Binding Characteristics of *N*-Acetylglucosamine-Specific Lectin of the Isolated Chicken Hepatocytes: Similarities to Mammalian Hepatic Galactose/*N*-Acetylgalactosamine-Specific Lectin†

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ABSTRACT: Binding characteristics of *N*-acetylglucosamine- (GlcNAc) specific lectin on the chicken hepatocyte surface were probed by an inhibition assay using various sugars and glycosides as inhibitors. Results indicated that the binding area of the lectin is small, interacting only with GlcNAc residues whose 3- and 4-OH's are open. The combining site is probably of trough-type, since substitution with as large a group as monosaccharide is permitted on the C-6 side of GlcNAc, and on the C-1 side, the aglycon of GlcNAc can be very large (e.g., a glycoprotein). These binding characteristics are shared with the homologous mammalian lectin specific for galactose/*N*-acetylgalactosamine, suggesting that tertiary structure of the combining area of these two lectins is similar. This is understandable, since there is ~40% amino acid sequence identitity in the carbohydrate recognition domain of these two lectins [Drickamer, K., Mannon, J. F., Binns, G., & Leung, J. O. (1984) *J. Biol. Chem. 259*, 770–778]. A series of glycosides, each containing two GlcNAc residues separated by different distances (from 0.8 to 4.7 nm), were synthesized. Inhibition assay with these and other cluster glycosides indicated that clustering of two or more GlcNAc residues increased the affinity toward the chicken lectin tremendously. Among the ligands containing two GlcNAc residues, the structure which allows a maximal inter-GlcNAc distance of 3.3 nm had the strongest affinity, its affinity increase over GlcNAc (monosaccharide) amounting to 100-fold. Longer distances slightly diminished the affinity, while shortening the distance caused substantial decrease in the affinity. Inhibition studies with reduced oligosaccharides and glycopeptides with multiterminal GlcNAc residues indicated that there is no increase in affinity at all when two GlcNAc residues are separated in space only by 0.8 nm. These results suggest that spatial arrangements of combining sites of the chicken and mammalian lectins are also quite similar.

Ever since its discovery nearly 20 years ago, the Gal/GalNAc-specific lectin (also known as asialoglycoprotein receptor) of mammalian liver has been the focus of many biochemical and cell biological studies. It has played a major role in our understanding of the steps involved in the receptor-mediated endocytosis [for a review, see Breitfeld et al. (1985)]. Though the physiological function of this lectin/receptor is not completely understood, the avidity by which this lectin binds and endocytoses desialylated proteins that carry complex-type, triantennary oligosaccharide chain(s) suggests that the clearance and catabolism of such aberrant serum glycoproteins may be its major function.

A homologous lectin/receptor that recognizes GlcNAc instead of Gal/GalNAc exists in avian livers (Lunney & Ashwell, 1976; Kawasaki & Ashwell, 1977). Though the subunit of chicken lectin is considerably smaller (26 kDa) than those of mammalian counterparts (40–60 kDa), all share remarkable homology in (1) the domain construct of polypeptide chain and primary structure in the carbohydrate recognition domain (Drickamer et al., 1984), (2) the hexameric subunit organization (Loeb & Drickamer, 1987; Halberg et al., 1987), (3) the increase in binding affinity by clustering of sugars (Lee, Y. C., & Lee, R. T., 1982), and (4) the cellular localization and endocytotic process (Kuhlenschmidt et al., 1984; Breitfeld et al., 1985).

In this paper we report syntheses and binding affinity of a series of divalent GlcNAc ligands, which have different intersugar distance. As in the case of the mammalian hepatic lectin (Lee, R. T., et al., 1984), binding affinity increased tremendously upon proper clustering of two and three GlcNAc residues. There is an optimal inter-GlcNAc distance for affinity enhancement, and this distance appears to be similar to that observed for the mammalian Gal/GalNAc binding systems. These and other results suggest that the tertiary structure in the carbohydrate recognition domain and the quaternary arrangement of binding sites of the mammalian and chicken hepatic lectins are quite similar.

Materials and Methods

The following compounds were obtained from the indicated sources: ethanolamine, 1-hydroxybenzotriazole hydrate, 1,3-dicyclohexylcarbodiimide (DCC),[1] *N*-(ethoxycarbonyl)-2-

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[1] Abbreviations: DCC, 1,3-dicyclohexylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; BSA, bovine serum albumin; MDE medium, modified Dulbecco's Eagle medium; AE, aminoethyl; AH, aminohexyl; DMR, *N,N*-dimethylformamide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; $GlcNAc_x$-AI-BSA, BSA containing on the average x residues of GlcNAc-$SCH_2C(=NH)$- linked to the amino group of BSA; $sugar_x$-AD-BSA, BSA containing x residues of sugar-$SCH_2CONH(CH_2)_2$- linked to the amino group of BSA.

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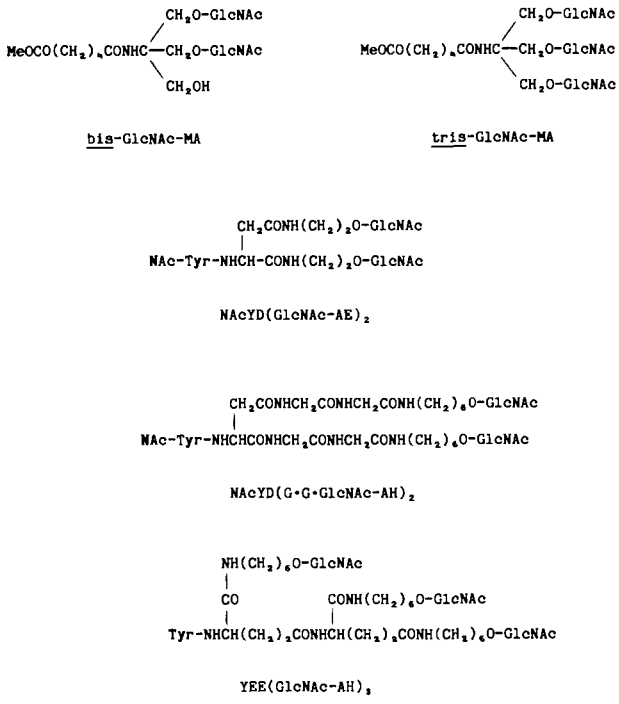
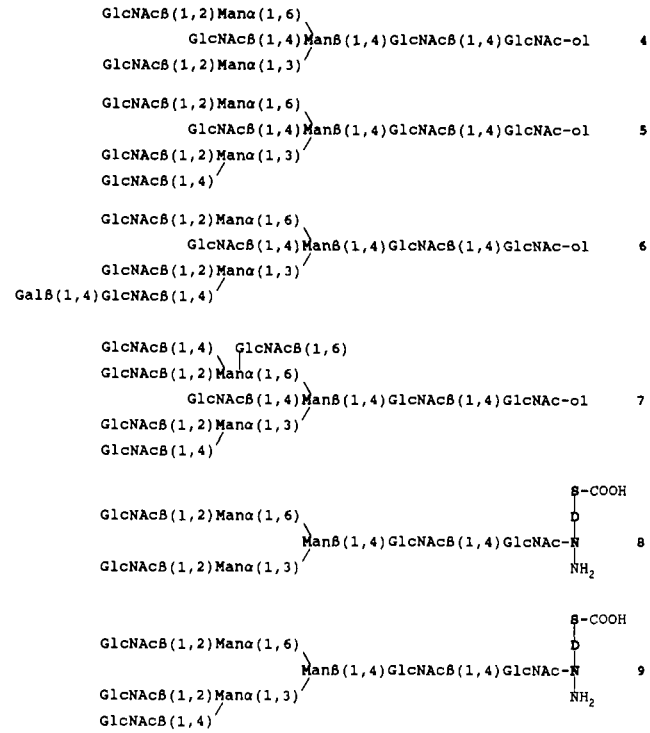
Chart I



