigen containing three β-linked thio-D-galactopyranosyl arms. MPR, minimal organic medium (13); PFP-β-D-Glc, *p*-(fluoresceinthioureidyl)-phenyl-β-D-glucopyranoside; PFP-α-D-Glc, *p*-(fluoresceinthioureidyl)-phenyl-α-D-glucopyranoside; β-D-Glc-Tyr, poly-*p*-β-D-glucosylphenylazo-L-tyrosine; α-D-Gal-Tyr, poly-*p*-α-D-galactosylphenylazo-L-tyrosine; TR-β-D-Glc-Tyr, Texas Red-labeled β-D-Glc-Tyr; TR-α-D-Gal-Tyr, Texas Red-labeled α-D-Gal-Tyr; DMF, *N*,*N*-dimethylformamide; Lac, lactose.

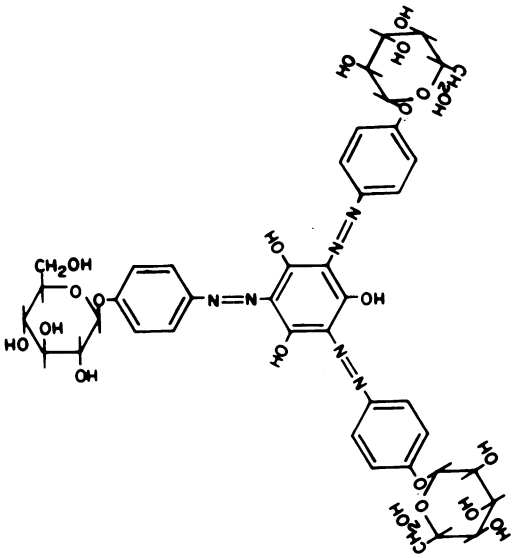

FIG. 1. Chemical structure of the $(\beta$-D-Glc$)_3$ Yariv antigen. Several similar molecules containing other sugars in either α- or β-anomeric linkage were also synthesized.

MATERIALS AND METHODS

Protoplasts. Suspension-cultured cells of 'Paul's Scarlet' rose (*Rosa* sp.) were derived from a callus culture kindly provided by John S. Fletcher (University of Oklahoma) and were grown in MPR medium formulated as described (13) except that molybdic acid and potassium iodide were present at 0.01 and 0.50 mg/L, respectively. All media used with protoplasts were based on MPR medium but were supplemented with sorbitol, additional sucrose, or other ingredients as indicated. Driselase from Plenum Scientific Research, Inc. was purified before use by column chromatography on Bio-Gel P-6. The components from Driselase that eluted in the void volume of this column were collected, lyophilized, and used as the cell wall-degrading enzyme for all experiments reported here.

Protoplasts were released from suspension-cultured cells that had been grown on a 3- or 4-d transfer cycle. At 36 to 40 h after the last transfer, the cells were washed with fresh MPR medium and then packed by centrifugation (2 min at 100*g*). The supernatant was removed down to the level of the cells, and then MPR medium supplemented with 950 mM sorbitol was added in a volume equal to two times the packed cell volume. After 15 min of this preplasmolysis, the enzyme mixture was added in a volume equal to the packed cell volume. The enzyme mixture consisted of 4% (w/v) purified Driselase, 1% (w/v) BSA, and 2 mM DTT, all dissolved together in MPR medium. After addition of the enzyme mixture, the cells were incubated in the dark at 26°C with gentle (30 cycles per s or less) shaking for 3.5 h. The suspension was then passed through a nylon net having 64 μm openings (Tetko, Inc., Monterey Park, CA) and then incubated for an additional 1 h. The protoplasts were purified from the suspension digest by centrifugation (5 min at 100*g*) onto a pad of MPR medium supplemented with an additional 450 mM sucrose. The protoplasts were collected from the interface, resuspended in MPR medium supplemented with 450 mM sorbitol, and washed by similar centrifugation four additional times, including one time in which the protoplasts were resuspended in the sucrose solution and then centrifuged up to the interface with the sorbitol solution. After the final wash, the protoplasts were suspended in MPR medium supplemented with 450 mM sorbitol.

Yariv Antigens. Yariv antigens were synthesized from phloroglucinol and *p*-aminophenyl-D-glycopyranoside precursors obtained from Sigma Chemical Co. or from Calbiochem-Behring. The reaction procedure was that of Yariv *et al.* (24). In our initial studies, we purified the Yariv antigens through several cycles of ethanol-induced precipitation from aqueous solutions (24). This precipitation procedure was later replaced by a purification procedure involving simple dialysis followed by lyophilization. Yariv antigens are retained by ordinary (mol wt cutoff of 12,000) dialysis bags (8) because these molecules strongly self-associate to form high mol wt complexes (22). Comparisons between precipitated and dialyzed batches of the same Yariv antigen revealed no differences in activity in any of the experiments reported here.

Yariv antigens containing thio-glycosidic linkages were synthesized from phloroglucinol and *p*-aminophenyl-thio-D-glycopyranoside precursors by an analogous procedure.

