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Protein–Carbohydrate Interaction. XVIII. The Preparation and Properties of Acetylated Concanavalin A, the Hemagglutinin of the Jack Bean*

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ABSTRACT: Concanavalin A, the phytohemagglutinin of the jack bean, was acetylated with sodium acetate and acetic anhydride; 84% of the amino groups and 31% of the phenolic hydroxyl groups were acetylated. The modified protein demonstrated no change in reactivity toward antibodies to the native protein as judged by two-dimensional agar gel diffusion. However, the electrophoretic mobility was changed and the ability of the protein to bind the Mn^{2+} ions (required for activity) was greatly diminished.

The activity of acetylated concanavalin A was investigated in detail. Agar gel and quantitative precipitation studies showed that the reaction of acetylated concanavalin A with the mannan from *Saccharomyces cerevisiae* was almost identical with that displayed by the native protein. However, the interaction with dextran B-1355-S was diminished slightly and that with the levan from *Aerobacter levanicum* was greatly decreased. Acetylated concanavalin A also failed to bind to Sephadex G-50 and a cross-linked levan even when these gels were equilibrated with 0.001 M $MnCl_2$. Inhibition studies with the acetylated protein using several representative inhibitors of the concanavalin A–dextran B-1355-S interaction revealed no change in the specificity of the modified protein. Unlike the native protein, acetylated concanavalin A required high NaCl concentrations (greater than 0.8 M NaCl) before displaying full activity. However, varying the NaCl concentrations from 0.2 to 0.6 M had little effect upon the specific viscosity of the modified protein. It is concluded from these studies that significant acetylation of free amino groups and phenolic hydroxyl groups of concanavalin A yields a modified protein which still retains considerable activity (capacity to precipitate specific polysaccharides) and whose specificity is not altered. This is suggested to mean that free amino groups and many of the tyrosyl residues are not important in maintaining the structural integrity of the protein and its combining sites. It would also appear that these residues are not directly involved in the binding of carbohydrates by concanavalin A.

The phytohemagglutinin of the jack bean, concanavalin A (Sumner and Howell, 1935), has been shown to form a specific precipitate with a variety of biological macromolecules. These include polysaccharides, such as glycogens, dextrans, yeast mannans, amylopectins, and certain levans (Sumner and Howell, 1936; Cifonelli *et al.*, 1956; Manners and Wright, 1962; Goldstein *et al.*, 1965a; Goldstein and So, 1965; So and Goldstein, 1968; L. L. So and I. J. Goldstein, 1968, manuscript in preparation), various serum glycoproteins (Nakamura *et al.*, 1960, 1965; Harris and Robson, 1963; Leon, 1967; I. J. Goldstein, L. L. So, Y. Yang, and Q. Callies, 1968, manuscript in preparation), and a number of carbohydrate–bovine serum albumin conjugates (Goldstein and Iyer, 1966).

The stereochemical requirements of the combining sites of concanavalin A have been investigated in detail by examining the extent to which a wide variety of low molecular weight carbohydrates inhibited the concanavalin A–polysaccharide interaction (Goldstein *et al.*,

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1965b; Smith and Goldstein, 1967; So and Goldstein, 1967a; Poretz and Goldstein, 1967a,b). These studies have demonstrated that the combining sites of concanavalin A accommodate sugars which possess the unsubstituted α-D-glucopyranosyl, α-D-mannopyranosyl, or β-D-fructofuranosyl residues, the common configurational features being the disposition of the hydroxyl groups at the C-3, C-4, and C-6 positions of the α-D-hexopyranosyl and the β-D-fructofuranosyl rings, respectively.

The availability of concanavalin A in large quantities and in a virtually homogeneous state (Agrawal and Goldstein, 1967a) have made it an ideal protein to study from a structure–function viewpoint.

Concanavalin A has been shown to be an associating–dissociating system of identical subunits of mol wt ~16,500 (Olson and Liener, 1967). At pH 7.5 the protein is maximally active and is completely excluded from a Bio-Gel P-100 column (Agrawal and Goldstein, 1968). In order to learn which amino acyl residues of concanavalin A might be crucial in maintaining the structural integrity of the protein, and in forming a part of the combining sites of the protein, we have initiated a program of specific chemical modifications of concanavalin A. This paper describes the effect of acetylation using sodium acetate–acetic anhydride on the structure and activity of concanavalin A. Further modification studies are in progress.

Materials and Methods

Chemicals. Glucose was purchased from Mann Research Laboratories, sucrose from Merck & Co., and methyl α-D-glucopyranoside and methyl α-D-mannopyranoside from Pfanstiehl Laboratories. Methyl β-D-fructofuranoside was synthesized by the method of Bose (1964). Isomaltose was a gift of Dr. Allene Jeanes. All sugars used were chromatographically pure. Hydroxylamine hydrochloride was obtained from the Eastman Organic Chemicals Co. *p*-Nitrophenyl acetate was the generous gift of Dr. J. A. Shafer. Sephadex was purchased from Pharmacia Fine Chemicals. Cross-linked levan was the generous gift of Dr. E. A. Kabat.