ethoxy-1,2-dihydroquinoline (EEDQ), and 1,12-dibromododecane from Aldrich; *N,N'*-diacetylchitobiose, *N,N',N''*-triacetylchitotriose, *N*-acetyl-α-D-glucosamine 1-phosphate, and *N*-acetyl-D-glucosamine 6-phosphate from Sigma; *N*-benzyloxycarbonylglycylglycine from Research Organics; benzyl chloroformate from Fluka; β-galactosidase (EC 3.2.1.23) from bovine testis from Boehringer-Mannheim. Preparation of GlcNAc glycosides has been reported [allyl α- and β- (Lee & Lee, 1974); 6-[*N*-(trifluoroacetyl)amino]hexyl α- (Lee et al., 1986); 6-[*N*-(trifluoroacetyl)amino]hexyl β- and 6-aminohexyl β- (Weigel et al., 1979); and cyanomethyl β-thio- (Lee et al., 1976)] as has preparation of (2-acetamido-2-deoxy-β-D-glucopyranosylthioglycolyl)aminoacetaldehyde dimethyl acetal (Lee & Lee, 1979a). The following disaccharides and their benzyl glycosides were prepared as described: Galβ(1,3)GlcNAc and Galβ(1,6)GlcNAc (Flowers, 1978); Galβ(1,4)GlcNAc (Johnson et al., 1975; Lee & Lee, 1979b); GlcNAcβ(1,6)Gal (Lee, R. T., & Lee, Y. C., 1982). Preparations of *N*-(trifluoroacetyl)glucosamine, a glycoside of 2-phthalimido-2-deoxyglucosamine, 6'-[*N*-(trifluoroacetyl)amino]hexyl 2-acetamido-2,6-dideoxy-β-D-glucopyranoside, and 6'-[*N*-(trifluoroacetyl)amino]hexyl 2-acetamido-2-deoxy-α-D-xylopyranoside will be described elsewhere. 1,12-Dithio-GlcNAc-dodecane [GlcNAc-S$(CH_2)_{12}$S-GlcNAc] was prepared by reacting 1,12-dibromododecane with 1-deoxy-1-thio-GlcNAc by the method previously described (Chipowsky & Lee, 1973). Preparation of bis- and tris-GlcNAc-MA (see Chart I) (Lee, 1978) and *N*-acetyl-L-tyrosyl-L-aspartic acid (NAcYD) and its derivative [NAcYD$(G)_2$] with a glycine residue attached to each of the carboxylic acid groups (Lee & Lee, 1987) has been reported. Two types of neoglycoproteins based on bovine serum albumin (BSA) were prepared: the AI-type BSA derivatives from thiosugar imidates (Lee et al., 1976) and the AD-type BSA derivatives by reductive alkylation using an ω-aldehydoglycoside (Lee & Lee, 1980).

General schemes for preparing iodinatable mono-, di-, and trivalent flexible cluster glycosides have been described (Lee, R. T., et al., 1984; Lee & Lee, 1987). The corresponding GlcNAc cluster ligands were prepared similarly, except that

Chart II

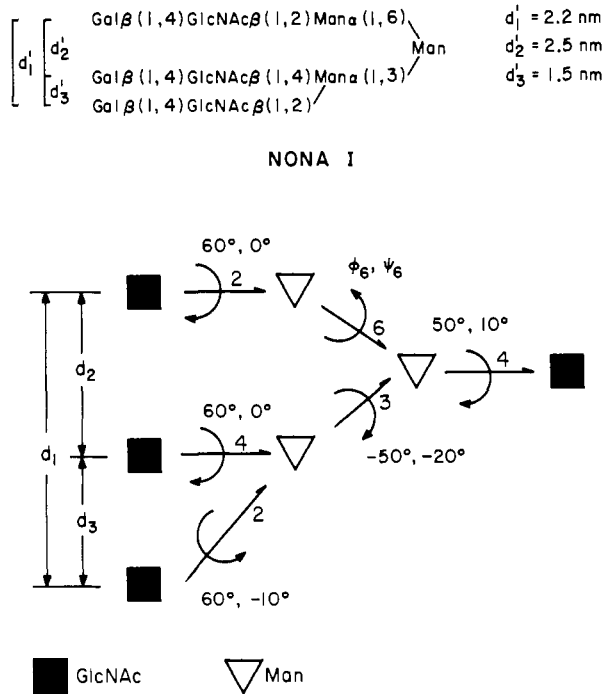


the method of Johnson and Coward (1987) was used for the conjugation of carboxylic acid and amino groups. An example of this method will be described under Results.