Fluorescent Phenyl-D-Glycosides. PFP-β-D-Glc was synthesized by reacting 29 mg of fluorescein-5-isothiocyanate (Molecular Probes, Inc., Junction City, OR) with 10 mg of *p*-aminophenyl-β-D-glucopyranoside in 2 ml of methanol plus 0.2 ml triethylamine. The reaction was run for 4 h at room temperature, and then the solvent was removed by evaporation. The crude product was analyzed by TLC on 5 × 10 cm analytical silica gel 60 F-254 plates (EM Science, Cincinnati, OH) with the solvent *n*-butanol:acetic acid:water (4:1:1 by volume). With this analytical system, R_F = 0.77 for PFP-β-D-Glc, R_F = 0.33 for *p*-aminophenyl-β-D-glucopyranoside, and R_F = 0.98 for fluorescein-5-isothiocyanate. The PFP-β-D-Glc was then purified from the reaction mixture by preparative TLC using the same solvent with 20 × 20 cm preparative silica gel 60 F-254 plates (EM Science, Cincinnati, OH). With this preparative system, PFP-β-D-Glc appeared in a band at R_F = 0.56 to 0.69 that was scraped from the plate and eluted with methanol. The recovered PFP-β-D-Glc was checked for purity by TLC on the analytical plates with the solvent dichloromethane:methanol:acetic acid (79:20:1 by volume). With this system, PFP-β-D-Glc appeared at R_F = 0.19, and only trace amounts of other components were detectable on a heavily loaded plate.

PFP-α-D-Glc was synthesized and purified by an analogous procedure. On analytical TLC plates with the *n*-butanol:acetic acid:water solvent, PFP-α-D-Glc appeared at R_F = 0.77, and *p*-aminophenyl-α-D-glucopyranoside appeared at R_F = 0.29. On preparative TLC plates with the same solvent, PFP-α-D-Glc appeared in a band at R_F = 0.57 to 0.71. After elution from the preparative plates, the PFP-α-D-Glc was checked for purity by TLC on analytical plates with the dichloromethane:methanol:acetic acid solvent. PFP-α-D-Glc appeared at R_F = 0.11 with only trace levels of impurities evident.

Phenylglycoside Derivatives of Polytyrosine. Poly-L-tyrosine of average mol wt 4000 (average degree of polymerization 24) was obtained from United States Biochemical Corp. This polymer was derivatized with azophenylglycosides by a reaction analogous to that used to synthesize Yariv antigens. To 2 ml of water were added 16.4 mg of poly-L-tyrosine (0.10 mmol of tyrosine residues). The resulting suspension was stirred on an ice bath and titrated with 0.5 N NaOH until the polymer dissolved at approximately pH 11. In a separate container on the ice bath, 32.5 mg (0.12 mmol) of *p*-aminophenyl-β-D-glucopyranoside were dissolved in 0.6 ml of 0.6 N HCl and then diazotized by the slow addition of 0.12 ml of 1.5 M $NaNO_2$. This solution was stirred on the ice bath for 1 h and was then slowly added to the cold poly-L-tyrosine solution which was maintained at pH 10 to 11 by addition of 0.5 N NaOH as needed. This reaction mixture was stirred on the ice bath for 2 h. The product, β-D-Glc-Tyr, was purified from the reaction mixture by five cycles of precipitation at acidic pH, decantation, solubilization of the precipitate at alkaline pH, and reprecipitation at acidic pH.

An analogous procedure was used to synthesize and purify α-D-Gal-Tyr.

Fluorescence-Labeled Phenylglycoside Derivatives of Polytyrosine. Poly-L-tyrosine of average mol wt 4000 was labeled with Texas Red, a rhodamine sulfonyl chloride dye. A suspension of 10 mg of Texas Red (Molecular Probes, Inc.) in 2.5 ml of anhydrous pyridine was added to a solution of 16 mg of poly-L-tyrosine dissolved in 0.5 ml of anhydrous pyridine. The reaction vessel was sealed, and the contents were stirred for 2 d in the dark at room temperature. The reaction was then stopped and the product precipitated by the addition of 20 ml of 1.8 N HCl. The purple precipitate was collected by centrifugation and purified through two cycles of solubilization at alkaline pH and reprecipitation at acidic pH to yield the Texas Red derivative of poly-L-tyrosine. This fluorescence-labeled polymer was reacted with diazotized phenylglycosides as described in the above section to yield TR-β-D-Glc-Tyr and TR-α-D-Gal-Tyr.

Protoplast Agglutination Assays. Protoplast agglutination assays were carried out in 15 × 60 mm diameter glass Petri dishes with MPR medium supplemented with 450 mM sorbitol. Approximately 1.5×10^6 protoplasts (corresponding to a packed protoplast volume of approximately 50 μl), the Yariv antigen or polytyrosine derivative to be tested, and sufficient medium to give a total volume of 1.5 ml were added to each dish. All of the dishes were incubated together at 25 to 27°C on a tray that was manually tilted once or twice every 5 to 10 min to gently swirl the protoplast suspensions. Protoplast agglutination was generally first visible within 10 min, maximal within 30 min, and then stable for at least several hours.

Because the thio-Yariv antigens and the polytyrosine derivatives were only sparingly soluble in the culture medium, these probes were usually added to the agglutination assays from highly concentrated solutions in DMF or DMSO. The protoplasts were quite sensitive to DMF, and hence this solvent could not be used at final concentrations greater than 0.3% (v/v). DMSO was used at final concentrations as high as 3% (v/v) with no deleterious effects evident. Whenever either DMF or DMSO was used in agglutination experiments, control experiments were also run with $(\beta\text{-D-Glc})_3$ in the presence of the same amount of the solvent to verify that protoplast agglutination was not altered by the solvent.

