

## Hemagglutinins from Two Influenza Virus Variants Bind to Sialic Acid Derivatives with Millimolar Dissociation Constants: A 500-MHz Proton Nuclear Magnetic Resonance Study<sup>†</sup>

Nicholas K. Sauter,<sup>†</sup> Mark D. Bednarski,<sup>§,||</sup> Beth A. Wurzburg,<sup>†</sup> John E. Hanson,<sup>†</sup> George M. Whitesides,<sup>§</sup>  
John J. Skehel,<sup>‡</sup> and Don C. Wiley<sup>\*,‡</sup>

*Department of Biochemistry and Molecular Biology, Howard Hughes Medical Institute, and Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.*

*Received April 18, 1989; Revised Manuscript Received June 19, 1989*

**ABSTRACT:** The equilibrium binding of influenza virus hemagglutinin to derivatives of its cell-surface ligand, sialic acid, was measured by nuclear magnetic resonance (NMR) spectroscopy. Binding was quantified by observing perturbations of sialic acid resonances in the presence of protein. The major perturbation observed was a chemical shift of the *N*-acetyl methyl resonance, presumably due to the proximity of the methyl group to tryptophan 153. X-31 hemagglutinin binds to the methyl  $\alpha$ -glycoside of sialic acid with a dissociation constant of 2.8 mM and does not bind to the methyl  $\beta$ -glycoside. Replacing the 4-hydroxyl group of sialic acid with an acetyl group has little effect, while replacing the 7-hydroxyl group with an acetyl prevents binding. Experiments with sialylated oligosaccharides confirm literature reports that mutations at amino acid 226 change the specificity of hemagglutinin for  $\alpha(2,6)$  and  $\alpha(2,3)$  glycosidic linkages. The NMR line broadening of sialyloligosaccharides suggests that sialic acid is the only component that contacts the protein. Saccharides containing two sialic acid residues appear to have two separate binding modes. Hemagglutinin that has undergone a low pH induced conformational change retains the ability to bind sialic acid.

**I**nfluenza virus infection is initiated by the attachment of virus particles to cell-surface receptors containing sialic acid [reviewed in Wiley and Skehel (1987)]. Sialic acid is recognized by the viral glycoprotein hemagglutinin, a membrane-bound trimer consisting of three HA1 and three HA2

polypeptide chains. Viral attachment is followed by receptor-mediated endocytosis, after which the viral and cell membranes fuse, allowing the viral nucleocapsid to enter the cytoplasm. Membrane fusion is thought to be mediated by a conformational change in the hemagglutinin, triggered at the pH of the endosome.

The recently published structure of hemagglutinin complexed with sialyllactose at 3-Å resolution (Weis et al., 1988) reveals that sialic acid binds to a shallow pocket in the HA1 polypeptide, a pocket containing several conserved amino acid residues. Amino acid mutations in or near the binding pocket are known to change the hemagglutinin's binding properties. Viruses that contain leucine at position 226, for example, preferentially agglutinate erythrocytes that possess sialic acids joined in an  $\alpha(2,6)$  linkage with galactose, whereas viruses

<sup>†</sup>This research was supported by National Institutes of Health Grants AI-13654 (D.C.W.) and GM-39589 (D.C.W. and G.M.W.). NMR instrumentation was supported by National Science Foundation Grant NSF-CHE-84-10774.

\*To whom correspondence should be addressed.

<sup>†</sup>Department of Biochemistry and Molecular Biology, Howard Hughes Medical Institute, Harvard University.

<sup>§</sup>Department of Chemistry, Harvard University.

<sup>||</sup>Present address: Department of Chemistry, University of California, Berkeley, CA 94720.

<sup>‡</sup>National Institute for Medical Research.

containing glutamine at that position prefer  $\alpha(2,3)$  linkages (Rogers et al., 1983). Such amino acid mutations are thought to confer species and tissue specificity to viruses, since naturally occurring sialic acids vary in type and in the glycosidic linkages that they form (Paulson, 1985; Schauer, 1982).

In this paper we show that differences in binding specificities can be observed in solution using mixtures containing only soluble hemagglutinin and small sialosides. We have used proton nuclear magnetic resonance (NMR)<sup>1</sup> spectroscopy to measure equilibrium dissociation constants in such mixtures and to obtain limited structural information about the hemagglutinin/sialoside interactions.

#### EXPERIMENTAL PROCEDURES

**Protein.** We studied two variants of influenza virus hemagglutinin, differing only in amino acid 226 of HA1: hemagglutinin from the recombinant viral strain X-31 (H3N2) containing leucine and hemagglutinin from the strain X-31/HS containing glutamine. The amino acid sequences are otherwise identical (Rogers et al., 1983). Virus was cultured in embryonated hens' eggs and purified as described (Skehel & Schild, 1971). The hemagglutinin trimer was released from the viral membranes by treatment with the protease bromelain (Brand & Skehel, 1972; Skehel & Waterfield, 1975) and isolated by density-gradient centrifugation (Skehel et al., 1982). Sucrose was then removed by dialysis against saline solution (0.15 M sodium chloride, 0.1% sodium azide). In order to remove residual neuraminidase activity, bromelain-released hemagglutinin (BHA) was passed through a column containing anti-neuraminidase antibodies attached to Protein A-Sepharose CL-4B (Pharmacia). This treatment reduced neuraminidase activity to a level that would result in less than 5% hydrolysis over the course of an NMR experiment, typically 5 h. Neuraminidase activity was assayed by incubating 15  $\mu$ g of BHA with 100 nmol of sialyllactose (from bovine colostrum, Sigma) at 37 °C and then testing for free sialic acid (Neu5Ac) by the thiobarbituric acid method (Warren, 1959, 1963). Finally, purified hemagglutinin was concentrated by vacuum dialysis.

Experiments were done on two different BHA conformers: the native conformation and the conformation that is irreversibly induced by lowering the pH to 5.0 (Skehel et al., 1982). BHA in the native conformation was prepared for NMR studies by dialyzing into a deuterated buffer [0.15 M sodium chloride, 0.1% sodium azide, 0.10 M sodium phosphate, 2–5 mM sodium (trimethylsilyl)propionate (TSP), 99.96 atom % D<sub>2</sub>O, pH 6.9–7.1]. The low-pH conformation was induced by dialyzing BHA into acetate-buffered saline (0.15 M sodium chloride, 0.1% sodium azide, 0.10 M sodium acetate, pH 4.9). NMR samples were then prepared by being dialyzed into deuterated phosphate buffer as above or into deuterated acetate buffer (0.15 M sodium chloride, 0.1% sodium azide, 0.10 M sodium acetate-*d*<sub>3</sub>, 2.5 mM TSP, 99.96 atom % D<sub>2</sub>O, pH 4.9). pH was measured on a Model MI-410 micro-combination pH probe (Microelectrodes, Inc.); meter readings were not corrected for the deuterated solvent.

The low-pH-induced conformational change was monitored by assaying the susceptibility of the HA1 polypeptide chain to tryptic cleavage (Skehel et al., 1982). After incubation at

pH 4.9, BHA solutions were adjusted to pH 7.3 with 1.0 M Tris and incubated with bovine trypsin [treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone, Sigma] for 2 min at 20 °C. The final BHA concentration was 0.8 mg/mL, and the trypsin concentration was 0.4 mg/mL. The reaction was quenched with soybean trypsin inhibitor, and products were reduced with  $\beta$ -mercaptoethanol, analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), and visualized with Coomassie blue or silver stain. The HA1 chain of native BHA was completely resistant to tryptic cleavage, while all of the HA1 chain from the low-pH-treated protein was cleaved to fragments of characteristic size (Skehel et al., 1982).

**Ligands (Table I).**  $\alpha(2,6)$ -Sialyllactose,  $\alpha(2,3)$ -sialyllactose, G<sub>M1</sub>-OS, LSTa, and DSL were purchased from Biocarb Chemicals (Lund, Sweden) and used without further purification. Integration of NMR spectra indicated that the  $\alpha(2,6)$ -sialyllactose contained about 20 mol % acetate ion and about 2% free Neu5Ac.  $\alpha(2,3)$ -Sialyllactose contained about 4% Neu5Ac.

