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Immunochemical Specificity of the Combining Site of *Wistaria floribunda* Hemagglutinin†

Shunji Sugii and Elvin A. Kabat*

ABSTRACT: The specificity of *Wistaria floribunda* hemagglutinin, purified by adsorption on insoluble polyleucyl hog gastric mucin blood group A + H (PL-hog A + H) substance, elution with lactose, and gel filtration on Sephadex G-200, was studied immunochemically by quantitative precipitin and precipitin inhibition assays. The purified hemagglutinin, with a molecular weight of 68 000 and an isoelectric point of 5.0, was homogeneous electrophoretically and immunochemically and was made up of two covalently linked subunits of molecular weight 32 000. It was precipitated by blood group A, BI, Le^a, and precursor I substances and by B substance subjected to mild acid hydrolysis (P1) but poorly by blood group B and H substances. It was also precipitated by asialoorosomucoid (ASOR) but not by agalactoorosomucoid (AGOR) and inactive antifreeze glycoprotein. Inhibition assays with various monosaccharides, glycosides, and oligosaccharides indicate that the hemagglutinin is most specific for terminal nonreducing α-linked DGalNAc. The best inhibitor was the disaccharide DGalNAcα(1→6)DGal which was 8.8 times more potent than DGalNAc. The A-active di-, tri-, and pentasaccharides were 3.3, 3.8, and 12.0 times less potent than DGalNAcα(1→6)DGal, respectively. Among the glycosides of DGalNAc tested, pNphβDGalNAc was most potent. It was 6.0 times more active than DGalNAc but 1.5 times less active than DGalNAcα(1→6)DGal. Although the best inhibitor was the α-linked disaccharide DGalNAcα(1→6)DGal and MeαDGalNAc was better than MeβDGalNAc, pNphβDGalNAc was more potent than pNphαDGalNAc. Molecular models showed pNphβDGalNAc and DGalNAcα-(1→6)DGal to be similar in shape and to differ from pNphαDGalNAc. These findings indicate that the combining site of the hemagglutinin is at least as large as a disaccharide and that hydrophobic interaction and shape are important for binding.

The hemagglutinating substances (lectins) obtained from plants and animals have specific receptor sites for glycoproteins (Kabat, 1976; Lis & Sharon, 1977; Goldstein & Hayes, 1978; Pereira & Kabat, 1979). They have been used extensively as reagents for detecting the distribution and mobility of glycoproteins on normal and malignant cell surfaces (Nicholson, 1974; Sharon & Lis, 1975) and for isolation of glycoproteins present on the cell surface (Kimura & Wigzell, 1978; Kimura et al., 1979).

Wistaria floribunda hemagglutinin and mitogen have been isolated separately from crude seed extracts by column chromatography (Toyoshima et al., 1971; Kurokawa et al., 1976) and by using immunoadsorbents (Cheung et al., 1979). The hemagglutinin is a glycoprotein which agglutinates human A, B, and O erythrocytes nonspecifically. Although some properties of the isolated hemagglutinin reported by different investigators have varied slightly, hemagglutination inhibition assays were in accord in that hemagglutination is inhibited best by DGalNAc[1] and synthetic DGalNAcα1→Tos-L-Ser

† From the Departments of Microbiology, Human Genetics and Development, and Neurology and the Cancer Center/Institute for Cancer Research, Columbia University College of Physicians and Surgeons, New York, New York 10032. *Received September 14, 1979.* Aided by grants from the National Science Foundation (BMS-72-02219 A04 and PCM 76-81029) and by a Cancer Center Support grant (CA 13696) from the National Cancer Institute to the Institute of Cancer Research, Columbia University.

[1] Abbreviations used: DGalNAc, 2-acetamido-2-deoxy-D-galactose; DGlcNAc, 2-acetamido-2-deoxy-D-glucose; DGal, D-galactose; DGlc, D-glucose; DFru, D-fructose; *p*, pyranoside; *f*, furanoside; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PL-hog A + H, polyleucyl hog gastric mucin A + H blood group substance; Ara, arabinose; ASOR, asialoorosomucoid; AGOR, agalactoorosomucoid; pNph, *p*-nitrophenyl; PhOH, phenol-insoluble fraction.

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(Kaifu & Osawa, 1979), that pNphβDGal was better than pNphαDGal, that MeαDGal was better than MeβDGal, and that lactose was better than melibiose (Toyoshima et al., 1971; Kurokawa et al., 1976; Cheung et al., 1979). Thus both α- and β-linked sugars were reacting with the hemagglutinin. This paper provides additional data on the immunochemical specificity of this hemagglutinin as determined by quantitative precipitin assays with blood group substances and glycoproteins and on its combining site as mapped by quantitative precipitin and inhibition assays with glycosides and mono- and oligosaccharides.

Materials and Methods

Materials. *W. floribunda* seeds were purchased from F. W. Schumacher, Co., Sandwich, MA. The blood group substances used were obtained from human ovarian cysts or salivas, from hog gastric mucin, and from horse stomach linings (Schiffman et al., 1964; Lloyd et al., 1968; Vicari & Kabat, 1969). Cyst 9 and Cyst 14 were previously described by Baer et al. (1959) and kindly made available; they were then used by Moreno et al. (1971). Beach phenol-insoluble P1 was the nondialyzable fraction of human ovarian cyst B substance subjected to mild acid hydrolysis (Allen & Kabat, 1959). The inactive antifreeze glycoprotein was provided by Dr. R. E. Feeney (de Vries et al., 1970) through Dr. M. Heidelberger. The asialoorosomucoid (ASOR) and agalactoorosomucoid (AGOR) have been described previously by Kawasaki & Ashwell (1976) and were provided by Dr. G. Ashwell. Various monosaccharides and lactose, melibiose, and raffinose were purchased from Eastman Organic Chemicals, Sigma Chemical Co., and Nutritional Biochemicals Co. The methyl α-D-galactoside and methyl β-D-galactoside were from Dr. A. B. Pardee. The four methyl anomers of DGalNAc were prepared in this laboratory (Sarkar & Kabat, 1979). Disaccharides DGalβ(1→4)DGlcNAc and DGalNAcα(1→6)-DGal were kindly provided by Drs. F. Zilliken (Kabat, 1962) and I. J. Goldstein, respectively. The oligosaccharides from blood group substances were described previously (Schiffman et al., 1962; Lloyd et al., 1966; Etzler et al., 1970; Lundblad et al., 1972).

