Chemical Modification and Hybridization of Wheat Germ Agglutinins†

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ABSTRACT: Four chromatographically distinct forms of wheat germ agglutinin were isolated from commercial wheat germ and shown to be similar in amino acid composition, molecular weight, and isoelectric point. Three of these forms were found to undergo subunit exchange with each other or with chemically modified electrophoretic variants to give hybrid agglutinins by exposure to denaturants, pH extremes, or high salt concentrations. One form was not observed to give hybrids, probably due to intersubunit disulfide bonding. Chemical modification studies employed in obtaining electrophoretic variants indicated that acetylation or succinylation of amino groups did not markedly change the lectin dimeric subunit structure or erythrocyte agglutinating ability, but the modified protein was unable to bind to ovomucoid-Sepharose columns. Acetylation of tyrosine residues, in conjunction with amino group acylation, produced a large change in protein conformation, probably involving subunit dissociation. Carbodiimide-mediated carboxyl group modification also produced a conformational change indicative of subunit dissociation, but some binding affinity to ovomucoid-Sepharose columns was retained.

Lectins are finding wide use in studies of animal cell surface structure by virtue of their ability to bind and cross-link membrane complex carbohydrates (for review see Lis and Sharon, 1973). Wheat germ agglutinin (WGA[1]) is a lectin that has received particular attention in demonstration of altered membrane fluidity of cultured cells upon transformation (Aub et al., 1963; Ozanne and Sambrook, 1971), in modulation of fat cell metabolism through insulin receptor binding (Cuatrecasas, 1973; Cuatrecasas and Tell, 1973), and in localization or isolation of specific membrane components (cf. Nicolson, 1974; Etzler and Branstrator, 1974).

Lectins have been isolated as closely related multiple forms from many sources (cf. Lis and Sharon, 1973; Waxdal, 1974; Carter and Etzler, 1975) including commercial wheat germ (Allen et al., 1973). In the present work, the multiple forms of WGA were compared in amino acid composition, molecular weight, isoelectric point, and ability to undergo mutual subunit interchange.

Hybridization of oligomeric proteins in vitro using naturally occurring (Markert, 1963) or chemically modified (Meighen and Schachman, 1970) electrophoretic variants has provided a generally applicable approach to investigation of subunit structure (Dawson et al., 1965; Penhoet et al., 1967) and interactions (Guidotti, 1967; Cardenas and Dyson, 1973; Bunn and McDonough, 1974; Gibbon et al., 1974). The dimeric subunit structure and dissociation–association properties of WGA (Nagata and Burger, 1974; Rice and Etzler, 1974) suggested that this approach might be suitable for demonstration of subunit interchange among the naturally occurring multiple forms of this lectin or chemically modified variants. In the present investigation, preparation of suitably modified variants has led to inferences about the relation of certain functional groups of this protein to its subunit structure and carbohydrate-binding ability. The results suggest the potential application of hybrid agglutinins to study of lectin subunit interactions and of cell surface complex carbohydrates.

Materials and Methods

Materials. Raw germ from soft white Idaho wheat was generously providied by General Mills Corp., Vallejo, Calif. [1-^{14}C]Glycine methyl ester (2 Ci/mol) was prepared by W. G. Carter of our laboratory as described by Hassing et al. (1971). *N*-Ethylmorpholine and triethylamine were redistilled and urea was recrystallized before use. Ovomucoid-Sepharose was prepared according to Marchesi (1972).

Lectin Isolation. Wheat germ agglutinins were prepared by generally used procedures (Marchesi, 1972; Allen et al., 1973) with minor modifications. Wheat germ was defatted with petroleum ether, air dried, and suspended in water (150 g/l.) at 4° with overnight stirring. The supernatant obtained after low-speed centrifugation was heated for 15 min at 63°, chilled, and clarified at low speed. The supernatant was adjusted to 0.1 *N* in acetic acid, clarified, and brought to neutral pH by addition of solid Tris base to 0.1 *M*. In some preparations, acetic acid treatment was replaced by precipitation and concentration of agglutinins with $(NH_4)_2SO_4$ (0.3 g/ml). The agglutinins were isolated by ovomucoid-Sepharose affinity chromatography and separated by ion-exchange column chromatography on SP- and QAE-Sephadex as described in the figure legends. The purified agglutinins appeared homogeneous by gel electrophoresis at pH 9.4 or pH 5.0 or in the presence of sodium dodecyl sulfate following reduction of disulfide bonds.

Analytical Methods. Lyophilized protein samples for amino acid analyses were sent to AAA laboratories (Seattle, Wash.) where they were hydrolyzed in 6 *N* HCl for 24, 48, or 96 hr and run on a Durrum single column amino acid analyzer. Hemagglutination experiments with 1% human

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[1] Abbreviations used are: WGA, wheat germ agglutinin; Tris, tris(hydroxymethyl)aminomethane; PBS, 0.01 *M* sodium phosphate buffer (pH 7.2) containing 0.15 *M* NaCl.

type A_1 erythrocytes were performed at room temperature by serial twofold dilutions in 0.15 *M* NaCl using microtiter test trays with Takatsy 0.025-ml loops. WGA concentrations were determined by OD_{277} using extinction coefficients of 1.70 (WGA I) and 1.60 (WGA IIa) cm^2/mg (Rice and Etzler, 1974). Sedimentation coefficients were obtained at 60,000 rpm and 0.5-mg/ml protein concentration as previously described (Rice and Etzler, 1974). Values were corrected for conditions of temperature (18–22°) and solvent (PBS) to density and viscosity of water at 20° ($s_{20,w}$).

