Wang, L. L., & Bryan, J. (1981) *Cell (Cambridge, Mass.)* *25*, 637–649.
Weeds, A. G. (1982) *Nature (London)* *296*, 811–815.
Wegner, A. (1976) *J. Mol. Biol.* *108*, 139–150.
Wegner, A. (1982) *J. Mol. Biol.* *161*, 607–615.
Wegner, A., & Neuhaus, J.-M. (1981) *J. Mol. Biol.* *153*, 681–693.
Yin, H. L., & Stossel, T. P. (1979) *Nature (London)* *281*, 583–586.
Yin, H. L., & Stossel, T. P. (1980) *J. Biol. Chem.* *255*, 9490–9493.
Yin, H. L., Zaner, K. S., & Stossel, T. P. (1980) *J. Biol. Chem.* *255*, 9494–9500.
Yin, H. L., Albrecht, J. H., & Fattoum, A. (1981a) *J. Cell Biol.* *91*, 901–906.
Yin, H. L., Hartwig, J. H., Maruyama, K., & Stossel, T. P. (1981b) *J. Biol. Chem.* *256*, 9693–9697.
Zechel, K. (1980) *Eur. J. Biochem.* *119*, 209–213.

Immunochemical Studies on the Combining Sites of Forssman Hapten Reactive Hemagglutinins from *Dolichos biflorus*, *Helix pomatia*, and *Wistaria floribunda*†

Donald A. Baker, Shunji Sugii, Elvin A. Kabat,* R. Murray Ratcliffe,‡ Peter Hermentin,§ and Raymond U. Lemieux

ABSTRACT: The lectin of *Dolichos biflorus*, a hemagglutinin previously considered to be blood group A specific, is now found to react much more strongly with the terminal disaccharide unit [αDGalNAc(1→3)βDGalNAc] of the Forssman antigenic determinant. In contrast, the relative reactions of the lectins of *Helix pomatia* (which also agglutinates A erythrocytes) and *Wistaria floribunda* (which agglutinates A, B, and O erythrocytes) with the Forssman pentasaccharide were substantially weaker than that of *Dolichos biflorus*. The combining site of the lectin of *Helix pomatia* has a broader affinity for terminal 2-acetamido-2-deoxy-α-D-galactopyranose (αDGalNAc) residues than does that of *Dolichos biflorus*. The reactions of the lectin with terminal αDGalNAc units are strongly dependent on the nature of the aglycon and remain ill defined. The lectin may also react with appropriately presented terminal 2-acetamido-2-deoxy-β-D-glucopyranose units. The broad affinity of the lectin of *Wistaria floribunda* which reacts both with a range of blood group specific glycoproteins (A, B, H, Le^a, and Le^b) and with non blood group glycoproteins [Sugii, S., & Kabat, E. A. (1980) *Biochemistry* *19*, 1192–1199] appears best assigned to a combining site that favors pauci- or multivalent cooperative effects of clustered terminal β-D-galactopyranose units. An attempt is made to rationalize certain of the inhibition data in terms of topographical features at the surfaces of the carbohydrate structures which are considered compatible for binding within essentially hydrophobic combining sites.

Hemagglutinins (lectins) isolated from various plants and animals have combining sites specific for carbohydrates (Goldstein & Hayes, 1978; Lis & Sharon, 1977; Nicolson, 1974; Pereira & Kabat, 1979). Some lectins induce lymphocyte blast transformation in addition to agglutinating animal red blood cells and malignant cells. These reactions are quite similar to antigen–antibody reactions and are specifically reversible with low molecular weight sugars. Thus, some hemagglutinins with apparently well-defined specificities have been extensively used as tools for detection, characterization, and isolation of materials containing carbohydrate moieties on cell membranes and cell surfaces (Allen & Johnson, 1976; Hudgin et al., 1974; Kawasaki & Ashwell, 1976; Kimura et al., 1979; Nicolson, 1974; Pereira & Kabat, 1976; Poste et al., 1979). Recently, the reactions of 12 lectins with a wide variety of *N*-glycosyl proteins were examined (Debray et al., 1981), and it was concluded "that lectins considered 'identical' in terms of monosaccharide specificity, possess the ability to recognize fine differences in more complex structures". It was also concluded that the results obtained with lectins in reactions with glycoproteins or cell-surface carbohydrates have to be very carefully interpreted.

This paper is concerned with a comparison of the affinities of the *Dolichos biflorus* hemagglutinin with those of *Helix pomatia* and *Wistaria floribunda* since these hemagglutinins all appeared to bind 2-acetamido-2-deoxy-D-galactopyranose (DGalNAc)[1] residues (Cheung et al., 1979; Kurokawa et al.,

† From the Departments of Microbiology, Human Genetics and Development, and Neurology and the Cancer Center/Institute for Cancer Research (S.S. and E.A.K.), Columbia University College of Physicians and Surgeons, New York, New York 10032, and Chembiomed Ltd. (D.A.B. and R.M.R.) and the Department of Chemistry (P.H. and R.U.L.), University of Alberta, Edmonton, Alberta T6G 2G2, Canada. *Received December 14, 1982.* This paper is dedicated to Professor Edgar Lederer on the occasion of his 73rd birthday. Supported by Grant PCM 81-02321 from the National Science Foundation to E.A.K., by Cancer Center Support Grant CA 13696 to Columbia University, and by National Science and Engineering Council of Canada Grant A172 to R.U.L.

* Correspondence should be addressed to this author at the Department of Microbiology, Columbia University College of Physicians and Surgeons.

‡ Professional Research Associate, 1977–1979, University of Alberta.

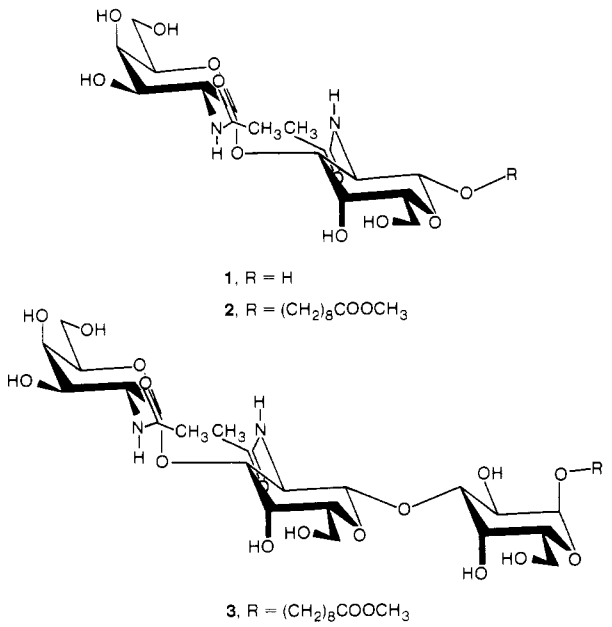
§ University of Alberta Postdoctoral Fellow, 1979–1981. Present address: Behringwerke AG, Abteilung Mikrobiologie, D-3350 Marburg 1, West Germany.

[1] Abbreviations: DGlc, D-glucopyranose; DGal, D-galactopyranose; LFuc, L-fucopyranose; DMan, D-mannopyranose; Fruc, fructofuranose; LAra, L-arabinose; DManNAc, 2-acetamido-2-deoxy-D-mannopyranose; DGalNAc, 2-acetamido-2-deoxy-D-galactopyranose; DGlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; MeαDGalNAc*p*, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside; MeαDGalNAc*f*, methyl 2-acetamido-2-deoxy-α-D-galactofuranoside; GalNAcol, *N*-acetyl-2-deoxy-D-galactitol; EDTA, ethylenediaminetetraacetic acid; MSS, 10% 2×, fraction of a human ovarian cyst blood group substance precipitated twice from 90% phenol by addition of ethanol to 10% final concentration (MS is the subject, S is serous fluid, and M is mucinous fluid); HSEA, hard sphere exoanomeric molecular orbital calculations (Thøgersen et al., 1982).

0006-2960/83/0422-2741$01.50/0

1976; Sugii & Kabat, 1980; Toyoshima et al., 1977). *Dolichos biflorus* and *Helix pomatia* hemagglutinins specifically agglutinate human type A erythrocytes (Bird, 1951, 1952; Boyd & Shapleigh, 1954). However, precipitin assays with different blood group substances, teichoic acids, and polysaccharides showed that while the *Dolichos biflorus* hemagglutinin appeared to react specifically with blood group A substances, the *Helix pomatia* hemagglutinin precipitated with a range of blood group substances as well as with teichoic acids and *Salmonella typhimurium* lipopolysaccharides (Etzler & Kabat, 1970; Hammarström & Kabat, 1969; Hammarström et al., 1972; Krupe & Pieper, 1966; Prokop et al., 1968). These findings indicated that the affinity of the *Helix pomatia* hemagglutinin is inhibited both by blood group A active oligosaccharides and by MeαDGalNAc (Etzler & Kabat, 1970; Hammarström & Kabat, 1969; Hammarström et al., 1972; Etzler et al., 1981). The agglutination of human type A red blood cells by human anti-A antibodies was shown to be inhibited by a Forssman-active glycolipid which possesses a terminal nonreducing αDGalNAc unit (Makita et al., 1966; Springer, 1971). This same glycolipid has also been observed to react with the two above-mentioned hemagglutinins (Ishiyama & Takatsu, 1970; Takatsu et al., 1971; Uhlenbruck et al., 1970).

