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S1. OLIGOSACCHARIDE CONFORMATION/CONFORMATION DES OLIGOSACCHARIDES

1.1
NMR SPECTROSCOPIC STUDIES ON GLYCOPROTEINS

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During the last decade NMR spectroscopy has been successfully used to determine the primary structure of the carbohydrate chains of numerous glycoproteins. Due to the complexity of most glycoproteins, manifested in several N- and/or O-glycosylation sites combined with microheterogeneity, it is usually necessary to conduct ^{1}H-NMR studies on isolated free oligosaccharides. However, NMR spectroscopy is also the sole method by which the solution structure of an intact glycoprotein can be derived. Attempts are now being made to use different NMR spectroscopic techniques for this purpose:

Laser photo-CIDNP studies have been performed on both an intact glycoprotein and on its enzymatically deglycosylated form, thereby giving information on the effect of the carbohydrate chain on the conformation of the protein.

In order to observe sequential NOE-contacts in a protein, NMR experiments have to be carried out in $^{1}H_2O$. For glycoproteins this implies that also carbohydrate amide protons are observed. Therefore, 2D NMR experiments have been conducted in aqueous solution on several differently branched glycoprotein glycans, including diantennary chains with and without intersecting GlcNAc, and tetrasialo tetraantennary oligosaccharides. Furthermore, the use of 3D NMR in $^{1}H_2O$ for structural studies on highly branched N-glycans will be illustrated.

1.2
PRINCIPLES OF SPATIAL ORGANIZATION OF O-GLYCOSYLPROTEINS

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At first on example of blood-group-specific glycoproteins (BGSG) by method of theoretical conformational analysis the general principles of spatial construction of O-glycosylated glycoproteins are concluded. These centauric macromolecules are characterised by high contention of carbohydrate component substituting side chains, practically, all of hydroxyaminoacide residues (Thr Ser). Calculation with consideration of nonbonded interaction shows, that the carbohydrat-carbohydrate (side-side) and short range carbohydrate-peptide (side-backbone) interactions are the dominant factors in spatial organization of this class glycoproteins.

For BGSG concretely we proposed "tunnel" structure (ø = 50Å, L = 300Å), in which the polypeptide backbone is covered with layer of closely packed carbohydrate chains. The presence of compact carbohydrate sheet in BGSG molecules shows an unique situation, when the carbohydrate-carbohydrate inter-actions, first of all, determine the spatial architecture of the molecules. The proposed structure are corresponded to experimental (aquametric) data.

1.3
TEMPERATURE DEPENDENT TIME-RESOLVED FLUORESCENCE ENERGY TRANSFER REVEALS ENERGY BARRIERS IN THE FLEXIBILITY OF TWO OF THE ANTENNA OF A TRIANTENNARY GLYCOPEPTIDE

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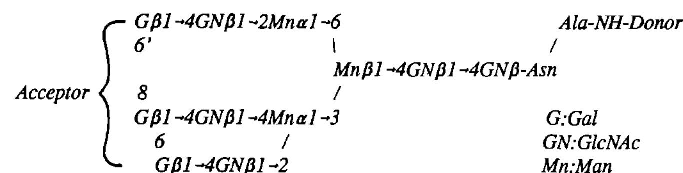
We have prepared three isomers of a triantennary glycopeptide (shown below) which have a fluorescence donor (naphthyl-2-acetyl) attached to the *N*-terminus of the peptide and differ in the location of a fluorescence acceptor (dansyl) which is attached to one of the terminal Gal residues (either 6′ 8, or 6). Fluorescence energy transfer measurements using the Förster equation provided an average distance separating the donor from the acceptor in each triantennary isomer. Time-resolved fluorescence energy transfer measurements revealed two populations of conformers for Gal 6 or 6′ isomers. One conformer contained the antenna folded back towards the core region, and a second was in an extended conformation. The antenna containing the acceptor attached to Gal 8 was found to be only in the extended conformation.



We have now determined the ratio of extended and folded conformers at temperatures varied from 0°C to 40°C. At 0°C the folded conformation dominated in both the Gal 6 and 6′ isomers. Increasing the temperature systematically shifted the equilibrium until at 40°C, the extended conformer predominated. The Gal 6 and 6′ isomer each showed different sensitivity to the temperature dependent conformational change, while the Gal 8 isomer remained only in the extended form within the temperature range tested. These data allowed calculation of $\triangle$H and $\triangle$S for the conformational change. Both the Gal 6 and 6′ isomer showed a positive $\triangle$S for unfolding, however the entropy change for the Gal 6′ isomer was twice that of Gal 6.

1.4
FAVOURED CONFORMATIONS AND ORIENTATIONS OF THE SACCHARIDE CHAIN OF GLYCOSPHINGO-LIPIDS AT THE MEMBRANE SURFACE: X-RAY ANALYSES AND THEORETICAL CALCULATIONS

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In glycosphingolipids (GSL) the conformation at the saccharide-ceramide linkage is crucial for the orientation of the saccharide chain at the membrane surface. As a complement to our x-ray single crystal investigations (1,2) theoretical calculations have been performed in order to define preferred conformations and possible orientations of the saccharide chain. The calculations (3) indicate that, especially for bulky and curved saccharide chains, there is a limited range of admissible saccharide chain orientations.

In the present study molecular mechanics calculations were applied on various monoglycosylceramides in order to obtain detailed information about the conformational energetics of the saccharide-ceramide linkage. Relaxed potential energy maps (ϕ,ψ,θ) and complete energy minimizations show a limited number of favoured conformations (4) which are compatible with x-ray and NMR data. According to these calculations intramolecular hydrogen bonds and dipole-dipole interactions play an important role in stabilizing certain conformations of the saccharide-ceramide linkage. Moreover, the admissible range of saccharide chain orientations is further restricted if steric interference of the saccharide chain with the membrane surface is accounted for (3,4).

The accumulated information on the conformation of the saccharide-ceramide linkage was applied to model the orientation of the oligosaccharide chains of different globo GSL with binding epitopes for *E. coli* G-adhesins. The calculations (5) indicate that for GSL in a membrane layer certain epitopes are efficiently exposed whereas others are inaccessible. This provides an explanation of crypticity phenomena which have been observed for epitopes on globo GSL (5,6,7).

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1.5
SYNTHESIS OF MODIFIED DI- AND TRI-SACCHARIDE ANALOGUES OF THE SHIGELLA FLEXNERI VARIANT O-ANTIGEN POLYSACCHARIDE

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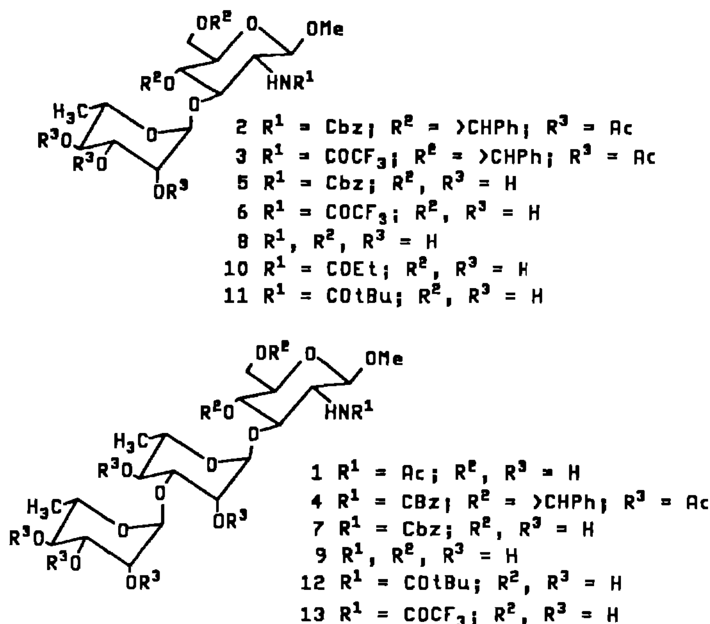
The fine details of the molecular recognition by monoclonal antibody of a trisaccharide epitope, α-L-Rha*p*-(1–3)-α-L-Rha*p*-(1–3)-β-D-GlcNAc*p*-1-OMe **1** that occurs in the Shigella flexneri Y antigen repeating unit[1]; [-2)-α-L-Rha*p*(1–2)-α-L-Rha*p*-(1–3)α-L-Rha*p*-(1–3)-β-D-GlcNAc*p*(1-] has been studied by functional group replacement. Here we deal with aspects of this work that involve synthesis of various amino derivatives of epitope **1**.

The strategy to obtain different N-acyl derivatives employed a key acceptor molecule protected by a N-carbobenzoxy group (Cbz). Glycosylations used a thioglycoside which was activated by in situ generation of iodonium ions[2]. Under these conditions the disaccharides **2** and **3** were prepared in excellent yields. Regioselective protection of **2** followed by glycosylation gave the trisaccharide **4**. The derivatives **5**, **6** and **7** were then obtained, in two steps, respectively from **2**, **3** and **4**. Upon hydrogenolysis, the N-carbobenzoxy derivatives **5** and **7** gave **8** and **9**. These derivatives were either isolated as their hydrochloride salt, or subsequently treated with various acylating agents to yield the derivatives **10–13**.

These analogues were used in competitive binding studies to determine the importance of the N-acetyl group to the interaction between the antigen and the combining site of the antibody SYA/J6. The conformation of these oligosaccharides was determined by nmr and potential energy calculation methods and the results obtained are being used to elaborate a molecular graphics based model of the antigen-antibody complex.



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1.6
N.M.R. AND CONFORMATIONAL ANALYSIS OF DERIVATIVES OF α-2,8-SIALYL OLIGOSACCHARIDES AND COMPARISON WITH THE NATIVE COMPOUNDS

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The capsular polysaccharides of goup B *N. meningitidis* and *E. coli K1* (1,2) and the glycoprotein antigen of fetal N-CAM (3) consist of linear chains of α-2-8-linked sialic acid. Based on this structure they share a

common epitope which is only stabilized by a minimum of ten contiguous sialic acid residues (1,3) and is helical in nature (4). In order to investigate the role of charges and the influence of N-acyl substituents on the stability of the helical epitope, extensive n.m.r. and conformational analyses were carried out with carboxyl-reduced and N-acyl derivatised oligomeric and polymeric α-2-8-linked sialic acids and comparisons were made with data obtained from their native precursors (2).

The ^{1}H- and ^{13}C-n.m.r. resonances were assigned and the $^{3}J_{H,H}$ coupling constants determined by conventional techniques. The conformation of the side-chain could be established from the coupling constants and was used together with the chemical shift as a sensitive indicator of conformational changes in the different derivatives. The $^{3}J_{C2,H8}$ coupling constant, from which the ψ angle can be estimated, was determined for some oligomers. N.O.e.-measurements were carried out in order to determine proton-proton proximities. Molecular modelling was performed using a combination of rigid potentials (GESA, PFOS) and Molecular Mechanics (MM2).

These analyses show that the conformation of the carboxyl-reduced oligo- and poly-sialic acids is drastically changed compared to the native compounds. The main changes occur in the rotamer distribution about the C7–C8 bond. Also $^{3}J_{C2,H8}$ coupling constants indicate a change in the ψ angle, in accord with potential energy calculations which is confirmed by the measurements of the . The change in acyl substituent, however, seems to have no or very small effect on the conformation, even for large groups such as N-isobutanoyl or N-hexanoyl. This implies that charges may be the important factor in keeping α-2-8-linked sialic acids in a more extended conformation as was shown previously (2,3).

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1.7
MOLECULAR DYNAMICS SIMULATION OF BLOOD GROUP OLIGOSACCHARIDES

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Molecular dynamics simulations without explicit inclusion of solvent molecules have been performed to study the motions of Lewis^{a} and Lewis^{b} blood group oligosaccharides and two blood group A tetrasaccharides having type I and type II core chains. The potential energy surface developed by Rasmussen and coworkers was used with the molecular mechanics code CHARMM. The global minima of the component disaccharide fragments were obtained from adiabatic conformational energy mapping. The global minima of these disaccharide fragments were used to build the tri- and tetrasaccharides which were further minimized before the actual heating/equilibration and dynamics simulations. The trajectories of the disaccharide fragments, e.g., Fuc α-(1→4)GlcNAc, Gal β-(1→3)GlcNAc etc., show transitions among various minima. However, the oligosaccharides were found to be dynamically stable and no transitions to other minimum energy conformations were observed in the time series of the glycosidic dihedral angles even during trajectories as long as 300 ps. The average fluctuations of the glycosidic angles were well within the range of $\pm15°$. The results of these trajectory calculations were consistent with the relatively rigid single-conformation models derived for these oligosaccharides from ^{1}H NMR data.

1.8
CONFORMATION OF BLOOD GROUP OLIGOSACCHARIDES BY 2D NOESY AND T_1 SIMULATIONS

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The 3-dimensional structures of the Lewis oligosaccharides Lacto-N-fucopentaose II and Lacto-N-difucohexaose I, Lacto-H-fucopentaose I (H type 1), and a blood group A type 1 and a type 2 penta- and hexasaccharide alditol, respectively, were studied using 2-dimensional nuclear Overhauser effect spectroscopy. NOE data from both carbon-bound and labile (acetamido) protons were obtained in D_2O (H_2O) and in mixtures of DMSO-D_2O(H_2O). Non-selective proton T_1 data were also obtained for isolated resonances. Using a complete relaxation matrix method NOESY intensities were calculated as a function of the internal coordinates of model oligosaccharides for comparison with experimental values. Simulation of NOE and T_1 data from carbon-bound protons as a function of geometry showed that the experimental data are consistent with NOEs for a small group of closely related conformations for each of these oligosaccharides. Based on the proposal of rigid single conformations for these oligosaccharides we have devised a method for quantitative simulation of NOEs from the amide protons of GlcNAc and GalNAc which incorporated the influence of the ^{14}H nucleus on the amide proton dipolar relaxation. Effects of chemical exchange were eliminated by adjusting solvent pH. Crosspeaks whose intensities depend on the glycosidic dihedral angles were observed between the amide protons and protons in adjacent residues. It was possible to match these intensities by simulations using the same model geometry deduced for these oligosaccharides from carbon-bound proton NOE, supporting the rigid model hypothesis. Crosspeaks that are sensitive to the orientation of the amide plane were observed between the amide protons and protons within the same residue, and values of the amide dihedral angles that are consistent with the data are also reported.

1.9
2D AND 3D HOMONUCLEAR ^{1}H-N.M.R. EXPERIMENTS AND CONFORMATIONAL STUDIES OF SIALYLATED OLIGOSACCHARIDES AND GLYCOPEPTIDES

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Comprehensive assignment of chemical shifts and nuclear Overhauser enhancement (NOE) effects have been achieved for oligosaccharides and glycopeptides using a combination of double quantum filtered correlated spectroscopy (DQCOSY), triple quantum filtered COSY and rotating frame NOE (ROESY) experiments. The conformational information given has been incorporated into computer graphics molecular models set up using Biosym software (Insight and Discover) incorporating data from AMBER, MOPAC and AMPAC forcefields.

The sialylated oligosaccharide (structure **1**) and glycopeptide (structure **2**) show multiple NOE effects demonstrating, in particular, through-space interactions of the sialic acids to the rest of the molecule. Whereas N0E's for the small N-linked glycopeptide GlcNAcβl-Asn could not be detected.

Oligosaccharide **1** was obtained from human milk. Related structures are found on N-linked chains of serum and cell surface glycoproteins where they have roles in molecular recognition and are important as antigenic determinants. The glycopeptide **2** was obtained from human

urine and is of interest as a model for determining the conformational effects of 0-linked glycosylation on the protein backbone.

1 Galβl-3GlcNAcβl-3Galβl-4Glc
 !2,3 !2,6
 NeuAcα NeuAcα

2 NeuAcα2-3Galβl-3GalNAcαl-Ser-Leu

1.10
MOLECULAR DYNAMICS SIMULATIONS OF SOLVATED MUCIN-LIKE GLYCOPEPTIDES: DETERMINATION OF INTRA-MOLECULAR INTERACTIONS IN MUCIN-LIKE MODELS WHICH VARY IN CARBOHYDRATE CHAIN LENGTH

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Computational models of carbohydrates and glycopeptides are potentially very sensitive to the treatment of solvation. Indeed, we have found that ignoring the effects of solvation leads to a qualitative misrepresentation of O-linked ovine submaxillary mucin (OSM) glycopeptide structure. We have analyzed a mucin-like peptide fragment with different extents of O-glycosylation using four distinct implicit solvent models. The results are compared with experimental conformational data from light scattering and NMR experiments on OSM and its sequentially deglycosylated derivatives (Gerken, et al. 1989, Shogren, et al. 1989). The experimental results indicate that the "extended random coil" conformation of these mucins is predominantly due to the O-linked GalNAc residue directly bound to the peptide. Through the use of molecular dynamics simulations we have identified specific interactions which induce the extended random coil conformation indicated by experiment. Inclusion of solvation energy through increasingly sophisticated solvation algorithms greatly improves the accuracy of molecular dynamic simulations without the vastly increased computational effort required for explicit solvent simulations. Supported by NIH grant DK39918 and a Cystic Fibrosis foundation post doctoral training grant.

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1.11
CONFORMATIONAL ANALYSIS OF GANGLIOSIDE OLIGOSACCHARIDES USING THE TRIPOS 5.2 MOLECULAR MECHANICS FORCE FIELD

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Gangliosides are sialic acid containing glycosphingolipids which are found in the plasma membranes of all mammalian cells, where they function as modulators of receptors and targets for pathogenic agents. Though much is known of the primary structure of ganglioside oligosaccharide moieties, little is known of their secondary structure. As an adjunct or alternative to often intractable X-ray and NMR experiments, we have employed molecular mechanics programs (Tripos 5.22 force field) to calculate (Iris 4D120GTX Silicon Graphics computer) the lowest energy conformations of ganglioside oligosaccharides. Though pyranose rings may be assumed to be rigid chair conformations, the rest of these oligosaccharide structures is potentially quite degenerate. Thus, we have considered the rotations about hydroxyl, hydroxymethyl, acetamido, carboxyl, and glycerol side-chain groups as well as ϕ and ψ, the torsion angles defined by the trans-glycosidic protons (and CO_2 of NeuAc residues). Another unique feature of our calculations has been the inclusion of an energy term for all charge interactions, which we find greatly restricts the number of possible conformations. Using this approach, we have found ϕ/ψ of the global minimum conformation for NeuAcα2-3Galβ1-4Glc (GM3) to be −60°/−4° and +58°/−5°, respectively, and for the added residue of the following: GM2 (+55°/+16°), GM1 (+62°/−14°) and GD1a (−60°/−4°). The four torsion angles for the NeuAcα2-8NeuAc unit of GD3, GD2, GD1b, GT1b and GQ1b as defined by the fragment C-1′/C-2′/0-8/C-8/C-7/C-6/H-6 are as follows: −164°, +12°, +118° and −58°. These findings are significant because these four angles impart a V-shaped conformation to the fragment in which the NeuAcα2-8NeuAc unit folds back on the lactose unit. In the cleft thus formed, there appears to be a site well-suited for divalent metal binding involving the internal NeuAc CO_2, Gal O-6, and external NeuAc O-7 and O-10. Other findings of this study are the possibility of conformation-stabilizing hydrogen bonds between: 1) Gal HO-2 and NeuAc O-6 in the disaccharide NeuAcα2-3Gal; 2) inner NeuAc CO_2 and outer NeuAc HO-7; and inner NeuAc HO-7, HO-9 and O-8 of NeuAcα2-8NeuAc. A large hydrophobic surface is possible for the trisaccharide Galβ1-3GalNAcβ1-4Gal. All of these calculated conformations agree with available NMR and X-ray crystal data better than previous calculations using HSEA, semi-empirical, or GESA-MM2 approaches.

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1.12
CONFORMATION OF THE OLIGOSACCHARIDE CHAIN OF GANGLIOSIDES

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The three-dimensional structures of the oligosaccharide chain of GM1 and GD1b gangliosides were modeled with the use of a distance-mapping procedure (*Acquotti et al., JACS 1990, 112, 7772*) and of molecular mechanics calculation respectively, on the basis of interresidue NOE contacts. It is vital to this approach that the NOE and proton-exchange cross-peaks can be discriminated by their opposite phase in rotating frame NOE spectra (ROESY), as this feature eliminates possible misinterpretations. Moreover, the virtual absence of spin diffusion in ROESY experiments excludes *de facto* another source of false contact information.

GM1 ganglioside. The glycosidic linkages of the branched core trisaccharide segment GalNAcβ1-4(Neu5Acα2-3)Galβ are significantly more rigid than the linkages of the external disaccharide Galβ1-3GalNac and Galβ1-4Glc segments. The conformation of the sialic acid side chain seems to be predominantly determined by the hydrogen bond between C(8)-OH proton and either the carboxilic or ring oxygen.

GDlb ganglioside. The tetrasaccharide GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ has a circular arrangement leaving a highly hydrophobic region with seven methyne protons pointing towards the center. At one side of this region the three electron rich groups GalNAc-NH, external Neu5Ac-OH4 and internal Neu5Ac-COO⁻ are grouped together; at the other side five polar groups (four hydroxy groups and the external Neu5Ac carboxylate) define a large annular hydrophilic region. The external Neu5Ac is close to the external Gal residue, and the external Neu5Ac-COO⁻ is within van der Waals contact with the inner Neu5Ac-OH9 group. The core trisaccharide GalNAcβ1-4(Neu5Acα2-3)Galβ is rigid as in GM1 but rearranges enlarging his

"mouth" to allow the presence of the external sialic acid. This unit will close this "mouth" as described.

1.13
CONFORMATIONAL STUDIES ON THE MUCIN-TYPE O-GLCOSYLATION SITE
1. CONFORMATIONAL STUDIES OF A MUCIN-TYPE FRAGMENT TETRASACCHARIDE GLYCOPROTEIN CONTAINING NEU5AC–(α2-6)-[NEU5AC-(α2-3)-GAL-(β1-3)]-GALNAC O-GLYCOSIDICALLY LINKED TO EITHER THREONINE OR SERINE

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The three dimensional structure of a tetrasaccharide O-linked to either threonine or serine, and containing both (α2-3) and (α2-6) linked sialic acid residues, has been investigated using a combination of NMR and computer modelling techniques. The two conclusions of biological significance that may be drawn are (i) that the glycosidic linkage between the GalNac-O-Thr/Ser is rather constrained due to strong H-bonding between the CH_3CONH group and the amino acid, and (ii) that the C2-C3 bond of the threonine or serine has low barriers to rotation between conformational states and can thus be thought of as relatively mobile and a possible "hinge" region. Preliminary computational data will also be presented concerning the conformational effects of substituting the threonine residue by a longer peptide moiety, as would occur in an intact glycoprotein. We also compare the utility of using HSEA and MM type calculations, using experimentally determined NOE distance constraints derived from 1H-NMR ROESY and NOESY measurements.

NMR structural and molecular modelling computational studies on the related hexasaccharide Neu5A-(α2-3)-Gal-(β1-3)-[Neu5Ac-(α2-3)-Gal(β1-4) -GlcNAc-(β1-6)-] -Gal-NAc-(α1-3) -O-L-Threonine/L-Serine will also be presented, including determinations of T_1 and T_2 values.

1.14
^{1}H- AND ^{13}C-NMR OF SULFATIDE AND SEMINOLIPID

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Recently, various roles of sulfoglycolipids, such as laminin and thrombospondin-mediated cell adhesion (1) and sperm-egg interaction (2), have been suggested. However, the physicochemical properties of these lipids and their ligand-receptor interactions on cell surface are still equivocal. To obtain some fundamental informations for deducing their conformation on the biomembrane, we carried out ^{1}H- and ^{13}C-NMR analyses of sulfatide (SM4s) and seminolipid (SM4g).

SM4s and SM4g were prepared from sea lion (or rat) kidney and porcine testis, respectively. After exchanging the labile protons with deuterons, the lipids were dissolved in d_6-DMSO/D_2O (98:2) (2-30 mM). The spectra were obtained with a JEOL GX-400 spectrometer at 60°C. By 2D-phase sensitive DQF-COSY, the ring protons (δ, ppm (^3J, Hz)) of SM4s (hFA) were determined as follows: H-1, 4.178 (7.6); H-2, 3.468 (9.5); H-3, 3.962 (3.4); H-4, 3.937 (1.1); H-5, 3.374 ($^3J_{5,6a}$=6.1, $^3J_{5,6b}$= 6.4); H-6a, 3.474; H-6b, 3.530 ($^2J_{6a,6b}$=−10.8). Roughly similar parameters were attained for SM4g, however, these values were slightly different from those for SM4g measured at a lower temperature (20°C) (3). In comparison with the desulfated lipids, about 0.7 ppm downfield shift of H-3 by a deshielding effect of sulfate was observed.

The ring carbons (δ, ppm) of SM4g were assigned by DEPT and C-H-COSY as follows: C-1, 103.7; C-2, 69.01; C-3, 79.17; C-4, 66.39; C-5, 75.01; C-6, 60.06. Almost the same data were obtained on SM4s. Similar results have also been reported on lyso-SM4s (4). The downfield shifts (ca. 6 ppm) by the electronegativity of sulfate were observed on C-3 of the sulfated lipids. Furthermore, it was found that the $^1J_{C,H}$ values of C-1 to C-5 of SM4s were 3-5.5 Hz larger than those of GalCer by INEPT experiment.

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1.15
CONFORMATION ANALYSIS OF A HEPTA-β-GLUCOSIDE ELICITOR BY GEGOP CALCULATIONS AND MONTE-CARLO SIMULATIONS

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The hepta-β-glucoside Glcβ(1→6)[Glcβ(1→3)]Glcβ(1→6)Glcβ(1→6) [Glcβ(1→3)]Glcβ(1→6)Glc has been characterized in this laboratory as a fungal elicitor of plant phytoalexin accumulation [1]. To gain insight into the interaction of the elicitor with its putative receptor [2], we are studying the energetically favored conformations of the hepta-β-glucoside.