Gel electrophoresis at pH 9.4 or 5.0 was performed in 6-cm polyacrylamide gels cast from a solution 7.5% in acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 0.25 mg/ml of $(NH_4)_2S_2O_8$, 1 μl/ml of *N,N,N',N'*-tetramethylethylenediamine, and 0.05 *M* 2-amino-2-methyl-1,3-propanediol-HCl (pH 9.4) or 0.03 *M* Tris (adjusted to pH 5.0 with acetic acid). Samples of 5–20 μg of protein in 20–50 μl of 3–5 m*M* buffer made 10% in glycerol were electrophoresed at 2 mA/gel for 3 hr (pH 9.4) or 12 hr (pH 5.0). The gels were stained in 12% trichloroacetic acid made 0.013% in Serva Coomassie G-250 (Gallard-Schlesinger, New York) as described by Diezel et al. (1972).

Isoelectric focusing gels (Wrigley, 1968) were prepared as above except for replacement of buffer by 2% ampholine, pH range 3–10, and were focused for 5–6 hr at 120 V. Samples containing 50–100 μg of protein in 50–100 μl of 30% sucrose made 2% in ampholine were applied to the tops of the gels and layered with 40 μl of 15% sucrose made 2% in ampholine. Usually the anode (top) solution was 2% H_2SO_4 and the cathode (bottom) solution was 2% ethylenediamine. In some runs the sample was applied to the basic side of the gels. After focusing, the gels were immersed in 12.5% trichloroacetic acid to visualize precipitated protein bands. The pH profile was obtained from nonfixed parallel gels, frozen in tightly stoppered tubes on Dry Ice immediately following the run, by fractionation into 1-mm slices each being eluted with 1 ml of water for 1 hr prior to pH measurement. For estimation of isoelectric points, positions of protein bands were normalized to the pH profile by gel length.

Sodium dodecyl sulfate gel electrophoresis in 10% polyacrylamide gels of 6 cm length was performed as previously described (Weber and Osborn, 1969; Rice and Etzler, 1974). Gels containing samples prepared without disulfide bond reduction were stained in 12% trichloroacetic acid with Coomassie G-250 or R-250, following removal of detergent by diffusion in 12% trichloroacetic acid, since 5–10% acetic acid was not sufficient to fix the protein bands.

Chemical Modifications. Reductive methylation of WGA amino groups was performed in an ice bath using formaldehyde in the presence of a slight excess of sodium borohydride (Means and Feeney, 1968). To 10 mg of protein dissolved in 2 ml of 0.2 *M* sodium borate (pH 9.2) containing a trace of 1-octanol to retard foaming, were added 30–35 μmol of sodium borohydride (in 50 μl of water) followed quickly by 65–70 μmol of formaldehyde (in 50 μl of water). The additions of borohydride and formaldehyde were repeated twice at 15-min intervals. After 1 hr, the protein was acidified and dialyzed against water or 2 m*M* HCl. By this procedure, 95% or more of the protein amino groups was blocked as determined by their reactivity with trinitrobenzenesulfonic acid (Habeeb, 1966). No free sulfhydryl groups resulting from the modification procedure were detected by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) at neutral pH in the presence or absence of 1% sodium dodecyl sulfate using protein dialyzed at pH 3.

Acetylation of WGA was performed in an ice bath using a 30–60-fold molar ratio of acetic anhydride to amino groups (Fraenkel-Conrat, 1957). Typically, 20 μl of acetic anhydride was added in 5-μl aliquots at 15-min intervals with constant stirring to 15 mg of protein dissolved in 5 ml of 0.3 *M* *N*-ethylmorpholine acetate buffer (pH 8.5). (WGA previously subjected to reductive methylation generally became opalescent during acetylation; the small amount of insoluble WGA was removed by low-speed centrifugation.) Small molecular weight components of the reaction solution were removed by dialysis or, when tyrosine O-acetylation was studied, by Sephadex G-25 gel filtration in 0.01 *M* sodium phosphate buffer (pH 6.6). Hydroxamate release (Hestrin, 1949; Agrawal et al., 1968) and change in protein absorption at 278 nm (Simpson et al., 1963) upon treatment of the modified protein with neutral hydroxylamine permitted estimation of tyrosine acetylation at 8–10 residues/subunit. Hybridization experiments were performed using acetylated lectin treated with 0.15 *M* hydroxylamine at pH 7 for 1.5 hr, after which time no further de-O-acetylation occurred. Succinylation of wheat germ agglutinin was performed at room temperature using a 30-fold molar ratio of succinic anhydride to amino groups essentially as above. Acylation with acetic or succinic anhydride was observed to yield about 95% amino group modification as judged by reaction with trinitrobenzenesulfonic acid (Habeeb, 1966).