In the course of synthetic studies related to the Forssman antigenic determinant, structure **2** [αDGalNAc(1→3)-βDGalNAcO$(CH_2)_8COOCH_3$] was synthesized (R. U. Lemieux, P. Hermentin, and R. M. Ratcliffe, unpublished experiments). Following the general procedure for the linking of such haptens to silylaminated Cristobolite (Ratcliffe et al., 1981), an immunoadsorbent was obtained which bound *Dolichos biflorus* hemagglutinin more strongly than the similarly linked blood group A active disaccharide [αDGalNAc-(1→3)βDGalO$(CH_2)_8$CONH–] and trisaccharide [αDGalNAc(1→3)[αLFuc(1→2)]βDGalO$(CH_2)_8$CONH–] (Lemieux & Ratcliffe, 1980). These findings led to the present investigation.



1, R = H

2, R = $(CH_2)_8COOCH_3$

3, R = $(CH_2)_8COOCH_3$

In the course of our synthetic studies leading to structures **1** [αDGalNAc(1→3)α,βDGalNAc], **2**, and **3** [αDGalNAc-(1→3)βDGalNAc(1→3)αDGalO$(CH_2)_8COOCH_3$], publications appeared on the synthesis of the disaccharide **1** and the Forssman antigenic determinant (Paulsen et al., 1978; Paulsen & Bunsch, 1980).

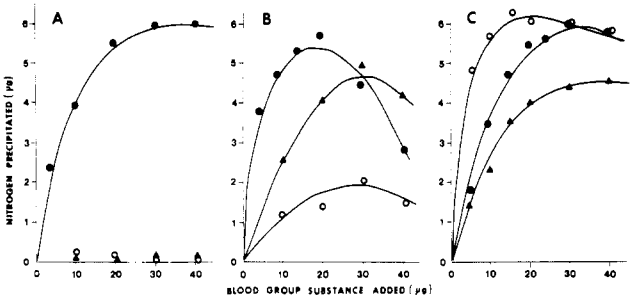


FIGURE 1: Quantitative precipitin curves of *Dolichos biflorus* (A) (6.6 μg of N), *Helix pomatia* (B) (5.75 μg of N), and *Wistaria floribunda* (C) (6.6 μg of N) hemagglutinins with native product (●), first Smith-degraded product (○), and second Smith-degraded product (▲) of blood group A substance (MSS, 10% 2×). The total volume was 250 μL for curves A and B and 200 μL for curve C.

Materials and Methods

Hemagglutinins. Dolichos biflorus, *Helix pomatia*, and *Wistaria floribunda* hemagglutinins were purified by the immunoadsorbent polyleucyl hog gastric mucin A + H (Kaplan & Kabat, 1966) according to the methods described previously (Cheung et al., 1979; Etzler & Kabat, 1970; Hammarström & Kabat, 1969; Sugii & Kabat, 1980).

Blood Group Substances and Glycoproteins. Blood group A substances and Smith-degraded products of blood group A substances were prepared as previously described (Beiser & Kabat, 1952; Feizi et al., 1971; Lloyd & Kabat, 1968; Kabat, 1956a). An additional set of Smith-degraded preparations was made by Drs. A. M. Wu and M. E. A. Pereira. The active and inactive antifreeze glycoproteins were obtained from Dr. R. E. Feeney (DeVries et al., 1970; Shier et al., 1975). Desialyzed ovine and porcine salivary glycoproteins which had been treated with 0.01 M HCl at 80 °C for 90 min (De Salegui & Pouska, 1969) were also kindly provided by Dr. A. M. Wu. Fetuin was purchased from GIBCO (Grand Island, NY) and desialyzed by exposing it to 0.01 M HCl at 80 °C for 90 min.

Sugar Inhibitors. Various monosaccharides were purchased from Eastman Organic Chemical Co. (Rochester, NY), Sigma Chemical Co. (St. Louis, MO), and Nutritional Biochemicals (Cleveland, OH). MeαDGalNAc was prepared in this laboratory (Sarkar & Kabat, 1979). The disaccharide αDGalNAc(1→6)DGal was from Dr. I. J. Goldstein, the disaccharides βDGal(1→3)DGlcNAc and βDGal(1→6)DGlcNAc were from F. Zilliken (Kabat, 1962), and the disaccharide βDGal(1→3)DGalNAc was from W. Gielen (Klenk et al., 1962). αDGalNAc(1→3)DGalNAc, αDGalNAc(1→3)-βDGalNAcO$(CH_2)_8COOCH_3$, and αDGalNAc(1→3)-βDGalNAc(1→3)αDGalO$(CH_2)_8COOCH_3$ were synthesized (R. U. Lemieux, P. Hermentin, and R. M. Ratcliffe, unpublished experiments). The blood group P active tetrasaccharide (globoside) βDGalNAc(1→3)αDGal(1→4)βDGal(1→4)DGlc and the Forssman-active pentasaccharide αDGalNAc(1→3)-βDGalNAc(1→3)αDGal(1→4)βDGal(1→4)DGlc were kindly provided by Drs. D. M. Marcus and S. Kundu (Marcus et al., 1978; Naiki et al., 1975).

Immunological Methods. Quantitative precipitin and precipitin inhibition assays were performed at 2 °C by a microprecipitin technique (Kabat, 1961). The total nitrogen in the washed precipitates was determined by the ninhydrin method (Schiffman et al., 1964).

Results

Quantitative Precipitin Assays. Figure 1 shows the precipitin reactions of the three hemagglutinins with native and with the first- and second-stage Smith-degraded products of

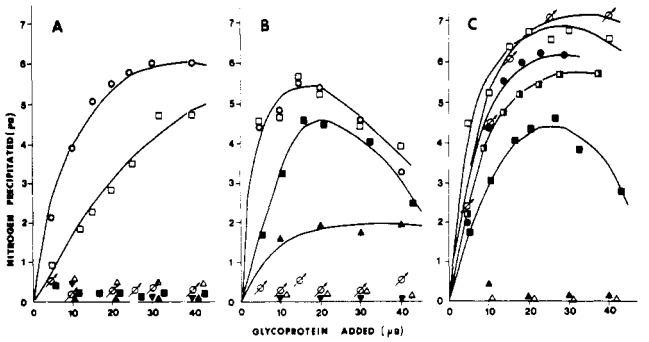


FIGURE 2: Quantitative precipitin curves of *Dolichos biflorus* (A), *Helix pomatia* (B), and *Wistaria floribunda* (C) hemagglutinins with blood group A substances (MSM, 10%, precipitate) (○) and cyst 9 (●), N-1 phenol insoluble blood group Le^a substance (right solid square), desialyzed ovine salivary glycoprotein (■), desialyzed porcine salivary glycoprotein (□), active antifreeze glycoprotein (▲), inactive antifreeze glycoprotein (▼), native fetuin (△), and desialyzed fetuin (ϕ). The amounts and total volumes were as described in Figure 1.

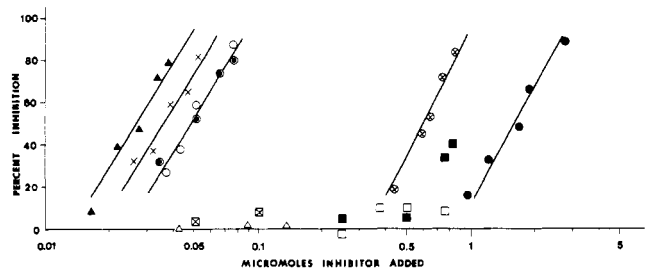


FIGURE 3: Inhibition by mono- and oligosaccharides of precipitation of *Dolichos biflorus* hemagglutinin (5.5 μg of N) by blood group A substance (MSM, 10%, precipitate) (20 μg). Inhibitors used were *p*-NO_2Ph-αDGalNAc (■), *p*-NO_2Ph-βDGalNAc (□), αDGalNAc-(1→6)DGal (crossed square), DGalNAc (●), MeαDGalNAc (⊗), αDGalNAc(1→3)DGalNAc (○), globoside (△), αDGalNAc(1→3)-βDGalNAcO$(CH_2)_8COOCH_3$ (◎), αDGalNAc(1→3)βDGalNAc-(1→3)αDGalO$(CH_2)_8COOCH_3$ (×), and the Forssman-active pentasaccharide (▲). Total volume was 250 μL at 2 °C.

blood group A substance (MSS, 10% 2×). The native substance was a good precipitinogen for *Dolichos biflorus* hemagglutinin but lost its precipitating ability after the first and remained inactive after the second Smith degradation (Figure 1A). On the other hand, *Helix pomatia* and *Wistaria floribunda* hemagglutinins were also precipitated with the blood group A substance subjected to the first and the second Smith degradations (Figure 1B,C). The best precipitinogen for the *Helix pomatia* hemagglutinin was the native blood group A substance, but the best precipitinogen for *Wistaria floribunda* hemagglutinin was the first Smith-degraded product. The second-stage Smith-degraded product was more active with *Helix pomatia* hemagglutinin than was the first-stage product.

