The role of sialic acid in the interaction of sialoglycoproteins with wheat germ agglutinin was investigated by using several well characterized saccharides and sialoglycoconjugates. N-Acetylneuraminic acid and neuramin 2→3 lactose, in addition to N-acetyl-d-glucosamine and its β1→4 oligomers were found to be inhibitors of wheat germ agglutinin-induced hemagglutination. Neuraminic acid-β-methyl ketoside, N-glycolyneuraminic acid and several other acidic sugars were noninhibitors. Both glycoporin and α1-acid glycoprotein were excellent inhibitors, whereas the corresponding asialo compounds were either very poor or noninhibitors. Among the mucins tested, all except porcine submaxillary mucin, which has N-glycolyneuraminic acid, were potent inhibitors. The results of double diffusion experiments, in which the ability of the glycoconjugates to form precipitin line with wheat germ agglutinin was tested, were in agreement with the agglutination inhibition data.

Affinity chromatography of labeled glycoconjugates on wheat germ agglutinin-Sepharose 4B columns confirmed that N-acetylneuraminyl residues play an important role in the binding of sialoglycoconjugates to this lectin. The results suggest that the binding of a glycopeptide to a wheat germ agglutinin-Sepharose 4B column may be influenced both by the density of the binding sugar residues (N-acetyl-d-glucosamine and N-acetylneuraminic acid) on the glycoprotein and by the density of the lectin molecules on the gel beads. In cell affinity chromatography, the cells interacted irreversibly with wheat germ agglutinin-Sepharose 6MB beads; this binding was partially lost on treatment of the cells with neuraminidase. Models for the interaction of wheat germ agglutinin with N-acetylneuraminic acid and polysialoglycoconjugates are presented.

Wheat germ agglutinin is also able to interact with glycoproteins containing multiple nonreducing terminals of N-acetylgalactosamine as are present, for example, in asialo ovine and bovine submaxillary mucins.

In recent years, wheat germ agglutinin (WGA) has been used extensively as a tool for the isolation and characterization of a variety of glycoconjugates, including cell surface components (1-4). Further, the cytotoxic properties of WGA have been utilized for selecting cells with altered cell surface oligosaccharide units (5, 6). In other studies, immunization of mice against the WGA receptor has been tried in an attempt to elicit an immune response in mice against possible wheat germ agglutinin specific tumor antigens (7). Even though a large number of such studies have been carried out with WGA, the true specificity of this lectin remains unclear.

Wheat germ agglutinin, a pure protein with a molecular weight of 36,000, is composed of two similar polypeptide chains, each possessing two binding sites for sugars (8, 9). The binding site consists of three subsites in an extended arrangement rather than in a single deep pocket in the protein (10). WGA is a very stable protein and its stability has been attributed to the presence of a large number of disulfide bridges (8). Several papers have been published on the sugar binding specificity of this lectin (9). It is generally accepted that this lectin binds specifically to 2-acetamido-2-deoxy-d-glucose (GlcNAc) and its β1,4-linked oligomers (8, 10-12). In addition, recent studies have provided either direct or indirect evidence for the interaction of sialic acid with WGA (13-20). However, there is no agreement regarding the nature of the interaction between sialic acid or sialy glycoconjugates and WGA (9, 21). Since WGA is a basic protein, three out of four isolectins having an isoelectric point of 8.7 ± 0.3 (22), the possibility exists of electrostatic interaction between sialic acid and WGA.

While investigating the glycoconjugates produced by murine melanoma cells, we isolated a sialoglycopeptide devoid of GlcNAc but capable of specifically interacting with WGA. It was shown that the interaction of this glycopeptide with WGA was due to the presence of clustered sialyl oligosaccharide groups on the peptide backbone (20). In order to obtain a better understanding as to the real specificity of WGA, a systematic study was undertaken using a large number of simple and complex model saccharides. The results of these investigations are presented in this paper. A preliminary report has been presented (23).

EXPERIMENTAL PROCEDURES

Raw wheat germ was obtained from local health food stores and from Sigma Chemical Co. Crude wheat germ lectin (B grade) was purchased from Calbiochem.

Ovalbumin (grade III), ovomucoid (trypsin inhibitor), bovine serum albumin, ceruloplasmin (type III), heparin (grade I), trypsin (type I), N-acetylneuraminic acid (type VI), N-glycolyneuraminic acid, and p-nitrophenyl-N-acetyl-β-d-galactosaminide were purchased from Sigma Chemical Co. Chitin, Vibrio cholerae neuraminidase, pronase-CB and p-nitrophenyl-N-acetyl-β-d-glucosaminide were from Calbiochem. Neuraminic acid-β-methyl ketoside (methoxyneuraminic acid) and bovine submaxillary mucin were obtained from Doelinger Manheim. Phenyl-N-acetyl-α-d-galactosaminide was from Nakarai acid; GalNAc, N-acetylgalactosamine; NeuNAc, N-glycolyneuraminic acid.
Isolation and Purification of Glycophorin

Erythrocytes obtained from the blood bank within 1 week of outdating were pooled according to blood group types and the ghosts prepared as described (32). The lyophilized erythrocyte ghosts were treated with lithium diiodosalicylate and the solubilized glycoproteins extracted with 0.05 M HCl, fractionated with ammonium sulfate, and butanol-treated (41). The lectin was purified by affinity chromatography on an ovomucoid or ovalbumin-Sepharose 4B column (42). To understand the process of elution of the lectin from the ovomucoid-Sepharose 4B column, we tested 0.1 M sodium acetate and 10 mM mercaptoethanol in phosphate buffer, pH 8.0, both of which failed to elute the lectin even though small amounts of inactive protein were eluted. Purified WGA purchased from Boehringer Mannheim was also used in our studies.