Preparation of Concanavalin A. Concanavalin A was prepared by the procedure of Agrawal and Goldstein (1967b) and stored at 4° in 1.0 M NaCl. Alternatively, the purified protein was dialyzed free of NaCl, lyophilized, and stored at 4°. Agar gel diffusion studies were performed as previously described (Goldstein and So, 1965).

Quantitative Precipitation and Inhibition. Quantitative precipitation studies of acetylated concanavalin A with dextran B-1355-S, the mannan from *Saccharomyces cerevisiae*, and levan from *Aerobacter levanicum* were conducted as described by So and Goldstein (1967b). The amount of polysaccharide precipitated at each stage of the precipitation curve was determined by the phenol–H_2SO_4 method of Dubois *et al.* (1956).

A previously described turbidimetric method was employed for hapten inhibition experiments on the acetylated concanavalin A–dextran system (So and Goldstein, 1967b). A typical reaction mixture (3.0 ml) contained 620 μg of acetylated concanavalin A and 525 μg of dextran B-1355-S in 1.0 M NaCl and 0.018 M phosphate (final pH 7.0) along with varying amounts of inhibitors. The turbidity at 420 mμ was compared with that of an uninhibited reaction and the percentage inhibition was calculated.

pH optimum studies on the acetylated protein were performed using a turbidimetric method previously described for native concanavalin A (Agrawal, 1967).

Acetylation of Concanavalin A. The procedure used was essentially as described by Fraenkel-Conrat (1955) and Kabat and Mayer (1961). Lyophilized concanavalin A (500 mg) was suspended in sodium acetate solution (10 ml) cooled in an ice bath. Acetic anhydride (0.6 ml) precooled to 0° was added in six 0.1-ml portions over a period of 1 hr, after which the suspension was dialyzed extensively against deionized water and lyophilized.

Antibody to Concanavalin A. Several rabbits were immunized with concanavalin A by periodic injection into the footpads of complete Freund's adjuvant containing 1–5 mg of the native protein. The adjuvant was prepared as follows. To a mixture containing 2.55 ml of Bayol F, 0.45 ml of Arlacel A, and 15 mg of heat-killed *Mycobacterium tuberculosis* H 37 Ra cells (Difco Laboratories lot no. 476464) was slowly added 3.0 ml of a buffered saline solution containing native concanavalin A (concanavalin A (10 mg/ml), 0.01 M phosphate (pH 7), and 0.14 M NaCl). This suspension was homogenized and stored at 4°. The rabbits were bled several times by cardiac puncture, the blood was allowed to clot (24 hr), and the clotted blood was centrifuged. Antisera from different bleeding times and different rabbits were not pooled. The sera were used without further purification and were stored at −10°.

Agar gel diffusion studies were employed as described previously except that the agar gel also contained 0.1 M methyl α-D-mannopyranoside, an inhibitor of the concanavalin A system. This was incorporated in order to eliminate the interaction of concanavalin A with various serum glycoproteins. Thus, the only interaction observed was the antibody to native concanavalin A reacting with concanavalin A.

Determination of the Extent of Chemical Modification. Modification of the amino groups was determined by the ninhydrin method (Rosen, 1957) using L-leucine as the standard. Comparison of the ninhydrin color of the modified and native protein gave an estimate of the extent of the modification.

O-Acetylation of tyrosine, threonine, and serine was determined by the alkaline hydroxamate method of Hestrin (1949). The reagents were: 2.0 M $NH_2OH \cdot HCl$, 3.5 N NaOH, concentrated HCl diluted 1:3, and 0.37 M $FeCl_3$ in 0.1 N HCl. Reactions which had an optical density too dense to measure were diluted with a solution which consisted of 0.07 M $FeCl_3$ in 0.1 N HCl. Alkaline NH_2OH reagent was prepared by mixing equal volumes of the $NH_2OH \cdot HCl$ and NaOH solutions. This solution (2.0 ml) was added to 0.5–3.0 ml of sample (0–2.0 μmoles of *O*-acetate) in 0–1.0 M NaCl and incubated for 15 min at 25°. The HCl solution (1.0 ml) was added followed by 1.0 ml of 0.37 M $FeCl_3$ solution. Protein which pre-

