Snyderman, R., Eds.) pp 129–137, Raven Press, New York.
Marcus, A. J. (1988b) in *Cecil Textbook of Medicine* (Wyngaarden, J. B., & Smith, L. H., Jr., Eds.) pp 1042–1059, W. B. Saunders, Philadelphia, PA.
Marcus, A. J., Weksler, B. B., Jaffe, E. A., & Broekman, M. J. (1980) *J. Clin. Invest. 66*, 979–986.
Marcus, A. J., Broekman, M. J., Safier, L. B., Ullman, H. L., Islam, N., Serhan, C. N., Rutherford, L. E., Korchak, H. M., & Weissmann, G. (1982) *Biochem. Biophys. Res. Commun. 109*, 130–137.
Marcus, A. J., Safier, L. B., Ullman, H. L., Islam, N., Broekman, M. J., Oglesby, T. D., & Gorman, R. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 903–907.
Marcus, A. J., Safier, L. B., Ullman, H. L., Islam, N., Broekman, M. J., & von Schacky, C. (1987) *J. Clin. Invest. 79*, 179–187.
Marcus, A. J., Safier, L. B., Ullman, H. L., Islam, N., Broekman, M. J., Falck, J. R., Fischer, S., & von Schacky, C. (1988) *J. Biol. Chem. 263*, 2223–2229.
Mathur, S. N., Field, F. J., Spector, A. A., & Armstrong, M. L. (1985) *Biochim. Biophys. Acta 837*, 13–19.
Nadler, J. L., Natarajan, R., & Stern, N. (1987) *J. Clin. Invest. 80*, 1763–1769.
Numano, F., Maezanva, H., Shurmato, T., & Adachi, K. (1976) *Ann. N.Y. Acad. Sci. 275*, 311–320.
Pomerantz, K. B., & Hajjar, D. P. (1989) *J. Lipid Res. 30*, 1219–1231.
Ross, R. (1981) *Arteriosclerosis 1*, 293–311.
Ross, R. (1986) *N. Engl. J. Med. 314*, 488–500.
Saito, H., Salmon, J. A., & Moncada, S. (1986) *Atherosclerosis, 61*, 141–148.
Sullivan, M. H. F., & Cooke, B. A. (1985) *Biochem. J. 232*, 55–59.
Tertov, V. V., Orekhov, A. N., Grigorian, E. Y., Korennaya, G. S., Kudryashov, S. A., Tkachuk, V. A., & Smirnov, V. N. (1987) *Tissue Cell 19*, 21–28.

Three Novel Oligosaccharides with the Sialyl-Le^a Structure in Human Milk: Isolation by Immunoaffinity Chromatography†

Hiroshi Kitagawa,‡ Hiroshi Nakada,§ Akira Kurosaka,§ Naoki Hiraiwa,‡ Yoshito Numata,‡ Shigeyuki Fukui,‡ Ikuo Funakoshi,‡ Toshisuke Kawasaki,‡ and Ikuo Yamashina*,‡

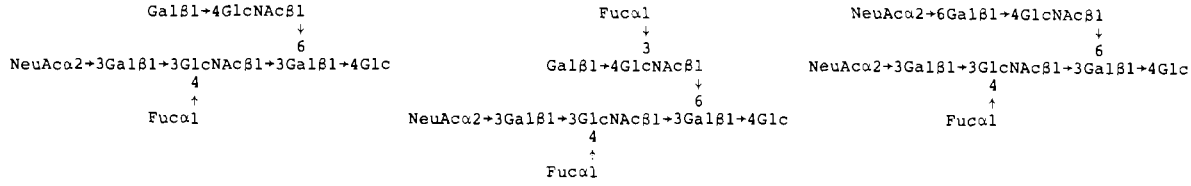
Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan, and Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, Kita-ku, Kyoto 603, Japan

Ichio Shimada and Fuyuhiko Inagaki

Department of Molecular Physiology, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan

Received May 2, 1989; Revised Manuscript Received June 29, 1989

ABSTRACT: We have determined the structures of three novel oligosaccharides isolated from human milk using the monoclonal antibody MSW 113. These oligosaccharides were purified by affinity chromatography on a column of the immobilized monoclonal antibody and by high-performance liquid chromatography. From the results of 500-MHz ^{1}H NMR spectroscopy and fast atom bombardment–mass spectrometry, their structures were deduced to be



These oligosaccharides bound to MSW 113 to nearly the same extent as sialyl-Le^a hexasaccharide but bound to another sialyl-Le^a structure-directed monoclonal antibody, NS 19-9, only weakly.

Many of the sialyloligosaccharides that occur in human milk have been found to correspond to the carbohydrate moieties of glycolipids and glycoproteins on cell surfaces. Therefore, they have been used as models for studies on the acceptor specificities of glycosyl transferases, the substrate specificities of glycosidases, and the structures of antigenic determinants (Kobata et al., 1968, 1969, 1972).

We have recently established several monoclonal antibodies directed toward mucin carbohydrates (Fukui et al., 1988; Kurosaka et al., 1987, 1988; Kitagawa et al., 1988a). During studies to determine the epitopic structure for one of these antibodies, MSW 113, we found that acidic oligosaccharides from human milk significantly inhibited the antigen–antibody reaction. By means of immunoaffinity chromatography with MSW 113, we were able to isolate the inhibitory oligosaccharides. One of the immunoreactive oligosaccharides was

† This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, by the Fugaku for Medicinal Research, and by a grant-in-aid from the Tokyo Biochemical Research Foundation.

* To whom correspondence should be addressed.
‡ Faculty of Pharmaceutical Sciences, Kyoto University.
§ Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University.

0006-2960/89/0428-8891$01.50/0

identified as a hexasaccharide with the sialyl-Le^a structure (Kitagawa et al., 1988b). This hexasaccharide was reported for the first time by Wieruszeski et al. (1985) and later briefly commented on by Smith et al. (1987). In this paper, we describe the isolation and identification of three immunoreactive oligosaccharides that had not been detected previously, either as free oligosaccharides in milk or as the carbohydrate moieties of glycolipids and glycoproteins.