Conjugation of Lectins and Glycoproteins to Sepharose 4B

Wheat germ agglutinin, ovalbumin, and ovomucoid were coupled to Sepharose 4B which was activated by cyanogen bromide by one of two methods (43, 44). WGA was reacted with the activated Sepharose in the presence of 0.1 M N-acetylglucosamine. The remaining active groups were blocked and the gels were washed as described previously (40). The amount of protein bound to Sepharose was determined by estimating the unbound protein in the supernatant and washes. WGA-Sepharose 6MB purchased from Pharmacia was also used in some of our experiments.

Affinity Chromatography of Glycoproteins on WGA-Sepharose 4B Columns

The sample, usually radioactively labeled, was applied in a volume of 100 to 500 μl to a lectin column (2.5 to 5 ml of gel) equilibrated with the elution buffer (50 mM Tris-Cl, pH 8.0, with or without 0.1% Triton X-100). The sample was washed twice into the column with about 0.5 ml of buffer and allowed to incubate for about 15 min. The column was then eluted with the above buffer followed by 0.1 M N-acetylglucosamine in the same buffer and fractions of 1 ml were collected. The flow rate of the column was controlled to about 10 to 15 ml/h. The radioactivity in the eluted fractions was measured after mixing with 10 ml of scintillation counting liquid. The total radioactivity recovered from the columns was better than 85% unless otherwise mentioned. The columns were routinely regenerated by washing with a minimum of 10 bed volumes of the starting buffer. Occasionally the columns were cleaned, particularly when the recoveries have been poor, by washing the column with 6 ml urea, followed by buffer containing 0.1% Triton X-100 and finally the starting buffer.

The columns were stored between experiments at 4°C in 0.1 M N-acetylglucosamine in buffer containing 0.02% sodium azide; most of our WGA-Sepharose 4B preparations have remained active over periods of 6 months to 1 year.

Other buffers, including those containing between 0.1 to 0.5 M NaCl, were also tried but had no significant effect on the results (45).

Agglutination and Agglutination-Inhibition Assays

Human blood group (O) erythrocytes were washed and a 2% suspension of cells in CaCl2- and MgCl2-free NaCl/P, was prepared. For the titration of lectin activity, serial 2-fold dilutions of the stock lectin...
solutions were prepared in siliconized microtest slides (A. H. Thomas, Philadelphia) in a volume of 50 µl of NaCl/P. An equal volume of the erythrocyte suspension was added to each well and the slide was incubated on a shaker at room temperature for 20 min. The degree of hemagglutination was scored on a serological scale from 0 (no agglutination) to +4 (maximum agglutination).

For agglutination inhibition assays, the inhibitors dissolved in NaCl/P, and pH adjusted to 7.0 if necessary, were serially diluted in the plates (in a volume of 40 µl). The minimum amount of WGA (for untreated erythrocytes, 1 µg in 10 µl of NaCl/P; giving a final concentration of 10 µg/ml) required to give complete agglutination in the absence of inhibitor was then added to each well and mixed, and 50 µl of the erythrocyte suspension was added and once again mixed. After shaking the mixture on a rotary shaker at room temperature for 20 min, the plates were examined under a microscope and the degree of agglutination in each well was assessed to determine the concentration of the inhibitor causing 50% inhibition. As pointed out by Allen et al. (10), such an assay involving visual scoring is only semiquantitative and is liable to give an error of ±20%.

Precipitation Analysis by Diffusion in Gel

Double diffusion was carried out in immunodiffusion plates (Miles) using 2% agar in 0.15 M phosphate buffer, pH 7.2 (46). The center well was filled with 20 µl of a solution of WGA, 2 mg/ml in NaCl/P, and outer wells were filled with glycoprotein solutions at 2 mg/ml in water or NaCl/P; certain glycoproteins were also tested at higher concentrations. The diffusion plates were incubated in a humid chamber for 18 to 48 h. Some plates were washed, dried, and stained with Amido black prior to visualization (47).

RESULTS

Further Purification of WGA

Attempts to purify further the lectin by a second chromatography on the same affinity column always resulted in extremely poor recovery. In a typical experiment, 186 mg of WGA was applied to an ovomucoid-Sepharose 4B column; elution with 0.1 N HOAc yielded only 25 mg of protein which had the same hemagglutinating activity as the original lectin. Elution of the column with 1 M acetic acid, 0.05 M HCl, or 0.1 M GlcNAc did not yield any additional protein. In another experiment, 200 mg of a crude wheat germ lectin preparation (Calbiochem, B grade, Lot 430031) having an activity equivalent to at least 50 mg of pure lectin yielded 3 mg of active protein after affinity chromatography using an ovomucoid column. Similar results were also obtained when repurification was attempted on a column of Sepharose substituted with p-amino phenyl-2-acetamido-2-deoxy-D-glucopyranoside (48).