Double-Diffusion Precipitation Assays. The procedure for double-diffusion precipitation assays was as described (8, 10) with minor modifications. The diffusion medium was a gel composed of 5 mg/ml electrophoresis-grade agarose (Bethesda Research Laboratories, Inc., Gaithersburg, MD) in a pH 7.0 buffer of 20 mM sodium phosphate and 10 mg/ml NaCl. Gels of 20 ml volume were cast into 15 × 100 mm diameter plastic Petri dishes and then punched with a hexagonal pattern of 4 mm diameter wells (number 1 cork borer) with center-to-center spacings of 1 cm. A 20 μl aliquot of 2 mg/ml gum arabic (Sigma Chemical Co.) was loaded into the center well, and the outer wells were loaded with 20 μl aliquots of Yariv antigen solutions that were routinely 1.03 mM (equivalent to 1 mg/ml $[\beta\text{-D-Glc}]_3$). Precipitation lines appeared faintly within 4 h at room temperature, intensified over several days, and then remained stable for several months.

Test compounds were generally loaded as solutions in the phosphate buffer that was used in casting the gels. The thio-Yariv antigens, however, were loaded into the wells as solutions in DMSO. Diffusion of these sparingly soluble compounds into the gel was improved when 0.1% (w/v) SDS was cast into the gel (8, 10). Control experiments with $(\beta\text{-D-Glc})_3$ showed that the presence of this DMSO and SDS did not alter the interaction between $(\beta\text{-D-Glc})_3$ and gum arabic.

Fluorescence-Labeled Phenylglycoside Stainings. Approximately 10^6 protoplasts were suspended together with TR-β-D-Glc-Tyr or TR-α-D-Gal-Tyr at a concentration corresponding to 50 μM phenylazotyrosine residues in 4.5 ml of MPR medium supplemented with 450 mM sorbitol. After incubation in this solution for 15 min at room temperature, the protoplasts were washed twice by centrifugation (2 min at 100*g*) and resuspended in fresh MPR medium supplemented with 450 mM sorbitol.

The MPR medium supplemented with 450 mM sorbitol, normally at a pH of approximately 5 after autoclaving, was titrated to pH 7.2 for use with PFP-β-D-Glc or PFP-α-D-Glc. This elevation of the pH above the pKa of fluorescein (18) reduced the membrane permeability of these fluorescein derivatives and prevented nonspecific staining of the protoplast cytoplasm. The treatment of protoplasts with PFP-β-D-Glc or PFP-α-D-Glc was otherwise analogous to the procedure used with the fluorescence-labeled polytyrosine derivatives. A 10 μM concentration of PFP-β-D-Glc or PFP-α-D-Glc was used during the 15 min incubation.

Hydrolysis and TLC of Yariv Antigens. Cleavage of the O-glycosidic linkages in Yariv antigens by acid hydrolysis leaves (1,3,5-tri-[*p*-hydroxyphenylazo]-2,4,6-trihydroxybenzene), a strong chromophore that is insoluble at acidic pH (24). Hydrolysis was accomplished by heating 1.0 mg of Yariv antigen with 3.5 ml of 2 N TFA in a screw-capped tube at 121°C. Hydrolysis was complete after 2 h, as judged by the loss of most color from the solution and the appearance of a large amount of dark precipitate. Yariv antigens containing thio-glycosidic linkages were more resistant to acid hydrolysis than were Yariv antigens containing O-glycosidic linkages. Thus, 1.0 or 2.0 mg of thio-Yariv antigen in 3.5 ml of 4 N TFA required 5 to 15 h of heating at 121°C to produce a colorless supernatant. Precipitates that formed during hydrolysis of Yariv antigens were collected by centrifugation. After decantation, the precipitates were suspended in methanol and evaporated to dryness three times. The final dried residues were dissolved or suspended in DMSO for loading onto TLC plates.

Intact and hydrolyzed Yariv antigens were compared by TLC on 5 × 20 cm analytical silica gel 60 F-254 plates (EM Science, Cincinnati, OH) with the solvent 95% ethanol:58% NH_4OH:water (70:18:12 by volume). These plates were first run overnight in this solvent and then air-dried before the samples were loaded and run. Intact and hydrolyzed Yariv antigens were loaded onto the washed plates from concentrated aqueous or DMSO solutions.

Microscopy. In the agglutination experiments, protoplasts were observed directly in the glass Petri dishes through a low-power brightfield objective on a Zeiss Universal microscope. In the fluorescence-staining experiments, protoplasts were placed under cover slips on microscope slides and examined by incident-light fluorescence microscopy using standard filter combinations for ultraviolet, blue, or green excitation light. Micrographs were recorded on 35 mm Kodak Tri-X film and developed in Kodak D-19 developer for 9 min at 26°C. This film/developer combination (14) has a film speed of approximately 3000 ASA.

Colorimetric Assays. Concentrations of aqueous solutions of Yariv antigens were determined spectrophotometrically using the published (24) extinction coefficient of $\epsilon_{485} = 4.18 \times 10^4\ M^{-1}\ cm^{-1}$. Concentrations of PFP-β-D-Glc or PFP-α-D-Glc in aqueous solutions at pH 7.2 were likewise determined spectrophotometrically using $\epsilon_{495} = 6.8 \times 10^4\ M^{-1}\ cm^{-1}$ as the extinction coefficient for coupled fluorescein-5-isothiocyanate (20). Quantitation of solutions of polytyrosine derivatives was based on the total concentration of phenylazotyrosine residues present, as calculated from A_{350} nm maximum. The extinction coefficient used in these calculations was $1.35 \times 10^4\ M^{-1}\ cm^{-1}$, as determined by Wofsy *et al.* (21) for the model compound *o*-(*o'*-azophenyl-β-D-galactopyranoside)-*N*-chloroacetyl-L-tyrosine in aqueous solution at pH 6.2.