Since the use of high-field ^{1}H NMR spectroscopy to determine oligosaccharide structures has been carefully developed by Vliegenthart and others (Hirdsgaul et al., 1982; Vliegenthart et al., 1983; Lamblin et al., 1984; Lönn, 1985a,b; Klein et al., 1988; Breg et al., 1988), this method was used to determine the structures of a set of closely related milk oligosaccharides like those isolated.

Materials and Methods

Materials. The human milk samples were gifts from the Department of Gynecology and Obstetrics, Faculty of Medicine, Kyoto University, Kyoto, and Hikone City Hospital, Hikone. A murine monoclonal antibody of the IgG_3 type, designated MSW 113 and established by Kitagawa et al. (1988a), was used. Another monoclonal antibody, NS 19-9 (Koprowski et al., 1979), of the IgG_1 type used was a gift from CENTOCOR EUROPE B.V., Leiden. DE 52 (microgranular, preswollen DEAE-cellulose) was purchased from Whatman Ltd., London. MSW 113–protein A–Sepharose CL-4B (6 mg of antibody/mL of resin) was prepared according to the method of Schneider et al. (1982). The column used for high-performance liquid chromatography (HPLC)[1] was of TSK-GEL NH_2-60, which was obtained from TOSOH Ltd., Tokyo. Sephadex G-25 and Sephadex G-50 were purchased from Pharmacia, Uppsala. Sialyl-Le^a hexasaccharide (sialyllacto-*N*-fucopentaose II) was prepared from human milk by affinity chromatography on a column of MSW 113–protein A–Sepharose CL-4B, as described previously (Kitagawa et al., 1988b).

Fractionation of Sialyloligosaccharides. Acidic oligosaccharides of human milk were prepared according to the method of Smith et al. (1978). The oligosaccharides obtained from 3 L of milk were dissolved in 50 mL of 2 mM acetic acid–pyridine buffer, pH 5.4, and the resultant solution was applied to a column (3.0 × 50 cm) of DE 52 previously equilibrated with the same buffer. The column was washed with the same buffer, and then the acidic oligosaccharides were eluted with 100 mM acetic acid–pyridine buffer, pH 5.4. The eluates were pooled and then lyophilized.

Affinity Chromatography. The dried material was dissolved in 20 mL of 50 mM phosphate buffer, pH 7.2, and the resultant solution was mixed with 20 mL of MSW 113–protein A–Sepharose CL-4B, which had been equilibrated with the same buffer. The resin was packed into a column and then washed with the same buffer. Antigens retained on the column were eluted with 50 mM diethylamine, pH 11.5. The eluates were neutralized with acetic acid, pooled, and then lyophilized. All procedures were carried out at 4 °C.

High-Performance Liquid Chromatography (HPLC). The oligosaccharides obtained by affinity chromatography were passed through a column of Sephadex G-25 equilibrated with 50 mM pyridine–acetic acid buffer, pH 5.0. The effluent was lyophilized. The oligosaccharides were further fractionated by HPLC, which was carried out with a Shimadzu Model LC-6A system on a TSK-GEL NH_2-60 column (4.6 × 25 cm). The initial solvent consisted of acetonitrile containing 20% 15 mM potassium phosphate, pH 5.2, the buffer concentration being subsequently increased to 100% over 80 min. The flow rate was 1.0 mL/min. Oligosaccharides were detected as to the absorbance at 195 nm. The oligosaccharides purified by HPLC were desalted by chromatography on Sephadex G-25 as described above.

500-MHz ^{1}H NMR Spectroscopy. NMR spectra of the sialyloligosaccharides corresponding to HPLC fractions 2–4 were measured with a JEOL JNM-GX500 500-MHz ^{1}H NMR spectrometer at 40 °C. NMR samples were dissolved in 150 μL of D_2O (99.96% CEA) in microcells, the pH of the sample solutions being ca. 7.0. Homogated decoupling was performed to suppress residual HDO resonance. The Gaussian function was applied for resolution enhancement of 1D spectra. For measurement of 2D homonuclear Hartmann–Hahn (2D HOHAHA) spectra (Bax & Davis, 1985), 64 scans were accumulated for each t_1, with a mixing time of 100 ms, and 512 × 2048 data points were used, with a spectral width of 3000 Hz and a relaxation delay of 1 s. The digital resolution was ca. 3.0 Hz/point in both dimensions, with zero-filling in the t_1 dimension. A phase-shifted sine bell was applied for both the t_1 and t_2 dimensions. 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard for chemical shifts.

Fast Atom Bombardment–Mass Spectrometry (FAB–MS). A few micrograms of an oligosaccharide mixed with 1 μL of triethanolamine was put on a stainless steel sample holder (1 × 5 mm) as the FAB ion source. The analysis was performed by bombardment with a neutral xenon beam, detecting negative ions with a mass spectrometer (JMS-HX 110; JEOL Ltd., Tokyo) equipped with a JMA-DA5000 computer system (JEOL).

Preparation of Glycopeptides from Human Colonic Cancer Cells. SW 1116 cells were treated with 1% Triton X-100 in 10 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl to solubilize cell membrane glycoproteins. The solubilized glycoproteins were then digested with Pronase P exhaustively, and the resulting glycopeptides were fractionated on Sephadex G-50 into mucin-type (G-50I) and serum-type (G-50II) glycopeptides according to the method of Funakoshi and Yamashina (1982).

Solid-Phase Radioimmunoassay. Antibodies were reacted with G-50I, coated on poly(vinylchloride) plates (Costar, Cambridge) according to the method of Fukui et al. (1988), and ^{125}I-labeled protein A (about 100000 dpm/12.5 ng) was added to the immune complex. The amount of antibodies bound was expressed as the radioactivity of bound ^{125}I-labeled protein A, as determined with a γ spectrophotometer (Gamma 5000; Beckman).

Other Materials. [^{125}I]NaI, carrier free, 16 mCi/mmol, was purchased from Amersham. Protein A (from *Staphylococcus aureus*) was from Nakarai Chemicals, Kyoto, and Pronase P (750000 units/g) from Kaken Kagaku Co., Tokyo. Preparation of ^{125}I-labeled protein A was carried out by the chloramine T method of Langone (1980), except that termination of the reaction was performed with saturated tyrosine.

Results

Isolation of Immunoreactive Oligosaccharides from Human Milk. The immunoreactive oligosaccharides of human milk were isolated by affinity chromatography on a MSW 113–protein A–Sepharose CL-4B column, as described previously

[1] Abbreviations: HPLC, high-performance liquid chromatography; 2D HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy; FAB–MS, fast atom bombardment–mass spectrometry; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine.