Preparation of reduced oligosaccharides **4**–**7** (Chart II) from hen riboflavin-binding protein will be described elsewhere (Piskarev et al., 1989). Bi- and triantennary, GlcNAc-terminated glycopeptides (**8** and **9**, Chart II) were prepared from the corresponding Gal-terminated glycopeptides by exhaustive treatment with β-galactosidase from bovine testis (Distler & Jourdian, 1973). The preparation of Gal-terminated bi- and triantennary glycopeptides from bovine fetuin will be described elsewhere (K. G. Rice, N. B. N. Rao, and Y. C. Lee, manuscript in preparation). Each of the Gal-terminated glycopeptides (2 μmol) in 25 μL of a buffer (0.1 M NaH_2PO_4–0.1 M citric acid, pH 4.3) was incubated with 100 milliunits of the β-galactosidase for 24 h at 37 °C, and for an additional 48 h with fresh enzyme (50 milliunits). The resulting GlcNAc-terminated glycopeptides were purified on a preparative C-8 RP-HPLC column (Phase Separations, Inc., Norwalk, CT) (2 × 25 cm) which was eluted with 5 mM phosphoric acid. The major 210-nm absorbing material eluting at 18 min (**8**) or 24 min (**9**) was collected, neutralized to pH 6 with dilute NaOH, and concentrated and desalted on a column (1.5 × 30 cm) of Sephadex G-10 in water. Glycopeptides **8** and **9** were characterized by monosaccharide compositional analysis (see below) and by ^{1}H NMR spectroscopy as described by Rice et al. (manuscript in preparation). Only Man and GlcN were obtained in the monosaccharide analysis, and the ^{1}H NMR spectra were consistent with the structures shown (Chart II). Analytical reverse-phase HPLC and anion-exchange HPLC on Dionex-BioLC as well as ^{1}H NMR spectra indicated both **8** and **9** to be >95% homogeneous.

Thin-layer chromatography (TLC) was performed on silica gel G-60 (F-254) precoated on an aluminum sheet (E. Merck). Solvent systems used are as follows: solvent A, 1:1 (v/v) toluene–ethyl acetate; solvent B, 1:4 (v/v) toluene–ethyl acetate; solvent C, 9:4:2 (v/v/v) ethyl acetate–2-propanol–water; solvent D, 8:2:1 (v/v/v) ethyl acetate–acetic acid–water; solvent E, 3:2:1 (v/v/v) ethyl acetate–acetic acid–water. After

Chart III



a thorough drying, plates were first examined under a UV lamp and then either sprayed with 15% sulfuric acid in 50% ethanol and charred at ~140 °C or sprayed with 0.2% ninhydrin in 95% ethanol and heated briefly for detection of the amino group. Melting points were determined with a Fisher-Johns apparatus, and elemental analyses were carried out by Galbraith Labs., Inc. (Knoxville, TN). Analysis of monosaccharides was carried out by high-performance anion-exchange chromatography using pulsed amperometric detector as described by Hardy et al. (1988). Radioiodination of $GlcNAc_{35}$-AI-BSA was carried out by using the chloramine T method (Greenwood et al., 1963) with 1 mCi of carrier-free $Na^{125}I$ (Amersham Corp.). Radioactivity was measured with a Packard Auto-gamma counter. ^1H NMR spectra were obtained with a Varian XL 400 spectrometer.

Empirical energy calculations of the terminal heptasacchride of **9** (Chart III) were carried out by the hard-sphere exoanomeric (HSEA) method (Lemieux & Bock, 1983). The energy terms included nonbonded, electrostatic, exoanomeric, and hydrogen-bond energy functions with parameters used by Cumming and Carver (1987). For all linkages except the $\alpha(1,6)$, ϕ and ψ were varied within ±30° of the minima for the corresponding disaccharide linkages. For the $\alpha(1,6)$ linkage, ϕ was varied by ±30°, while ψ was varied from 70° to 210° for rotamers of $\omega = -60°$ and $\omega = 180°$. A rotamer with $\omega = 60°$ was energetically unfavorable. Computations were carried out on a microVax II computer with VMS operating system. The glycosidic torsion angles are defined as follows: $\phi = \chi(H1\text{-}C1\text{-}O1\text{-}Cx)$ and $\psi = \chi(C1\text{-}O1\text{-}Cx\text{-}Hx)$ were x is the aglycon. For the $\alpha(1,6)$ linkage, $\psi = \chi(C1\text{-}O1\text{-}C6\text{-}C5)$ and $\omega = \chi(H5\text{-}C5\text{-}C6\text{-}O6)$.

Chicken hepatocytes were isolated by the collagenase perfusion method (Seglen, 1976) as described before (Kuhlenschmidt et al., 1982). Hepatocyte preparations were typically 85–90% viable, as determined by trypan-blue exclusion. Inhibition assay for the estimation of binding affinity of a ligand toward GlcNAc-specific lectin on the chicken hepatocyte surface was performed as described for the Gal/GalNAc-lectin of mammalian hepatocytes (Connolly et al., 1982). The incubation mixture contained in 1 mL of MDE ~7×10^{-11} M

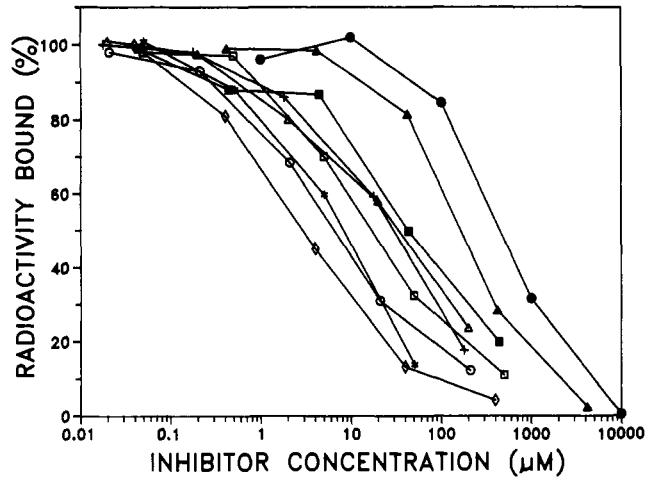


FIGURE 1: Inhibition curves of GlcNAc and various divalent GlcNAc ligands. Inhibition assays were carried out as described in the text, and ^{125}I-$GlcNAc_{35}$-AI-BSA bound to chicken hepatocytes (percent of the control tube without inhibitor) was plotted against inhibitor concentration. Inhibitors used were (●) GlcNAc, (*) dithio-GlcNAc-dodecane, (▲) bis-GlcNAc-MA, (■) NAcYD(GlcNAc-AE)$_2$, (□) NAcYD(GlcNAc-AH)$_2$, (◇) YD(G-GlcNAc-AH)$_2$, (○) NAcYD(G-GlcNAc-AH)$_2$, (△) NAcYD(G-G-GlcNAc-AH)$_2$, (+) NAcYD(G-G-G-GlcNAc-AH)$_2$.