Modification of WGA carboxyl groups was carried out in 8 *M* urea in the presence of a water-soluble carbodiimide (Hoare and Koshland, 1967). Wheat germ agglutinin (5 mg), urea (0.4 g), and [1-^{14}C]glycine methyl ester HCl (126 mg) or glycinamide HCl (111 mg) were dissolved in one solution by addition of 0.5 ml of water. Following addition of 20 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide in 0.2 ml of 8 *M* urea, the solution was adjusted with 0.1 *N* NaOH and maintained with 0.1 *N* HCl in the pH range of 4.6–4.9 for 1 hr at room temperature. The sample was dialyzed 16 hr against water, treated with 0.5 *M* hydroxylamine at pH 7 for 7 hr (Carraway and Koshland, 1968), during which time the protein absorption at 278 nm increased 10–20%, and dialyzed extensively against water. Measurements of radioactive glycine methyl ester incorporated into the protein indicated that 7–8 carboxyl groups were modified by this procedure.

Results

Isolation and Characterization of Multiple Forms of Wheat Germ Agglutinin. Four chromatographically distinct forms of WGA were obtained from the commercial wheat germ used in these experiments. Figure 1 illustrates the separation of three agglutinin peaks by ion-exchange column chromatography on SP-Sephadex following the isolation of purified WGA by ovomucoid-Sepharose affinity chromatography. The forms have been numbered I, II, and III by order of elution from SP-Sephadex columns following the nomenclature of Allen et al. (1973). The material in two of these three peaks, labeled I and III in Figure 1, appeared to be homogeneous upon ion-exchange column chromatography on QAE-Sephadex. The material in the largest peak, labeled II in Figure 1, gave rise to two agglutinin forms, IIa and IIb, as illustrated in Figure 2. Purified WGA amounted to 200–250 mg/kg of raw wheat germ and was distributed

Table I: Amino Acid Compositions of Wheat Germ Agglutinins.[a]

	I	IIa	IIb	III
Asp	14.9	15.1	15.4	15.1
Thr[b]	5.0	4.0	4.1	4.0
Ser[c]	13.7	13.5	13.0	13.5
Glu	16.4	16.0	16.6	15.8
Pro	5.2	6.2	6.3	6.0
Gly	41.0	40.5	41.1	40.5
Ala	10.1	9.4	9.4	9.8
½-Cys[d]	37.7	36.6	38.7	38.8
Val	0.9	1.1	1.0	1.0
Met	2.0	2.0	2.0	2.0
Ile	2.1	2.1	2.2	2.1
Leu	4.0	4.0	4.0	4.0
Tyr	8.0	7.4	8.0	7.6
Phe	3.3	2.9	2.4	2.9
His	0.0	2.0	1.8	2.0
Lys	6.8	7.4	7.8	7.4
Arg	4.0	3.4	3.5	4.0
Trp[e]	3.2[f]	2.9[g]	3.3	3.0

[a] The reported values were obtained by analyses of 24-hr hydrolysates of two independently purified preparations of each form. The compositions have been normalized to 4.0 leucine residues, giving a protein subunit molecular weight of 18,000; 48- and 96-hr hydrolyses of WGA I and IIa gave no further release of amino acids including leucine, isoleucine, and valine. [b] Corrected for 5% destruction in 24 hr, consistent with extrapolation from 48- and 96-hr hydrolysates of WGA I and IIa. [c] Corrected for 15% destruction in 24 hr. The corrected values may be uncertain by ±0.5 residue due to an apparent destruction rate greater than first order in 48- and 96-hr hydrolysates of WGA I and IIa, as observed for carboxypeptidase A (Bargetzi et al., 1963). [d] Determined as cysteic acid after performic acid oxidation. [e] Determined spectrophotometrically at 280 and 288 nm by the procedure of Edelhoch (1968) using tabulated values above for tyrosine and disulfide content. [f] The value of 3.2 was also obtained by oxidation with *N*-bromosuccinimide at pH 4.0 (Spande and Witkop, 1967). [g] The value of 3.3 was obtained following reduction and carboxymethylation.

among the forms approximately as 35% I, 50% IIa, 5% IIb, and 10% III. The finding of chromatographically distinct forms appeared not to be affected by minor alterations in purification procedure, including extraction of defatted wheat germ in 0.05 *N* HCl (Bloch and Burger, 1974) with subsequent $(NH_4)_2SO_4$ precipitation prior to affinity chromatography.

Amino acid compositions of the four chromatographically distinct forms, presented in Table I, indicated that they were similar to each other and to preparations obtained by others (Allen et al., 1973; Nagata and Burger, 1974). The characteristically high content of half-cystine, probably responsible for the great stability of this lectin, and a high content of glycine were observed in all the forms. However, a form of this agglutinin lacking histidine, as seen here in form I, has not previously been reported. Free sulfhydryl groups were not detectable in the purified forms with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) in the absence or presence of 1% sodium dodecyl sulfate. Preliminary studies of carbohydrate content done as previously described (Carter and Etzler, 1975) indicated little if any sugar in the purified lectin preparations, consistent with observations of others that WGA does not appear to be a glycoprotein (Allen et al., 1973; Nagata and Burger, 1974).

The four agglutinin forms were indistinguishable in their ability to agglutinate human type A_1 erythrocytes. They appeared to have the same dimeric subunit structure as judged by sedimentation velocity ($s_{20,w}$ = 3.7 ± 0.1). Results of electrophoresis in sodium dodecyl sulfate polyacrylamide gels following disulfide bond reduction indicated the subunits had identical mobilities corresponding to approximately 18,000 daltons. However, WGA IIb appeared to differ from I, IIa, and III in subunit dissociation properties.

