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Ultraviolet Difference Spectral Studies on Concanavalin A

Carbohydrate Interaction

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The interaction of concanavalin A, the phytohemagglutinin of the jack bean, with a variety of glycosides has been studied by the technique of ultraviolet difference spectroscopy. Whereas methyl α-D-gluco- and manno-pyranoside gave rise to relatively low intensity difference spectra, *p*-nitrophenyl α-D-mannopyranoside and α-D-glucopyranoside yielded large difference spectra upon interaction with concanavalin A. Using this technique as a measure of concanavalin A activity, it was demonstrated that the protein specifically binds low molecular weight carbohydrates at much lower pH values (*e. g.* pH 2.4) than previously believed. Although polysaccharides are also bound at these low pH values, they are not precipitated by concanavalin A. Molecular weight studies in acid media indicate that the protein does not dissociate and it is suggested that electrostatic repulsion of the protein molecules due to their high net positive charge prevents protein-polysaccharide lattice formation and hence failure of the complex to precipitate.

The interaction of certain specific carbohydrates and carbohydrate-containing macromolecules with concanavalin A, the lectin present in jack bean, has been studied extensively. This protein has been shown to interact to form a precipitate with various polysaccharides, *e.g.* glycogens, mannans, and dextrans, and with certain serum glycoproteins [1—12]. Extensive inhibition studies, using mono- and oligosaccharides, have demonstrated that the specificity of the saccharide binding sites of concanavalin A are directed towards the unmodified hydroxyl groups at the C-3, C-4 and C-6 positions of α-D-glucopyranosyl and α-D-mannopyranosyl residues [14—17]. Equilibrium dialysis studies have indicated that one mole of monosaccharide (methyl α-D-glucopyranoside or methyl α-D-mannopyranoside) is bound per 32000 to 34000 g of concanavalin A [18,19]. Furthermore, the linearity of the Scatchard plots suggests a homogeneity of binding constants for a given ligand.

Other studies have indicated the complexity of structure of concanavalin A. Olson and Liener [20] found that in 8 M urea at pH 7, concanavalin A dissociated into three fractions, with molecular weights corresponding to 16500, 42000 and in excess of 200000, each with the same peptide map, amino acid composition, and N-terminal amino acid (alanine).

The molecular weight of native concanavalin A was originally reported by Sumner to be 96000 [21]. More recent estimates range from 55000 to 68000 to 71000 [22—24]. Bivalent metals are required for the protein to bind carbohydrates. It has been established that either Mn^{++} or Ni^{++} and Ca^{++} are necessary for full activity [25,26].

Recently we have directed our efforts toward ascertaining which amino acyl residues are involved in the carbohydrate binding sites of concavalin A. It has been established that free amino groups are not apparently essential since concanavalin A displays considerable carbohydrate-binding activity even when almost completely acetylated [27]. However, free carboxyl residues appear to be essential for the carbohydrate-binding activity of concanavalin A [28,29].

Recently Doyle *et al.* [30] demonstrated that when D-glucose, an inhibitor of concanavalin A—polysaccharide precipitation, was added to concanavalin A, an ultraviolet difference spectrum was obtained. This report represents a detailed study of the carbohydrate-binding activity of concanavalin A, using the technique of ultraviolet difference spectroscopy.

MATERIALS AND METHODS

Concanavalin A, prepared as described previously [31] was stored in 1.0 M NaCl at 4°. Heavy metal-free concanavalin A was prepared by dialysis of the native protein against 0.1 N HCl, followed by dialysis against 0.05 M NaCl in the cold. The demetallized protein did not form a precipitate with dextran B-1355-S [11].

All carbohydrates used were chromatographically pure. D-Glucose, and methyl α-D-gluco and manno-pyranoside were purchased from Pfanstiehl Labora-

tories (Waukegan, Illinois). *p*-Nitrophenyl α-D-glucopyranoside was purchased from Koch-Light Laboratories, England. Phenyl α-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl α-D-mannopyranoside and *p*-aminophenyl α-D-mannopyranoside were prepared in this laboratory by Dr. R. N. Iyer. Methyl α-D-galactopyranoside was a gift of Dr. D. M. Myhre. *p*-Nitrophenyl α- and β-D-galactopyranoside were obtained from Dr. D. Aminoff. *p*-Nitrophenyl 2-*O*-methyl-α-D-mannopyranoside was the gift of Dr. M. J. Jermyn.