Methyl glycoside derivatives of Neu5Ac were synthesized as follows. The benzyl ester of Neu5Ac $\alpha$ 2Me was prepared from the corresponding methyl ester (van der Vleugel et al., 1982) by treatment with catalytic potassium *tert*-butoxide in benzyl alcohol. The benzyl ester was removed with 10% Pd/C and H<sub>2</sub> to yield Neu5Ac $\alpha$ 2Me. In a similar fashion, Neu5Ac $\beta$ 2Me was prepared from its methyl ester (Kuhn et al., 1966).

Treatment of the benzyl ester of Neu5Ac $\alpha$ 2Me with 2,2-dimethoxypropane and acetone in the presence of Dowex 50W (H<sup>+</sup>) gave the 8,9-acetonide, which was either selectively acetylated at the 4-position or diacetylated at the 4- and 7-positions. Removal of the acetonide with acetic acid followed by hydrogenolysis to remove the benzyl ester, afforded Neu4,5Ac $\alpha$ 2Me and Neu4,5,7Ac $\alpha$ 2Me. Neu4,5Ac $\alpha$ 2Me contained about 11 mol % of an impurity thought to be another *O*-acetyl derivative of Neu5Ac $\alpha$ 2Me.

G<sub>D1a</sub>-OS was isolated from purified G<sub>D1a</sub> ganglioside (a gift from Dr. Yasuo Suzuki) as described by Ong and Yu (1984), except that the crude oligosaccharide was purified by silica flash chromatography using a 4:1 1-propanol/water solvent. The final product contained about 5% unreacted G<sub>D1a</sub> ganglioside.

**Binding Experiments.** Equilibrium binding constants were measured as described for other systems [Kronis and Carver (1982) and references cited therein]. When a ligand rapidly exchanges between a protein binding site and solvent, the observed chemical shifts ( $\delta_{\text{obs}}$ ) and line widths ( $\Delta\nu_{\text{obs}}$ ) of various ligand resonances can differ from the shifts and line widths seen in the absence of protein ( $\delta_{\text{free}}$  and  $\Delta\nu_{\text{free}}$ ). Under conditions where the fraction of ligand bound to protein is small, the dissociation constant  $K_D$  is given by either of the following equations:

$$[L]_{\text{TOT}} = \frac{[P]_{\text{TOT}} \Delta\delta_{\text{B}_{\text{app}}} - K_D}{\Delta\delta} \quad (1a)$$

$$[L]_{\text{TOT}} = \frac{[P]_{\text{TOT}} \Delta\Delta\nu_{\text{B}_{\text{app}}} - K_D}{\Delta\Delta\nu} \quad (1b)$$

where  $\Delta\delta = \delta_{\text{obs}} - \delta_{\text{free}}$  and  $\Delta\Delta\nu = \Delta\nu_{\text{obs}} - \Delta\nu_{\text{free}}$ ,  $[P]_{\text{TOT}}$  is the total concentration of protein binding sites,  $[L]_{\text{TOT}}$  is the total ligand concentration, and  $\Delta\delta_{\text{B}_{\text{app}}}$  and  $\Delta\Delta\nu_{\text{B}_{\text{app}}}$  are the apparent chemical shift and line-width changes for the ligand in the bound state.

In order to determine dissociation constants from the  $y$  intercept in plots of  $[L]_{\text{TOT}}$  vs  $1/\Delta\delta$  or  $[L]_{\text{TOT}}$  vs  $1/\Delta\Delta\nu$ ,

<sup>1</sup> Abbreviations: NMR, nuclear magnetic resonance; BHA, bromelain-released hemagglutinin; TSP, sodium (trimethylsilyl)propionate; Neu5Ac, *N*-acetylneuraminic acid (sialic acid); Neu5Ac $\alpha$ 2Me,  $\alpha$ -2-*O*-methyl-*N*-acetylneuraminic acid; Neu4,5Ac $\alpha$ 2Me,  $\alpha$ -2-*O*-methyl-*N*-acetyl-4-*O*-acetylneuraminic acid; Neu4,5,7Ac $\alpha$ 2Me,  $\alpha$ -2-*O*-methyl-*N*-acetyl-4,7-di-*O*-acetylneuraminic acid; Neu5Ac $\beta$ 2Me,  $\beta$ -2-*O*-methyl-*N*-acetylneuraminic acid.

Table I: Dissociation Constants, 297 ± 1 K

hemagglutinin variant	structural formula of ligand	common name of ligand	$K_D$ (mM) <sup>a</sup>	[protein] ( $\mu$ M)	protein saturation range (%)
X-31 (226 Leu)	Neu5Ac $\alpha$ 2Me	$\alpha$ -methylsialic acid	2.8 ± 0.3	33	16-82
	Neu5Ac $\beta$ 2Me	$\beta$ -methylsialic acid	>200	150	
	Neu4,5Ac $\alpha$ 2Me	4-O-acetylsialic acid	2.1 ± 0.3	42	29-90
	Neu4,5,7Ac $\alpha$ 2Me	4,7-di-O-acetylsialic acid	>100	63	
	Neu5Ac $\alpha$ (2,6)Gal $\beta$ (1,4)Glc	$\alpha$ (2,6)-sialyllactose	2.1 ± 0.3	25, 49	28-85
	Neu5Ac $\alpha$ (2,3)Gal $\beta$ (1,4)Glc	$\alpha$ (2,3)-sialyllactose	3.2 ± 0.6	37	22-76
	Gal $\beta$ (1,3)GalNAc $\beta$ (1,4)Gal $\beta$ (1,4)Glc	G <sub>M1</sub> -OS	>40	23	
	Neu5Ac $\alpha$ (2,3)				
	Neu5Ac $\alpha$ (2,3)Gal $\beta$ (1,3)GalNAc $\beta$ (1,4)Gal $\beta$ (1,4)Glc	G <sub>D1a</sub> -OS	11.4 ± 2 <sup>c</sup>	44	3-43
	15 mM <sup>b</sup>				
X-31/HS (226 Gln)	Neu5Ac $\alpha$ (2,3)				
	44 mM <sup>b</sup>				
	Neu5Ac $\alpha$ (2,3)Gal $\beta$ (1,3)GlcNAc $\beta$ (1,3)Gal $\beta$ (1,4)Glc	LSTa	3.8 ± 0.8	24	7-42
	Neu5Ac $\alpha$ (2,3)Gal $\beta$ (1,3)GlcNAc $\beta$ (1,3)Gal $\beta$ (1,4)Glc	DSL	1.2 ± 0.2 <sup>d</sup>	24	26-77
	4.4 mM <sup>b</sup>				
	Neu5Ac $\alpha$ (2,6)				
	1.7 mM <sup>b</sup>				
	Neu5Ac $\alpha$ (2,3)Gal $\beta$ (1,3)GlcNAc $\beta$ (1,3)Gal $\beta$ (1,4)Glc	DSL	4.2 ± 1.1 <sup>d</sup>	32	10-60
	6.8 mM <sup>b</sup>				
	Neu5Ac $\alpha$ (2,6)				
11 mM <sup>b</sup>					
X-31 (low-pH-induced conformation)		$\alpha$ -methylsialic acid	4.7 ± 0.5	34	7-74
		$\alpha$ (2,6)-sialyllactose	5.9 ± 0.7	33, 53	5-67
		$\alpha$ (2,3)-sialyllactose	2.9 ± 0.3	17, 33	11-79
		DSL	4.2 ± 1.1 <sup>d</sup>	32	10-60
		pH 4.9, $\alpha$ -methylsialic acid	2.2 ± 0.5	35	14-85
		pH 7.1, $\alpha$ -methylsialic acid	2.4 ± 0.3	39	12-82

<sup>a</sup>Dissociation constants are based on the *N*-acetyl chemical shift of sialic acid unless otherwise indicated. <sup>b</sup>Intrinsic dissociation constants for individual sialic acid residues. <sup>c</sup>Overall effective dissociation constant based on the *N*-acetyl chemical shift of the terminal sialic acid moiety. <sup>d</sup>Overall effective dissociation constant based on the  $\alpha$ (2,6)-linked H<sub>3ax</sub> line width.

titrations were performed during which protein concentration was fixed and ligand concentration was varied over a wide range. For these titrations, we mixed solutions of protein alone (P) and protein plus ligand (PL), each containing about a 0.5-mL volume (equal aliquots of protein from a common stock solution were mixed with either an aliquot of ligand solution or an aliquot of buffer). NMR spectra were obtained for the P and PL solutions, and then the PL solution was pipetted into the P solution in a series of small steps (5–50  $\mu$ L).