Hemagglutination-Inhibition Assays

The results of the hemagglutination-inhibition experiments are summarized in Tables I to III. The results reported were reproducible in several experiments. There were no significant differences in the inhibitory effect of the saccharides using either our WGA preparations or those obtained from Boehringer Mannheim. The glycoconjugate inhibitors listed in Tables II and III did not cause agglutination or lysis of human erythrocyte suspension. Similar results were also obtained when repurification was attempted on a column of Sepharose substituted with p-aminophenyl-2-acetamido-2-deoxy-D-glucopyranoside (48).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration needed for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc</td>
<td>12.5</td>
</tr>
<tr>
<td>N-Tosylglucosamine</td>
<td>&gt;400 µM</td>
</tr>
<tr>
<td>GlcNAcβ1 → 4GlcNAc</td>
<td>0.15 µM</td>
</tr>
<tr>
<td>GlcNAcβ1 → 4GlcNAcβ1 → 4GlcNAc</td>
<td>0.03 µM</td>
</tr>
<tr>
<td>GlcNAcβ1 → CH2NO2(p)</td>
<td>0.10 µM</td>
</tr>
<tr>
<td>Galβ1 → 6 GlcNAcβ1 → CH2NO2(p)</td>
<td>0.125 µM</td>
</tr>
<tr>
<td>Galβ1 → 4GlcNAcβ1 → CH2NO2(p)</td>
<td>2.0 µM</td>
</tr>
<tr>
<td>ManNAcβ1 → 6Galβ1 → 4GlcNAcβ1 → 4GlcNAc</td>
<td>0.125 µM</td>
</tr>
<tr>
<td>GalNAc</td>
<td>200 µM</td>
</tr>
<tr>
<td>GalNAcβ1 → phenyl</td>
<td>&gt;400 µM</td>
</tr>
<tr>
<td>GalNAcβ1 → CH2NO2(p)</td>
<td>&gt;3.2 µM</td>
</tr>
<tr>
<td>NeuNAcα2 → 3 lactose</td>
<td>6.25 µM</td>
</tr>
<tr>
<td>NeuNAc</td>
<td>25 µM</td>
</tr>
<tr>
<td>Neuraminic acid β-methyl ketoside</td>
<td>&gt;800 µM</td>
</tr>
<tr>
<td>NeuNGc</td>
<td>200 µM</td>
</tr>
<tr>
<td>Lactose</td>
<td>&gt;200 µM</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>&gt;160 µM</td>
</tr>
<tr>
<td>Glucosaminic acid</td>
<td>&gt;125 µM</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>&gt;260 µM</td>
</tr>
<tr>
<td>Gluconic acid</td>
<td>&gt;332 µM</td>
</tr>
</tbody>
</table>

* Higher concentrations could not be tested due to poor solubility.

Table II

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration needed for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-Acid glycoprotein*</td>
<td>1.4, 4.5 µM, 62.5, 200 µg/ml</td>
</tr>
<tr>
<td>Asialo α1-acid glycoprotein</td>
<td>&gt;20.8 µM, &gt;800 µg/ml</td>
</tr>
<tr>
<td>Fetuin</td>
<td>20.8 µM, 1000 µg/ml</td>
</tr>
<tr>
<td>Asialo fetuin</td>
<td>&gt;52.7 µM, &gt;2160 µg/ml</td>
</tr>
<tr>
<td>Fetuin glycopeptides A</td>
<td>&gt;200 µg/ml</td>
</tr>
<tr>
<td>Fetuin glycopeptides B and C</td>
<td>&gt;400 µg/ml</td>
</tr>
<tr>
<td>Alkaline borohydride-resistant fetuin</td>
<td>&gt;88.9 µM, &gt;4000 µg/ml</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>2.2 µM, 62.5 µg/ml</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>4.2 µM, 187.5 µg/ml</td>
</tr>
<tr>
<td>Ribonuclease B (Worthington)</td>
<td>&gt;54.4 µM, &gt;800 µg/ml</td>
</tr>
<tr>
<td>Ribonuclease B (purified)</td>
<td>4.25 µM, 62.5 µg/ml</td>
</tr>
</tbody>
</table>

* Two different preparations, one from Dr. Karl Schmid and the other from the American Red Cross, gave two different values.

The fraction bound on concanavalin A-Sepharose 6B column and eluted with α-methyl mannoside.

potent inhibitor than NeuNAc. The effect was specific for NeuNAc, since NeuNGc, neuraminic acid β-methyl ketoside and other acidic sugars, such as muramic, glucuronic, and glucosaminic acids, were noninhibitory. Of the glycoproteins with mainly N-glycosidically linked carbohydrates (Table II), the inhibitory activity of ovomucoid, ovalbumin, and ribonuclease B is due to GlcNAc residues in these molecules. On the other hand, the inhibition by α1-acid glycoprotein is probably due to the terminal NeuNAc residue since the asialo compound was not inhibitory at 4 times the concentration. Fetuin was a weak inhibitor compared to α1-acid glycoprotein and ovomucoid. Various derivatives of fetuin including asialo fetuin were all noninhibitors. The weak inhibition by fetuin was surprising in view of the fact that all other results indicate an efficient interaction between fetuin and WGA. For example, WGA could be isolated using a column of fetuin-Sepharose 4B (50), fetuin gave a precipitin line with WGA and labeled fetuin bound to affinity columns of WGA-Sepharose 4B (Table IV).