Figure 2 shows the quantitative precipitin curves of the hemagglutinins with blood group A substance and glycoproteins. *Dolichos biflorus* hemagglutinin precipitated with blood group A substance (MSM, 10%, precipitate) and desialyzed porcine salivary glycoprotein but reacted very poorly or not at all other glycoproteins (Figure 2A). With *Helix pomatia* hemagglutinin, blood group A substance (MSM, 10%, precipitate) and desialyzed ovine and porcine salivary glycoproteins were good precipitinogens. Active antifreeze glycoproteins reacted poorly while inactive antifreeze glycoprotein (Feeney & Yeh, 1978) and fetuin before and after desialization did not react (Figure 2B). *Wistaria floribunda* hemagglutinin reacted well with blood group A substance (cyst 9), desialyzed fetuin, and desialyzed ovine and porcine salivary glycoproteins. It reacted weakly with active antifreeze glycoprotein but did not precipitate with native fetuin and inactive antifreeze

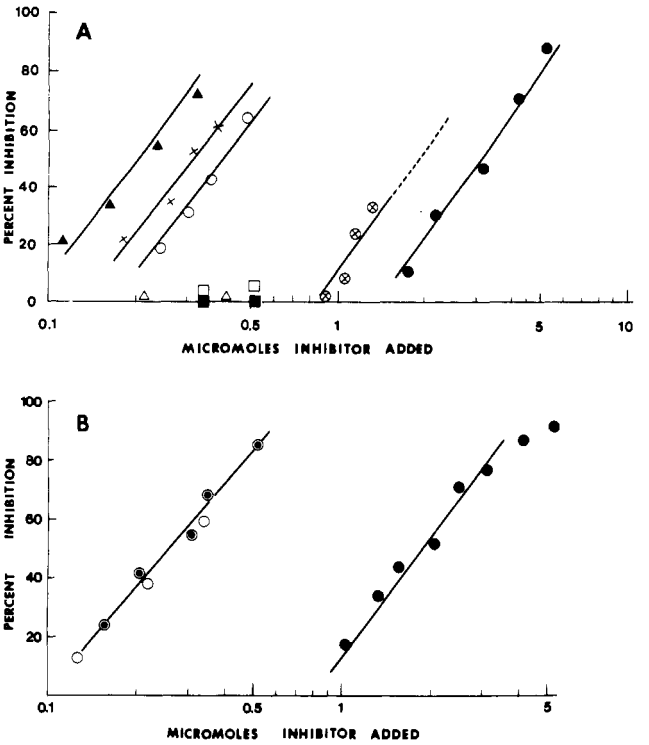


FIGURE 4: Inhibition of different mono- and oligosaccharides of precipitation of *Helix pomatia* hemagglutinin (5.75 μg of N) by blood group A substance (MSM, 10%, precipitate) (15 μg). Symbols for inhibitors are the same as those shown in Figure 3. The data plotted in (B) were obtained in a different experiment, and the values are presented in brackets in Table I.

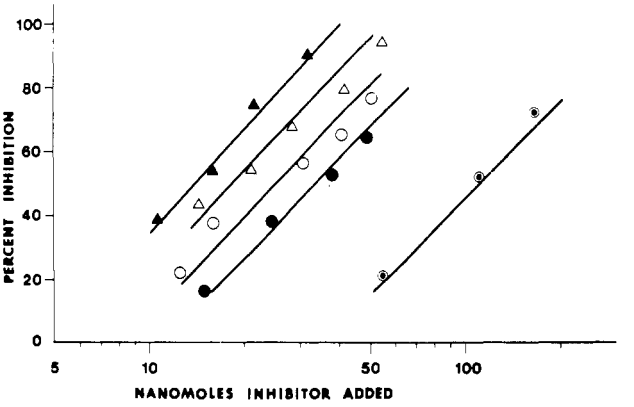


FIGURE 5: Inhibition by different mono- and oligosaccharides of precipitation of *Wistaria floribunda* hemagglutinin (6.6 μg of N) by blood group Le^a substance (N-1 phenol insoluble) (15 μg). Symbols for inhibitors are the same as those shown in Figure 3 except for βDGal(1→3)DGlcNAc (◎).

glycoprotein (Figure 2C) (Sugii & Kabat, 1980).

Precipitin Inhibition Assays. The abilities of various sugars to inhibit the precipitation reaction are shown in Figures 3–5, and their relative inhibitory potencies are summarized in Table I for comparison with previous reports (Etzler & Kabat, 1970; Hammarström & Kabat, 1969; Sugii & Kabat, 1980). As shown in Figure 3 and Table I, the Forssman-active pentasaccharide [αDGalNAc(1→3)βDGalNAc(1→3)αDGal(1→-4)βDGal(1→4)DGlc] was the best inhibitor for *Dolichos biflorus* hemagglutinin and was more active than blood group A active reduced tetrasaccharide (R_L = 0.52)[2] [αDGalNAc-(1→3)[αLFuc(1→2)]βDGal(1→4)βDGlcNAc(1→3)-3-hexene

[2] $R_L = R_{lactose}$ on 6:4:3 BuOH:pyridine:water.

Table I: Activity and $\Delta\Delta G^\circ$ Relative to α,βDGalNAc of Various Sugars in Inhibiting Precipitation of *Dolichos biflorus* and *Helix pomatia* Lectins by Blood Group A (MSM, 10%, Precipitate) and of *Wistaria floribunda* Lectin by Le[a] Substance (N-1 Phenol Insoluble)

	relative potency to α,βDGalNAc			$\Delta\Delta G^\circ$		
	Dolichos biflorus	*Helix pomatia*	*Wistaria floribunda*	*Dolichos biflorus*	*Helix pomatia*	*Wistaria floribunda*
αDGalNAc(1→3)βDGalNAc(1→3)αDGal(1→4)βDGal(1→4)-α,βDGlc, Forssman-specific pentasaccharide	62.5	15.12	2.61	−2.3	−1.5	−0.5
αDGalNAc(1→3)βDGalNAc(1→3)αDGalO$(CH_2)_8COOCH_3$	48.6	9.69		−2.1	−1.2	
αDGalNAc(1→3)βDGalNAcO$(CH_2)_8COOCH_3$	36.4	[7.3]		−2.0	−1.1	
αDGalNAc(1→3)α,βDGalNAc	36.4	7.56 [7.3]	1.30	−2.0	−1.1	−0.2
αDGalNAc(1→6)α,βDGal	10% inhibn at 0.1 μmol		(8.8)			−1.2
αDGalNAc(1→3)[αLFuc(1→2)]βDGal(1→4)βDGlcNAc-(1→6)R (R_L = 0.52), A-specific oligosaccharide	(4.51)[a]	36% inhibn at 1.25 μmol	(0.73)	−0.8	−0.1[b]	+0.2
αDGalNAc(1→3)βDGal(1→3)βDGlcNAc (A_5II)	(2.69)	(1.91)	(2.32)	−0.5	−0.4	−0.5
αDGalNAc(1→3)α,βDGal (R_L = 1.85)	(2.69)	2% inhibn at 0.14 μmol	(2.67)	−0.5		−0.5
MeαDGalNAc*p*	(3.04) 2.84	(1.98) 1.63	(1.69)	−0.6	−0.4	−0.3
α,βDGalNAc[c]	(1.0) 1.0	(1.0) 1.0 [1.0]	(1.0) 1.0	0	0	0
EtβDGlcNAc		(0.165)				
EtβDGalNAc	(0.42)	6% inhibn at 4.2 μmol		+0.5	+1.0	+0.7[d]
p-NO_2Ph-αDGalNAc*p*	40% inhibn at 0.86 μmol	5.2% inhibn at 0.518 μmol	(1.69)			−0.3
p-NO_2Ph-βDGalNAc*p*	11% inhibn at 0.86 μmol	7.5% inhibn at 0.518 μmol	(6.03)			−1.0
βDGalNAc(1→3)αDGal(1→4)βDGal(1→4)DGlc, globoside	4% inhibn at 0.125 μmol	no inhibn at 0.42 μmol	1.79			−0.3

[a] Values in parentheses: *Dolichos biflorus*, from Etzler & Kabat (1970); *Helix pomatia*, from Hammarström & Kabat (1969); *Wistaria floribunda*, from Sugii & Kabat (1980). [b] By extrapolation. [c] α,βDGalNAc giving 50% inhibition: *Dolichos biflorus*, 1.75 nmol; *Helix pomatia*, 3.10 and 1.90 μmol; *Wistaria floribunda*, 34.0 nmol. Conditions used given in legends to Figures 3-5. [d] MeβDGalNAc.

tetrols] and DGalNAc, respectively. The disaccharides αDGalNAc(1→3)DGalNAc and αDGalNAc(1→3)-βDGalNAcO$(CH_2)_8COOCH_3$ were slightly less active than the Forssman pentasaccharide but much more potent than the blood group A active disaccharide (R_L = 1.85) [αDGalNAc(1→3)DGal] and DGalNAc. The Forssman-active trisaccharide [αDGalNAc(1→3)βDGalNAc(1→3)αDGalO-$(CH_2)_8COOCH_3$] was slightly better than both αDGalNAc-(1→3)DGalNAc and αDGalNAc(1→3)βDGalNAcO-$(CH_2)_8COOCH_3$ but slightly less active than the Forssman-active pentasaccharide. *p*-NO_2Ph-αDGalNAc, *p*-NO_2Ph-βDGalNAc, αDGalNAc(1→6)DGal, and globoside [βDGalNAc(1→3)αDGal(1→4)βDGal(1→4)DGlc] were much less active.