The colorimetric orcinol assay (6) was used to verify the presence of carbohydrate in the synthesized phenylglycosides. This assay suffered from two sources of interference that limited

its accuracy in these measurements. First, all of the phenylglycosides were themselves chromophores that contributed unpredictable absorbances at the orcinol assay wavelength of 665 nm. Second, several of the phenylglycosides formed precipitates during the course of the assay, and it was necessary to remove these precipitates by filtration before measuring the A_{665} nm. Because appropriate standards were not available to correct for these interferences, the results from this assay are only semiquantitative and must be considered as lower limits on the amounts of carbohydrate actually present.

RESULTS

Protoplast Yield. The yield of protoplasts from suspension-cultured cells of "Paul's Scarlet" rose (*Rosa* sp.) was typically 0.5 ml of packed protoplasts (approximately 1.5×10^7 protoplasts) per initial 5 ml of packed cells. These protoplasts were checked for residual cell wall by applying 0.001% (w/v) Calcofluor White M2R for 10 min and then washing away the excess dye by centrifugation. Comparison of densities on film negatives recorded under controlled conditions through the fluorescence microscope showed that the fluorescence from Calcofluor-stained protoplasts was only about two times more intense than the autofluorescence from unstained protoplasts. For additional comparison, the fluorescence from the wall of Calcofluor-stained whole cells was at least 1000 times more intense than the fluorescence from stained protoplasts.

Agglutination of Protoplasts by Yariv Antigens. Protoplasts were readily agglutinated by either the $(\beta$-D-Gal$)_3$ or $(\beta$-D-Glc$)_3$ Yariv antigen. Figure 2A shows the extent of agglutination induced by $(\beta$-D-Gal$)_3$. This tight agglutination contrasts with the results shown in Figure 2B for $(\alpha$-D-Gal$)_3$. This Yariv antigen, which differs only in the anomeric configuration, induced no agglutination beyond the low level normally observed in protoplast suspensions containing no Yariv antigens (not shown). Likewise, no agglutination was observed if sulfur replaced oxygen in the glycosidic linkage (Fig. 2C). A Yariv antigen carrying β-linked disaccharides, $(\beta$-D-Lac$)_3$, induced only weak agglutination under these conditions (Fig. 2D).

The dependence of protoplast agglutination on Yariv antigen concentration was investigated for a number of Yariv antigens. The results presented in Table I show that $(\beta$-D-Glc$)_3$ and $(\beta$-D-Gal$)_3$ were equally effective and could agglutinate protoplasts when applied at concentrations as low as 3 μM. The $(\beta$-D-Lac$)_3$ Yariv antigen required a much higher concentration of 75 μM to induce even modest agglutination. The strong agglutination that was characteristic of $(\beta$-D-Glc$)_3$ and $(\beta$-D-Gal$)_3$ (Fig. 2A) was never observed with $(\beta$-D-Lac$)_3$. Although tested over a range of concentrations, neither (Thio-β-D-Glc$)_3$ nor (Thio-β-D-Gal$)_3$ induced any protoplast agglutination (Table I).

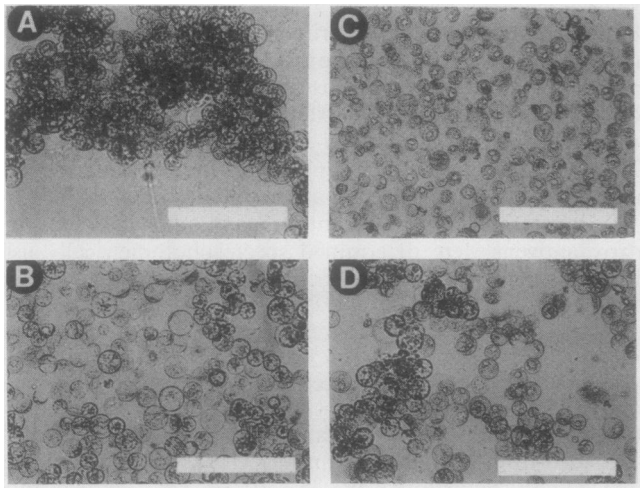

FIG. 2. Agglutination of protoplasts by various Yariv antigens containing galactosyl residues. A, Strong agglutination induced by $(\beta$-D-Gal$)_3$; B, no agglutination induced by $(\alpha$-D-Gal$)_3$; C, no agglutination induced by (Thio-β-D-Gal$)_3$; D, weak agglutination induced by $(\beta$-D-Lac$)_3$. Each Yariv antigen was applied at 31 μM concentration. Bars, 300 μm.

The agglutination assays reported in Table I were carried out with approximately 1.5×10^6 protoplasts in the 1.5 ml assay volume. The $(\beta$-D-Glc$)_3$ Yariv antigen was tested in similar experiments where as few as 2.5×10^5 or as many as 8×10^6 protoplasts were used. Yariv antigen-induced agglutination was observed throughout this range of protoplast densities (results not shown).

Other Experients with Yariv Antigens. The interactions between gum arabic and various Yariv antigens were tested in double-diffusion precipitation assays. The results from one set of such assays are shown in Figure 3A. Single precipitation lines formed between gum arabic and the β-linked Yariv antigens $(\beta$-D-Glc$)_3$ and $(\beta$-D-Gal$)_3$ but not between gum arabic and the α-linked Yariv antigen $(\alpha$-D-Gal$)_3$.

The dependence of this double-diffusion precipitation on Yariv antigen concentration is shown in Figure 3B where $(\beta$-D-Glc$)_3$ was effective down to 206 μM. The Yariv antigens $(\beta$-D-Lac$)_3$, (Thio-β-D-Glc$)_3$, and (Thio-β-D-Gal$)_3$ were also tested over this 20.6 μM to 2.06 mM concentration range. None of these latter three Yariv antigens formed a precipitation line with gum arabic under any of the conditions tested (results not shown).