Heavy metal-free NaCl was purchased from Fisher Scientific Company.

Difference spectra were recorded on a dual beam Cary 14 Recording Spectrophotometer. Temperatures were maintained at ±0.1° by a Lauda K-2/R Circulator (Brinkman Instruments, Inc., Westbury, N.Y.). Unless otherwise specified, all runs were performed at 8°. Matched Yankeelov cuvettes [32] (Pyrocell Manufacturing Co., Westwood, N.Y.) with an effective path length of 9 mm were employed. Into one compartment of a Yankeelov cuvette was placed 0.80 ml of an appropriate concanavalin A solution; 0.80 ml of a saccharide solution was pipetted into the second compartment. A second cuvette was prepared in exactly the same manner. In all experiments, except where noted, the buffer, pH and ionic strength of both solutions were identical. After obtaining a satisfactory baseline with the two cuvettes, the sample cuvette was mixed by inverting and the ultraviolett difference spectrum recorded.

All binding constants were determined as follows: a series of glycoside solutions of varying concentrations were mixed individually with a concanavalin A solution as described above in order to generate a series of difference spectra. Using the absorption change at the maximum wavelength, the data obtained were treated according to the method of Dahlquist *et al.* [33].

The pH optima of the interaction of concanavalin A with *p*-nitrophenyl α-D-mannopyranoside and *p*-nitrophenyl α-D-glucopyranoside were determined as follows: a stock solution of concanavalin A (8 mg/ml) in 0.2 M NaCl was diluted to 2 mg/ml with each of the buffer solutions used. Stock solutions of the glycosides were diluted ten-fold with the appropriate buffer such that the final pH and ionic strength were identical to the corresponding concanavalin A solution. Ultraviolet difference spectra were then obtained as described above.

The binding constant at pH 2.4 of *p*-nitrophenyl α-D-mannopyranoside with concanavalin A was also determined by equilibrium dialysis using the method of So and Goldstein [18] except that the concentrations of *p*-nitrophenyl α-D-gluco- and α-D-mannopyranoside were measured by determining the absorbance of the solutions, inside and outside the dialysis bag, at 325 nm (using ε_M of 7500, determined on a dry weight basis for the stock sugar solution at pH 2.4). This wavelength was employed in order to preclude interference due to protein absorption. The concentration of concanavalin A was 2.19 mg/ml.

Molecular weight determinations were performed on a calibrated gel column. Biogel P-60 (Calbiochem, Los Angeles, Calif.) was swelled in water and packed in a column (1×46 cm). The column was washed extensively with the appropriate buffer and calibrated under each of the conditions employed. The standard proteins used were lysozyme, soybean trypsin inhibitor, pepsin, ovalbumin and bovine serum albumin.

RESULTS

Fig. 1 shows the relatively small perturbations observed in the 260—290 nm region when concanavalin A was allowed to interact with methyl α-D-glucopyranoside and methyl α-D-mannopyranoside.

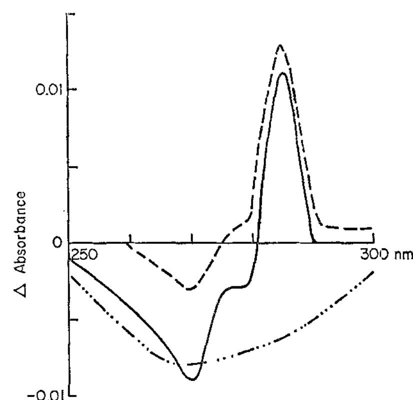

Fig. 1. *Ultraviolet difference spectra of concanavalin A (3.1 mg/ml).* 5 mM methyl α-D-mannopyranoside, (——); 5 mM methyl α-D-glucopyranoside, (– – – –); 5 mM D-glucose, (-·····-). All solutions were 0.05 M NaCl in 0.01 M phosphate buffer, pH 6.5

Methyl α-D-galactopyranoside which does not bind to concanavalin A did not produce an observable perturbations of concanavalin A. D-Glucose, which afforded a difference spectrum qualitatively different from that of the methyl glycosides, was similar to that observed by Doyle *et al.* [30]. Both methyl α-glycosides exhibited a positive maximum difference at 285 nm, an inflection point between 265 and 271 nm, and a negative difference maximum at 270 nm, whereas D-glucose displayed a broad negative difference spectrum between 260 and 290 nm.