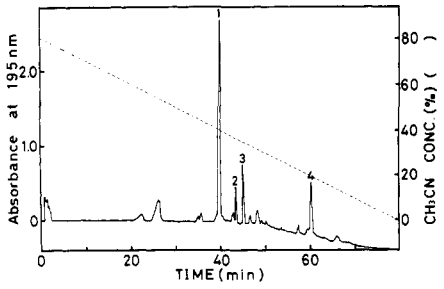


FIGURE 1: High-performance liquid chromatography of the immunoreactive oligosaccharides isolated from human milk by affinity chromatography on a MSW 113–protein A–Sepharose CL-4B column. The oligosaccharides were detected as to the absorbance at 195 nm.

(Kitagawa et al., 1988b). Only about 0.1% of the total sialic acid from human milk was retained and eluted. The eluate was further fractionated by HPLC on a column of TSK-GEL NH_2-60. As shown in Figure 1, the eluate gave one major peak accompanied by several minor peaks. The fractions corresponding to four peaks (peaks 1–4) were pooled, and then each pooled fraction was submitted to gel filtration on Sephadex G-25 to remove inorganic ions in the buffer used for the HPLC. The structure of the major peak material, fraction 1, was previously identified as sialyl-Le^a hexasaccharide (Kitagawa et al., 1988b). Fractions 2–4 were subjected to structural characterization as described below.

Structural Characterization of the Oligosaccharides by 500-MHz ^1H NMR Spectroscopy. Figure 2 shows the 500-MHz ^1H NMR spectra of fractions 2–4. The spectra were closely related to the spectrum of sialyl-Le^a hexasaccharide, reported previously (Kitagawa et al., 1988b), showing that these oligosaccharides have a common sialyl-Le^a structure.

In a previous paper, we reported that subspectra extraction for individual sugar components is helpful for analysis of the NMR spectra of glycolipids (Inagaki et al., 1987a,b). In the present study, we applied the 2D HOHAHA method (Bax & Davis, 1985) to extract the subspectrum of each sugar component of the oligosaccharides. The 2D HOHAHA spectrum of fraction 2 is shown in Figure 3a, which shows the cross-peak regions of the anomeric proton and other sugar proton resonances. Cross peaks due to magnetization transfer from the anomeric proton resonances were observed and are connected by horizontal lines in the figure. It should be noted that the magnetization transfer in the case of HOHAHA spectra is insufficient beyond H4 for galactose, so the H5 and H6 resonances of β-linked galactose were not detected in the present study. On the basis of the chemical shifts and splitting patterns observed in the 1D NMR spectrum (Figure 2a) together with those observed in the 2D HOHAHA spectrum (Figure 3a), the type and the anomeric configuration of each sugar component of fraction 2 were determined, as shown in Figure 2a and 3a. Fraction 2 was found to contain one β-linked glucose (Glcβ) residue, three β-linked galactose [Galβ(I), Galβ(II), and Galβ(III)] residues, two β-linked *N*-acetylglucosamine [GlcNAcβ(I) and GlcNAcβ(II)] residues, one α-linked fucose [Fucα(I)] residue, and one α-linked *N*-acetylneuraminic acid [NeuAcα(I)] residue. In a similar manner, 2D HOHAHA spectra were measured for fractions 3 and 4, and the sugar components were identified, as shown in Figure 2b, 3b, and 2c, 3c, respectively. On comparison with the 2D HOHAHA spectrum of sialyl-Le^a hexasaccharide (Kitagawa et al., 1988b), fraction 2 was found to contain one β-linked *N*-acetylglucosamine [GlcNAcβ(II)] residue and one β-linked galactose [Galβ(III)] residue in addition. Fractions 3 and 4 contain additionally one α-linked fucose [Fucα(II)] residue and one

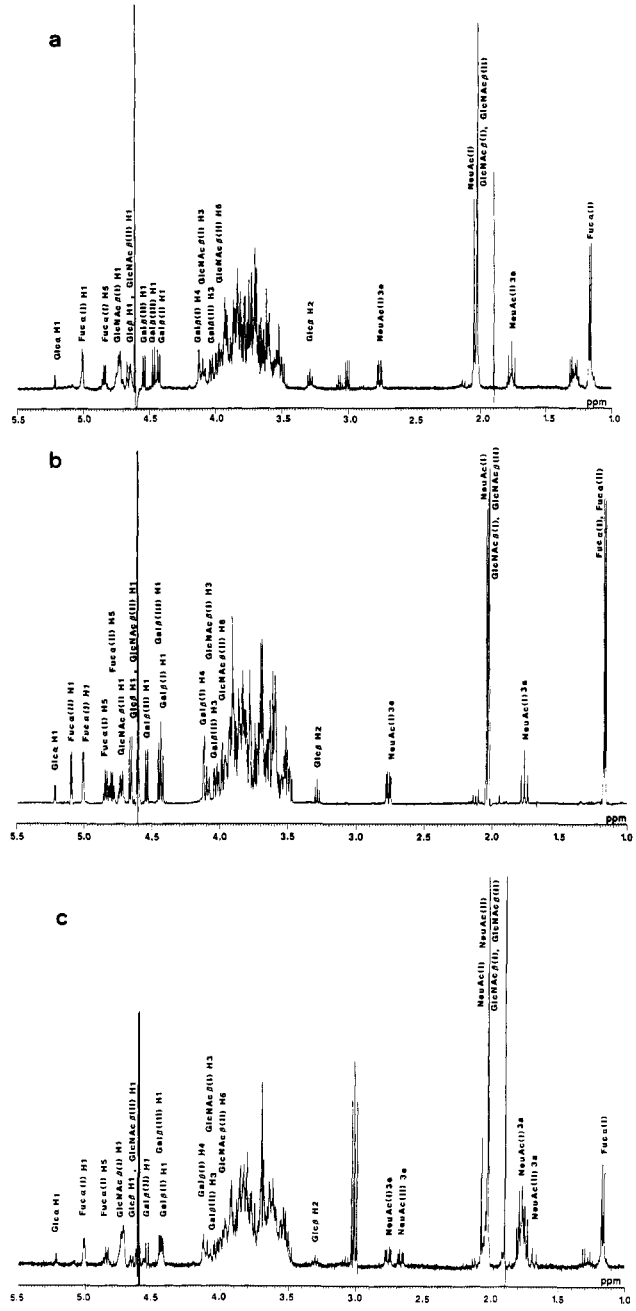


FIGURE 2: 500-MHz ^1H NMR spectra of the three oligosaccharides found in HPLC fractions (a) 2, (b) 3, and (c) 4; 300, 500, and 250 μg of fractions 2, 3, and 4, respectively, were dissolved in 150 μL of D_2O in microcells. Base-line correction was performed to remove the background around 4.7 ppm. Due to water suppression by homogated decoupling, the resonances around HDO were suppressed.

