Interaction of Wheat Germ Agglutinin with Sialic Acid†

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ABSTRACT: Wheat germ agglutinin (WGA) specifically binds *N*-acetylneuraminic acid and *N*-acetyl-D-galactosamine in addition to *N*-acetyl-D-glucosamine and its β-(1→4)-linked oligosaccharides. Both ovine submaxillary mucin (OSM) and asialo ovine submaxillary mucin, glycoconjugates lacking *N*-acetyl-D-glucosamine units, precipitate WGA. The precipitation of WGA by OSM is inhibited completely by D-GlcNAc as well as by the following saccharides (potency relative to GlcNAc (1.0) in parentheses): *N,N'*-diacetylchitobiose (32); *N*-acetylneuraminic acid (0.30); *N*-acetylneuraminic acid methyl ester (0.27); NeuNAc-7, 5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid (0.80); *N*-acetyl-D-galactosamine (0.20). Selective conversion of the *N*-acetylneuraminic acid groups of fetuin and of OSM to NeuNAc-7 (two fewer carbon atoms) by periodate oxidation–borohydride reduction yielded analogue glycoproteins that reacted more strongly with WGA than the parent glycoproteins. Analogue fetuin, for example, precipitated 2.5 times more WGA from solution than native fetuin. A threefold higher concentration of *N,N'*-diacetylchitobiose also was required to inhibit the analogue fetuin–WGA interaction than to inhibit equivalently the native fetuin–WGA interaction. Sialic acid groups appear to be immunodominant in fetuin–WGA interaction. Removal of sialic acid from fetuin or analogue fetuin by mild acid hydrolysis abolished the ability of the resulting substrates to precipitate WGA but had no effect on their interaction with the phytohemagglutinin from *Phaseolus vulgaris*. These results are interpreted in terms of the configurational similarity of NeuNAc and D-GalNAc with D-GlcNAc at positions C-2 (*N*-acetamido group) and C-3 (hydroxyl group) of the pyranose ring; these are the positions critical to productive contact with the WGA combining site.

The carbohydrate binding site of wheat germ agglutinin (WGA)[1] is complementary to *N*-acetyl-D-glucosamine (D-GlcNAc) and its β-(1→4)-linked oligosaccharides (Allen et al., 1973; Goldstein et al., 1975). Several observations suggest that WGA may also bind *N*-acetylneuraminic acid (NeuNAc) (Burger & Goldberg, 1967; Adair & Kornfeld, 1974; Greenaway & LeVine, 1973; Redwood et al., 1975; Redwood & Polefka, 1976; Jordan et al., 1977; Bhavanandan et al., 1977; Bhavanandan, 1978; Sheik Fareed et al., 1978). We have investigated the molecular basis for WGA–NeuNAc interaction by quantitative precipitation and hapten inhibition analyses. Our results indicate that NeuNAc, several of its derivatives, and *N*-acetyl-D-galactosamine (D-GalNAc) are capable of combining with WGA.

Materials and Methods

WGA (grade A) was purchased from Calbiochem. Navy bean phytohemagglutinin (Plouffe et al., 1979) and *Bandeiraea simplicifolia* I agglutinin (Hayes & Goldstein, 1974) were prepared as described. *Helix pomatia* agglutinin was the kind gift of Dr. S. Hammarström (Wenner-Gren Institute, University of Stockholm, Sweden). Ovine submaxillary mucin (OSM) was the generous gift of Dr. Don Carlson (Purdue University) and Dr. David Aminoff (University of Michigan). Fetuin was purchased from Grand Island Biologicals. NeuNAc, colominic acid, sialyllactose, and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma Chemical Co. D-GlcNAc, D-GalNAc, and erythritol were obtained from Pfanstiehl. The methyl α- and β-*N*-acetylneuraminic acids, *N*-acetylneuraminic acid methyl ester, and 2,3-dehydro-*N*-acetylneuraminic acid were prepared and characterized as described (Miller et al., 1978).

Periodate–Borohydride Treatment of Fetuin. The sialic acid groups of fetuin were selectively modified to the seven-carbon analogue (5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid, NeuNAc-7) by mild periodate oxidation, followed by borohydride reduction (Van Lenten & Ashwell, 1971; Spiro, 1964). Fetuin (500 mg) was dissolved in cold sodium acetate buffer (0.05 M, pH 5.0, 100 mL), and 5 mL of sodium metaperiodate (0.1 M in sodium acetate buffer) was added dropwise to the stirred solution. The initial reactant concentrations were 5 mM $NaIO_4$ and 1 mM bound sialic acid. After 1-h incubation in the dark at 4 °C, oxidation was terminated by the addition of 2 mL of 5% glycerol in acetate buffer. The oxidized fetuin was then dialyzed against 4 L of cold distilled water.

Sodium borohydride (85 mg) was dissolved in 5 mL of cold sodium borate buffer (0.25 M, pH 8.2) and added dropwise with stirring to the solution of oxidized fetuin. Reduction was continued for 2 h at 4 °C. The reaction mixture was dialyzed 4 times against 4 L of cold distilled water and lyophilized.

Periodate–Borohydride Treatment of OSM. A solution of sodium metaperiodate (20 mM in 0.05 M sodium acetate buffer, pH 5.0, 1.0 mL) was added to OSM (4 mg/mL in the same buffer), and the reaction was conducted in the dark at 4 °C. At time points from 1 min to 52 h, aliquots (5 μL) were added to 2.5 mL of the TPTZ reagent (Avigad, 1969) for the determination of remaining periodate. From the periodate consumption curve (Figure 1), a 32-min oxidation period was considered optimum for the selective oxidation of sialic acid groups. A second periodate oxidation was conducted as before, except that the oxidation was terminated after 32 min by the addition of 50 μL of a 20% solution of glycerol in sodium acetate buffer.