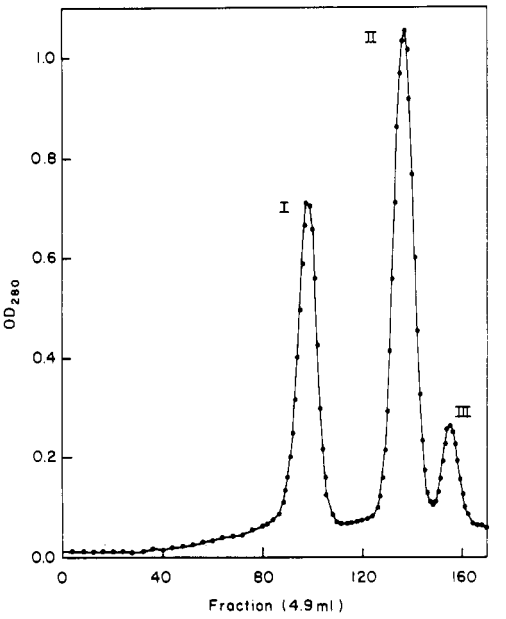

FIGURE 1: WGA (195 OD_{277} units) prepared by ovomucoid-Sepharose affinity chromatography was applied to a column 1.9 × 51 cm of SP-Sephadex maintained in 0.1 *M* sodium acetate buffer (pH 3.8) made 0.1 *M* in NaCl. The column was then washed with two volumes of buffer and the protein eluted with a series of two gradients of increasing NaCl concentration in 0.1 *M* sodium acetate (pH 3.8). The first gradient was linear from 0.1 to 0.25 *M* in NaCl (200 ml of each solution). The second gradient was nonlinear from 0.25 to 0.5 *M* in NaCl (300 ml of 0.25 *M* and 150 ml of 0.5 *M* in beakers of different diameter such that the solutions were of equal height). The three agglutinin peaks I, II, and III eluted at NaCl concentrations between 0.25 and 0.40 *M* according to conductivity measurements. The material in each peak was pooled individually and salt was removed by dialysis against water or 0.01 *N* acetic acid. The column was often scaled up to dimensions of 3.1 × 54 cm to accommodate agglutinin derived from 1 kg of raw wheat germ (about 750 OD_{277} units).

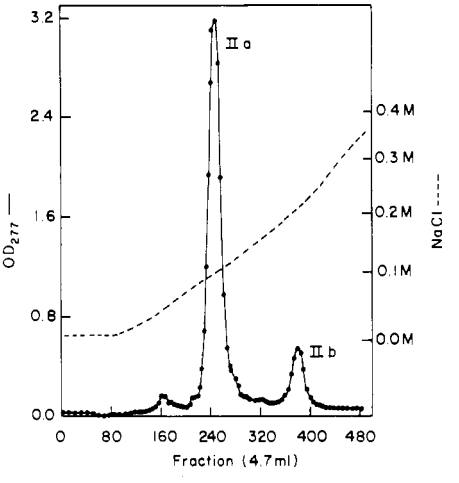

FIGURE 2: WGA II obtained from SP-Sephadex columns (580 OD_{277} units) was adjusted to 0.05 *M* in 2-amino-2-methyl-1,3-propanediol (pH 9.4) and applied to a 3.1 × 54 cm column of QAE-Sephadex maintained in this buffer. The agglutinin peaks were eluted with a gradient of increasing NaCl concentration consisting of 1.6 l. of this 0.05 *M* buffer and 0.9 l. of 0.4 *M* NaCl in the buffer (in beakers of different diameter such that the solutions were of equal height). Buffer background absorption of 0.1 unit was subtracted in the figure. In parallel runs, WGA I eluted at about 0.10 *M* in NaCl (slightly later than WGA IIa). WGA III eluted at about 0.13 *M* in NaCl, and was run on columns scaled down to 1.9 cm diameter due to the lower amounts of protein available. The material in the individual peaks was concentrated by precipitation with ammonium sulfate (0.4 g/ml), dialyzed extensively against water, and lyophilized.