The backbone of the heptaglucoside contains four β(1→6) linkages which cause great flexibility in the conformation of the molecule. To obtain information on the conformational preferences of the heptaglucoside we pursued two strategies. First, we used a variety of different potential starting conformations and optimized their geometry by energy minimization using the GEGOP program [3]. As expected, we obtained a multitude of local energy minima (>35 conformations within 10 kcal/mole from the global minimum). Second, we used Metropolis Monte-Carlo calculations at 300 K and 500 K to characterize the dynamics of the molecule. We found that the β(1→3) branches and the ϕ angles of the backbone show only moderate flexibility. However, the ψ and ω angles around the β(1→6) linkages in the backbone show high flexibility with the ψ angles having values from 60° to 280°, while conformations around the ω angles cover all three staggered rotamers. Interestingly, the average overall conformation in a 300,000 step Monte-Carlo run shows that the two β(1→3)-linked glucosyl residues should be close in space for a significant proportion of the time. We calculate a 15% NOE between H4 of one and H2 of the other β(1→3)-linked residue. This indicates that the two branches have fairly well-defined relative orientations. [Research supported in part by DOE grant DE-FG09-85ER13810.]

[1]. J.K. Sharp, B. Valent and P. Albersheim (1984) *J. Biol. Chem.* **259**: 11312-11320.
[2]. J.J. Cheong and M.G. Hahn (1991) *The Plant Cell* (in press).
[3]. R. Stuike-Prill and B. Meyer (1991) *Eur. J. Biochem.* (in press).

1.16
CONFORMATIONAL INTERACTIONS OF SULFATE WITH CARBOXYLATE GROUPS IN SULFATED DISACCHARIDES

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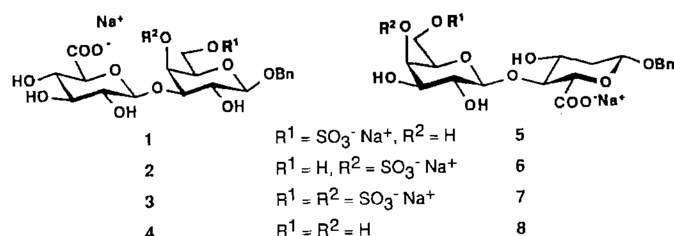
We synthesized mono- and disulfated disaccharides derived from b-D-GlcA-(1-3)-b-D-Gal (**4**) [1] and b-D-Gal-(1-4)-b-D-GlcA (**8**) [2], respectively. Starting from central precursor disaccharides, we synthesized the 6-O-sulfate **1**, 4,6-di-O-sulfate **2** and 4-O-sulfate **3** derived from **4** as well

as the 6′-O-sulfate **5**, 4′,6′-di-O-sulfate **6** and 4′-O-sulfate **7** derived from **8**.

Conformational analysis of the sulfated disaccharides and their unsulfated parent compounds by NMR spectroscopy revealed, that the conformation of the glycosidic bond changes with the pattern of sulfation. Calculations with the GEGOP program show that steric interactions of the ionic groups are not responsible for the conformational changes observed. The direction of the observed changes can be interpreted as being caused by repulsive or attractive interactions of the carboxylate and sulfate groups. Attractive or repulsive interactions between sulfate and carboxylate groups are determined by the distance and relative orientation of the charged groups.

[1] Marianne Zsiska and Bernd Meyer, Carbohydr. Res. submitted.
[2] Marianne Zsiska and Bernd Meyer, Carbohydr. Res. in press.



1.17
RECENT IMPROVEMENTS IN THE SOLUTION CONFORMATION ANALYSIS OF CARBOHYDRATES

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We present, for the first time, an NMR spectroscopy strategy for detailed characterization of the solution conformations of oligosaccharides, a strategy that generates a sufficiently large number of interglycosidic spatial constraints so that the glycosidic linkage conformations can be determined with a precision heretofore unachievable. In addition to the commonly used {^1H,^1H} N0E contacts between carbon-linked protons, our constraints are: (a) homonuclear NOEs of OH protons in H_2O to other protons in the oligosaccharide [1], (b) heteronuclear {^1H,^{13}C} N0Es [2], and (c) long-range heteronuclear scalar couplings across glycosidic bonds [3]. We will illustrate the approach for a trisaccharide, namely, NeuAcα(2→6)Galβ(1→4)Glc. Also, we characterized the internal dynamics of the glycosidic bonds in an octasaccharide, NeuAcα(2→3)Galβ(1→3)-[Galα(1→4)Galβ(1→4)Galβ(1→4) GlcNAcβ(1→6)]GalNAcα(1→3)GalNAc-ol, by measurements of the {^1H,^1H} cross relaxation rates in the laboratory frame and the rotating frame, at various temperatures and magnetic field strengths. The consequences of our experimental results for force-field methods will be discussed.

[1] L. Poppe and H. van Halbeek (1991) *J. Am. Chem. Soc.* **113**: 363–365.
[2] L. Poppe and H. van Halbeek (1991) *Magn. Reson. Chem.* (in press).
[3] L. Poppe and H. van Halbeek (1991) *J. Magn. Reson.* (in press).

1.18
CARBOHYDRATE-PROTEIN INTERACTIONS IN GLYCOPHORIN A AND IMMUNOGLOBULIN G_1

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The preferred conformations of glycoproteins, their dynamics. and their carbohydrate-peptide interactions were evaluated using the GEGOP program [1]. We calculated the C_H2 domain of IgG_1 including the N-type biantennary decasaccharide as well as the N-terminal triglycosylated heptapeptide of glycophorin A^N.

We calculated between $3*10^5$ and 10^6 conformations using Monte Carlo techniques to determine time averaged properties of the glycoproteins. Local minima were determined by optimization techniques. In case of the Fc-fragment we obtained the same oligosaccharide conformatlon as found in the X-ray crystal structure – even if starting from randomly selected starting points. Here the protein dominates the carbohydrate conformatlon.

For glycophorin A, we found that the carbohydrate structures can adopt a large number of conformatlons. We found 40 conformations within 20 [kcal/mol] of the global minimum. However, the peptide backbone seems to be significantly restricted in its flexibility by the clustered oligosaccharides. Here the carbohydrate seems to determine the preferred threedimensional structure of the peptlde backbone, opposite to what is found in the Fc fragment described above.

[1] R. Stuike-Prill and B. Meyer, Eur. J. Biochem. **194**, 903 (1990).

1.19
STEREOCHEMICAL FEATURES OF SULFATED CARBOHYDRATES

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The three dimensional structures of crystalline α-D-galactopyranoside 2-sulfate sodium, 3-sulfate sodium, 4-sulfate sodium and 4-sulfate potassium have been determined by X-ray crystallography. These results extend our knowledge of the stereochemical features such as ring conformation, sulfate geometry, hydrogen bonding and cation coordination which characterize sulfate monosaccharides.

This work provides structural features and first detailed experimental information on sulfated monosaccharides which are found in naturally occurring sulfated carbohydrates and glycoconjugates. In particular, it is shown that the substitution with sulfate slightly affects the chair conformation and results both in a lengthening of the C-O bond and the opening of the COS angle at the point of attachment of the sulfate moiety. The coordination numbers and geometries of the cations are of the type to be expected, but no definite rules can be formulated with regard to which of the oxygen atoms of the sulfate, hydroxyl, sugar ring and waters, contribute to the coordination. The results obtained show that the conformation of the sulfate group does not depend on environmental factors such as position, cation coordination, hydrogen bonding and packing. These observations are supported by energy calculations in which the van der Waals interactions alone account for the spatial orientation of the sulfate group.

Accordingly, a forcefield has been derived from the present experimental data, and it may have applications in the structural investigations of more complex cases such as sulfated polysaccharides and glycoconjugates.

1.20
MOLECULAR DYNAMICS SIMULATIONS OF THE GLYCANS OF THE N-ACETYLLACTOSAMINIC TYPE

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Molecular dynamics simulations were carried out to explore the conformational mobility of the antennae of N-linked glycans. 200 picosecondes molecular dynamics simulations were performed i) on Man(α1–3)[GlcNAc)(β1–2)Man(α1–6)]Man (β1–4)GlcNAc(β) structure both *in vacuo* and with the explicit inclusion of water molecules and ii) on the complete disialylated monofucosylated biantennary glycan of the N-acetyllactosaminic type *in vacuo*. Starting from one of the Y-conformations the 3D-structure evolved through 8 successive transitional phases to a new, compact and energetically favourable conformation which had never been previously described. During the simulation, the T- and broken wing-conformations were observed as transitional states.

All the glycosidic linkages of the disialylated monofucosylated biantennary glycan, except the Fuc(α1–6)GlcNAc(β) one, were concerned by the phase transitions. Particularly, the Man(β1–4)GlcNAc(β) linkage, which was previously described by NMR and X-ray diffraction as a rigid one, was involved in numerous conformational changes during 83 picosecondes, before the first transition phase. The freedom of motion of the torsional angles of the Man(α1–6)Man(β) linkage was limited, under these simulation conditions, to the angle psi which took three values: 30°, 90° and 180°. Moreover, from 150 picosecondes up to the end of the simulation, the value of the torsional angle omega of the NeuAc(α2–6)Gal(β) linkage of the α–I, 6-antenna continuously swung between +60° and −60°.

Finally, we observed that the values of the torsional angles of the three linkages: NeuAc(α2–6)Gal(β), Gal(β1–4)GlcNAc(β) and GlcNAc (β1–2)Man(β) of the two antennae were different, demonstrating their asymmetric conformation.

1.21
MOLECULAR MODELING OF FREE AND CONJUGATED GLYCANS: TRIDIMENSIONAL STRUCTURE AND GLYCAN PROTEIN INTERACTION

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On the basis of experimental data and of computer calculations, using the Tripos 5.3 force field, in order to examine the tridimensional structures which are sterically feasible and the conformations which are energetically the most favourable, we have calculated the conformation of free biantennary glycans of the N-acetyllactosaminic type (complex type). In a first step, the low energy conformations of each of the glycosidic linkages were calculated when included in a disaccharide unit. The obtained results were in a good agreement with the literature data obtained by X-ray crystallography and ^{1}H NMR analysis. Then, the calculations were performed on each glycosidic linkages when included in higher oligosaccharide structures (tri- to undecasaccharides). In fact, the conformation of a branched glycan does not result from the simple addition of the different low energy conformers of each of the glycosidic linkages constituting the glycan structure. In absence of any interaction with the protein, a high number of glycan conformations exists which can be classified into five basic conformations of which four have been already described. In fact, in addition to the Y-, T-, bird-, broken wing-conformations, a "back folded wing"-conformation is energetically feasible.

In contrast, in the case of rabbit serotransferrin, the glycan linked to the protein is immobilized into only one conformation: the broken wing-conformation which fits well the space available between the two lobes of the peptide chain, the glycan conjugated to the C-terminal lobe interacting with the N-terminal lobe through its antennae. The obtained results strongly suggest that, in the case of rabbit serotransferrin, the glycan contributes in maintaining the protein moiety in a biologically active tridimensional conformation.

1.22
THE INFLUENCE OF THE MEMBRANE SURFACE ON GLYCOLIPID STRUCTURE AND MOTION

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Glycolipids constitute an important class of biomolecules which are involved in biomolecular recognition. The importance of carbohydrate head-group conformation in such processes is well recognized. Glycolipids typically occur as minor components of the complex heterogeneous matrix of a biological membrane. As a result, the membrane surface may not only influence head-group conformation but also serves as a spatial reference frame in which the glycolipid is oriented and recognized.

Previous work has used ^{2}H nuclear magnetic resonance (^{2}H NMR) to examine the conformational and motional properties of membrane associated glycolipids. The results established the average orientation of the carbohydrate residue relative to the membrane surface, and the amplitudes and rates of molecular motion. Previous studies of the simple lipid 1,2-di-O-tetradecyl-3-O-(β-D-glucopyranosyl)-sn-glycerol (*β*-DTGL) have shown that there is conformational exchange about the glycerol C3-C2 bond, as well as about the glycosidic linkage. However, there are details of these processes which remain unclear. In order to investigate this head-group motion in more detail, energy calculations have been performed to identify possible low energy conformations for *β*-DTGL which may be involved in the exchange process. An attempt has been made to include the influence of the membrane surface on the conformational energy. The results of such calculations have been used to analyze further ^{2}H NMR lineshape and longitudinal relaxation data for *β*-DTGL in bilayer membrane systems.

1.23
CARBOHYDRATE BASED METAL CHELATES

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As part of an ongoing project to develop carbohydrate based metal chelates we have investigated heavy metal binding to two synthetic disaccharides: methyl 2-**O**-sulfo-4-**O**-(α-L-idopyranosyluronic acid)-2-deoxy-2-sulfamido-D-glucopyranoside, *1a*, and methyl 2-**O**-sulfo-4-**O**-(α-L-idopyranosyluronic acid)-2-deoxy-2-sulfamido-6-**O**-sulfo-D-glucopyranoside, *1b*. Disaccharides *1a* and *1b* were found to bind $^{67}Cu(II)$ and $^{63}Ni(II)$ using chromatographic assays. Further NMR studies involving titrations with $Zn(OAc)_2$ allowed for the determination of Zn(II) binding constants. These constants strongly suggest chelation by comparison to the known constant for the monosaccharide sodium, methyl-

α-L-idopyranosiduronate (D.M. Whitfield and B. Sarkar, J. Inorg. Biochem., *41* 157, 1991). Molecular modelling in conjunction with the determination of NOE's and T_1's of these two disaccharides was used to compare the metal free and metal bound conformations. Evidence is found for coordination by the idopyranosiduronate ring and either O3 for *1a* or the O6 sulfate group for *1b* of the glucosamine. This is the first example of chelation to Zn(11) by a simple disaccharide. These results are being used to design novel carbohydrate based metal chelates.

1.24
INTERACTIONS OF ASPARAGINE-LINKED CARBOHYDRATES WITH CONCANAVALIN A. NUCLEAR MAGNETIC RELAXATION DISPERSION AND CIRCULAR DICHROISM STUDIES

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Using near-UV circular dichroism (CD) and solvent proton relaxation dispersion (NMRD) measurements, three different conformational states have been detected in Ca^{2+}-Mn^{2+}-concanavalin A (ConA) upon binding a variety of asparagine-linked carbohydrates. Two of these transitions have been previously described: one for the binding of monosaccharides such as methyl α-*D*-mannopyranoside and oligosaccharides with terminal α-Glc or α-Man residues; and the second for the binding of oligomannose and complex type carbohydrates (Brewer, C.F. & Bhattacharyya, L (1986) *J. Biol. Chem.*, *262*, 7306–7310). The third transition occurs upon binding a bisected biantennary complex type carbohydrate with terminal GlcNAc residues. Temperature dependent NMRD and CD measurements have identified regions of the protein near the two metal ion binding sites that are associated with the conformation changes, and Tyr 12, which is part of the monosaccharide binding site, as responsible for the CD changes. The results support our previous conclusions that the rotamer conformation of the α(1–6) arm of bisected complex type oligosaccharides binds to ConA with dihedral angle $\omega = -60°$, while nonbisected complex oligosaccharides bind with $\omega = 180°$ (Bhattacharyya, L, Haraldsson, M. & Brewer, C.F. (1987) *J. Biol. Chem.*, *262*, 1294–1299). The present findings also explain the effects of increasing chain length of bisected complex type carbohydrates on their interactions with the lectin.

S2. X-RAY CRYSTALLOGRAPHY OF GLYCOPROTEINS/CRISTALLOGRAPHIE PAR RAYONS X DES GLYCOPROTÉINES

2.1
EXPERIMENTAL NMR EVIDENCE FOR INTERNAL MOTION IN A OLIGOSACCHARIDES

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The primary tools for the determination of the three-dimensional structures of oligosaccharides are X-ray diffraction and NMR. The latter experimental technique makes use of the nuclear Overhauser effect (NOE) which yields information on the distances between hydrogens in the molecule. When these distances span a glycosidic linkage, information regarding the torsional angles about that linkage can be deduced. However, a major problem with this otherwise ideal approach is internal flexibility. Because the NOE builds up over hundreds of milliseconds, any flexibility on this time scale will result in fluctuations in trans-glycosidic H-H distances and influence the final NOE value. To deduce three-dimensional structure from NOE measurements, one must, therefore, be able to model the internal flexibility of the oligosaccharide both in terms of the relative populations of confomers and in terms of the rate of interconversion between these. Molecular dynamics calculations provide such information. From such simulations using CHARMM, it is straightforward to calculate the predicted ensemble average steady state NOE and NOESY intensities. Detailed consideration of the observed NMR parameters for a specifically designed hexa-deuterio Manα1-3ManβOCD$_3$ disaccharide demonstrate the importance of internal flexibilty. Comparisons between the molecular dynamics derived values and over twenty observed NMR parameters will be presented.

2.2
THE CRYSTAL STRUCTURE OF A DODECASACCHARIDE-ANTIBODY FAB COMPLEX

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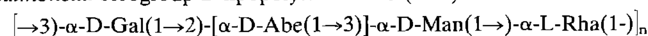
The 2.05Å resolution crystal structure of a Fab, from a mouse monoclonal antibody, complexed with a dodecasaccharide fragment of the *Salmonella* serogroup B lipopolysaccharide (LPS)



[→3)-α-D-Gal(1→2)-[α-D-Abe(1→3)]-α-D-Man(1→)-α-L-Rha(1-)]$_n$

reveals a unique carbohydrate recognition site. It is defined by aromatic amino acids and a water molecule rather than the carboxylic acid and amide side chains that are a feature of transport and other carbohydrate binding proteins. A branched trisaccharide fills this 8Å deep by 7Å wide pocket in an entropy assisted association ($K_A = 2.05 \times 10^5$, $\Delta H = -20.5$ kJ/mole and $T\Delta S = 10.0$ kJ/mole) and adopts a low energy conformation in the bound state that is within *ca.* 14kJ/mole of the global minimum energy conformer. The branched abequose (3,6-dideoxy-D-galactose) unit, an immunodominant sugar of the LPS, provides most contacts with the Fab and participates in two saccharide-protein hydrogen bonds, two saccharide–water hydrogen bonds and intramolecular hydrogen bonds between the abequose and galactose sugar residues.

Functional group replacement supports the hydrogen bonding pattern deduced from interatomic distances and geometry. The stringent requirements of this scheme that relies on neutral amino acid hydrogen bonding partners and a highly complementary protein surface impose Gal→Man glycosidic torsional angles that favour the intramolecular sugar-sugar hydrogen bonds. These in turn compensate for the *ca.* 40° shift at this linkage away from ϕ values favoured by the *exo*-anomeric effect. Recently the structure of the unliganded form of the Fab has been solved, as well as a complex of the Fab with a synthetic, branched tetrasaccharide.

Crystal structure data and synthetic gene technology have been used to design and express in *E. coli* (Narang *et. al.* unpublished work) a fully active Fab and single chain Fv fragment. These results provide a rational basis for engineering carbohydrate binding sites and exploring the importance of the different hydrogen bonding types for the stability of carbohydrate-protein complexes.

2.3
SITE–DIRECTED MUTAGENESIS OF MURINE ANTI-CARBOHYDRATE ANTIBODIES

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The objective of this project is to elucidate the structural basis of the specificity and affinity of anti-carbohydrate Ab. As a model system we are studying Ab against the 3-fucosyllactosamine (3-FL) and galactosylgloboside (GalGb4) antigens. Both sets of Ab are encoded by the VH441 gene, which codes for a glycosylation sequence at Asn58 in CDR2. This sequence is retained in all known VH441-encoded Abs except anti-GalGb4. Previous data indicate that the presence of carbohydrate in a variable domain can affect the affinity of Ab for antigen. Our data also suggest that the heavy chain CDR3 plays an important role in Ab specificity and affinity. We are conducting site-directed mutagenesis studies of Abs against 3-FL and GalGb4 to determine the structural basis of their Ag-binding specificity. Wild-type and mutated VH domains were ligated into an expression vector that contains a human IgG1 constant domain, and these constructs were expressed in mutant anti-3-FL and anti-GalGb4 hybridoma cells that synthesize only light chains. The anti-3-FL mutations include elimination of the glycosylation site, and alteration of the CDR3 sequence. Changes in the anti-GalGb4 Abs include replacement of the somatically mutated V_H441 gene with the germline V_H44I sequence and with the closely related germline sequence VHX24, which encodes two glycosylation sites. Preliminary data indicate that the Ab encoded by the V_H441 germline gene binds antigen with an affinity roughly comparable to the original monoclonal antibody, but the antibody encoded by VHX24 had no detectable antigen-binding activity. The recombinant Ab produced by these cells, and from five other transfections, are now being purified in order to analyze their binding specificity.

2.4
NEW TOOLS FOR MODELING LECTIN/CARBOHYDRATE INTERACTIONS. COMPARISON WITH X-RAY STRUCTURE OF A BIANTENNARY OCTOSACCHARIDE-LECTIN COMPLEX

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New tools have been designed for the modeling of protein/carbohydrate interactions: 1/ A set of appropriate energy parameters have been developed and added to the Tripos force-field used in Sybyl graphic software. This new force-field allows for the energy minimization of carbohydrates, either in the isolated state or in interaction with proteins. 2/ A geometrical procedure, the "crankshaft" method, has been developed for the docking of carbohydrate in lectin binding site. This allows for a systematic search of all the positions and orientations of a ligand in the protein recognition site. These methods have been applied for modeling the interaction of concanavalin A with mannose and glucose. The systematic search reveals that these two monosaccharides can adopt several orientations in the binding site. Evaluation of the energy of interaction and comparison with experimental data have been performed.

For the study of oligosaccharides interacting with proteins, we made use of two data bases previously developed: the data base of three-dimensional structure of monosaccharides containing in its present version 35 hexopyranoses, and the data base of the energy surfaces and low energy conformations of the glycosidic linkage for 25 disaccharides present in N-linked oligosaccharides.

Simulated conformations of isolated oligosaccharides are compared with the one observed in the crystal structure of a lectin/octosaccharide complex. The isolectin I of *Lathyrus ochrus*, a member of the mannose/glucose leguminous lectins, has been crystallized in the presence of a biantennary octosaccharide of the N-acetyllactosamine type, and the structure has been solved to a resolution of 2.3A. The oligosaccharide conformation is unambiguously defined and displays some original features. At each glycosidic linkage the observed conformation corresponds to one among the several predicted low energy domains. Nevertheless, drastic variations, away from the arrangements corresponding to the stabilizing influence of the exo-anomeric effect are experienced. The present work provides an illustration of oligosaccharide flexibility as well as new understanding of the energy forces involved in recognition phenomena.

2.5
X-RAY STRUCTURES OF BI- AND TRIANTENNARY SACCHARIDE-LECTIN COMPLEXES

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We describe the refined X-ray structures of bi- and triantennary saccharides of the N-acetyllactosamine type, complexed to isolectin I from *Lathyrus ochrus* (LOL I)

Crystals of LOL I complexes with biantennary octa, undeca and dodeca saccharides, as well as with a triantennary decasaccharide, have been obtained in the same conditions: lectin concentration was 20 mg/ml; precipitant was MPD 35–45 %; pH 7.5; saccharide/lectin ratio = 4. The crystals are monoclonic with the same space group, C2. Cell dimensions are a=78.3Å, b=75.4Å, c=104.0Å and β=94°.

Crystals of a complex between Concanavalin A and the same undecasaccharide as above have been obtained from MPD 30–40%, pH 6.5. Space group is C222, with cell dimensions a=96.4Å, b=102.8Å and c=59.7Å. X-ray study is underway.

In the case of the biantennary octasaccharide LOL I complex, the two saccharide moieties are located in clefts at each end of the long axis of the lectin. The complex is stabilized by numerous hydrogen bonds, half of them involving water molecules and van der Waals interactions, including some with aromatic residues. The structure analysis of the biantennary undecasaccharide-LOL I complex will be discussed and specially the role of the sialic acids. A binding mechanism is proposed on the basis of our results and of the one of previous studies. A more general schematic model of a possible lectin-glycoprotein interaction is also proposed.

2.6
THE THREE DIMENSIONAL STRUCTURE AT HIGH RESOLUTION OF *ERYTHRINA CORALLODENDRON* LECTIN AND ITS COMPLEX WITH LACTOSE

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Erythrina corallodendron lectin (EcorL) is a dimer of a glycosylated subunit, m.w. of 30,000. It is specific for Gal/GalNAc, and also binds

lactose. It's primary structure has been established by chemical sequencing (1), and from its cDNA (2), and the structure of its carbohydrate shown as Manα3 (Manα6) (Xylβ2) Manβ4 GlcNAcβ4 (Fucα3) GlcNAc (3), linked to Asn-17 of the protein. Comparison of the primary sequence of EcorL with those of other legume lectins reveals >10% of invariant amino acids (4). These include essentially all the residues that ligate the tightly bound Ca^{2+} and Mg^{2+} required for binding of carbohydrate, as well as an Asp and an Asn, that form key polar contacts with MeαMan in the high resolution x-ray structure of concanavalin A (5) and the *Lathyrus ochrus* lectin (6).

The structure of EcorL and its complex with lactose, have been determined and refined at 2.0 Å resolution. A unique packing of molecules in the crystal allows the N-linked heptasaccharide to settle into a single conformation stabilized by a number of interactions with the protein. Consequently, the whole carbohydrate chain can be seen with a clarity hitherto not observed in crystallographic studies of glycoproteins. The conformation of the carbohydrate is within the range of accessible conformations in solution as determined by NMR and energy calculations. The glycosylation site is located at a position of the monomer-monomer interface in the other legume lectins of known 3-D structure. Disruption of the normal monomer-monomer assembly by the bulky carbohydrate chain forces the EcorL to form a dimer whose quaternary structure differs drastically from that of other members of the family.