The immunoadsorbent hog gastric mucin A + H blood group substance (PL-hog A + H) was prepared by copolymerization of the purified hog A + H with the *N*-carboxyanhydride of L-leucine by the technique of Tsuyuki et al. (1956) as described by Kaplan & Kabat (1966).

Purification. Finely powdered seeds (100 g) were suspended in 1000 mL of 0.01 M phosphate-buffered saline, pH 7.2, containing 0.02% sodium azide (PBS). The suspension was allowed to stand overnight at 4 °C with occasional stirring, and the supernatant was obtained by centrifugation at 2000 rpm for 2 h at 4 °C and was filtered through a Millipore filter (0.45 μm). For separation of the hemagglutinin, the immunoadsorbent was prepared by mixing 800 mg of PL-hog A + H and 4 g of washed Celite as described previously (Pereira & Kabat, 1974). The hemagglutinin in the clear crude extract was adsorbed onto the immunoadsorbent PL-hog A + H at 4 °C for 1 h with stirring. The unadsorbed substances were separated by centrifugation at 2000 rpm at 4 °C for 1 h and used for purification of the mitogen. The hemagglutinin–PL-hog A + H complex was extensively washed with PBS. Elution of the hemagglutinin was carried out by mixing with 50 mL of 1% lactose and stirring at 4 °C for 2 h. The eluate was obtained by centrifugation at 2000 rpm for 1 h at 4 °C. This procedure was repeated until the optical density at 280 nm of the eluate decreased to 0.05. The eluate was collected and filtered through a Millipore filter to remove insoluble small particles. Further purification of the eluate to remove lactose was performed by gel filtration on Sephadex G-200 with 0.1 M Tris-HCl, pH 8.5. The main peak was collected and concentrated in a diaflow PM-10 membrane (Amicon Co., Lexington, MA). The hemagglutinin was also purified by the methods of Cheung et al. (1979) using ethanol precipitation before the immunoadsorbent.

For purification of the mitogen, the crude extract was used after removal of the hemagglutinin on PL-hog A + H. As described (Toyoshima et al., 1971), the mitogen was partially purified by sequential steps involving ammonium sulfate precipitation between 40 and 70% saturation, chromatography on SP-Sephadex C-50 at pH 5.0, and gel filtration on Sephadex G-200 at pH 5.0. The main peak having the mitogenic activity was collected and concentrated in a diaflow PM-10 membrane.

Determination of Mitogenic Activity. As described by Phillips et al. (1976), peripheral human lymphocytes were separated by Ficoll-Hypaque and washed 3 times in RPMI 1640 (GIBCO). After the lymphocytes were washed, a suspension of 10^6 lymphocytes/mL was prepared in RPMI 1640 containing 10% heat-inactivated fetal calf serum. Twenty microliters of the sample at an appropriate concentration was mixed with 200 μL of lymphocytes. The culture was incubated at 37 °C for 72 h in 5% CO_2 in air in a microtest plate. At the end of the culture period, the cells in each well were incubated for 2 h with 2 μCi of [^{3}H]thymidine. The cells were collected on the filters with a MASH II cell harvester using 0.9% NaCl followed by cold 5% trichloroacetic acid and by cold 80% ethanol. After the filters dried, they were transferred into scintillation vials containing 3% BBOT in toluene and counted in a Packard Tricarb liquid scintillation counter.

Electrophoresis. Disc electrophoresis was run in 7.5% polyacrylamide gel according to the methods of Davis (1964) for pH 8.9 gel and those of Reisfeld et al. (1962) for pH 4.5 gel, respectively. The molecular weight of the hemagglutinin was determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5% gel) as described previously by Weber & Osborn (1969). Protein markers used for determination of molecular weight were γ-globulin (M_r 160 000), bovine serum albumin (M_r 68 000), egg albumin (M_r 43 000), pepsin (M_r 35 000), chymotrypsinogen (M_r 25 500), trypsin (M_r 23 300), and lysozyme (M_r 14 300). Analytical isoelectrofocusing was run in 5% polyacrylamide gel containing 2% carrier ampholytes (Ampholine pH 3.5–10, LKB) according to the methods of Righetti & Drysdale (1971). The gels were stained overnight in 0.05% Coomassie brilliant blue R-250 in 7% acetic acid and destained in 7% acetic acid.

Immunological Methods. Hemagglutination was performed at room temperature with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, VA) using 25-μL loops and 25 μL of a 2% human A, B, and O erythrocyte suspension. Hemagglutination titer was determined 1 h after incubation at room temperature.