^{125}I-$GlcNAc_{35}$-AI-BSA, 7–15 million chicken hepatocytes, and a test inhibitor at various concentrations. Incubation was carried out in 5-mL polyethylene tubes which were rotated in an end-over-end fashion at 2 rpm in an ice–water bath. After 2 h, 200-μL duplicate aliquots were placed on top of the oil mixture (4:1, silicon oil–mineral oil) in 0.4-mL microfuge tubes and centrifuged for 5 min at full speed in a Beckman microfuge. The bottom of the tube which contained the cell pellet was clipped off and counted. Nonspecific binding was assessed by including 50 mM GlcNAc in the incubation. Counts were converted to percent of the counts associated with cell pellet in the absence of inhibitor, and these values were plotted agaist inhibitor concentration as shown in Figure 1. The concentration which caused 50% reduction in bound radioactivity (I_{50}) was obtained from such a plot. Under the described conditions of incubation the I_{50} value closely approximates the K_D value (Jacobs et al., 1975).

Results

Preparation of 2′-Aminoethyl 2-Acetamido-2-deoxy-β-D-glucopyranoside (GlcNAc-AE). 2-[*N*-(Benzyloxycarbonyl)-amino]ethanol (**1**) was prepared by reacting benzyl chloroformate with ethanolamine as described for the analogous 6-aminohexanol derivative (Chipowsky & Lee, 1973). The product was extracted into chloroform, and after evaporation of chloroform, it was crystallized from ethyl acetate–hexane, yielding crystalling product in 49% yield; mp 54 °C, homogeneous by TLC in solvent A (R_F = 0.18) when visualized by UV absorption and by charring (light brown).

Anal. Calcd for $C_{10}H_{13}NO_3$ (195.21): C, 61.52; H, 6.71; N, 7.18. Found: C, 62.03; H, 6.91; N, 7.24.

Formation of the glycosidic linkage between GlcNAc and **1** and eventual production of GlcNAc-AE were carried out as reported for the preparation of 6′-aminohexyl 2-acetamido-2-deoxy-β-D-galactopyranoside (Lee et al., 1986). The scheme involves (1) production of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-α-glucopyranosyl chloride, "acetochloro-GlcNAc", from GlcNAc (Horton, 1972); (2) Königs–Knorr-type coupling of "acetochloro-GlcNAc" with **1** and purification of the product, 2′-[*N*-(benzyloxycarbonyl)-amino]ethyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-

glucopyranoside (**2**), on a column (5 × 190 cm) of Sephadex LH-20 in 95% ethanol; (3) de-O-acetylation in 10 mM NaOMe in dry methanol to produce 2′-[*N*-(benzyloxycarbonyl)amino]ethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**3**); and (4) hydrogenolysis (Brown & Brown, 1966) using 10% Pd/C as catalyst to obtain the product, GlcNAc-AE. In the present reaction sequence, **2** was obtained in crystalline form from 95% ethanol: mp 169–172 °C, homogeneous by TLC in solvent B (R_f = 0.15) when visualized by UV absorption and by charring with sulfuric acid.

Anal. Calcd for $C_{24}H_{32}H_2O_{11}$ (524.52): C, 54.75; H, 6.15; N, 5.34. Found: C, 55.23; H, 6.17; N, 5.35.

After de-O-acetylation of **2**, **3** was obtained as a gelatinous solid from 100% ethanol: mp 182–184 °C, homogeneous by TLC in solvent C (R_f = 0.54). Hydrogenolysis of **3** produced the final product, GlcNAc-AE, which was crystallized from 95% ethanol: mp 190–191 °C, homogeneous by TLC in solvent E (R_f = 0.22) when examined by charring and ninhydrin staining (orange). The ^{1}H NMR spectrum was consistent with the proposed structure and indicated the anomeric configuration of GlcNAc to be β (4.52 ppm, J = 8.49 Hz).

Anal. Calcd for $C_{10}H_{20}N_2O_6 \cdot {}^1/_2H_2O$ (273.29): C, 43.94; H, 7.75; N, 10.25. Found: C, 44.78; H, 7.76; N, 10.28.

Preparation of GlcNAc-O(CH$_2$)$_6$NHCOCH$_2$NHCOCH$_2$NH$_2$ (GlcNAc-AH·G·G). GlcNAc-AH (1.1 equiv) was coupled to *N*-benzyloxycarbonylglycylglycine (1 equiv) with the help of EEDQ (1.15 equiv) in 70% ethanol at ~45 °C overnight. The product, GlcNAc-AH·G·G·Z, which crystallized out upon cooling, was filtered and recrystallized from hot 80% ethanol: mp 193–194 °C, homogeneous by TLC in solvent D (R_f = 0.18) and solvent E (R_f = 0.66) was visualized by UV absorption and charring with sulfuric acid. Concentration of the filtrate produced more solid, which was crystallized similarly. Combined yield of crystalline product was 36%. Acid hydrolysis followed by monosaccharide analysis of this crystalline product yielded 106% of the expected amount of GlcN. Hydrogenolysis of this product in 60% ethanol using 10% Pd/C in a micro Brown hydrogenator produced GlcNAc-AH·G·G in 95% yield. The product, which was homogeneous by TLC in solvent E (R_f = 0.19) by charring and ninhydrin staining, was recrystallized from 95% ethanol–ethyl ether; mp 182–183 °C.

Anal. Calcd for $C_{18}H_{34}N_4O_8$ (434.48): C, 49.76; H, 7.89; N, 12.90. Found: C, 49.54; H, 7.77; N, 12.69.