Fig. 2 shows the absorption spectra of the phenyl glycosides studied in this work. Note the high extinction coefficient of the *p*-nitrophenyl α-D-glycopyranosides (ε_M = 10000 at 305 nm). All *p*-nitrophenyl glycosides displayed identical spectra. In contrast, *p*-aminophenyl α-D-mannopyranoside and

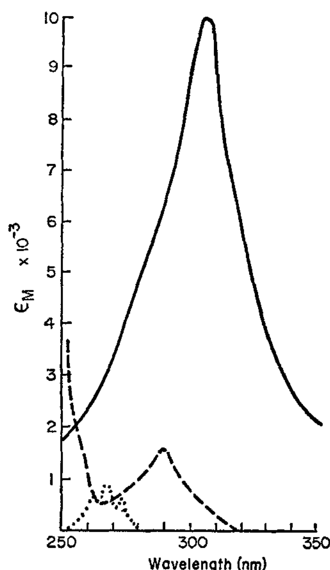

Fig. 2. *Ultraviolet absorption spectra of* p-*nitrophenyl glycosides,* (——); p-*aminophenyl* α-D-*mannopyranoside,* (– – –); *phenyl* α-D-*glucopyranoside* (······)

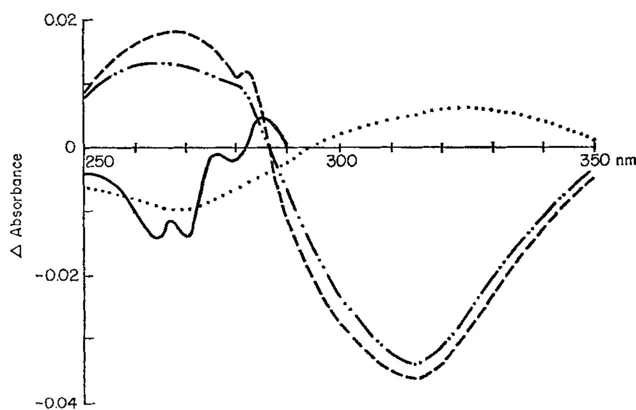

Fig. 3. *Ultraviolet difference spectra of concanavalin A (1.1 mg/ml).* 50 μM *p*-nitrophenyl α-D-mannopyranoside, (– – – –); 200 μM *p*-nitrophenyl α-D-glucopyranoside, (–····–); 50 μM *p*-nitrophenyl β-D-glucopyranoside, (······); 500 μM phenyl α-D-glucopyranoside, (——). All solutions were 0.5 M NaCl in 0.01 M phosphate buffer, pH 6.5

phenyl α-D-glucopyranoside afforded less intense absorption with maxima at lower wavelengths.

Fig. 3 presents the difference spectra obtained when concanavalin A was allowed to interact with *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl β-D-glucopyranoside, and phenyl α-D-glucopyranoside. Note the intense difference spectra produced by the two *p*-nitrophenyl α-D-glycosides. In contrast, *p*-nitrophenyl β-D-glucopyranoside gave a difference spectrum much less intense and qualitatively different in displacement (negative below and positive above

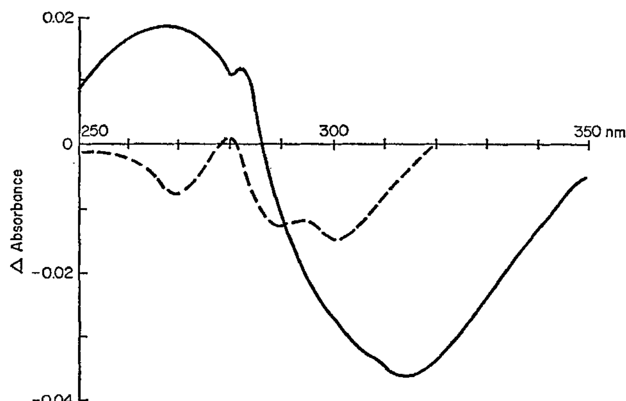

Fig. 4. *Ultraviolet difference spectra of concanavalin A (1.1 mg/ml).* 50 μM *p*-nitrophenyl α-D-mannopyranoside, (——); 50 μM *p*-aminophenyl α-D-mannopyranoside, (– – – –). All solutions were 0.5 M NaCl in 0.01 M phosphate buffer, pH 6.5

300 nm). On the other hand, the perturbations displayed by phenyl α-D-glucopyranoside were more similar to those observed when concanavalin A was mixed with the α-methyl glycosides (Fig. 1). When *p*-nitrophenyl 2-*O*-methyl-α-D-mannopyranoside was mixed with concanavalin A it was found to yield qualitatively the same difference spectrum as *p*-nitrophenyl α-D-mannopyranoside and *p*-nitrophenyl α-D-glucopyranoside at similar concentrations (0.1 mM).