Some of the Yariv antigens were subjected to acid hydrolysis and TLC analysis to obtain at least some evidence verifying the expected chemical structure of these molecules. Some of the data from these experiments are summarized in Table II. In their intact forms, the $(\beta$-D-Glc$)_3$, $(\beta$-D-Lac$)_3$, (Thio-β-D-Glc$)_3$ and (Thio-β-D-Gal$)_3$ antigens could all be distinguished from each other by TLC. After limited acid hydrolysis, the residual chromophores from $(\beta$-D-Glc$)_3$ and $(\beta$-D-Lac$)_3$ could not be distinguished from each other but could be distinguished from their respective precursors. These results are consistent with the cleavage of the glycosidic linkages during hydrolysis and the release of the same (1,3,5-tri-[*p*-hydroxyphenylazo]-2,4,6-trihydroxybenzene) core chromophore from both $(\beta$-D-Glc$)_3$ and $(\beta$-D-Lac$)_3$.

The chromophores released by acid hydrolysis of (Thio-β-D-Glc$)_3$ and (Thio-β-D-Gal$)_3$ were immobile in this TLC system and as such were distinguishable both from their respective precursors and from the chromophores released by hydrolysis of $(\beta$-D-Glc$)_3$ and $(\beta$-D-Lac$)_3$ (Table II). These chromophores released from (Thio-β-D-Glc$)_3$ and (Thio-β-D-Gal$)_3$ were extremely insoluble, and further TLC analysis of these chromophores proved intractable.

Additional evidence regarding the chemical structures of (Thio-β-D-Glc$)_3$ and (Thio-β-D-Gal$)_3$ was obtained by using the colorimetric orcinol method to assay for soluble carbohydrate released during the hydrolysis of these Yariv antigens. Correction for carbohydrate degradation during the extended hydrolysis of these thio-Yariv antigens (see "Materials and Methods") was estimated by subjecting glucose and galactose standards to the same hydrolysis conditions. After this correction, the orcinol assay indicated the release of 2.1 glucose equivalents per (Thio-β-D-Glc$)_3$ and 1.6 galactose equivalents per (Thio-β-D-Gal$)_3$. As explained in the "Materials and Methods" section, these results represent lower limits, and the actual amount of carbohydrate present may be nearer the expected 3.0 equivalents per thio-Yariv antigen.

Fluorescent Phenyl-D-Glycosides. The expected chemical structure of PFP-β-D-Glc is shown in Figure 4. The expected chemical structure of PFP-α-D-Glc is identical except for the

Table I. *Agglutination of Protoplasts by Yariv Antigens*

Protoplasts were incubated in culture medium containing the indicated concentration of the specified Yariv antigen. Protoplast agglutination was scored on a semiquantitative scale.

Yariv Antigen Concentration	Protoplast Agglutination[a]				
	(β-D-Glc)$_3$	(β-D-Gal)$_3$	(β-D-Lac)$_3$	(Thio-β-D-Glc)$_3$	(Thio-β-D-Gal)$_3$
μM					
0.3	–	–	NT[b]	NT	NT
3	+	+	–	–	–
31	++	++	±	–	–
75	NT	NT	+	–	–
225	++	++	+	–	–

[a] Semiquantitative agglutination scale: –, no agglutination, *e.g.* as in Figure 2B; ±, weak agglutination, *e.g.* as in Figure 2D; +, medium agglutination; ++, strong agglutination, *e.g.* as in Figure 2A. [b] NT, not tested.

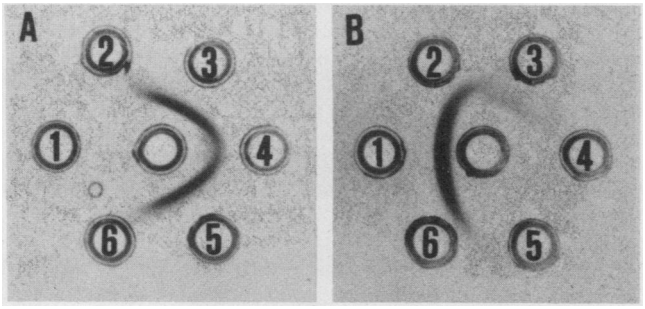

FIG. 3. Double-diffusion precipitation assays of Yariv antigens. Each well was loaded with 20 μl of a solution of the indicated compound dissolved in phosphate buffer. A, Wells 1, 3, and 5 loaded with 1.03 mM solutions of (α-D-Gal)$_3$, (β-D-Gal)$_3$, and (β-D-Glc)$_3$, respectively. B, Wells 1, 3, and 5 loaded with solutions of (β-D-Glc)$_3$ at 2.06 mM, 206 μM and 20.6 μM, respectively. In both A and B, the center well was loaded with 2 mg/ml gum arabic, and wells 2, 4, and 6 were loaded with phosphate buffer alone.

Table II. *TLC Analyses of Intact and Hydrolyzed Yariv Antigens*

Intact and acid-hydrolyzed Yariv antigens were analyzed by TLC on silica gel plates with the solvent system described in the text. Spots were directly visible because both intact and hydrolyzed Yariv antigens are strong chromophores.