α-linked *N*-acetylneuraminic acid [NeuAcα(II)] residue, respectively, attached to the fracction 2 octasaccharide. In Table I, the chemical shifts of the structural reporter group protons of the constituent monosaccharides of sialyl-Le^a hexasaccharide and fractions 2–4 are summarized. The linkages of GlcNAcβ(II), Galβ(III), NeuAcα(II), and Fucα(II) in the oligosaccharides were subsequently examined on the basis of the 2D HOHAHA spectra by use of the structural reporter groups, as established by Vliegenthart et al. (van Halbeek et al., 1982; Vliegenthart et al., 1983; Lamblin et al., 1984; Klein et al., 1988; Breg et al., 1988). The H2, H3, and H4 resonances of GlcNAcβ(II) shifted downfield when Fucα(II) was attached to fraction 2 (see Figure 3a,b), suggesting that this fucose residue is attached to GlcNAcβ(II) in fraction 3. The chemical shift of Fucα(II) H1 in fraction 3 was 5.093 ppm,

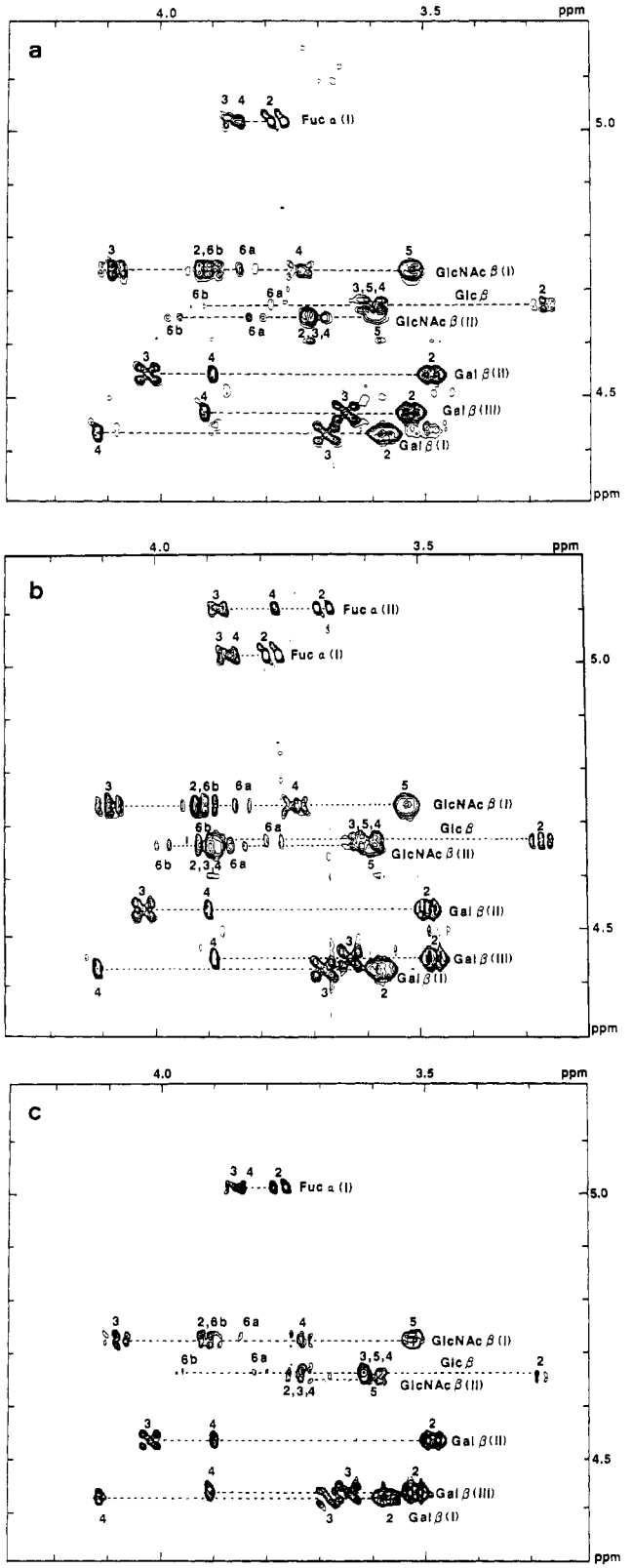
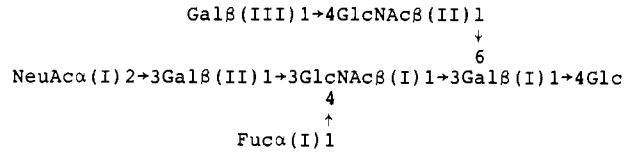


FIGURE 3: Cross-peak regions of anomeric proton resonances and other sugar proton resonances of 2D HOHAHA spectra of fractions (a) 2, (b) 3, and (c) 4. The mixing time was 100 ms. Cross peaks due to magnetization transfer from the anomeric proton resonances of individual sugar components are connected by horizontal lines with the assignments of cross peaks.