Comparison of the differences in perturbations which resulted when concanavalin A was mixed with *p*-aminophenyl and *p*-nitrophenyl α-D-mannopyranoside is shown in Fig. 4. Recalling the less intense ultraviolet absorption which *p*-aminophenyl α-D-mannopyranoside displayed (Fig. 2), it is apparent that the perturbations are less intense and at lower wavelength, even though inhibition studies with these two compounds showed that *p*-aminophenyl α-D-mannopyranoside was a three-fold more potent inhibitor of polysaccharide precipitation by concanavalin A [34]. No difference spectra were obtained with *p*-nitrophenyl α-D- and β-D-galactopyranoside or *p*-nitrophenol.

The results of Fig. 3 and 4 demonstrate the extremely large difference spectra obtained when concanavalin A interacted with *p*-nitrophenyl α-D-glucopyranoside and *p*-nitrophenyl α-D-mannopyranoside. These large differences allowed additional quantitative studies to be performed.

The ultraviolet spectrum of *p*-nitrophenyl α-D-mannopyranoside in the presence and absence of concanavalin A is shown in Fig. 5. Subtraction of the two spectra (in the presence and absence of concanavalin A, using Yankeelov cuvettes) produces an ultraviolet difference spectrum very similar to that seen in Fig. 3 and 4. Thus the large ultraviolet

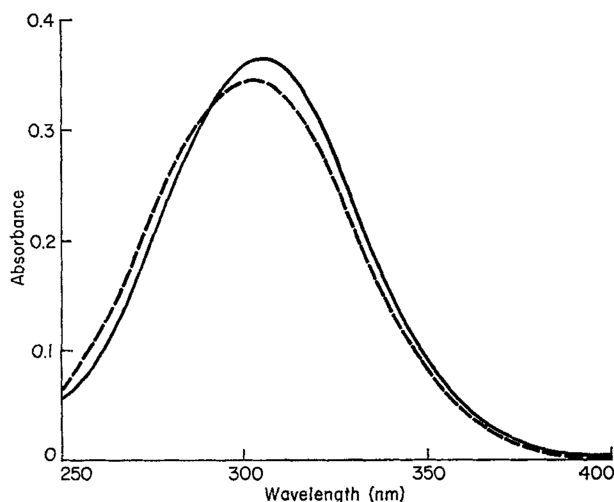

Fig. 5. *Ultraviolet spectra of* p-*nitrophenyl α-D-mannopyranoside in the presence and absence of concanavalin A.* 33.3 µM *p*-nitrophenyl α-D-mannopyranoside in 1 M NaCl, (——); 33.3 µM *p*-nitrophenyl α-D-mannopyranoside in 1 M NaCl containing concanavalin A, 1.32 mg/ml, (----)

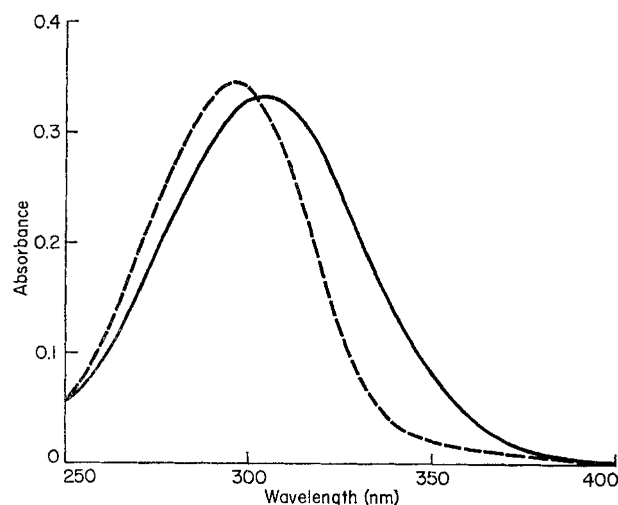

Fig. 6. *Ultraviolet spectra of* p-*nitrophenyl α-D-mannopyranoside in water and dioxane.* 33.3 µM *p*-nitrophenyl α-D-mannopyranoside in water (——); and in dioxane, (----)

difference spectra displayed by the *p*-nitrophenyl α-D-glycosides specifically bound to concanavalin A is most likely due to a perturbation of the chromophoric aglycone by the protein. The spectrum of *p*-nitrophenyl α-D-mannopyranoside in dioxane and in water is presented in Fig. 6.