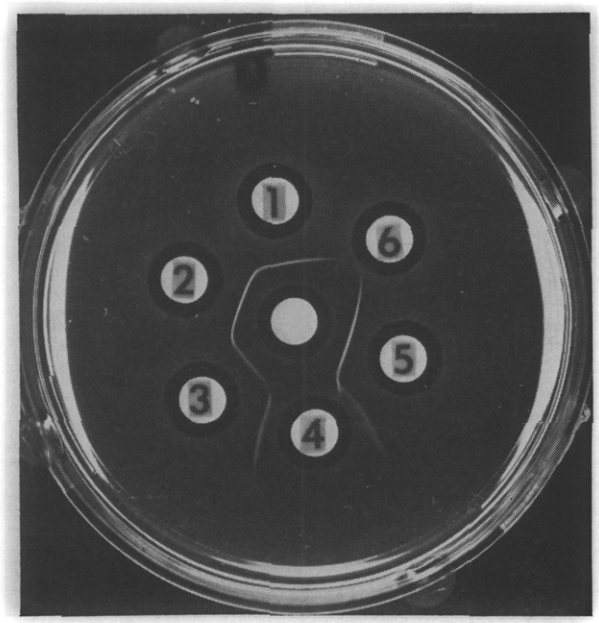

FIGURE 1: Reaction of native and acetylated concanavalin A with rabbit antisera to the native protein. Center well contains antiserum from rabbit 3. Peripheral wells: (1 and 3) acetylated concanavalin A, 100 μg/ml; (2 and 5) native concanavalin A, 100 μg/ml; (4) antiserum from rabbit 4; and (6) 0.2 M NaCl. All volumes, approximately 0.1 ml.

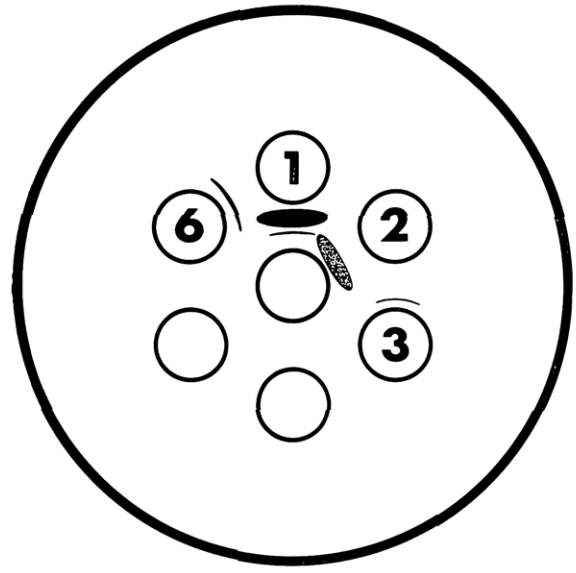

FIGURE 2: Reaction of native and acetylated concanavalin A with levan B-1662 and dextran B-1355-S. Center well contains levan B-1662, 2 mg/ml. Peripheral wells: (3 and 6) dextran B-1355-S; (1) native concanavalin A, 3 mg/ml; and (2) acetylated concanavalin A, 3 mg/ml. All volumes, approximately 0.1 ml.

cipitated was centrifuged and the supernatant solution was decanted into matched Bausch and Lomb Spectronic 20 tubes (0.5-in. diameter). The absorbance at 540 mμ was measured in a Spectronic 20 colorimeter. *p*-Nitrophenyl acetate was used as standard. The color at 540 mμ was stable up to 60 min. *N*-Acetylglycine gave no color under these conditions, whereas ethyl acetate was quantitatively transformed to the corresponding hydroxamate. It was also possible to measure quantitatively the *O*-acetyl content of ethyl β-D-glucopyranoside tetraacetate.

A modified procedure was employed for measuring hydroxamate formed at pH 7. $NH_2OH \cdot HCl$ (2.0 M) was mixed with NaOH solution (1:0.57, v/v). Neutral hydroxamate solution (1.6 ml) was added to the sample, which also contained 0.5 ml of 0.1 M phosphate buffer (pH 7.0). The pH of the solution was always 7.0 ± 0.1. After incubating at 25° for 15 min, 1.0 ml of HCl was added followed by 0.4 ml of NaOH and 1.0 ml of $FeCl_3$ solutions. Under these conditions the same standard curve was obtained with *p*-nitrophenyl acetate but ethyl acetate gave no color at 540 mμ.

O-Acetylation of tyrosine, threonine, and serine was determined by the alkaline procedure. Since only phenyl acetates are hydrolyzed by NH_2OH at pH 7, the determination at pH 7 gave an accurate measure of *O*-acetylated tyrosyl residues. The number of *O*-alkyl acetyl groups was determined by the difference between these two values.

Viscometry Determinations. An Oswald viscometer was used for these determinations. A 2.5% solution of acetylated concanavalin A (3.0 ml), 1.0×10^{-4} M $MnCl_2$, and 0.2–0.6 M NaCl, was added to the viscometer. Times for flow in a 40° bath were measured and, after performing the appropriate solvent blanks, the specific viscosity was calculated.