Quantitative precipitin and precipitin inhibition assays (Kabat, 1961) were carried out by a microprecipitin technique using a final volume of 200 μL of quantitative precipitin assays and 400 μL for inhibition assays, respectively; 6.9 μg of hemagglutinin N was mixed with varying amounts of blood group substances or glycoproteins in quantitative precipitin assays; for inhibition assays known quantities of sugars were added to 6.9 μg of hemagglutinin N and 15 μg of blood group Le^a substance (N-1 phenol insoluble), an amount giving almost maximum precipitation in the absence of inhibitor. The mixtures were incubated at 37 °C for 1 h and then kept at

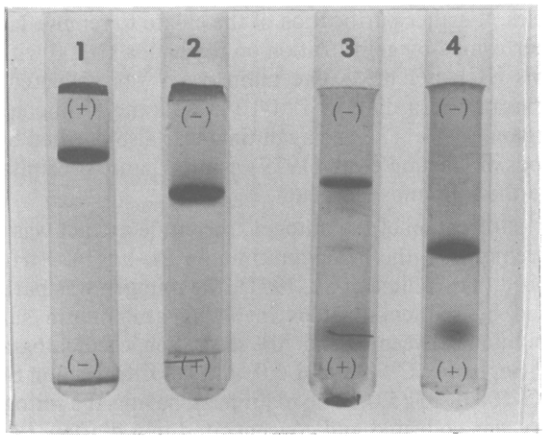

FIGURE 1: Polyacrylamide gel electrophoresis of *W. floribunda* hemagglutinin. Disc electrophoresis patterns of 4.75 μg of N of hemagglutinin at pH 8.9 (1) and pH 4.5 (2). $NaDodSO_4$ electrophoresis patterns of 4.16 μg of N of unreduced (3) and reduced (4) hemagglutinin with dithiothreitiol. A thin wire has been inserted to locate the position of the tracking dye.

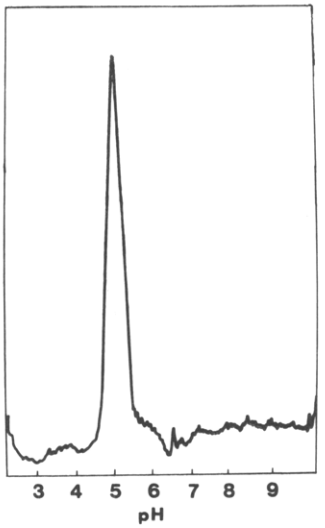

FIGURE 2: Densitometric pattern of 2.8 μg of N of hemagglutinin on analytical isoelectrofocusing.

4 °C for 1 week. The nitrogen content in the washed precipitates was determined by the ninhydrin procedure (Schiffman et al., 1964).

Antiserum to a crude extract of *W. floribunda* seeds was produced in a rabbit by injecting an emulsion of 0.5 mL of the crude extract in Freund's complete adjuvant H37Ra (Difco, Detroit, MI) into the footpads once a week for 3 weeks. The rabbit was bled on 3 successive days beginning 1 week after the final injection, and these sera were pooled.

Two-dimensional gel diffusion and immunoelectrophoresis were carried out in 1.5% agar (Special Noble Agar, Difco, Detroit, MI) containing 0.05 M sodium barbital, pH 8.3, according to the methods of Ouchterlony (1948) and of Grabar & Williams (1953), respectively. Immunoelectrophoresis was run at 150 V for 1 h at room temperature. The gels were stained for 20 min in 1% Amido Black 10B in 7% acetic acid and destained in 7% acetic acid.

Results

Both hemagglutinating and mitogenic activities were found in the crude extract of *W. floribunda* seeds. Almost all of the hemagglutinating substance was bound to the PL-hog A + H immunoadsorbent, but the mitogen was not bound. About 130 mL of crude extract was required to saturate 100 mg of PL-hog A + H. The lactose eluate from PL-hog A + H usually emerged in a single symmetrical peak by gel filtration on Sephadex G-200; occasionally a small faster eluting peak was seen. The retarded main peak had the hemagglutinating activity, whereas the small faster moving peak was inactive. Approximately 60–80 mg of hemagglutinin was isolated from 100 g of seed powder. The purified hemagglutinin showed a single homogeneous band on polyacrylamide gel electrophoresis at pH 4.5 and 8.9 (Figure 1) and also gave a single band of isoelectric point pH 5.0 on analytical isoelectrofocusing (Figure 2). On $NaDodSO_4$–polyacrylamide gel electrophoresis, the hemagglutinin was resolved into two bands corresponding to molecular weights of 68 000 and 32 000, respectively, the proportions varying with different batches. When reduced with dithiothreitol at 10^{-2} M, a single band was found of molecular weight 32 000 (Figure 1). The same results were also obtained with the hemagglutinin isolated by the methods of Cheung et al. (1979).

The mitogen purified by column chromatography gave one major band and a few minor bands on polyacrylamide gel electrophoresis at pH 4.5 but showed several bands on polyacrylamide gel electrophoresis at pH 8.9. On $NaDodSO_4$–polyacrylamide gel electrophoresis, the mitogen was resolved into several bands under both reduced and unreduced conditions.

On gel diffusion and immunoelectrophoresis, the hemagglutinin gave a single precipitin line against the rabbit antiserum to the crude extract, whereas the crude extract gave three major and one or two minor precipitin lines on gel diffusion and three major and four minor precipitin lines on immunoelectrophoresis, respectively (Figure 3). As shown in Figure 3, the precipitin line of the hemagglutinin crossed that of the partially purified mitogen; both lines fused with their corresponding lines of the crude extract. On immunoelectrophoresis, the hemagglutinin gave a single arc but the partially purified mitogen showed one major and one minor arc. These arcs were different in electrophoretic mobility. Sera from preimmunization bleedings from the rabbits used gave no bands on gel diffusion with the materials used in Figure 3, indicating that neither the hemagglutinin nor the mitogen, nor other components in the crude extract, reacted with normal serum.

The purified hemagglutinin nonspecifically agglutinated human type A, B, and O erythrocytes to the same extent. The minimal amount required for positive hemagglutination of an equal quantity of 2% erythrocytes was 28 μg/mL. For induction of mitogenic activity, more than 1.44 mg/mL of the hemagglutinin was required whereas the partially purified mitogen was active at 1 μg/mL.