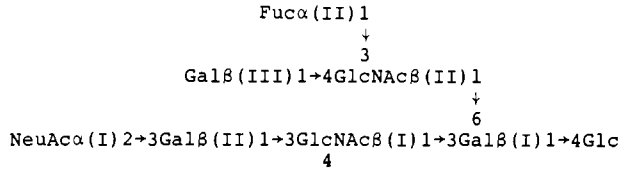
which is characteristic of the Fucα1→3 linkage (van Halbeek et al., 1982; Lamblin et al., 1984; van Halbeek, 1984; Klein et al., 1988; Breg et al., 1988). Moreover, the chemical shift of GlcNAcβ(II) H6b was observed at 3.994 ppm, which is consistent with the Galβ(III)(1→4)GlcNAcβ(II) linkage (van Halbeek et al., 1982; Lamblin et al., 1984; van Halbeek, 1984). Taking these observations together, it was concluded that fractions 2 and 3 comprise the groups Galβ(III)(1→4)-GlcNAcβ(II) and Galβ(III)(1→4)[Fucα(II)(1→3)]-GlcNAcβ(II), respectively, attached to sialyl-Le^a hexasaccharide.

The linkage between GlcNAcβ(II) and sialyl-Le^a hexasaccharide was then studied. All the sugar proton resonances of Glcβ and GlcNAcβ(I), and the H1–4 proton resonances of Galβ(I) and Galβ(II) in fractions 2–4 were found to be quite similar to those in sialyl-Le^a hexasaccharide. However, a significant upper field shift of H4 of Galβ(I), i.e., from 4.139 (sialyl-Le^a hexasaccharide) (Kitagawa et al., 1988b) to 4.123 (fraction 2), was observed, which indicates the presence of the GlcNAcβ(II)(1→6)Galβ(I) linkage in fraction 2, as pointed out by van Halbeek (1984). This linkage has been reported to occur in the neutral oligosaccharides from human milk (Kobata & Ginsburg, 1972; Yamashita et al., 1976; Tachibana et al., 1978). NeuAcα(II) is present in fraction 4. The chemical shifts of H3e and H3a of NeuAcα(II) (2.667 and 1.693 ppm, respectively) are characteristic of the α2→6 linkage (Vliegenthart et al., 1983). A small but significant difference was observed in the signal patterns between fractions 2 and 4 for Galβ(III) (Figure 3a,c), indicating the occurrence of the NeuAcα(II)(2→6)Galβ(III) linkage in fraction 4. It should be noted that the occurrence of Fucα(II)(1→3)GlcNAc(II) in fraction 3 also causes a similar downfield shift of H1 of Galβ(III). Thus, the structures of fractions 2–4 were deduced to be

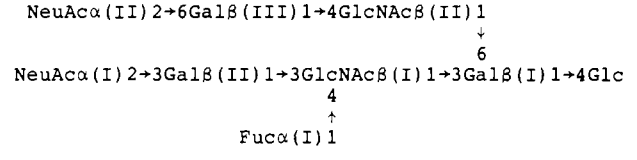
fraction 2



fraction 3



fraction 4



Analysis of the Oligosaccharides by Fast Atom Bombardment–Mass Spectrometry (FAB–MS). Negative ion FAB–MS (Figure 4) of the three oligosaccharides corresponding to HPLC fractions 2–4 gave molecular ions $(M - H)^-$ of 1508, 1654, and 1799, respectively. These results are consistent with the structures proposed on the basis of the results of 500-MHz ^{1}H NMR spectroscopy described above. However, it was not possible to obtain fragment ions. Bombardment of a reducing oligosaccharide with a xenon beam seems to result in random fragmentation of the whole oligosaccharide molecule.

Immunoreactivity of the Milk Oligosaccharides. By use of G-50I-coated assay plates, the immunoreactivities of the oligosaccharides with the antibodies MSW 113 and NS 19-9

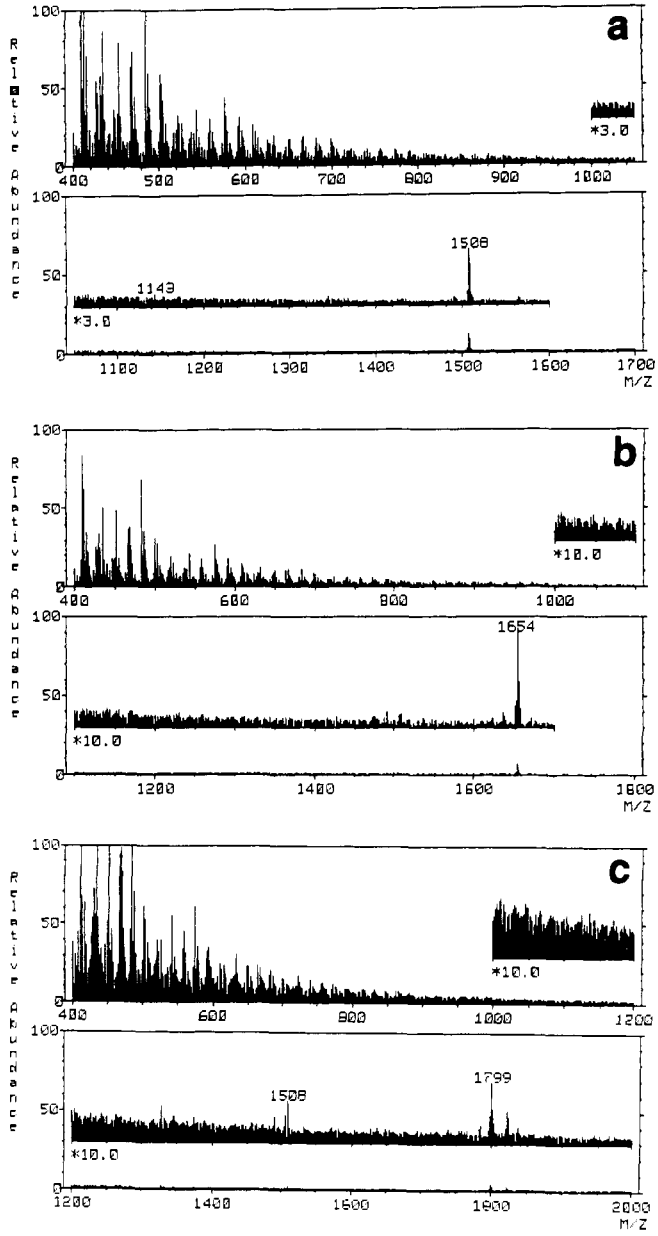


FIGURE 4: Negative ion fast atom bombardment–mass spectra of the three oligosaccharides found in HPLC fractions (a) 2, (b) 3, and (c) 4.

were compared with respect to their inhibitory activities toward the antigen–antibody reaction, as shown in Figure 5. Sialyl-Le^a hexasaccharide and the three novel oligosaccharides with the sialyl-Le^a structure reacted with MSW 113 to similar extents. In contrast, the reactivities of these three novel oligosaccharides with NS 19-9 were at least 30-fold less on a molar basis compared to sialyl-Le^a hexasaccharide.