Yariv Antigen	R_F	
	Intact	Hydrolyzed
(β-D-Glc)$_3$	0.65	0.88
(β-D-Lac)$_3$	0.00	0.88
(Thio-β-D-Glc)$_3$	0.71	0.00
(Thio-β-D-Gal)$_3$	0.59	0.00

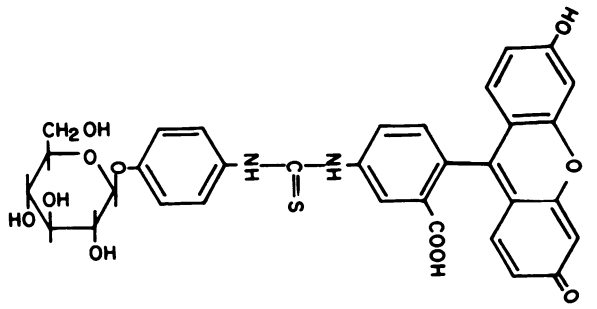

FIG. 4. Chemical structure of the fluorescent phenylglucoside PFP-β-D-Glc. The left end of the molecule is comparable to one arm of a Yariv antigen (see Fig. 1). The right end of the molecule is fluorescein, a strong fluorophore.

anomeric configuration of the glucosidic linkage. The carbohydrate/fluorescein ratios were 0.38 glucose equivalents per fluorescein for PFP-β-D-Glc and 0.44 glucose equivalents per fluorescein for PFP-α-D-Glc, as estimated by spectrophotometric quantitation of fluorescein and colorimetric determination of carbohydrate. Although lower than the ratio of 1.0 glucose equivalents per fluorescein expected for the structure shown in Figure 4, these estimated ratios represent only lower limits on the amount of carbohydrate actually present. Taken together with the results of analytical TLC where both PFP-β-D-Glc and PFP-α-D-Glc appeared to be essentially homogeneous in two different solvent systems, these results support the chemical structure shown in Figure 4.

Brightfield and fluorescence micrographs are shown in Figure 5, A and B, respectively, for protoplasts that had been incubated with PFP-β-D-Glc. This fluorescent phenyl-β-glucoside produced no detectable staining of intact protoplasts. The dim fluorescence evident from the cytoplasm of the intact protoplasts in Figure 5B was not brighter than the autofluorescence observed from control protoplasts that had not been exposed to PFP-β-D-Glc (not shown).

The field of view shown in Figure 5, A and B, was intentionally

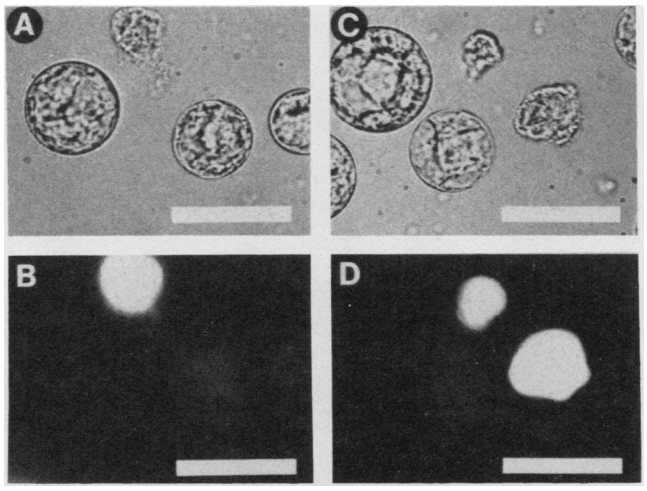

FIG. 5. Brightfield and fluorescence micrograph pairs showing protoplasts after treatment with fluorescent phenylglucosides. A, Brightfield and B, fluorescence micrographs of protoplasts after treatment with PFP-β-D-Glc. C, Brightfield and D, fluorescence micrographs of protoplasts after treatment with PFP-α-D-Glc. In both cases the intensity of fluorescence from intact protoplasts was comparable to the intensity of autofluorescence from unlabeled protoplasts (not shown). Although neither of the fluorescent probes bound to intact protoplasts, both of the probes were readily visible when bound nonspecifically to debris from ruptured protoplasts. Bars, 50 μm.

selected to include cytoplasmic debris from a ruptured protoplast. In our experience, a variety of fluorophores will bind to such debris through unidentified interactions. Thus, the binding of PFP-β-D-Glc to the debris shown in Figure 5B was probably nonspecific, *i.e.* the binding occurred through mechanisms not involving the carbohydrate portion of the molecule. Nevertheless, the intense staining of the debris serves as a positive control showing that the sensitivity of the experiment was adequate to reveal bound PFP-β-D-Glc.

Figure 5, C and D, shows the results of a parallel control experiment in which protoplasts were incubated with PFP-α-D-Glc instead of PFP-β-D-Glc. The results obtained with PFP-α-D-Glc were indistinguishable from those obtained with PFP-β-D-Glc.

Poly-L-Tyrosine Derivatives. A hypothetical structure for the amino-terminal region of β-D-Glc-Tyr appears in Figure 6A where every tyrosine side chain is shown derivatized with a single azophenyl-β-D-glucoside. Analysis by the colorimetric orcinol assay indicated that 39% of the tyrosine side chains in β-D-Glc-Tyr carried a glucosyl residue. Since this polymer did not dissolve in the orcinol reagent solution, it is likely that some of the glucosyl residues were not hydrolyzed and, therefore, not detected in the assay. Thus, the 39% derivatization is a lower limit, and the actual degree of derivatization might have been more like that suggested in Figure 6A. Even at only 39% derivatization, however, this polymer of about 24 tyrosine residues would have carried about nine glucosyl residues.

This poly-L-tyrosine derivative, β-D-Glc-Tyr, and a similar α-anomeric derivative, α-D-Gal-Tyr, were both tested for activity in protoplast agglutination experiments. Neither of these phenylglycosides induced protoplast agglutination above the low level normally observed in control protoplast suspensions containing no polymer (not shown). The concentrations of phenylazotyrosine residues used in these experiments ranged from 0.8 μM to 93 μM. Concentrations higher than 93 μM could not be tested because of the limited solubilities of these polymers in the protoplast culture medium.