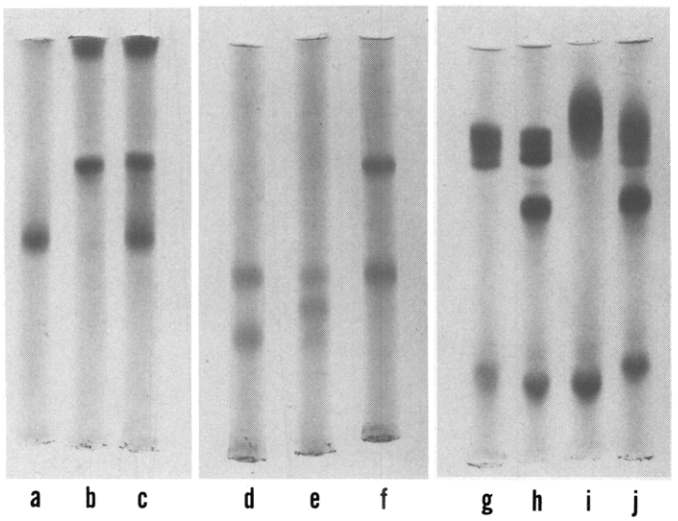

FIGURE 3: Acrylamide gel electrophoresis. Gels a–c were run in the presence of sodium dodecyl sulfate with no disulfide bond reduction, d–f at pH 5.0, and g–j at pH 9.4. Direction of migration in each case was from top to bottom. (a) WGA IIa; (b) WGA IIb; (c) WGA IIa and IIb; (d) WGA I (upper band) and IIa (lower band), combined after individual treatment in 0.1 *N* acetic acid; (e) WGA I (upper band), hybrid form (middle band), and IIa (lower band), where the pattern was obtained by treatment of I and IIa together in 0.1 *N* acetic acid; (f) WGA IIb (upper band) and I (lower band) treated together in 0.05 *N* HCl; (g) WGA III (upper band) and acetylated WGA I (lower band), combined after individual treatment in 4 *M* Tris-HCl; (h) WGA III (upper band), hybrid (intermediate band), and acetylated WGA I (lower band), where the pattern was obtained by treatment of WGA III and acetylated WGA I together in 4 *M* Tris-HCl; (i) reductively methylated WGA IIa (upper band) and acetylated WGA I (lower band), combined after individual treatment in 4 *M* Tris-HCl; (j) reductively methylated WGA IIa (upper band), hybrid (intermediate band), and acetylated WGA I (lower band), where the pattern was obtained by treatment of reductively methylated WGA IIa and acetylated WGA I together in 4 *M* Tris-HCl.

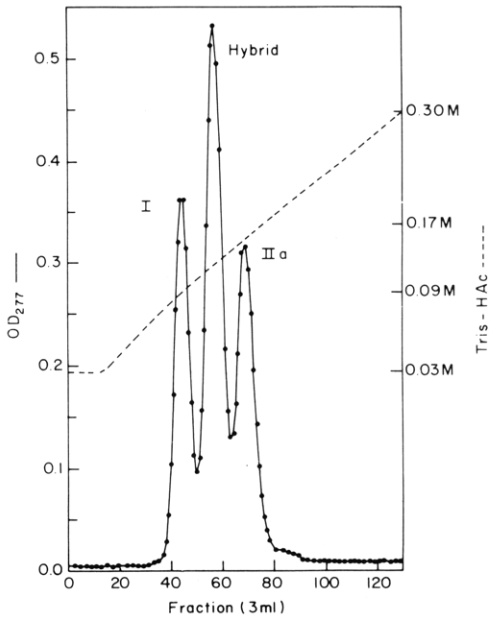

FIGURE 4: Equal amounts of wheat germ I and IIa agglutinins (14 OD_{277} units each) were pooled and incubated in 0.1 *N* acetic acid (5 ml) at room temperature for 20 hr. The solution was dialyzed for 12 hr against 0.03 *M* Tris-acetate buffer (pH 5.0) and applied to a column 1.0 × 46 cm of SP-Sephadex maintained in 0.03 *M* Tris-acetate buffer (pH 5.0). The agglutinins were eluted with a linear gradient of Tris-acetate using 180 ml each of 0.03 and 0.3 *M* buffer (pH 5.0).

The sedimentation coefficient of IIb in 0.05 *N* HCl ($s_{20,w}$ = 3.4) was considerably greater than that observed for I or IIa ($s_{20,w}$ = 2.1) in this solvent (Rice and Etzler, 1974). Also, the mobility of IIb in sodium dodecyl sulfate gels without protein disulfide bond reduction, unlike I, IIa, and III, corresponded approximately to that expected for the dimeric lectin. The gels presented in Figure 3a–c illustrate the relative mobilities of IIa and IIb and indicate that the IIb form also contained aggregated material which remained at the top of the gel. This aggregated material was probably due to extensive disulfide cross-linking since it was not observed after reduction. The inability of some WGA dimers to be dissociated into subunits by sodium dodecyl sulfate in the absence of reducing agent has been noted by others (Allen et al., 1973).

Hybridization of Agglutinin Forms. WGA I and IIa were noted to undergo subunit interchange under a variety of conditions. Gel electrophoresis at pH 5.0 permitted analytical separation of these two agglutinins and observation of hybrid molecules of intermediate mobility, as seen in Figure 3d and e. Overnight incubation in 8 *M* urea, in extremes of pH (0.05 *N* HCl, 0.1 *N* acetic acid, or 0.05 *M* triethylamine HCl (pH 11)), or in high concentrations of various buffer salts at pH 7–9 (4 *M* Tris-HCl, *N*-ethylmorpholine acetate, or 2-amino-2-methyl-1,3-propanediol-HCl) followed by dialysis against 3 m*M* Tris-acetate (pH 5.0) prior to electrophoresis was sufficient to promote considerable subunit interchange. As a control, forms I and IIa were treated individually by the same procedure as above; when these samples were recombined, no hybridization was detected by electrophoresis. WGA IIb migrated with about one-half the mobility of WGA I on these gels, but no hybridization between I and IIb was detected (Figure 3f) under the various treatment conditions. At pH 5.0, WGA III migrated in the gels with mobility between I and IIa; hence, subunit interchange was not investigated with this form due to difficulties in hybrid detection.