The oxidized OSM was dialyzed against 2 L of cold distilled water and treated with 200 μL of a solution of sodium borohydride, 19 mg/mL in 0.2 M sodium borate buffer, pH 8.2. Reduction was carried out for 2.5 h at 4 °C, during which time the pH never exceeded 8.8. The reaction mixture was then

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[1] Abbreviations used: WGA, wheat germ agglutinin; OSM, ovine submaxillary mucin; D-GlcNAc, *N*-acetyl-D-glucosamine; D-GalNAc, *N*-acetyl-D-galactosamine; D-Gal, D-galactose; NeuNAc, *N*-acetylneuraminic acid; NeuNAc-7, 5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid; TPTZ, 2,4,6-tri-2-pyridyl-*s*-triazine.

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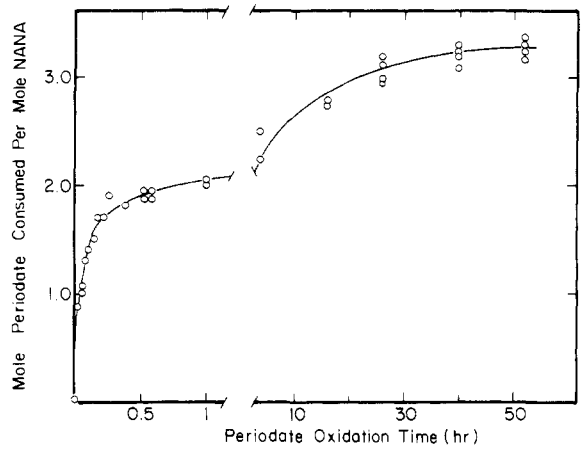

FIGURE 1: Periodate consumption by OSM. Reaction was conducted in the dark at 4 °C at 0.010 M $NaIO_4$ and 2 mg/mL OSM (from Dr. Aminoff).

dialyzed 4 times against 4 L of cold distilled water and lyophilized.

Isolation of NeuNAc-7 from Analogue Fetuin. Analogue fetuin (430 mg) was dissolved in 130 mL of distilled water and 1.3 mL of 6 N H_2SO_4 was added. The solution was heated in a water bath at 80 °C for 20 min. The hydrolysate was chilled on ice, and the pH was adjusted to 5.0 with the careful addition of 1 N NaOH to the vigorously stirred solution. Liberated NeuNAc-7 was separated from protein by filtration through an Amicon PM 10 ultrafiltration membrane. The neutralized hydrolysate was concentrated from 130 to 20 mL, collecting the filtrate. Ultrafiltration was repeated twice, reconstituting the retentate each time to 130 mL with water. As determined by Svennerholm's resorcinol assay (Svennerholm, 1957), the first filtrate contained 60% of the NeuNAc-7, the second filtrate contained 5.4% of the NeuNAc-7, and the third filtrate contained no detectable NeuNAc-7; 23% of the NeuNAc-7 remained in the retentate.

Filtrates 1 and 2 were pooled and lyophilized, and half the material was dissolved in water and spotted on a Brinkman 200-μm silica gel G preparative thin-layer plate (20 × 20 cm). After development with 1-propanol–water (7:3), the plate was dried and scraped into 2-cm bands, and the silica gel from each band was extracted with 200 mL of water. The resorcinol-positive fractions were pooled and lyophilized, and the product was dissolved in 500 μL of water.

Standardization of Sialic Acid Derivatives. The concentration of stock solutions of the sialic acid derivatives was measured as described by Peters & Aronson (1976). Briefly, aliquots of each derivative were oxidized with excess sodium metaperiodate, and the liberated formaldehyde (1 mol/mol of derivative) was quantitated by the chromotropic acid procedure (Hay et al., 1965) employing erythritol as the standard.

Preparation of Asialoglycoconjugates. Fetuin, analogue fetuin, or OSM was dissolved in water at a concentration of 2.5 mg/mL and acidified with 0.025 volume of 6 N H_2SO_4 (final concentration 0.15 N H_2SO_4). Hydrolysis was performed at 80 °C for 90 min, and the hydrolysates were chilled on ice, dialyzed 4 times against 4 L of cold distilled water, and lyophilized. No resorcinol-positive material was found in samples (1.25 mg) large enough to detect 5% of the original NeuNAc content.

Quantitative Precipitation Analysis. Quantitative precipitation was performed by the modified procedure of So & Goldstein (1967). After incubation at room temperature for 2 days, the precipitates were washed twice with 200 μL of phosphate-buffered saline (0.01 M sodium phosphate, pH 7.0, 0.15 M sodium chloride, and 0.04% sodium azide). The amount of protein in the precipitates was determined by a semimicro Lowry procedure (Mage & Dray, 1965).

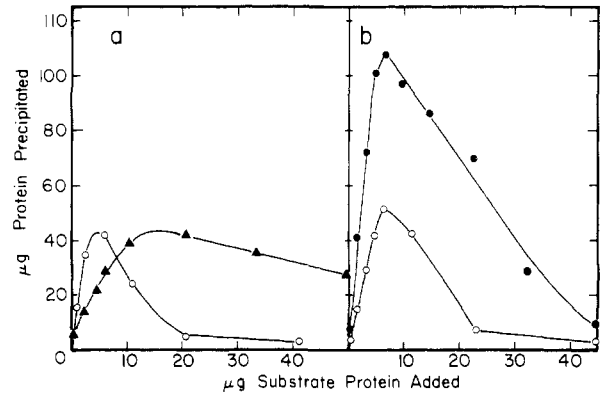

FIGURE 2: Quantitative precipitation of untreated and treated ovine submaxillary mucin by WGA. (a) Each tube contained 90 μg of WGA and increasing amounts of glycoprotein (from Dr. Carlson) in a total volume of 300 μL: OSM (○); asialo-OSM (▲). (b) Each tube contained 115 μg of WGA and increasing amounts of glycoprotein (from Dr. Aminoff) in a total volume of 300 μL: OSM (○); analogue OSM (●).