Discussion

Many monoclonal antibodies that recognize cellular antigens associated with differentiation and malignant transformation are directed toward oligosaccharides constituting glycoproteins or glycolipids on the cell surface (Magnani, 1986). Isolation of these antigens generally requires laborious, multistep chemical and/or physical separation procedures, with monitoring of the immunological reactivities of the antigens at each purification step (Magnani et al., 1987). Recently, affinity purification of oligosaccharides from complex mixtures using monoclonal antibodies attached to a solid matrix was successfully achieved (Zopf et al., 1987; Kitagawa et al., 1988b; Dakour et al., 1988; Mårtensson et al., 1988). This includes our work, in which a monoclonal antibody, MSW 113, was successfully used for the isolation of an antigenic oligosaccharide from human milk. This work was further extended to the isolation of a family of novel oligosaccharides from pooled human milk.

Previously, we determined the structure of one of the oligosaccharides that reacted with MSW 113, on the basis of the results of 500-MHz ^{1}H NMR and FAB–MS analyses, which

Table I: Chemical Shifts (ppm) of Structural Reporter Group Protons of the Constituent Monosaccharides for Sialyl-Le^a Hexasaccharide and the Oligosaccharides Found in HPLC Fractions 2–4

		chemical shift in			
residue	^{1}H	sialyl-Le^a	2	3	4
Glcβ	H1	4.656	4.658	4.658	4.660
Galβ(I)	H1	4.434	4.424	4.426	4.428
	H4	4.139	4.123	4.118	4.122
GlcNAcβ(I)	H1	4.729	4.725	4.726	4.723
	H3	4.098	4.095	4.095	4.095
Galβ(II)	H1	4.539	4.537	4.536	4.537
	H3	4.032	4.031	4.031	4.031
Fucα(I)	H1	5.002	5.005	5.005	5.008
	H5	4.837	4.836	4.836	4.837
	H6	1.168	1.168	1.167	1.168
NeuAcα(I)	H3e	2.762	2.762	2.762	2.762
	H3a	1.761	1.760	1.763	1.762
GlcNAcβ(II)	H1		4.651	4.648	4.654
	H6b		3.984	3.994	3.989
Galβ(III)	H1		4.464	4.443	4.437
Fucα(II)	H1			5.093	
	H5			4.795	
	H6			1.167	
NeuAcα(II)	H3e				2.667
	H3a				1.693

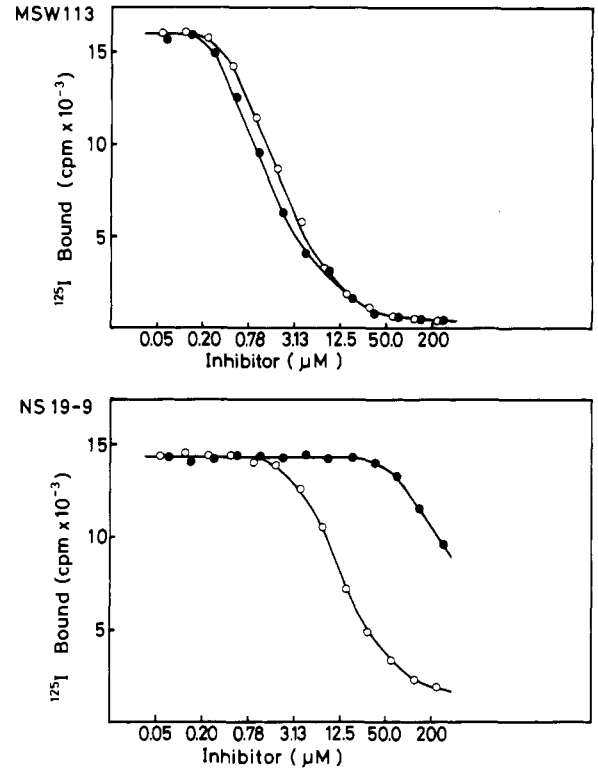
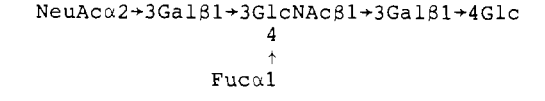


FIGURE 5: Reactivities with MSW 113 and NS 19-9 of the oligosaccharides with the sialyl-Le^a structure from human milk. (○) Sialyl-Le^a hexasaccharide (NeuAcα2→3Galβ1→3(Fucα1→4)-GlcNAcβ1→3Galβ1→4Glc); (●) novel oligosaccharides with the sialyl-Le^a structure (see the structures in the Abstract). The reactivities of these three oligosaccharides were indistinguishable.

led to its identification as sialyl-Le^a hexasaccharide.



The same procedure was applied to the determination of the structures of the three newly isolated oligosaccharides. The results showed unambiguously that they are novel oligosaccharides. All of them have the sialyl-Le^a structure, and one of them (fraction 3), interestingly, has both the sialyl-Le^a and Le^x structures. These structures have separately been found for mucin-type glycoproteins and glycolipids in a variety of human cancers (Yang & Hakomori, 1971; Magnani et al., 1982; Hakomori et al., 1984; Blaszczyk et al., 1984; Kitagawa et al., 1988a).

The amounts of these oligosaccharides in human milk vary widely among individuals. It is roughly estimated that 1 L of pooled milk contains about 1 mg of the MSW 113 reactive oligosaccharides. The amounts of the immunoreactive oligosaccharides found in HPLC fractions 1–4 (Figure 1) were estimated to be as follows: 1, 500–800 μg/L; 2, 50–100 μg/L; 3, 100–250 μg/L; and 4, 50–100 μg/L.