* V. P. Bhavanandan and A. Katlic, unpublished results.
The most powerful inhibitors of WGA agglutinations are mucins or glycoporin, which has a mucin-type structure having several clustered sialyl oligosaccharides linked O-glycosidically to the peptide backbone (51) (Table III). Ovine submaxillary mucin and glycoporin were 50,000 and 70,000 times more potent than GlcNAc in inhibiting WGA. Of the mucins tested, the only one which did not show inhibition was pig submaxillary mucin. This is interesting since about 90% of the sialic acid in this mucin is N-glycolyl (52). Glycopeptides isolated from glycoporin were also inhibitory, but it is not possible to compare their potency against glycoporin and GlcNAc because of the lack of information on their molecular weights. The noninhibitory nature of polyanions such as hyaluronic acid, heparan sulfate, and keratan sulfate (all GlcNAc-containing) confirms the specific nature of the inhibition by NeuNAc-containing polyanionic mucins. The nonac- cidual desulfated keratan sulfate has been shown to form a precipitate with WGA (53). This is attributable to the alternating 1 → 4-linked GlcNAc in this polysaccharide; desuf- fatsion would also remove the NeuNAc from this polymer (54). A sialic acid polymer, colominic acid, was also noninhibitory. Colominic acid is a linear polysaccharide of sialic acid linked to the peptide backbone (51) (Table III). Ovine cidic desulfated keratan sulfate has been shown to form a precipitate with WGA, not inhibit WGA-induced hemagglutination.

Asialo ovine submaxillary mucin and asialo bovine submax- 
illary mucin also inhibited agglutination by WGA, although at a slightly higher concentration than the native mucins (see also Ref. 21). These results would tend to suggest that NeuNAc has no role in the inhibition of WGA agglutination by these mucins. On the other hand, it is likely that the large numbers of nonreducing terminal GalNAc in these asialo mucins are responsible for inhibition. It should be pointed out that GalNAc is an inhibitor, even though only at higher concentrations (200 mM) compared to NeuNAc (25 mM) and GlcNAc (12.5 mM). Thus, a peptide with multiple, some clustered, GalNAc would be expected to be a good inhibitor. In order to confirm the role of GalNAc in the asialomucin in the interaction with WGA, in preliminary experiments, we tested bovine submaxillary mucin and ovine submaxillary mucin which were deglycosylated by treatment with hydrogen fluoride (55). It was found that these preparations free of sialic acid and about 90% of the GalNAc did not inhibit WGA agglutination up to about 7 μM. Higher concentrations could not be tested due to the poor solubility of the deglycosylated samples.

Asialoglycoporin was a poor inhibitor compared to the asialo mucins. This is consistent with the fact that the non- 
reducing terminus in this compound is galacose. The weak inhibition by this compound could be due to the internal GlcNAc residues present in the O-glycosidically linked pros- 
thetic group, even though the possibility of contribution from the terminal galactose residues to the interaction cannot be completely discounted (19). Interaction between the internal GlcNAc in carcinoembryonic antigen and WGA was suggested by Goldstein et al. (12).

**Double Diffusion Experiments**

The results of a few typical double diffusion experiments using wheat germ agglutinin and various glycoproteins are shown in Fig. 1.

**Table III**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration needed for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoporin (MM)</td>
<td>0.16</td>
</tr>
<tr>
<td>Glycoporin (NN)</td>
<td>0.32</td>
</tr>
<tr>
<td>Glycoporin glycopptides (trypt-</td>
<td>100</td>
</tr>
<tr>
<td>sin)</td>
<td></td>
</tr>
<tr>
<td>Glycoporin glycopeptides (pro-</td>
<td>200</td>
</tr>
<tr>
<td>nase)</td>
<td></td>
</tr>
<tr>
<td>Asialoglycoporin</td>
<td>16.3; &gt;32.5</td>
</tr>
<tr>
<td>Ovine submaxillary mucin</td>
<td>0.23</td>
</tr>
<tr>
<td>Bovine submaxillary mucin</td>
<td>0.32-0.65</td>
</tr>
<tr>
<td>Porcine submaxillary mucin</td>
<td>&gt;0.52</td>
</tr>
<tr>
<td>Porcine submaxillary mucin gly-</td>
<td>800</td>
</tr>
<tr>
<td>copeptides</td>
<td></td>
</tr>
<tr>
<td>Asialo ovine submaxillary mucin</td>
<td>1.85; 0.23</td>
</tr>
<tr>
<td>Asialo bovine submaxillary mucin</td>
<td>0.32; 0.92</td>
</tr>
<tr>
<td>Human bronchial mucin</td>
<td>25</td>
</tr>
<tr>
<td>Dog tracheal mucin</td>
<td>50</td>
</tr>
<tr>
<td>Monkey cervical mucin</td>
<td>30</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Keratan sulfate II</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Colominic acid</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>Di- and trisialogalactosides</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

**Table IV**

<table>
<thead>
<tr>
<th>WGA-Sepharose 4B preparation number a</th>
<th>Glycoporin</th>
<th>Asialoglycoporin</th>
<th>Fetuin</th>
<th>Asialo fetuin</th>
<th>Ovomucoid</th>
<th>α-Acid glycoprotein</th>
<th>Pig submaxillary mucin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>N.T. a</td>
<td>30; 33; 35</td>
<td>N.T.</td>
<td>16; 30; 21</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>47</td>
<td>63; 66</td>
<td>1.5</td>
<td>40; 46</td>
<td>52</td>
<td>N.T.</td>
</tr>
<tr>
<td>3</td>
<td>99; 100</td>
<td>37</td>
<td>73; 80</td>
<td>0</td>
<td>96</td>
<td>100</td>
<td>34</td>
</tr>
</tbody>
</table>