BHA concentration of the P solution was determined by absorbance of a 40-fold diluted sample. The extinction coefficient at 280 nm was estimated to be 1.5 cm<sup>-1</sup> (mg/mL)<sup>-1</sup> (Cantor & Schimmel, 1980), by use of the known amino acid sequence and a molecular mass of 69 474 daltons per monomer (Verhoeven et al., 1980). The ligand concentration of the PL solution (typically 10 mM) was calculated by weight, taking into account the impurities noted above, and the ligand concentration during the titration was calculated by assuming accurate pipetting. We found that protein could be reisolated after an NMR titration by extensive dialysis against saline and used again for NMR binding studies. Dissociation constants from reisolated material were identical with those measured with freshly prepared BHA.

**Instrumental Conditions.** Proton magnetic resonance spectroscopy was performed at the Chemistry Department of Harvard University on a 500-MHz Bruker AM spectrometer. Free induction decays were collected at 297 ± 1 K, with quadrature detection. We acquired 16K complex data points, with sweep widths ranging from 3205 to 5000 Hz. Depending

on the ligand concentration, between 64 and 1024 scans were collected. Samples were analyzed in 5-mm NMR tubes (No. 528PP, Wilmad Glass Co., Inc.).

**Data Processing.** Data were transferred to a VAX 8700 and processed with a modified version of the program NMR1 (New Methods Research, Inc.). Free induction decays were zero-filled to 32K complex points and Fourier transformed. The resulting spectra had digital resolutions from 0.098 to 0.153 Hz/point. Positions and line widths of resonances were determined by modeling singlets with Lorentzian lines and multiplets with a sum of Lorentzians. The trimethylsilyl resonance of internal TSP was used as a chemical shift and line-broadening standard. For experiments with BHA in the low-pH-induced conformation, acetone (0.6 mM) was used as an internal standard, since the chemical shift of the TSP resonance is known to be sensitive to small changes in pH near its pK<sub>a</sub> of 5.0 (De Marco, 1977). In spectra with a high protein/ligand ratio, a broad protein resonance at 2.05 ppm (Figure 1a) interfered with the modeling of the *N*-acetyl resonances of sialosides. In these cases, line widths and chemical shifts were estimated from hard-copy plots.

## RESULTS

**Determination of Dissociation Constants.** Proton NMR revealed that in the presence of X-31 BHA the chemical shifts and line widths of certain Neu5Ac $\alpha$ 2Me resonances were perturbed. Figure 1 shows that the *N*-acetyl resonance was broadened and shifted upfield and that the resonance of the axial proton in the 3-position (H<sub>3ax</sub>) was also broadened. At

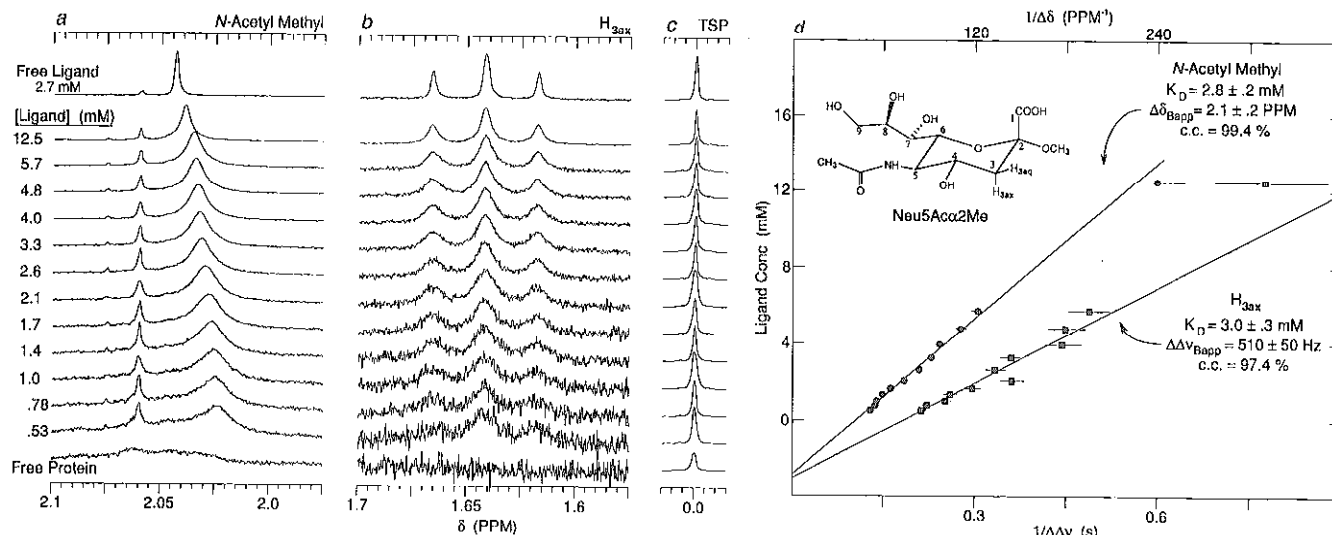


FIGURE 1: 500-MHz proton NMR spectra showing the *N*-acetyl methyl (a) and  $H_{3ax}$  (b) resonances of Neu5Ac $\alpha$ 2Me free in solution (top trace) and in the presence of 33  $\mu$ M X-31 BHA. The *N*-acetyl resonance of the  $\beta$ -anomer of Neu5Ac (present as an 11% impurity by weight) is seen at 2.06 ppm. The chemical shift scales of these spectra are aligned on the basis of the trimethylsilyl resonance of internal TSP (c). TSP also served as a control for  $\Delta\nu$ , the full width at half-maximum. For example, the broadening of the  $H_{3ax}$  resonance due to the presence of protein was calculated with  $\Delta\nu = (\Delta\nu_{H_{3ax}} - \Delta\nu_{TSP})_{protein} - (\Delta\nu_{H_{3ax}} - \Delta\nu_{TSP})_{free}$ . The  $H_{3ax}$  ( $\circ$ ) and  $H_{3ax}$  ( $\square$ ) data are plotted in panel d. The error in the chemical shift of the *N*-acetyl resonance was defined to be one-fourth of the full width at half-maximum divided by the square root of the signal-to-noise ratio. The line-broadening values presented for the  $H_{3ax}$  resonance are averages over the three components of the triplet, and the error is defined to be equal to the digital resolution of the spectra. Linear regression (Bevington, 1969) yielded the dissociation constants, apparent bound parameters, and correlation coefficients shown.

Table II: Apparent Bound Chemical Shift Changes of *N*-Acetyl Resonance

	X-31		X-31/HS	
	$\Delta\delta_{BAPP}$ (ppm)	$[P]_{TOT}$ ( $\mu$ g)	$\Delta\delta_{BAPP}$ (ppm)	$[P]_{TOT}$ ( $\mu$ g)
Neu5Ac $\alpha$ 2Me	$2.1 \pm 0.2$	33	$2.6 \pm 0.3$	34 <sup>a</sup>
$\alpha(2,6)$ -sialyllactose	$1.5 \pm 0.2$	25	$2.2 \pm 0.3$	53
$\alpha(2,3)$ -sialyllactose	$1.9 \pm 0.2$	49	$1.2 \pm 0.2$	33
		37	$2.1 \pm 0.2$	33
Neu4,5Ac $\alpha$ 2Me	$1.5 \pm 0.2$	42	$1.3 \pm 0.2$	17

<sup>a</sup> Protein recovered from previous experiments by extensive dialysis.

a fixed protein concentration, these perturbations were dependent on the ligand concentration, reflecting differences in the fraction of ligand which was bound to protein. A smaller fraction was bound at high ligand concentrations, resulting in resonances that more closely resembled those of the free ligand. A separate control (not shown) indicated that in the absence of protein these ligand resonances did not shift or broaden as a function of concentration. Data from the *N*-acetyl and  $H_{3ax}$  resonances yielded dissociation constants which were within experimental error of each other (Figure 1d), consistent with the idea that a single binding mode results in the perturbation of both resonances. Due to the complexity of the spectra, other protons could not be analyzed to give quantitative binding data. Because the *N*-acetyl resonance had a higher signal-to-noise ratio than the  $H_{3ax}$  resonance, we have used the *N*-acetyl chemical shift data to compute most of the dissociation constants presented in Table I. Errors quoted for the dissociation constants in Table I include both the error from the scatter of the NMR measurements (e.g., Figure 1d) and the error in ligand concentration, which we estimate to be  $\pm 10\%$ .