Figure 6B shows the expected chemical structure of TR-β-D-Glc-Tyr. Prior to reaction with diazotized phenylglycosides, the Texas Red-labeled poly-L-tyrosine preparation was characterized spectrophotometrically by the ratio of absorbances at the Texas Red maximum and the tyrosine maximum, $A_{594}/A_{276} = 1.19$. After phenylglycosylation, the ratio of absorbances at the Texas Red maximum and the phenylazotyrosine maximum was $A_{594}/A_{350} = 0.106$ for TR-β-D-Glc-Tyr and $A_{594}/A_{350} = 0.100$ for TR-α-D-Gal-Tyr.

Brightfield and fluorescence micrographs are shown in Figure 7, A and B, respectively, for protoplasts that had been incubated with TR-β-D-Glc-Tyr. The washes after the incubation were not quite complete, so Figure 7B shows a faint background fluorescence due to residual TR-β-D-Glc-Tyr in the medium. The intact protoplasts shown in Figure 7B did not bind TR-β-D-Glc-Tyr and, in fact, appeared negatively stained because of the faint fluorescence from the medium. Cytoplasmic debris was brightly stained, however, and demonstrated that nonspecifically bound TR-β-D-Glc-Tyr could be readily visualized under the conditions of the experiment.

Figure 7, C and D, shows the results of a parallel control experiment in which protoplasts were incubated with TR-α-D-Gal-Tyr instead of TR-β-D-Glc-Tyr. The results obtained with TR-α-D-Gal-Tyr were indistinguishable from those obtained with TR-β-D-Glc-Tyr.

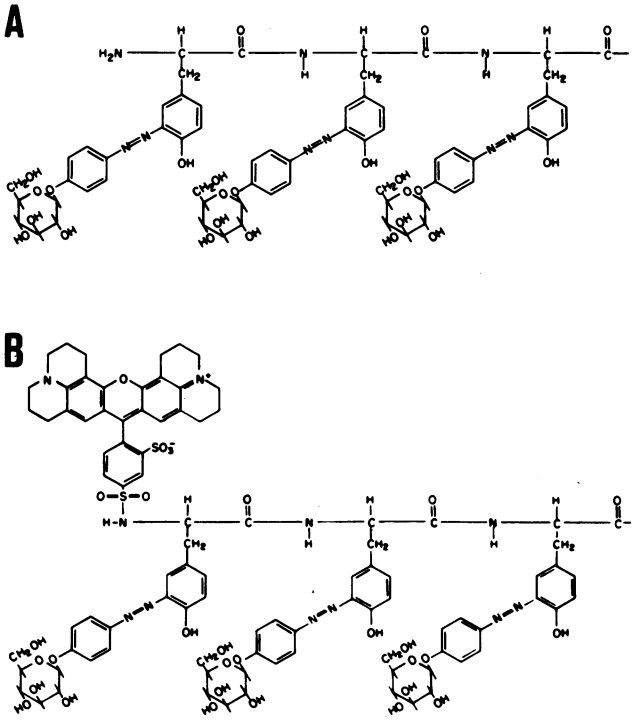

FIG. 6. Hypothetical chemical structures of poly-L-tyrosine derivatives. A, The amino-terminal region of β-D-Glc-Tyr shown with every tyrosine side chain carrying a single azophenyl-β-D-glucoside. Carbohydrate analysis showed that glucosides were present on at least 9 of the approximately 24 tyrosine residues in this polymer. B, TR-β-D-Glc-Tyr, a poly-L-tyrosine derivative carrying the Texas Red fluorophore at the amino-terminal. Each derivatized tyrosine side chain in these polymers is a close structural analog of one arm of a Yariv antigen (see Fig. 1).

DISCUSSION

The results of our initial experiments on protoplast agglutination induced by Yariv antigens confirm the results of Larkin (11, 12). Yariv antigens containing monosaccharides in β-*O*-glycosidic linkage strongly agglutinate protoplasts (Fig. 2). Yariv anti-

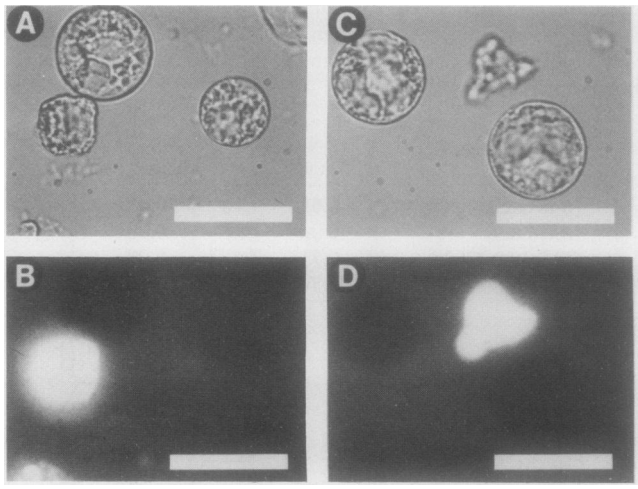

FIG. 7. Brightfield and fluorescence micrograph pairs showing protoplasts after treatment with fluorescent poly-L-tyrosine derivatives. A, Brightfield and B, fluorescence micrographs of protoplasts after treatment with TR-β-D-Glc-Tyr. C, Brightfield and D, fluorescence micrographs of protoplasts after treatment with TR-α-D-Gal-Tyr. In each case, the intact protoplasts appeared negatively stained against a faint background fluorescence due to traces of the fluorescent probe in the medium. Although neither of the probes bound to intact protoplasts, both of the probes were visible when bound nonspecifically to debris from ruptured protoplasts. Bars, 50 μm.

gens containing the same glycosyl residues linked in the α-anomeric configuration do not agglutinate protoplasts. While we also qualitatively confirm Larkin's observation (12) that protoplasts are agglutinated by (β-D-Lac)$_3$, we find in our system that the agglutinating activity of this disaccharide-containing Yariv antigen is substantially lower than the activities of Yariv antigens carrying the same sugars as monosaccharides (Table I).