Quantitative Precipitin Assays. The precipitin curves of the purified hemagglutinin with various blood group substances and glycoproteins are shown in Figure 4, and the quantity of blood group substance and glycoprotein giving 50% precipitation and the maximum amount of the added hemagglutinin which could be precipitated by different blood substances and glycoproteins are given in Table I. Blood group A, I, B (horse 4), BI (Tij II) fractions, Le^a, and precursor I substances precipitated the hemagglutinin added, but B (Beach phenol insoluble) and H substances did poorly. When used with A substances, more than 85% of the hemagglutinin added was precipitated (Table I); the order of potency giving 50% precipitation was hog 75 > Cyst 9 > Cyst 14 > MSM 10% and hog A + H. With blood group B substances, more than 75% of the hemagglutinin added was precipitated with Tij II fractions and horse 4 25%. Although the reactivity of Beach

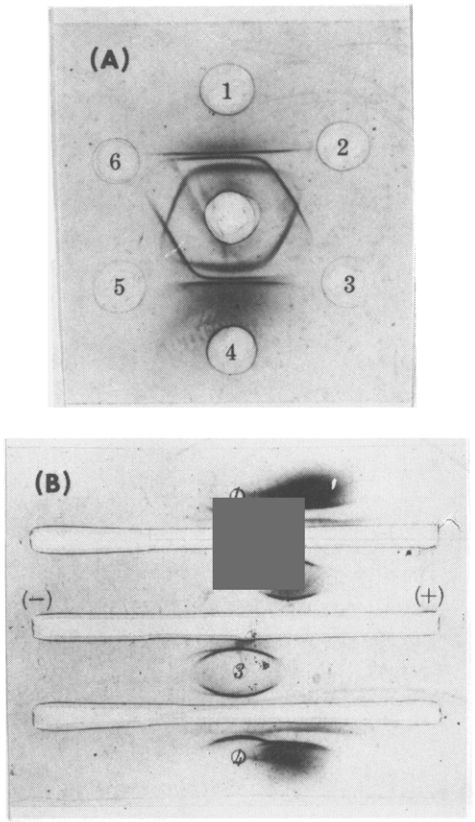

FIGURE 3: Immunodiffusion and immunoelectrophoresis with *W. floribunda* crude extract, hemagglutinin, and partially purified mitogen. Center wells of immunodiffusion (A) and trough of immunoelectrophoresis (B) contain undiluted rabbit antiserum to *W. floribunda* crude extract. Peripheral wells for immunodiffusion (A): (1 and 4) undiluted crude extract; (2 and 5) purified hemagglutinin, 104 μg of N/mL; (3 and 6) partially purified mitogen, 96 μg of N/mL. Wells for immunoelectrophoresis (B): (1 and 4) undiluted crude extract; (2) purified hemagglutinin, 104 μg of N/mL; (3) partially purified mitogen, 96 μg of N/mL.

phenol insoluble was poor, its mild acid-hydrolyzed fraction (P1) showed similar activity to Tij 20% 2X (Figure 4 and Table I). The blood group Le^a substances also precipitated more than 80% of the hemagglutinin added, and the precursor I substance OG was as potent as Beach phenol-insoluble P1. When used with glycoproteins, asialoorosomucoid (ASOR) precipitated about ~80% of the added hemagglutinin but agalactoorosomucoid (AGOR) and inactive antifreeze glycoprotein were inactive.

Quantitative Inhibition Assays. Figure 5 and Table II present the ability of various sugars to inhibit the precipitation reaction between the hemagglutinin and blood group Le^a substance which is N-1 phenol insoluble. Among the monosaccharides tested, DGalNAc was the best monosaccharide inhibitor and was 52 times more potent than DGal. With glycosides of DGalNAc, the order of inhibitory potency was pNphβDGalNAc > MeαDGalNAc*p* = pNphαDGalNAc > PhαDGalNAc > DGalNAc > MeβDGalNAc*p* > MeαDGalNAc*f* > MeβDGalNAc*f*. The *p*-nitrophenyl β-glycoside of DGalNAc was 6.0 times more potent than DGalNAc. With glycosides of DGal, similar results were obtained: pNphβDGal > PhβDGal = MeαDGal > pNphαDGal > DGal > MeβDGal. The *p*-nitrophenyl β-glycosides of DGalNAc and DGal were more potent than the corresponding α-anomers, whereas their methyl α-anomers were more potent than their corresponding β-anomers. With other monosaccharides, DFuc and LAra gave 50% inhibition at 16 000 and 31 000 nmol, respectively. DGlcNAc, LGal, DAra, and LFuc gave no inhibition at the highest concentrations tested. No inhibition was also observed with *p*-nitrophenol at up to 5350 nmol.

Table I: Comparative Precipitating Activities of Various Blood Group Substances and Glycoproteins

		blood group substance		lectin	
symbol	BGS and glycoprotein	μg giving 50% pptn	μg giving max pptn	max μg pptd	recovery (%)
●	Cyst 9 (A_1)	6.0	25.8	6.28	91.0
○	MSM 10% (A_1)	9.8	20.0	5.86	85
■	Cyst 14 (A_2)	7.3	19.8	6.56	95
□	hog 75 (A)	3.5	19.6	6.45	93
◑	hog A + H	9.8	26.2	6.55	95
⊙	Beach PhOH (B)	>47.5	28.5	1.86	27
◓	Beach PhOH P1 (B)	5.5	19.6	6.23	90
◒	Tij II PhOH (BI)	9.0	24.6	5.17	75
▪	Tij II 20% 2X (BI)	10	39.3	6.04	88
◘	horse 4 25% (B)	8.0	22.0	5.35	78
▲	JS PhOH (H)	>43.2	43.2	3.19	46
△	Tighe 20% 2X (H)	>48.8	48.8	1.79	26
◨	N-1 PhOH (Le^a)	9.0	20.4	6.16	89
◑	N-1 20% 2X (Le^a)	9.0	30.0	6.13	89
▼	N-1 10% 2X (Le^a)	7.5	24.9	5.56	81
⊠	OG (precursor) (I)	5.0	20.0	6.15	89
◪	inactive antifreeze glycoprotein	>50	>50	0	0
☆	ASOR	5.5	29.6	5.43	78.6
●	AGOR	>50.0	2		0