Cellulose Acetate Electrophoresis. Electrophoresis an cellulose acetate was performed using the Gelman apparatus. The duration for each run was 1 hr at a constant voltage of 250 V (18 V/cm), acetate buffer (pH 5.0), ionic strength 0.05. The strips were stained with ponceau S.

Results

Acetylation of concanavalin A by treatment with sodium acetate and acetic anhydride resulted in extensive modification of the protein. The ninhydrin determination indicated that there was an 84% reduction in ninhydrin color yield of the acetylated protein. Acetylation could occur on both α-amino and ε-amino groups and this result does not differentiate the amount of modification of each type of residue. However, it does indicate that considerable modification of free amino groups did occur (there are 47 lysyl residues/100,000 g of concanavalin A). Repeated acetylations never yielded more than an 89% reduction in ninhydrin color. The hydroxamate determination at pH 13 indicated that there were 10 moles of *O*-acetyl residues/100,000 g of concanavalin A. When this determination was performed at pH 7, 9 moles of *O*-acetyl groups was found. These results indicated that, within experimental error, there were 9 ± 1 *O*-acetyltyrosyl residues/100,000 g of protein (there are 29 tyrosyl residues/100,000 g of protein).

Further evidence for alteration of the concanavalin A molecule was demonstrable by cellulose acetate electrophoresis at pH 5.0. The results showed that whereas untreated concanavalin A migrated to the cathode, the the acetylated preparation migrated toward the anode,

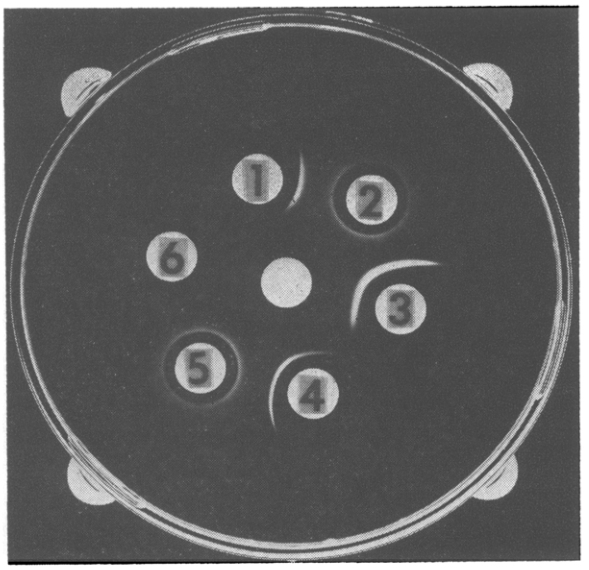

FIGURE 3: Interactions in agar gel of native and acetylated concanavalin A with various polysaccharides. Center well contains acetylated concanavalin A, 5 mg/ml. Peripheral wells: (1) levan from *A. levanicum*, 5 mg/ml; (2 and 5) native concanavalin A, 5 mg/ml; (3) rabbit liver glycogen, 5 mg/ml; (4) dextran B-1355-S, 5 mg/ml; and (6) 1 M NaCl. All volumes, approximately 0.1 ml.

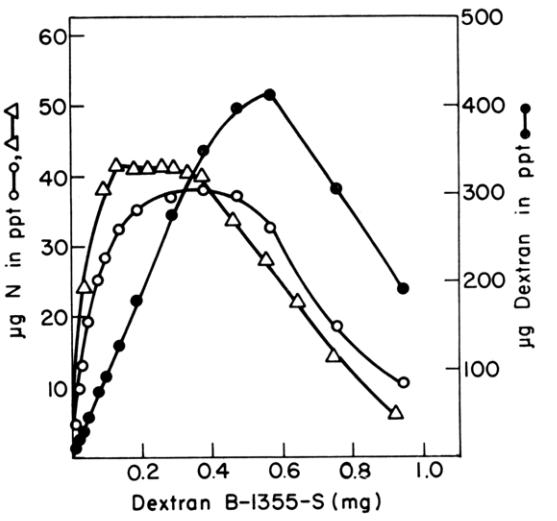

FIGURE 4: Quantitative precipitation curve of dextran B-1355-S with native (△) and acetylated (○) concanavalin A. The total amount of dextran in the precipitate with the acetylated protein is also illustrated. Native concanavalin A, 42 μg of nitrogen; acetylated concanavalin A, 46 μg of nitrogen.

although a trace of material remained at the origin. The acetylated product was readily washed from cellulose acetate strips by washing with 5% acetic acid. Whereas it requires extensive dialysis at pH 2 to remove Mn^{2+} from native concanavalin A, the Mn^{2+} was readily removed from the acetylated protein by dialysis even at neutral pH. Thus, activity (and viscometry) determinations necessitated the addition of $MnCl_2$. Furthermore, acetylated concanavalin A had lost its capacity to bind to cross-linked dextran (*e.g.*, Sephadex G-50) and levan gels. Even when the gels were equilibrated with low concentrations (0.001 M) of $MnCl_2$, all of the protein emerge with the void volume of the column.