The extent of subunit interchange between WGA I and IIa was measured by SP-Sephadex ion-exchange column chromatography at pH 5.0. Figure 4 illustrates the result obtained following overnight incubation of equal amounts of the forms in 0.1 *N* acetic acid. From the relative areas of the three peaks obtained, it is seen that the hybrid agglutinin consisted of 45–50% of the eluted protein, consistent with essentially random subunit distribution under these conditions. No hybrid peak was observed upon chromatography of WGA I and IIa prior to treatment promoting subunit interchange. Similar attempts to separate the hybrid form at pH 3.8 on SP-Sephadex columns were unsuccessful. Although subunit interchange may occur during purification procedures employing acid elution from affinity resins, as in the present work, the multiple forms isolated at pH 3.8 on SP-Sephadex appear not to represent hybrid agglutinins, which are apparently unstable under these chromatographic conditions.

Chemical Modification. Table II summarizes the effect of chemical modifications on subunit structure and carbohydrate-binding ability of the WGA I and IIa agglutinins as measured by sedimentation velocity, erythrocyte agglutinating ability, and binding to ovomucoid-Sepharose. Succinylation of the available lectin amino groups did not detectably alter the protein sedimentation coefficient of erythrocyte agglutinating ability. In contrast, acetylation of the protein, modifying available amino and phenolic groups, greatly lowered both its sedimentation coefficient and

Table II: Effect of Chemical Modification on Sedimentation Coefficient and Activity of WGA I and IIa.

Modification	$s_{20,w}$	Relative Agglutination[a]	Binding to Ovomucoid-Sepharose[b]
None (native protein)	3.7	1	+
Succinylation	3.7	1	–
Acetylation	2.5	<0.03	–
Acetylation, then de-O-acetylation	3.7	1	–
Succinylation, then acetylation	2.4	<0.03	–
Reductive methylation	3.7	1	+
Reductive methylation, then acetylation	3.4 (WGA I)	<0.03	–
	2.7 (WGA IIa)	<0.03	–
Carbodiimide-mediated amide formation	2.4	<0.03	+

[a] The values represent the ratio of end point titer of the modified protein to the end point titer of native WGA of the same concentration tested in parallel. Differences less than one serial dilution were not detectable. [b] Tested by passage through 0.4 × 4.5 cm columns of ovomucoid-Sepharose. Derivatives whose passage through the column was only slightly retarded would have scored negative. Native protein and bound derivatives were eluted by 0.1 *N* acetic acid.

agglutinating ability. After regeneration of free tyrosine residues by brief hydroxylamine treatment or by incubation at alkaline pH, the derivative with acetylated amino groups regained the sedimentation coefficient and agglutinating ability of the native protein. Consistent with these results, acetylation of tyrosine residues also caused a marked decrease in sedimentation rate and agglutinating ability of the succinylated protein. To investigate the effect of tyrosine O-acetylation on lectin retaining positively charged amino groups, the acetylation was performed on protein which was first subjected to reductive methylation. By itself, the methylation of available amino groups had no detectable effect on the lectin sedimentation coefficient or agglutinating ability. Subsequent O-acetylation caused a detectable decrease in sedimentation rate and a large decrease in agglutinating ability, reversible by hydroxylamine treatment. In other experiments, carboxyl group modification with glycine methyl ester or glycinamide in the presence of a water-soluble carbodiimide resulted in great reduction of sedimentation coefficient and agglutinating ability. Binding ability to ovomucoid-Sepharose, presumably not as dependent on lectin subunit structure as agglutination, was noted to be lost upon acylation of amino or phenolic groups (or both) but to be retained upon methylation of amino groups or upon carboxyl group modification.

WGA III resembled the I and IIa forms in losing agglutinating ability upon treatment with acetic anhydride and regaining this ability upon subsequent hydroxylamine treatment. The IIb form, however, lost agglutinating ability upon acetylation and did not regain it when de-O-acetylated. The sedimentation coefficient of the fully acetylated IIb form was the same as the native protein, consistent with the apparent inability of this form to dissociate without disulfide bond reduction. Both the WGA IIb and III forms lost their agglutinating ability upon carbodiimide-mediated modification of carboxyl groups with glycine methyl ester.

Hybridization of Native and Acetylated Forms. Native agglutinin forms were observed to undergo subunit interchange with derivatives having acetylated amino groups under conditions similar to those promoting subunit interchange of the native I and IIa forms. Judging by its sedimentation rate, native WGA retained its dimeric subunit structure at pH 9.4. Gel electrophoresis at this pH was used for detection of hybrid agglutinin species. Under the conditions of electrophoresis, the native agglutinins formed broad bands near the gel tops while acetylated derivatives formed sharper less intense bands migrating near the gel bottoms. Figure 3g shows the pattern obtained with the acetylated WGA I and native WGA III forms. Overnight incubation of these two species together in 4 *M* Tris-HCl (pH 8.0) or 5 *M* 2-amino-2-methyl-1,3-propanediol-HCl (pH 9.6) followed by dialysis against 5 m*M* 2-amino-2-methyl-1,3-propanediol (pH 9.4) prior to electrophoresis gave rise to a band of apparent hybrid dimers with intermediate mobility (Figure 3h). Similarly, WGA I, IIa, and III were all observed to hybridize with acetylated derivatives of any of these forms. Reductively methylated derivatives also were observed to hybridize with acetylated derivatives. Figure 3 shows acetylated WGA I and methylated WGA IIa forms treated individually (i) or together (j) in 4 *M* Tris-HCl (pH 8.0) prior to electrophoresis. In addition to high concentrations of buffer salts, 8 *M* urea and high pH (0.05 *M* triethylamine-HCl (pH 11)) were effective in promoting subunit interchange. Overnight dialysis at pH 9.4 in 5 m*M* 2-amino-2-methyl-1,3-propanediol was also noted to yield a faint intermediate band on the gels probably indicative of a slow rate of hybridization. Combinations of WGA IIb or its acetylated derivative with any of the other forms or their derivatives gave no clear evidence for subunit interchange. Faint bands were occasionally noted on the gels near the position of hybrid bands, possibly due to traces of nonspecific aggregates.