The Forssman-active pentasaccharide was also the best inhibitor of *Helix pomatia* hemagglutinin (Table I) and substantially more potent than both DGalNAc and MeαDGalNAc. With the three different Forssman-active oligosaccharides, findings were similar to those obtained with *Dolichos biflorus* hemagglutinin (compare Figures 3 and 4). The Forssman-active trisaccharide was a better inhibitor than the disaccharide but was weaker than the pentasaccharide. Globoside was a very poor inhibitor relative to αDGalNAc-(1→3)DGalNAc and gave no inhibition up to 0.42 μmol. *p*-NO_2Ph-αDGalNAc was not a strong inhibitor, and, surprisingly, *p*-NO_2Ph-βDGalNAc was somewhat more potent. Also, EtβDGlcNAc was a much stronger inhibitor than EtβDGalNAc.

With *Wistaria floribunda* hemagglutinin, the Forssman-active pentasaccharide was only slightly more potent than DGalNAc but less active than the best inhibitor, αGalNAc-(1→6)DGal (Figure 5 and Table I). However, *p*-NO_2Ph-βDGalNAc was nearly as potent. As summarized in Table I, globoside, which has a terminal βDGalNAc residue, and αDGalNAc(1→3)DGalNAc (**1**) had very nearly the same potency, and both were slightly more potent than DGalNAc. The blood group A active disaccharide (R_L = 1.85) [αDGalNAc(1→3)DGal] was twice as active as αDGalNAc-(1→3)DGalNAc (**1**). Of the disaccharides with terminal DGal, βDGal(1→3)DGlcNAc was as potent as βDGal(1→4)-DGlcNAc, but βDGal(1→3)DGlcNAc and βDGal(1→4)-DGlcNAc were about equally potent and one-third as active as DGalNAc; βDGal(1→6)DGlcNAc and βDGal(1→3)-DGalNAc were substantially weaker.

Effects of EDTA and Metal Ions on the Precipitation and Hemagglutination. As shown in Figure 6, the precipitating ability of *Dolichos biflorus* hemagglutinin was entirely abolished by addition of EDTA at concentrations above 0.1 mM. Its precipitating ability was restored by addition of Ca^{2+} at more than 0.5 mM after previous exposure to 3.2 mM EDTA at room temperature for 1 h. Its precipitating and hemagglutinating abilities were not affected by metal ions such as Ca^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, and Ni^{2+} at less than 8.8 mM. Fe^{2+} at 1 mM significantly enhanced hemagglutinating activity but inhibited precipitating activity, indicating that Fe^{2+} induced nonspecific agglutination.

The precipitating abilities of *Dolichos biflorus*, *Helix pomatia*, and *Wistaria floribunda* hemagglutinins were not abolished by EDTA up to 8.8 mM, the highest concentration tested (Figure 6).

Discussion

The specificity of an agglutinin is considered to be derived from that property of the protein which is responsible for its ability to bind strongly with the particular structure which it recognizes. With lectins and with myeloma proteins, unlike antibodies, the substances for which a given protein shows specificity are generally discovered by chance, and there is no assurance that one will not turn up a more active compound. Indeed, the present paper is the result of just such an occurrence. An important feature of these binding reactions is that having identified one such reactive compound, one can study the pattern of reactivity with structurally related substances and map the specificity of its combining site.

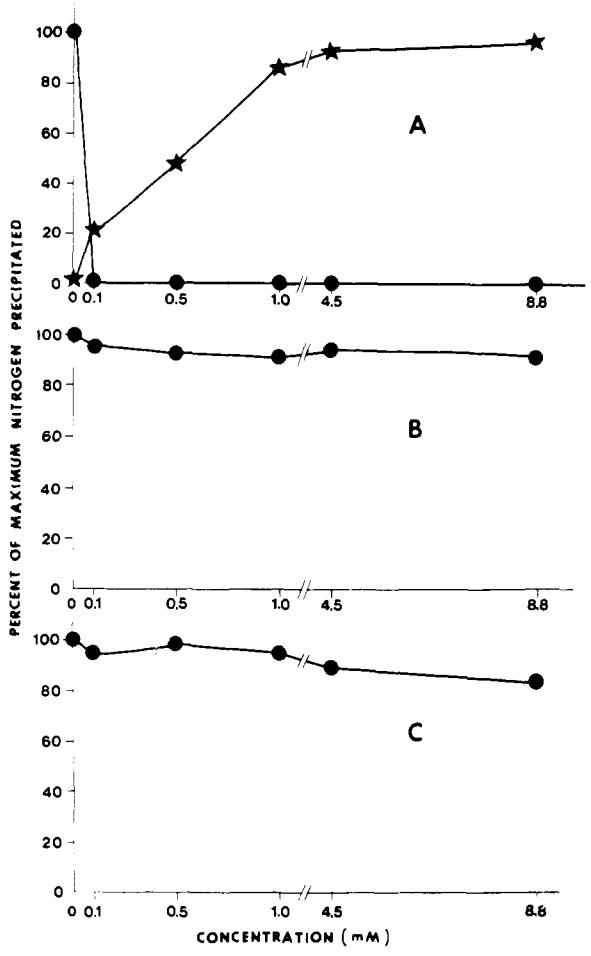


FIGURE 6: Effect of the concentration of EDTA (●) and Ca^{2+} (solid star) on the precipitin reaction between 5.5 μg of N of *Dolichos biflorus* hemagglutinin and 20 μg of blood group A substance (MSM, 10%, precipitate) (A), between 5.75 μg of N of *Helix pomatia* hemagglutinin and 15 μg of blood group A substance (MSM, 10%, precipitate) (B), and between 6.6 μg of N of *Wistaria floribunda* hemagglutinin and 15 μg of blood group Le^a substance (N-1 phenol insoluble) (C).

Specificity differences among various lectins and among monoclonal antibodies essentially depend upon the overall findings from such studies. In recent years, definition of such patterns has been helped greatly by the ability to synthesize oligosaccharides to test hypotheses as to the nature of the forces involved (Kabat et al., 1981).

It is now recognized that a broad specificity arises because the binding reaction involves an adaptation by a conformationally labile portion of the protein to the formation of a specific complex and that it is this conformational adaptation (induced fit) which enables the accommodation of other related structures in thermodynamically favorable complexing reactions. This behavior of proteins has been termed the conformative response [Citri, 1973; Lerner, 1982; see Karush (1950)]. The recent studies (Lerner, 1982) with 27 monoclonal antibodies to an influenza virus HA1 peptide with 36 amino acids showed 14 of the monoclonals to react with intact HA1 virus; this was considered a surprisingly high number if the antibody combining sites were restricted to one or to a few conformations. The broader specificities of lectins and their many similarities to antibodies suggest that their combining sites might also assume different conformations in reacting with one or another carbohydrate determinant. Whether lectin or antibody sites of narrow specificity are more limited in induced fit is unknown but is worthy of study.

Normally, the various structures which are detectably accepted by a given protein have common structural features; for example, the hemagglutinins of *Dolichos biflorus*, *Helix pomatia*, and *Wistaria floribunda* all appear to have an affinity for DGalNAc residues (see Table I). However, it is to be recognized that complex formation involves interactions between surfaces and not sugar units per se. For example, the lectin 1 of *Ulex europaeus* binds αLFuc(1→2)βDGlc(1→4)-βDGlcNAc very nearly as strongly as it does αLFuc(1→2)-βDGal(1→4)βDGlcNAc (Lemieux et al., 1979). Furthermore, this lectin strongly binds αLFuc(1→2)βDGal(1→4)βDGlc (Pereira et al., 1979). What these three structures have in common is not sugar units, but, instead, they present the same topographical feature to the lectin (Lemieux, 1982; Hindsgaul et al., 1982).

Since the binding of a substrate by a protein involves the essential freezing of a portion of the protein into a specific conformation which has accepted the substrate to form a complex, it can be argued that the inhibition of the reaction of a protein with a given substrate by another structure need not necessarily involve complex formation at the same region of the protein (Schubert et al., 1970).

However, when the various structures have similar structural features and since, in the case of antibodies, the site reactive with the spectrum of related compounds studied can be localized to a particular domain of the molecule, it is generally accepted that all are reacting at the same region of the molecule defined as the active site.

It is to be expected that the conformative response of the site which takes place in the binding of one inhibitor may not be precisely the same as that when another inhibitor is bound at the same site.

That a given lectin or antibody can display a wide range of affinities for carbohydrate structures that have only partial topographical features in common is not necessarily surprising since it has become clearly apparent that much of the driving force for the binding *may* be hydrophobic in nature (Kabat et al., 1981; Lemieux, 1982). Hydrophobic bonding is not atom–atom specific but, instead, is related to the complementarity of shapes and their hydrophobicities. This fact was well displayed by the observation (Hindsgaul et al., 1982) that the lectin 1 of *Ulex europaeus* binds both αLFuc(1→2)-βDGal(1→4)βDGlcNAc (H type 2) and αLFuc(1→2)-βDGal(1→3)βDGlcNH$_2$ which, although structurally very different, do offer similar topographies for the binding by the lectin. Acetylation of the amine to form the H type 1 determinant drastically changes the relevant topography, and, indeed, the product is not bound by the lectin. It is noteworthy in these regards that the 6-deoxy derivative of the H type 2 determinant was a much superior inhibitor (Hindsgaul et al., 1982).