The broad pH optima for the interaction between concanavalin A and the two *p*-nitrophenyl α-D-glycosides is shown in Fig. 7. Note that the maximum extends from pH 3—8 and that considerable interaction still occurs at pH 2.4. This is in marked contrast to the pH optimum for the precipitation reaction which occurs when concanavalin A interacts with polysaccharides. Dextrans and α-mannans, for example, are optimally precipitated between pH 5.7 and 7.5 [11,12].

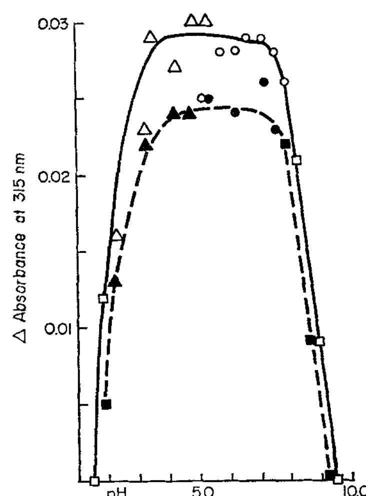

Fig. 7. *pH optima of binding of* p-*nitrophenyl glycosides to concanavalin A (1 mg/ml).* 50 µM *p*-nitrophenyl α-D-mannopyranoside, (——); 200 µM *p*-nitrophenyl α-D-glucopyranoside, (----). Glycine buffers, (□, ■); acetate buffers, (△, ▲); phosphate buffers, (○, ●). All solutions were 0.025 M NaCl and 0.075 M buffer

Table. *Summary of binding constants for concanavalin A-glycoside interaction*

Glycoside	Conditions	*K* (assoc.)
p-Nitrophenyl α-D-mannopyranoside	0.5 M NaCl[a], 0.01 M phosphate, pH 6.5	3.9×10^4
p-Nitrophenyl α-D-mannopyranoside	0.05 M NaCl, 0.01 M phosphate, pH 6.5	3.6×10^4
p-Nitrophenyl α-D-mannopyranoside	0.02 M NaCl, 0.08 M acetic acid, pH 2.4	2.3×10^4
p-Nitrophenyl α-D-mannopyranoside	0.02 M NaCl[b], 0.08 M acetic acid, pH 2.4	1.1×10^4
p-Nitrophenyl α-D-glucopyranoside	0.5 M NaCl, 0.01 M phosphate, pH 6.5	1.0×10^4
Methyl α-D-mannopyranoside	1.0 M NaCl, pH 6.2 [18]	2.1×10^4

[a] A value of $K = 4.2 \times 10^4$ was calculated from the inhibition data of Poretz [34].
[b] Determined by equilibrium dialysis according to So and Goldstein [18].

Binding constants for *p*-nitrophenyl α-D-mannopyranoside and *p*-nitrophenyl α-D-glucopyranoside were determined under various conditions. The results are presented in the Table. Note that ionic

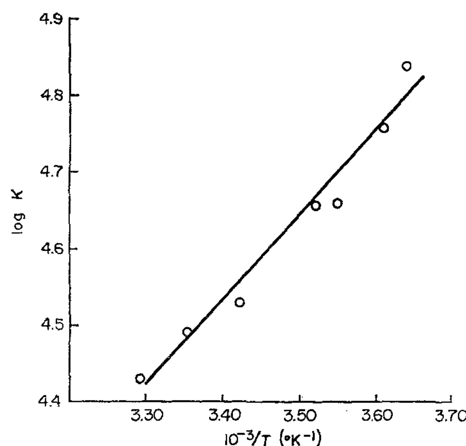

Fig.8. *Effect of temperature on binding of* p-*nitrophenyl* α-D-*mannopyranoside to concanavalin A.* Equilibrium constants were determined at the temperatures indicated using 50 µM *p*-nitrophenyl α-D-mannopyranoside and concanavalin A (1.1 mg/ml), assuming $\Delta\varepsilon_M$ at 315 nm of 4700 (calc. from binding studies). All solutions were 0.5 M NaCl in 0.01 M phosphate buffer, pH 6.5