Table I: Inhibition of Wheat Germ Agglutinin–Ovine Submaxillary Mucin Precipitation by Various Sugar Haptens

compd	inhibitor concn required for 50% inhibn (nmol)
N,N'-diacetylchitobiose	5
N-acetyl-D-glucosamine	160
N-acetyl-D-galactosamine	810
N-acetylneuraminic acid (NeuNAc)	540
N-acetylneuraminyl methyl α-ketoside	540
N-acetylneuraminyl methyl β-ketoside	470
N-acetylneuraminic acid methyl ester	600
7-carbon analogue of *N*-acetylneuraminic acid (NeuNAc-7)	200
2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid	700
sialyllactose	370
lactose	2.7% at 10 000[a]

[a] Indicates the percentage inhibition for the nanomoles of inhibitor noted.

Quantitative Hapten Inhibition Analysis. Quantitative hapten inhibition studies were conducted by determining the extent to which various carbohydrates of low molecular weight inhibited WGA–OSM precipitation. Each tube contained 62 μg of WGA, 4 μg of OSM, and increasing amounts of inhibitor in a total volume of 300 μL of phosphate-buffered saline.

Results

OSM Precipitates WGA. The carbohydrate chains of OSM comprise units of α-D-NeuNAc-(2→6)-α-D-GalNAc-(1→0)-serine; there are 350 such oligosaccharide side chains for each 1000 amino acid residues (Hill et al., 1977). Even though it lacks D-GlcNAc units (less than 1% by weight in this preparation; D. Carlson, personal communication), OSM nevertheless precipitated WGA, 5 μg of the mucin bringing down 42 μg (44%) of total protein (Figure 2).

Hapten Inhibition of WGA–OSM Precipitation. WGA–OSM precipitation was inhibited completely by each of the haptens tested (Table I), with the exception of lactose, a non-inhibitor of WGA. Hapten inhibition curves were parallel and typically sigmoidal. Of the monosaccharides tested, D-GlcNAc was the best inhibitor; NeuNAc was 3 times and D-GalNAc was 5 times poorer than D-GlcNAc. Equipotent with NeuNAc were three derivatives: the methyl α- and β-ketosides, the

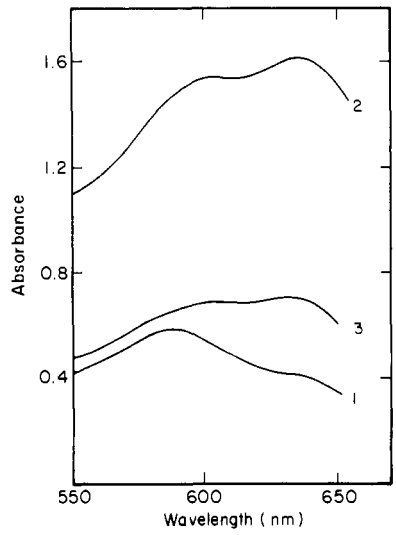

FIGURE 3: Visible absorption spectra of the resorcinol chromogens arising from 500 μg of the following: (1) fetuin; (2) periodate-oxidized fetuin; (3) periodate-oxidized, borohydride-reduced analogue fetuin.

methyl ester, and 2,3-dehydro-NeuNAc. Sialyllactose [α-D-NeuNAc-(2→3 or 6)-β-D-Gal-(1→4)-D-Glc] was twofold more potent than NeuNAc. NeuNAc-7, the seven-carbon analogue of NeuNAc, displayed an inhibitory potency 2.7 times that of NeuNAc and comparable to that of D-GlcNAc.

Asialo-OSM Precipitates WGA. Mild acid hydrolysis of OSM liberated 95% of its sialic acid content as measured by the resorcinol assay. The presence of newly exposed D-GalNAc groups was substantiated by the observation that *H. pomatia* lectin (1.5 mg/mL) reacted strongly with asialo-OSM (2.1 mg/mL) but very weakly with OSM (2.7 mg/mL) by the capillary precipitation test. The *B. simplicifolia* I-A_4 isolectin (2.5 mg/mL) also reacted strongly with asialo-OSM but not with OSM.

The precipitation of WGA by asialo-OSM is presented in Figure 2a. Although a concentration of asialo-OSM 3 times that of OSM was required to maximally precipitate WGA, the asialo-OSM precipitated the same amount of total protein.

Analogue OSM Precipitates More WGA Than Native OSM. The periodate consumption by OSM (Figure 1) of 2 mol of periodate per mol of NeuNAc over the first 0.5 h, followed by 1 additional mol of periodate per mol of NeuNAc over the subsequent 52 h, is consistent with the rapid, selective oxidation of the exocyclic chain of NeuNAc groups, followed by the slow oxidation of the 6-substituted D-GalNAc residues of OSM (Suttajit & Winzler, 1971). Termination of the oxidation after 32 min gave a product which produced a chromogen in the resorcinol assay for sialic acid with an absorption maximum shifted to 630 nm from the 585-nm maximum characteristic of NeuNAc. This shift in absorption maximum is a consequence of the oxidation of the exocyclic chain of NeuNAc (Jourdian et al., 1971).