General reaction schemes for the preparation of divalent GlcNAc glycosides having varying inter-GlcNAc distances are shown in reactions 1–4. The shortest homologue was prepared

$$\text{NAcYD} + 2\text{GlcNAc-AH} \rightarrow \text{NAcYD(GlcNAc-AH)}_2 \quad (1)$$

$$\text{NAcYD} + 2\text{GlcNAc-AH·G·G} \rightarrow \text{NAcYD(G·G·GlcNAc-AH)}_2 \quad (2)$$

$$\text{NAcYD(G)}_2 + 2\text{GlcNAc-AH} \rightarrow \text{NAcYD(G·GlcNAc-AH)}_2 \quad (3)$$

$$\text{NAcYD(G)}_2 + 2\text{GlcNAc-AH·G·G} \rightarrow \text{NAcYD(G·G·G·GlcNAc-AH)}_2 \quad (4)$$

by using GlcNAc-AE instead of GlcNAc-AH in reaction 1. The two longest homologues were prepared by substituting GlcNAc-AH·G·G for GlcNAc-AH (reactions 2 and 4). Formation of the amide linkage was accomplished by using in situ activation of carboxylic acid with 1-hydroxybenzotriazole and DCC (Johnson & Coward, 1987). Preparation of NAcYD(GlcNAc-AH)$_2$ (reaction 1) will be detailed below.

N-Acetyl-L-tyrosyl-L-aspartic acid (NAcYD) (1.2 mmol, 440 mg) and 1-hydroxybenzotriazole (2.4 mmol, 324 mg) were dissolved in 5 mL of dry DMF and cooled in ice. DCC (2.57 mmol, 530 mg) was added, and the mixture was stirred in an ice–NaCl mixture (−10 °C). DCC dissolved rapidly to form initially a clear solution which gradually became turbid. After 1 h, a solution of GlcNAc-AH (2.57 mmol, 820 mg) in Me_2SO and *N*-methylmorpholine (2.4 mmol, 265 μL) was added to the mixture. The mixture was stirred in ice–water for $^1/_2$ h and then stirred overnight at room temperature. The precipitate (precipitate 1) copiously formed was filtered and washed with cold DMF. To the filtrate was added enough toluene till opacity persisted. After overnight storage, the precipitate (precipitate 2) was filtered and washed with ether. Both precipitate 1 and precipitate 2 contained mainly the desired product, NAcYD(GlcNAc-AH)$_2$ (R_f = 0.35 in solvent E). Precipitate 1, which contained a high-R_f, UV-absorbing material, was suspended in an ethanol–ethyl acetate (1:1) mixture, stirred overnight, and filtered. Both precipitate 2 and washed precipitate 1 were stirred in 10 mL of 50% ethanol for several hours. The insoluble contaminant, 1,3-dicyclohexylurea, was filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in hot 90% ethanol, from which the product came out as a gelatinous solid in 71% yield. ^{1}H NMR data were consistent with the structure: σ (in D_2O) 1.3 (8 H, CCH_2C), 1.45 and 1.50 (8 H, CCH_2C), 1.99 (s, 3 H, COCH_3 of Tyr), 2.03 (s, 6 H, COCH_3 of GlcNAc), 2.6 (m, 2 H, β-H of Asp), 2.97 (d, 2 H, β-H of Tyr), 3.12–3.92 (m, ring protons and CH_2O), 4.49 (d, 2 H, anomeric H, J = 8.31 Hz), 4.55 (t, 1 H, α-H of Asp), 6.83 and 7.13 (both d, 4 H, aromatic H). The cluster glycosides shown in reactions 2–4 were prepared similarly.

In this coupling method, conjugation of the carboxylic acid and the amino group was essentially complete with only 7% excess of the amino compound, producing only disubstituted desired products and nearly no monosubstituted products. Thus, it was unnecessary to purify the product by gel filtration. In contrast, the coupling was not complete, even with 50% excess of the amino group over the carboxylic acid, when the chloroformate method (Lee & Lee, 1987) or EEDQ method (Lee, R. T., et al., 1984) was used for similar preparations. All the cluster glycosides were characterized by UV absorption of tyrosyl group and GlcN content to establish the validity of structure. The results are shown in Table I.

Various sugars, simple glycosides, cluster glycosides, multibranched oligosaccharides, and glycopeptides, as well as neoglycoproteins were used as inhibitors in the inhibition assay. In general, at least two independent determintions were made, and the average I_{50} values are shown in Tables II–IV. Variation in I_{50} values was less than 2-fold, and the deviation is indicated when multiple determinations were available.

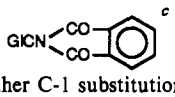
The following conclusions can be made from the results presented in Table II. (1) The hierarchy of the inhibitory potency of monosaccharides (GlcNAc ≫ ManNAc > Man, Glc > L-Fuc ≫ Gal, GalNAc) is the same as reported for the Triton-solubilized, purified chicken hepatic lectin (Kuhlenschmidt & Lee, 1984). (2) When C-1 is substituted, the presence of aglycon does not improve affinity. The fact that the negatively charged phosphate group increased the affinity while an amino group had the reverse effect suggests that the lectin may have positively charged group(s) in this vicinity. (3) In C-2 substitution, an equatorial acetamido group is essential for strong affinity. Paradoxically, the axial acetamido group (i.e., ManNAc) also contributed somewhat to the binding. Since Glc, Man, and 2-deoxy-Glc all had similar I_{50} values, both equatorial and axial OH groups make negligible contributions to the binding. Replacement of an equatorial

Table I: Characterization of New GlcNAc-Containing Ligands

compound	mol wt	expected content[a] Tyr[b]	GlcN[c]	GlcN/Tyr (mol/mol)
monovalent				
NAcY(GlcNAc-AH)$_1$	525.58	99	102	1.03
divalent				
NAcYD(GlcNAc-AE)$_2$	830.84	98.6	105	2.13
NAcYD(GlcNAc-AH)$_2$	943.08	98.6	100.4	2.04
YD(G·GlcNAc-AH)$_2$	1015.28	100[d]	110	2.2
NAcYD(G·GlcNAc-AH)$_2$	1057.18	103	105.6	2.05
NAcYD(G·G·GlcNAc-AH)$_2$	1171.38	101	101.3	2.01
NAcYD(G·G·G·GlcNAc-AH)$_2$	1285.48	105	105.5	2.01
trivalent				
YEE(GlcNAc-AH)$_3$	1345.56	100[d]	103	3.09

[a] Expected content of Tyr and GlcN in each solution was calculated on the basis of the molecular weight of the proposed structure. [b] Tyr content was calculated from absorbance at 278 nm, using a molar absorption value of 1330. [c] GlcN was determined by anion-exchange chromatography of acid-hydrolyzed samples as described by Hardy et al. (1988). [d] Since these compounds were obtained as amorphous solid, the concentration was calculated on the basis of Tyr content and this value was set as 100.