Koprowski et al. established a monoclonal antibody by injecting SW 1116 cells into mice and showed that the antibody is useful in the diagnosis of pancreas cancer inter alia (Herlyn et al., 1982; Ritts et al., 1984; Kitagawa et al., 1988a). The epitopic structure of this antibody, NS 19-9, was then determined to be sialyl-Le^a oligosaccharide by isolating sialyllacto-*N*-fucopentaosyl (II) ceramide (CA 19-9) (Magnani et al., 1982). Recently, a hexasaccharide with this determinant was isolated from human milk (Wieruszeski et al., 1985; Kitagawa et al., 1988b). Since our antibody, MSW 113, seemed to be directed toward the sialyl-Le^a structure (Kitagawa et al., 1988b), we compared the immunoreactivities of various oligosaccharides with these two antibodies, as reported in a previous paper (Kitagawa et al., 1988a). These antibodies differed in that NS 19-9 is directed more toward a fucose-containing internal structure (or Le^a structure), whereas MSW 113 is directed more toward a sialylated terminal structure. However, this difference did not appear to account for the fact that MSW 113 is far more reactive with mucin carbohydrates from SW 1116 cells compared to NS 19-9 (Kitagawa et al., 1988a), although they were similar with respect to the reactivity with glycolipids from the same cells. Thus, the marked difference in the reactivity with the novel oligosaccharides, fractions 2–4, between MSW 113 and NS 19-9 might account for the difference in their reactivities with mucin carbohydrates. The structures of the carbohydrate moieties of mucins from cancer cells remain to be investigated.

Acknowledgment

We thank Yurika Yakura and Tomoko Saito for their excellent secretarial assistance.

References

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–360.
Blaszczyk, M., Ross, A. H., Ernst, C. S., Marchisio, M., Atkinson, B. F., Pak, K. Y., Steplewski, Z., & Koprowski, H. (1984) *Int. J. Cancer 33*, 313–318.
Breg, J., van Halbeek, H., Vliegenthart, J. F. G., Klein, A., Lamblin, G., & Roussel, P. (1988) *Eur. J. Biochem. 171*, 643–654.
Dakour, J., Lundblad, A., & Zopf, D. (1988) *Arch. Biochem. Biophys. 264*, 203–213.
Fukui, S., Numata, Y., Kurosaka, A., Kitagawa, H., Nakada, H., Funakoshi, I., Kawasaki, T., Takahashi, Y., Hayashi, K., & Yamashina, I. (1988) *Gann 79*, 1119–1129.
Funakoshi, I., & Yamashina, I. (1982) *J. Biol. Chem. 257*, 3782–3787.
Hakomori, S., Nudelman, E., Levery, S. B., & Kannagi, R. (1984) *J. Biol. Chem. 259*, 4672–4680.
Herlyn, M., Sears, H. F., Steplewski, Z., & Koprowski, H. (1982) *J. Clin. Immunol. 2*, 135–140.
Hirdsgaul, O., Norberg, T., Le Pendu, J., & Lemieux, R. U. (1982) *Carbohydr. Res. 109*, 109–142.
Inagaki, F., Kohda, D., Kodama, C., & Suzuki, A. (1987a) *FEBS Lett. 212*, 91–97.
Inagaki, F., Kodama, C., Suzuki, M., & Suzuki, A. (1987b) *FEBS Lett. 219*, 45–50.
Kitagawa, H., Nakada, H., Numata, Y., Kurosaka, A., Fukui, S., Funakoshi, I., Kawasaki, T., & Yamashina, I. (1988a) *J. Biochem. 104*, 817–821.
Kitagawa, H., Nakada, H., Numata, Y., Kurosaka, A., Fukui, S., Funakoshi, I., Kawasaki, T., Shimada, K., Inagaki, F., & Yamashina, I. (1988b) *J. Biochem. 104*, 591–594.
Klein, A., Lamblin, G., Lhermitte, M., Roussel, P., Breg, J., van Halbeek, H., & Vliegenthart, J. F. G. (1988) *Eur. J. Biochem. 171*, 631–642.
Kobata, A., & Ginsburg, V. (1969) *J. Biol. Chem. 244*, 5496–5502.
Kobata, A., & Ginsburg, V. (1972) *J. Biol. Chem. 247*, 1525–1529.
Kobata, A., Grollman, E. F., & Ginsburg, V. (1968) *Arch. Biochem. Biophys. 124*, 609–612.
Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., & Fuhrer, P. (1979) *Somatic Cell Genet. 5*, 957–972.
Kurosaka, A., Fukui, S., Kitagawa, H., Nakada, H., Numata, Y., Funakoshi, I., Kawasaki, T., & Yamashina, I. (1987) *FEBS Lett. 215*, 137–139.
Kurosaka, A., Kitagawa, H., Fukui, S., Numata, Y., Nakada, H., Funakoshi, I., Kawasaki, T., Ogawa, T., Iijima, H., & Yamashina, I. (1988) *J. Biol. Chem. 263*, 8724–8726.
Lamblin, G., Boersma, A., Klein, A., Roussel, P., van Halbeek, H., & Vliegenthart, J. F. G. (1984) *J. Biol. Chem. 259*, 9051–9058.
Langone, J. J. (1980) *Methods Enzymol. 70*, 365–375.
Lönn, H. (1985a) *Carbohydr. Res. 139*, 105–113.
Lönn, H. (1985b) *Carbohydr. Res. 139*, 115–121.
Magnani, J. L. (1986) *Chem. Phys. Lipids 42*, 65–74.
Magnani, J. L., Brockhaus, M., Zopf, D., Steplewski, Z., Koprowski, H., & Ginsburg, V. (1982) *J. Biol. Chem. 257*, 14365–14369.
Magnani, J. L., Spitalnik, S. L., & Ginsburg, V. (1987) *Methods Enzymol. 138*, 195–207.
Mårtensson, S., Due, C., Pahlsson, P., Nilsson, B., Eriksson, H., Zopf, D., Olsson, L., & Lundblad, A. (1988) *Cancer Res. 48*, 2125–2131.
Ritts, R. E., Jr., Del Villano, B. C., Go, V. L. W., Herberman, R. B., Klug, T. L., & Zurawski, V. R., Jr. (1984) *Int. J. Cancer 33*, 339–345.
Schneider, C., Newman, R. A., Sutherland, D. R., Asser, V., & Greaves, M. F. (1982) *J. Biol. Chem. 257*, 10766–10769.
Smith, D. F., Zopf, D. A., & Ginsburg, V. (1978) *Anal. Biochem. 85*, 602–608.
Smith, D. F., Prieto, P. A., McCrumb, D. K., & Wang, W.-C. (1987) *J. Biol. Chem. 262*, 12040–12047.
Tachibana, Y., Yamashita, K., & Kobata, A. (1978) *Arch. Biochem. Biophys. 188*, 83–89.
van Halbeek, H. (1984) *Biochem. Soc. Trans. 12*, 601–605.
van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Hull, W. E., Lamblin, G., Lhermitte, M., Boersma, A., &