Conversion of the NeuNAc groups of OSM to NeuNAc-7 produced an analogue OSM which precipitated 86% of WGA from solution in contrast to 41% precipitated by native OSM (Figure 2b).

Analogue Fetuin Also Precipitates More WGA Than Native Fetuin. The mild periodate oxidation conditions employed selectively convert the NeuNAc groups of fetuin to the 7-aldehydo derivative (Peters, 1973).

The visible absorption spectra of the resorcinol chromogens arising from fetuin, periodate-oxidized fetuin, and borohydride-reduced analogue fetuin are depicted in Figure 3. The oxidized fetuin displayed increased extinction in the resorcinol

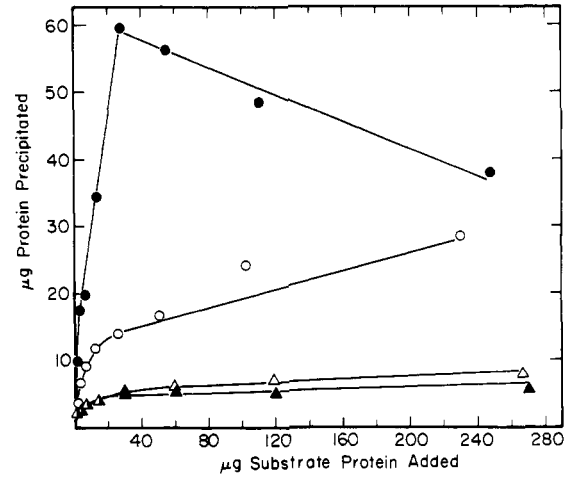

FIGURE 4: Quantitative precipitation of untreated and treated fetuin by WGA. Each tube contained 98 μg of WGA and increasing amounts of glycoprotein in a total volume of 300 μL: fetuin (○); asialofetuin (△); analogue fetuin (●); asialo analogue fetuin (▲).

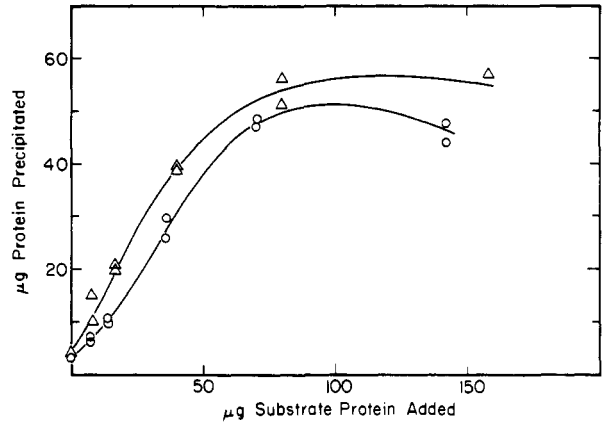

FIGURE 5: Quantitative precipitation of fetuin (○) and asialofetuin (△) by *P. vulgaris* phytohemagglutinin (PHA). Each tube contained 107 μg of PHA and increasing amounts of glycoprotein in a total volume of 300 μL.

assay with concomitant shift in the wavelength of maximum absorption from 585 to 630 nm as described by Jourdian et al. (1971). Upon borohydride reduction, the extinction returned to near the original value (at 585 nm), but the wavelength of maximum absorption remained at 630 nm.

The material liberated from the analogue fetuin by mild acid hydrolysis migrated on thin-layer chromatography as a single spot (silica gel G; propanol–water, 7:3) with R_{NeuNAc} = 1.2, visualized either by chromic acid charring or with the resorcinol reagent spray.

The precipitation of WGA by native and analogue fetuin is presented in Figure 4. Whereas native fetuin reacted weakly with WGA, precipitating only 14% of the total protein, analogue fetuin was capable of precipitating 50% of the total protein. Hydrolysis of the sialic acid groups with mild acid rendered both native and analogue fetuin incapable of precipitating WGA (Figure 4). The inability of asialofetuin to react with WGA was not simply a nonspecific effect of the mild acid hydrolysis since, as shown in Figure 5, fetuin and acid-treated fetuin reacted identically with the phytohemagglutinin (PHA) from *Phaseolus vulgaris*.

In order to verify that analogue fetuin interacts more strongly than native fetuin with WGA, we tested the ability of the hapten *N,N'*-diacetylchitobiose to inhibit both precipitation systems (Figure 6). A threefold higher concentration of hapten was required to inhibit by 50% analogue fetuin–

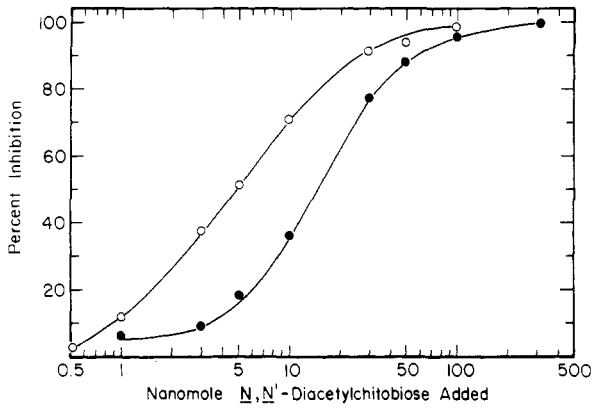

FIGURE 6: Inhibition of WGA–fetuin (○) and WGA–analogue fetuin (●) precipitation by *N,N'*-diacetylchitobiose. Each tube contained 98 μg of WGA, 26 μg of fetuin, or 28 μg of analogue fetuin and increasing amounts of *N,N'*-diacetylchitobiose in a total volume of 300 μL.

WGA precipitation over native fetuin–WGA precipitation.