In an attempt to evaluate more precisely the role of hydrophobic interactions, we have made the assumption that the combining sites of the hemagglutinins of *Dolichos biflorus*, *Helix pomatia*, and *Wistaria floribunda* preferentially interact via topographical features compatible with basic hydrophobic regions within their combining sites. Complex formation occurs when sufficient complementarity is found in the absence of substantial conflict with polar hydrophilic groups. The engagement of hydroxyl groups of the oligosaccharide in the formation of the complex is not precluded and may well occur. However, in view of the above-mentioned experimental evidence, our working hypothesis is that such engagements may not dominate the driving force for the complex formation. This view is to be contrasted to that of Ochoa (1981), who has

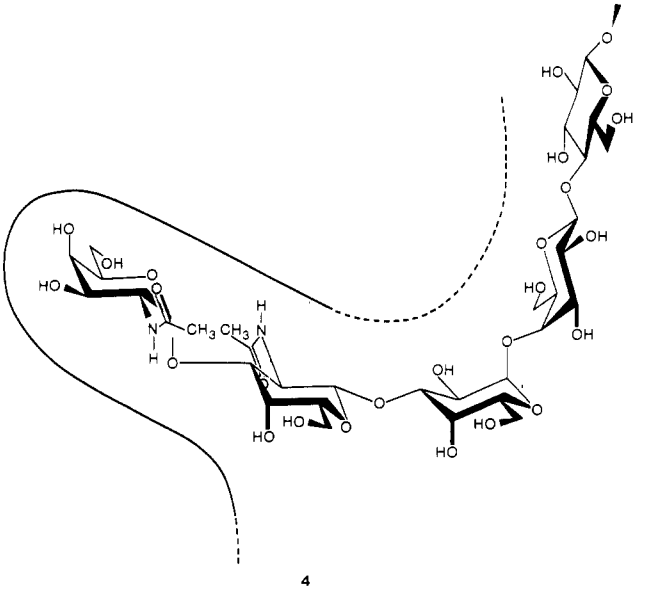
proposed that hydrogen-bonding and charge-transfer interactions are the main sources of complex stability in the association between lectins and carbohydrates. Certainly, we expect that a proper disposition of hydrophilic groups within the complex is necessary for complex stability, and therefore, for example, hydrogen bonding with the complex would contribute importantly to the specificity of the reaction. It is in this context that an attempt is made to rationalize the inhibition data presented in Table I. We consider it useful to assess selected differences in the potencies of inhibitors in terms of differences in binding free energies ($\Delta\Delta G°$ values; Kabat, 1956b, 1961; Pressman & Grossberg, 1968) as presented in Table I since to do so provides an appreciation of the structure–activity relationships in terms of differences in the driving forces for the reaction which are being compared. It should be emphasized that no clear dividing line can be drawn as to whether a given slight differences in $\Delta\Delta G°$ is specific or nonspecific. Such interferences can only be made when the sequence of the protein, the site structure, and the way the ligand fits into the site are established by X-ray crystallography.

Table I lists selected $\Delta\Delta G°$ values for the binding reactions studied in this and previous papers (Etzler & Kabat, 1970; Hammarström & Kabat, 1969; Sugii & Kabat, 1980). In all cases, the anomeric mixture of DGalNAc was used as the reference inhibitor. For the hemagglutinins of *Dolichos biflorus* and *Helix pomatia*, the reference precipitinogen was a blood group A substance (MSM, 10%, precipitate). This substance was also precipitated by the nonblood group A, B, and O specific *Wistaria floribunda*, but in this case, in view of the results in Figure 1, the inhibition reactions were examined by using a blood group Le^a substance (N-1 phenol insoluble). It may be noted that with the lectin of *Helix pomatia* and with the less strongly bound guaran, the precipitation inhibition assays (Hammarström et al., 1977) had much higher sensitivity. Earlier studies were extended, and these suggested that DGal as well as DGlcNAc (Hammarström & Kabat, 1969) were bound.

As reported previously (Kocourek et al., 1979), the hemagglutinating activity of *Dolichos biflorus* hemagglutinin was abolished by EDTA, suggesting that *Dolichos biflorus* hemagglutinin is metal dependent. Recently, Borrebaeck and coworkers (Borrebaeck et al., 1981) have shown this lectin to require a single calcium cation. The present study confirms these findings by hemagglutination and precipitin assays. *Helix pomatia* and *Wistaria floribunda* hemagglutinins are probably metal independent since their precipitating abilities were not abolished by EDTA. The ability to study the metal ion dependency by quantitative precipitin assays makes for more precise probing of the lectin site than is possible by hemagglutination studies.

Lectin of Dolichos biflorus. The specificity of the *Dolichos biflorus* hemagglutinin was first appreciated (Etzler & Kabat, 1979) when the precipitation of a blood group A substance was found to be inhibited by the A-specific oligosaccharide (R_L = 0.52). However, since the $\Delta\Delta G°$ value for this oligosaccharide and MeαDGalNAc is only 0.2 kcal/mol, it seemed possible that this oligosaccharide might not be the best inhibitor although its A specificity and the specificity of the lectin for A_1 erythrocytes were consistent. The Forssman determinant which has always been associated with blood group A (Kabat, 1961) specificity (Siddiqui & Hakomori, 1971), in view of its terminal αDGalNAc, was evidently a candidate for binding with *Dolichos biflorus*. Indeed, the synthetic structures αDGalNAc(1→3)α,βDGalNAc (**1**), αDGalNAc-(1→3)βDGalNAcO$(CH_2)_8COOCH_3$ (**2**), and αDGalNAc-(1→3)βDGalNAc(1→3)αDGalO$(CH_2)_8COOCH_3$ (**3**) were found to bind this hemagglutinin much more strongly (>1.2 kcal/mol; see Table I). As expected, the Forssman-specific pentasaccharide was also found to strongly inhibit the reaction between *Dolichos biflorus* and the blood group A substance. The very slightly stronger inhibition provided by the trisaccharide than the disaccharide suggests that the αDGal unit of the Forssman determinant is not appreciably involved in the binding reaction. This also is likely the case for the αDGal(1→)βDGal(1→4)α,βDGlc portion of the Forssman pentasaccharide. The 0.3 kcal/mol greater difference in binding free energy exhibited by this pentasaccharide as compared to the simple αDGalNAc(1→3)α,βDGalNAc disaccharide could well result from nonspecific hydrophobic interactions about the fringe of the combining site, although these interactions might equally well be specific. Since the Forssman pentasaccharide is more potent than its terminal disaccharide with all three lectins, it is also possible that part or all of the third sugar plays some role in the site of one or more of the lectins. The remaining two sugars of the pentasaccharide might well contribute some nonspecific binding since it seems unlikely that each of the three lectins possesses a combining site which accepts all five sugar units of the pentasaccharide in a specific manner.

As mentioned above, evidence is accumulating that the binding of oligosaccharides to antibodies and lectins appears to be mainly hydrophobic in nature. Also, it has become apparent that the hydrophobicity of the combining sites may extend to the surface about the combining site (Lemieux et al., 1978; Debray et al., 1981). It is of interest in this regard to note that HSEA calculations (Thøgersen et al., 1982) of the conformation which is expected to be preferred for the Forssman determinant set the structure in an L-shaped conformation as is roughly depicted in the conformational formula **4**. Thus, it can be expected that the lactose portion of the



4

Forssman pentasaccharide is in close proximity to the acetamido group of the βDGalNAc unit and therefore well oriented for superficial nonspecific engagements with the surface of the hemagglutinin near the combining site.

In a preliminary communication, Lemieux & Hermentin (1980) discussed in detail the high-resolution proton magnetic resonance spectrum for αDGalNAc(1→3)-βDGalNAcO$CH_2C_6H_5$ which showed that the relative nuclear

Overhauser enhancements found for H-3 and H-4 of the βDGalNAc unit on saturation of H-1 of the αDGalNAc residue were in excellent accord with those calculated (HSEA calculations; Thøgersen et al., 1982) for the disaccharide unit in the conformation $\phi^H = 50°$, $\psi^H = 35°$ (Lemieux & Koto, 1974). These data place the terminal Forssman disaccharide unit in a conformation which has the two acetamido groups on opposite sides of the structure as is depicted in **1**.