**a** WGA isolated by affinity chromatography on immobilised ovomucoid was used in the preparations. Based on the estimations of the protein not bound to Sepharose, the Preparations 1, 2, and 3 should have approximately 1, 2, and 5 mg of bound protein/ml of gel. WGA-

**b** N.T., not tested.

c Ten times more sample applied.

d Fifty times more sample applied.
At concentrations of 2 mg/ml, ovine submaxillary mucin, bovine submaxillary mucin, human bronchial mucin, dog tracheal mucin, monkey cervical mucin, glycophorin, and glycoprotein glycopeptides all showed strong precipitin bands. In the case of glycophorin, a band was detectable even at a concentration of 200 µg/ml. Glycoconjugates which gave weak bands at 2 mg/ml include porcine submaxillary mucin, asialo submaxillary mucins, and asialglycophorin. Ovalbumin and fetuin gave strong bands at 15 mg/ml and very weak bands at 5 mg/ml; in comparison, ovomucoid and α-acid glycoprotein formed strong bands at 5 mg/ml and weak bands at 2 mg/ml.

Several other components failed to produce a precipitin band against WGA at concentrations as high as 15 mg/ml. These include asialo fetuin, bovine serum albumin, hyaluronic acid, chondroitin 4-sulfate, heparin, and di- and trisialoglycosides.

**Affinity Chromatography of Glycoconjugates on WGA-Sepharose Columns**

Except for those experiments reported in Table IV, all others were carried out using fully active WGA-Sepharose 4B columns, comparable to Preparation 3 in Table IV.

**Glycophorin**—Glycophorin isolated from human erythrocytes of blood group MM or NN and labeled either in sialic acid or in the protein was completely retained on various WGA-Sepharose columns (Table IV and Fig. 2). The results obtained when a typical column was eluted with various solutions prior to elution with 0.1 M GlcNAc is shown in Fig. 3. Asialoglycophorin preparations isolated either by neuraminidase or acid treatment of the glycophorin were only partially (35 to 50%) retained on the WGA columns (Table IV and Fig. 2). All four major glycopeptides isolated from [NeuNac-3H]glycophorin by pronase or trypsin digestion were retained on the WGA column (Fig. 2). When [NeuNac-3H]glycophorin was treated with alkaline borohydride and chromatographed on a Bio-Gel P6 column, a large included peak was obtained eluting in the position of tetrasaccharides isolated from fetuin by similar treatment (36). These tetrasaccharides were not retained on the WGA column.

**Fetuin**—Different proportions of fetuin labeled in the protein either by reductive methylation or acetylation were retained on different WGA-Sepharose 4B columns as can be seen from Table IV. Once again prior elution of the column with salts or acetic acid did not displace a significant portion of the fetuin (Fig. 3). Fetuin glycopeptides A, B, and C labeled by reductive methylation were not bound to the WGA column which completely retained the native fetuin labeled by the same method. The alkaline borohydride-resistant portion of fetuin also was not retained on the affinity column. Asialo glycophorin glycopeptides (pronase). C, 1, porcine submaxillary mucin (a weak band was visible soon after development); 2, di- and trisialoglycosides; 3, asialo ovine submaxillary mucin; 4, ovine submaxillary mucin; 5, asialo bovine submaxillary mucin; 6, bovine submaxillary mucin. Other details are given under “Experimental Procedures.”
Interaction of Sialic Acid with Wheat Germ Agglutinin

**Fig. 3. Chromatography of [3H]acetyl fetuin (○ - ○) and [3H]acetyl glycoporphorin (■ - ■) on WGA-Sepharose 4B columns.** After application of the sample and a 15-min incubation, the columns were sequentially washed with 50 mM Tris-HCl buffer, pH 8.0, 1 M NaCl in buffer, 0.1 M NaOAc, pH 8.1, 0.1 M acetic acid, and finally 0.1 M GlcNAc in buffer. Fractions of 1 ml were collected and radioactivity was determined.

**Fig. 4. Affinity chromatography of [14C]acetyl fetuin (○ - ○) and [14C]acetyl glycoporphorin (■ - ■) on a WGA-Sepharose 4B column.** The mixed samples were applied on the column and eluted with 50 mM Tris-HCl, pH 8.0, followed by a linear gradient (---) of buffer alone (10 ml) to 0.1 M GlcNAc in buffer (10 ml). Fractions of 1 ml (1 to 25) and 0.5 ml (26 to 65) were collected and analyzed for radioactivity.

Chondroitin sulfate and heparan sulfate, isolated from human melanoma HM-2 cells grown in the presence of [3H]glucosamine, were not retained on the WGA columns. NeuNAc-2→3 [3H]actylactitol was also not retained on the WGA column. [14C]Dimethyl ribonuclease B, purified on concanavalin A-Sepharose 4B column, was partially (35%) retained on the WGA column. [14C]Acetil ovalbumin glycopeptides were not bound to this column.

**Elution of the WGA-Sepharose 4B Columns with GlcNAc Gradient**

Since the various glycoproteins appeared to interact differently with the immobilized WGA, the possibility of separating those glycoproteins by affinity chromatography using a gradient (0 to 100 mM) of GlcNAc was tested. Fetuin and α1-acid glycoprotein appeared to elute at the same position (45 mM GlcNAc) in the gradient. Glycoprophorin, however, appeared to elute a few fractions after fetuin and α1-acid glycoprotein. The result of a mixed experiment using [14C]acetyl fetuin and [1H]acetyl glycoporphorin is shown in Fig. 4.