Table II: I_{50} Values of Various Sugars and Glycosides

compound	I_{50} (mM)
monosaccharide	
GlcNAc	0.44 ± 0.06
ManNAc	~12
Glc	~130
Man	~120
L-Fuc	~200
2-deoxy-Glc	~130
Gal	NI[a]
GalNAc	NI[a]
simple glycoside	
-OCH$_2$CH=CH$_2$ (α)	0.44
-OCH$_2$CH=CH$_2$ (β)	0.20
-O(CH$_2$)$_6$NHCOCF$_3$ (α)	0.51
-O(CH$_2$)$_6$NHCOCF$_3$ (β)	0.69
-O(CH$_2$)$_6$NH$_2$ (β)	1.4
-SCH$_2$C≡N	0.44
-SCH$_2$CONHCH$_2$CH(OMe)$_2$ (β)	0.39
Gal substitution at various positions	
Galβ(1,3)GlcNAc	NI[b]
Galβ(1,3)GlcNAcα-OCH$_2$Ph	NI[b]
Galβ(1,4)GlcNAc	NI[b]
Galβ(1,6)GlcNAc	2.0
Galβ(1,6)GlcNAcα-OCH$_2$Ph	0.6
substitution of C-2 N	
GlcNHCOCF$_3$	2.5
 [c]	NI at 10
other C-1 substitution	
GlcNAcβ(1,6)Gal	0.44
GlcNAcβ(1,4)GlcNAc	0.62
GlcNAcβ(1,4)GlcNAc-β(1,4)GlcNAc	0.40
GlcNAc α-1-P	0.04 ± 0.003
C-5 substitution	
6-deoxy-GlcNAcβ-OR[d]	0.28
XylNAcα-OR[d]	1.0 ± 0.2
GlcNAc-6-P	0.6

[a] Not inhibitory at 80 mM. [b] Not inhibitory at 20 mM. [c] Tested as a β-glycoside, -SCH$_2$CONHCH$_2$CH(OMe)$_2$. [d] R = 6-[*N*-(trifluoroacetyl)amino]hexyl.

N-acetyl group with an *N*-trifluoroacetyl group weakened the affinity somewhat, while replacement with a phthalimido group abolished the binding. (4) A bulky substituent at both C-3 and C-4 is not permitted. (5) When C-5 is substituted, removal of this substituent altogether [i.e., XylNAc-O-(CH$_2$)$_6$NHCOCF$_3$] weakened the binding somewhat, while replacement of 6-OH with H [i.e., 6′-[*N*-(trifluoroacetyl)-amino]hexyl 2-acetamido-2,6-dideoxy-β-D-glucopyranoside] increased the affinity. A bulky substituent on 6-OH [e.g., Galβ(1,6)GlcNAc, GlcNAc-6-P] did not affect the binding affinity much, even when the substituent was negatively charged.

Table III: I_{50} of GlcNAc-Containing Cluster Glycosides

compound	I_{50} (μM)	inter-GlcNAc distance (nm)[a] parallel	maximum
monovalent			
GlcNAc	440		
NAcYD(GlcNAc-AH)$_1$	500		
divalent			
bis-GlcNAc-MA	160	NP[b]	0.8
NAcYD(GlcNAc-AE)$_2$	40 ± 20	0.8	1.8
1,12-dithio-Glc-NAc-dodecane	8	1.2	2.04
NAcYD(GlcNAc-AH)$_2$	15 ± 3	1.3	2.65
YD(G·GlcNAc-AH)$_2$	3.0 ± 0.4	2.1	3.3
NAcYD(G·GlcNAc-AH)$_2$	9.0 ± 2.5	2.1	3.3
NAcYD(G·G·GlcNAc-AH)$_2$	28	2.5	4.0
NAcYD(G·G·G·GlcNAc-AH)$_2$	32	2.9	4.7
trivalent			
tris-GlcNAc-MA	85	NP	0.8
YEE(GlcNAc-AH)$_3$	0.17	2.2, 2.4, 2.7	2.7, 3.3, 3.4

[a] Fisher–Herschfelder–Taylor atomic models were used to determine the approximate distances between the GlcNAc residues. Maximum: when the molecule is stretched out in a near-straight line with two GlcNAc residues at the opposite end pointing in the opposite direction. Parallel: approximate maximal separation of two GlcNAc residues when they are oriented parallel, pointing in the same direction. [b] Not possible.

Table IV: I_{50} of GlcNAc-Terminated Oligosaccharides and Glycopeptides

compound[a]	I_{50} (μM)
4, (GlcNAc)$_3$(Man)$_3$-X[b]	~200[d]
5, (GlcNAc)$_4$(Man)$_3$-X	~300[d]
6, (Gal)(GlcNAc)$_4$(Man)$_3$-X	~500[d]
7, (GlcNAc)$_6$(Man)$_3$-X	≫500
8, (GlcNAc)$_2$(Man)$_3$-Y[c]	10
9, (GlcNAc)$_3$(Man)$_3$-Y	12

[a] See Chart II for complete structures. [b] X: -GlcNAc-GlcNAc-ol. [c] Y: -(GlcNAc)$_2$-Asn-Asp-Ser. [d] Due to the limited amounts available, complete inhibition curves could not be obtained. I_{50} values werre obtained by extending the partial inhibition curves.

Table III shows that clustering of two and three GlcNAc residues could bring about as much as 100- and 3000-fold increase in affinity, respectively. Results of inhibition assay using divalent glycosides are shown graphically in Figure 1. In the divalent series, there appeared to be an optimal length of spatial separation between the GlcNAc residues for the cluster effect. YD(G·GlcNAc-AH)$_2$ had the highest affinity, while both shorter and longer homologues had lower affinity. Affinity of dithio-GlcNAc-dodecane, which was higher than

expected, may be due to the higher flexibility of its connecting chain as well as to the presence of an S-glycosidic linkage. Homologous Gal/GalNAc-lectins of mammalian liver show somewhat higher affinity toward thiogalactosides than toward the corresponding *O*-galactosides (unpublished results).