Figure 1 shows the agar gel patterns obtained when antisera to native concanavalin A were treated with native and acetylated concanavalin A, using the double diffusion method. Antisera from two different rabbits were used. Reactions of immunological identity resulted when either native or acetylated concanavalin A was treated simultaneously with the two antisera. The same finding was obtained when native and acetylated concanavalin A were treated with each of the antisera separately. Evidently all of the antigenic determinants on the native protein are still present after the protein has been extensively modified by acetylation.

Acetylated concanavalin A gave a precipitation band with dextran B-1355-S on agar gel diffusion. Levan B-1662 gave two precipitation bands with native concanavalin A, an intense rather broad band and a second weak but sharp band. It was suggested on the basis of chemical evidence that the levan was contaminated with a small quantity of dextran (Goldstein and So, 1965). On acetylation of the protein only one band (broad) of approximately the same mobility as the sharp lower moving one obtained with native concanavalin A was observed. It is possible that only the dextran gives a band with the acetylated protein. However, the band formed between acetylated concanavalin A and dextran B-1355-S is identical in position (although somewhat diminished in intensity) with the band between native concanavalin A and dextran B-1355-S (Figure 2).

Figure 3 shows further interactions in agar gel. The band formed between acetylated concanavalin A and rabbit liver glycogen fuses with the band formed between native concanavalin A and rabbit liver glycogen to afford what has been referred to as a line of identity in immunochemical terms. A similar observation resulted when dextran B-1355-S was used as the precipitating polysaccharide, although the portion of the band due to acetylated concanavalin A interacting with the dextran was somewhat less intense. Under the experimental conditions employed, the weak interaction of acetylated concanavalin A with the levan from *A. levanicum* was not observed.

The pH optimum of the acetylated concanavalin A–dextran B-1355-S interaction was shown to be between 6.2 and 7.2, which is similar to that for native concanavalin A (So and Goldstein, 1967b).

Quantitative precipitation analyses of the acetylated concanavalin A were performed using three different polysaccharides, dextran B-1355-S, the levan from *A. levanicum*, and the yeast mannan from *S. cerevisiae*. Figure 4 shows the results obtained with dextran B-1355-S. All assays with the acetylated protein were performed in 0.001 M $MnCl_2$. For comparison, the precipitation curve of native concanavalin A with dextran B-1355-S is shown on the graph. No significant difference in the activity between native and acetylated concanavalin A was observed. Figure 4 also shows the total amount of dextran precipitated at each stage of the precipitation

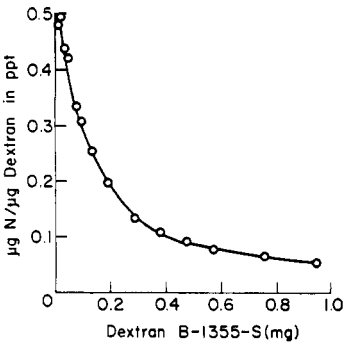

FIGURE 5: Composition of acetylated concanavalin A–dextran precipitates in weight ratios. Acetylated concanavalin A, 46 μg of nitrogen.

curve. Up to and throughout the equivalence zone, almost all of the dextran added was precipitated by acetylated concanavalin A. Figure 5 presents a plot of the ratio of protein nitrogen to dextran in the precipitate. Again, the results are similar to those obtained with native concanavalin A.

When the levan from *A. levanicum* was used as the precipitating polysaccharide longer periods of time were required for precipitation and only 38% of the total protein nitrogen was precipitated at the end of 10 days (Figure 6). In contrast, 70% of native concanavalin A was precipitated by this polysaccharide in 48 hr (So, 1967).

The mannan from *S. cerevisiae* interacted very strongly with acetylated concanavalin A, as illustrated in Figure 7; 90% of the acetylated concanavalin A was precipitated by the mannan.

The inhibition of acetylated concanavalin A–dextran B-1355-S interaction by several representative sugars is shown in Figure 8, in which percentage inhibition is plotted against the micromoles of inhibitor added on a logarithmic scale. D-Galactose (6% inhibition at 123 μmoles) and melibiose ($Gal_p1\xrightarrow{\alpha}6G_p$) (0% inhibition at 101 μmoles) were noninhibitors of acetylated concanavalin A–polysaccharide interaction. The inhibition curves of D-glucose and sucrose are essentially identical. Methyl α-D-mannopyranoside (0.5 μmole for 50% inhibition) was about four times more potent as an inhibitor than methyl α-D-glucopyranoside (2.0 μmoles for 50% inhibition) and displayed ten times the inhibition potency of methyl β-D-fructofuranoside (5.0 μmoles for 50% inhibition). It should also be noted that isomaltose ($G_p1\xrightarrow{\alpha}6G_p$) is as good an inhibitor as methyl α-D-glucopyranoside, precisely what was noted in the case of the native protein.