Discussion

WGA Combines with Glycoconjugates Lacking D-*GlcNAc Residues.* The most compelling arguments that WGA binds sugars other than D-GlcNAc are founded on the ability of WGA to react with glycoconjugates devoid of D-GlcNAc (Maget-Dana et al., 1977; Bhavanandan et al., 1977; Bhavanandan, 1978). We have verified by quantitative precipitation analysis the observation of Bhavanandan (1978) that both OSM, bearing about 200 α-D-NeuNAc*p*-(2→6)-α-D-GalNAc*p*-(1→0)-serine or -threonine groups per molecule, and asialo-OSM, bearing 200 α-linked D-GalNAc groups per molecule (Hill et al., 1977), are able to precipitate WGA. The OSM preparation used contained less than 1% D-GlcNAc by weight.

Inhibitors of WGA, *N,N'*-diacetylchitobiose, and D-GlcNAc completely inhibit WGA–OSM precipitation and exhibit a relative potency in the OSM system comparable to that observed in other systems (Allen et al., 1973; Goldstein et al., 1975). Lactose, a noninhibitor of WGA, had no effect on WGA–OSM precipitation. These data show that the interaction of OSM with WGA is a specific one mediated by the WGA carbohydrate combining site.

The interaction between WGA and OSM is significantly weaker than the interaction between WGA and a *p*-azophenyl-β-D-GlcNAc–bovine serum albumin conjugate reported by Goldstein et al. (1975). Whereas 45 nmol of *N,N'*-diacetylchitobiose was required to inhibit by 50% the precipitation of WGA by the β-D-GlcNAc–BSA conjugate, only 5 nmol of the same inhibitor was required to achieve comparable inhibition of the precipitation of WGA by OSM. The weaker interaction of OSM with WGA in comparison to the interaction of *p*-azophenyl-β-D-GlcNAc–bovine serum albumin with WGA (Goldstein et al., 1975) also reflects the relative potencies of NeuNAc and *p*-nitrophenyl-β-D-GlcNAc as inhibitors of WGA. NeuNAc is a threefold less potent inhibitor of WGA than D-GlcNAc (Table I), and *p*-nitrophenyl-β-D-GlcNAc is a fivefold better inhibitor of WGA than D-GlcNAc. We have taken advantage of the sensitivity to inhibition of the WGA–OSM system in order to probe the combining site of WGA by hapten inhibition analysis.

N-Acetylneuraminic Acid and D-*GalNAc Inhibit WGA–OSM Precipitation.* Both NeuNAc and D-GalNAc inhibited completely the precipitation of WGA by OSM. NeuNAc was threefold less potent and D-GalNAc was fivefold less potent than D-GlcNAc. Glycoconjugates bearing NeuNAc or D-GalNAc groups, such as OSM and asialo-OSM, precipitate

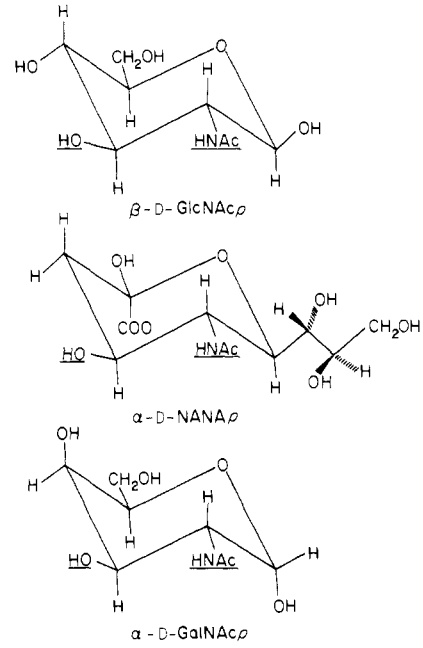

FIGURE 7: Comparison of common structural features shared by D-GlcNAc*p*, D-NeuNAc*p*, and D-GalNAc*p*. The substituents critical to interaction with WGA are underlined.

WGA because NeuNAc and D-GalNAc are hapten inhibitors of WGA. As pointed out by Bhavanandan (1978), the reactivity of WGA with NeuNAc and D-GalNAc in addition to D-GlcNAc is explained by the superimposability of these three molecules at the equatorial C-2 acetamido group and at the equatorial C-3 hydroxyl group of the pyranose ring (Figure 7). Substitution or epimerization of either of these two groups abolishes reactivity with WGA (Allen et al., 1973; Goldstein et al., 1975), whereas substitution of the hydroxyl groups at position 4 or 6 has no effect (Allen et al., 1973; Ebisu et al., 1977). These three molecules are identical with regard to the groups critical to proper contact with the WGA carbohydrate combining site. They differ at positions not essential to WGA binding.

All but Two of the NeuNAc Derivatives Tested Were Equipotent with NeuNAc as Inhibitors of WGA–OSM Precipitation. Whereas WGA exhibits some anomeric specificity for D-GlcNAc, reacting more strongly with the methyl β-glycoside than with the methyl α-glycoside (Allen et al., 1973; Goldstein et al., 1975), both methyl α- and methyl β-ketosides of NeuNAc were equivalent to NeuNAc as inhibitors of WGA–OSM precipitation. As can be seen from Figure 7, the anomeric carbon of D-GlcNAc is in close proximity to the critical contact face of the molecule where its substituents can interact with the WGA molecule. In contrast, the anomeric carbon of NeuNAc is on the opposite side of the molecule from the WGA contact face; substituents at the anomeric carbon atom of NeuNAc play no significant role in interfacing with the WGA molecule. A similar argument can be made for the observation that NeuNAc and its 2,3-dehydro derivative inhibit to the same extent.