As seen in Table I, the inhibition provided by αDGalNAc(1→3)α,βDGal (R_L = 1.85) was somewhat inferior to those provided by either the A-specific oligosaccharide (R_L = 0.52) or MeαDGalNAc and much inferior (1.5 kcal/mol, Table I) to that by αDGalNAc(1→3)α,βDGalNAc. This result suggests an inability of the combining site to accommodate the strongly hydrophilic hydroxyl group at C-2 of the βDGal unit. The fact that a change in the configuration of C-4 of **1**, to provide αDGalNAc(1→3)α,βDGlcNAc, results in a basically inactive compound is also noteworthy since this latter structure projects the highly hydrophilic 4-OH toward the αDGalNAc residue. Thus, the hydration of this hydroxyl group may strongly prohibit the accommodation of this group into a hydrophobic combining site. These results do not necessarily define the complete size and specificity of the combining site of *Dolichos biflorus*, but highly favorable involvement of the two terminal DGalNAc residues of the Forssman determinant appears established. On this basis, the compounds are accepted into the combining site somewhat as depicted in structure **4** where the solid line is meant to indicate that portion which offers maximum stability to the complex. We plan to synthesize appropriate variations of this structure to serve as probes for the further investigation of the nature of the combining site.

Lectin of Helix pomatia. The results obtained with this lectin (Figures 1B and 2B and Table I) do not allow a conclusion as the affinity of this lectin beyond that it reacts with *appropriately* presented terminal αDGalNAc residues including the A determinant [αDGalNAc(1→3)[αLFuc(1→2)]βDGal], the Forssman terminal unit [αDGalNAc(1→3)-βDGalNAc(1→3)αDGal], and MeαDGalNAc. However, although it displays a good affinity for the A_5II [αDGalNAc(1→3)βDGal(1→3)α,βDGlcNAc] trisaccharide, the related αDGalNAc(1→3)α,βDGal disaccharide proved to be a relatively very weak inhibitor. This result may arise from the presence of DGlcNAc in the trisaccharide since MeαDGlcNAc was only 4.7 times less potent as an inhibitor than MeαDGalNAc, and this difference in activity was also observed for the simple sugars, α,βDGalNAc and α,-βDGlcNAc. It would be extremely surprising (Lemieux et al., 1978) if the same region of a combining site would react with the C-4 to C-6 portions of both DGalNAc and DGlcNAc as terminal units. These two sugar units, of course, have common structural features about their C-1 to C-3 portions.

Structures which terminate with βDGal units such as the βDGal(1→3)α,βDGlcNAc, βDGal(1→3)α,βDGalNAc, and βDGal(1→4)α,βDGlcNAc disaccharides proved to be inactive (Table I). With reference to Figure 2B, the lectin was unreactive with desialyzed fetuin in which three βDGal(1→3)-DGalNAc and nine βDGal(1→4)DGlcNAc residues per mole are exposed as terminal nonreducing ends. This result may be ascribable to the low total carbohydrate content of this glycoprotein (Graham, 1972; Nilsson et al., 1979; Spiro & Bohyroo, 1974) but more likely to the absence of terminal DGlcNAc or DGalNAc units.

Since the A_5II trisaccharide [αDGalNAc(1→3)βDGal(1→3)α,βDGlcNAc] proved quite active, it may have been expected that the terminal disaccharide of A_5II, namely, αDGalNAc-(1→3)α,βDGal, would display an activity at least superior to that of the simple MeαDGalNAc. However, this was not the case. As seen in Table I, this disaccharide is a relatively very poor inhibitor and less active than MeαDGalNAc, EtβDGlcNAc, and MeαDGlcNAc. Even the simple sugars α,βDGalNAc and α,βGlcNAc are superior inhibitors. It is apparent, therefore, that the combining site of this lectin cannot accommodate a free hydroxyl group at C-1 of the βDGal unit of the αDGalNAc(1→3)α,βDGal disaccharide. On the other hand, the structurally related terminal disaccharide of the Forssman determinant [αDGalNAc(1→3)βDGalNAc], which has a hydrophobic acetamido group at C-2, proved to be an excellent inhibitor. It is remarkable in these regards that the αDGlc-NAc(1→3)α,βDGalNAc disaccharide is a much weaker inhibitor than αDGalNAc(1→3)α,βDGalNAc and even less potent than either MeαDGalNAc, MeαDGlcNAc, and EtβGlcNAc. It seems likely, therefore, that these latter simple compounds are acting at different regions of a rather large combining site.

The choice of potential inhibitors listed in Table I was influenced by the early indications that this lectin has an affinity for a structure which terminates with an αDGal unit. Consequently, oligosaccharides that terminate with βDGlcNAc and βDGalNAc units were not examined. The results presented in Figure 1B suggest that such compounds should be tested prior to any conclusion being drawn as to the innate specificity of the lectin of *Helix pomatia*. It is seen that this lectin caused a weak precipitation of the first product of Smith degradation of the blood group A substance but a much stronger precipitation of the product of a second Smith degradation. The first product is expected to be rich in terminal βDGal units. Judging from the results listed in Table I and mentioned above for the disaccharides that terminate with βDGal units, it does not appear likely that the precipitation occurred because of the terminal βDGal units in the first product of Smith degradation. On the other hand, the possibility exists that terminal αDGalNAc residues were formed from either βDGal(1→3)αDGalNAc (T determinants; Kim & Uhlenbruck, 1966) or H type 3 determinants (Donald, 1981) which may have been present in the blood group A substance. In support of this possibility is the fact that over 75% of the carbohydrate side chains in desialyzed ovine salivary glycoproteins are αDGalNAc linked to serine or threonine of the protein core (Herp et al., 1979; Tettamanti & Pigman, 1968), and this protein precipitated 100% of the added lectin of *Helix pomatia*.

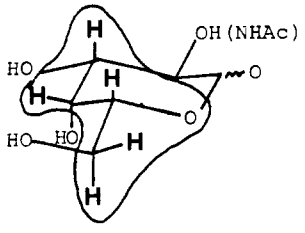
Unless the blood group A substance contained A type 3 (Donald, 1981) terminal units, the strong precipitation exhibited by the product of the second Smith degradation would be expected to be related to the terminal βDGlcNAc units which are undoubtedly abundant in this product. It may be relevant in this regard that MeβDGlcNAc caused a weak but discernible inhibition which was substantially enhanced by changing the methyl group to the more hydrophobic ethyl group to provide EtβDGlcNAc. For these reasons, it is considered that conclusions regarding the precipitation of the product of the second Smith degradation must await the examination of structures which terminate with βDGlcNAc-(1→3)βDGal and βDGlcNAc(1→6)βDGal units since such structures may be present (Lloyd et al., 1968) and responsible for the precipitation. Meanwhile, the innate specificity of the lectin of *Helix pomatia* remains obscure.

Lectin of Wistaria floribunda. In the case of this lectin, the inhibition data using simple compounds (Sugii & Kabat,

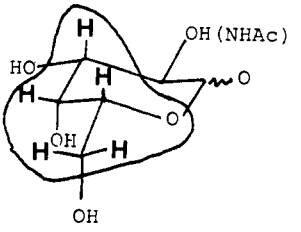
1980) show a substantially greater affinity for a DGalNAc than for a DGal terminal unit. Thus, *N*-acetyl-D-galactosamine was about 50 times more effective than D-galactose and MeαDGalNAc about 21 times more potent than MeαDGal. A preference for the α configuration is suggested by the fact that MeαDGalNAc was about 6.3 times more effective than its β anomer, and, although they are very weak inhibitors, MeαDGal appeared about 8 times more potent than its β anomer. These indications are, however, confused by the finding that *p*-NO_2Ph-βDGalNC was 3.6 times more potent than its α anomer. Also, the oligosaccharide A_5II which terminates in an αDGalNAc unit appeared only very slightly (about 1.3 times) more active than globoside which terminates with a βDGalNAc residue. Also, lactose was more potent than either melibiose or raffinose.

Although the data show a greater affinity for a DGalNAc than a DGal unit, the reverse appears to be the case for the precipitin data presented in Figure 1C. It is seen that the *Wistaria floribunda* hemagglutinin reacted substantially more strongly with the first product of the Smith degradation than with the A blood group substance itself. As was discussed above, the first product of Smith degradation must be expected to be essentially devoid of αDGalNAc residues but rich in βDGal terminal units. Also, as seen in Figure 2C, this lectin reacted strongly with desialyzed fetuin in which three βDGal(1→3)DGalNAc and nine βDGal(1→4)DGlcNAc residues per mole are exposed at the terminal nonreducing ends. This dichotomy appears best rationalized by assuming that the combining site of this lectin has an affinity for both DGalNAc and DGal units but that this affinity is best expressed when these are presented in the form of pauci- or multivalent clusters of determinants. Blood group substances are well established (Lloyd et al., 1968) to possess branched structures which could, in principle, lead to pauci- or multivalent clusters as do the *N*-acetyllactosamine-type glycans studied by Lee and co-workers (Lee, 1978).

It should be noted in this regard that a DGal unit can present a substantial hydrophobic surface in its preferred conformer, **5** (Bock & Lemieux, 1982), or in the intramolecularly hydrogen-bonded form displayed in structure **6** (Lemieux et al., 1978). Should the combining site of the lectin of *Wistaria floribunda* bind with the topography described by the hydrogen atoms displayed in these structures, then this topography would be present in virtually all blood group substances and blood group related glycolipids. Thus, the broad affinity of the lectin of *Wistaria floribunda* in the sense that it not only reacts with the A blood group substance and its first and second products of Smith degradation (Figure 1C) but also reacts with desialyzed porcine salivary glycoprotein, desialyzed fetuin, cyst 9 blood group A substance, desialyzed ovine salivary glycoprotein, and active antifreeze glycoprotein could be rationalized. As already mentioned, the lectin agglutinates A, B, and O erythrocytes. The fact that it did not react with inactive antifreeze glycoprotein may be related to an inability of this low molecular weight substance to form appropriate clusters. The lack of reaction by native fetuin can be assigned to the presence of the anionic α-sialoside groups as previously observed for asialoglycopeptides (Baenziger & Fiete, 1980).