Of the oligosaccharides tested, those with terminal α-linked DGalNAc were much more potent than those with terminal α- and β-linked DGal. The disaccharide DGalNAcα(1→6)-DGal was the best inhibitor and was 1.5 times more potent than pNphβDGalNAc. It was also 3.3 times more active than the A-active disaccharide DGalNAcα(1→3)DGal (R_L 1.85) which was 2.7 times more active than DGalNAc. When used with A-active trisaccharide (A_5II) DGalNAcα(1→3)DGalβ(1→4)DGlcNAc and pentasaccharide (AR_L 0.52) DGalNAcα(1→3)[LFucα(1→2)]DGalβ(1→4)DGlcNAcβ(1→6)R, A_5II was as potent as R_L 1.85 but AR_L 0.52 was 3.7 times less active than R_L 1.85. Of the disaccharides with terminal DGal, DGalβ(1→4)DGlcNAc and DGalβ(1→4)DGlc (lactose) were 17 and 7 times more potent than DGal, respectively. Melibiose, DGalα(1→6)DGlc, was 2.2 times less active than lactose. The B-active disaccharide DGalα(1→3)DGal gave only 14% inhibition at up to 450 nmol. Raffinose, a trisaccharide with a terminal α-linked DGal, was as potent as DGal.

Discussion

The crude extract of *W. floribunda* seeds shows both hemagglutinating and mitogenic activities (Boyd et al., 1958; Barker & Farnes, 1969). The separation of the hemagglutinin from the mitogen in the crude extract has been successful by column chromatography (Toyoshima et al., 1971; Kurokawa et al., 1976) and by immunoadsorbent PL-hog A + H (Kaladas & Poretz, 1978; Cheung et al., 1979). The immunoadsorbent PL-hog A + H is preferable for isolating the hemagglutinin because it binds only the hemagglutinin in the crude extract (Kaladas & Poretz, 1979; Cheung et al., 1979). The present results confirmed these findings. The hemagglutinin isolated by PL-hog A + H following gel filtration was electrophoretically and immunologically homogeneous. It had a molecular weight of 68 000 and an isoelectric point of 5.0 and is made up of two subunits of molecular weight 32 000. It was capable of agglutinating human erythrocytes nonspecifically and had essentially no mitogenic activity. Gel diffusion and immunoelectrophoresis also demonstrated that

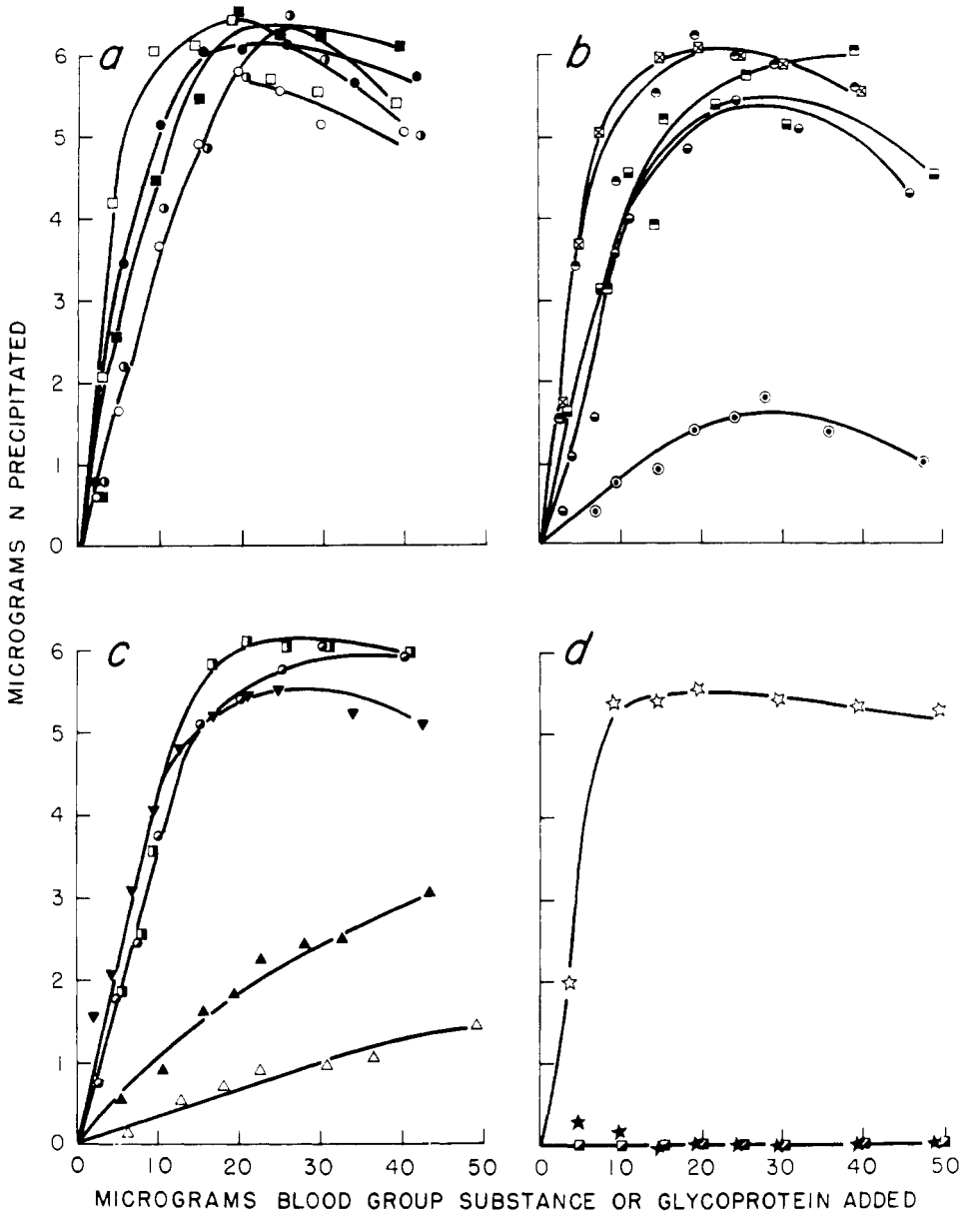

FIGURE 4: Quantitative precipitin curves of *W. floribunda* purified hemagglutinin (6.9 μg of N) with various blood group substances and glycoproteins. Total volume in all cases was 200 μL. Symbols are shown in Table I.