**Cell Affinity Chromatography**

In an attempt to find out whether cultured cells could be fractionated, cell affinity chromatography was carried out using a K8/15 column with 80-μm nylon net (Pharmacia) packed with 2 ml of WGA-Sephrose 6MB gel. Human breast cells (HBL-100 and MDA-321) (56) grown in the presence of [3H]thymidine were harvested, washed three times with NaCl/P, and fixed with formaldehyde (10% HCHO in NaCl/P; 1 h at room temperature). A suspension of the cells in NaCl/P was applied to the column, and the column was washed with NaCl/P and eluted with 0.1 M GlcNAc. The recoveries were extremely low as can be seen in Table V. The cells recovered from the column increased more than 5-fold when the fixed MDA-231 cells were treated with V. cholerae neuraminidase (37°C, 24 h) prior to affinity chromatography. This suggested that the binding of the cells to the WGA-Sepharose gels was probably mediated by cell surface sialyl residues. On applying formaldehyde fixed MDA-321 cells to the column which was pre-equilibrated with 0.1 M GlcNAc, 52% of the radioactivity was not retained. Similar results were obtained with unfixed cells.

**DISCUSSION**

Wheat germ agglutinin was isolated by affinity chromatography on ovomucoid (or ovalbumin) Sepharose 4B columns. The yields of lectin varied from batch to batch of crude wheat germ. Usually between 50 to 200 mg of the lectin/200 g of wheat germ were obtained; however, certain batches of crude wheat germ gave extremely poor yields (<10 mg). The length of storage of the crude wheat germ prior to use may be one of the reasons for this variation in yield since one batch of raw wheat germ which initially gave about 100 mg of lectin/200 g after storage for about a year at 4°C yielded only 7 mg. In addition to this, it is not clear whether the strain of the wheat, condition, and length of storage of wheat before isolation of the germ, and other unknown factors influence the yield. A most puzzling aspect of WGA isolation was the loss of material incurred during attempted repurification. On rechromatography of the isolated lectin on affinity columns, the protein apparently bound to the ligand irreversibly and could not be displaced. This aspect was not further investigated.

WGA has been purified by affinity chromatography on immobilized ovomucoid, fetuin, synthetic GlcNAc-based adsorbents or on chitin (9, 50). We found that immobilized bovine submaxillary mucin (which lacks GlcNAc but contains NeuNAc) was also effective for the isolation of WGA. In control experiments, WGA did not bind to columns packed with immobilized bovine serum albumin confirming the specificity of the interaction between WGA and GlcNAc or NeuNAc-containing ligands, or both.

The inability of sodium acetate to elute the WGA from affinity columns indicates that even though N-acetyl groups of the hapten sugars (GlcNAc, NeuNAc, and GalNAc) are important for the interaction, acetate ions are not capable of disrupting the complex between WGA and the ligand. Mercaptoethanol, which was expected to reduce the large number of —S—S— bonds in WGA, also failed to disrupt the inter-
action between the lectin and the ligand under the conditions used.

The hemagglutination inhibition experiments, while confirming earlier reports that GlcNAc-containing compounds are good inhibitors of WGA induced hemagglutination, provide clear evidence for the inhibitory ability of NeuNAc-containing compounds. Nonspecific interaction between NeuNAc and WGA could be excluded by the inability of several other acidic sugars to inhibit agglutination. The importance of the N-acetyl group in NeuNAc (and NeuNAc 2 \( \rightarrow 3 \) lactose) is shown by the lack of inhibition by neuraminic acid \( \beta \)-methyl ketoside with a free \( \text{NH}_2 \) group and by NeuNGc.

In double diffusion experiments, the macromolecular glycoconjugates which were good inhibitors of hemagglutination were also the ones which gave precipitation lines with WGA.

Previous studies with sialoglycopeptides isolated from mouse and human melanoma cells (20, 57) indicated that glycopeptides having clustered \( O \)-glicosidically linked sialyl oligosaccharides were capable of interacting specifically with WGA. It was also apparent that the interaction was through sialic acid residues since the removal of sialic acid abolished the interaction. The major sialoglycoprotein of the human erythrocyte membrane is known to have 15 \( O \)-glicosidically linked sialyl tetrasaccharides in addition to 1 \( N \)-glicosidically linked oligosaccharide (51). Fetuin has three each of \( N \)-glycosidically and \( O \)-glicosidically bound oligosaccharides (36).

Therefore, in order to confirm our previous findings, we chose these and other well characterized glycoproteins for further studies using affinity chromatography. The results obtained with fetuin, glycophorin, and \( \alpha_1 \)-acid glycoprotein confirm the role of NeuNAc in the binding of these components to WGA. Asialo fetuin and asialo \( \alpha_1 \)-acid glycoprotein were not retained on the lectin column; thus, the terminal sialic acids are the sites of interactions. The specificity of the binding is illustrated by the inability of sodium chloride, acetic acid, or sodium acetate to displace the bound fetuin or glycophorin (Fig. 3).