5



6

Should the above hypothesis for the mode of binding by the lectin be correct, then the erratic nature of the inhibition data reported in Table I could be attributed to contributions to binding of a nonspecific nature by hydrophobic groupings about the C-2 to C-1 atoms such as the acetamido group of a DGalNAc residue and the *p*-nitrophenyl group of *p*-NO_2-βDGalNAc. Specific binding of an aglycon appears precluded since the difference in binding free energy (Table I) between α,βDGalNAc and MeαDGalNAc is only 0.3 kcal/mol. It would be remarkable, indeed, should the methoxy group be involved in the binding, that its substitution by a strongly hydrophilic hydroxyl group would be accompanied by such a small change in binding energy. On this basis, the substantially greater (0.9 kcal/mol) potency exhibited by αDGalNAc(1→6)DGal over MeαDGalNAc may be a mere coincidence arising from the hydrophobic nature of the α side of the DGal unit (structure **5** with H-6 = αDGalNAc) and may further exemplify the hazards that can be encountered in efforts to probe the combining site of a lectin by using only low molecular weight inhibitors. Fortunately, synthetic carbohydrate chemistry has developed sufficient power (Lemieux, 1978) to enable the planning of synthetic programs to test the hypothesis presented for the affinity of the lectin of *Wistaria floribunda*. Such studies would be modeled along the lines of the elegant syntheses described by Lönngren and co-workers [see Arnapp et al. (1982)].

Registry No. αDGalNAc(1→3)βDGalNAc(1→3)αDGal(1→4)-βDGal(1→4)α,βDGlc, 71937-76-3; αDGalNAc(1→3)βDGalNAc-(1→3)αDGalO$(CH_2)_8COOCH_3$, 85318-24-7; αDGalNAc(1→3)-βDGalNAcO$(CH_2)_8COOCH_3$, 85304-85-4; αDGalNAc(1→3)α,-βDGalNAc, 62026-07-7; αDGalNAc(1→6)α,βDGal, 40895-89-4; αDGalNAc(1→3)βDGal(1→3)βDGlcNAc, 85304-86-5; αDGalNAc(1→3)α,βDGal, 66781-75-7; MeαDGalNAc*p*, 6082-22-0; α,βDGalNAc, 1811-31-0; EtβDGlcNAc, 2495-96-7; EtβDGalNAc, 6082-23-1; *p*-NO_2Ph-αDGalNAc*p*, 23646-68-6; *p*-NO_2Ph-βDGalNAc*p*, 14948-96-0; βDGalNAc(1→3)αDGal(1→4)βDGal(1→-4)DGlc, 75660-79-6; αDGalNAc(1→6)DGal, 40895-89-4; βDGal-(1→3)DGlcNAc, 50787-09-2; βDGal(1→4)DGlcNAc, 32181-59-2; βDGal(1→6)DGlcNAc, 50787-10-5; βDGal(1→3)DGalNAc, 3554-90-3.

References

Allen, H. J., & Johnson, E. A. J. (1976) *Biochim. Biophys. Acta 436*, 557–566.

Arnapp, J., Haraldson, M., & Lönngren, J. (1982) *J. Chem. Soc., Perkin Trans. 1*, 1841–1844.

Baenziger, J. V., & Fiete, D. (1980) *Cell (Cambridge, Mass.) 22*, 611–620.

Beiser, S. M., & Kabat, E. A. (1952) *J. Immunol. 68*, 19–40.

Bird, G. W. G. (1951) *Curr. Sci. 20*, 298.

Bird, G. W. G. (1952) *Nature (London) 170*, 674.

Bock, K., & Lemieux, R. U. (1982) *Carbohydr. Res. 100*, 63–74.

Borrebaeck, C., Lonnerdal, B., & Etzler, M. (1981) *Biochemistry 20*, 4119–4122.

Boyd, W. C., & Shapleigh, E. (1954) *Science (Washington, D.C.) 119*, 419.

Cheung, C. T., Haratz, A., Kabar, M., Skrokov, R., & Poretz, R. D. (1979) *Biochemistry 18*, 1646–1650.

Citri, N. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 37*, 397–648.

Debray, H., Decout, D., Strecker, G., Spik, G., & Montreuil, J. (1981) *Eur. J. Biochem. 117*, 41–55.

De Salegui, M., & Pouska, H. (1969) *Arch. Biochem. Biophys. 129*, 49–56.

DeVries, A. L., Komatsu, S., & Feeney, R. E. (1970) *J. Biol. Chem. 245*, 2901–2908.

Donald, A. S. R. (1981) *Eur. J. Biochem. 120*, 243–249.

Etzler, M. E., & Kabat, E. A. (1970) *Biochemistry 9*, 869–877.

Etzler, M. E., Gupta, S., & Borrebaeck, C. (1981) *J. Biol. Chem. 256*, 2367–2370.

Feeney, R. E., & Yeh, Y. (1978) *Adv. Protein Chem. 32*, 191–282.

Feizi, T., Kabat, E. A., Vicari, G., Anderson, B., & Marsh, W. L. (1971) *J. Exp. Med. 133*, 39–52.

Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 128–340.

Graham, E. R. B. (1972) in *Glycoproteins* (Gottschalk, A., Ed.) p 719, Elsevier, Amsterdam, The Netherlands.

Hammarström, S., & Kabat, E. A. (1969) *Biochemistry 8*, 2696–2705.

Hammarström, S., Lindberg, A. A., & Robertsson, E. S. (1972) *Eur. J. Biochem. 25*, 274–282.

Hammarström, S., Murphy, L. A., Goldstein, I. J., & Etzler, M. E. (1977) *Biochemistry 16*, 2750–2755.

Herp, A., Wu, A. M., & Moschera, J. (1979) *Mol. Cell. Biochem. 23*, 27–43.

Hindsgaul, O., Norberg, T., Le Pendu, J., & Lemieux, R. U. (1982) *Carbohydr. Res. 109*, 109–142.

Hudgin, R. L., Pricer, W. E., Jr., Ashwell, G., Stockert, R. J., & Morell, A. G. (1974) *J. Biol. Chem. 249*, 5536–5543.

Ishiyama, I., & Takatsu, A. (1970) *Z. Immunitaetsforsch. 140*, 189–202.

Kabat, E. A. (1956a) *Blood Group Substances. Their Chemistry and Immunochemistry*, pp 135–139, Academic Press, New York.

Kabat, E. A. (1956b) *J. Immunol. 77*, 377–385.

Kabat, E. A. (1961) *Kabat and Mayer's Experimental Immunochemistry*, 2nd ed., Charles C. Thomas, Springfield, IL.

Kabat, E. A. (1962) *Arch. Biochem. Biophys., Suppl. 1*, 181–186.

Kabat, E. A., Liao, J., Burzynska, M. H., Wong, T. C., Thøgersen, H., & Lemieux, R. U. (1981) *Mol. Immunol. 18*, 873–881.

Kaplan, M. E., & Kabat, E. A. (1966) *J. Exp. Med. 123*, 1061–1081.

Karush, F. (1950) *J. Am. Chem. Soc. 72*, 2714–2718.

Kawasaki, T., & Ashwell, G. (1976) *J. Biol. Chem. 251*, 1296–1302.

Kim, Z., & Uhlenbruck, G. (1966) *Z. Immunitaetsforsch. 130*, 88–99.

Kimura, A., Wigzell, H., Holmquist, G., Ersson, B., & Carlsson, B. (1979) *J. Exp. Med. 149*, 473–483.

Klenk, E., Hendriks, V. W., & Gielen, W. (1962) *Hoppe-Seyler's Z. Physiol. Chem. 330*, 140–144.

Kocourek, J., Jamieson, G. A., Votruva, T., & Hořejší, V. (1979) *Biochim. Biophys. Acta 500*, 344–360.

Krüpe, M., & Pieper, H. (1966) *Z. Immunitaetsforsch., Allerg. Klin. Immunol. 130*, 296–300.

Kurokawa, T., Tsuda, M., & Sugino, T. (1976) *J. Biol. Chem. 251*, 5685–5893.

Lee, Y. T. (1978) *Carbohydr. Res. 67*, 509–514.

Lemieux, R. U. (1978) *Chem. Soc. Rev. 7*, 423–452.

Lemieux, R. U. (1982) *Frontiers in Chemistry*, pp 3–24, Pergamon Press, Oxford.

Lemieux, R. U., & Koto, S. (1974) *Tetrahedron 30*, 1933–1944.

Lemieux, R. U., & Hermentin, P. (1980) IUPAC Xth International Symposium on Carbohydrate Chemistry, July 7–11, Sydney, Australia.

Lemieux, R. U., & Ratcliffe, R. M. (1980) U.S. Patent 4 195 174.