**Dissociation Constants Are Independent of Protein Concentration.** Figure 2 shows the *N*-acetyl chemical shift data that were used to calculate the binding constants of  $\alpha(2,6)$ -sialyllactose and  $\alpha(2,3)$ -sialyllactose to X-31 and X-31/HS BHA. Three of these experiments were repeated at different

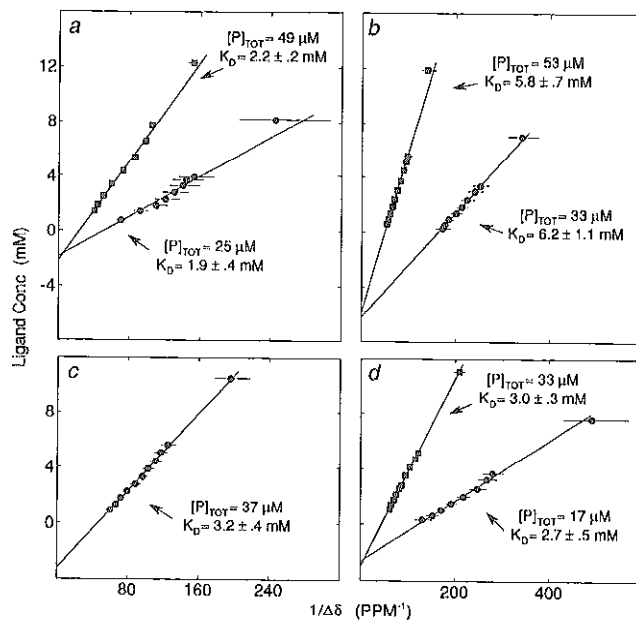


FIGURE 2: Chemical shift of the *N*-acetyl resonance of sialyllactose isomers is plotted according to eq 1a. Protein concentrations and calculated dissociation constants are indicated. In all cases, correlation coefficients from linear regression were greater than 98.5%. (a)  $\alpha(2,6)$ -Sialyllactose plus X-31 BHA; (b)  $\alpha(2,6)$ -sialyllactose plus X-31/HS BHA; (c)  $\alpha(2,3)$ -sialyllactose plus X-31 BHA; (d)  $\alpha(2,3)$ -sialyllactose plus X-31/HS BHA. Experiments in panels a, b, and d were performed twice; dissociation constants listed in Table I are weighted averages of the two trials.

protein concentrations, which is reflected in the fact that the plots have different slopes. In all cases the  $K_D$ 's derived from these duplicate experiments are in good agreement. This suggests that the assumption of simple bimolecular association is valid for this system.

**Interpretation of the Bound Chemical Shift of the *N*-Acetyl Methyl Resonance.** In all sialosides which bound to BHA, the *N*-acetyl group of the sialic acid moiety exhibited a significant upfield chemical shift. Using eq 1a, we can extrapolate

$\Delta\delta_{B,pp}$ , the apparent upfield shift of the resonance in the state in which all of the ligand is bound to protein. The apparent bound chemical shifts calculated in this manner (Table II) were typically on the order of 2 ppm. The recent structure determination of BHA complexed with  $\alpha(2,6)$ - and  $\alpha(2,3)$ -sialyllactoses allows us to identify protein/ligand interactions that might account for this large chemical shift. Around the binding site residues, we constructed a surface (Richards, 1977) accessible to a probe of radius 1 Å, the approximate van der Waals radius for methyl hydrogens. For each point on this surface, we calculated the chemical shift which a methyl proton would experience due to protein ring currents (Johnson & Bovey, 1958; Perkins, 1982) and protein electrostatic charges (Buckingham, 1960). These calculations indicate that a methyl proton located directly over the six-membered ring of tryptophan 153 would experience a large (>2 ppm) upfield chemical shift due to the tryptophan ring current (the five-membered ring is not accessible). No other aromatic residue in the binding site is exposed in such a manner as to cause an upfield shift. Similarly, there are no positive electrostatic charges in the binding site that could cause such a shift. Even if histidine 183 were doubly protonated and therefore positively charged between pH 5 and pH 7, it would still be too far away to cause a large upfield shift with the Buckingham model. Other diamagnetic anisotropy effects (Schmalz et al., 1973) have been implicated in chemical shifts in some peptide systems (Sternlicht & Wilson, 1967; Clayden & Williams, 1982), but existing models predict effects that are also too small to account for our observed chemical shift. These considerations strongly suggest that the *N*-acetyl methyl group of sialic acid contacts the six-membered ring of tryptophan 153. This supports the model of  $\alpha$ -Neu5Ac, built into the 3 Å resolution electron density map (Weis et al., 1988), which also places the *N*-acetyl methyl group over tryptophan 153.

This interpretation of the upfield *N*-acetyl chemical shift appears to be reasonable even in view of the following factors. The protein extinction coefficient used [ $1.5 \text{ cm}^{-1} (\text{mg/mL})^{-1}$ ] is an estimate; Weis (1988) reported a value of  $0.95 \text{ cm}^{-1} (\text{mg/mL})^{-1}$  on the basis of Lowry and Bradford protein assays with BSA standards. The effect of using the latter value would be to divide all of the  $\Delta\delta_{B,pp}$ 's in Table II by a factor of 1.58, in which case the tryptophan ring current would still be the most likely explanation for the upfield chemical shift. In addition, Table II shows that the  $\Delta\delta_{B,pp}$ 's from duplicate titrations with the same protein/ligand combination are significantly different. This unexpected result might be explained if the number of competent binding sites per BHA molecule varied between different preparations of BHA. This prevents us from accurately comparing the apparent bound parameters in different titrations, but it in no way invalidates the equilibrium dissociation constants, since they derive from the *y* intercept and not the slope in eq 1. Finally, the apparent bound chemical shift  $\Delta\delta_{B,pp}$  depends on the dissociation rate of ligand from protein (Swift & Connick, 1962). However, this effect and the possibility that some of the protein is inactive would cause  $\Delta\delta_{B,pp}$  to be an underestimate of the true bound chemical shift, which is the proper quantity to compare with ring current calculations.

We note that the *N*-acetyl resonance is the only resonance that exhibited a large apparent bound chemical shift in the presence of BHA. The glycosidic methyl resonance of Neu5Ac $\alpha$ 2Me exhibited a downfield apparent bound shift of 0.3 ppm, but this value was too small to attribute to any structural feature of the binding site.

**Binding Affinities of Methyl Glycosides.** Of the methyl glycosides studied, only Neu5Ac $\alpha$ 2Me and Neu4,5Ac $_2\alpha$ 2Me bound to X-31 BHA with a measurable affinity (Table I). The other glycosides, Neu5Ac $\beta$ 2Me and Neu4,5,7Ac $_3\alpha$ 2Me, interacted too weakly with BHA for us to measure dissociation constants. Neu5Ac $\beta$ 2Me resonances are not broadened in the presence of protein, and unlike the  $\alpha$ -anomer, the *N*-acetyl resonance exhibits a small downfield chemical shift (0.002 ppm) which was concentration independent at the concentrations tested, 0.9–85 mM. These factors allow us to place a lower bound on the dissociation constant of 200 mM. This assumes that if Neu5Ac $\beta$ 2Me bound to protein, it would exhibit concentration-dependent line-broadening or chemical shift effects of about the same magnitude as those observed with Neu5Ac $\alpha$ 2Me. In Neu4,5,7Ac $_3\alpha$ 2Me, one of the acetyl groups (not assigned) exhibited a 0.002 ppm upfield shift which did not vary at concentrations from 0.2 to 15 mM, again indicating that the  $K_D$  is well above this range. In both cases, we were able to show that the protein contained competent binding sites by adding Neu5Ac $\alpha$ 2Me and observing perturbations of its resonances (not shown).