Table IV shows that the presence of a large number of terminal GlcNAc residues did not necessarily generate high affinity. Spatial separation of one GlcNAc residue from the others seemed to be very important. As explained in the footnote to Table IV, precise I_{50} values for **4**–**7** could not be obtained due to limited quantities of these compounds. It is obvious, however, that these oligosaccharides are much worse inhibitors than the flexible cluster glycosides shown in Table III. In fact, I_{50} values of oligosaccharides **4**–**6** were quite similar to that of GlcNAc, which suggests that mainly one GlcNAc residue may be interacting with the lectin. This GlcNAc residue probably is the uncluttered GlcNAc on the 6-linked arm of the branching Man, since **4**–**6** all have this GlcNAc while noninhibitory **7** does not. On the other hand, **4** and **5** may actually have slightly higher affinities than GlcNAc, in which case it might mean that the second GlcNAc residue in **4** and **5** can also bind. The binding of the first GlcNAc residue increases the local GlcNAc concentration and thus produces enhancement in affinity. This type of affinity enhancement is observed quite often in the lectin–oligosaccharide interactions. If one assumes that the peptide portion does not contribute to the binding, comparison of **4** with biantennary glycopeptide **8** suggests that the bisecting GlcNAc interferes strongly with binding.

Inhibition data obtained with neoglycoproteins showed that the I_{50} values of GlcNAc-AI- and GlcNAc-AD-BSA that contained more than 10 mol of GlcNAc per mol of BSA were all in the nanomolar range. This result parallels the similar finding observed with the Gal/GalNAc-lectin on the mammalian hepatocyte surface. In the mammalian system, the I_{50} value increased exponentially with a linear increase in Gal residue up to 15–20 mol per mol of BSA, beyond which I_{50} values remained in the nanomolar range (Lee et al., 1989). Though I_{50} of monosaccharide Glc in the chicken system is impractically high for accurate measurement, measurable I_{50} is attained when Glc is clustered on a BSA molecule (Kuhlenschmidt & Lee, 1984). The I_{50} value of Glc_{30}-AI-BSA was 1.1 μM, while Xyl_{29}-AI-BSA was not inhibitory at 2 μM, again indicating the importance of the C-5 substituent, $-CH_2OH$.

Discussion

The GlcNAc-specific lectin of chicken liver and the Gal/GalNAc-specific lectin of rat/rabbit liver are homologous proteins. Both lectins are membrane-bound proteins with three domains: a short N-terminal cytosolic domain, a membrane-spanning hydrophobic domain, and a large C-terminal domain facing the outside of the cell (162 and 218 amino acid residues for the chicken and rat lectin, respectively); this last domain includes a carbohydrate recognition domain and the neck region (Drickamer, 1981; Drickamer et al., 1984). While there is no obvious homology between the corresponding cytosolic and transmembrane domains, there is ~40% sequence identity in the C-terminal region. There are other homologies as well. For instance, both lectins appear to form a stable hexamer in the Triton-solubilized, purified state, as well as possibly on the respective hepatocyte surface (Loeb & Drickamer, 1987; Halberg et al., 1987).

There has been a considerable amount of information accumulated on the binding specificity of the Gal/GalNAc-lectin (Sarkar et al., 1979; Lee, Y. C., & Lee, R. T., 1982; Lee, 1982; Lee et al., 1982). On the galactose moiety itself, it is known that (1) the presence of an aglycon larger than a Me group increases affinity, (2) a C-2 equatorial acetamido or acetoxy group binds much tighter than an hydroxy group, (3) both equatorial 3-OH and axial 4-OH should be free, and (4) of the C-5 substituent CH_2OH, the methylene group is needed, but OH can be absent or substituted with as large a group as another monosaccharide. There are other interesting features. The presence of negative charge(s) in the binding area of aglycon is implicated, since a positively charged group in aglycon greatly enhanced the binding affinity while the negatively charged Gal-1-phosphate had lower affinity (Lee et al., 1982; Lee & Lee, 1987). Similarly, the presence of negative charge(s) is postulated in the binding area for C-6 of Gal, since negatively charged derivatives were poor ligands (Lee, 1982; Lee et al., 1982).

In the present study, we found some similarities and differences in the binding specificity of chicken vs rabbit/rat lectins. The similarities are as follows: (1) Large aglycons are permitted in both lectins. (2) A C-2 equatorial acetamido group enhances binding affinity. (3) A large substituent is permitted on 6-OH, and C-6 in the form of $-CH_2-$ or $-CH_3$ contributes to affinity. (4) Substitution at C-3 and C-4 hydroxyls is not permitted. The differences are as follows: (1) Chicken lectin requires equatorial 4-OH, while the mammalian lectins require axial 4-OH. (2) For the chicken lectin, the presence of aglycon had little effect on binding affinity, and a negatively charged GlcNAc-1-phosphate actually enhanced (rather than diminished) the affinity. (3) A negatively charged group on 6-OH did not affect the affinity of the chicken lectin. From the similarities, we can deduce that the overall topology of binding sites of the two lectins must be quite similar. Binding sites of these lectins are rather small, binding mainly the terminal sugar residues. The combining site is most likely of trough type rather than of pocket type, since the C-6 end can accommodate a large group and the C-1 side must be open-ended in order for the lectin to accommodate a macromolecular ligand. Most of the differences between the two lectins, on the other hand, involve some sort of charge effect, which does not necessarily require change in tertiary structure. Proper point substitutions of certain amino acids in the carbohydrate recognition domain would easily produce such change in behavior toward charged groups.

Another important homology between the two lectins is that the clustering of GlcNAc (or Gal) residues augmented a tremendous increase in the binding affinity. In this study as well as in earlier studies (Lee et al., 1989; Kuhlenschmidt, 1983), we found that affinity of both mammalian and chicken lectins on their respective hepatocyte surface for BSA carrying Gal (for mammalian lectin) and GlcNAc (for chicken lectin) increased exponentially up to ca. $K_a = 10^9\ M^{-1}$ with a linear increase in the number of sugar residues on BSA. In this study, we showed that the similarity extends to a finer level of binding-site topography. Divalent, lactose-containing glycosides with maximal inter-Gal separation of 2.8 nm had an affinity comparable to that of the best divalent oligosaccharide ligand toward the mammalian hepatic lectin (Lee, R. T., et al., 1984; Lee & Lee, 1987). The best synthetic divalent ligand for the chicken lectin obtained in this study, $YD(G\text{-}GlcNAc\text{-}AH)_2$, has similar maximal GlcNAc separation, suggesting that the distance between two adjacent sugar-combining sites of the chicken and mammalian lectins is similar. These similarities in the cluster effects of the mammalian and chicken lectins suggest that homology between the two lectins exists not only at the individual sugar-combining site but also in the tertiary and quaternary arrangement of these