Although thio-Yariv antigens have been previously reported (8) to precipitate carrot β-lectins (AGPs), agglutination of protoplasts by thio-Yariv antigens has not, to our knowledge, been investigated. In our protoplast system, Yariv antigens containing thioglycosides had no detectable agglutinating activity when tested over a wide range of concentrations (Fig. 2C; Table I).

The interactions between Yariv antigens and gum arabic were tested in double-diffusion precipitation assays. Although a variety of plants have been extracted to obtain buffer-soluble AGPs that precipitate with Yariv antigens (1, 3, 4, 10, 16), the first identified proteoglycan precipitated by a Yariv antigen was gum arabic from *Acacia senegal* (23). In our experiments, protoplast-agglutinating activities of the various Yariv antigens were closely correlated with gum arabic-precipitating activities (Figs. 2 and 3; Table I). The only exception to this correlation was (β-D-Lac)$_3$ which did not precipitate gum arabic but did weakly agglutinate protoplasts.

This close correlation between protoplast agglutination and gum arabic precipitation supports the idea (2, 7) that the plasma membrane components mediating protoplast agglutination are AGPs or AGP-like proteoglycans. This correlation does not appear to be universal, however, because (β-D-Lac)$_3$, (Thio-β-D-Glc)$_3$, and (Thio-β-D-Gal)$_3$ have all been reported to precipitate other AGPs (8, 10) but have little or no protoplast-agglutinating activity in our system.

To further characterize the binding of phenyl-β-glycosides to the protoplast surface, we synthesized and tested several phenylglycosides that were not Yariv antigens. Previous studies of phenyl-β-glycoside binding to protoplasts (11, 12, 17) have generally relied on Yariv antigens as probes. Indirect evidence that simple phenyl-β-glycosides may also bind to the protoplast surface and to soluble AGPs has come from observations that *p*-nitrophenyl-β-D-glucoside (12, 17), salicin (5, 12), certain other phenyl-β-glycosides (12), and a flavonol glycoside (9) can inhibit Yariv antigen-induced protoplast agglutination or AGP precipitation. Relatively high concentrations (10–150 mM) of these simple phenylglycosides are required, however, to achieve this inhibition.

One of the probes that we synthesized in this work, PFP-β-D-Glc, was designed to enable a direct test of simple phenyl-β-glycoside binding at the protoplast surface. Although the two phenyl rings adjacent to the glycosyl residue in this probe are joined by thioureidyl- rather than azo-linkage, PFP-β-D-Glc (Fig. 4) is a somewhat closer analog of a Yariv antigen (Fig. 1) than is *p*-nitrophenyl-β-D-glucoside or salicin. Nevertheless, we found that PFP-β-D-Glc had no detectable affinity for the protoplast surface (Fig. 5).

Because Yariv antigens self-associate in aqueous solutions to form complexes of 10 to 50 molecules (22), these probes may have a very high effective valency. The poly-L-tyrosine derivatives synthesized in this work (Fig. 6) have both a high valency and a very close structural resemblance to Yariv antigens (Fig. 1). In particular, the poly-L-tyrosine derivatives carry a hydroxyl group in the ortho position relative to the azo-linkage, a structural feature thought to be important in Yariv antigen activity (8, 9). Moreover, the spatial density of these phenyl-β-glycosides on β-D-Glc-Tyr is significantly higher than what was probably achieved with a similarly-glycosylated porcupine quill protein that failed to precipitate carrot AGPs (8).

When tested over a wide range of concentrations, however, β-D-Glc-Tyr had no detectable protoplast-agglutinating activity. Since it seemed possible that β-D-Glc-Tyr might be binding to the protoplast surface without inducing agglutination, we synthesized a fluorescence-labeled version of this probe (Fig. 6B) to enable a direct test of binding. Results obtained in fluorescence-staining experiments with TR-β-D-Glc-Tyr (Fig. 7) showed that this poly-L-tyrosine derivative had no detectable affinity for the protoplast surface.

The results presented in this paper contribute to our understanding of the structural requirements for binding of phenyl-β-glycosides to the protoplast surface. Although the plasma membrane components responsible for this binding have been called β-lectins (10–12), it is now clear that these molecules (probably some form of AGP) should not be thought of as lectins in the usual sense. Although Yariv antigens interact with these membrane components to induce tight protoplast agglutination, thio-Yariv antigens and other mono- and poly-phenyl-β-glycosides have no detectable affinity for the protoplast surface. It has been previously suggested (2, 7) that the self-association of Yariv antigens provides the multi-valency property that is necessary for cross-linking in protoplast agglutination and AGP precipitation experiments. The results of the present work suggest that the self-association of Yariv antigens may be a prerequisite for binding to AGPs *i.e.* AGPs may bind stacked Yariv antigens but not simple phenylglycosides or even unassociated, single Yariv antigens. The weaker protoplast-agglutinating activity observed with (β-D-Lac)$_3$ might occur if the usual self-association is disrupted by the increased polarity or bulkiness of this disaccharide-containing Yariv antigen. Likewise, the poly-L-tyrosine derivatives may lack activity because the relative spatial orientation of adjacent phenylglycosides on this polymer is different from the spatial orientation of stacked Yariv antigens.

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