**Binding Affinities of Monosialylated Oligosaccharides.** Of the oligosaccharides studied, the  $\alpha(2,6)$ - and  $\alpha(2,3)$ -sialyllactose isomers possessed the simplest spectra; consequently, they yielded the most precise dissociation constants (Table I). Resonances on the Neu5Ac moiety of LSTa showed the same qualitative behavior as those of Neu5Ac $\alpha$ 2Me in the presence of protein, indicating that LSTa binds to X-31 BHA with approximately the same affinity. It was difficult to quantify the *N*-acetyl chemical shift, since the compound contained two overlapping acetyl resonances (Sabesan & Paulson, 1986). None of the  $G_{M1}$ -OS resonances were perturbed by protein, except the *N*-acetyl signal of Neu5Ac which showed a small upfield chemical shift of 0.0011 ppm, which did not change at ligand concentrations from 0.1 to 3.3 mM. The shift was diminished by addition of  $\alpha(2,6)$  sialyllactose, suggesting that  $G_{M1}$ -OS binds very weakly to X-31 BHA and is displaced from the binding site by the sialyllactose.

**Bound Line Widths Suggest That the Non Sialic Acid Portion (Asialo Portion) of a Sialoside Makes No Specific Protein Contacts.** In sialosides which bind to BHA [Neu5Ac $\alpha$ 2Me, Neu4,5Ac $_2\alpha$ 2Me,  $\alpha(2,6)$ -sialyllactose,  $\alpha(2,3)$ -sialyllactose,  $G_{D1a}$ -OS, LSTa, and DSL], resonances of protons on the sialic acid moiety exhibited significant line broadening in the presence of protein. Since the *N*-acetyl,  $H_{3ax}$ , and  $H_{3eq}$  resonances were resolved, we were able to estimate the apparent line broadening for the bound ligand ( $\Delta\Delta\nu_{B,pp}$ ). The value of  $\Delta\Delta\nu_{B,pp}$  was always on the order of 500 Hz. In the case of Neu4,5Ac $_2\alpha$ 2Me, the  $H_4$  resonance at 4.941 ppm was also resolved, and it exhibited similar line broadening. In contrast, proton resonances from the asialo portions of all of these molecules were much less broadened ( $\Delta\Delta\nu_{B,pp} < 100 \text{ Hz}$ ). This marked difference in line broadening is seen in the lactose resonances of  $\alpha(2,6)$ -sialyllactose (galactose,  $H_1$ ; glucose,  $H_{2'}$ ,  $H_{1'}$ , and  $H_{1''}$ ), shown in Figure 3, and also in all of the resolved resonances (Dorland et al., 1986; Sabesan & Paulson, 1986) from the neutral sugar moieties of  $\alpha(2,3)$ -sialyllactose,  $G_{D1a}$ -OS, LSTa, and DSL. The pattern of line broadening is consistent with the idea that the Neu5Ac moiety makes specific contacts with the protein surface and is thus restrained in position, while the asialo portion is more mobile. Restrained protons would experience more rapid dipole-dipole relaxation (Solomon, 1955) due to an increased rotational correlation time (Creighton, 1983) or due to interactions with protons on the protein.

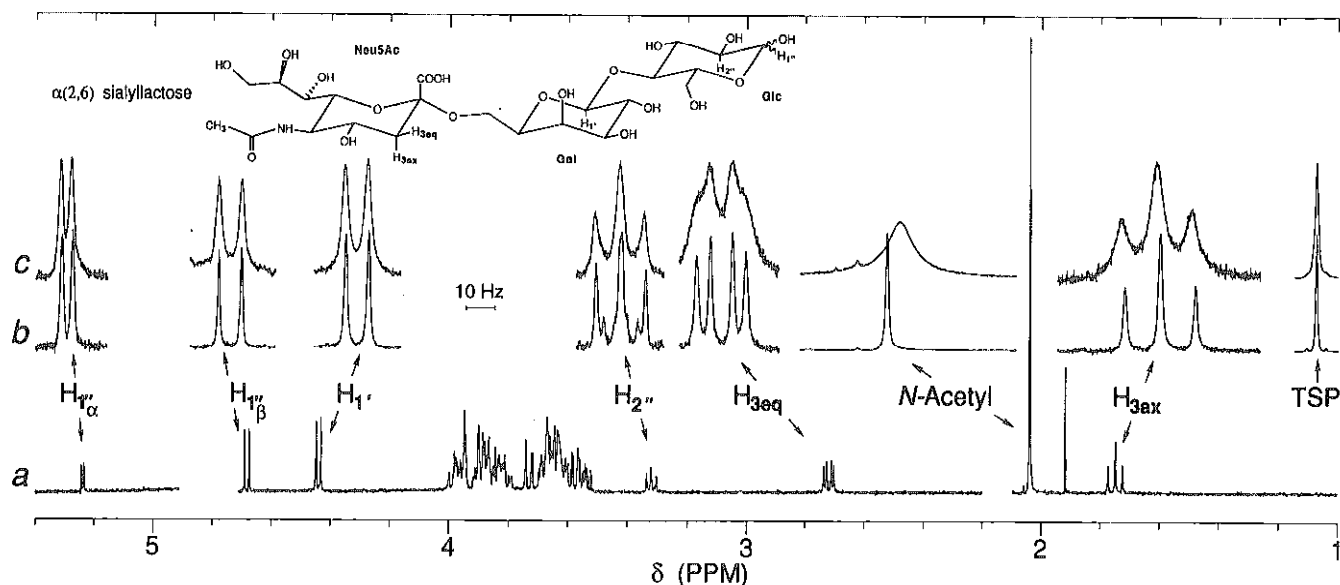


FIGURE 3: 500-MHz proton NMR spectra of  $\alpha(2,6)$ -sialyllactose free in solution (a and b) and in the presence of  $49 \mu\text{M}$  X-31 BHA (c). The concentration of  $\alpha(2,6)$ -sialyllactose in spectrum c is  $6.5 \text{ mM}$ ;  $0.6\%$  of the ligand is bound to protein. The glucopyranose moiety of sialyllactose exists as a mixture of two anomeric forms, consequently the  $H_{1''\alpha}$  and  $H_{1''\beta}$  signals have relative intensities of  $0.40$  and  $0.60$  proton (integrated areas compared to the *N*-acetyl resonance). Despite this anomeric mixture, proton signals on the Neu5Ac moiety appear as single resonances. Note the signal due to acetate ion at  $1.92 \text{ ppm}$ .

**Binding Affinities of Disialylated Oligosaccharides.** We studied two compounds,  $G_{D1a}$ -OS and DSL, which contained two Neu5Ac residues. In both cases, resonances from both Neu5Ac residues were perturbed by BHA. These results suggest that the bivalent compounds bind to BHA in two competing ways, corresponding to each of the Neu5Ac moieties filling the binding site. This interpretation is supported by several qualitative factors. First, our experiments with sialyllactose show that both  $\alpha(2,6)$ - and  $\alpha(2,3)$ -linked sialic acids bind to hemagglutinin, so it is likely that the two corresponding Neu5Ac residues of DSL also bind to BHA. Second, all of the perturbations observed are qualitatively similar to those observed with Neu5Ac $\alpha$ 2Me: the *N*-acetyl resonances broaden and shift upfield, and the  $H_{3ax}$  and  $H_{3eq}$  resonances broaden. Finally, in all of the monosialylated saccharides that we studied, none of the resonances of the asialo portion were significantly broadened. Therefore when a given Neu5Ac residue on a disialylated molecule contacts the hemagglutinin binding site, there is no reason to believe that this contact would cause line broadening in the resonances of the other Neu5Ac residue.