The necessity for the presence of several sialyl oligosaccharides on a peptide backbone for stable binding is shown by the failure of neuraminyl lactitol, sialyl tetrasaccharide (isolated by alkaline borohydride treatment of glycophorin or fetuin), and fetuin glycopeptide Fractions A, B, and C to bind to the affinity column. It should be noted that, whereas neuraminyl lactitol and sialyl tetrasaccharide have 1 and 2 NeuNAc residues, respectively, fetuin glycopeptide Fraction A could have up to 3 terminal NeuNAc residues and 5 internal GlcNAc residues (58). In contrast, the glycopeptides isolated from glycophorin all bound to the WGA column in agreement with the fact that these glycopeptides would have multiple sialyl oligosaccharides (51). These results indicate clearly the requirement of the macromolecular structure and the polyvalent binding sites of the glycoconjugates for their stable binding to WGA-Sepharose column. Further work is necessary before the minimum valency required for stable interaction can be defined.

The role of internal GlcNAc residues in the interaction between glycoconjugates and WGA columns is not clear since asialo fetuin and asialo \( \alpha_1 \)-acid glycoprotein were not retained on WGA columns but asialoglycophorin was partially retained. The failure of asialo galectofetuin having nonreducing termini of GlcNAc and GalNAc to be retained on WGA columns was unexpected and difficult to understand. One possible explanation for this finding is that the spacing of GlcNAc and GalNAc terminals on the protein is unsuitable for stable binding to the WGA immobilized on Sepharose beads.

In agreement with the results from agglutination inhibition experiments, porcine submaxillary mucin was only partially retained on the lectin column probably because of the \( N \)-glycoxylineuraminic acid present on this glycoprotein. The retained portion may be molecules with human blood group A activity and thus having nonreducing GalNAc terminals. Contributions from the NeuNAc residues, 10% of the total sialic acid, to this binding is also possible. The porcine submaxillary mucin used in these studies was from pooled pig submaxillary glands.

The finding that the proportions of fetuin, \( \alpha_1 \)-acid glycoprotein, and ovomucoid binding to different WGA-Sepharose 4B preparations varied, in contrast to glycophorin which bound completely to all the preparations, is difficult to interpret (Table IV). Since applying 10 and 50 times more of the samples to the lectin columns 1 and 2 did not alter the results, it is clear that the partial binding of fetuin and ovomucoid was not due to overloading the columns. The variation in the concentration of the lectin on the gel could be a factor influencing the binding of different proportions of glycoproteins to different preparations. In other words, the interaction between a sialoglycoprotein and immobilized WGA could be influenced by both the density of GlcNAc or NeuNAc residues on the glycoprotein and the density of WGA molecules on the gel bead. Thus, a WGA-Sepharose 4B preparation with high density of lectin on the gel beads is capable of binding several glycoproteins completely, including those with a low density of NeuNAc or GlcNAc on the protein (fetuin, \( \alpha_1 \)-acid glycoprotein, and ovomucoid). In contrast, an affinity gel preparation with a low density of lectin on the gel will bind completely those glycoproteins with a high density of NeuNAc, such as glycophorin, and interact only partially with the other glycoproteins. The proportions of fetuin and ovomucoid retained on these columns apparently reflects the heterogeneity of these molecules with regard to distribution of terminal NeuNAc and GlcNAc available for interaction. Further investigation of this aspect is necessary to test this hypothesis.

The results of this study indicate that the interaction of glycoproteins with WGA is complex. A well recognized feature of this interaction is the role of GlcNAc and its \( \beta \rightarrow 4 \) linked oligomers in glycopeptides which bind to subsites postulated by Allen and co-workers (10). These workers also reported that, for efficient binding, the equatorial acetamido group at position 2 and the equatorial hydroxy group at position 3 of the hexosamine are essential. In an analogous manner, NeuNAc could interact at the same site via the acetamido group at position 5 and the hydroxy group at position 4 as illustrated in Fig. 5. Since the sialoglycopeptides labeled by modifying the NeuNAc residues to 8- and 7-carbon analogs still interacted with WGA, the side chains involving carbon atoms 7, 8, and 9 in NeuNAc are most probably not involved in the binding and in fact may even bind more efficiently to WGA-Sepharose column. Further work is necessary before the minimum valency required for stable interaction can be defined.

In the case of sialylglycoproteins, up to 3 sialyl residues present in a suitable arrangement could interact at the sugar binding regions, thus leading to the formation of an eventually stable complex between the glycoprotein and WGA as illustrated in Fig. 6. It is not clear whether 3 sialic acid residues as presented by the oligosaccharides attached to consecutive amino acids, for example, as in glycophorin, or the terminal sialic acids present in the branched \( N \)-glycosidically linked oligosaccharides could interact at three consecutive subsites without hindrance. If such an interaction is prevented due to the larger size of the NeuNAc residue, compared to the GlcNAc residue, than a stable complex could still be formed by the interaction of only 1 or 2 NeuNAc residues at each receptor region of WGA. Further studies are needed to obtain additional information as to the exact mechanism of the interaction between sialoglycoconjugates and WGA.
interaction of sialic acid with wheat germ agglutinin

FIG. 5. Structures of NeuNAc and GlcNAc in the IC and C1 conformations, respectively. The similar (trans) relationship between the acetamido (position 5 of NeuNAc and 2 of GlcNAc) and the hydroxyl (position 4 of NeuNAc and 3 of GlcNAc) groups in both structures is evident.