the hemagglutinin is distinct from the partially purified mitogen. With regard to the molecular forms of the isolated hemagglutinin, the previous reports did not agree (Toyoshima et al., 1971; Kurokawa et al., 1976; Cheung et al., 1979). Either a dimeric or a tetrameric hemagglutinin molecule was isolated by column chromatography (Toyoshima et al., 1971; Kurokawa et al., 1976), whereas a mixture of octomeric, tetrameric, and dimeric hemagglutinin molecules containing mainly tetrameric molecules were purified by using PL-hog A + H (Cheung et al., 1979). The hemagglutinin molecule isolated by us according to the methods of Cheung et al. (1979) was entirely dimeric. Cheung et al. (1979) reported that both tetrameric and octameric hemagglutinin molecules were found be gel filtration but not by $NaDodSO_4$–polyacrylamide gel electrophoresis because they are noncovalently associated with the dimeric hemagglutinin molecules, whereas Kurokawa et al. (1976) found no molecular weight difference by these two methods. The hemagglutinin isolated in this study is partially reduced and is a mixture of dimeric and monomeric hemagglutinin molecules. Such a hemagglutinin molecule has never been isolated from crude extracts of *W. floribunda* seeds, suggesting that the discrepancy in molecular weight may be due to the materials used rather than the methods of molecular weight determination. The octameric, tetrameric, and dimeric molecules gave positive hemagglutination, but the monomeric molecule did not (Toyoshima et al., 1971; Kurokawa et al., 1976; Cheung et al., 1979).

The quantitative inhibition assays provide information as to the specificity and size of the combining site of the hemagglutinin. They show the site to be strikingly specific for a terminal nonreducing DGalNAc which is 52 times more active than DGal, 44 nmol as compared with 2300 nmol being required for 50% inhibition. Nevertheless, appreciable inhibitory power can be obtained with oligosaccharides of DGal since DGalβ(1→4)DGlcNAc had 33% of the activity of DGalNAc, whereas DGalα(1→3)DGal was much less active. DGlcNAc was inactive at 2200 nmol. The D isomer and an OH on carbon 6 are essential since LGal and LFuc were almost completely inactive and DFuc was 7 times less active than DGal. These inhibition data account for the quantitative precipitin data. The A substances are reacting because of the terminal nonreducing DGalNAcα. Le^a N-1, precursor OG, and BI (Tij

Table II: Activities of Glycosides and Mono- and Oligosaccharides Inhibiting Precipitation of *W. floribunda* Hemagglutinin by Blood Group Substance

symbol	inhibitor	nmol needed to give 50% inhibn	rel potency
⊙	DGalNAcα(1→6)DGal	5.0	8.8
⋇	pNphβDGalNAc	7.3	6.0
✦	DGalNAcα(1→3)DGal (R_L 1.85)	16.5	2.7
○	DGalNAcα(1→3)DGalβ(1→3)DGlcNAc (A_5II)	19.0	2.3
☆	MeαDGalNAc*p*	26.0	1.7
✲	pNphαDGalNAc	26.0	1.7
○	PhαDGalNAc	32.0	1.4
●	DGalNAc	44.0	1.0
□	DGalNAcα(1→3)[LFucα(1→2)]DGalβ(1→4)DGlcNAcβ(1→6)R (MSS AR_L 0.52)	60.0	0.73
⊹	DGalβ(1→4)DGlcNAc	135	0.33
★	MeβDGalNAc*p*	165	0.27
△	MeαDGalNAc*f*	250	0.18
◒	DGalβ(1→4)DGlc (β-D-lactose)	320	0.14
✲	pNphβDGal	460	0.09
●	PhβDGal	580	0.08
▽	MeαDGal	580	0.08
◓	DGalα(1→6)DGlc (melibiose)	690	0.06
✲	pNphαDGal	1050	0.04
⊠	DGalα(1→6)DGlcβ(1⇄2)DFru (raffinose)	1750	0.03
◨	DGal	2300	0.02
▼	MeβDGal	2500	0.02
■	DFuc	16000	0.002
◆	LAra	31000	0.001
◓	DGalα(1→3)DGal	tested up to 450 nmol	14% inhibn
▲	MeβDGalNAc*f*	tested up to 2600 nmol	2% inhibn
◒	DGlcNAc	tested up to 22000 nmol	no inhibn
◧	LGal	tested up to 27000 nmol	no inhibn
◇	DAra	tested up to 31000 nmol	no inhibn
▬	LFuc	tested up to 31000 nmol	no inhibn
⊛	pNph	tested up to 5350 nmol	no inhibn