Roussel, P. (1982) *Eur. J. Biochem. 127*, 7–20.
Vliegenthart, J. F. G., Dorland, L., & van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.
Wieruszeski, J.-M., Chekkor, A., Bouquelot, S., Montreuil, J., Strecker, G., Peter-Katalinic, J., & Egge, H. (1985) *Carbohydr. Res. 137*, 127–138.
Yamashita, K., Tachibana, Y., & Kobata, A. (1976) *Biochemistry 15*, 3950–3955.
Yang, H.-J., & Hakomori, S. (1971) *J. Biol. Chem. 246*, 1192–1200.
Zopf, D., Schroer, K., Hansson, G., Dakour, J., & Lundblad, A. (1987) *Methods Enzymol. 138*, 307–313.

Structure and Protein Environment of the Retinal Chromophore in Light- and Dark-Adapted Bacteriorhodopsin Studied by Solid-State NMR†

Steven O. Smith,‡,§ Huub J. M. de Groot,‡,∥ Ronald Gebhard,⊥ Jacques M. L. Courtin,⊥ Johan Lugtenburg,⊥ Judith Herzfeld,∥ and Robert G. Griffin*,‡

Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, Department of Chemistry, Rijksuniversiteit te Leiden, 2300 RA Leiden, The Netherlands, and Department of Chemistry, Brandeis University, Waltham, Massachusetts 02254-9110

Received March 13, 1989; Revised Manuscript Received June 20, 1989

ABSTRACT: Our previous solid-state ^{13}C NMR studies on bR have been directed at characterizing the structure and protein environment of the retinal chromophore in bR_{568} and bR_{548}, the two components of the dark-adapted protein. In this paper, we extend these studies by presenting solid-state NMR spectra of light-adapted bR (bR_{568}) and examining in more detail the chemical shift anisotropy of the retinal resonances near the ionone ring and Schiff base. Magic angle spinning (MAS) ^{13}C NMR spectra were obtained of bR_{568}, regenerated with retinal specifically ^{13}C labeled at positions 12–15, which allowed assignment of the resonances observed in the dark-adapted bR spectrum. Of particular interest are the assignments of the ^{13}C-13 and ^{13}C-15 resonances. The ^{13}C-15 chemical resonance for bR_{568} (160.0 ppm) is upfield of the ^{13}C-15 resonance for bR_{548} (163.3 ppm). This difference is attributed to a weaker interaction between the Schiff base and its associated counterion in bR_{568}. The ^{13}C-13 chemical shift for bR_{568} (164.8 ppm) is close to that of the *all-trans*-retinal protonated Schiff base (PSB) model compound (~162 ppm), while the ^{13}C-13 resonance for bR_{548} (168.7 ppm) is ~7 ppm downfield of that of the 13-cis PSB model compound. The difference in the ^{13}C-13 chemical shift between bR_{568} and bR_{548} is opposite that expected from the corresponding ^{15}N chemical shifts of the Schiff base nitrogen and may be due to conformational distortion of the chromophore in the $C_{13}{=}C_{14}{-}C_{15}$ bonds. We have also obtained spectra of dark-adapted bR regenerated with retinal ^{13}C labeled at positions 1–4, 16, and 17. The chemical shifts of the ^{13}C-1 (34.5 ppm), ^{13}C-2 (42.7 ppm), ^{13}C-3 (18.6 ppm), and ^{13}C-4 (34.6 ppm) resonances in bR are very close to their values in the all-trans PSB model compound. This indicates that the perturbation previously observed near the β-ionone ring is localized near C-5. The spin–lattice relaxation times (T_1) of the ^{13}C-16,17 bR resonances of ~1.2 s are closer to the T_1's of 6-s-trans model compounds than to those of 6-s-cis model compounds, confirming the previous solid-state NMR determination of a 6-s-trans chromophore in the protein.

Absorption of light by the retinal chromophore in bacteriorhodopsin (bR)[1] initiates a cyclic photochemical reaction which drives the transport of protons across the cell membrane of *Halobacterium halobium* [for reviews, see Birge (1981) and Stoeckenius and Bogomolni (1982)]. The structure of bR's retinal prosthetic group and its interaction with specific binding site protein residues determine in large part its efficiency as a light-energy converter. Spectroscopic methods have revealed many of the details of the proton translocation process. Resonance Raman (Smith et al., 1985; Terner & El-Sayed, 1985; Stockburger et al., 1986) and FTIR spectroscopy (Engelhard et al., 1985; Dollinger et al., 1986; Braiman et al., 1987) have been used to study chromophore structural changes and protein protonation changes during the bR photocycle, while visible absorption studies of bR regenerated with dihydro derivatives of retinal have provided evidence for protein charges (or dipoles) near the β-ionone ring of the chromophore (Nakanishi et al., 1980; Lugtenburg et al., 1986; Spudich et al., 1986). Solid-state NMR spectroscopy has also been used to probe the structure of bR and has provided specific information on the chromophore's C_6–C_7 single-bond conformation and C=N double-bond configuration, as well as the location of specific protein perturbations in the retinal binding site

† This research was supported by the National Institutes of Health (GM-36810, GM-23289, RR-00995), the Netherlands Foundation for Chemical Research (SON), and the Netherlands Organization for the Advancement of Pure Research (ZWO). S.O.S. was supported by a USPHS postdoctoral fellowship (GM-10502).

‡ Massachusetts Institute of Technology.
§ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511.
∥ Brandeis University.
⊥ Rijksuniversiteit te Leiden.

[1] Abbreviations: bR, bacteriorhodopsin; bR_{568}, light-adapted bacteriorhodopsin; bR_{548}, 13-cis component of dark-adapted bacteriorhodopsin; MAS, magic angle spinning; PSB, protonated Schiff base; ppm, parts per million; SB, unprotonated Schiff base; TMS, tetramethylsilane.

0006-2960/89/0428-8897$01.50/0