In the complex with lactose, hydrogen bonds occur between the carboxylate of Asp 89 and the 4-OH and 3-OH of the galactose, and between the amide of Asn 133 and the 3-OH.

Thus, the ability of individual legume lectins to distinguish between mannose and galactose cannot be ascribed to the nature of the contact amino acids, but must be related to their spatial disposition, as well as to other, yet unknown, steric and conformational factors. Supported in part by a grant from BARD to N.S.

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2.7
X-RAY CRYSTAL STRUCTURE OF A PEA LECTIN-TRIMANNOSIDE COMPLEX

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The structure of a complex between pea lectin and the methyl glycoside of a fragment of the N-linked oligosaccharide core structure, methyl 3,6-0-(α-D-mannopyranosyl)-α-D-mannopyranoside, has been determined at 2.6 Å resolution. The structure was solved by molecular replacement using the native pea lectin structure as a model.

A single terminal carbohydrate residue of this trisaccharide has been positioned in electron density defining the pea lectin monosaccharide binding site. Interpretable electron density for the second and third carbohydrate residues is not seen suggesting that these residues are disordered. The complex is stabilized by the hydrogen bond pairs, Gly 99 NH – Man 03, Asn 125 ND2 – Man 04, Ala 217 NH – MAN 05, Glu 218 NH – Man 06, Man 04 – Asp 81 OD2, Man 06 – Asp 81 OD1, as well as van der Waals interactions, involving Phe 123 in particular. The proposed hydrogen bonding scheme is in complete agreement with binding data obtained with a series of chemically modified glucose analogues (1).

The model also allows for an interpretation, in structural terms, of some of the differences in carbohydrate binding specificity shown by other members of this plant family. These results are consistent with the fact that the binding site geometry for this complex is very similar to that reported for the Con A – methyl α-D-mannopyranoside complex (2).

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S3. GLYCOPROTEIN FOLDING AND INTRA-CELLULAR TRANSPORT/REPLIEMENT ET TRANSPORT INTRACELLULAIRE DES GLYCOPROTÉINES

3.1
FUNCTIONAL CHARACTERIZATION OF BINDING PROTEIN (BiP) IN *S. CEREVISIAE*

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BiP (GRP78) is a member of the HSP70 family of proteins and it is located within the lumen of the endoplasmic reticulum. BiP has been shown to associate transiently with a variety of newly synthesized membrane and secretory proteins or permanently with misfolded proteins that fail to leave the ER. It has been suggested that BiP may be involved in the folding or assembly of nascent proteins or it may function to retain misfolded or incompletely folded proteins either for degradation or until folding/assembly is complete.

We previously cloned a homologue of the BiP gene from *Saccharomyces cerevisiae* and showed that it is essential for vegetative growth [Nicholson *et al.* (1990) PNAS *86* 1159]. As an approach to study BiP function, the yeast gene was placed under the control of a glucose-repressible promoter and the effects of BiP depletion were examined following transfer of cells to glucose-containing media. Reduction of BiP protein to about 15% of normal levels did not affect cell viability or protein synthesis, but it led to a profound decrease in secretion of α-factor and invertase. In parallel with the secretion defect, unglycosylated precursor forms of the secretory proteins accumulated intracellularly. The predominant form of invertase precursor lacked an N-terminal signal sequence but surprisingly was located in the cytosol. For α-factor, only the prepro- form which has an intact signal sequence was detected. Prepro-α-factor sedimented with microsomal membranes and was exposed on the cytoplasmic face in a protease-resistant form. The properties of the accumulated precursors suggest that, in yeast, BiP is required for the translocation of proteins across the ER membrane at a stage beyond the initial nascent chain-membrane interaction.

BiP may interact with nascent chains as they penetrate the lumen of the ER, preventing premature aggregation and allowing time for productive folding and assembly events to occur. In such a model, BiP depletion should lead to aggregation of nascent chains, a state that could block further translocation. Alternatively, BiP may not interact directly with nascent chains but instead may function to maintain the activity of some component of the translocation machinery.

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3.2
THE ROLE OF N-LINKED GLYCOSYLATION IN FOLDING AND INTRACELLULAR TRANSPORT OF A MODEL PLASMA MEMBRANE PROTEIN

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The G protein of vesicular stomatitis virus is a well-characterized glycoprotein that has been used extensively as a model in studies of membrane protein biosynthesis and transport in animal cells. The G protein contains two sites for asparagine-linked oligosaccharide addition, both of which are glycosylated. G protein forms a noncovalently associated homotrimer soon after synthesis in the endoplasmic reticulum (ER), prior to its transport through the Golgi complex to the plasma membrane. We have studied the role of N-linked glycosylation in the folding, assembly, and intracellular transport of G protein by site-directed mutagenesis of the coding sequence and expression of G proteins with altered glycosylation sites in transfected cells. By mutating the normal glycosylation sites, we found that only one oligosaccharide at either site resulted in efficient transport of G protein to the plasma membrane, whereas elimination of both sites resulted in G protein aggregates which did not exit the ER. New sites were created in the mutant G protein which lacked the normal glycosylation sites to ask if carbohydrate played a direct role in intracellular transport of G protein. Glycosylation at some, but not all, of these new sites resulted in transport of the altered G proteins to the plasma membrane. Although somewhat flexible, the position of the oligosaccharide in the polypeptide chain determined the ability of G protein to fold and trimerize correctly. The efficiency of transport of many of the mutant G proteins was increased at lower temperature, where proper folding occurred more efficiently. The G proteins with altered glycosylation sites which were retained in the ER did not form correct intrachain disulfide bonds, and instead formed aggregates with aberrant interchain disulfide bonds. These aggregated G proteins were stably associated with the ER resident BiP (GRP78). Thus, N-linked glycosylation plays a critical role in the initial folding of the G protein monomer. The position of these oligosaccharides in the growing polypeptide chain influences proper intrachain disulfide bond formation, and thus effects the downstream events of trimerization and transport to the plasma membrane. The presence of carbohydrate did not enhance the rate of transport once proper folding had occurred.

3.3
ROLE OF N-LINKED GLYCANS IN THE FOLDING OF HIV-1 ENVELOPE GLYCOPROTEINS

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Our recent results indicate that carbohydrates (CHO) present on mature *env* glycoproteins (gp) do not play a major role for HIV-1 infectivity (J. Exp. Med. 1989, March; J. Virol. 1990, June). However, sugar analogs like I deoxynojirimycin (dNM) that interfere with the glycosylation process of nascent gp160 markedly reduce virus infectivity. To resolve this apparent discrepancy we approached the mechanisms by which glycan processing interplays with gp160 tridimensional structure.

We investigated the effect of dNM (4mM) on glycosylation, production, bioactivity and immunoreactivity of gp160 produced by recombinant vaccinia virus-infected BHK21 cells. Glycosylation was studied by endoglycosidase analysis. Binding to CD4+ cells was determined by quantitative indirect immunofluorescence using anti-HIV-1 human antibodies. Because it is thought that exposed V3 loop of gp120 plays an essential role in post CD4-binding events, accessibility of V3 to mAb 110-4 (Genetic Systems) was studied by ELISA capture and by immunoaffinity purification. Non-glycosylated gp120 obtained in E. coli (ec gp120) and mannosylated gp160 from recombinant baculovirus-infected insect cells (bcv gp160) were used as controls.

dNM did not affect the amount of rgp160 recovered nor its secretion from the cells. As already described, dNM effect was incomplete, resulting in the production of molecules the glycosylation of which was heterogeneous with respect to their apparent MW and to sensitivity to Endoglycosidase H. Strongly reduced binding to CD4+ cells was noted with rgp160 produced in dNM treated cells. bcv gp160 bound to CD4 cells, but not ec gp120. Similarly, dNM treatment dramatically altered (by at least 10 fold) the accessibility of V3 to 110-4, whereas ec gp120 and bcv gp160 presented unchanged immunoreactivity.

Proper processing of CHO of nascent gp160 is essential for normal folding. dNM profoundly affects the glycosylation, bioactivity and conformation of rgp160. This may account for impaired HIV-1 infectivity elicited by dNM, while glycans present on the mature molecule are not of paramount importance.

3.4
POST-TRANSLATIONAL MODIFICATIONS OF HUMAN CD8 GLYCOPROTEIN CONSTITUTIVELY EXPRESSED IN AN EPITHELIAL RAT CELL LINE

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CD8 is a cell surface glycoprotein expressed on subsets of T lymphocytes, which carries only *O*-linked chains. We studied its *O*-glycosylation in a permanently transformed clone (FRT-U10) expressing high levels of human CD8 glycoprotein in order to correlate the post-translational modifications with the intracellular transport from the endoplasmic reticulum to the Golgi complex. SDS-PAGE analysis showed (i) a low M_r unglycosylated precursor, (ii) a palmitylated intermediate form carrying predominantly nascent *O*-linked GalNAc, (iii) two forms of the mature glycoprotein varying in the extent of sialylation.

Characterization of the glycomoiety indicates the occurrence of 4–6 chains, probably clustered in the NH_2-terminus. Beside the sialylation step, the addition of *N*-acetyllactosamine sequences and non-reducing terminal GalNAc (in α–anomeric linkage) are responsible for the M_r increment of the mature forms. The CD8 intermediate form is generated immediately after the block of the intracellular transport induced by incubation at 15°C, thus in an intermediate location between the endoplasmic reticulum and Golgi or in an early Golgi area. At this time the *O*-linked moiety included, beside nascent GalNAc, elongated chains heterogeneous in size with GlcNAc and GalNAc as outer sugars. These results suggest that the initial sequential addition of carbohydrates to *O*-linked GalNAc at different *O*-glycosylation sites does not occur as a synchronous event.

3.5
EFFECTS OF THE ANTI-VIRAL COMPOUND N-BUTYL DEOXYNOJIRIMYCIN ON CELLULAR GLYCOPROTEINS

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The N-butyl derivative of deoxynojirimycin (NB-DNJ) exhibits potent anti-viral activity against HIV *in vitro* (Fleet *et al.* 1988 FEBS Lett. 237:128-132 and Karpas *et al.* 1988 P.N.A.S. 85:9229-9233) NB-DNJ is

a glucose analogue and an inhibitor of the isolated enzyme ER-glucosidase I. One possible mechanism for the anti-viral effect of the compound is via the inhibition of glucosidase I, resulting in incomplete processing of the HIV envelope protein gp 120. However, to date there is no experimental evidence that the anti-viral activity of NB-DNJ results from inhibition of glucosidase I. An understanding of the mode of action of compounds such as NB-DNJ is critical for their potential use as anti-viral therapeutics.

To investigate the effect(s) of NB-DNJ in a cellular system we have performed *in vitro* studies with potential HIV host cells. Using flow cytometry we investigated the cell surface expression of a range of glycoproteins of the $CD4^+$ T cell line MOLT-4. We observed that while several cell surface glycoproteins, such as CD3, CD4 and CD5, were unaffected by NB-DNJ treatment one protein, the transferrin receptor (TR) was selectively affected. Treatment at the anti-viral concentration (0.5 mM) resulted in approximately 50% reduction of cell surface TR expression implying either a total cellular reduction of TR or an altered distribution of this receptor. The effect was dose dependent using both fluoresceinated mAb and ligand and was reflected in a reduction of iron uptake by the cells.

We are currently investigating the biochemical basis for the altered cell surface expression of the TR. We wish to establish if treatment of cells in culture with NB-DNJ results in the production of unprocessed, glucosylated glycoproteins and if such a modification is responsible for the observed effects on the TR. The receptor from treated and untreated cultures has been purified using ligand affinity chromatography. The TR from untreated cultures, as analysed by SDS-PAGE, shows heterogeneity which may reflect differential glycosylation. Following NB-DNJ treatment this heterogeneity is reduced. To elucidate if the observed heterogeneity is due to glycosylation two approaches are in progress: 1) Investigation of the purified TR by SDS-PAGE following endoglycosidase digestion and 2) Detailed analysis of the carbohydrates of the TR following hydrazinolysis and reduction with NaB_3H_4.

3.6
ANALYSIS OF A SPECIFIC GLYCOSYLATION SITE OF THE HUMAN TRANSFERRIN RECEPTOR

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The cellular uptake of iron is mediated by interaction of transferrin with the transferrin receptor (TfR). Human TfR is a homodimeric cell surface glycoprotein with a monomeric M_r of 95 kDa. The deduced amino acid sequence of human TfR contains three consensus sites for N linked glycosylation in the extracellular domain of the receptor (Asn 251, Asn 317, and Asn 727). Indirect studies indicate that human TfR contains two high mannose and one complex side chain, suggesting that all three glycosylation sites are occupied. Treatment of A431 cells with tunicamycin has shown that glycosylation is required for formation of a functional receptor. In conjunction with studies on the role of each glycosylation site in receptor function, we are mapping the carbohydrate structures present at each of the individual glycosylation sites in the human TfR. Human TfR, isolated from placental membranes by affinity chromatography, was reduced, carboxymethylated and digested with trypsin (1:25 w/w). Tryptic peptides were subjected to chromatography on Biogel P-2 and peptides eluting in the void volume were separated by reverse phase HPLC using a 0 to 40% linear gradient of CH_3CN. Individual peptides were applied to slot blots and glycopeptides were detected with digoxigenin conjugated lectins and alkaline phosphatase linked to anti-digoxigenin. The glycosylation site at Asn 251 was identified by amino acid sequence analysis of one of the HPLC purified peptides (Asp-Phe-Glu-Asp-Leu-Tyr-Thr-Pro-Val-Xxx-Gly). The inability to detect Asn 251 predicted at the tenth sequencing cycle is consistent with the peptide being glycosylated. The peptide was subjected to acid hydrolysis (2M TFA, 100°C, 5h) and the hydrolysate analyzed by high performance anion exchange chromatography. Glucosamine, galactose and mannose were detected in the hydrolysate, confirming that the peptide is glycosylated. The detailed structure of oligosaccharides at this site are currently under investigation. [Supported by NIH grant GM 43111]

3.7
ASSOCIATION OF GLOBOSIDE (Gb_4) WITH INTERMEDIATE FILAMENTS (IF) OF MESENCHYMAL, MUSCLE, GLIAL AND EPITHELIAL CELLS

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We showed previously that Gb_4 and GM_3 are present both on the cell surface of endothelial cells and inside the cell in association with the vimentin IF of the cytoskeleton. To determine if association of GSLs with IF is a general phenomenon, we have examined other cells. Colocalization of GSLs with IF was analyzed by double-label immunofluorescence of untreated and colchicine-treated cells. Our results, summarized in the Table, demonstrated association of Gb_4 with IF Types I, II and III but not Type IV. The intensity of filamentous staining with anti-Gb_4 varied both among cells within the same culture, and among different cell types. Gb_4 colocalized with the desmin and vimentin IF in primary dog smooth muscle cells, but not in BC3H1 and C2C12 muscle cells. In epithelial cells, which contain separate vimentin and keratin networks, Gb_4 colocalized with vimentin and not keratin. Association of Gb_4 with neurofilaments (NF) was not detected. The association of Gb_4 with IF depends on both the cell type and the type of IF. We are now investigating the possibility that intermediate filaments play a role in the intracellular transport and sorting of glycosphingolipids.

IF Type		Cell Type	Co-localization with IF
I,II	Keratin	Hepatoma, keratinocytes	Yes
I,II,III	Ker & Vim	Epithelial HeLa, MDCK	Yes with vim, not ker
III	Vim	Endothelial, fibroblasts	Yes
III	Vim & Des	Primary smooth muscle	Yes
		Muscle lines BC3H1 & C2C12	No (high surface Gb_4)
III	GFAP	Glial cells	Yes
IV	NF	Primary neuronal cells	No
III,IV	NF & Vim	PC12 pheochromocytoma cells	No (Gb_4 on surface)

3.8
TEMPERATURE-SENSITIVE MUTANT OF CHINESE HAMSTER OVARY CELLS WITH A REVERSIBLE BLOCK IN PROTEIN SECRETION, GOLGI APPARATUS DISASSEMBLY AND REDISTRIBUTION OF GOLGI APPARATUS COMPONENTS INTO THE ENDOPLASMIC RETICULUM

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The Chinese hamster ovary (CHO) cell mutant DS28-6 exhibits a pleiotropic defect in protein secretion. Our study was aimed at the further characterization of this mutant and the identification of the subcellular basis for the secretion defect.

The analysis of DS28-6 cell lysates showed a normal protein synthesis at the permissive and non-permissive temperature. Wild-type (at 37°C) and mutant DS28-6 (at permissive temperature of 33°C) showed identical fine structure with well developed perinuclear located Golgi apparatus. Mutant DS28-6 shifted to non-permissive temperature (39.5°C) exhibited a time-dependent disassembly and loss of the Golgi apparatus. The

disorganization of the Golgi apparatus into vesicles was complete after 1–2 h at 39.5°C. Transitional elements of the ER, mitochondria, nuclei and the nuclear envelope appeared structurally unaltered. Complete reorganization of Golgi apparatus structure was observed within 1–2 h following shift to permissive temperature. Immunofluorescence for Golgi mannosidase II and galactosyltransferase as well as fluorescent NBD-ceramide (a trans Golgi apparatus marker in CHO cells) produced a pattern characteristic of the Golgi apparatus in both wild-type and mutant DS28-6 grown at permissive temperature. Shifting mutant DS28-6 to non-permissive temperature changed the perinuclear immunofluorescence dramatically into a fine reticular fluorescence present throughout the cytoplasm. This pattern coincided with $DiOC_6$ fluorescence, a marker of the ER. Pretreatment of mutant DS28-6 with 2-deoxy-D-glucose and NaN_3 or with nocodazole prior to the shift to 39.5°C prevented redistribution of Golgi mannosidase II immunofluorescence. Similarly, NBD-ceramide fluorescence remained localized in a Golgi apparatus pattern after depletion of cellular ATP. In contrast, nocodazole pretreatment did not prevent redistribution of NBD-ceramide fluorescence into an ER pattern. When mutant DS28-6 was shifted back to permissive temperature, the ER staining pattern changed within 1-2 h to a localized perinuclear pattern indistinguishable from that for Golgi mannosidase II, galactosyltransferase and NBD-ceramide in control cells.

Thus, this mutation exhibits a striking similarity to the cellular effects caused by Brefeldin A (BFA). The discovery of such a mutation provides further evidence for the existence of a Golgi apparatus-ER recycling pathway as proposed on the basis of the BFA experiments. Hence, the CHO mutant DS28-6 may prove important in the further characterization of this recycling pathway and may aid the identification of the principle regulatory molecule(s) involved.

3.9

INDUCTION OF GLUCOSE REGULATED PROTEINS AT NONPERMISSIVE TEMPERATURE IN AN FM3A MUTANT, G258, HAVING A TEMPERATURE-SENSITIVE MUTATION ON OLIGOSACCHARIDE-LIPID SYNTHESIS

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I have isolated a temperature-sensitive(*ts*) Asn-linked glycosylation mutant, designated G258, from the mouse mammary carcinoma cell line FM3A by [2–^{3}H]mannose suicide selection. G258 is not only *ts* for Asn-linked glycosylation, but also *ts* for cell growth(1,2). The biochemical defect resides in the formation of oligosaccharide-lipid(OL). At 33°C, G258 cells synthesize $Glc_3Man_9GlcNAc_2$-PP-Dol, but at 39°C, G258 cells are able to synthesize merely the medium-sized OL (up to $Man_3GlcNAc_2$-PP-Dol), but are unable to synthesize the larger OL(2). Spontaneous revertants for cell growth were isolated from G258 cells. All growth-revertants also showed reversions on the Asn-linked glycosylation(3,4) and on the synthesis of OL(4). These results imply that both temperature-sensitivities of G258 cells were derived from a single gene mutation(3,4). This characteristic will enable me to clone human cDNA on the OL synthesis by screening its activity to let G258 cells recover from their temperature-sensitivity for cell growth. At 39°C, VSV-infected G258 cells could not perform Asn-linked glycosylation of G (glyco)protein(5). This result suggests that such medium-sized OLs would not be recognized by oligosaccharyltransferase, for enzymological or topographical reasons. Moreover, syntheses of GRP78 (Bip) and GRP94 were induced at 39°C in G258 cells(5). This result implies that aberrant foldings and aberrant assemblies of (glyco)proteins occur at 39°C in G258 cells. Thus, at 39°C, G258 cells cannot perform Asn-linked glycosylation of (glyco)protein(s) that are needed for cell growth. Thus, the functional expression of such essential (glyco)proteins [perhaps receptors for growth factors, *etc.* (4,5)] may be impaired at 39°C by association with GRP78(Bip). This may lead G258 cells to stop their growth at 39°C.

(1) Cell Struct. Funct. *7*:412 (1982); (2) J. Cell. Physiol. *119*:260 (1984); (3) Cell Struct. Funct. *13*:676 (1988); (4) Biochim. Biophys. Acta in press; (5) submitted.

3.10

RETENTION SIGNAL FOR THE TRANS-GOLGI LOCALISED BOVINE β1,4-GALACTOSYLTRANSFERASE

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β1,4 Galactosyltransferase (Gal T) shares a common domain structure with all Golgi glycosyltransferases cloned to date, namely, a short amino terminal cytoplasmic tail, a signal/anchor domain, a stem region and a large carboxy catalytic domain (Paulson & Colley, *J. Biol. Chem.* 264; 17615:1989). Truncated forms of Gal T are secreted into body fluids which lack the cytoplasmic, transmembrane and stem domains of the membrane-bound form; these domains are therefore likely to contain the dominant signal required for the retention of Gal T within the Golgi complex.

Constructs containing the full length cDNA for bovine Gal T were transiently expressed in COS-1 cells. The product of the transfected COS-1 cells was detected with bovine-specific anti-Gal T antibodies, affinity purified using a recombinant fusion protein. The expressed product was localised to the Golgi by immunofluorescence. In addition, these transfected COS cells have a 15–25 fold elevation in Gal T activity. Stable expression of bovine Gal T in murine L cells, using an SV4O based expression vector, also resulted in a Golgi localisation of the expressed product. No cell surface expression of bovine Gal T was detected.

To identify the retention signal responsible for the Golgi localisation of Gal T, a series of cDNA constructs have been generated with deletions corresponding to the amino terminal domains. The removal of the cytoplasmic tail did not alter the Golgi localisation of the product transiently expressed in COS cells or stably expressed in L cells. In constrast, deletion of both the cytoplasmic and transmembrane domains of Gal T and incorporation of the cleavable signal sequence from influenza haemagglutinin, resulted in the secretion of the expressed bovine Gal T from transfected cells. Further deletion mutants of Gal T, and the generation of hybrid constructs, are being prepared to further assess the roles of the transmembrane and stem domains in Golgi retention.

3.11

CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR): A SURFACE MEMBRANE GLYCOPROTEIN OF AIRWAY EPITHELIAL CELLS

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CFTR was predicted to be a membrane glycoprotein from the deduced amino acid sequence (Science, 245, 1066, 1989). Alterations in glycosylation have been described in some, but not all CF glycoproteins, and in secreted as well as in integral membrane glycoproteins (Clin. Chim. Acta, 188, 193, 1990). To examine the subcellular localization and

glycosylation of CFTR in CF and non-CF cells, polyclonal antibodies were prepared from peptides of two different regions of the proposed sequence of CFTR: 1) the nucleotide binding fold containing the most common CF mutation, ΔF_{508}, and 2) the R domain which is thought to be a functional domain. By western blot analysis of immortalized airway epithelial cells, both antibodies detect a protein, M_r 170,000, which is not seen with pre-immune serum. The membrane location of CFTR was established by isolating whole surface membranes from the immortalized epithelial cells by the Zn^{++} procedure. Both whole cells and isolated surface membranes of airway cells from a CF individual heterozygous for ΔF_{508} and from a non-CF individual who is homozygous normal were examined. As predicted, the surface membranes from the non-CF cells contained CFTR. The CF cells also contained surface membrane-localized CFTR. This is in contrast to a recent report which suggested that as a result of the common mutation in the nucleotide binding fold, CFTR remains primarily in the ER and has altered glycosylation (Cell, 63, 827, 1990). Both antibodies will be used to immune precipitate CFTR from airway epithelial cells which have been labeled with radioactive oligosaccharide and protein precursors. These studies are the first phase of a detailed characterization of this important cell surface membrane glycoprotein. Supported in part by the CF Foundation.

3.12
TRANSPORT ACTIVITY OF DEGLYCOSYLATED BAND 3, THE ANION EXCHANGE PROTEIN OF THE ERYTHROCYTE MEMBRANE

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The single N-linked oligosaccharide chain on Band 3, the anion transport protein of the erythrocyte membrane, could be removed by treatment with glycopeptidase F. Deglycosylation was assayed by the sharpening of the Band 3 band on SDS polyacrylamide gels, loss of tomato lectin binding on blots and analysis of glucosamine in hydrolysed samples. Purified Band 3 in detergent solution (0.1% n-dodecyl octaethylene glycol monoether) could be deglycosylated using 1 U glycopeptidase F/mg protein for 18 h at room temperature. Deglycosylation of Band 3 in red cell ghost membranes required 10 U/mg ghost protein (about 50 U/mg Band 3), for 18 h at 37°C and pretreatment of red cells with trypsin to cleave the extracellular portion of glycophorin. Anion transport was assayed in resealed ghosts loaded with Cl^- and the Cl^--sensitive, fluorescent compound, 6-methoxy-N-(3-sulfo-propyl) quinilinium (SPQ). Chloride efflux in exchange for SO_4^{2-} was proportional to the increase in SPQ fluorescence. Net transport rates, after subtracting the leak rates in the presence of the anion exchange inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), were identical for control and deglycosylated ghosts. The polylactosaminyl moiety of the Band 3 carbohydrate structure could be removed by treatment with endo-β-galactosidase. Control red cells had a halftime of phosphate uptake in exchange for internal Cl^- of 30 minutes while the halftime for endo-β-galactosidase-treated cells was 29 minutes. The secondary structure, oligomeric state, detergent and inhibitor binding of Band 3 did not change upon deglycosylation. We conclude that the carbohydrate chain at asparagine 642 of human Band 3 is not required for anion transport. Supported by the Medical Research Council of Canada.