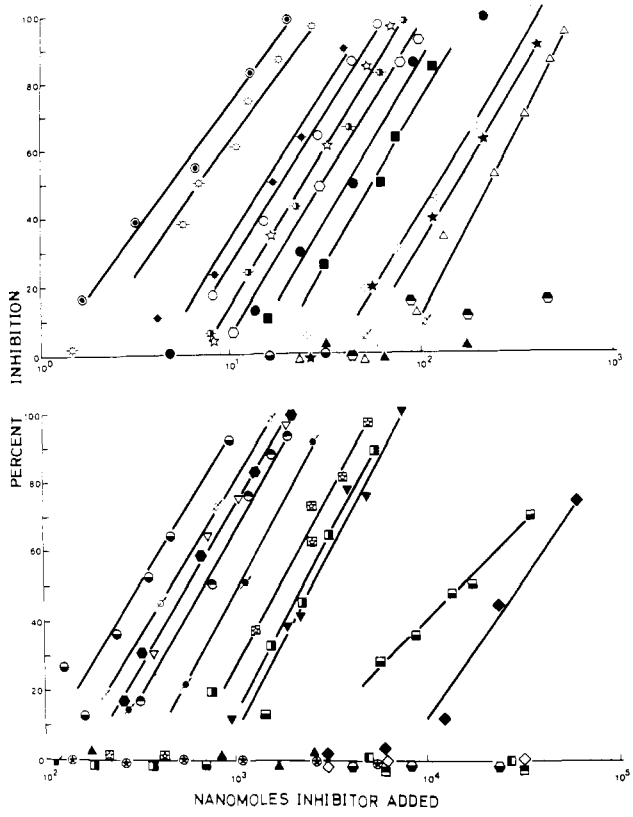

FIGURE 5: Inhibition of glycosides and mono- and oligosaccharides of precipitation of *W. floribunda* (6.9 μg of N) with Le^a substance, N-1 phenol insoluble (15 μg). Total volume was 400 μL. Symbols are shown in Table II.

II) are reacting because of the presence of many DGalβ(1→4)DGlcNAcβ(1→6) (I determinants) (Maisonrouge-McAuliffe & Kabat, 1976; Feizi et al., 1971), and the low reactivities of Beach phenol insoluble and of the two H substances JS phenol insoluble and Tighe phenol insoluble are due to the poor reactivity of DGalα(1→3)[LFucα(1→2)]DGal and DGalβ(1→4)[LFucα(1→2)]DGlcNAc, respectively. Mild acid hydrolysis of Beach phenol insoluble to expose internal DGalβ(1→4)DGlcNAc activity increased the reactivity to those of the A, Le^a, and BI substances. Asialoorosomucoid glycoproteins are also reacting because of the terminal disaccharide DGalβ(1→4)DGlcNAc. The disaccharides and glycosides provide further insight into the combining site of the *W. floribunda* hemagglutinin.

The best inhibitor was the disaccharide DGalNAcα(1→6)DGal, which was 8.8, 3.3, 3.8, and 12 times more potent than DGalNAc and the blood group A-active disaccharide DGalNAcα(1→3)DGal (R_L 1.85), trisaccharide DGalNAcα(1→3)DGalβ(1→3)DGlcNAc (A_5II), and pentasaccharide DGalNAcα(1→3)[LFucα(1→2)]DGalβ(1→4)DGlcNAcβ(1→6)R (MSS AR_L 0.52) studied, respectively. These findings show that the site is at least as large as a disaccharide and is most specific for an α(1→6) linkage of DGalNAc to DGal; nevertheless, DGalNAcα(1→3)DGal can fit quite well; further linear extension of this chain has a negligible effect, but an LFucα(1→2) on the subterminal DGal reduces its interaction with the combining site.

The *p*-nitrophenyl glycosides were all more active than the methyl glycosides, indicating an important hydrophobic interaction between the subterminal sugar moiety and the combining site as has been found for other hemagglutinins (Poretz & Goldstein, 1970; Poretz et al., 1974; Bretting & Kabat, 1976; Bretting et al., 1976; Pereira et al., 1976; Kabat, 1978). Since the pNphαDGalNAc and PhαDGalNAc had about the same potency as did pNphβDGal and PhβDGal, the NO_2 group does not contribute to binding. The inhibitory potency of pNphβDGalNAc was 1.5 times lower than that of DGalNAcα(1→6)DGal but 3.6 times higher than that of MeαDGalNAc*p*. MeβDGalNAc*p* was 6.3 times less active than MeαDGalNAc*p*. Thus, the region of the site of joining

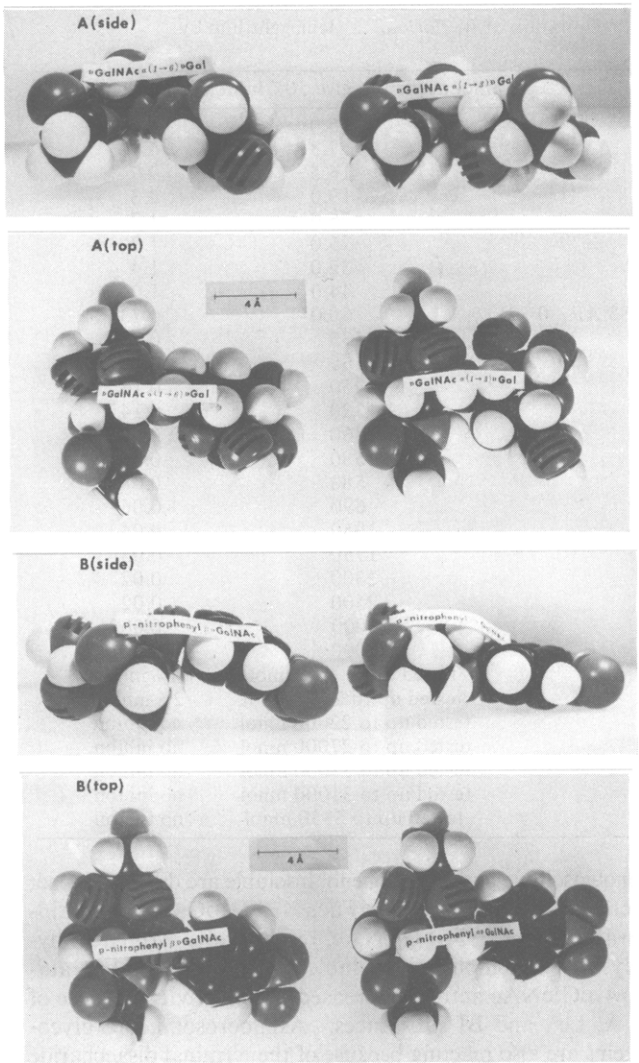

FIGURE 6: Molecular models of various sugars (side and top views). (A) DGalNAcα(1→6)DGal (left) and DGalNAcα(1→3)DGal (right). (B) pNphβDGalNAc (left) and pNphαDGalNAc (right).