Figure 9 shows the effect of increasing NaCl concentration on the reactivity of acetylated concanavalin A with dextran B-1355-S as assayed turbidimetrically. From essentially zero turbidity (*i.e.*, interaction) after 12 min in 0.1 M NaCl, there is a dramatic rise in turbidity in the concentration range 0.2–0.6 M NaCl. This observation is the same at both 25 and 40°. Figure 9 also shows that the specific viscosity, η_{sp}, for a 2.5% solution of acetylated concanavalin A *increased* slightly when the NaCl concentration is raised from 0.2 to 0.6 M.

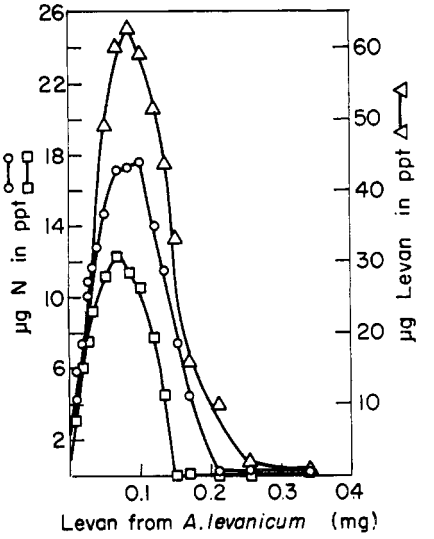

FIGURE 6: Quantitative precipitation study of acetylated concanavalin A with levan from *A. levanicum:* (□) 140-hr precipitation and (○) 235-hr precipitation. Acetylated concanavalin A, 46 μg of nitrogen.

Discussion

One of the prime interests in our study of concanavalin A–carbohydrate interaction has been an attempt to elucidate in detail the precise manner in which carbohydrate binds to the active sites of the protein. Such studies could be of primary importance in furthering our understanding of how carbohydrate moieties bind to the combining sites of enzymes, immune antibodies, and possibly systems concerned in the transport of carbohydrate. To this end we have embarked on a program of specific chemical modification of concanavalin A in an effort to identify those amino acyl residues which are involved in noncovalent interaction with the glycosyl residues with which this protein interacts.

The free amino groups of concanavalin A were extensively modified by treatment with acetic anhydride in sodium acetate solution. Somewhat surprising was the acetylation of the phenolic hydroxyls of tyrosyl residues. This was estimated chemically by means of the hydroxamic acid test. There was also a slight decrease in the

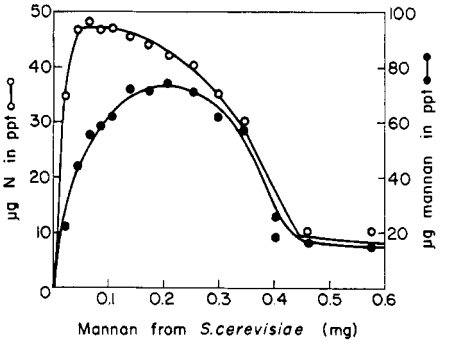

FIGURE 7: Quantitative precipitation curve of mannan from *S. cerevisiae* with acetylated concanavalin A. The total amount of mannan in the precipitation is also illustrated. Acetylated concanavalin A, 54 μg of nitrogen.

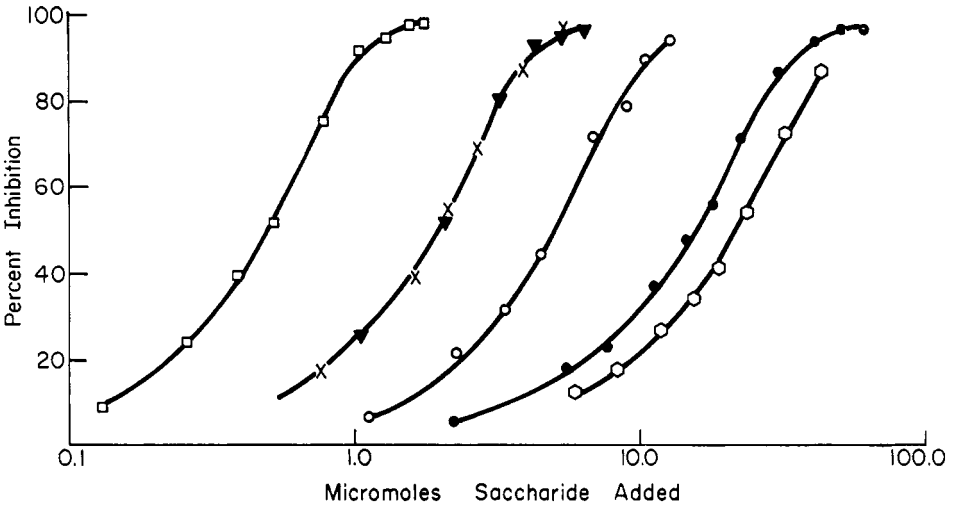

FIGURE 8: Inhibition of acetylated concanavalin A–dextran precipitation by representative carbohydrates. (—□—) Methyl α-D-mannopyranoside, (—×—) isomaltose, (—▼—) methyl α-D-glucopyranoside, (—○—) methyl β-D-fructofuranoside, (—●—) D-glucose, and (—○—) sucrose. Reaction mixture contained 620 μg of acetylated concanavalin A and 525 μg of dextran B-1355-S.