3.13
THE ROLE OF GLYCOSYLATION IN SURFACE EXPRESSION OF HUMAN GLYCOPHORIN A cDNA BY CHINESE HAMSTER OVARY CELLS

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Glycophorin A (CpA), the most abundant membrane sialoglycoprotein of human red blood cells (RBCs), carries several blood group antigens. To study the role of glycosylation in translocation and antigenicity of this highly glycosylated protein (1 N-glycan and 15 O-glycans), normal and mutant forms of GpA cDNA were expressed in wild-type and lectin-resistant CHO cells.

GpA was expressed on the surface of transfected wild-type CHO cells and, on immunoblots, monomers (38 kD) and dimers were seen which co-migrated with human RBC forms of GpA. Tunicamycin treatment of these CHO cells did not block surface expression of GpA, suggesting that N-linked glycosylation is not required for this process. To confirm this result, the Mi.I (Thr_{28}➤Met) and Mi.II (Thr_{28}➤Lys) mutants of GpA were constructed by site-directed mutagenesis, preventing N-linked glycosylation at Asn_{26}. Following transfection of Mi.I GpA, the variant protein was expressed on the surface of wild-type CHO cells, and faster migrating monomers (36 kD) and dimers were seen on immunoblots. Transfection experiments with Mi.I GpA are currently underway.

To study O-linked glycosylation, GpA cDNA was transfected into the lectin-resistant CHO cell lines Lec 2, Lec 8, and IdID. Partially O-glycosylated GpA was expressed on the surface of Lec 8 and Lec 2 cells cultured in complete media, as well as on IdID cells cultured in the presence of GalNAc alone, with monomers of 25, 25, and 33 kD, respectively. In contrast, non-O-glycosylated GpA (19 kD monomer) was poorly expressed on the surface of IdID cells cultured in the absence of both Gal and GalNAc.

Since blocking N-linked glycosylation with tunicamycin does not affect GpA surface expression, the results with the lectin-resistant cells suggest that O-linked oligosaccharides are required for appropriate surface expression of transfected GpA in CHO cells.

3.14
DIFFERENTIAL EFFICIENCY OF N-LINKED GLYCOSYLATION AT SPECIFIC SEQUONS IN RABIES VIRUS GLYCOPROTEIN

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The sequon Asn-X-Ser/Thr is a necessary but not sufficient signal for N-linked glycosylation. To further define the signals on a polypeptide which regulate its N-linked glycosylation, the efficiency of glycosylation at specific sequons on rabies virus glycoprotein (rabgp) was compared. Rabgp is the only glycoprotein synthesized by the virus and glycosylation is necessary for the appropriate expression of rabgp on the surface of transfected CHO cells. Mature ERA strain rabgp is a transmembrane protein with 505 amino acids and sequons at Asn residues 37, 247, and 319 on the extracellular domain. Biochemical studies using purified virions suggest that glycosylation normally occurs only at Asn 247 and 319. To further study rabgp glycosylation, a series of mutants was produced by site-directed mutagenesis; the changes were confirmed by DNA sequencing. In each mutant, one or more sequons was abolished by replacing the Ser/Thr residue with Ala. Glycosylation was studied in a cell-free transcription/translation/glycosylation system using rabbit reticulocyte lysate and dog pancreatic microsomes. This system supports initial core glycosylation but not subsequent processing. Proteins were labeled with ^{35}S-Met and analyzed by SDS-PAGE and autoradiography. In the absence of microsomes, both wild-type and mutant transcripts directed the synthesis of 58 kD unglycosylated rabgps which contain the

signal sequence. Glycosylation of wild-type rabgp in vitro yielded two products, a major species with two oligosaccharides and a minor species with three oligosaccharides. Similar studies with mutant rabgps revealed efficient, complete glycosylation of the sequons at Asn 247 and 319 and inefficient glycosylation at Asn 37. In addition, the efficiency of glycosylation at individual sequons was not influenced by glycosylation of the other sequons. Future studies will define the structural features of these sequons which determine glycosylation efficiency in both cell-free systems and transfected cells.

3.15
THE POLY-*N*-ACETYLLACTOSAMINES OF LYSOSOMAL MEMBRANE GLYCOPROTEINS, LAMP-1 AND LAMP-2, ARE INCREASED BY THE PROLONGED ASSOCIATION WITH THE GOLGI COMPLEX

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Lysosomal membrane glycoproteins (lamps) are the major glycoproteins carrying polylactosaminoglycans. We have analyzed the detailed carbohydrate structures of lamps, and demonstrated that the increase of poly-*N*-acetyllactosamine can be caused by an increased activity of β-D-Gal-β1,3*N*-acetylglucosaminyltransferase, "extension enzyme" (*J. Biol. Chem. 265*, 20476). In order to further understand the reason why only certain glycoproteins can be modified by poly-*N*-acetyllactosamine, we have utilized the incubation condition at 20°C, which was previously shown to cause the accumulation of glycoproteins at the *trans*-Golgi.

HL-60 cells were labeled with [^{3}H]galactose at 21°C or 37°C for 6 hr or 24 hr, and lamp-1 and lamp-2 were immunoprecipitated. Upon examination by SDS-polyacrylamide gel electrophoresis, each lamp from HL-60 cells incubated at 21°C exhibited a much broader and slower band than those isolated from the cells incubated at 37°C. The number of *N*-glycans containing poly-*N*-acetyllactosamine, estimated by their binding to tomato-lectin column, increased approximately 50% after incubation at 21°C than incubation at 37°C. The analysis of oligosaccharides released by endo-β-galactosidase digestion demonstrates that the amount of side chains containing three or more *N*-acetyllactosamine repeats increased about 100% after incubation at 21°C. The methylation analysis confirmed the above results. The same analysis and the results obtained by ion-exchange chromatography also provide the evidence that the *N*-glycans of lamps are sialylated at 21°C as much as at 37°C. These results therefore indicate that the incubation at 21°C render the lamps residing longer with the Golgi complex, and such longer residing in the Golgi complex allows lamps to acquire more polylactosaminoglycan. These results suggest that the time necessary for moving through the Golgi complex is a critical factor for poly-*N*-acetyllactosaminyl modification. (Supported by CA48737 and DK37016).

3.16
THE STABILITY OF N-LINKED GLYCOPROTEINS IN HT-29 CELLS IS A FUNCTION OF THEIR STATE OF ENTEROCYTIC DIFFERENTIATION

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When the human colon cancer HT-29 cells undergo an enterocytic differentiation, they correctly process their N-glycans whereas their undifferentiated counterpart are unable to process Man_{9-8}-$GlcNAc_2$ species, the natural substrate of mannosidase I. As this enzyme is fully active and that its substrate (Man_8-$GlcNAc_2$) has the same structure in both HT-29 cell populations, we hypothesize that N-glycoproteins are unable to reach the cis Golgi, the site where mannosidase I has been localized. In the presence of 1-deoxymannojirimycin, a specific inhibitor of mannosidase I, differentiated HT-29 cells as expected accumulate Man_{9-8}-$GlcNAc_2$ species whereas in undifferentiated HT-29 cells these compounds continue to be rapidly degraded. In contrast, the use of leupeptin, a specific inhibitor of thiol and serine proteases, lead to the accumulation of these oligosaccharides in undifferentiated HT-29 cells. Monensin, a carboxylic ionophore that perturbs the distal Golgi functions, is unable to stabilize these compounds. However, brefeldin A as well as low temperature (15°C) treatments which interfere with the endoplasmic reticulum to Golgi traffic prevent the high mannose bearing glycoproteins from degradation in undifferentiated cells. These results favor the hypothesis that a direct pathway should exist between the rough endoplasmic reticulum and a leupeptin-sensitive degradative compartment in undifferentiated HT-29 cells. The emergence of this new pathway could explain why protein stability and N-glycan processing may vary as a function of the state of cell differentiation.

3.17
SEPARATION BY ELECTROPHORETIC LEVITATION OF CELL ORGANELLES MEDIATING PROTEIN TRAFFICKING

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A separation device was constructed consisting of a column (5 cm length, 3.8 cm^2) that contained a Ficoll density gradient. Circular (3.8 cm^2) electrodes of platinum and palladium respectively generated an electrical field (3 Volts/cm) and caused particles to migrate upwards. By way of special layering and fractionation measures, resolution was very high. Thus mixtures of rabbit, rat and human erythrocytes were separated (within 60 min.) as well as human red cells from NaNase-treated red cells (within 10 min.). After Dounce homogenization of human hepatoma cells (HepG2), lysosomes were readily separated from other vesicular constituents of the post nuclear supernatant using β-hexosaminidase as a marker. Also early endosomes, monitored as ^{125}I–transferrin containing vesicles, could be separated in two clear-cut populations at some distance from the peak of free soluble transferrin. Putative Golgi vesicles derived from the 1.2 M sucrose interphase of a Mg^{2+} containing sucrose gradient – after buoying density centrifugation – were electrophoresed and a separation on N-linked complex type transferrin and α_1-antitrypsin containing vesicles from high-mannose proteins containing vesicles was effectuated. Separation was improved by prior treatment of the vesicles with small amounts of trypsin. This particular separation may represent the separation of post-ER smooth vesicles from trans Golgi network vesicles. In preliminary experiments, a separation of galactosyltransferase containing vesicles was obtained from sialyltransferase containing vesicles. In forthcoming experiments emphasis will be laid on the feasibility of separating cis, medial, trans, and trans Golgi network compartments.

3.18
PHOSPHORYLATION OF α-L-FUCOSIDASE IN A LYMPHOID CELL LINE OF AN I-CELL DISEASE PATIENT

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Previously, we investigated the phosphorylation of fucosidase in lymphoid cell lines of a healthy individual and an I-cell disease patient

(Glycoconj. J. 7:498, 1990). Phosphoserine was identified in fucosidase of both control and I-cell lymphoid cells while mannose 6-phosphate (M6P) was found only in enzyme from control cells. M6P is the recognition marker found on many newly made acid hydrolases required for their transport to lysosomes. I-cell lymphoid cells retain normal intracellular levels of fucosidase despite lack of M6P. The absence of M6P in fucosidase of I-cell lymphoid cells is the result of a deficiency of N-acetylglucosamine-1-phosphotransferase. The presence of phosphoserine in fucosidase was novel. Therefore, we investigated the metabolism of ^{32}Pi in fucosidase. During a 1.5 h pulse with ^{32}Pi, fucosidase was synthesized by control cells as an intracellular form (M_r = 58,000). Companion cultures chased with Pi from 2 h to 21 h revealed ^{32}P-fucosidase both intracellularly and extracellularly. After the 21 h chase, 68% of the ^{32}P-fucosidase was intracellular and 32% was extracellular. Incorporation of ^{32}Pi into fucosidase of I-cell lymphoid cells was not detected employing labeling times of 1 h, 2 h, or 4 h. However, in I-cell cultures incubated with ^{32}Pi for 6 h, synthesis of an intracellular form of fucosidase (M_r = 58,000) was detected. All of the fucosidase labeled with ^{32}Pi during the 6 h pulse was absent from cultures after a 4 h chase with Pi. ^{32}P-fucosidase was never found in the medium of I-cell cultures. In contrast, ^{35}S-methionine-fucosidase was found in cells and medium. Since phosphoserine occurred in intracellular but not in extracellular fucosidase of I-cell cultures, we speculate that phosphoserine may be involved in intracellular retention of fucosidase in I-cell lymphoid cells. Supported by DK32161 and NS12138.

3.19
STRUCTURAL INFORMATION IN β-HEXOSAMINIDASE REQUIRED FOR SORTING OF PROTEINS TO *DICTYOSTELIUM DISCOIDEUM* LYSOSOMES

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In order to locate elements of the primary structure of β-hexosaminidase which are necessary for the sorting of proteins to lysosomes, we prepared vector constructs which contain portions of the 5′-coding sequence of the enzyme fused in frame with the coding sequence for the yeast SUC2 gene, invertase. These were expressed in *Dictyostelium discoideum* and transformants were analyzed. As a positive control we transformed the organism with a similar vector construct which contained an insert coding for the entire sequence of β-hexosaminidase. Transformants expressed active β-hexosaminidase, with the subunit molecular mass of 68 kDa expected if the enzyme was normally processed. β-hexosaminidase activity in transformants was 1–200 times the level in axenic strain.

The over-expressed enzyme co-localized with other acid hydrolases, in the lumen of the lysosome. Over-expression did not alter the fate of other hydrolases. The fusion products contained 22 (Hex 22-Inv) to 532 (Hex 532-Inv) amino-terminal amino acids from β-hexosaminidase, fused to invertase. The molecular masses of fusion proteins ranged from 84 kDa to 150 kDa. Only Hex 22-Inv was rapidly ($t_{1/2}$ < 30 min) and quantitatively secreted. All others were slowly ($t_{1/2}$ > 5 h) and partially secreted. All expressed invertase activity. During growth, the invertase activity of all fusions, except that of Hex 22-Inv, was retained to the same extent as endogenous lysosomal enzymes. All of those retained, except Hex 532-Inv, localized in intermediary vesicles (d = 1.05).

The fusion containing 532 amino acids localized in denser vesicles (d = 1.08) together with lysosomal enzymes. The bulk of intracellular Hex 22-Inv, Hex 70-Inv and Hex 532-Inv was soluble but most of Hex 97-Inv, Hex 314-Inv, Hex 397-Inv and Hex 469-Inv was membrane associated. The release of membrane associated fusion products was not induced by starvation but soluble fusion products were subject to induced secretion. The results provide evidence for a linear array of sorting information, beginning at the amino terminus of the enzyme, including: a trans-membrane sorting signal, a signal for entrance into pre-lysosomal sorting vesicles, a sequence which is required for membrane association and a carboxy-terminal sequence required for transfer to dense lysosomes.

3.20
THE GLYCOSYLATION OF LYSOSOMAL CARBOXYPEPTIDASE B FROM BOVINE SPLEEN

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A number of common structural protein and carbohydrate recognition signals in lysosomal enzymes have been shown to influence the targeting of these glycoproteins after translation to the lysosome and subsequently to determine their regulatory function *in situ* (1). In the lysosomes, they undergo the final processing steps which may include exo- and/or endoproteolytic trimming by proteinases (2). The structural and biosynthetic parameters for a large group of thiol-dependent endoproteinases, the cathepsins, have been extensively studied (3): on the other hand those from the exo-proteinases are virtually unknown.

Lysosomal carboxypeptidase B [EC 3.4.18.1.] has been purified recently from bovine spleen (4). Since only controversial data has been obtained experimentally, or none at all, about its molecular size and glycosylation patterns, we developed a new strategy to get more direct information on its structural parameters. A combination of mass-spectrometric techniques, involving the matrix-assisted UV-induced desorption (UVI-LD-MS) of complete, of partially and of totally deglycosylated enzyme, as well as fast atom bombardment (FAB-MS) of its carbohydrate portion, has been applied on the microscale level.

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3.21
PLATELET ARYLSULFATASE A IN ALCOHOLIC AND NON-ALCOHOLIC HUMANS

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Human platelet arylsulfatase A (ASA) is a lysosomal enzyme which displays electrophoretic variants. A normal North American population exhibits four predominant isoforms (IV_A pattern), while enzyme from some documented alcoholics (III_A pattern) differs from this profile. We have undertaken studies to understand the role of protein linked glycan on the electrophoretic mobilities of the ASA isoforms from normal and alcoholic individuals. Alkaline phosphatase treatment of ASA from normal individuals results in conversion of the multiple band pattern into a single component exhibiting a mobility equal to the mobility of the slowest moving isoform. Apparently, phosphate esters are responsible for the production of different electrophoretic forms of the enzyme. The separation of bound and unbound forms of the enzyme on a mannose-6-phosphate receptor affinity column verifies that some, if not all, of the bound phosphate is as 6-phosphomannosyl groups. Only the slowest moving, non-phosphorylated isoform of ASA does not bind to the receptor.

The electrophoretic heterogeneity of the variant enzyme from indi-

vidual alcoholics is also simplified upon dephosphorylation of the enzyme. However, the mobility of the dephosphorylated enzyme from the alcoholic individual, differs from that of dephosphorylated ASA from the normal population. Interestingly, both the native, and dephosphorylated enzyme yield at least two separable populations on a *Lens culinaris* affinity column. Both populations from native ASA produce identical electrophoretic patterns, each indistinguishable from the original sample. Glycosidase studies indicate that the enzyme probably lacks sialic acid, and the inability of ASA to bind to a *R. communis* affinity adsorbant is consistent with the enzyme possessing one or more oligomannosyl glycans.

We conclude that human platelet arylsulfatase A exhibits electrophoretic heterogeneity due to different levels of phosphorylation, and that the enzyme from alcoholic individuals differs from that obtained from a non-alcoholic population either in aminoacid sequence, and/or in post-translational modifications probably unrelated to the glycan moieties. (This research was supported in part by grants from the USDHHS-NIAAA and the UNICO foundation.)

3.22

CATABOLIC PATHWAYS OF OLIGOMANNOSIDIC AND N-ACETYL-LACTOSAMINIC TYPE GLYCANS BY THE LYSOSOMAL AND CYTOSOLIC GLYCOSIDASES OF RAT LIVER. A STUDY 400 MHz-^{1}H NMR STUDY.

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The stepwise degradation of oligomannosidic and N-acetyllactosaminic type glycans by lysosomal and/or cytosolic glycosidases was investigated using a method which combines the HPLC separation of isomers formed after enzymatic digestion and their structural determination by 400 MHz-^{1}H-NMR spectroscopy.

1 – Hydrolysis of oligomannosidic N-glycans appears to follow different but possibly complementary pathways in lysosomes and cytosol. We demonstrate the existence of a specific and ordered pathway inside the lysosomes, completely different from that realized by the Golgi and RER α-mannosidases. The degradation is realized in two stages: the first one leads from $Man_9GlcNAc$ to $Man_5GlcNAc$ by preferential cleavage of the four α1,2 linked mannose residues, and the second one, Zn^{2+} dependant, leads from $Man_5GlcNAc$ to Man(β1,4)GlcNAc by hydrolysis of α-1,3- and α-1,6-linked residues. On the contrary the cytosolic pattern leads by a quite different pathway to an unique hexasaccharide $Man_5GlcNAc$ which has curiously the same structure as one of the polyprenolic intermediates occuring in the cytosol during biosynthesis of N-glycans. Man(α1–2)Man(α1–2)Man(α1–3)[Man(α1–6)]Man(β1–4)-GlcNAc.

2 – Hydrolysis of N-acetyl lactosaminic glycans is realized randomly by sequential degradation by the different exo-glycosidases. Nevertheless hydrolysis of the α-1,3-mannose linked antenna is realized faster than the α-1,6-linked branch. Influence of additive branches or intersecting GlcNAc was also studied. All these results are explained in terms of glycan conformation.

3.23

TRANSIENT GLUCOSYLATION OF GLYCOPROTEINS IN THE ENDOPLASMIC RETICULUM.

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The UDP-Glc:glycoprotein glucosyltransferase is a soluble protein of the endoplasmic reticulum. It has been detected in mamalian, plant, fungal and protozoan cells and catalyzes the formation of protein-linked GlclMan9GlcNAc2, GlclMan8GlcNAc2 and GlclMan7GlcNAc2 from the respective unglucosylated compounds.The reaction products are immediately deglucosylated in vivo by glucosidase II. It was found, in cell-free assays, that the Man8GlcNAc2 and Man7GlcNAc2 isomers having the mannose unit to which the glucose is added were glucosylated by the rat liver glucosyltransferase at 50% and 15%, respectively, of the rate of Man9GlcNAc2 glucosylation. This indicated that processing by endoplasmic reticulum mannosidases decreases the extent of glycoprotein glucosylation.

All five different glycoproteins tested as glucose acceptors (bovine and porcine thyroglobulins, Phytohemagglutinin, soybean agglutinin and bovine pancreas ribonuclease B) were found to be poorly or not glucosylated unless they were subjected to treatments that modified their native conformations. The effect of glycoprotein denaturation was not to make oligosaccharides accessible to the glucosyltransferase but to expose protein determinants, the interaction of which with the enzyme appeared to be required for the transfer reaction to occur because a) cleavage of denatured glycoproteins by unspecific (pronase) or specific (trypsin) proteases abolished their glucose acceptor capacities almost completely except when the tryptic peptides were held together by disulfide bonds and b) high mannose oligosaccharides in native glycoproteins, although poorly or not glucosylated, were accessible to macromolecular probes as ConcanavalinA-Sepharose and endo-β-N-acetylglucosaminidase H. In addition, denatured, endo-β-acetylglucosaminidase H-de-glycosylated glycoproteins were found to be potent inhibitors of denatured glycoprotein glucosylation. It is suggested that in vivo, only unfolded, partially folded and malfolded glycoproteins are glucosylated and that glucosylation stops upon adoption of the correct conformation, a process that hides the protein determinants (hydrophobic amino acids ?) from the glucosyltransferase.

3.24

ACTIVITIES OF GLYCOSYLTRANSFERASES IN PLATELETS SHOULD BE PROPORTIONAL TO THEIR GOLGI COMPLEX CONTENTS AND POLYPLOIDIZATION OF MEGAKARYOCYTES

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Platelets release α-6-L-fucosyltransferase (6-FL) during coagulation of blood (1) or treatment of isolated platelets with agonists which cause them to change shape, aggregate, and secrete (2). We have shown that under similar conditions other glycosyltransferases are also released; β-4-D-galactosyltransferase, β-3-D-, and β-6-D-N-acetylglucosaminyltransferase. We have studied in detail the release of the activity of 6-FL by platelets in humans and rats and found it to be high or low when the pace of thrombopoesis was presumably increased or decreased respectively. Measurement of the serum enzyme activity/platelet count ratio may be even a sensitive diagnostic test for the pace of thrombopoesis; in blood donors who frequently donate platelets this ratio is significantly increased. Elevation of the activity of 6-FL in platelets under conditions of stimulated thrombopoesis is not paralleled by the rise of carbohydrates in platelet glycoproteins. We have found that platelets are quite heterogenous with respect to the activity of 6-FL and in platelets separated according to buoyant density the enzyme activity was largely missing in light platelets and significantly reduced in very heavy platelets. Distribution of 6-FL activity in fractions of platelet lysates subjected to ultracentrifugation in 27% sucrose closely parallelled that of β-4-D-galactosyltransferase which is a marker of the Golgi complex. Both enzymes were missing from platelet membranes and granules. We conclude that 6-FL activity in platelets parallels their Golgi complex

contents and should be proportional to polyploidization of megakaryocytes. It is widely assumed that the latter – at all stages of maturation – may fragment to platelets.

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3.25
THE SUBCELLULAR LOCALIZATION OF GANGLIOSIDE O-ACETYL TRANSFERASE

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O-Acetylated G_{D3} is a developmentally regulated oncofetal ganglioside frequently expressed in melanoma tumors. It is synthesized by transfer of an acetyl group from acetyl CoA to the terminal α-2-8 linked sialic acid residue of G_{D3}. We have used two separate approaches to spatially and temporally define this enzymatic activity in melanoma cells. Others have shown that brefeldin A(BFA) disrupts the Golgi apparatus and imposes a block on ganglioside biosynthesis distal to the compartment in which G_{D3} synthesis takes place, yet proximal to ganglioside N-acetylgalactosamine transferase, thus inhibiting the synthesis of more complex gangliosides. Three melanoma cell lines were radiolabeled with [^{3}H]galactose in the presence of BFA. Gangliosides were analyzed by DEAE-HPLC, lipid ELISA using specific mAB's, and HPTLC with and without saponification of O-acetyl esters. In the Melur cell line, which synthesizes G_{D3} and O-acetyl G_{D3}, BFA completely inhibits synthesis of disialogangliosides, leading to an accumulation of G_{D3}. In M14 cells, which produce G_{D2} as well as G_{D3} and O-acetyl G_{D3}, BFA partially reduces G_{D3} and O-acetyl G_{D3} synthesis, and completely inhibits G_{D2} synthesis. In M21 cells, which synthesize more G_{D2} than M14 cells, BFA increases the labeling of G_{D3} while inhibiting completely the formation of G_{D2}. When O-acetyl G_{D3} was analyzed in M14 and M21 cells, the ratio of O-acetyl G_{D3} to G_{D3} was unchanged by BFA. Therefore, the enzymatic activity responsible for O-acetylation of G_{D3} resides in a compartment proximal to the biosynthetic block imposed by BFA and cannot be separated from G_{D3} synthase activity. Biosynthesis in isolated Golgi-enriched membrane preparations from melanoma cells is dependent upon the co-localization within intact discrete vesicles of the appropriate ganglioside precursor, sugar nucleotide transporter and glycosyl transferase. Light membrane fractions enriched 10–15× in a Golgi marker were incubated with radiolabeled sugar nucleotides or [^{3}H]acetyl CoA. When vesicles were incubated with CMP-[^{3}H]Neu5Ac, 10–11% of the resulting G_{D3} was O-acetylated, increasing to 20% when 500μM Acetyl CoA is added. When vesicles were incubated with [^{3}H]acetyl CoA, O-[^{3}H]acetyl G_{D3} was formed. The amount of O-acetylated G_{D3} could not be increased by adding saturating concentrations of non-labeled CMP-Neu5Ac to the incubation mixture. One plausible explanation is that the melanoma associated ganglioside O-acetyl-transferase functions via an acetyl intermediate analogous to the O-acetyl transferase in rat liver Golgi. Thus, a subset of the O-acetyl transferase molecules may be occupied with pre-existing acetyl donor groups from the melanoma cytosol, while the remainder would arise from added acetyl CoA. Taken together, the data suggest that at least a portion of the enzymatic activity responsible for the addition of O-acetyl esters to G_{D3} resides within the same discrete vesicle(s) as that of G_{D3} synthase and that both activities are proximal to the location of G_{D2} synthase.