The binding of a bivalent ligand to a protein is described by two intrinsic dissociation constants, both of which are greater than the effective dissociation constant  $K_{D,eff}$  for the whole molecule [case II in Perkins et al. (1981)]. In the NMR experiments described here, we determine  $K_{D,eff}$  by plotting perturbations in resonances from either Neu5Ac residue according to eq 1. We calculate the intrinsic dissociation constants by considering the relative magnitudes of the perturbations from the two residues (see Figure 4 caption). Figure 4 shows that the terminal Neu5Ac residue of  $G_{D1a}$ -OS binds to X-31 BHA approximately 3 times tighter than the branched residue. Figure 5 shows that the  $\alpha(2,6)$ -linked residue of DSL binds more tightly to X-31 BHA than the  $\alpha(2,3)$ -linked residue, but that the order is reversed for binding to X-31/HS BHA.

**BHA Retains Sialic Acid Binding Activity in the Low-pH Conformation.** Neu5Ac $\alpha$ 2Me binds to the low-pH-induced form of X-31 BHA both at pH 4.9 and at pH 7.1 (Table I). The dissociation constants,  $2.2$  and  $2.4 \text{ mM}$ , respectively, are

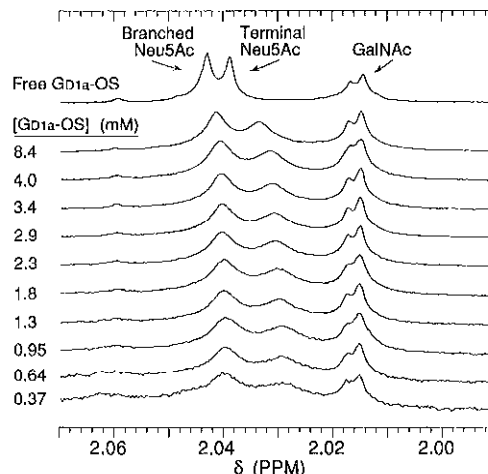
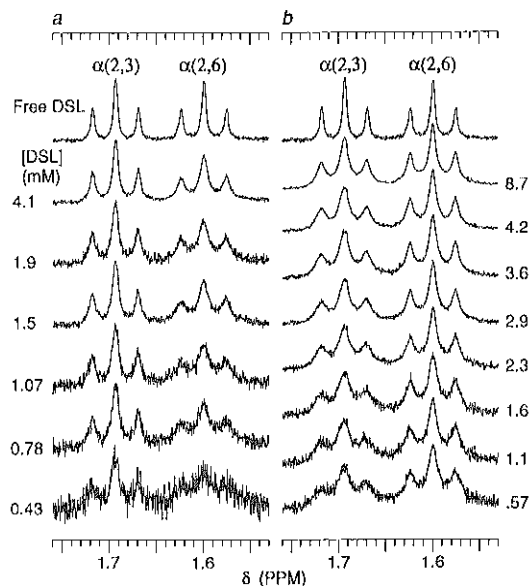


FIGURE 4: 500-MHz proton NMR spectra showing the *N*-acetyl methyl resonances of  $G_{D1a}$ -OS in the absence of protein (top trace) and in the presence of  $44 \mu\text{M}$  X-31 BHA. The fact that both Neu5Ac resonances are shifted suggests that there are two competing ways in which the ligand can bind to the same protein site, one way causing the branched *N*-acetyl to shift and the other way causing the terminal *N*-acetyl to shift. We can define two intrinsic dissociation constants for the system:  $K_b = [P][L]/[PL]_{\text{branched}}$  and  $K_t = [P][L]/[PL]_{\text{terminal}}$ , where  $[PL]_{\text{branched}}$  and  $[PL]_{\text{terminal}}$  are the concentrations of the protein/ligand complexes in which the branched and terminal *N*-acetyl resonances are shifted. A plot  $K_{D,eff} [L]_{TOT}$  vs  $1/\Delta\delta$  yields an overall effective dissociation constant  $K_{D,eff}$  given by  $1/K_{D,eff} = 1/K_b + 1/K_t$ . Estimates of  $K_{D,eff}$  from the terminal ( $11.4 \pm 2 \text{ mM}$ ) and branched ( $9 \pm 3 \text{ mM}$ ) *N*-acetyl resonances are within experimental error of each other. We can solve for the intrinsic dissociation constants,  $K_b$  and  $K_t$ , if we assume that the *N*-acetyl resonances experience identical true bound chemical shifts in the two respective binding modes (this assumption would be justified if the two binding modes corresponded to the two Neu5Ac moieties filling the binding pocket with congruent geometries). In this case,  $K_b/K_t = (\Delta\delta)_{\text{terminal}}/(\Delta\delta)_{\text{branched}} = 2.9 \pm 0.1$ , so that  $K_t \approx 15 \text{ mM}$  and  $K_b \approx 44 \text{ mM}$ . The value  $2.9$  was obtained by averaging the ratio in the preceding equation over all spectra in the series. Note that the glucopyranose moiety exists as a mixture of two anomeric forms; consequently, the *N*-acetyl resonance of the GalNAc moiety is split into two signals.

within experimental error of each other, and with the dissociation constant measured with protein in the native conformation. The apparent upfield bound chemical shift of the



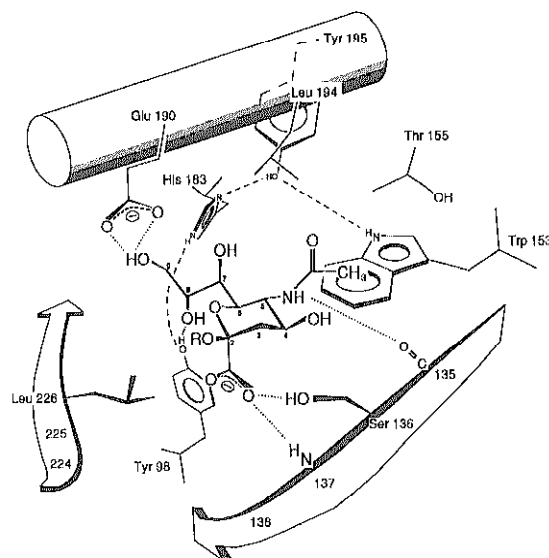
**FIGURE 5:** 500-MHz proton NMR spectra of the  $H_{3,ax}$  resonances of DSL in the presence of  $24 \mu\text{M}$  X-31 BHA (a) and  $32 \mu\text{M}$  X-31/HS BHA (b). Line broadening of the resonances corresponding to the  $\alpha(2,6)$ - and  $\alpha(2,3)$ -linked Neu5Ac moieties can be plotted independently according to eq 1b, yielding overall effective dissociation constants which are within experimental error of each other:  $1.2 \pm 0.2 \text{ mM}$  and  $1.5 \pm 0.7 \text{ mM}$ , respectively, for binding to X-31 BHA;  $4.2 \pm 1.1 \text{ mM}$  and  $6.6 \pm 1.5 \text{ mM}$ , respectively, for binding to X-31/HS BHA. The fact that resonances from both Neu5Ac moieties broaden suggests that there are two competing ways in which DSL can bind to the same protein site: one way causing broadening of the  $\alpha(2,6)$ -linked  $H_{3,ax}$  signal and the other causing broadening of the  $\alpha(2,3)$ -linked  $H_{3,ax}$  signal. We can define two intrinsic dissociation constants for the system,  $K_{\alpha(2,6)}$  and  $K_{\alpha(2,3)}$ , analogously to the case of  $G_{D1a}\text{-OS}$  (Figure 4). By making the same assumptions as in Figure 4, we can calculate the ratio of these intrinsic dissociation constants from the relative line broadening of the two resonances,  $K_{\alpha(2,6)}/K_{\alpha(2,3)} = (\Delta\Delta\nu)_{\alpha(2,3)}/(\Delta\Delta\nu)_{\alpha(2,6)}$ . By averaging this ratio in the spectra shown, we obtain  $K_{\alpha(2,6)}/K_{\alpha(2,3)} = 0.38 \pm 0.02$  for the binding of DSL to X-31 BHA and  $K_{\alpha(2,6)}/K_{\alpha(2,3)} = 1.6 \pm 0.2$  for the binding of DSL to X-31/HS BHA. The resulting intrinsic dissociation constants are shown in Table I next to the structural formula of DSL.

*N*-acetyl resonance is  $1.7 \pm 0.2 \text{ ppm}$  in both cases, also the same as with native protein.