extinction coefficient at 278 mμ in 1 M NaCl but the use of this measurement to determine *O*-acetyltyrosyl formation (Simpson *et al.*, 1963) is very difficult because of the high tryptophan content (Agrawal and Goldstein, 1966) and correspondingly high over-all extinction coefficient at 278 mμ.

The acetylation of tyrosyl hydroxyl groups using sodium acetate–acetic anhydride has been reported. Tabachnick and Sobotka (1960) demonstrated that they could acetylate 15 of the 19 tyrosyl residues of bovine serum albumin with acetic anhydride if they had previously acetylated 87% of the free amino groups with this same reagent. Recently, Fraenkel-Conrat and Colloms (1967) have shown both with glycyltyrosine and tobacco mosaic virus protein that *O*-acetylation of tyrosyl residues occurs almost as readily as *N*-acetylation of amino groups. In fact, they attributed the presumed specificity of acetic anhydride for acetylation of amino groups to the fact that in most proteins more of the amino groups are on the surface of the protein whereas many of the tyrosyl residues are buried in the interior of the molecule.

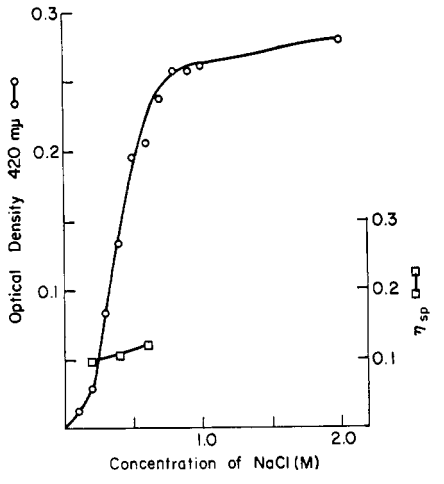

FIGURE 9: Effect of NaCl concentration on interaction of acetylated concanavalin A with dextran B-1355-S as measured by turbidity formation. Reaction mixtures contain 600 μg of acetylated concanavalin A and 600 μg of dextran B-1355-S. Also shown is effect of NaCl concentration on specific viscosity of acetylated concanavalin A.

The chemical modification of concanavalin A was also evident from its physical behavior. The electrophoretic mobility of the acetylated protein was, as expected, markedly different from the native protein. Its affinity for Mn^{2+} was so greatly reduced that Mn^{2+} could be easily removed upon dialysis against 1 M NaCl or deionized water. However, Mn^{2+} was still required for interaction with polysaccharides and was therefore included routinely in all incubations with acetylated concanavalin A. Finally it was not possible to specifically adsorb acetylated concanavalin A onto cross-linked dextran or levan gels even when these gels were equilibrated with $MnCl_2$.

Evidently, acetylation of concanavalin A does not introduce gross changes in the protein, as the immunochemical studies show. The acetylated protein apparently retains all of the antigenic determinants of the native protein by the criterion of double diffusion in agar gel. If this were not the case, one would expect spur formation (Crowle, 1961) due to a loss of certain antigenic determinants as a consequence of acetylation. Thus it would appear that acetylation of ε-amino groups of lysyl residues and certain phenolic hydroxyl groups of tyrosyl residues does not considerably alter the antigenic properties of concanavalin A. A similar situation exists in the case of bovine serum albumin and many other protein antigens (for an extensive review of the literature, see Ram *et al.*, 1962). Extensive acetylation of amino groups resulted in relatively little change in the reaction with the homologous antibodies for ovomucoid, insulin, ovalbumin, ribonuclease, β-lactoglobulin, gelatin, and bovine serum albumin.

Agar gel diffusion studies indicated that acetylated concanavalin A still interacts strongly with rabbit liver glycogen. However, the reaction of the modified protein with dextran B-1355-S is definitively diminished when compared to the interaction of native concanavalin A with this polysaccharide. The interaction of native concanavalin A with the levan from *Aerobacter levanicum* was quite weak and the acetylated protein displays a greatly reduced affinity for this polysaccharide as can be seen from the agar gel studies.