In gel electrophoresis patterns obtained at pH 9.4, the bands of hybrid dimers were not midway between the native and acetylated forms, but instead closer to the gel tops. This feature may be attributable to slight interaction of native but not acetylated subunits with the polyacrylamide support. Allen et al. (1973) noted that wheat germ agglutinin eluted much later from Bio-Gel P-100 acrylamide gel filtration columns than expected for a 36,000-dalton protein. This apparent slight sticking to Bio-Gel P-100 has also been noted by us with the native but not the acetylated protein, the latter showing minimal if any such retardation.

The extent of subunit interchange between native and acetylated agglutinins was examined by DEAE-Sephadex ion-exchange column chromatography at pH 7.9. Under conditions of high salt treatment WGA I and its acetylated derivative appeared to undergo essentially random subunit interchange. As illustrated in Figure 5, the peak of hybrid molecules contained 50% of the material applied to and eluting from the column. (Experiments employing the acetylated WGA IIa form were not quantitated in this way, however, since this derivative appeared to precipitate on the column and was eluted only in trace amounts.) The hybrid molecules consisting of native and acetylated WGA I subunits resembled the native form in having the same sedimentation coefficient and specific activity of erythrocyte agglutination and in ability to bind to ovomucoid-Sepharose columns. This hybrid agglutinin rechromatographed as a homogeneous peak shortly after its initial isolation, but appeared to disproportionate to the extent of several percent if incubated at room temperature for 1 day in 0.3 *M* Tris-HCl (pH 7.9) prior to rechromatography. Disproportionation appeared complete within 30 days under these conditions, rechromatography of the hybrid agglutinin yielding 50% of the material in the hybrid fraction and 25% each in the native and acetylated fractions. In similar hybridization ex-

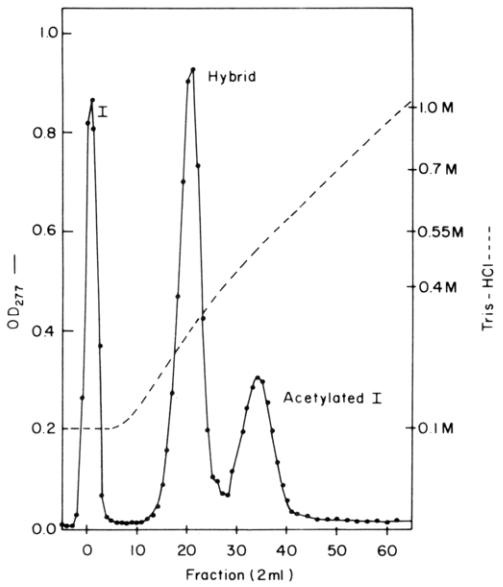

FIGURE 5: Equal amounts of WGA I and its acetylated (and de-O-acetylated) derivative (9 OD_{277} units each) were pooled in 7 ml and dialyzed first for 5 hr against 5 *M* 2-amino-2-methyl-1,3-propanediol (pH 9.6), then for 17 hr against 0.015 *M* triethylamine acetate (pH 10.4), and finally 4 hr against 0.1 *M* Tris-HCl buffer (pH 7.9). The sample was applied to a DEAE-Sephadex column 1.0 × 12 cm and eluted with a linear gradient of increasing Tris-HCl concentration made from 60 ml each of 0.1 and 1.0 *M* Tris-HCl buffer (pH 7.9).

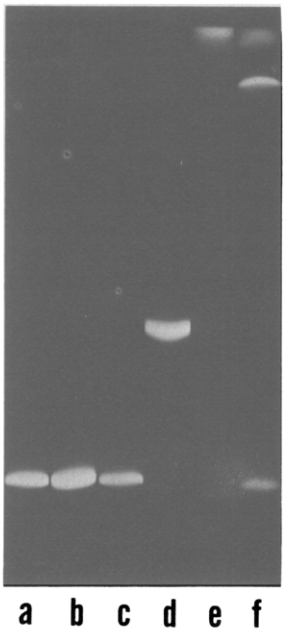

FIGURE 6: Isoelectric focusing. The samples for the isoelectric focusing gels were (a) WGA I; (b) IIa; (c) III; (d) IIb; (e) acetylated I; (f) acetylated I (upper band), hybrid (intermediate band), and native I (lower band), where the pattern was obtained after treatment of I and acetylated I in 0.05 *M* triethylamine-HCl (pH 11). The samples were applied to the acid side (top) of the gels.

periments using equal amounts of native and succinylated WGA I, a hybrid peak containing 50% of the applied material was obtained. The hybrid peak, eluting at a 0.7 *M* NaCl concentration, had a markedly skewed distribution, probably due to slow subunit interchange in moderate salt concentrations during the column chromatography.