3.26
THE DIFFERENT FATES OF THE OLIGOSACCHARIDE MOIETIES OF LIPID INTERMEDIATES

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We have previously described that the N-glycosylation process was accompanied by the release of oligosaccharide-phosphates and neutral oligosaccharides, both originating from lipid intermediates. We have examined the synthesis and fate of lipid intermediates in a glycosylation mutant of CHO cells which does not synthesize Man–P–Dol (B3F7 cell line). The relationship between the oligosaccharide-PP-Dol and their metabolic products (glycoproteins, oligosaccharide-phosphates and neutral oligosaccharides) was investigated on the basis of the structure of their oligosaccharide moieties and of their kinetic behaviors (pulse and chase experiments).

Evidence were obtained for the presence of two pools of oligosaccharide-PP-Dol: 1) the major one is not glucosylated, is rapidly labelled (and immediately chased by mannose) and generates the oligosaccharide-phosphate species; 2) the second one is glucosylated, exhibits a lag time (5–10 min) to be labelled and chased with mannose and to be utilized in the glycosylation of proteins or the production of neutral oligosaccharides.

Thus, in our model, the cleavage of lipid intermediates into oligosaccharide-phosphates is restricted to the nonglucosylated species and represents a "by-pass" in the dolichol cycle which allow direct regeneration of P-Dol. This "by-pass" may control the availability and the structural suitability of lipid intermediates for protein glycosylation. The glucosylation of lipid intermediates channels them through the glycosylation of proteins and through the formation of neutral oligosaccharides which could be, at some steps, related to the protein glycosylation process itself.

3.27
GLYCOGEN BIOGENESIS IN THE RAT-BRAIN ASTROCYTE

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Extracts from several tissues and cells contain a low-molecular-weight trichloroacetic acid (TCA)-insoluble form of glycogen which we have named proglycogen (Lomako et al,. FEBS Lett. in the press). Proglycogen has M_r approx. 400 Kda and contains at its core the M_r 37 Kda protein glycogenin the primer for glycogen synthesis which is an autocatalytic, self-glucosylating protein (SGP) (Lomako et al. FASEB J. 2, 3097, 1988). The cultured rat-brain astrocyte is a very suitable system in which to study the interrelations of SGP, proglycogen and glycogen. This is because in the astrocyte, especially after exposure to NH_4^+ we can study the conversion of SGP into proglycogen. This is not possible in muscle in which proglycogen is already present and no SGP, the progenitor of proglycogen remains. The intracellular concentration of glycogen varies with the extracellular concentration of glucose (Passonneau and Crites, J. Biol. Chem. 25, 2015, 1976; Swanson et al. J. Neurochem. 52, 1359, 1989). Ammonia suppresses the formation of intracellular glycogen (Dombro et al. Amer. Soc. Neurochem. 21, 216, 1990). We now report that newborn rat-brain astrocytes, shortly before refeeding with glucose, contain the M_r 37 Kda SGP. Glucose feeding causes the rapid formation of proglycogen with the disappearance of SGP. NH_4Cl (5mM) retards the process. In a pulse-chase experiment, a small amount of [^{14}C]glucose was provided 5 minutes before the cells were washed and bathed in unlabelled 5mM glucose. There was an immediate formation of proglycogen followed, after 30 min., by the appearance of ^{14}C in glycogen. The precursor-product relationship of

SGP to proglycogen to glycogen is thereby established. Cells treated with ammonia before the pulse-chase displayed many sharply defined intermediates between SGP and proglycogen (seen by TCA precipitation, SDS-PAGE and radioautography) but the label did not pass into glycogen. We suggest that two different types of glycogen synthase cause the synthesis of proglycogen and glycogen, the form synthesizing glycogen being inactive in presence of ammonia. We can now interpret our earlier work on the stimulation of glycogen synthesis in adipocytes by insulin (Lomako et al. Biochem. Int. 21, 507, 1990), to conclude that the stimulation is primarily directed to the synthase that forms proglycogen. Supported by NIH DK37500 (WJW), AM38153 (MDN), Fla. Affiliate, Amer. Heart Assn. (WJW), Juvenile Diabetes Foundation International (WJW) and VA Medical Research Service (MDN).

3.28

LOCALIZATION AND TOPOLOGY OF GLYCOSPHINGOLIPID GLYCOSYLTRANSFERASES IN RAT LIVER

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We have previously shown that the glycosyltransferases involved in the elongation and terminal glycosylations of the oligosaccharide chain of gangliosides are distributed in different density membranes of the Golgi apparatus in rat liver (1, 2). Now, we attempt to investigate the glycosyltransferase activities involved in the early steps of glycosphingolipid biosynthesis, namely UDP-Glc: ceramide β1→1'glucosyltransferase, GlcT, and UDP-Gal: glucosylceramide β1→4galactosyltransferase, GalT-2, (3). For this purpose, we prepared different subcellular fractions from rat liver, including an extremely purified Golgi apparatus fraction, which contains intact cisternal stacks with a 140-fold enrichment of ganglioside and glycoprotein glycosyltransferases. Then we characterized GlcT and GalT−2, and established their subcellular localization. Results indicate they are both localized in the Golgi apparatus, that is the only subfraction where substrate dependent formation of the reaction products is actually demonstrated. This indicates that glycosphingolipid glycosylation takes place completely in the Golgi, where ceramide is made available from the endoplasmic reticulum (4). On this regard, it becomes important to unambiguously establish the topology of the two enzymes in the Golgi apparatus. Incubation of the above-mentioned intact Golgi cisternae with liposomal dispersed acceptor substrates appears a good tool for investigating this problem. In fact, we found that in such a condition enzymes known to have a luminal catalytic domain are not detectable, unless the Golgi cisternae are previously disrupted.

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3.29

CELL SURFACE GLYCOCONJUGATE MODIFICATIONS USING GLYCOSYLHYDRAZINES

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We have developed a method for attaching desired carbohydrate structures covalently into glycoconjugates of living cells (1) based on making a hydrazine derivative of a reducing sugar and reacting it with glycoconjugates that have been specifically oxidized in their terminal residues (2, 3).

From recent work (4) with fluorescent oligosaccharides (5) we know that the "new" sugars attached to cell surface can be internalized into cells. In the present work we have attached the disaccharide Gal-β4-[1-^{3}H]GlcNAc to the glycans of cell surface glycoconjugates in living cells in order to study the intracellular fates of the added structure. The disaccharide was synthesized enzymatically from [1-^{3}H]GlcNAc and UDP-Gal. After derivatization with hydrazine it was attached to oxidized K562 erythroleukemia cells.

After the attachment reaction at 0°C the cells were cultured at 37°C. At later time points the cells were lysed, the hydrazine-linked sugars were released from glyconjugates by treatment at pH 3 and analyzed by gel filtration and paper chromatography in order to assess degradation and/or elongation. The effect of transport perturbants was analyzed.

References:

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3.30

ROLE OF *ENV* PROTEIN GLYCOSYLATION IN THE PATHOGENICITY OF FRIEND SPLEEN FOCUS-FORMING VIRUS

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Friend spleen focus-forming virus (F-SFFV) is a replication-defective, highly pathogenic retrovirus causing an acute erythroleukemia in mice. The induction of the disease presupposes the presence and expression of the viral envelope (*env*) gene which encodes for a glycoprotein with four *N*-glycosylation sites at N-11, -26, -264 and -296. In the case of the polycythemia-inducing variant (F-SFFV$_{P}$), two different glycoforms can be observed: a primary *env* gene product, gp52, which carries solely high-mannose type glycans and accumulates intracellularly, and small amounts of a processed form, gp65, which is expressed at the surface of the virus-infected cell.

In order to study potential biological functions of the carbohydrate moieties of these glycoproteins, we have used site-directed mutagenesis and the polymerase chain reaction to construct *env* gene mutants of F-SFFV$_{P}$ lacking distinct *N*-glycosylation sites. Wild-type and mutant glycoproteins obtained from transfected rat-1 cells after metabolic labelling with [6-^{3}H]GlcN or [^{35}S]Met and immunoprecipitation were analysed by SDS/PAGE before and after limited digestion with endoglycosidases. The results revealed a reduction of their apparent molecular masses according to the number of eliminated carbohydrate attachment sites. Similar to wild-type F-SFFV$_{P}$, two glycoforms were observed in the case of *env* gene products deficient in N-11, N-26 or N-11/26, whereas only the primary gene product was found in the case of mutant protein lacking N-264.

Infection of DBA-2 mice with wild-type and mutant pseudotypes revealed no significant change in the pathogenicity of mutants encoding for glycoproteins lacking N-11 or N-11/26 with regard to enlargement of spleens and formation of cytopathic foci. The mutant encoding for a N-264-deficient glycoprotein, however, exhibited reduced pathogenicity.

Thus, our results indicate that glycosylation of the F-SFFV$_{P}$ *env* gene products at N-264 might be important for further processing of the virus-encoded glycoprotein and also for viral pathogenicity.

3.31

PRODUCTION, MOLECULAR AND BIOLOGICAL PROPERTIES OF RAT TRANSFERRIN BEARING A HYBRID GLYCAN

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Rat transferrin (rTf) contains a single N-linked glycan which, under normal circumstances, is diantennary and is di-, tri-, or tetrasialylated. We induced the production of rTf having a hybrid glycan (Man_5-rTf) both in intact rats and in the isolated perfused rat liver by the adminstration of swainsonine, an inhibitor of mannosidase II. Man_5-rTf, obtained in good yield, was separated from normal rTf by ConA chromatography.

Man_5-rTf produced by the perfused liver contained 0 (4–6% of total), 1 (70–72%) or 2 (17–19%) residues of Neu5Ac. The latter form, in which the second Neu5Ac is linked to GlcNAc in the Man 3 branch, was more electronegative than standard diantennary rTf with both Neu5Ac in the antennae. The core fucose content of Man_5-rTf was not significantly different from that of diantennary rTf.

Man_5-rTf was degraded in vivo with a half-life of 14.2 ± 0.3 hrs, this value being only approx. one-third of the half-life of normal rTf. It is also considerably shorter than the half-lives of asialo and aglyco rTf. Iodotyraminecellobiose, a label that is trapped in lysosomes, was used to trace the catabolic sites of Man_5-rTf. Just as with rTf, the liver, bone marrow, spleen and lungs were the most active in degrading Man_5-rTf. However, the liver accumulated disproportionately more Man_5-rTf than rTf.

Studies with cell suspensions (bone marrow, pulmonary macrophages) showed that Man_5-rTf can be taken up by two independent mechanisms. One of them was inhibited by ovalbumin, mannopyranoside and, to a lesser extent, by EGTA. The other was inhibited by human transferrin, a high-affinity ligand for the rTf receptor. In the former case, the intracellular degradation of Man_5-rTf was reduced, and in the latter case it was enhanced. These data suggest that Man_5-rTf can be endocytosed, in addition to conventional Tf receptors, also by the mannose-specific lectin. Thus two exposed Man, as in a hybrid glycan, constitutes a stronger in vivo ligand than two exposed Gal in a diantennary glycan.

3.32

EXPRESSION AND GLYCOSYLATION OF YEAST ACID PHOSPHATASE IN ANIMAL CELLS

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In an effort to understand the factors influencing protein glycosylation, we have studied expression and glycosylation of acid phosphatase from *Saccharomyces cerevisiae* in a heterologous system. The structural gene for the repressible acid phosphatase, PHO5, was subcloned into the animal cell expression vector, containing strong human -actin promoter. The transfected baby hamster kidney fibroblast cells (BHK) secreted active acid phosphatase in the medium. The active enzyme was also detected inside the cells, by immunocytological assay. Western blott and immunoprecipitation experiments showed that the protein reactive with antiphosphatase antibody was the most abundant protein secreted by the transfected cells. On the other hand, acid phosphatase activity found in the medium did not correspond to the amount of the secreted immunoreactive protein, indicating that partially inactive enzyme was secreted, or that inactivation of the enzyme took place after the secretion into the cells growth medium.

On the basis of the estimated molecular mass of the secreted enzyme it could be concluded that only few out of 12 potential glycosylation sites are glycosylated by BHK cells. This is significantly lower than in yeast, where on average 8 to 9 oligosaccharide chains are attached to the protein. The experiments dealing with the estimation of the exact number, as well as the type and structure of the attached carbohydrate chains of the recombinant acid phosphatase, are in progress.

3.33

THE CONVERSION OF HIGH MANNOSE TO COMPLEX GLYCANS ON A PLANT PROTEIN DOES NOT REQUIRE SPECIFIC INFORMATION IN THE POLYPEPTIDE DOMAIN

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We are interested in the determinants for protein sorting and glycan modification in plant cells. To study this problem, we chose a protein which would presumably lack any specific (selected for) information to direct the glycan conversion. The cytosolic pea seed albumin, ALB, is a 27kD polypeptide with a cryptic N glycosylation site. We introduced a modified form of this cytosolic protein into the secretory system of plant cells by creating and expressing in transformed cells a chimeric gene, called *phalb*. This chimeric gene encodes the signal peptide and first three amino acids of the bean vacuolar protein phytohemagglutinin (PHA), two other amino acids added during the cloning steps, and the amino acid sequence of ALB. The protein product of *phalb*, called PHALB, contains three potential sites for high mannose glycan addition, but one of these sites is lost upon cleavage of the signal peptide.

We placed *phalb* under control of two different promoters and studied the nature of the glycans of PHALB which accumulated in transformed tobacco suspension cultured cells and also in the seeds of transgenic tobacco plants. We found that in both tissues, PHALB received high mannose glycans at the two glycosylation sites of the polypeptide. In suspension cells, the high mannose glycans were fully converted to Endo H resistant forms, but in tobacco seeds, PHALB displayed a heterogeneous population of Endo H sensitive and Endo H resistant glycoforms. In addition we found that the suspension cultured cells efficiently secreted PHALB into the extracellular space. These results show that secretion is the bulk flow or default pathway in plant cells, and that the efficiency of glycan modification in the Golgi apparatus is tissue dependent. Since PHALB has not evolved as a glycoprotein, and is therefore presumably devoid of structural motifs which would positively direct glycan modification, we also conclude that complex modification of high mannose glycans in plants is not mediated by structural determinants in the amino acid sequence of the glycoprotein as is the case in the modification of high mannose glycans to mannose-6-phosphate containing glycans of lysosomal enzymes.

3.34

2-DEOXY-2-FLUORO-D-GALACTOSE INHIBITS N-GLYCOSYLATION OF MEMBRANE AND SECRETORY PROTEINS

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2-Deoxy-2-fluoro-D-galactose (dGalF) is taken up by hepatocytes and

metabolized via the Leloir pathway. We demonstrate that in primary cultured rat hepatocytes and in hepatoma cells dGalF inhibits N-glycosylation of membrane and secretory proteins (membrane glycoproteins: dipeptidylpeptidase IV, gp 120; secretory: α1- and α2-macroglobulin, transferrin). dGalF reduces ^{3}H-glucosamine incorporation into glycoproteins of both cell types in a dose-dependent manner. Moreover, glycoproteins, synthesized in the presence of dGalF, have a lower apparent M_r when separated by SDS-PAGE. In hepatocytes the inhibitory effect of dGalF (1–5 mmol/l) was not discernible from tunicamycin-treated cells. In contrast to hepatocytes hepatoma cells were more resistant to dGalF in that complete inhibition of N-glycosylation was not achieved. In hepatoma cells, N-glycans formed in the presence of dGalF, became endoglycosidase H-resistant despite inhibition of mannosidase I by deoxymannojirimycin. This indicates that dGalF may either cause formation of aberrant oligosaccharide structures or may interfere with oligosaccharide processing in hepatoma cells.

3.35
OLIGOSACCHARIDE-REPROCESSING OF THE TRANSFERRIN-RECEPTOR AND OF DIPEPTIDYLPEPTIDASE IV IN Hep G2 CELLS

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During recycling cell surface glycoproteins may encounter intracellular compartments containing glycosidases and glycosyltransferases. Since the oligosaccharide units of cell surface glycoproteins are potential substrates for these enzymes, the question arises as to whether glycoprotein glycans may undergo further processing during recycling. Whereas resialylation of cell surface glycoproteins in the trans-Golgi network has been proven, return to enzymes involved in early sequences of the oligosaccharide processing pathway is a matter of controversy. Here we report experiments designed to examine whether the transferrin receptor and the serine peptidase dipeptidylpeptidase IV (DPP IV) of Hep G2 human hepatoma cells recycle to mannosidase I in the distal ER/cismedial Golgi and to compartments containing fucosyltransferases. Glycoproteins were cell-surface labelled with NHS-SS-biotin. Using the biotin-label as a tag, the glycoproteins could thereafter be recovered while recycling. In order to study return to and reprocessing by mannosidase I, this enzyme was inhibited by deoxymannojirimycin. As a result the newly synthesized glycoproteins pulse-chase labelled with L-(35S)methionine retained oligomannosidic N-glycans. Upon additional labelling with biotin at the cell surface inhibition of mannosidase I was raised by washout of the inhibitor and the glycoproteins were monitored for conversion of their oligomannosidic glycans to glycans of the complex type. No such conversion was found for both the transferrin receptor and DPP IV. Recycling to fucosyltransferases was traced measuring the incorporation of L-(3H)fucose into the glycoproteins that had been surface-labelled with biotin. Seven electrophoretically separable surface glycoproteins including DPP IV acquired L-fucose, whereas the biotinylated transferrin receptor was not labelled even after 12 h of incubation in the presence of (3H)fucose. These results suggest that recycling cell surface glycoproteins do not return to distal ER or cis Golgi compartments. Hence, re-processing of oligomannosidic N-glycans to complex-type glycans during recycling is very unlikely. By contrast, selected glycoproteins may acquire terminal sugars during recycling.

S4. MOLECULAR BIOLOGY OF GLYCOSYLTRANSFERASES AND OTHER PROCESSING ENZYMES/BIOLOGIE MOLÉCULAIRE DES GLYCOSYLTRANSFÉRASES ET D'AUTRES ENZYMES DE MATURATION

4.1
CONTROL OF GLYCOSYLATION AND ITS BIOLOGICAL IMPLICATIONS

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Recent cloning of glycosyltransferase cDNAs and the corresponding genomic sequences are beginning to yield insights into the basis of cell type specific glycosylation. The control of glycosylation by cells is of current interest as it relates to understanding the roles of carbohydrate groups as signals in cell–cell communication and cell adhesion. Results relevant to the regulation of sialyltransferase and fucosyltransferase expression as a means of controlling cell–cell interactions will be presented.

4.2
STRUCTURAL AND FUNCTIONAL DIVERSITY IN HUMAN FUCOSYLTRANSFERASE GENES

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The surfaces of mammalian tissues display a variety of distinct and structurally-related glycan molecules containing fucose. Some of these molecules play important roles in processes involving adhesion between immune cells and the vascular endothelium. Mechanisms that control the tissue-specific expression patterns of such molecules are poorly understood. It can be expected, however, that they are largely a function of tissue-specific expression of specific fucosyltransferases (FTs), that also each exhibit distinct patterns of acceptor substrate specificities. To investigate the molecular basis for the tissue-specific expression patterns and substrate specificities of these enzymes, we have isolated and characterized a number of human fucosyltransferase genes and cDNAs. These enzymes each appear to maintain a type II transmembrane topology that places their catalytic domains within the Golgi lumen. Comparative analysis of their primary sequences and substrate specificities, however, suggests that they may be segregated into two distinct classes. Primary sequence comparisons indicate that the human Lewis blood group α(1,3/1,4)FT is structurally similar to an α(1,3)FT expressed in cells of the myeloid lineage. Functional analyses indicate that the latter enzyme can construct only a subset of the linkages made by the Lewis enzyme. By contrast, these two structurally-related enzymes maintain primary sequences distinct from the human H α(1,2)FT, despite the fact that all three FTs utilize the sugar nucleotide substrate

GDP-fucose, and can operate efficiently on the disaccharide acceptor substrate N-acetyllactosamine. Recently, we have used cross-hybridization procedures to isolate a third human α(1,3)F, with primary structure and enzymatic properties distinct from the two α(1,3)Fs noted above. This α(1,3)F shares 95% amino acid sequence identity with the Lewis α(1,3/1,4)FT, but can catalyze the synthesis of only a subset of the linkages made by the Lewis enzyme. PCR analysis confirms that the DNA sequence encoding this enzyme is not an allele of the Lewis locus and thus further confirms that it represents a distinct α(1,3)F gene. Based upon preliminary analysis of the enzyme's acceptor requirements, this α(1,3)FT represents a candidate for the "plasma" type α(1,3)FT. Experiments are in progress to confirm this hypothesis, and to further define the functional properties of this enzyme.

4.3
TISSUE SPECIFIC ALTERNATIVE SPLICING OF THE α2,6 SIALYLTRANSFERASE GENE

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The tissue-specific and developmentally regulated terminal carbohydrate structures have been implicated as important mediators of cell–cell recognition and differentiation. Since the expression of glycosyltransferases are responsible for the terminal oligosaccharides biosynthesis, to understand the organization and regulation of the expression of glycosyltransferases genes is of current interest. Previously, we have found that β-galactoside α2,6 sialyltransferase gene exhibits striking differential expression with three different sized mRNAs (4.7, 4.3 and 3.6 Kb) expressed at different levels in different rat tissues. In order to understand the organization and the regulation of the expression of the α2,6 sialyltransferase gene, we have cloned the 4.7 and 3.6 Kb cDNAs from rat kidney and the corresponding genomic sequences. The nucleotide sequences of the 4.7 Kb mRNA and the 4.3 Kb mRNA, which has been cloned from rat liver previously, were found different only at the 5'-untranslated end. The extended sequence at the 5' end of the 4.7 Kb mRNA was shown expressed only in the 4.7 Kb mRNA. This unique sequence was found to be one exon of the α2,6 sialyltransferase gene and was located 15 Kb upstream of the promoter responsible for the transcription of the 4.3 Kb liver specific α2,6 sialyltransferase mRNA. The results suggested that an alternative promoter is utilized for the production of the 4.7 Kb constitutively expressed α2,6 sialyltransferase mRNA. We have begun an initial analysis of this promoter. In addition, three transcripts of about 3.6 Kb in size were cloned from rat kidney. They were shown expressed only in kidney and were found generated from the α2,6 sialyltransferase gene by alternative splicing and alternative promoter utilization. The results reveal the complexity of the α2,6 sialyltransferase gene which contains at least three promoters and produces at least five transcripts in a tissue-specific fashion.

4.4
THE INCREASED EXPRESSION OF β-GALACTOSIDE α-2,6-SIALYLTRANSFERASE IS INDUCED BY THE c-Ha-ras ONCOGENE IN RAT FIBROBLASTS (FR3T3) CELLS

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We have studied the modification of β-galactoside α-2,6-sialyltransferase (Gal-α-2,6-ST) activity in rat fibroblast cell lines (FR3T3) transformed by various oncogenes as c-Ha-*ras* or v-*myc*. We found that, in contrast to v-*myc*, c-Ha-*ras* induces a striking increase of Gal-α-2,6-ST activity in FR3T3 cells. Cells transfected with both v-*myc* and c-Ha-*ras* did not shown a larger increase of enzymatic activity when compared to cells transfected with c-Ha-*ras* alone. In order to determine if this increase was correlated with cellular transformation, we test other cellular oncogenes such as v-*src*, middle T (MT) of polyoma virus and the transforming BPV1 virus. Surprisingly, we found that none of these oncogenes were able to induce an increase of Gal-α-2,6-ST activity which seems specific to c-Ha-*ras* transformed cells.

This increase of Gal-α-2,6-ST activity could be correlated with an elevation of the expression of this enzyme since immunofluorescence studies with anti-Gal-α-2,6-ST antibodies revealed a sharp perinuclear signal present in c-Ha-*ras* transformed cells but absent in control cells. In addition, immunoprecipitation with these antibodies showed that the expression of the enzyme was nearly 5 fold greater in c-Ha-*ras* transformed cells when compared with reference cells. Moreover, in fluorescence studies with fluorescein-labelled *Sambuccus nigra* agglutinin, we detected an increase of Neu5Ac(α2–6)Gal sequence on membrane glycoproteins in cells transformed by c-Ha-*ras* oncogene. Finally, we showed, by northern blot experiments#, that there is increased levels of Gal-α-2,6-ST mRNA in c-Ha-*ras* transformed cells, confirming the results obtained with antibodies. All these data permit us to conclude that c-Ha-*ras* specifically induces, in FR3T3 cells, an increase of the expression of the Gal-α-2,6-ST and that this differential expression produces a supplementary terminal α-2,6-sialylation.

#The authors are indebted to Dr. J.C. PAULSON for kind gift of anti-Gal-α-2,6-ST antibodies and cDNA probe.