FIG. 6. Illustration representing the multivalent interaction of a glycoprotein with WGA. The WGA molecule is depicted as a dimeric protein with four carbohydrate binding regions, each capable of interacting with up to 3 NeuNAc or GlcNAc residues. The glycoprotein molecules have oligosaccharides linked both O-glycosidically and N-glycosidically to the protein. The model is not drawn to scale; only some of the sugar residues are indicated by ● (terminal NeuNAc residues) and by ○ (internal GlcNAc residues in the N-glycosidic linkage region interacting with WGA). Maximum interaction is shown, however, this may not be possible always due to stereochemical reasons. For example, interactions of only 1 sugar residue (NeuNAc or GlcNAc)/binding region may be sufficient to lead to a stable complex.

possible to explain how a glycoprotein having only GalNAc, such as asialo ovine submaxillary mucin or asialo bovine submaxillary mucin, could lead to the formation of a stable complex with WGA. It is known that the configuration at position 4 of an N-acetylhexosamine is important for the interaction with WGA (10). When the 4-hydroxyl group is equatorial with or without substitution, as in GlcNAc and its 4-substituted derivatives, favorable binding occurs. Replacement by an axial 4-hydroxyl group as in GalNAc leads to weak binding, since GalNAc had 1/6 of the potency of GlcNAc (Table I). Asialo ovine submaxillary mucin having several GalNAc on the protein backbone (on an average one in six amino acids are substituted (62)) will interact through these sugar residues, leading to a favorable stable complex. Thus, it is clear that the specificity of WGA and of course of other lectins is relative rather than absolute. In the case of WGA, at the present at least three sugars (GlcNAc, NeuNAc, and GalNAc) commonly found in glycoconjugates appear capable of interacting with different affinities. Depending on several other factors glycoconjugates containing these sugars could form stable complexes. The situation of WGA binding to the cell surface would thus be more complicated consisting for example of low and high affinity binding sites as suggested earlier (4, 6).

Four forms (isolectins) of WGA having similar properties have been isolated (10, 22). It will be interesting to determine whether these isolectins have different specificities with respect to GlcNAc, NeuNAc, and GalNAc.

In preliminary experiments, we have observed that the recovery of labeled glycoproteins is usually poor from lectin columns with a high (>5 mg/ml) concentration of lectin on the gel (compare the 6% recovery of WGA receptor activity from erythrocytes (4)). This suggests the possibility of strong irreversible interaction between glycoproteins and gels with a very high density of lectin molecules. The multivalent interaction between a glycoprotein and WGA will vary from very weak to very strong, depending on the factors discussed above and the possible cooperativity of the interaction. Positively cooperative binding of WGA to fat cells has been reported by Cuatrecasas (14). Thus, a monovalent hapten inhibitor, such as GlcNAc, would not be capable of interrupting a strong interaction between a polyvalent glycoprotein and WGA, thus explaining the poor recovery mentioned above. The results of the cell affinity chromatography are in agreement with this; the very high density of sialyl and or N-acetylglucosaminyl receptor sites on the cell surface will lead to an extremely strong interaction with the WGA immobilized on gel beads which will be difficult to break up by GlcNAc, whereas treatment of the cells with neuraminidase, thereby removing some of the binding sites or application of the cells in the presence of the inhibitor GlcNAc, improved the recovery of the cells from the columns considerably (Table V).

If the forces of interaction between glycoproteins and WGA are variable, then depending on the nature of the glycoprotein, it may be possible to fractionate mixtures of various glycoproteins on WGA column by elution with a gradient of the hapten sugar. We were able to obtain partial separation between fetuin or ovomucoid and glycoporphin, since fetuin and ovomucoid apparently have weaker interactions with WGA in contrast to glycoporphin as discussed above. This is another aspect which needs further investigation before developing it as a possible method for fractionation of cell membrane glycoproteins.

Greenaway and Levine (13) noted that approximately 9 mol of NeuNAc and 1 mol of GlcNAc were bound/mol of agglutinin when a 50-fold molar excess of the hapten over WGA was used. They concluded that, at this concentration, WGA had a higher affinity for binding NeuNAc than GlcNAc. Under optimum conditions, a maximum of 12 mol of NeuNAc would be expected to bind/mol of WGA, since it is proposed that WGA has four binding sites/mol, each of which has three subsites. Jordan et al. (18) investigated the binding of GlcNAc and NeuNAc to WGA by proton magnetic resonance and found that $K_m$ was $238 \text{ m}^{-1}$ for GlcNAc and $560 \text{ m}^{-1}$ for NeuNAc. Adair and Kornfeld (4) provided evidence indicating that sialic acid residues are a component of the WGA receptor on human erythrocytes. Several studies (14, 15, 59) have indicated that treatment of cells with neuraminidase decreased their binding affinity for WGA and also abolished WGA-induced agglutination. Not all cells are affected this way since neuraminidase digestion had no effect on WGA-induced agglutination of Novikoff tumor cells (60). WGA-resistant clones isolated from Chinese hamster ovary cells have shown defects in the sialylation of surface glycoproteins (5, 6). These and other reports (16, 17, 19) are consistent with our results.

In conclusion, the data provided in this paper clearly show that the N-acetylneuraminic acid residues of sialoglycopro-
teins are directly involved in specific interaction with WGA. Rather than the usually internal GlcNAc residues, further, it is clear from our data that the topography of the NeuNAc and GlcNAc residues on the glycoconjugates and the cell surface is important for the formation of a stable association between the lectin and the glycoconjugates or the cell.

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