Of great importance is the fact that the methyl ester of NeuNAc is equipotent with NeuNAc, ruling out nonspecific electrostatic effects between negatively charged NeuNAc molecules and the positively charged WGA molecule (Rice & Etzler, 1975) as the basis for WGA–NeuNAc interaction.

A comparison of NeuNAc, lactose, and sialyllactose (85% 2→3 isomer and 15% 2→6 isomer; data supplied by manufacturer) as inhibitors of WGA reveals that, although lactose by itself does not measurably interact with WGA, the lactosyl

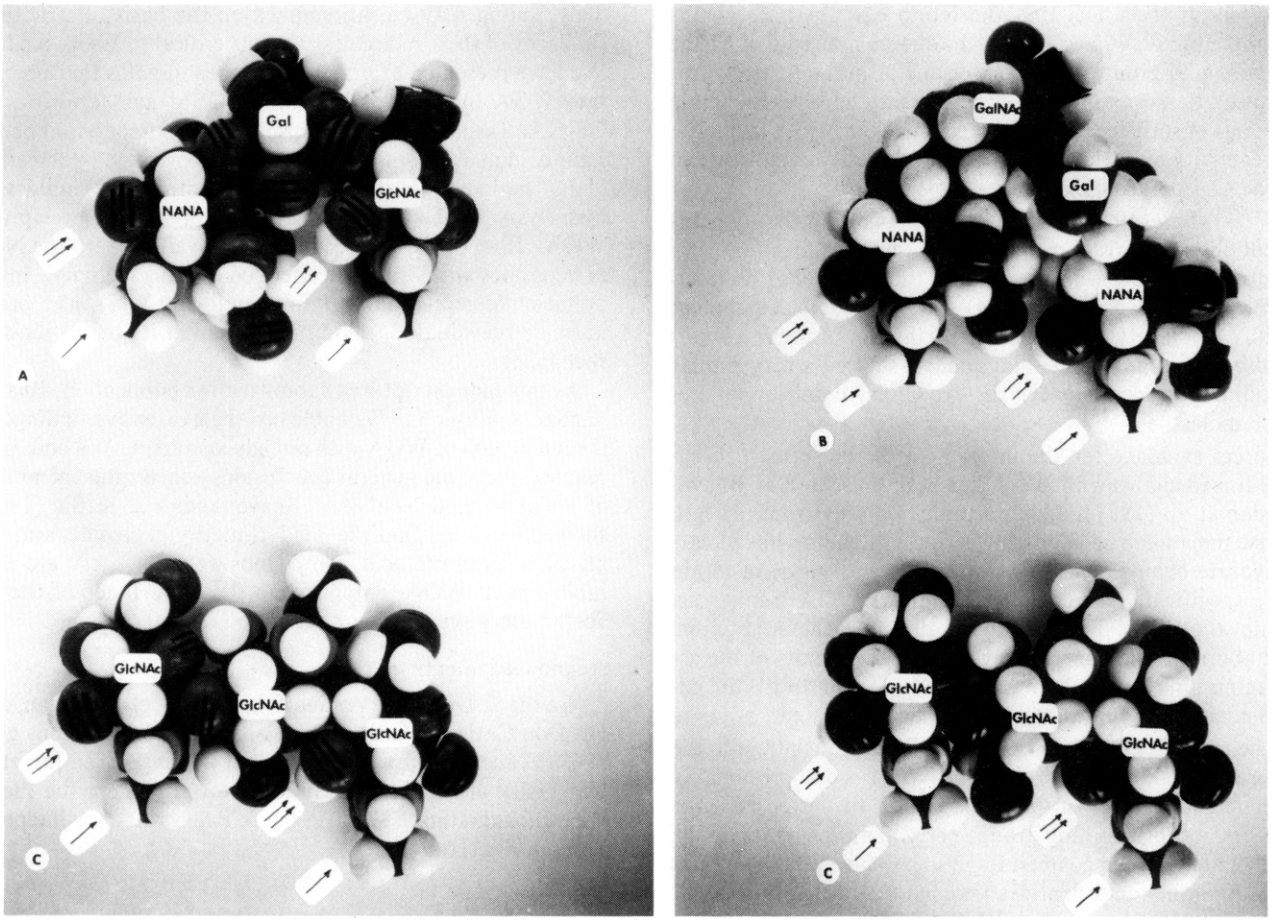

FIGURE 8: Space-filling models compare *N,N′,N″*-triacetylchitotriose (C) to (A) the trisaccharide α-D-NeuNAc-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→core) and to (B) the tetrasaccharide α-D-NeuNAc-(2→3)-β-D-Gal-(1→3)-[α-D-NeuNAc-(2→6)]-α-D-GalNAc-(1→0)-serine or -threonine. The superimposability of the critical C-2 acetamido (↑) and C-3 hydroxyl (↑↑) groups of the first and third GlcNAc residues of *N,N′,N″*-triacetylchitotriose with equivalent groups on the fetuin saccharides is emphasized.

residue of sialyllactose is capable of contributing to the interaction. Some productive contact of the lactosyl residue of the trisaccharide must occur with the extended carbohydrate combining site (Allen et al., 1973) of the WGA molecule.

The Exocyclic Chain of NeuNAc Interferes with Optimal NeuNAc–WGA Interaction. Conversion of NeuNAc to its seven-carbon analogue NeuNAc-7 by periodate–borohydride treatments results in a threefold increase in the inhibitory potency of the molecule, to a level comparable to that of D-GlcNAc. The exocyclic chain of NeuNAc, as seen in Figure 8, represents a potential source of steric hindrance to optimal contact with the WGA combining site. In this regard, colominic acid, an α-(2→8)-linked homopolymer of NeuNAc, neither inhibits nor precipitates WGA (data not shown).