Isoelectric Point Estimation. The column chromatographic and gel electrophoretic behavior of WGA I, IIa, and III suggested that they were basic proteins with similar isoelectric points in the pH range of 7.9–9.4. A similar observation has been made by others (Allen and Neuberger, 1972). Consistent with this behavior, these agglutinins focused in sharp bands at pH 8.7 ± 0.3 in gel isoelectric focusing experiments. WGA I, IIa, and III were not distinguishable from each other in isoelectric point (Figure 6a–c). WGA IIb focused at pH 7.7 ± 0.3 in parallel runs (Figure 6d), often containing a faint minor band focusing about 0.5 pH unit lower. In contrast, the acetylated agglutinins focused in the pH range 3.9–4.4, sometimes forming several discrete bands. WGA I, illustrated in Figure 6e, focused at the lower end of this range, while the other forms were at the upper end of the range, the difference probably being due to the absence of histidine in WGA I. Hybrid agglutinins containing native and acetylated subunits were observed to focus on the gels 0.5–1.0 pH unit higher than the constituent acetylated derivative. Figure 6f illustrates the focusing of native, hybrid, and acetylated WGA I in one gel.

Discussion

Following purification of WGA by affinity chromatography on columns of ovomucoid-Sepharose, four forms of this lectin were isolated by ion-exchange column chromatography. These chromatographically distinct forms appeared to be quite similar in physical properties as indicated by amino acid composition, molecular weight, and isoelectric point. However, the lack of histidine in WGA I and the inability of WGA IIb to undergo subunit dissociation in the absence of reducing agent were important structural differences observed. The origin of multiple forms of this agglutinin might appear related to the mixture of wheat strains in the commercial germ preparation employed, but multiple forms have been obtained from one defined hexaploid strain in preliminary experiments. The various forms might also arise by enzymatic modification before or during purification, though evidence for this process was not obtained by minor variation in purification procedure. The similarity in amino acid composition of WGA IIa and IIb suggests the possibility that IIb might have arisen by intersubunit thiol oxidation in IIa before or during purification. In that case, however, the difference in charged residues revealed by isoelectric focusing of these lectin forms requires further explanation.

Further evidence for structural similarities in three forms of wheat germ agglutinin (I, IIa, III) was obtained by hybridization experiments. Native WGA I and IIa were shown to undergo subunit interchange under a variety of conditions including treatment with denaturants, high salt, or pH extremes. It was of interest to note that 0.1 *N* acetic acid and 0.05 *N* HCl promoted hybridization, indicating that this phenomenon probably occurs during low pH elution from affinity adsorbants employed in purification. Agglutinin derivatives with acetylated or methylated amino groups were able to form hybrid molecules as well, indicating that the modifications produced minimal disturbance in the intersubunit binding region. The finding of a single hybrid species in each experiment is consistent with the reported dimeric subunit structure of native agglutinin (Rice and Etzler, 1974; Nagata and Burger, 1974). Isolation of the hybrid species indicates the feasibility of this approach for study of subunit interactions in lectin carbohydrate binding or study of hybrid variants in cell surface modification.

Acylation of WGA amino groups with acetic or succinic anhydrides did not appear to disturb the native subunit

structure, but did appear to alter the lectin carbohydrate-binding specificity. This effect may be explained by alteration of the association constant perhaps due to partial obstruction of binding sites, which may be large enough in the native protein to accommodate a tetrasaccharide (Allen et al., 1973); however, blocking of one of two different binding sites cannot be ruled out. The large alterations in protein net electrostatic charge upon amino group acylation, as shown by isoelectric focusing experiments, might also influence carbohydrate-binding specificity toward glycoprotein substrates. Experiments using defined carbohydrate substrates (in progress) may help choose between such alternatives. For example, in a careful study of acetylated concanavalin A (Agrawal et al., 1968), the modified lectin appeared to retain its carbohydrate specificity in inhibition experiments but to have reduced affinity for certain polysaccharides, including Sephadex G-50 to which it no longer bound.

The substantial decrease in sedimentation coefficient of WGA with modified carboxyl groups probably involves dissociation of the dimer to subunits and may be analogous to the effect of carboxyl group titration at low pH, where the sedimentation rate is similarly lowered (Rice and Etzler, 1974; Nagata and Burger, 1974). Apparent retention of some carbohydrate-binding ability is suggested by binding of the modified lectin to ovomucoid-Sepharose, but the strength and specificity of residual carbohydrate-binding ability remain to be determined. The effect of modification in the absence of denaturants may also be of interest. Under nondenaturing conditions, carbodiimide-mediated carboxyl modification of concanavalin A with glycine methyl ester has been shown to give progressive loss of carbohydrate-binding activity, though not apparently causing large disturbance of lectin subunit structure (Hassing et al., 1971).

Reductive methylation of WGA amino groups had no detectable effect on the protein sedimentation coefficient or carbohydrate-binding ability. Subsequent tyrosine O-acetylation prevented binding of the protein to ovomucoid-Sepharose and greatly lowered its erythrocyte agglutinating ability. Detectable reduction in sedimentation coefficient was observed. This result indicated that the large decrease in sedimentation rate of fully acetylated agglutinin, presumably due to subunit dissociation, was due to modification of both phenolic and amino groups, of which the contribution of tyrosine acetylation appeared to have the largest effect.

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