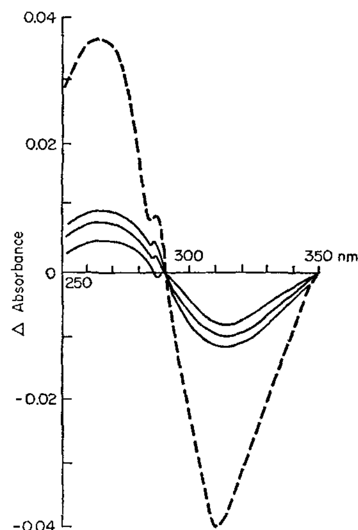

Fig.9. *Effect of cations on the ultraviolet difference spectrum of* p-*nitrophenyl* α-D-*mannopyranoside (50 µM) and metal free concanavalin A (1 mg/ml) in 0.05 M NaCl, pH 5.5.* Spectra obtained after successive additions of 1 µl 0.1 M $MnCl_2$ to mannoside-protein solution, (——); spectrum obtained after addition of 1 µl of 0.1 M $CaCl_2$, (----) to above solution

strength appears to have no effect upon the value of K (association constant) obtained; $K = 3.9 \times 10^4$ in 0.5 M NaCl, pH 6.5, and $K = 3.6 \times 10^4$ in 0.05 M NaCl, pH 6.5. Note also that the mannoside was bound very strongly even at pH 2.4 in 0.08 M acetic acid. These findings (at pH 2.4) were substantiated by equilibrium dialysis experiments. The binding data showed that even at pH 2.4 concanavalin A has approximately one binding site per 32000 g of protein as found previously [18,19]. It is also indicated in the Table that the ratio of the binding constants determined for the mannoside and glucoside under identical conditions (0.01 M phosphate, pH 6.5, 0.5 M NaCl) is 3.9 to 1. This is similar to results obtained by inhibition of concanavalin A—polysaccharide precipitation. Finally, note in the Table the excellent correlation between the calculated and observed binding constant for the mannoside at neutral pH.

Fig.8 shows the effect of temperature on the interaction of *p*-nitrophenyl α-mannoside with concanavalin A. $\Delta H°$ was determined graphically to be -4.76 kcal/mole.

An investigation of the metal requirement of concanavalin A showed that Mn^{++} or Ni^{++} and Ca^{++} were necessary for full protein activity [25,26,35] as measured by the ultraviolet difference spectrum obtained with *p*-nitrophenyl α-D-mannopyranoside. Metal-free concanavalin A was obtained as described previously [25]. Fig.9 shows that the addition of consecutive 1 µl aliquots of 0.1 M $MnCl_2$ did not greatly enhance the interaction between *p*-nitrophenyl α-D-mannopyranosides and concanavalin A. However, the addition of 1 µl of 0.1 M $CaCl_2$ to the above reaction mixture greatly increased the difference peak at 315 nm. When $NiCl_2$ was substituted for $MnCl_2$ exactly the same results were obtained. When the order of addition of $NiCl_2$ and $CaCl_2$ was reversed, the results were identical, namely, additions of several 1 µl aliquots of 0.1 M $CaCl_2$ elicited a small response but the addition of 1 µl of 0.1 M $NiCl_2$ greatly enhanced the difference spectra.

It was also found that small additions of methyl α-D-glucopyranoside and methyl α-D-mannopyranoside (about 1 mg) completely obliterated a difference spectrum generated between concanavalin A and *p*-nitrophenyl α-D-mannopyranoside in both neutral (pH 7.0) and acidic (pH 2.4) media, demonstrating that these methyl glycosides bind to the protein at these pH values.

In order to determine if polysaccharide is bound to concanavalin A at pH 2.4, the following experiment was performed: concanavalin A (2 mg/ml) was mixed with *p*-nitrophenyl α-D-mannopyranoside (0.2 mM) in 0.075 M acetic acid, pH 2.4, and this solution was placed into one compartment of each of two Yankeelov cells. Into the second compartment of each cell was placed a solution (4 mg/ml) of yeast mannan (*Saccharomyces cerevisiae*) in 0.075 M acetic acid. The interaction of this polysaccharide with concanavalin A is well known [35—37,12] and the pH optimum of precipitation with concanavalin A is 5.7 to 6.7. After tipping the sample cuvette a difference spectrum was obtained which was the mirror image of that observed with the *p*-nitrophenyl

glycosides (due to the mannan displacing the *p*-nitrophenyl glycoside from concanavalin A whereas the interaction in the reference cuvette was undisturbed). Thus the yeast mannan is shown to bind to concanavalin A at pH 2.4 despite failure to precipitate with the protein even after incubation for 8 days.