Selective Modification of the NeuNAc Groups of Fetuin and OSM by Periodate–Borohydride Treatment Enhances Reactivity with WGA. That NeuNAc-7 reacts more strongly with WGA than NeuNAc is reflected by the ability of the analogue glycoproteins to precipitate at equivalence more than twice the amount of WGA from solution as compared to the native glycoproteins. The greater binding affinity of WGA for analogue fetuin compared to native fetuin is verified by the fact that a threefold greater concentration of *N,N′*-diacetylchitobiose is required to inhibit the precipitation of WGA by analogue fetuin than to inhibit to a comparable degree the precipitation of WGA by native fetuin.

NeuNAc Is Immunodominant in Fetuin–WGA Interaction. Mild acid hydrolysis of fetuin and analogue fetuin abolished their reactivity with WGA (Figure 4). The mild acid treatment removed >95% of the sialic acid from these molecules without perturbing their reactivity with *P. vulgaris* phytohemagglutinin, ruling out a possible nonspecific effect of the hydrolysis on lectin reactivity. Even though asialofetuin has twelve 4-substituted D-GlcNAc residues and three *N,N′*-diacetylchitobiose groups per molecule (Spiro, 1964; Baenziger & Fiete, 1979), these groups are silent toward WGA.

We speculate that the molecular basis for the immunodominance of NeuNAc in the interaction of fetuin with WGA is the structural similarity of the carbohydrate chains of fetuin with the trisaccharide *N,N′,N″*-triacetylchitotriose. The fetuin molecule possesses 12 nonreducing trisaccharide units [α-D-NeuNAc*p*-(2→3 or 6)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→-carbohydrate core)] distributed over three asparagine-linked carbohydrate chains (Baenziger & Fiete, 1979) as well as 3 units of α-D-NeuNAc*p*-(2→3)-β-D-Gal*p*-(1→3)-[α-D-NeuNAc*p*-(2→6)]-α-D-GalNAc*p*-(1→0)-serine or -threonine per 55 000 daltons (Spiro & Bhoyroo, 1974). Space-filling models of both carbohydrate chains are shown in Figure 8 along with *N,N′,N″*-triacetylchitotriose, the smallest, most potent inhibitor capable of filling the extended site of WGA (Allen et al., 1973; Goldstein et al., 1975). Although the conformation of these oligosaccharides is not known, the space-filling models illustrate the potential superimposability of both types of fetuin chains with *N,N′,N″*-triacetylchitotriose. Inhibition studies of Allen et al. (1973) and Goldstein et al. (1975) single out as critical to WGA–*N,N′,N″*-triacetylchitotriose interaction the C-2 acetamido and C-3 hydroxyl groups of the pyranose rings occupying subsites 1 and 3 of each WGA binding site. It is

precisely at these loci that the fetuin carbohydrate chains resemble the *N,N',N''*-triacetylchitotriose molecule by virtue of sialic acid groups filling either one or both of the critical subsites. Removal of the sialic acid groups deprives the fetuin molecule of sufficient productive contact with WGA to abolish its reactivity even though 12 D-GlcNAc residues substituted at the C-4 position remain per molecule.

Wood et al. (1978) first made the suggestion that two different oligosaccharides could present the same face for lectin binding. These workers found the trisaccharide α-D-GlcNAc-(1→4)-β-D-Gal-(1→4)-D-GlcNAc to be equipotent with *N,N'*-diacetylchitobiose as an inhibitor of the *B. simplicifolia* II lectin. Space-filling models showed a very similar spatial relationship between the two GlcNAc units in both of these molecules.

Direct evidence for the interaction of the serine-, threonine-linked chains with WGA has been presented by Bhavanandan et al. (1977). Glycopeptides isolated from the B16 mouse melanoma cells bearing serine-, threonine-linked carbohydrate chains identical in structure with those of fetuin were specifically bound to a column of WGA–Sepharose. Removal of the sialic acid groups abolished the interaction. The ability of the nonreducing trisaccharide units of the asparagine-linked carbohydrate chains of fetuin to fill the extended site of WGA remains to be demonstrated.

The reactivity of asialo-OSM with WGA in contrast to the failure of asialofetuin to react with WGA is due presumably to the higher valency of the asialo-OSM: the latter possesses 200 single subsite-filling groups per 600 amino acid peptide (Hill et al., 1977) in contrast to 12 single subsite-filling groups per 400 amino acid peptides on asialofetuin (Spiro, 1964).

Role of WGA–Sialic Acid Interaction with Whole Cells. Several studies on the ability of WGA to agglutinate intact cells may be explained on the basis of the contribution of sialic acid groups to WGA binding. Burger & Goldberg (1967) reported that L1210 cells, agglutinated by WGA, were no longer agglutinated after treatment of the cells with protease-free, glycosidase-free neuraminidase. The cells recovered their agglutinability after 3 min, suggesting new synthesis or repair had taken place. Nonspecific WGA–NeuNAc interaction or an indirect effect of surface charge alteration was suggested as a possibility. The WGA binding sites of rat adipocytes (Cuatrecasas, 1973) and of human erythrocytes (Adair & Kornfeld, 1974) were also labile to neuraminidase digestion. Scatchard analysis by Adair & Kornfeld (1974) revealed that neuraminidase treatment of erythrocytes reduced the affinity of WGA binding without altering the number of combining sites per cell. Although internal GlcNAc residues remain on the surface of neuraminidase-treated red cells as binding sites for the lectin, the loss of the productive contacts contributed by NeuNAc groups seriously curtails the affinity of the interaction.