of the terminal nonreducing end to the subterminal sugar is capable of accepting β-linked aglycons and sugars although its specificity seems to be predominantly α. This has been encountered previously with *Bandeiraea simplicifolia* lectin II (Wood et al., 1978). The pyranose ring is clearly required for binding because Meα- and MeβDGalNAc*p* were much more potent than Meα- and MeβDGalNAc*f*, respectively. It differs strikingly from the combining site of the rabbit hepatic lectin with which MeαDGalNAc*p* was only about twice as active as MeαDGalNAc*f* (Sarkar et al., 1979).

Molecular models were constructed in an attempt to account for the difference in reactivity of the *p*-nitrophenyl β-glycoside of DGalNAc in comparison with disaccharides with terminal nonreducing α-linked DGalNAc (Figure 6). When the models were placed in similar conformations, the difference was found to be the angle of the *p*-nitrophenyl ring relative to the DGalNAc (side view of Figure 6B). In the model of pNphβDGalNAc, the ring is at an angle to the sugar plane while the α-anomer is in the same plane and the molecule is flat. In the models (Figure 6A) of the disaccharide DGalNAcα(1→6)DGal, the two sugars are also at an angle as in pNphβDGalNAc while in the DGalNAcα(1→3)DGal the angle is less, and DGalNAcα(1→3)DGal is intermediate in activity between pNphβDGalNAc and pNphαDGalNAc. In both disaccharides there is a cluster of hydrophobic hydrogens in the region of the *p*-nitrophenyl ring. Thus, within the limitations imposed by the unavailability of other related oligosaccharides, the site would appear to be complementary to the entire DGalNAc and the region of the hydrophobic hydrogens of the subterminal sugar, the angle between the terminal and subterminal residues being most important for proper contact (top views of parts A and B of Figure 6).

The present investigation confirmed the previous studies (Toyoshima et al., 1971; Kurokawa et al., 1976; Cheung et al., 1979; Kaifu & Osawa, 1979) and provides important new information on the combining site of *W. floribunda* hemagglutinin by quantitative precipitin and precipitin inhibition assays. This interaction can prove useful in its reactivity with cell surface receptors.

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Maltosyl Isothiocyanate: An Affinity Label for the Glucose Transporter of the Human Erythrocyte Membrane. 1. Inhibition of Glucose Transport†

Richard E. Mullins‡ and Robert G. Langdon*

ABSTRACT: Maltosyl isothiocyanate (MITC) has been synthesized from maltose with an overall yield of 88%. It has been found to be a potent irreversible inhibitor of zero trans influx of glucose with human erythrocytes. Kinetic analysis of glucose transport after treatment of erythrocytes with MITC revealed that V_T was diminished while K_T was unchanged. Transportable sugars and competitive inhibitors of monosaccharide transport protected against MITC inhibition, while carbohydrates which do not interact with the transporter gave no protection. Covalent inhibitors of anion transport were without effect on glucose transport. MITC fulfilled the kinetic requirements for an affinity label of the glucose transporter of human erythrocytes [Groman, E. V., Schultz, R. M., & Engel, L. L. (1977) *Methods Enzymol. 46*, 54].

Monosaccharide transport through the plasma membranes of mammalian cells has been studied vigorously during the past 30 years, and an extensive body of kinetic evidence supports the conclusion that passive entry into cells such as erythrocytes, adipocytes, and muscle cells is a facilitated diffusion process (LeFevre, 1961; Stein, 1967; Deuticke, 1977; Wilson, 1978). However, the answers to the key questions of the identity of the membrane transporter and the mechanism by which translocation is effected still remain uncertain.

Two general approaches have been used to identify the glucose transporter of human erythrocytes and rat adipocytes. The first of these has employed transport inhibitors to label the transporter. The types of compounds used have been substrate analogue covalent affinity labels (Taverna & Langdon, 1973b; Trosper & Levy, 1977), nonspecific covalent inhibitors such as maleimides (Batt et al., 1976) and fluorodinitrobenzene (Jung & Carlson, 1975), and a bound reversible inhibitor, cytochalasin B, in conjunction with differential membrane extraction and fluorodinitrobenzene labeling (Lienhard et al., 1977; Zoccoli et al., 1978; Jung & Rampal, 1977; Pinkovsky et al., 1978). The second general approach has been to extract, purify, and isolate a specific membrane protein which, when reassociated with a lipid bilayer, conferred upon it stereospecific glucose transport (Kasahara & Hinckle, 1976, 1977; Kahlenberg, 1976; Zala & Kahlenberg, 1976; Jones & Nickson, 1978; Goldin & Rhoden, 1978; Shanahan & Czech, 1977a,b; Phutrakul & Jones, 1979).

Although some investigators have reported evidence which suggests that other proteins are involved, most evidence has supported the view that the transporter is an integral membrane protein having a M_r of either 90 000–100 000 [band 3 of the erythrocyte membrane in the terminology of Fairbanks

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received July 20, 1979.* This work was supported by Research Grant GM-19319 from the National Institutes of Health.

‡ Material for this publication is from a dissertation submitted by R.E.M. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences, University of Virginia.

0006-2960/80/0419-1199$01.00/0