Molecular weight determinations of concanavalin A on a calibrated Biogel P-60 column showed concanavalin A to be eluted as a single component at an elution volume identical to that of ovalbumin using 0.1 N HCl in 0.2 M NaCl and 0.08 M acetic acid (pH 2.4) as eluants. Metal-free and native concanavalin A behaved identically under these conditions. Ultracentrifuge studies (Model E Spinco) using the same conditions as above and measuring sedimentation velocity gave substantially the same results.

DISCUSSION

Ultraviolet perturbation studies on the interaction of carbohydrases with carbohydrate substrates are now well documented. Since the primary and three dimensional structure of hen egg white lysozyme is now known in great detail, the studies on lysozyme are especially noteworthy [33,38]. Petterson [39] also has observed difference spectra when cellobiose was allowed to interact with the cellulase from *Penicillium notatum*. Both carbohydrase enzymes displayed sharp difference spectral maxima in the 292—293 nm region as well as much less intense maxima at lower wavelengths (280—285 nm) when allowed to interact with low molecular weight carbohydrate substrates or inhibitors.

Certain alkyl α-glycosides and D-glucose, known to be specifically bound by concanavalin A, interacted with the protein to generate very small but distinct ultraviolet difference spectra (Fig. 1). Methyl α-D-glucopyranoside and methyl α-D-mannopyranoside perturb concanavalin A differently than D-glucose. The maxima observed in the case of the glycoside difference spectra are in the 270—282 nm region, suggesting that perhaps tyrosyl residues (but not tryptophanyl residues) are being perturbed because of the specific interaction. The fact that methyl α-D-glucopyranoside (and the corresponding α-mannoside) gave more distinct and resolvable spectra than D-glucose (which produced a broad, nondescript maximum between 260—290 nm) is hardly surprising; the α-glycosides of these sugars which possess the specifically bound α-anomeric oxygen atom are bound 8—30 times more strongly than free D-glucose.

Chemical modification work, however, appears to eliminate the direct involvement of tyrosyl residues in the binding of carbohydrates by concanavalin A. Acetylation of this protein with acetic anhydride, in addition to acylating 83% of the ε-NH_2 groups of lysine, also resulted in the acetylation of 30% of the tyrosyl residues with very little loss of protein activity [27]. This was also the case when tyrosyl residues were acetylated with *N*-acetyl imidazole [28]. These results differ from those of Doyle and Roholt [40] who found more extensive modification, and a partial loss of activity, of concanavalin A upon treatment with *N*-acetyl imidazole. Tetranitromethane treatment and iodination of concanavalin A resulted in a less pronounced affect on protein activity and these workers also concluded that carbohydrates do not bind directly to tyrosyl residues of concanavalin A [40].

Observations of ultraviolet difference spectra generated by phenyl glycosides suggest that the maxima and minima observed are due to perturbations of the chromophoric aglycone portion of these glycosides by amino acyl residues in or near the carbohydrate binding sites of concanavalin A. Consideration of these results is assisted by reference to the absorption spectra of the phenyl, *p*-aminophenyl, and *p*-nitrophenyl glycosides shown in Fig. 2. Thus, phenyl α-D-glucopyranoside, which has absorption maxima of very low intensity at 261, 268 and 273 nm, interacted with concanavalin A to generate an ultraviolet difference spectrum which is similar to those obtained with the methyl α-glycosides (which displayed a small positive maximum at 285 nm and negative maxima at 265 and 270 nm) all of which are of low intensity. The difference spectrum obtained upon mixing concanavalin A with *p*-aminophenyl α-D-mannopyranoside (which had an absorption maximum near 290 nm) showed negative maxima at 270, 289 and 300 nm. Note that the ultraviolet difference spectrum exhibits large effects near the absorption maximum of the aglycone.

The observation that the 2-*O*-methyl ether of *p*-nitrophenyl α-D-mannopyranoside gave the same ultraviolet difference spectrum as the parent glycoside is not surprising inasmuch as these compounds inhibited concanavalin A—polysaccharide interaction to the same extent and it has been shown that it is the oxygen atom of the C-2 hydroxyl group of D-mannose which binds to the protein [34].