Lemieux, R. U., Boullanger, P. H., Bundle, D. R., Baker, D. A., Nagpurkar, A., & Venot, A. (1978) *Nouv. J. Chim. 2*, 321–329.

Lemieux, R. U., LePendu, J., & Hindsgaul, O. (1979) *Jpn. J. Antibiot. 32*, Suppl. S21–S31.

Lerner, R. A. (1982) *Nature (London) 299*, 592–596.

Lis, H., & Sharon, N. (1977) in *The Antigens* (Sela, M., Ed.) Vol. IV, pp 429–529, Academic Press, New York.

Lloyd, K. O., & Kabat, E. A. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1470–1477.

Lloyd, K. O., Kabat, E. A., & Licerio, E. (1968) *Biochemistry 7*, 2976–2990.

Makita, S., Suzuki, C., & Yoshizawa, Z. (1966) *J. Biochem. (Tokyo) 60*, 502–513.

Marcus, D. M., Naiki, M., & Kundu, S. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3263–3267.

Naiki, M., Fong, J., Ledeen, R., & Marcus, D. M. (1975) *Biochemistry 14*, 4837–4846.

Nicolson, G. L. (1974) *Int. Rev. Cytol. 39*, 89–190.

Nilsson, B., Norden, N. E., & Svensson, S. (1979) *J. Biol. Chem. 254*, 4545–4553.

Ochoa, J.-L. (1981) *J. Chromatogr. 215*, 351–360.

Paulsen, H., & Bünsch, A. (1980) *Angew. Chem., Int. Ed. Engl. 19*, 902–903.

Paulsen, H., Kolar, C., & Stenzel, W. (1978) *Chem. Ber. 111*, 2358–2369.

Pereira, M. E. A., & Kabat, E. A. (1976) *J. Exp. Med. 143*, 422–436.

Pereira, M. E. A., & Kabat, E. A. (1979) *CRC Crit. Rev. Immunol. 1*, 33–78.

Pereira, M. E. A., Gruezo, F., & Kabat, E. A. (1979) *Arch. Biochem. Biophys. 194*, 511–525.

Poste, G., Allen, H. J., & Matta, K. L. (1979) *Cell. Immunol. 44*, 89–98.

Pressman, D., & Grossberg, A. L. (1968) *The Structural Basis of Antibody Specificity*, W. A. Benjamin, New York.

Prokop, O., Uhlenbruck, G., & Köhler, W. (1968) *Vox. Sang. 14*, 321–333.

Ratcliffe, R. M., Baker, D. A., & Lemieux, R. U. (1981) *Carbohydr. Res. 93*, 35–41.

Sarkar, M., & Kabat, E. A. (1979) *Carbohydr. Res. 69*, 143–149.

Schiffman, G., Kabat, E. A., & Thompson, W. (1964) *Biochemistry 3*, 113–120.

Schubert, D., Roman, A., & Cohn, M. (1970) *Nature (London) 225*, 154–158.

Shier, W. T., Lin, Y., & deVries, Y. L. (1975) *FEBS Lett. 54*, 135–138.

Siddiqui, B., & Hakomori, S. (1971) *J. Biol. Chem. 246*, 5766–5769.

Spiro, R. J., & Bohyroo, V. D. (1974) *J. Biol. Chem. 249*, 5704–5717.

Springer, G. F. (1971) *Prog. Allergy 15*, 9–77.

Sugii, S., & Kabat, E. A. (1980) *Biochemistry 19*, 1192–1199.

Takatsu, A., Mukaida, M., & Ishiyama, I. (1971) *Jpn. J. Exp. Med. 41*, 411–421.

Tettamanti, G., & Pigman, M. (1968) *Arch. Biochem. Biophys. 124*, 41–50.
Thøgersen, H., Lemieux, R. U., Bock, K., & Meyer, B. (1982) *Can. J. Chem. 60*, 44–57.
Toyoshima, S., Akiyama, Y., Nakano, K., Tonomura, S., & Osawa, T. (1977) *Biochemistry 10*, 4457–4463.
Uhlenbruck, G., Sprenger, I., Leseney, L. M., Font, J., & Bouvillon, R. (1970) *Vox Sang. 19*, 488–495.

Characterization of Cytochalasin B Photoincorporation into Human Erythrocyte D-Glucose Transporter and F-Actin†

Michael F. Shanahan

ABSTRACT: The photoincorporation of cytochalasin B into the human erythrocyte glucose transporter and purified G-actin previously reported by this laboratory [Shanahan, M. F. (1982) *J. Biol. Chem. 257*, 7290–7293] was investigated. [^{3}H]Cytochalasin B photolabeled polypeptides of M_r ~43 000–73 000, as determined by polyacrylamide gel electrophoresis, in a concentration-dependent manner with maximum incorporation occurring at 5 μM [^{3}H]cytochalasin B and a half-maximum value of 0.63 μM. This incorporation, previously shown to be partially blocked in the presence of D- but not L-glucose, did not occur in the absence of photolysis and increased linearly with a photolysis time up to 30 s. The reaction was relatively insensitive to pH in the range of pH 6–9, but apparent nonspecific labeling significantly increased at pH 5. The effect of cytochalasin B photoincorporation on D-glucose uptake in intact erythrocytes was also examined. Purified chicken muscle F-actin was also photolabeled with this ligand, but at a specific activity of incorporation (pmol/mg of protein) ~50 times lower than that of the erythrocyte transporter polypeptides. D-Glucose had no effect on this incorporation while 10^{-4}M cytochalasin E completely blocked actin photolabeling. The efficiency of photoincorporation for both the transporter and F-actin was around 1%. Extraction of [^{3}H]cytochalasin B labeled membranes with Triton X-100 resulted in the selective elution of labeled polypeptides from the transporter region while cytochalasin B labeled polypeptides in the region of red cell actin remained in the extracted pellet.

Entry of monosaccharides into human erythrocytes is a facilitated diffusion process mediated by proteins located in the plasma membrane [see Jones & Nickson (1981) for review]. In recent years these transporter polypeptides have been identified by a number of laboratories using a variety of approaches. Several laboratories have isolated this transport system and reconstituted glucose transport activity in liposomes using purified transporter polypeptides. These investigators have identified the glucose transporter as a broad band of heterogeneously, glycosylated proteins of M_r 43 000–70 000 by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis (Kasahara & Hinkle, 1977; Kahlenberg & Zala, 1977; Baldwin et al., 1981). Other methods employing differential labeling (Batt et al., 1976; Lienhard et al., 1977; Shanahan & Jacquez, 1978) and immunological techniques (Sogin & Hinkle, 1980; Baldwin & Lienhard, 1980) have also identified these polypeptides as the glucose transporter.

Cytochalasin B is a potent reversible inhibitor of erythrocyte monosaccharide transport (Taverna & Langdon, 1973; Lin et al., 1974; Jung & Rampal, 1977). Binding of this ligand to the transporter is blocked by D-glucose but not by L-glucose, and this effect has been used to characterize and purify the glucose transporter (Zoccoli et al., 1978; Baldwin et al., 1979; Pinkofsky et al., 1978; Gorga & Lienhard, 1981). Two laboratories have recently developed a method for irreversibly photoincorporating [^{3}H]cytochalasin B into the human erythrocyte glucose transporter (Shanahan, 1982; Carter-Su et al., 1982). Herein we describe a more detailed characterization of the conditions and kinetics of this reaction.

Experimental Procedures

Materials. Cytochalasins B and E were purchased from Aldrich Chemical Co. Electrophoresis reagents were obtained from Bio-Rad. Sequanal grade (lauryl) $NaDodSO_4$ was from Pierce Chemical Co. [^{3}H]Cytochalasin B (radiochemical purity >98%) was obtained from Amersham at a specific activity of 10.3 Ci/mmol. All other reagents were obtained from Sigma Chemical Co. Cytochalasins B and E were routinely prepared in an ethanolic stock solution (10 mg/mL) and diluted to their appropriate final concentration in buffer as described below. Silicone oil (density 1.01–1.02 g/cm^3) was obtained from Contour Chemical Co., Woburn, MA. D-[^{3}H]Glucose (30 Ci/mmol) and L-[1-^{14}C]glucose (54.8 mCi/mmol) were purchased from New England Nuclear.

Preparation of Plasma Membranes. Outdated blood was provided by the American Red Cross Regional Blood Band, Madison, WI. Washed human erythrocytes and membrane ghosts were prepared by the method of Steck & Kant (1974). For ghosts, this method was modified by using the procedure of Fröman et al. (1980) in which osmotically lysed red cells are separated from hemoglobin by gel chromatography using a Sepharose CL-6B column (9 × 30 cm) equilibrated with 5 mM sodium phosphate buffer, pH 8.0 (5P8). Ghost membranes in 5P8 buffer were quick frozen in liquid nitrogen and stored at −100 °C until use. For experimental conditions involving different buffers, the membrane samples were pel-

† From the Department of Physiology, University of Wisconsin Medical School, Madison, Wisconsin 53706. *Received August 2, 1982; revised manuscript received December 30, 1982.* This research was supported by Grant AM26093 from the National Institutes of Health.

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline, 150 mM $NaCl_2$–5 mM sodium phosphate, pH 7.4.

0006-2960/83/0422-2750$01.50/0