## DISCUSSION

Sialic acids are the only components of cell-surface receptors known to be necessary for influenza virus attachment. Early experiments [reviewed in Gottschalk (1959)] showed that when sialic acid was enzymatically removed from cell surfaces, the cells were less susceptible to infection by influenza virus. Later, Pritchett et al. (1987) showed that the presence of soluble sialosides reduced the rate at which the A/Memphis/102/1972 virus attached to sparsely resialylated erythrocytes, indicating that the soluble sialosides were competing with the erythrocyte receptors for the hemagglutinin binding site. The present NMR experiments have allowed us to measure dissociation constants in equilibrium mixtures of soluble hemagglutinin and sialosides by observing perturbations of sialic acid resonances. Dissociation constants measured in this manner were the same when two different resonances were analyzed (Figure 1) and were constant over a wide range of protein saturations (Table I and Figure 2), demonstrating that binding is a simple bimolecular association.

In crystals of sialyllactose isomers complexed with BHA, Weis et al. (1988) found that the *N*-acetyl group of Neu5Ac is located over the indole ring of tryptophan 153. Our results confirm this model, since we observe a large upfield chemical



**FIGURE 6:** Schematic diagram of sialic acid bound to X-31 hemagglutinin, based on the crystal structure determined at a resolution of  $3 \text{ \AA}$  (Weis et al., 1988). Dotted lines indicate possible hydrogen bonds between sialic acid and hemagglutinin, while dashed lines show potential hydrogen bonds within the protein.

shift in the *N*-acetyl methyl resonance, presumably due to ring current effects. X-ray diffraction yielded no electron density corresponding to the lactose component of sialyllactose, possibly because the lactose was spatially disordered. None of our experiments with monosialylated oligosaccharides indicate that the asialo portion makes any specific contacts with protein, since the NMR resonances from this portion were not significantly broadened nor shifted in the presence of BHA.

Many different variants of the basic sialic acid moiety (Neu5Ac) exist in nature. We have synthesized the methyl  $\alpha$ -glycoside of the 4-*O*-acetylated moiety, which is found in equine species (Schauer, 1982). Our binding experiments indicate that Neu4,5Ac<sub>2</sub>α2Me binds to X-31 BHA with about the same affinity as Neu5Acα2Me. The 4-*O*-acetyl methyl resonance of Neu4,5Ac<sub>2</sub>α2Me is not broadened in the presence of protein, suggesting that the methyl group does not contact the protein. These results are in agreement with the three-dimensional model of sialic acid in the X-31 hemagglutinin binding site (Weis et al., 1988; diagrammed in Figure 6), in which the equatorial substituent at the carbon 4-position is pointing toward solution. Assuming that Neu4,5Ac<sub>2</sub>α2Me binds with approximately this orientation, we would not expect the acetyl group to make contact with the protein.

Our studies show that when an *O*-acetyl group is added to the 7-position of Neu4,5Ac<sub>2</sub>α2Me, the resulting molecule (Neu4,5,7Ac<sub>3</sub>α2Me) interacts very weakly (if at all) with X-31 BHA. The low binding affinity is consistent with the observation that, in the three-dimensional model of sialyllactose complexed with hemagglutinin, it is impossible to model a 7-*O*-acetyl group which does not overlap either with the leucine 194 side chain or with some of the remaining atoms of the sialic acid moiety. We also find that the  $\beta$ -anomer of Neu5Ac2Me interacts very weakly (if at all) with X-31 BHA. This confirms the result of Pritchett (1987) and Pritchett et al. (1987) that Neu5Acβ2Me does not significantly inhibit the attachment of viruses to sparsely resialylated erythrocytes, and it is consistent with the model (Figure 6), which suggests that if the 2-carboxylate is placed in the equatorial position, it cannot make favorable hydrogen bonds with the serine 136 hydroxyl and the main-chain NH group at position 137 (Weis et al., 1988). Thus, although we have found derivatives of

Neu5Ac $\alpha$ 2Me that have the same or reduced binding affinity for X-31 BHA, none of the derivatives assayed by NMR or by the method of Pritchett et al. (1987) binds significantly better.

Although there is no evidence that the asialo portions of sialylated molecules make specific interactions with hemagglutinin, previous reports show that the type of glycosidic linkage affects virus attachment to cells. Rogers et al. (1983) and Daniels et al. (1987) found that X-31 virus could agglutinate erythrocytes that were derivatized with a Neu5Ac $\alpha$ -(2,6)Gal linkage but not with a Neu5Ac $\alpha$ -(2,3)Gal linkage. The specificity was reversed in X-31/HS virus, which contains a single amino acid substitution, leucine 226 to glutamine. Pritchett (1987) found that attachment of X-31 virus to erythrocytes was preferentially inhibited by  $\alpha$ -(2,6)-sialyllactose, whereas attachment of X-31/HS virus was inhibited by  $\alpha$ -(2,3)-sialyllactose. Our NMR results show that, under equilibrium binding conditions, mixtures of soluble hemagglutinin and sialyllactose exhibit the same specificity (Table I). In addition, we performed a set of experiments on a saccharide, DSL, which contains both a 2,6- and a 2,3-linked Neu5Ac moiety. Our observations (Figure 5) suggest that both moieties independently occupy the hemagglutinin binding pocket, with the 2,6-linked moiety preferentially binding to X-31 BHA and the 2,3-linked moiety preferentially binding to X-31/HS BHA. The three-dimensional models of bound sialic acid (Weis et al., 1988) do not explain why the substitution at position 226 should change the binding specificity for the type of glycosidic linkage. Equally puzzling are the results from Daniels et al. (1987), which indicate that viruses with a 193 Ser to Arg mutation and viruses with a 224–230 deletion also change their linkage specificity when their hemagglutinating activity is compared to that of X-31 viruses.

It is striking that differences in binding affinities that we measure are relatively small. Under our equilibrium conditions, X-31 BHA prefers to bind to  $\alpha$ -(2,3)- over  $\alpha$ -(2,6)-sialyllactose by a factor of about 1.5, which corresponds to a free energy difference of only 0.25 kcal/mol. It is possible that further studies may indicate that the statistical cooperativity of many hemagglutinin molecules on a virus interacting with many sialic acids on a cell could reconcile these solution studies with those employing cell surfaces. We note that Roy et al. (1987, 1988) have synthesized polymers containing multiple sialic acid moieties, which were successful in inhibiting hemagglutination by influenza A.

Bergelson et al. (1982) found that gangliosides can be incorporated into the membranes of desialylated cells, whereupon the cells are newly susceptible to virus binding. When the agglutination activity of X-31 virus was measured, Suzuki et al. (1986) found that  $G_{M3}$  ganglioside was a more effective cell-surface receptor than  $G_{D1a}$  ganglioside and that  $G_{M1}$  ganglioside conferred almost no agglutinating activity. This specificity is reflected in our experiments with the oligosaccharides contained in those gangliosides.  $\alpha$ -(2,3)-Sialyllactose, which is the oligosaccharide component of  $G_{M3}$  ganglioside, binds to X-31 BHA with a  $K_D$  of 3.2 mM.  $G_{D1a}$ -OS has an overall effective  $K_D$  (Figure 4) of 11.4 mM, and  $G_{M1}$ -OS interacts too weakly (>40 mM) with X-31 BHA for us to precisely measure the binding affinity. Although we cannot offer an explanation for why  $G_{D1a}$ -OS binds less tightly than  $\alpha$ -(2,3)-sialyllactose on the basis of their molecular structures, there is a possible explanation for the low affinity of  $G_{M1}$ -OS. Sabesan et al. (1984) have proposed a conformation for  $G_{M1}$ -OS on the basis of nuclear Overhauser enhancement experiments and molecular mechanics calculations.

If the Neu5Ac moiety of their model is placed in the hemagglutinin binding site, the Gal $\beta$ (1,3)GalNAc portion of  $G_{M1}$ -OS overlaps with protein residues 224–226. Binding would only be possible if the saccharide adopted a conformation different from that of the proposed model, which might be energetically unfavorable. It is possible that the branched Neu5Ac of  $G_{D1a}$ -OS would be similarly constrained and that it too would possess a weak intrinsic binding affinity for BHA, compared to the terminal moiety. Indeed, the considerations in Figure 4 suggest that the branched Neu5Ac binds to BHA more weakly by a factor of 3, so that its intrinsic dissociation constant (44 mM) is of the same order of magnitude as the  $K_D$  of  $G_{M1}$ -OS.