4.5
OVEREXPRESSION, MEMBRANE LOCALIZATION, AND SEQUENCING OF THE POLYSIALYLTRANSFERASE FROM *ESCHERICHIA COLI* K1.

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We are investigating mechanisms of *Escherichia coli* K1 poly-α2,8-sialic acid (PSA) expression and here describe molecular characterization of the polysialyltransferase (ST). PSA capsules synthesized by neuro-invasive bacteria structurally mimic polysialyl moieties on the vertebrate adhesion molecule NCAM. We recently showed that PSA expression is a complex process carried out by 12–15 genes located in a 17-kb cluster designated *kps* (J. Bacteriol. 1989, 171: 1106–17). Similar complexity may exist for vertebrate PSA synthesis. *E. coli* K1 is thus a general model for investigating PSA biosynthesis.

Certain *kps* genes, designated *neu* are located in an 5.8-kb central biosynthetic cassette referred to as region 2. This cassette, encoding functions for sialic acid synthesis, activation, and polymerization, is flanked by region 1 and 3 genes that encode general functions for sialoconjugate assembly, regulation, and possibly translocation. Region 2 genes are organized as a unidirectionally transcribed complex containing multiple internal promoters for at least three *neu* operons. The last gene of this complex, *neuS*, encodes ST, as shown by insertion mutagenesis, complementation, Southern, deletion, and biochemical analysis (Mol. Microbiol. 1990, 4: 603–11). ST was overproduced 25-fold by subcloning in pTTQ18, thereby placing *neuS* under control of the IPTG-inducible *tac* promoter. ST activity cofractionated with membranes, demonstrating *neuS* contained information for proper ST localization

independent of other *kps* gene products. However, overproduced ST was incapable of *de novo* PSA synthesis and could only transfer sialic acid from CMP-sialic acid to exogenous colominic acid acceptors. These results demonstrate that other components of a putative ST complex are required for physiologically relevant activity.

To better define ST and identify other members of a putative ST complex, a 2.9–kb *kps* subclone that included *neuS* was sequenced. The results showed ST lacked hydrophobic membrane spanning domains but contained numerous Lys and Arg residues that could mediate enzyme localization. ST was not homologous to α2,3 or α2,6 vertebrate ST's, but a *neuS* probe did crosshybridize with the ST gene from *E. coli* K92, bacteria which synthesize an α2,8/2,9-sialyl copolymer. Two other genes, designated *neuE and kpsS* were located immediately 5′ and 3′ of *neuS*, respectively. We suggest the *neuE* gene product, which contains one membrane spanning domain at its C-terminus, participates in PSA synthesis as endogenous acceptor or initiase. The region 1 *kpsS* gene product is hydrophilic with a predicted signal peptide, suggesting an extracytoplasmic location. Together, these results begin to identify components of an ST complex and suggest new approaches for genetic and biochemical reconstitution of PSA biosynthesis. ST probes are also being used to screen vertebrate cells for the cognate ST that functions in NCAM PSA expression.

4.6

A CHO CELL LINE TRANSFECTED WITH cDNA FOR THE HUMAN α1,2-FUCOSYLTRANSFERASE SYNTHESIZES H ANTIGEN PRIMARILY ON THE POLY-N-ACETYL-LACTOSAMINE CHAINS OF LAMPs

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Chinese hamster ovary (CHO) cells, transfected permanently with cDNA encoding the human GDPFuc:β-D-Gal α1,2 fucosyltransferase (2-FT) were designated NeoH CHO. NeoH CHO cells were metabolically radiolabeled with [6-^{3}H]galactose and the proteins were extracted with Triton X-100. Approximately 5% of the radioactivity in the NeoH CHO protein extracts was bound to a *Ulex europaeus*-I-agarose affinity column and subsequently eluted with fucose (4 mg/ml). The affinity purified, galactose-labeled protein fraction contained primarily Lamp1 and Lamp2 as determined by immunoprecipitation and analysis by SDS–PAGE.

The galactose-labeled glycopeptides from CHO and NeoH CHO cells, obtained by pronase treatment, were fractionated by chromatography on columns of immobilized Concanavalin A and *Ulex europaeus* I. Approximately 10% of the total radioactivity in glycopeptides from NeoH CHO cells bound to UEA-1. No parental CHO glycopeptides were bound by this column. The affinity purified glycopeptides were structurally characterized, and the H determinant (Fucα1-2Galβ1-4GlcNAc-R) was found primarily on oligosaccharides sensitive to endo-β-galactosidase, indicating its association with poly-N-acetyllactosamine-containing structures. Negligible amounts of the H-determinant were found on biantennary, complex-type H-linked oligosaccharides.

Since the 2-FT uses lactose and N-acetyllactosamine as acceptors *in vitro*, we anticipated that all of the glycoproteins terminating with Galβ1-4GlcNAcβ1-R synthesized by NeoH CHO cells would contain the H antigen. However, the H-antigen was found to be restricted primarily to LAMP-1 and by LAMP-2 synthesized by the NeoH CHO cells and to occur in the poly-N-acetyllactosamine chains which we have shown to be restricted to the LAMPs. These results suggest the possibility that 2-FT in intact cells prefers extended N-acetyllactosamine sequences as acceptor. This work was supported by NIH Grant CA 37626 to RDC and DK 30331 to DFS.

4.7

POLYMORPHISM OF THE SERUM α-3-FUCOSYL-TRANSFERASE IN INDONESIA AND ITS RELATIONS WITH OTHER FUCOSYLTRANSFERASES (FT)

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Only four serum α-3-FT deficient individuals have been reported (Greenwell et al. *Blood Transf Immunohemat.* **29**: 233, 1986; Caillard et al. *Exp Clin Immunogenet.* **5**: 15, 1988). After a transfusion problem, serum samples from a large Indonesian family, with three H α-2-FT deficient individuals in red cells (*h/h*) and normal ABH secretions in saliva (*Se/-*), were sent to Paris. The three H deficient phenotypes were confirmed as *h/h*, *Se/-*, but one of them, plus 7 other H normal individuals, of the same family, were found to be α-3-FT deficient in serum and Lewis negative on red cells (*le/le*). The coexistence of the very rare α-3-FT and H α-2-FT deficient phenotypes in the same family, suggested that one of them might have a particular high incidence in Indonesia. Two series of 100 random serum samples were collected, one from the original area of this family (Surakarta) and the other from Jakarta. No other H deficient individuals were found, but, 18 serum samples (9%) were found to be α-3-FT deficient (10 from Jakarta and 8 from Surakarta). All the α-3-FT deficient samples were from individuals with Lewis negative phenotype on red cells. The overall frequency of Lewis negatives (*le/le*) was 26%. Since the Lewis gene is linked to C3, which has been localized on chromosome 19, our working hypothesis is that the Lewis and the serum α-3-FT are encoded by two closely linked loci on this chromosome and both are probably quite far away from *H* and *Se* since no linkage has been detected between *Le* and (*H-Se*) loci (Oriol et al. *Cytogenet Cell Genet.* **37**: 564, 1985). Another α-3-FT was described in myeloid cells (Mollicone et al. *Eur J Biochem.* **191**: 169, 1990) and the gene encoding for this enzyme was located on chromosome 11 (Tetteroo et al. *J Biol Chem.* **262**: 15984, 1987; Couillin et al. *Cytogent Cell Genet.* 1991, in press), suggesting that different α-3-FT genes might be located on different chromosomes.

4.8

CLONING AND EXPRESSION OF A HUMAN GENOMIC DNA FRAGMENT ENCODING α(1,3)FUCOSYLTRANSFERASE ACTIVITY

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This laboratory has previously reported the transfection of human genomic DNA from HL-6O cells into CHO cells and the selection of primary transfectants (1°T) that express a human α(1,3)fucosyltransferase (α(1,3)Fuc-T) activity (Potvin *et al.*, J. Biol. Chem. (1990) *265* 1615). Five, independent 2°T were obtained following a second round of transfection with 1°T genomic DNA and shown to express HL-6O-like α(1,3)Fuc-T activity as well as an ~7.5kb common EcoRI DNA fragment that hybridized to a human *Alu* repeat sequence. This result indicated that the human gene encoding α(1,3)Fuc-T was linked to *Alu* and could be rescued by screening a 2°T genomic DNA library with an *Alu* probe. Therefore, EcoRI-digested, size-fractionated 2°T DNA was ligated into λZAPII to obtain a library of ~5.5 × 1O^{5} independent recombinants. Following three rounds of purification, DNA from one isolate with an insert of ~6.3kb was found to confer α(1,3)Fuc-T activity when transfected into CHO cells. Restriction analysis showed that this DNA fragment includes an ~3.6kb PstI DNA fragment that was cloned

from a human peripheral blood lymphocyte genomic library and shown to encode α(1,3)Fuc-T activity in transfected cells (Lowe et al., Cell (1990) *63*, 475). Comparisons between the α(1,3)Fuc-T activity of CHO cells transfected with the ~6.3kb DNA from HL-6O cells and the 3.6kb DNA from human lymphocytes indicated that they express α(1,3)Fuc-T enzymes with very similar properties. In addition, the 3.6kb fragments from both sources exhibit identical restriction maps for nine different restriction enzymes. Unique restriction fragments from this region are being used to probe blots of genomic DNA and RNA from 2°T, HL-6O cells and CHO mutants that express α(1,3)Fuc-T activity.

4.9
5-AZACYTIDINE INDUCES EXPRESSION OF TWO, APPARENTLY NOVEL, α(1,3)FUCOSYLTRANSFERASE ACTIVITIES IN CHO CELLS

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Pro^-5 Chinese hamster ovary (CHO) cells do not express detectable α(1,3)fucosyltransferase(α(1,3)Fuc-T) activity (Campbell, C. and Stanley, P (1983) Cell *35* 303). However, two, rare dominant mutants, LEC11 and LEC12, isolated after mutagenesis, each express a distinct α(1,3)Fuc-T activity (Howard *et al.*, (1987) J. Biol. Chem. *262* 16830). The LEC11 α(1,3)Fuc-TI synthesizes carbohydrates terminating in Galβ(1,4)[Fucα(1,3)]GlcNAcβ1 (Le^x) and NeuNAcα(2,3)Galβ(1, 4) [Fucα1,3)]GlcNAcβ1 ($SALe^x$) while the LEC12 α(1,3)Fuc-TII synthesizes only the Le^x structure. Since both the Le^x and $SALe^x$ structures have been implicated in the binding of leukocytes by LEC-CAM molecules, the identification of additional α(1,3)Fuc-T activities is of current interest. Therefore 5-azacytidine (5AzaC), which is known to induce expression of silent genes, was investigated as an inducer of α(1,3)Fuc-T activities in CHO cells.

Treatment with varying concentrations of 5AzaC (up to 1.5μg/ml) caused an increase in Pro^- to Pro^+ reversion frequency which correlated with an increase in the frequency of CHO colonies that survived 3.5μg/ml wheat germ agglutinin (WGA) and bound an anti-Le^x monoclonal antibody conjugated to sheep red blood cells (α-SSEA−1/sRBC). A cloned isolate (LEC29) exhibited a novel lectin-resistance (Lec^R) phenotype and bound α-SSEA-1 antibody similarly to LEC11 and LEC12 cells. LEC29 cells possessed low levels of α(1,3)Fuc-T activity with LacNAc as substrate. The LEC29 phenotype was dominant in hybrids.

Another dominant, α(1,3)Fuc-T expressing clone (LEC3O) was isolated from 5AzaC-treated CHO cells that were mutagenized with N-methyl-N-nitrosoguanidine (MNNG) and selected for resistance to 13.3μg/ml WGA. LEC3O had very high α(1,3)Fuc-T activity with a variety of glycoprotein and glycolipid substrates (~12 times the specific activity of α(1,3)Fuc-TII with LacNAc as substrate), and also had a novel Lec^R phenotype. These cell lines should be useful for investigating carbohydrate ligands involved in adhesion to LEC-CAM molecules.

4.10
A NOVEL GLYCOSYLATION PHENOTYPE EXPRESSED BY Lec23, A CHO MUTANT DEFICIENT IN α-GLUCOSIDASE I

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Lec23 is a new, recessive, Chinese hamster ovary (CHO) cell mutant that has a novel lectin-resistance (Lec^R) phenotype (Stanley *et al.*, (1990) Som. Cell Molec. Genet. *16* 211). When vesicular stomatitis virus (VSV) was grown in Lec23 cells in the presence of labeled sugars, Lec23/VSV G glycopeptides possessed four species of carbohydrates that could be separated by chromatography on Con A-sepharose. Compared with CHO/VSV that contains branched and biantennary carbohydrates, Lec23/VSV had, in addition, species that eluted with hybrid and oligomannosyl, N-linked structures. A similar profile was obtained for glycopeptides from CHO/VSV produced in the presence of 5 μg/ml swainsonine and labeled with 2-^{3}H-Mannose. However, monosaccharide analysis of the glycopeptides from Lec23/VSV that eluted with α-methylmannoside revealed that both species contained glucose residues. Therefore, labeled $Glc_3Man_9GlcNAc_2Asn$ and $Glc_2Man_9GlcNAc_2Asn$ were prepared to assay α-glucosidase I and α-glucosidase II activities in cell free extracts. Under assay conditions that gave linear release of glucose with time and protein, Lec23 cell extracts had α-glucosidase II activity equivalent to CHO cells but ≤5% of CHO α-glucosidase I activity. Specific inhibitors of the glucosidases and product analysis by ion suppression HPLC were used to characterize the oligomannosyl carbohydrates synthesized by Lec23/VSV and the products of the *in vitro* assays. The combined data provide several lines of evidence that the primary defect in Lec23 cells is a lack of α-glucosidase I. Lec23 cells provide a mammalian cell line that can be used to clone or to characterize cloned α-glucosidase I by complementation with transfected DNA.

4.11
CHARACTERIZATION OF A MOUSE GENE ENCODING N-ACETYLGLUCOSAMINYLTRANSFERASE I

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The medial Golgi enzyme N-acetylglucosaminyltransferase I(GlcNAc-TI) catalyzes the transfer of GlcNAc from UDP-GlcNAc to the oligomannosyl acceptor $Man_5GlcNAc_2Asn$ and is therefore the key enzyme in initiating the synthesis of hybrid and complex N-linked carbohydrate structures. In our previous report (Kumar *et al.*, (1990) Proc. Natl. Acad. Sci. USA *87*, p. 9948), we used the human repetitive sequence *Alu* as a probe to rescue human genomic DNA sequences that confer GlcNAc-TI activity on transfectants of Lecl Chinese hamster ovary-(CHO) cell mutants which lack GlcNAc-TI activity. Full length cDNAs that encode GlcNAc-TI activity have been isolated from human and mouse cDNA libraries by cross-hybridization to a unique probe generated from the rescued human DNA sequences. The protein sequence deduced from the human cDNA contains 445 amino acid residues and has an overall domain structure similar to other type II transmembrane Golgi glycosyltransferases. We have now sequenced a full-length mouse cDNA. It encodes a protein of 447 amino acids and the deduced sequence shows similar structural features to and a high degree of homology with human GlcNAc-TI. By using probes generated from different regions of the mouse cDNA, several mouse genomic DNA clones spanning the entire 5′ to 3′ ends of the full length cDNA have been isolated from a mouse liver genomic DNA library. Restriction analyses of the clones and characterization of mouse liver genomic DNA using the polymerase chain reaction(PCR) are being applied to characterize the 5′ promoter region and the intron/exon organization of the mouse GlcNAc-TI gene. The nature of the RNA products of the mouse GlcNAc-TI gene is being determined by primer extension studies using RNA from mouse tissues. The number of copies of the GlcNAc-TI gene in the mouse genome and its expression in different mouse tissues are being determined by Southern and Northern blot hybridizations using probes from mouse GlcNAc-TI cDNA. Preliminary results indicate that there is a single GlcNAc-TI gene in the mouse genome and that mouse liver expresses one RNA species (M.W~3kb) that hybridizes to mouse GlcNAc-TI cDNA.

4.12
THE HUMAN UDP-*N*-ACETYLGLUCOSAMINE: α-3-D-MANNOSIDE β-1,2-*N*-ACETYLGLUCOSAMINYLTRANSFERASE 1 GENE

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UDP-*N*-acetylglucosamine:α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101) is a medial-Golgi enzyme essential for the synthesis of hybrid and complex N-glycans. The cDNAs for rabbit (1) and human (2) GnT I have recently been cloned. We have isolated two overlapping genomic DNA clones (13 and 15 kb) which span 18 kb containing the gene for GnT I. Comparison of 4.7 kb of human genomic DNA sequence with the cDNA sequence for human GnT I shows that a 34 bp sequence at the 5′-end of the cDNA sequence is not present in the genomic DNA. The gene therefore has at least two exons, an exon more than 5 kb upstream of the intron-exon junction and a 2.5 kb 3′-terminal exon which contains most of the 5′-untranslated region, the complete coding sequence for GnT I (445 amino acids) and the complete 3′-untranslated region. There is a 3′-splice junction consensus sequence at the point of divergence of the genomic and cDNA sequences. Southern blot analysis indicates that the gene exists in single copy in the human genome. Chromosome blots containing *Eco* RI-digested DNA from 25 human-hamster somatic cell hybrids were hybridized with a randomly primed probe; the gene is located on human chromosome 5. The human and rabbit enzymes are 85% similar at the nucleotide sequence level and 92% similar at the amino acid sequence level. GnT I shows no sequence homology to other previously cloned glycosyltransferases but GnT I appears to have a domain structure typical of these enzymes, i.e., a short cytoplasmic aminoterminal domain, a non-cleavable signal-anchor trans-membrane domain, a "neck" region and a large carboxy-terminal catalytic domain. Neither the rabbit nor the human protein has an Asn-Xaa-Ser (or Thr) sequence indicating the absence of N-glycans. Transient transfection of the 13 or 15 kb clone into Lec 1 Chinese hamster ovary cell mutants (which lack GnT I) results in expression of GnT I activity. The data indicate that the GnT I gene has two promoters, one preceding the upstream exon(s) and a second promoter in the intron preceding the 3′-terminal exon; both promoters appear to produce the same protein and may therefore function as a quantitative rather than a qualitative control mechanism (Supported by grants from the MRC of Canada to H.S. and R.D.).

(1) Sarkar,M., *et al.* Proc. Natl. Acad. Sci. USA, 1991. **88**(1), 234–238.
(2) Kumar, R., *et al.* Proc. Natl. Acad. Sci. USA, 1990. **87**(24), 9948–9952.

4.13
HEMPAS DISEASE: MUTATION IN α-MANNOSIDASE II GENE

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Congenital dyserythropoietic anemia type II is a rare genetic anemia in humans inherited by an autosomally recessive mode. This anemia is characterized by *h*ereditary *e*rythroblastic *m*ultinuclearity associated with a *p*ositive *a*cidified *s*erum lysis test (HEMPAS). Band 3 and Band 4.5 glycoproteins isolated from HEMPAS erythrocyte membranes lack polylactosamines made of galactose and N-acetylglucosamine repeats, and contain unusual truncated hybrid structures, while normal erythrocyte membranes contain polylactosaminoglycans (*Brit. J. Haemat. 56*, 55–68, 1984). Therefore, in HEMPAS the synthetic pathway of Band 3 glycoprotein could result from a defect in some processing enzymes. In fact, one case of HEMPAS has been shown to be caused by a gene defect encoding α-mannosidase II (α-M II), an enzyme involved in the synthesis of Asn-linked oligosaccharides (*Proc. Natl. Acad. Sci. USA 86*, 5276–5280, 1989; *Proc. Nat. Acad. Sci. USA 87*, 7443–7447, 1990).

Recently, we have found a significantly lowered activity of α-M II in a different HEMPAS case. Lymphocytes isolated from HEMPAS patient B.S. were transformed by the Epstein-Barr virus. Poly(A)+ mRNA was isolated from immortalized lymphocytes and, by reverse-transcriptase, cDNAs were synthesized. The gene encoding α-M II was amplified by polymerase chain reaction (PCR) using these cDNAs as template. PCR products were subcloned in Bluescript II KS- and the nucleotide sequence of B.S. α-M II gene was analyzed. Comparison of the α-M II gene sequence between B.S. and normal has revealed that B.S. α-M II has an extra T at nucleotide 1337. This insertion mutation in B.S. has been confirmed in all (ten) subclones derived from two independent gene amplification reactions. The mutation found in B.S. would predict a shift in the reading frame of α-M II, resulting in an enzymatically inactive α-M II peptide, introducing a termination codon at approximately the middle of the coding region of full-length α-M II. (Supported by NIH grant DK37016).

4.14
DEMONSTRATION OF A NOVEL α-MANNOSIDASE GENE IN MAMMALIAN CELLS

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The gene from *Saccharomyces cerevisiae*, encoding an α-mannosidase of unique specificity which catalyzes the removal of one mannose from $Man_9GlcNAc$ to form a single isomer of $Man_8GlcNAc$, was isolated, sequenced and overexpressed (1). The deduced amino acid sequence of this yeast gene is highly homologous to that of cDNAs isolated from rabbit liver and mouse 3T3 cells encoding a ManDJN-sensitive, calcium-dependent α-1,2-mannosidase which converts $Man_9GlcNAc$ to $Man_5GlcNAc$ (2). To identify a mammalian gene which may encode an α-mannosidase with the same specificity as the yeast enzyme, regions of perfect homology between the yeast and mammalian sequences were chosen to design degenerate oligonucleotide primers which were used for polymerase chain reactions (PCR) on templates of mouse and rabbit liver cDNAs. PCR products of the expected sizes were obtained, but the mouse PCR product appeared heterogeneous by restriction enzyme analysis. It was subcloned into M13, and random clones were sequenced in both orientations. Two populations of PCR products were distinguished: PCR_1 had the sequence of the previously isolated rabbit and mouse cDNAs, whereas PCR_2 was about 70% homologous. Hybridization of Southern blots of mouse genomic DNA with labeled PCR_1 and PCR_2 showed different restriction maps with the two probes, indicating the existence of two different genes. Different transcripts were revealed by the two PCR probes on Northern blots, and were differentially expressed in different mouse tissues. Several positive clones which hybridized with PCR_2 were isolated from a 3T3 cDNA library in λZAP, and these are being characterized.

(1) Camirand, A. *et al.* (1990) Glycoconjugate J. **7**, 408.
(2) Moremen, K.W. *et al.* (1990) Glycoconjugate J. **7**, 401.

4.15
HETEROLOGOUS EXPRESSION OF β(1–4)-GALACTOSYLTRANSFERASE IN E. COLI AND SACCHAROMYCES CEREVISIAE

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Topogenesis and localization of glycosyltransferases in higher eukaryotic cells along their secretory pathway is of current interest. Galactosyltransferase (GT, EC 2.4.1.22) exists intracellularly as a type II membrane-bound Golgi enzyme and is released from cells in a soluble form by proteolytic cleavage. This is the predominant form in human breast milk. Heterologous expression in E. coli of GT as a fusion protein with β-galactosidase was carried out to obtain a non-glycosylated enzyme protein against which polyclonal antisera were elicited: Two hybrid fusion proteins were prepared to immunize rabbits: one coding for the 339 C-terminal amino acids corresponding to the milk GT and a shorter form with only 197 C-terminal amino acids. While the immune responses to β-galactosidase were comparable, only the long form of GT proved to be specifically immunogenic. Antibodies to the GT-part of the fusion protein were strong reagents for the immunocytochemical identification of the Golgi apparatus in a variety of cell lines. Furthermore, a full length form of GT (from HeLa cells) which included the membrane-spanning domain was expressed in Saccharomyces cerevisiae under the control of the PH05 promotor (by courtesy of A. Hinnen, Ciba-Geigy). Specific activity determined by using free GlcNAc or ovalbumin as acceptor substrates was comparable to that of HeLa cell lysates. Membrane-association of recombinant yeast-GT was suggested by stimulation of activity by Triton-X-100 and enrichment in a 16000g pellet. The enzyme was detected by immunofluorescence in yeast cells using antibodies to the fusion protein. Further work is aimed at investigating the intracellular pathway of rGT in yeast cells and the expression of a soluble form of GT. Supported by grant 3.136.088 to EGB of the SNSF and grant 1781 of KWF to EGB and Ciba-Geigy.

4.16
β1→4-GALACTOSYLTRANSFERASE: GOLGI RETENTION IS DEPENDENT ON THE PRESENCE OF AN AMINO-TERMINAL SEGMENT WHICH CONTAINS THE TRANSMEMBRANE DOMAIN

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β1→4-Galactosyltransferase (β1→-GT) is a Golgi resident, type II membrane-bound glycoprotein that functions in the coordinate biosynthesis of complex oligo-saccharides. Additionally, β1→4-GT has been localized to the cell surface of a variety of cell types and tissues where it is proposed to function in intercellular recognition and/or adhesion. Thus β1→4-GT is an appropriate molecule to analyze the structural determinants for retention of a membrane-bound enzyme in the Golgi complex and transport to the cell surface.