The most marked spectral differences were obtained when concanavalin A was interacted with *p*-nitrophenyl α-D-glucopyranoside and *p*-nitrophenyl α-D-mannopyranoside. Fig. 5 demonstrates that interaction of concanavalin A with *p*-nitrophenyl α-D-mannopyranoside alters the spectrum of the glycoside (causing a blue shift) thus leading to the type of ultraviolet difference spectrum observed in Fig. 3 and 4. The blue shift observed in the ultraviolet spectrum when *p*-nitrophenyl α-D-mannopyranoside is dissolved in dioxane is presented in Fig. 6. At 25° the dielectric constant of dioxane is 2.101 [41] and is probably not greatly different from that of the nonpolar region of protein molecules. It is suggested that

this shift is similar to the ultraviolet difference spectrum observed when concanavalin A interacts with *p*-nitrophenyl α-glucoside and mannoside.

It is interesting to note that the ultraviolet difference spectrum generated upon mixing *p*-nitrophenyl β-D-glucopyranoside with concanavalin A, was considerably less intense and qualitatively different from the interaction with *p*-nitrophenyl α-D-manno- and glucopyranosides (Fig. 3). However, it is known that β-linked phenyl glucosides and mannosides are bound to concanavalin A much less strongly and that these glycosides interact with a different region of the protein molecule [34].

Extensive spectral studies of dinitrophenyl and trinitrophenyl haptens with purified antibodies demonstrated that these highly chromophoric haptens will generate difference spectra (when allowed to interact with purified antibodies specific for the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl groups) near the absorption maxima of the haptens [42]. Since the difference spectra observed were obtained at wavelengths above 300 nm, these workers were able to use high concentrations of antibody protein or amino acids (as model compounds) to further explore these results. Interactions of these haptens with high concentrations of tryptophan gave rise to difference spectra similar to those obtained when hapten was bound to antibody, and the authors concluded that tryptophanyl residues in or near the antibody combining sites are interacting with the dinitrophenyl and trinitrophenyl chromophoric haptens, leading to the large difference spectra observed. However, they did not conclusively rule out the possibility that these large spectral changes in antibody bound hapten are caused by relatively nonspecific solvent effects attributable to a difference between the dielectric environments of the antibody combining site and the bulk solvent.

By exploiting the strong interaction of the *p*-nitrophenyl α-D-glycosides with concanavalin A, several parameters of concanavalin A—carbohydrate interaction were studied. Thus, the necessity of Mn^{++} and Ca^{++} for maximum protein activity was confirmed by the ultraviolet spectral difference technique (Fig. 9).

Binding constants were determined for the interaction of *p*-nitrophenyl α-D-glucopyranoside and *p*-nitrophenyl α-D-mannopyranoside. The Table shows that the results obtained were similar to the values predicted from an analysis of previous inhibition studies [34]. Thus, inasmuch as *p*-nitrophenyl α-D-mannopyranoside is, on a molar basic, twice as potent as methyl α-D-mannopyranoside in inhibiting the interaction of concanavalin A with levans, one would except its binding constant to be twice as large. Within experimental error this result was obtained. Also, the *p*-nitrophenyl α-D-mannopyranoside has a binding constant which is approximately 4-fold larger than that of the glucoside, a result which has been obtained repeatedly in this system [14, 16, 17].

One of the most interesting aspects of this study was an investigation of the pH optimum of the binding of *p*-nitrophenyl glycosides by concanavalin A. Fig. 7 shows the rather broad pH optimum obtained. As previously stated, concanavalin A does not precipitate polysaccharides between pH 2 and pH 4. Nevertheless, the protein binds *p*-nitrophenyl α-D-glucopyranoside and *p*-nitrophenyl α-D-mannopyranoside at these low pH values. The protein also binds yeast mannan, as demonstrated by the ultraviolet difference experiment described above.

The fact that concanavalin A is capable of binding simple carbohydrate haptens as well as polysaccharide at low pH values, but does not precipitate polysaccharides at low pH values is unexplained at this time. Gel filtration and ultracentrifuge studies indicate that the protein did not dissociate into monovalent subunits. It is possible that electrostatic repulsion of the protein molecules due to their high net positive charge (concanavalin A has an isoelectric point of 7.1 ± 0.1) [22] prevents protein-polysaccharide lattice formation and hence failure of the complex to precipitate.

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