Because of the extensive modification of the native protein and the corresponding changes produced in over-all charge distribution on the protein in the neutral pH range, it might be expected that the pH characteristics of the interaction of acetylated concanavalin A with polysaccharides would change somewhat. However, examination of this effect showed that the pH optimum was identical with that of native concanavalin A. Any charge effect that acetylation may have conferred on concanavalin A is apparently not sufficient to cause disruption of the saccharide combining sites.

Quantitative precipitation analyses of acetylated concanavalin A with the three polysaccharides used in this study reveal a remarkable similarity to that observed for the native protein (So, 1967). The greatest change in reactivity occurred in the interaction of the modified protein with the levan from *A. levanicum*. The low amount of nitrogen precipitated and the extensive time intervals required for this interaction show that acetylation of concanavalin A has diminished somewhat its capacity to react with this polysaccharide. However, since the native protein also interacted quite weakly with this levan, a slight reduction of an already weak interaction resulted in this low reactivity.

With the two more reactive polysaccharides, dextran B-1355-S and the mannan from *S. cerevisiae*, the precipitation curves obtained were almost identical with those obtained with native concanavalin A. Also, the characteristically continuous variation in the ratio of protein nitrogen to carbohydrate in the precipitate (Figure 5) remains the same as that for native concanavalin A.

Hapten inhibition studies conducted on the acetylated concanavalin A–dextran system indicate quite definitely that acetylation of the protein did not alter its *specificity*. The order of reactivity of the various inhibitors tested was identical with that found with native concanavalin A–dextran precipitation (So and Goldstein, 1967a), *i.e.*, methyl α-D-mannopyranoside > methyl α-D-glucopyranoside > methyl β-D-fructofuranoside. However smaller quantities of these sugars were needed to effect the same percentage inhibition, indicating that the interaction of polysaccharides with acetylated concanavalin A is weaker than that between native concanavalin A and polysaccharides. Thus, whereas it required 1.65 μmoles of methyl α-D-mannopyranoside to inhibit native concanavalin A–dextran interaction by 50% (So and Goldstein, 1968), it required only 0.5 μmole to achieve 50% inhibition of acetylated concanavalin A–dextran interaction.

Since the preparation of acetylated concanavalin A studied had an 84% reduction in ninhydrin color, considerable free amino groups must have been acetylated and at neutral pH there was a considerable reduction of positive charges. Thus at pH 7 concanavalin A has a much higher net negative charge than in the native state.

When a macromolecule attains a considerable net charge, one would expect it to possess a lower electrostatic free energy in an expanded configuration (Tanford, 1961). If this is indeed the case in the concanavalin A system, then the expanded configurations of the protein could lead to a disruption of the integrity of the combining sites. However, the effect of repulsion by the net negative charges at neutral pH may be "masked" by an increase in the supporting electrolyte concentration. Thus, the expansion of bovine serum albumin at acid pH (as measured by an increase in the intrinsic viscosity) is initiated at a net positive charge of +15 on the protein molecule at ionic strength 0.01, but at an ionic strength of 0.15, a net positive charge of +35 is required to initiate the expansion of its configuration (Tanford, 1961). If at neutral pH the negative charge on concanavalin A is "smeared" by a high salt concentration, then it would be expected that the protein would return to its native, preferred configuration. If this were the case, one might also expect that the combining sites on the protein molecule would remain intact and that the protein would retain its activity. That this is in fact true can be seen from Figure 9.

The specific viscosity of acetylated concanavalin A was measured at a protein concentration of 2.5% in sodium chloride solutions varying in ionic strength from 0.2 to 0.6 in order to ascertain if an increase in specific viscosity could be observed at the lower ionic strengths. The specific viscosity increased very slightly when the ionic strength was varied from 0.2 to 0.6. Since the activity of acetylated concanavalin A also increased dramatically over this range of ionic strength, the specific viscosity apparently is not a sufficiently sensitive parameter for determining a marked change in the protein configuration. Lack of solubility of the protein prevented the use of a higher protein concentration or lower salt concentrations in these studies. If the loss of activity was due to a configurational change in acetylated concanavalin A due to electrostatic repulsions, the effect was not very great. An alternative explanation is that the region on the protein surface in close proximity to the combining sites was disrupted locally but that the over-all configurational change of the protein in the range of ionic strength 0.2–1.0 is too small to detect by viscometric procedures.

In summary, these studies demonstrate that extensive acetylation of concanavalin A caused no gross disruption of association of protein subunits or of the individual combining sites. Despite the rigorous modification, the protein is remarkably similar to native concanavalin A in its activity and in the nature of its antigenic determinants. This is strongly suggestive evidence for the nonparticipation of free amino groups and some of the tyrosyl residues in important structural features or in binding of carbohydrates at the combining site of concanavalin A.

Additional modification studies in progress should yield further information on the precise amino acyl res-

idues which may be involved in concanavalin A–carbohydrate interaction.

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