Previously we have shown that the gene for bovine and murine β1→4-GT is unusual in that it specifies a long (LGT) and short (SGT) form of the enzyme (Russo, R.N., et al., J. Biol. Chem. *265*:3324, 1990). The only difference between the two related forms is the primary structure of the respective cytoplasmic domains. LGT has an NH_2-terminal extension of 13 amino acids. Since cytoplasmic domains can specify the final subcellular destination of a protein, we have tested the hypothesis that LGT and SGT are differentially retained in the Golgi or directed to the cell surface. LGT, SGT and chimeric proteins containing either the complete NH_2-terminal cytoplasmic/transmembrane domain (TMD) or truncated NH_2-terminal cytoplasmic/TMD of LGT and SGT fused to the cytoplasmic protein pyruvate kinase, were independently expressed in Chinese Hamster Ovary cells (CHO). Proteins expressed from each construct were localized by immunofluorescence staining, exclusively to a perinuclear region, identified as the Golgi by co-localization with wheat germ agglutinin. Furthermore each of the SGT and LGT constructs were localized to the trans-Golgi as determined by EM-immunocytochemical localization. These data suggest that neither form of β1→4-GT is differentially directed to the cell surface and that a short NH_2-terminal segment containing the TMD of LGT or SGT (29 amino acids) is sufficient for retention of these membrane-bound proteins in the Golgi region of CHO cells. (Supported by NIH grants CA45799 and GM38310)

4.17
CHARACTERIZATION OF A UNIQUE MURINE SPERMATID β1,4-GALACTOSYLTRANSFERASE TRANSCRIPT

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β1,4-Galactosyltransferase (β1,4-GT) is a Golgi resident type II membrane-bound glycoprotein that participates in the biosynthesis of the carbohydrate moieties of glycoproteins and glycolipids. The gene for β1,4-GT is unusual in that it specifies two size sets of mRNA transcripts in somatic cells of 3.9 kb and 4.1 kb. Translation of each mRNA set results in the predicted synthesis of two related forms of the enzyme that differ only in the length of their NH_2-terminal, cytoplasmic domains.

Expression of β1,4-GT during murine spermatogenesis has also been examined. In contrast to all somatic tissues examined to date, male germ cells are predicted to synthesize only the long form of the β1,4-GT polypeptide. Futhermore, we have observed that both the amount and structure of the mRNA are regulated during spermatogenesis (Shaper et al. PNAS, 791 [1990]). The characteristic 3.9 kb and 4.1 kb mRNAs detected in somatic cells are replaced by two truncated transcripts of 2.9 kb and 3.1 kb in round spermatids. These two specific germ cell transcripts encode the same open reading frame, utilize alternative polyadenylation signals, but have a significantly longer 5′-untranslated region (~600 bp) when compared to somatic cell transcripts. We have isolated and characterized germ cell specific cDNA clones (from round spermatids) containing this 5′ extension and the complete coding sequence for murine β1,4-GT. An analysis of these cDNA clones indicates the presence of two distinct groups which differ in the sequence of their 5′-untranslated region. At least one of these groups arises as a consequence of the use of one or more germ cell specific exons. This implies that regulation of β1,4-GT expression in germ cells differs from the regulation of β1,4-GT gene expression in somatic cells. We are presently determining the different regulatory sequences governing the expression of β1,4-GT transcripts in both germ cells and somatic cells. Supported by an EMBO postdoctoral fellowship ALTF329 (A.H.-L.) and NIH Grant GM CA45799 (J.H.S.).

4.18
MURINE α1→3-GALACTOSYLTRANSFERASE: ALTERNATIVE SPLICING OF A SINGLE GENE RESULTS IN THE SYNTHESIS OF FOUR DIFFERENT PROTEIN ISOFORMS

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Previously, we have isolated and expressed a cDNA encoding bovine $\alpha1\rightarrow3$-galactosyltransferase (α1,3-GT) (Joziasse et al. (1989) J. Biol. Chem *264*, 14290–14297; (1990) Eur. J. Biochem. *191*, 75–83). The bovine cDNA hybridized to murine genomic DNA sequences, which allowed us to map the murine α1,3-GT gene to the centromeric region of mouse Chromosome 2 (Joziasse et al. (1991) Somatic Cell & Mol. Genet., in press). Using the bovine α1,3-GT cDNA as a probe, we isolated a cDNA clone (4A) encoding murine α1,3-GT. While clone 4A encodes a protein similar to bovine α1,3-GT, the murine protein is shorter, due to an apparent 34 amino acid deletion in the "stem" region of the molecule (the domain that links the membrane anchor to the catalytic domain of the enzyme).

Analysis of mouse C127 RNA by RNA-PCR, using primers that span the "stem" region of α1,3-GT, yielded four products that ranged in size from 180 to 290 bp. Sequence analysis showed that the 180 bp product represents cDNA 4A and a 250 bp product represents the α1,3-GT cDNA isolated by Larsen et al (PNAS (1989) *86*, 8227–8231). To determine how the different transcripts were generated from a single gene, we screened a mouse genomic DNA library, and isolated 3 overlapping clones that span the entire coding sequence of murine α1,3-GT. This sequence is contained within 6 exons, which span $\geqslant$ 20 kb of genomic sequence. The size of the individual exons ranges from 36 to $>$ 2500 bp. The different PCR products that we detected represent transcripts that are generated by alternative splicing through the use of different combinations of the 3 exons that encode the stem region of α1,3-GT. The four transcripts encode proteins that have the same catalytic domain, but differ in the lengths of their stem region. This results in the synthesis of four protein isoforms of 371, 359, 349 and 337 amino acids, respectively. A cell/tissue survey by RNA-PCR detected the same splicing pattern in all murine cell types studied. The potential relevance of the existence of different protein isoforms will be discussed. Supported by NATO grant CRG 890490 (D.H.J.), and NIH grants GM-38310 and CA-45799(J.H.S)

4.19
CHARACTERIZATION OF A GENE WHICH DETERMINES EXPRESSION OF A-BLOOD GROUP

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Malignancies often exhibit loss or change from the anticipated blood group expression. These alterations may result from changes in regulation of expression of one glycosyl-transferase. The structural changes observed have most often been identified in epithelial tumors of the gastro-intestinal and uro-genital tissues and have been reported to have prognositic significance. Most of the observed, altered blood group structures include the Lewis or A-like antigens, most interesting is the identification of A-antigen on the tumors of genetically incompatible individuals.

A gene element, *ala*, isolated from a human genomic library constructed from DNA of CLL lymphocytes confers the ability to produce ALA(A-like antigen) when transfected into murine L-cells. When the appropriate primate blood group precursors are provided by transfection into monkey cells (O-blood type) a subset of A-blood group structures are observed. This induced expression of surface A-antigen is accompanied by a coordinate increase in α-GalNAc transferase activity over controls.

Structural analysis of *ala* reveals a 7.07 kbp genomic element. Within the element have been identified potential unique coding regions whose nucleotide sequence does not demonstrate any significant homology to the various reported glycosyltransferases. Using these unique regions as probes for Northern blots, it has been shown that *ala* is expressed as 3.2 kb message in A-expressing tissues only. Upstream of potential coding regions are a number of regulatory sequences including active enhancer/promoter element(s) homologous to that found for the β-globin locus.

The genomic structure of *ala* demonstrates a gene which appears to exist in 2-forms – lymphoid(6.6 kbp) and non-lymphoid(7.0 kbp) which may relate tissue-specific expression of blood group antigens.

4.20
CLONING AND SEQUENCING OF AN *ACTINOMYCES VISCOSUS* SIALIDASE GENE

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The cell-bound sialidase of *A. viscosus* has been previously studied (1). It exhibits a relatively high molecular mass of 150 kDa but is not composed of subunits. In order to obtain information on the relatedness of sialidases, the gene encoding the enzyme of *A. viscosus* was cloned, and the primary structure obtained after sequencing was compared with those of clostridial sialidases.

Cloning was performed by restriction of chromosomal DNA of *A. viscosus*, ligation of the fragments with pUC-vectors, and transformation of *E. coli* cells. Recombinants were screened for sialidase activity by spraying the colonies with the fluorogenic substrate 4-methylumbelliferyl-α-D-N-acetylneuraminic acid. Positive clones contained an insert of about 4,800 basepairs, which was further characterized by restriction mapping. For sequencing, the insert was stepwise reduced by exonuclease Bal-31 digest and introduced in the M-13 system.

The enzyme expressed by the *E. coli* host exhibits the same properties as the natural sialidase isolated from *A. viscosus*. Remarkably, the amino acid sequence deduced from nucleotide sequencing exhibits a short stretch of amino acids, which is repeated fourfold in the protein, and is described to be conserved in all bacterial sialidases sequenced so far (2). Similarities with other sialidase proteins were also found with respect to the distances between these four "Asp"-blocks. The amino acid sequence could be aligned with clostridial sialidase proteins, indicating a common origin of these bacterial enzymes.

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4.21
"PIR", A GENE REQUIRED FOR MANNOSYLATION OF DOLICHOL-LINKED OLIGOSACCHARIDES

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Our laboratory has been studying a mutation, termed PIR, in Chinese hamster ovary cells which results in a block at the $Man_5GlcNAc_2$-P-P-dolichol biosynthetic intermediate. PIR is recessive and genetically distinct from the Lec9 (dolichol-phosphate biosynthesis) and Lec 15 (mannose-P-dolichol biosynthesis) mutations. Furthermore, mannose-P-dolichol and the appropriate mannosyltransferases appear to be present in PIR cells, and the defect can be corrected by disrupting the cells or by isolating membrane fractions. Thus, all of the known biosynthetic components are present in PIR cells, but for an unknown reason biosynthesis fails to proceed past the pentamannosyl intermediate.

We are now taking three approaches towards elucidating the PIR defect. First, we have developed a new cDNA transfection/selection

strategy that is being used in an effort to clone the PIR gene. Second, we have now identified a mannolipid which is synthesized by membranes from PIR cells, but cannot be detected in membranes from normal or Lec15 cells. This lipid, which is soluble in chloroform/methanol (2:1) and appears to be more hydrophilic than mannose-P-dolichol by TLC, may be directly related to the PIR defect. Third, we have identified conditions for *in vitro* biosynthesis of dolichol-linked oligosaccharides under which the PIR defect is maintained and can be studied directly.

4.22
TRANSLOCATION OF DOLICHYLPHOSPHORYL MANNOSE ACROSS THE ENDOPLASMIC RETICULUM

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Dolichylphosphoryl mannose (Dol-P-Man) is synthesized by eukaryotic cells to act as a membrane-bound donor of mannosyl residues during the process of N-linked glycosylation, and during O-linked glycosylation in fungi. The transfer of mannose from Dol-P-Man occurs in the endoplasmic reticulum (ER), where the lipid-linked sugar itself is synthesized from Dol-P and GDP-Man by the membrane-bound enzyme, dolichylphosphoryl mannose synthase. Surprisingly, Dol-P-Man is synthesized on the cytoplasmic face of the ER, but transfer of the mannosyl residue takes place in the lumen. It is of interest to determine the mechanism of translocation of Dol-P-Man across the ER membrane, as the same type of mechanism may be used by other lipid-linked sugar donors involved in N-linked glycosylation that are also synthesized and used on opposite faces of the membrane. In the case of Dol-P-Man translocation, Dol-P-Man synthase has been implicated as the factor that catalyzes translocation as well as synthesis of the molecule.

Dol-P-Man synthase from *Saccharomyces cerevisiae* has been cloned and sequenced, and can be expressed in a bacterial host. The bacterial system is being used for purification of the enzyme, so that no contaminating factors that may influence translocation in yeast may be copurified. To aid in purification, the enzyme is being overexpressed in *E. coli* on a vector containing a strong bacterial promoter and ribosomal binding site. PCR (polymerase chain reaction) was used to engineer restriction sites flanking the coding region of the yeast Dol-P-Man synthase gene for correct placement of the gene in the expression vector.

A translocation assay utilizing liposomal vesicles has been used to determine the amount of radioactive mannose that can traverse the membrane with and without the presence of the embedded enzyme. Dol-P-Man synthase partially purified from a bacterial host was shown to translocate the labeled sugar to the interior of the vesicles. To confirm these studies, the purified enzyme from both yeast and the *E. coli* host will be subjected to the translocation assays. Also, the possibility of a covalent mannosyl-enzyme intermediate is being probed since previous studies have not indicated the steps along the reaction pathway that translocation is occurring.

4.23
AMINO ACID SUBSTITUTION BY SITE-DIRECTED MUTAGENESIS OF HUMAN TISSUE PLASMINOGEN ACTIVATOR RESULTED IN THE ALTERED PROCESSING OF ASPARAGINE-LINKED OLIGOSACCHARIDE

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Tissue plasminogen activator (tPA), a glycoprotein with a molecular weight of about 68,000, consists of five structural domains. There are three potential glycosylation sites at Asn 117, 184, and 448. Oligosaccharide chains linked to Asn 184 and 448 are of complex type while the chain linked to Asn 117 is of high mannose type. We have prepared five structurally modified recombinant tPAs (mfr-tPAs) with prolonged *in vivo* half life (1). In order to investigate the structure-function relationship of these tPAs, we analyzed the structures of the N-linked oligosaccharides of mfr-tPA #9200 (a variant with a substitution of Ser 84 for Cys) and compared them with those of native recombinant tPA (r-tPA) expressed in baby hamster kidney cells (BHK).

The N-linked oligosaccharide chains of r-tPA and mfr-tPA were quantitatively liberated as radioactive oligosaccharides by hydrazinolysis followed by *N*-acetylation and NaB^3H_4 reduction. Native r-tPA produced in BHK was shown to contain complex type oligosaccharides linked to Asn 184 and 448 and high mannose type oligosaccharides linked to Asn 117. In contrast to it, mfr-tPA produced in BHK is devoid of high mannose type oligosaccharide and all oligosaccharide chains in the molecule were complex type.

Thus, single amino acid substitution distant from the glycosylation site alters the normal N-glycosylation. It is suggested that the prolonged *in vivo* half life of mfr-tPA is ascribed to the altered N-glycosylation.

1) Yuzuriha, T., et al: Thrombos Haemostas., **62**, 543 (1989)

4.24
MOLECULAR CLONING AND EXPRESSION OF THE 118 kDa GLYCOPROTEIN COMPONENT OF RAT INTESTINAL MUCIN

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Mucins in the gastrointestinal tract are thought to play an important role in mucosal protection and lubrication. Intestinal mucins from man, rat and rabbit are complex, polymeric molecules comprised of a number of large, highly-glycosylated (> 80% by wt carbohydrate) glycoprotein monomers and a smaller glycoprotein of 118 kDa held together by disulphide bonds. The 118 kDa glycoprotein components of these mucins show strong immunological cross-reactivity and remarkable compositional similarities, containing ~50% by wt carbohydrate (of which, ~9 mol% is mannose) and a peptide core enriched in Asx, Glx, Gly, Cys and Ser. At present, the function of the 118 kDa glycoproteins is not known. The aim of this study was to use molecular biology techniques to obtain further information on the peptide moeity of the 118 kDa glycoprotein with the ultimate view of developing a better understanding of its role in the mucin polymer. *Methods and Results*: A λZAP11 cDNA library from rat intestine was screened with a highly specific polyclonal antibody developed against the purified 118 kDa component of human intestinal mucin. After screening ~150,000 recombinant plaques, a positive clone was identified. Following *in vivo* excision, the pBluescript SK- phagemid was introduced into *E. coli* XL1-Blue. Subsequent analysis of the plasmid revealed the presence of a 1.45 Kb insert. By transforming the plasmid into *E. coli* DH5 α and analysing bacterial proteins by SDS-PAGE, a fusion protein of 52 kDa was detected. This suggests that the cDNA insert is essentially fully expressed and encodes for a protein of ~48 kDa. Nucleotide sequencing was performed to obtain sequences (~ 160 nucleotides in length) at both the 5′ and 3′ ends of the insert. In both sequences, a single open-reading frame was

detected. The deduced amino acid sequence of the 5′ end indicated a high proportion of Glu, Leu, Cys, Gly, and Ser while the 3′ end contained high levels of Leu and Asn. Potential N-glycosylation sites were not observed in these sequences. No homology with known amino acid sequences was detected, including those reported for the peptide cores of the glycoprotein monomers of human intestinal, porcine and bovine submaxillary mucin. *Conclusions*: We have isolated a cDNA clone which we believe encodes for the majority of the peptide core of the 118 kDa glycoprotein of rat intestinal mucin. The protein product of this clone reacts strongly with anti-118 kDa glycoprotein antibodies and appears to have unique, as yet unrecorded, amino acid sequences. (Supported by CCFF and CFIC)

4.25

TRANSMEMBRANE TRANSLOCATION OF POLYSIALIC ACID CHAINS ACROSS THE INNER MEMBRANE OF NEUROINVASIVE *E. COLI* K1

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Introduction. The poly-α-2,8-linked sialic acid (polySia) capsule of *E. coli* K1 serotypes is a determinant of neuroinvasive *E. coli* disease. The capsule is synthesized by a membrane-associated CMP-Sia:poly-α-2,8-sialyltransferase (polyST) complex whose catalytic domain is located on the cytoplasmic surface of the inner membrane. This implies that either oligo- or polySia residues must be translocated across the inner membrane before being exported to the outer membrane.

Objectives. The objectives of this study were two-fold: 1) To determine the length of the polySia chains synthesized inside the cell before they are translocated across the inner membrane, i.e. are sialyl oligomers first translocated and then polymerized on the periplasmic surface of the IM (Model I), or are the chains fully polymerized on the cytoplasmic face of the IM and then translocated (Model II)? 2) To determine if the proton electrochemical potential gradient ($\triangle\mu H^+$) is required for translocation of polySia chain across the inner membrane.

Experimental procedures. An *in vivo* ^{14}C-Sia labeling method that allowed us to determine the length of polySia chains in the cytoplasm or periplasm, and to assess the role of the $\triangle\mu H^+$ in translocation, was developed. The procedure uses K1 cells that are unable to degrade Sia (*nan*A mutation). The crucial experiment to answer the first objective was to isolate periplasmic and cytoplasmic chains from ^{14}C-Sia labeled cells, and to determine their chain length. Objective 2 was studied by pulse-labeling spheroplasts with ^{14}C-Sia and the transmembrane movement of ^{14}C-polySia chains was followed kinetically in the presence and absence of protonophores. Translocated polySia chains were differentiated from chains remaining inside by their sensitivity to depolymerization by Endo-N-acylneuraminidase.

Results. 1) ^{14}C-polySia chains with a degree of polymerization (DP) $>$ 85–200 Sia residues were synthesed and found in the cytoplasmic fraction from both wild type and a translocation-defective mutant. 2) After ^{14}C-polySia chain synthesis, translocation across the IM was inhibited by 5μM CCCP by which dissipated the $\triangle\mu H^+$.

Conclusions. 1) The internal location of fully polymerized polySia chains supports Model II and requires that polySia chain polymerization must precede translocation across the IM; 2) polySia chain polymerization is not coupled to chain translocation; 3) translocation of polySia chains across the IM requires energy provided by $\triangle\mu H^+$. Energized membranes may be required to integrate conformational changes and cross-talk among various components of the polyST-polySia translocation apparatus. (Supported by NIH Grant AI–09352.)

4.26

DETECTION OF CYTIDINE MONOPHOSPHO-2-KETO-3-DEOXY-D-GLYCERO-D-GALACTO-NONONIC ACID (CMP-KDN) SYNTHETASE AND CMP-SIA:POLY-α-2,8-SIALYLTRANSFERASE(POLYST) IN UNFERTILIZED EGGS OF RAINBOW TROUT

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Introduction. A unique structural feature of the poly(α2,8)NeuGc chains in unfertilized rainbow trout (R_T) eggs is that they are capped at their nonreducing termini by deaminated neuraminic acid (KDN). In spite of a number of elegant structural studies on the polySia moieties of fish egg polysialoglycoproteins (PSGP's), and cloning and sequencing of cDNAs coding for the apoPSGPs, there are no published reports on the biosynthesis of polySia chains in fish egg PSGPs.

Objectives. As a first step in elucidating the biosynthetic pathway and in possibly cloning of the poly ST responsible for polysialylation of R_T PSGPs, the aim of this work was to determine if we could detect CMP-KDN synthetase and poly ST activities in embryonic R_T eggs.

Experimental Procedures. Soluble and membrane fractions were prepared by French Press disruption of immature oocytes obtained from R_T. Membranes were sedimented at 180,000 × x g/1hr/4°C. The supernatant was dialyzed against 50 mM MOPS buffer, pH 7.0 containing 25 mM $MgCl_2$ and 1 mM DTT before use.

Results. 1) [^{14}C]KDN was synthesized from [^{14}C]Man and pyruvate in 89% yield using an immobilized Sia aldolase. 2) *Detection of CMP-KDN Synthetase and Synthesis of CMP-[^{14}C]KDN-* R_T eggs were shown to contain CMP-KDN synthetase, based on the conversion of [^{14}C]KDN to CMP-[^{14}C]KDN in the presence of CTP. Concomitant with a loss of radioactivity in KDN was the formation of a new nucleotide-containing component with the chromatographic properties expected for CMP-[^{14}C]KDN. The enzyme had a pH optimum of 9.0. CMP-KDN appears to be more labile than CMP-Neu5Ac. 3) *Detection of polyST Activity-* Poly ST activity was detected in both the membrane and supernatant fractions using CMP-[^{14}C]Neu5Ac as substrate. A nearly two-fold increase in poly ST activity occurred when PSGPs from R_T were added as exogenous acceptors.

Conclusions. 1) [^{14}C]KDN was synthesized from [^{14}C]Man plus pyruvate in 89% yield using an immobilized Sia aldolase; 2) Urfertilized R_T eggs contain CMP-KDN synthetase that was used to synthesize CMP-[^{14}C]KDN; 3) R_T eggs also contain a soluble and membrane-bound poly-α2,8 sialyltransferase(s) that catalyzes polysialylation of PSGPs, added as exogenous acceptors. (Supported by NIH Grant AI-09352 and Grant 02455022 and 022592024 from the Ministry of Education, Science and Culture of Japan).

4.27

USE OF THE E COLI K1 POLYSIALYLTRANSFERASE AS A SYNTHETIC REAGENT FOR THE SYNTHESIS OF STRUCTURALLY UNIQUE POLYSIALYLATED GLYCOSPHINGOLIPIDS

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Introduction. A number of glycosphingolipids (GSLs) are developmentally regulated heterophile antigens that function as surface receptors for bacterial cells and toxins, and are also tumor-associated antigens. The

bifunctional role of GSLs as modulators for transmembrane signaling and as mediators for cell-cell interactions has also been proposed. Some cell adhesion processes appear to involve a complementary GSL to GSL interaction that is mediated by specific carbohydrate residues in each GSL.In our studies to map the acceptor sugar requirements of the *E. coli* K1 poly-α-2,8-sialyltransferase (polyST), we made the unexpected finding that the enzyme recognized a trisialoganglioside as an exogenous sugar acceptor. This membrane-bound enzyme is responsible for synthesis of the poly-α-2,8-linked polysialic acid (polySia) capsule that is a neurovirulence determinant in these human pathogens.

Objectives. The objectives of this study were two-fold: 1) to use GSLs of defined chemical structures to map the active site of the *E. coli* K1 polyST for exogenous acceptor sugar requirements; and 2) to use the enzyme as a synthetic reagent for synthesis of structurally unique polysialylated GSLs.

Experimental Procedures. Sealed inside-out-vesicles (IOV) in which the catalytic domain of the polysT was expressed on the external surface were incubated with CMP-[^{14}C]Neu5Ac and different GSLs, added as exogenous acceptors. The polysialylated oligosaccharides were released from the GSLs by ceramide glycanase and the approximate length of Sia residues estimated by PAGE.

Results. 1) A common structural feature of the preferred acceptors ($G_{D3} = G_{T1a} > G_{Q1b} = G_{T1b} > G_{D2} = G_{D1b} = G_{D1a} > G_{M1}$)) was the disialyl unit, Siaα2-8Sia, linked α2–3 to Gal; 2) Monosialyl residues linked α2–3 to Gal [G_{D1a} and G_{M1}) were also acceptors. Thus, α2–8-linked polysialylation can occur in the absence of preexisting α2–8 sialyl residues, and may have relevance for initiation of poly-α-2,8 sialylation in eucaryotic systems; 3) A linear oligosaccharide containing a minimum of 3 monosaccharide residues and a terminal Sia residue is required for acceptor activity; 4) Surprisingly, approximately 100 Sia residues were transferred onto the oligosaccharide moiety of G_{D3}.

Final Product: Siaα2(−8Siaα2)$\sim_{100}$−G_{D3}.

Conclusions. 1) The *E. coli* K1 polyST can polysialylate several structurally related acidic GSL as exogenous acceptors; 2) The enzyme can be used as a synthetic reagent to polysialylate and remodel the structure of GSLs. Such "designer" GSLs may have enhanced or novel biological and pharmacological properties. (Supported by NIH Grant AI-09352.)

4.28

POLYSIALIC ACID SYNTHESIS IN NEUROINVASIVE *E. COLI* K1: THE FUNCTIONAL DOMAIN OF THE POLYSIALYLTRANSFERASE COMPLEX IS LOCALIZED ON THE CYTOPLASMIC SURFACE OF THE INNER MEMBRANE

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Introduction. The poly-α-2,8-linked sialic acid (polySia) capsule is a virulence determinant in neuroinvasive *E. coli* K1 strains. The *kps* gene cluster that codes for proteins involved in polySia chain synthesis and export is surprisingly complex, requiring 17 kB of DNA. The genes are organized in three coordinately regulated clusters and encodes for ca. 12 proteins, including proteins of the membrane-bound polysialyltransferase (polyST) complex. Undecaprenyl-P functions is an intermediate carrier of Sia residues, and a 20 kDa sialylated protein has been implicated in polySia chain translocation. In spite of this extensive genetic and biochemical knowledge, there is a paucity of information regarding the topological organization of the enzyme in the membrane, and the molecular mechanism of chain translocation.

Objective and Experimental Procedure. The purpose of this study was to determine the topology of the polyST in the inner membrane of *E. coli* K1. Membrane vesicles of defined orientation were used to assay the enzyme activity. Sealed right-side-out vesicles (ROV) and inside-out-vesicles (IOV) were prepared and used to assay for polyST activity. The sidedness and impermeability of ROV and IOV were verified using voltage-sensitive fluorescent probes.