Variant CHO cells able to resist the cytotoxic effects of WGA were isolated by Briles et al. (1977) and by Stanley & Siminovitch (1977). The prominent plasma membrane modification detected in Briles' clone 1021 was an 80% reduction in sialic acid content relative to that of the parental cell clone. This variant appears similar to the $Wga^{R_{II}}$ variant of Stanley that also exhibits defective cell surface sialylation (Stanley & Carver, 1977). WGA binding to both of these lines is markedly impaired due to the absence of NeuNAc groups from a class of WGA binding sites that mediate the cytotoxic effect of the lectin.

In summary, we have demonstrated that, in addition to D-GlcNAc, NeuNAc and D-GalNAc are inhibitors of WGA. This finding may be rationalized on the basis of structural similarity of these molecules at points critical to WGA binding. We have presented examples of glycoconjugates that precipitate WGA by virtue of NeuNAc (OSM and fetuin) or D-GalNAc (asialo-OSM) groups. The immunodominance of sialic acid in WGA interaction was demonstrated by the effect of (1) periodate–borohydride treatment and (2) mild acid hydrolysis on the ability of glycoconjugates to precipitate WGA. Both of these procedures selectively perturb the NeuNAc groups with dramatic effect on WGA reactivity. Interpretation of cell binding studies employing WGA as a probe must be reevaluated, taking into account its broad binding specificity.

As this manuscript was submitted for publication, Bhavanandan & Katlic (1979) published their extensive findings on the interaction of WGA with sialoglycoproteins. We both have reached the same general conclusions concerning the ability of WGA to bind NeuNAc. Bhavanandan & Katlic (1979) showed that neuraminic acid β-methyl ketoside and *N*-glycolylneuraminic acid, compounds we did not test, are noninhibitors of WGA, emphasizing the importance of the *N*-acetamido group of NeuNAc to WGA interaction.

Acknowledgments

We thank Dr. David Aminoff, University of Michigan, and Dr. Don Carlson, Purdue University, for their generous gifts of OSM and Dr. Sten Hammarström, Wenner-Gren Institute, University of Stockholm, Sweden, for supplying the *Helix pomatia* agglutinin. We also thank Paula Kane for the preparation of PHA.

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Affinity-Directed Cross-Linking of Membrane-Bound Acetylcholine Receptor Polypeptides with Photolabile α-Bungarotoxin Derivatives†

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ABSTRACT: Photolabile derivatives of [^{125}I]-α-bungarotoxin that retain specific binding to *Torpedo californica* acetylcholine receptor have been utilized as structural probes of the receptor complex of polypeptide components in its membrane-associated form. The derivatized toxins contained aryl azide side chains poised to form covalent cross-links to both associated and adjacent polypeptides following toxin–receptor complex formation. The results demonstrate that, depending on the possible radius of extension of the photoactivated group from the parent toxin, either (1) both the polypeptide to which the toxin derivative binds and an adjacent polypeptide can be derivatized upon photolysis or (2) only the adjacent polypeptide is labeled. The results lend strong support to the notion that the nicotinic receptor from *T. california* is composed of a complex of different polypeptides.

Torpedo californica electric tissue has been one of the main sources of both highly enriched membrane preparations of AcChR[1] (Duguid & Raftery, 1973) in addition to providing starting material for the purification of detergent-solubilized receptor preparations (Raftery, 1973; Schmidt & Raftery, 1973a). Purified *T. californica* receptor is considered to consist of four types of polypeptide chains (Raftery et al., 1974, 1975; Weill et al., 1974; Karlin et al., 1975; Vandlen et al., 1976, 1979; Flanagan et al., 1975; Hamilton et al., 1977; Chang & Bock, 1977; Chang et al., 1977; Hucho et al., 1978; Lindstrom et al., 1978). The same polypeptides occurring in the same apparent ratios, as defined by Coomassie Blue stained bands in $NaDodSO_4$ gel electrophoresis, are present in enriched membrane preparations (Duguid & Raftery, 1973; Raftery et al., 1974; Reed et al., 1975; Witzemann & Raftery, 1977, 1978a,b). In addition, these membranes prepared by centrifugation procedures or by additional steps such as affinity partitioning (Flanagan et al., 1975) still contain a few additional polypeptides, notably those of molecular weight 43 000 and 90 000. It has recently been shown that highly enriched membranes can be depleted of polypeptides except those characteristic of purified AcChR by brief treatment at pH 11 under conditions of low ionic strength (Neubig et al., 1979; Elliott et al., 1979) following the procedures of Steck & Yu (1973) for selective extraction of red blood cell membrane proteins. Despite the identical subunit composition of detergent-extracted *T. californica* receptor purified by affinity chromatographic procedures and the membrane preparations, the question still remains as to whether these polypeptides are in actual physical contact within the postsynaptic membrane and naturally whether more than one of these is involved in synaptic transmission at the molecular level. We have adopted two main approaches in an attempt to answer this question. The first of these involves detection of conformational changes

† From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering (Contribution No. 5988), California Institute of Technology, Pasadena, California 91125. *Received May 3, 1979.* Supported by U.S. Public Health Service Grant NS 10294, by a grant from the Muscular Dystrophy Association of America, by a grant from the American Heart Foundation, and by a grant from the Deutscheforschungsgemeinschaft (to V.W.). A preliminary account of part of this research has been given [see Witzemann & Raftery (1978a)].

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[1] Abbreviations used: AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; Me_2SO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; $IAcNH_2$, iodoacetamide; DEAE, diethylaminoethyl; Carb, carbamylcholine.

0006-2960/79/0418-5511$01.00/0