Membrane-bound hemagglutinin acquires the ability to fuse membranes when the pH is lowered to 5.0. Skehel et al. (1982) found that BHA incubated at this pH becomes susceptible to tryptic cleavage, indicating that a conformational change has occurred. A number of studies have been interpreted to indicate that the membrane-distal domains of HA1 containing the sialic acid binding sites may dissociate during this conformational change (Skehel et al., 1982; Daniels et al., 1983; Yewdell et al., 1983; Webster et al., 1983; White & Wilson, 1987). Data in Table I indicate that BHA in the low-pH-induced conformation retains the ability to bind to the methyl  $\alpha$ -glycoside of Neu5Ac. It may therefore be possible to conduct ligand binding studies with a monomeric subfragment of the hemagglutinin, such as the HA1 polypeptide chain. However, it is possible that a subfragment without an intact trimer interface will not exhibit the same ligand specificity as the intact trimer. Crystallographic studies (Weis et al., 1988) show that the amino acid substitution 226 Leu  $\rightarrow$  Gln, which changes the binding specificity for glycosidic linkages, causes small structural changes which are transmitted across the subunit boundary. In addition, studies with modified erythrocytes (Daniels et al., 1987) showed that the glycosidic linkage specificity was changed by the amino acid substitution 218 Gly  $\rightarrow$  Glu, which is at the subunit boundary.

In future research, we hope to design and synthesize Neu5Ac derivatives with a higher affinity for hemagglutinin; these derivatives might be useful in antiviral therapy. As a first step toward accomplishing this objective, we have begun to establish the parts of the sialic acid group that are essential for binding. We know, for example, that the molecule must be in the  $\alpha$ -anomeric form. Since the 4-*O*-acetyl derivative binds as well as Neu5Ac $\alpha$ 2Me, it seems that the equatorial substituent at position 4 is unimportant. We are currently synthesizing a series of Neu5Ac derivatives in order to probe the contribution of the various substituents to the free energy of binding.

#### ACKNOWLEDGMENTS

We thank David Stevens for excellent technical assistance. We thank Dr. Reinhard Brossmer (Institut für Biochemie II, Universität Heidelberg) for gifts of  $\alpha$ -2-*O*-benzyl-*N*-acetylneuraminic acid and its corresponding methyl ester, which helped initiate this work. We also thank Dr. Yasuo Suzuki (Department of Biochemistry, School of Pharmaceutical Science, University of Shizuoka) for the generous gift of purified  $G_{D1a}$  ganglioside from which the oligosaccharide was prepared.

#### REFERENCES

- Bergelson, L. D., Bukrinskaya, A. G., Prokazova, N. V., Shaposhnikova, G. I., Kocharov, S. L., Shevchenko, V. P., Kornilava, G. V., & Pominova-Ageeva, E. V. (1982) *Eur. J. Biochem.* 128, 467–474.



- Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp 92-118, McGraw-Hill, New York.
- Brand, C. M., & Skehel, J. J. (1972) *Nature* 238, 145-147.
- Buckingham, A. D. (1960) *Can. J. Chem.* 38, 300-307.
- Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Vol. II, pp 380-381, W. H. Freeman, San Francisco.
- Clayden, N. J., & Williams, R. J. P. (1982) *J. Magn. Reson.* 49, 383-396.
- Creighton, T. E. (1983) *Proteins: Structures and Molecular Properties*, pp 182-185, W. H. Freeman, New York.
- Daniels, R. S., Douglas, A. R., Skehel, J. J., & Wiley, D. C. (1983) *J. Gen. Virol.* 64, 1657-1662.
- Daniels, R. S., Jeffries, S., Yates, P., Schild, G. C., Rogers, G. N., Paulson, J. C., Wharton, S. A., Douglas, A. R., Skehel, J. J., & Wiley, D. C. (1987) *EMBO J.* 6, 1459-1465.
- De Marco, A. (1977) *J. Magn. Reson.* 26, 527-528.
- Dorland, L., van Halbeek, H., & Vliegthart, J. F. G. (1986) *Carbohydr. Res.* 151, 233-245.
- Gottschalk, A. (1959) in *The Viruses* (Bumet, F. V. M., & Stanley, W. V. M., Eds.) Vol. 3, pp 51-61, Academic, New York.
- Johnson, C. E., & Bovey, F. A. (1958) *J. Chem. Phys.* 29, 1012-1014.
- Kronis, K. A., & Carver, J. P. (1982) *Biochemistry* 21, 3050-3057.
- Kuhn, R., Lutz, P., & MacDonald, D. L. (1966) *Chem. Ber.* 99, 611-617.
- Laemmli, U. K. (1970) *Nature* 227, 680-685.
- Ong, R. L., & Yu, R. K. (1984) *J. Neurosci. Res.* 12, 377-393.
- Paulson, J. C. (1985) in *The Receptors* (Conn, P. M., Ed.) Vol. 2, pp 131-219, Academic, Orlando, FL.
- Perkins, S. J. (1982) *Biol. Magn. Reson.* 4, 193-336.
- Perkins, S. J., Johnson, L. N., Phillips, D. C., & Dwek, R. A. (1981) *Biochem. J.* 193, 553-572.
- Pritchett, T. J. (1987) Ph.D. Thesis, University of California, Los Angeles.
- Pritchett, T. J., Brossmer, R., Rose, U., & Paulson, J. C. (1987) *Virology* 160, 502-506.
- Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng.* 6, 151-176.
- Rogers, G., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., & Wiley, D. C. (1983) *Nature* 304, 76-78.
- Roy, R., Laferrière, C. A., Gamian, A., & Jennings, H. J. (1987) *J. Carbohydr. Chem.* 6, 161-165.
- Roy, R., Gamian, A., & Chomik, M. (1988) in *Sialic Acids: Proceedings of the Japanese-German Symposium on Sialic Acids* (Schauer, R., & Yamakawa, T., Eds.) pp 264-265, Bärble Mende, Kiel, FRG.
- Sabesan, S., & Paulson, J. C. (1986) *J. Am. Chem. Soc.* 108, 2068-2080.
- Sabesan, S., Bock, K., & Lemieux, R. U. (1984) *Can. J. Chem.* 62, 1034-1045.
- Schauer, R. (1982) *Adv. Carbohydr. Chem.* 40, 132-235.
- Schmalz, T. G., Norris, C. L., & Flygare, W. H. (1973) *J. Am. Chem. Soc.* 95, 7961-7967.
- Skehel, J. J., & Schild, G. C. (1971) *Virology* 44, 396-408.
- Skehel, J. J., & Waterfield, M. D. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 93-97.
- Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A., & Wiley, D. C. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 968-972.
- Solomon, I. (1955) *Phys. Rev.* 99, 559-565.
- Sternlicht, H., & Wilson, D. (1967) *Biochemistry* 6, 2881-2892.
- Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K., & Nobusawa, E. (1986) *J. Biol. Chem.* 261, 17057-17061.
- Swift, T. J., & Connick, R. E. (1962) *J. Chem. Phys.* 37, 307-320.
- van der Vleugel, D. J. M., van Heeswijk, W. A. R., & Vliegthart, J. F. G. (1982) *Carbohydr. Res.* 102, 121-130.
- Verhoeyen, M., Fang, R., Jou, W. M., Devos, R., Huylebroeck, D., Saman, E., & Fiers, W. (1980) *Nature* 286, 771-776.
- Warren, L. (1959) *J. Biol. Chem.* 234, 1971-1975.
- Warren, L. (1963) *Methods Enzymol.* 6, 463-465.
- Webster, R. G., Brown, L. E., & Jackson, D. C. (1983) *Virology* 126, 587-599.
- Weis, W. (1988) Ph.D. Thesis, Harvard University.
- Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., & Wiley, D. C. (1988) *Nature* 333, 426-431.
- White, J. M., & Wilson, I. A. (1987) *J. Cell Biol.* 105, 2887-2896.
- Wiley, D. C., & Skehel, J. J. (1987) *Annu. Rev. Biochem.* 56, 365-394.
- Yewdell, J. W., Gerhard, W., & Bachi, T. (1983) *J. Virol.* 48, 239-248.