Table V: Inter-GlcNAc Distances in Different Local Minima of the Heptasaccharide (Chart III)

minima	torsional angles of α(1,6) linkage[a] (deg)			inter-GlcNAc distance[b] (nm)			rel energy (kcal/mol)
	ω	ϕ_6	ψ_6	d_1	d_2	d_3	
1	−60	−60	190	1.6	1.8	0.8	0[c]
2	180	−50	120	1.5	1.5	0.8	1.6
3	180	−50	190	1.5	1.7	0.8	4.2

[a] See Materials and Methods for the definition of ϕ, ψ, and ω angles. Torsional angles of other glycosidic linkages for each of the minima are as shown in Chart III. [b] Distances are between the two C-2 atoms and are calculated for each of the local minima of the α(1,6) linkage with allowable energy limit set at 5 kcal/mol for the lowest energy. [c] These energy minima had the lowest energy, which was set at 0.

combining sites. Presumably the lectin binds two or more GlcNAc (or Gal) residues simultaneously in a concerted fashion, producing tremendous enhancement of binding affinity. Recently we have determined the stoichiometry of binding of both rabbit and rat hepatic lectins using the equilibrium gel filtration method and found that there are, on the average, two Gal/GalNAc-combining sites per monomeric unit (Lee & Lee, 1988). This means that the stable hexamer state of lectins would provide 12 potential combining sites, which is probably more than enough sites to produce the highest affinity observed ($I_{50} = \sim 10^{-10}$ M) with these lectins.

For the mammalian lectins on the hepatocyte surface, oligosaccharide NONA I (Chart III) with only three terminal Gal residues produced binding affinity ($K_a = 2 \times 10^8$ M^{-1}) 10^5-fold tighter than that of simple galactosides ($K_a = 2 \times 10^3$ M^{-1}) (Lee et al., 1983). Interestingly, other structural isomers of NONA I, which either had the third Galβ(1,4)-GlcNAc unit attached to a different branch or had an isomeric Galβ(1,3)GlcNAc structure on the third branch, had affinity greatly inferior (as much as 100-fold lower) to that of NONA I (Lee et al., 1983; Townsend et al., 1986). Therefore, it was postulated that the three neighboring Gal-combining sites of the rabbit lectin are arranged complementarily to the preferred conformation of NONA I to allow concerted binding of all three Gal residues simultaneously. This means that the three sites are located at the apexes of a triangle of 1.5-, 2.2-, and 2.5-nm sides with correct orientation at each of the sites (Lee, Y. C., et al., 1984; Lee, 1989).

When Gal residues are removed from the NONA I structure, the GlcNAc-terminated oligosaccharide structure present in glycopeptide **9** is produced. Inter-GlcNAc distances of the terminal heptasaccharide structure of **9** (see Chart III) were calculated by molecular modeling as described under Materials and Methods, and the results are given in Table V. In accordance with solution studies of the complex biantennary oligosaccharide (Brisson & Carver, 1983; Homans et al., 1986), there were two preferred ω values, −60° and 180°, for the flexible α(1,6) linkage. Energy minima for $\omega = -60°$ were deep and narrow, while minima for $\omega = 180°$ were broad and shallow and contained two local minima. For this reason, when $\omega = 180°$, d_1 can vary by 1.5 ± 0.2 nm. Though there were altogether three local energy minima for the heptasaccharide structure, inter-GlcNAc distances (d_1, d_2, and d_3) calculated at each minima were quite similar: d_1 varied by 0.1 nm, d_2 varied by 0.3 nm, and d_3 had no variation. The corresponding inter-Gal distances of the NONA I structure obtained by 1H NMR and molecular modeling (Bock et al., 1982) are also shown in Chart III. Comparison of the two structures shows that d_1 and d_2 of **9** are similar to the shortest distance (d_3') of NONA I, but considerably shorter than the other two distances (d_1' and d_2'), while d_3 of **9** (0.8 nm) is shorter than any of the distances. Experimentally some cluster effect was observed in the biantennary structure **8** (~50-fold increase in binding affinity over GlcNAc), but there was absolutely no increase in affinity upon addition of the third GlcNAc in **9**. Perhaps inter-GlcNAc distance in **8** (d_1) is not quite correct or the orientation of the sugar rings is not optimal for a strong cluster effect to be generated, while d_3 in **9** (0.8 nm) is much too short for simultaneous binding of the third GlcNAc residue. If there exists in nature a high-affinity, GlcNAc-terminated triantennary ligand such as exemplified by NONA I in the mammalian system, it must have longer inter-GlcNAc distances than **9**.

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Effects of Hydrostatic Pressure on the Location of PRODAN in Lipid Bilayers: A FT-IR Study†

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ABSTRACT: The effects of hydrostatic pressure on the location of 6-propionyl-2-(dimethylamino)naphthalene (PRODAN), an environmentally sensitive fluorescent probe, in phosphatidylcholine lipid bilayers have been studied by Fourier-transform infrared spectroscopy (FT-IR) over the pressure range of 0.001-25 kbar. The results derived from the PRODAN C=O stretching band, the correlation field splitting of the methylene scissoring mode, and the methylene symmetric stretching mode as well as the absorption of the naphthalene ring show that in the sample of 4% (w/w) PRODAN in dimyristoyl-L-α-phosphatidylcholine (DMPC) at pH 6.8, most of the PRODAN molecules are embedded in the bilayers. In contrast, at pH 3.0, PRODAN was found to reside either on the membrane surface or dispersed in water. Compared to DMPC, egg yolk phosphatidylcholine (egg PC), which contains a substantial amount of unsaturated fatty acyl chains, is more susceptible to PRODAN permeation. The present study shows that the pressure dependence of the location of PRODAN in lipid membranes is different from that of tetracaine, a local anesthetic, in lipid bilayers. The model regarding the PRODAN location in lipid bilayers derived from the present infrared data has been compared with that obtained with previous fluorescence studies.

Interactions of small molecules with membranes are important issues in membrane biology [reviewed by Gennis (1989)]. Understanding the role of small molecules in modulating the structure and function of biological membranes requires knowledge of (1) the location of small molecules in membranes and (2) the degree of perturbation of membranes caused by these molecules. Many aspects of this subject have been investigated by physical techniques in model membrane systems. While most investigations were conducted at atmospheric pressure, a few were done at high pressures. Knowledge regarding the effects of pressure on the interaction between small molecules and membranes, particularly the aspect of pressure dependence of the location of small molecules in lipid bilayers, is fundamentally important for understanding membrane events at high pressures [reviewed in MacDonald (1984) and Wong (1984)]. In addition, pressure

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