Results. 1) There was no polyST activity in ROV above a background level attributed to vesicles that were inverted during preparation. In contrast, IOV showed a 5-fold increase in enzyme activity. 2) There was only a slight decrease in polyST activity when ROV were treated with trypsin, and then inverted for assay. In contrast, >90% of the polyST activity was lost in IOV, or in ROV that were inverted before trypsinolysis.

Conclusions. These data confirm that the functional domain of the polyST is located on the cytoplasmic surface of the inner membrane. These results imply that polySia chains must be translocated across the inner membrane before being exported to the outer membrane. (Supported by NIH Grant AI-09352.)

4.29

LECTIN SELECTION EXPRESSION-CLONING OF A cDNA WHICH CONFERS WGA RESISTANCE TO POLYOMA LT-PRODUCING CHO CELLS (CHOP CELLS)

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The cytosolic enzyme sialic acid hydroxylase catalyzes the hydroxylation of CMP-N-acetylneuraminic acid (CMP-NeuNAc) to form CMP-N-glycolylneuraminic acid (CMP-NeuNGc) in the presence of O_2, NAD(P)H and Fe^{+}. Expression of NeuGc in glycoproteins and glycolipids is developmentally regulated in rodents and is an oncofetal antigen in humans. We have attempted to expression-clone a cDNA encoding murine sialic acid hyroxylase using a polyoma LT-producing CHO cell line described previously (Nucleic Acids Research 19(1): 85–92, 1991). Based on the premise that cell lines expressing a high ratio of NeuNGc/NeuNAc are resistant to the toxic effects of wheat germ agglutinin (WGA) in tissue culture, the WGA sensitive CHOP-C4 cell line was transiently transfected with a cDNA library generated from a murine cell line (D33W25) which synthesizes abundant NeuNGc. The transfected cells were placed under selection in WGA-containing medium and surviving colonies were harvested after 2–3 weeks. Five of 20 independant isolates contained episomal plasmid, consistent with the autonomous plasmid replication capable in the CHOP cell line. All five isolates possessed cDNA which encode an identical 475 amino acid protein with features indicating cytosolic localization. Stable transfection of either MDAY-D2 cells or CHO cells with the cDNA results in high level expression of NeuNGc. Further characterization of the cDNA is in progress. This system serves as a model for expression-cloning through lectin selection cDNAs which confer lectin resistant phenotypes.

S5. TRANS-MEMBRANE SIGNALLING/SIGNALISATION MEMBRANAIRE

5.1
STUDIES ON THE PURIFICATION AND REGULATION OF CMP-SIALIC ACID: LACTOSYLCERAMIDE α2,3-SIALYLTRANSFERASE

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We have proposed a model for the regulation of cell growth based on the synthesis and degradation of endogenous GM_3. According to our model, the inhibitory effect of GM_3 on the EGF-receptor kinase activity is abolished by a cell cycle-dependent sialidase-catalyzed conversion of GM_3 to LacCer. LacCer (or further catabolites) may be internalized and recycled back to GM_3 within the Golgi by cell-cycle-dependent activation of SAT-1, possibly by a mechanism involving phosphorylation/ dephosphorylation, GM_3 expression and SAT-1 activity are maximal late M/early G_1 of the cell cycle. Apparently, the cyclic accumulation of GM_3 at G_1, prior to DNA synthesis, may inhibit progression through the cell cycle.

GM_3 synthesis in rat liver Golgi may involve the post-translational modification of CMP-sialic acid: lactosylceramide α2,3-sialyltransferase (SAT-1). Recent purification of SAT-1 to apparent homogeneity allows studies to be made probing its regulation. Analysis of immunoaffinity-purified rat liver Golgi SAT-1, by SDS-PAGE and immunodetection on Western blots with a monoclonal antibody specific for phosphotyrosine residues, indicates that SAT-1 is a phosphotyrosine-containing protein. The expression of this tyrosine-phosphorylated form may regulate SAT-1 activity.

Detection of phosphotyrosine residue(s) in rat hepatic SAT-1 implicates phosphorylation as a potential post-translational regulatory mechanism for the cell cycle-dependent expression of SAT-1. Studies are in progress in human KB cells to determine the turnover of SAT-1, GM_3 sialidase activity and levels of GM_3 in substantiating our model of cell growth through the recycling of GM_3 and the regulation of GM_3 synthesis by cell-cycle-dependent phosphorylation of SAT-1.

5.2
FUNCTIONS OF SPHINGOLIPID BREAKDOWN PRODUCTS IN CELLULAR PROLIFERATION AND SIGNAL TRANSDUCTION

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Sphingosine, a metabolite of membrane sphingolipids, has recently been shown to stimulate DNA synthesis and to act synergistically with known growth factors and phorbol esters inducing proliferation of quiescent Swiss 3T3 fibroblasts. Sphingosine, which has been proposed to be a physiological inhibitor of protein kinase C, does not bind to protein kinase C at mitogenic concentrations and still stimulates DNA synthesis in cells made protein kinase C deficient by prolonged treatment with phorbol ester. Thus, sphingosine may play an important role as a positive modulator of cell growth acting in a fundamentally different, protein kinase C-independent pathway; other targets for its action still remain to be discovered. Mitogenic concentrations of sphingosine induce early increases in cytosolic phosphatidic acid, which is a potent mitogen for Swiss 3T3 cells. Sphingosine not only stimulated DNA synthesis with similar efficiency and kinetics as phosphatidic acid, it also induced similar morphological alterations. Although both sphingosine and phosphatidic acid acted synergistically with a wide variety of growth factors, there was no additive or synergistic effect in response to a combination of sphingosine and phosphatidic acid. Furthermore, similar to the actions of phosphatidic acid in these cells, mitogenic concentrations of sphingosine also inhibit cAMP accumulation, trigger the hydrolysis of polyphosphoinositides and increase cytosolic free calcium concentration. In view of the prominent role of phosphatidic acid in signal transduction and cellular proliferation, the observation that sphingosine, at mitogenic concentrations, increases the levels of phosphatidic acid and also mimics the effects of phosphatidic acid on signal transduction has important implications for the mechanism of action of sphingosine. Our results further suggest that sphingolipid turnover could regulate the diacylglycerol cycle and that crosstalk between these lipid metabolites which have been proposed to serve as intracellular second messengers may be important.

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5.3
THE INFLUENCE OF THE CARBOHYDRATE GROUPS OF RHODOPSIN ON ITS REGENERABILITY

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Rhodopsin is an asparagine-linked glycoprotein containing two short oligosaccharide chains composed of mannose and GlcNAc. The ability of photobleached rhodopsin (opsin) to combine non-enzymatically with 11-cis-retinaldehyde, thus reforming rhodopsin (regeneration), is a key aspect of the visual process and an important criterion to evaluate the native conformation of the apoprotein. Previous studies have revealed that alterations in the amino acid composition of rhodopsin have resulted in altered glycosylation patterns and an inability to regenerate (for example, Karnik, Sakmar, Chen, & Khorana (1988) PNAS *85*, 8459). We have examined the influence that the carbohydrate groups specifically might have on the regeneration process. Bovine rhodopsin was enzymatically deglycosylated with peptide-N-glycosidase F (PNGase-F). This was carried out on rhodopsin in rod outer segments and also after purification. Deglycosylation was verified by (1) SDS-PAGE patterns; (2) carbohydrate compositional analysis using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD); (3) analysis of the cleaved oligosaccharides by HPAEC-PAD and FAB-mass spectrometry; (4) absence of reactivity with con A and WGA. The following properties were observed: Deglycosylated rhodopsin remained immunoreactive toward a polyclonal antirhodopsin antibody. Deglycosylated rhodopsin exhibited the same absorption spectrum as the native material. After photobleaching, deglycosylated rhodopsin reacted with 11-cis-retinaldehyde in a manner similar to the native material, restoring the spectral properties lost upon light-exposure. The oligosaccharides of rhodopsin therefore are not required for expressing its spectral properties nor to maintain the conformation required for the binding of vitamin A. Supported in part by U.S. Public Health Service Grants EY 00393, EY 03685, EY 06571, and the Ohio Lions Eye Research Foundation.

5.4
REGULATION OF CAPILLARY ENDOTHELIAL CELL SPECIFIC PROTEIN FACTOR VIII:C GLYCOSYLATION BY TRANSMEMBRANE SIGNALING

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Angiogenesis is a complex phenomenon and is less understood. This is primarily because of not being able to disect between a simple metabolic event and a metabolic manifestation of the genetic consequences. We have therefore, examined the role of a signal pathway in growth and differentiation of capillary endothelial cells. When these cells are exposed to either B-agonist isoproterenol or cAMP analogue or cholera toxin or forskolin, the growth rate is enhanced. Examination of the cell specific protein Factor VIII:C, a M_r 270,000 dalton Asn-linked constitutive glycoprotein in which the heavy (M_r 215,000) and light (M_r 46,000 dalton) chains are held together by S-S bridge(s) indicated its synthesis is directly coupled to the cell proliferation. B-adrenoreceptor and other cAMP-related stimuli though enhanced the Factor VIII:C biosynthesis, the response is not mediated by the expression of new Factor VIII:C gene(s). Monitoring its glycosylation event in the presence of isoproterenol indicated that the glycosylation is enhanced by two-fold. This is highly correlated with the increased oligosaccharide-PP-dolichol biosynthesis and turnover. The isoproterenol effect is specific, reproduced with 8Br-cAMP/cholera toxin/forskolin and can be completely abolished by pre-treating the cells with B_1 or B_2-antagonists. The observed isoproterenol effect on oligosaccharide-PP-dolichol is neither due to increase in Dol-P level nor due to increased sugar transport. The effect however, is due to enhanced Man-P-Dol synthase activity. In order to provide further support we have analyzed oligosaccharide-PP-dolichol as well as the Man-P-Dol synthase activity in wild type and cAMP-dependent protein kinase deficient (cADepPK) mutant Chinese hamster ovary (CHO) cell lines. The results suggested that the rate of oligosaccharide-PP-Dol biosynthesis in mutant cells is much lower than that of wild type cell with no appreciable change in the turnover rate. Measurement of Man-P-Dol synthase in the isolated membrane preparations indicated that the enzyme activity in the mutant cells is not only low, the K_m for GDP-mannose is also high. Taking all these informations into consideration it is proposed that protein N-glycosylation serves as an indicator for angiogenesis. Supported by USPHS Grant S06RR08224 and AHA.

5.5

THE ROLE OF GLYCOSYLATION IN THE IMMUNOLOGICAL AND BIOLOGICAL POLYMORPHISM OF HUMAN THYROTROPIN (hTSH)

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Pituitary glycoprotein hormones like TSH (Thyroid-Stimulating Hormone) and gonadotropins (LH and FSH) have long been recognized to display variable biological (B) to immunological (I) ratio due to their natural polymorphism. Epitope mapping of enzymatically deglycosylated hTSH (DG-hTSH) has recently shown that most of the epitopes located in the β subunit of the hormone are lost upon CHO removal while the antigenic domains of the α-subunit and a few of those specific for the αβ dimer are preserved (1). At least 5 distinct epitopes were thus identified in DG-TSH, suggesting that α-glycosylation modulates the expression of β-specific domains through conformational changes. To correlate these alterations to bioactivity, we compared the action of hTSH and DG-hTSH on FRTL-5 cells and found that DG-hTSH retained its ability to stimulate cAMP production but lost that of promoting thyroid cell growth.

Resolving intrapituitary polymorphism of hTSH by isoelectrofocusing further confirmed that the various glycoforms of hTSH differ in both immunoreactivity and biological activity. Anti-α antibodies revealed up to 11 isoforms of TSH (pI = 8.6, 8.3, 8.0, 7.5, 7.0, 6.5, 6.0 5.8, 5.5, 4.8) while most of the anti-β antibodies only bound 7 (pI = 8.6 – 6.0) (2). Three different groups of TSH glycoforms could be distinguished on the basis of their bioactivity: alkaline forms (pI = 8.6 – 7.5) were definitely more potent than neutral forms (pI 7.5 – 6.0) both in cAMP release and growth-promoting activity while these two effects were poorly related in acidic forms of hTSH (pI = 5.8 – 4.5).

Altogether the current findings show that 1) the expression of both the immunoreactive and bioactive domains in hTSH is under the control of glycosylation; 2) CHO chains act to modulate hormone domains involved in post-receptor events, thus conferring variable biological activity to the different glycoforms of by hTSH.

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5.6

THE ROLE OF GLYCOSYLATION IN HORMONE ACTION: STRUCTURE-FUNCTION STUDIES OF RECOMBINANT THYROTROPIN

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Thyrotropin (TSH) belongs to the family of pituitary and chorionic glycoprotein hormones, each of which consist of two subunitsα and β. The α subunit contains two Asn-linked complex type oligosaccharide chains and the β subunit of TSH has one such chain. Studies from our and other laboratories have shown that the oligosaccharide chains play an important role at several steps of the hormone synthesis and function. Pituitary human (ph) TSH uniquely contains oligosaccharide chains terminating in SO4-GalNAc-GlcNAc- as well as the common NeuNAc-Gal-GlcNAc. We have previously documented alterations in the ratio of sulfated to sialylated oligosaccharide chains in various physiological, developmental and pathological conditions, resulting in changes in the hormone activity and metabolic clearance. We have now used a preparation of recombinant human (rh) TSH to study further the role of differentially terminating oligosaccharide chains in TSH. The rhTSH, in contrast to phTSH contains only sialic acid terminating oligosaccharide chains and contains no GalNAc. The immunologic and receptor binding activities of the rhTSH were 3–4 fold lower compared to phTSH. In two different in vitro bioassays, rhTSH displayed the same V max as phTSH, but had 3–5 fold higher EC_{50} values. Desialylation of rhTSH caused an increase in its potency 2.4, 2.6 and 26.7 fold in receptor binding, adenylyl cyclase and FRTL-5 assays, respectively. Desialylated rhTSH showed a 7.5 fold greater metabolic clearance than phTSH, whereas untreated rhTSH had a 2 fold lower clearance than phTSH. These studies clearly show that the terminal sugar moieties of TSH oligosaccharides influence hormonal bioactivity as well as metabolic clearance. Detailed studies on the structure-function relationships of oligosaccharides in recombinant glycoproteins are essential to assess their use for biochemical or therapeutic purposes.

5.7

REMODELING OF THE CARBOHYDRATE CHAINS OF hCG BY USE OF GLYCOSYL-TRANSFERASES: EFFECTS ON BIOLOGICAL ACTIVITY AND RECEPTOR BINDING

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Human chorionic gonadotropin (hCG) is a glycoprotein hormone which

contains both *N*- and *O*-linked carbohydrate chains. It consists of two subunits, α and β. The α-subunit contains two major *N*-linked carbohydrate chains: a mono-antenna and a non-fucosylated di-antenna. The β-subunit contains two *N*- and four *O*-linked carbohydrate structures. Both of the *N*-linked carbohydrate chains are di-antennary, one of which is fucosylated.

It has been shown that the carbohydrate chains of glycoprotein hormones influence the biological properties (circulation time, tissue targeting, cell proliferation and hormone action). In order to investigate the exact biological role of the different carbohydrates and to eliminate the unwanted side effects in the clinical use of hCG we attempt to modulate the activity of hCG by remodeling of the carbohydrate chains. This is being carried out using glycosyltransferases as specific tools. The enzymes are of particular use because of their high specificity.

Native hCG, asialo-hCG and partially resialylated asialo-hCG preparations were used to investigate the effect of the degree of sialylation and the sialic acid (NeuAc) linkage type on the biological activity in a Leydig cell *in vitro* bioassay. The bioactivity of as-hCG appeared to be only 45% compared to that of native hCG, and as-hCG showed a reduced affinity for its receptor. Resialylation of the O-linked chains on the β-subunit of asialo-hCG using the Galβ1-3GalNAc α2–3-sialyltransferase did not restore the biological activity. By contrast, 55% α2–6 resialylation of the N-linked chains yielded a preparation which was approximately as active as native hCG (known to carry exclusively α2–3-linked NeuAc on these chains), while the affinity of this preparation for its receptor remained unchanged in comparison to as-hCG. Interestingly, further sialylation with the α2–6-sialyltransferase resulted in a decrease of the activity to a level lower than that obtained with asialo-hCG. This decrease was accompanied by a further decrease in receptor affinity. Replacement of sialic acid by terminal α3-Gal resulted in a decrease of the bioactivity.

Thus it appears that the lectin-carbohydrate binding, which is part of the process that triggers the biological response of the target cell, can be mimicked by α2–6-linked NeuAc-containing chains. However, too high a density of such residues interferes with this interaction.

5.8
GLUCOSYLCERAMIDE AND ITS ROLE IN SIGNAL TRANSDUCTION IN RENAL EPITHELIA

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We have recently reported that changes in endogenous glucosylceramide (GlcCer) content are associated with alterations in cellular proliferation and hormone-stimulated inositol trisphosphate formation in MDCK cells. The metabolism of ceramide, a precursor and metabolite of GlcCer, is associated with the formation of two putative mediators of protein kinase C (PKC), diacylglycerol and sphingosine. We therefore measured these products as well as PKC activity in MDCK cells under conditions of GlcCer depletion by D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D–PDMP), a specific GlcCer synthase inhibitor, and of GlcCer excess with conduritol B epoxide (CBE), a β-glucosidase inhibitor. Sphingosine and related long chain bases (sphinganine and phytosphingosine) were measured by HPLC following derivatization with o-phthalaldehyde; ceramide and diglyceride were assayed with bacterial diglyceride kinase; and PKC activity was measured by histone phosphorylation. GlcCer synthase inhibition with D-PDMP was associated with a time and concentration dependent decrease in GlcCer and increased ceramide, diglyceride and sphingosine. Under these conditions a corresponding decrease in PKC activity was observed. β-Glucosidase inhibition with CBE resulted in a time and concentration dependent increase in GlcCer, decreased sphingosine, and unchanged diglyceride and ceramide levels. Under these conditions PKC activity was increased. While PKC has been reported to be inhibited by exogenous sphingosine the concentration of sphingosine endogenously formed was markedly lower than that which would be predicted to have an inhibitory effect. These studies raise the possible existence of a GlcCer mechanism, independent of sphingosine, which modulates signalling events.

5.9
REGULATION OF ANTI-HEMOPHILIA FACTOR A GLYCOSYLATION IN CAPILLARY ENDOTHELIAL CELLS BY INSULIN

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Anti-hemophilia Factor A (Factor VIII:C) is a cofactor in the conversion of Factor X to Factor X_a in the presence of Factor IX_a, Ca^{2+} and phospholipid during blood coagulation and is absent in hemophilia A patients, a X-linked genetic disorder. Upon establishing that the Factor VIII:C gene is expressed in capillary endothelial cells from bovine adrenal medulla as a 270,000 dalton Asn-linked glycoprotein, we became interested in studying its biosynthesis and regulation by extracellular signaling such as insulin. It is now accepted that insulin mediates cellular regulation by immediate activation of intrinsic tyrosine kinase upon binding to its receptor which in turn activates serine/threonine protein kinases responsible for phosphorylating serine and threonine residues in proteins. When examined, we have observed that [^{125}I]-insulin binds to the confluent monolayer culture with multiple affinities. The dissociation constants (K_d's) and B_{max} values thus calculated were K_{d1} (high affinity) = 0.04 nM; B_{max1} = 14 nmol/mg protein and K_{d2} (low affinity) = 4.7 nM; B_{max2} = 0.28 nmol/mg protein, respectively and accounted for approximately 210,000 high affinity receptors per cell. Metabolic labeling of cells with [^{35}S]-methionine and [^{3}H]-mannose in the presence of insulin (1 μg/ml) followed by analysis of immunoprecipitated double-labelled FVIII:C on SDS-PAGE indicated that the glycosylation is enhanced by ~2-fold. This was supported by increased [^{3}H]-mannose incorporation into Man-P-Dol and mannosylated oligosaccharide-PP-Dol (OSL) and was independent of Dol-P level in the cell. Sizing of OSL suggested the presence of both full-length and shorter oligosaccharide chains. Furthermore, the turnover of OSL ($t_{1/2}$) was reduced to 5 min in insulin treated cells as compared to 20 min in untreated control. This raised a possibility that insulin could increase OSL biosynthesis by increasing the (i) sugar-nucleotide pool; or (ii) glycosyltransferase activity. To answer the first question, we have carried out a detail study on 2-deoxy-D[^{3}H]-glucose (2DG) transport in these cells. In summary, kinetic studies indicated that the K_m and V_{max} for 2DG were 0.24 mM and 0.88 nmol/mg protein/min under normal condition but changed to 0.32 mM and 1.44 nmol/mg protein/min in the presence of insulin. These results though support that insulin could mediate its effect by increasing the sugar-nucleotide pool, but activation of "key" glycosyl-transferases of the Dol-P pathway remains a distinct possibility. RR08224/AHA.

5.10
ELUCIDATION OF BIOLOGICAL FUNCTIONS OF CELL-SURFACE GLYCOSPHINGOLIPIDS IN SITU BY ENDOGLYCOCERAMIDASES MADE POSSIBLE USING THEIR ACTIVATOR PROTEINS

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Endoglycoceramidase (EGCase) cleaves the linkage between oligosac-

charides and ceramides of various glycosphingolipids [Ito, M., and Yamagata, T. (1986) J. Biol. Chem. 261, 14278–14282]. Recently, by extensive purification, it was separated from cell-lytic factor (hemolysin) and found to consist of three molecular species each with its own specificity (EGCases I, II and III)[Ito, M., and Yamagata, T. (1989) J. Biol. Chem. 264, 9510–9519]. A detergent was required for EGCases to express full activity, possibly due to their hydrophobic nature, and thus EGCases cannot be used for research on live cells. This paper presents findings on activator proteins in the culture supernatant of *Rhodococcus* sp. M-777 regarding the stimulation of EGCase activity in the absence of detergents. The activator protein, exhaustively purified and designated as activator II in this study, showed a single protein band on SDS-, native-, and isoelectrofocussing-polyacrylamide slab gel electrophoresis after being stained with Coomassie Brilliant Blue. Its molecular weight and pI were 69.2 KD and 4.0, respectively. The activator protein enhanced the hydrolysis of glycosphingolipids *in vitro* and on the cell-surface by EGCase II in the absence of detergents in a concentration-dependent manner. Interestingly, activator II stimulated the activity of EGCase II much more than that of EGCase I on using asialo GM_1 as the substrate. This activator protein was found non-specific to substrates susceptible to hydrolysis with EGCase II. Besides activator II, strain M-777 produced a second minor molecular species of activator protein designated as activator I which appeared specific for stimulating the activity of EGCase I in contrast to activator II.

Following the addition of activator II, EGCase II hydrolyzed cell-surface glycosphingolipids quite efficiently at neutral pH at which hydrolysis hardly occurred at all in its absence. When using activator II in place of Triton X-100 for stimulating EGCase II activity, it was also noted to cause no damage to intact cells. It is thus possible by activator proteins to elucidate the biological functions of endogenous glycosphingolipids *in situ* by EGCases.

5.11
REGULATION OF THE EGF RECEPTOR TYROSINE KINASE BY GANGLIOSIDE G_{M3}

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Addition of ganglioside G_{M3} to cultured human foreskin fibroblasts inhibits cell growth and that cells ability to respond to the epidermal growth factor (EGF). The inability to respond to EGF can be directly related to decreased autophosphorylation of the EGF receptor (EGF-R). G_{M3} does not alter EGF binding to the cells or EGF-R expression. It is the signal transduction mechanism that G_{M3} blocks since the kinase activity is inhibited.

G_{M3} induced inhibition of the EGF-R/kinase is persistent after partial purification of the EGF-R, suggesting that G_{M3} has a direct effect on the receptor. Kinetic analysis of G_{M3} inhibition indicated that G_{M3} is non-competitive with respect to the kinase substrates. G_{M3} appears to inhibit activation of the kinase with an apparent Ki of approximately 150 μM. G_{M3} inhibition of the EGF-R/kinase also appears to be carbohydrate specific. The kinetic data and carbohydrate specificity data imply a binding site for the G_{M3} oligosaccharide on the EGF-R. To test for such a site, EGF-R rich membranes were incubated in ganglioside coated microtiter wells and binding was detected with either anti-EGF-R antibodies or ^{125}I-EGF. In both cases, significantly more EGF-R was detected in G_{M3} coated wells as compared to wells coated with other gangliosides. These results suggest that ganglioside G_{M3} may bind directly to the EGF-R and regulate the growth factor specific tyrosine kinase by controlling its activation.

The putative G_{M3} binding site on EGF-R is most likely on the external domain of the EGF-R. The ability of G_{M3} to inhibit EGF-R kinase activity has been tested on two EGF-Rs with deletions in the external domain. The first deletion mutant was similar to the erb-B gene product in that the entire external domain is deleted. The kinase activity of this deletion mutant was not regulated by G_{M3}. The second deletion mutant was missing a small part of external domain IV and was also not responsive to G_{M3}. The kinase activity of the second mutant could be stimulated by EGF addition, indicating that the EGF binding site is present. The lack of inhibition by G_{M3} suggests that G_{M3} may interact with external domain IV of the EGF-R.

G_{M3} is also able to inhibit dimerization of the EGF-R. When G_{M3} is preincubated with the EGF-R, significantly less dimer formation can be detected. G_{M1}, on the other hand, has no effect on the ability of the EGF-R to form dimers. Thus, G_{M3} may prevent the activation of the EGF-R kinase by inhibition of dimer formation and hence provide an explanation for the inhibition of cell growth by G_{M3}. These findings are also consistent with the idea that G_{M3} may serve as regulatory molecule for the EGF-R.

5.12
GLYCOSYLATION OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER IS ESSENTIAL FOR GLUCOSE TRANSPORT ACTIVITY

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The human erythrocyte glucose transporter was purified and reconstituted in proteoliposomes. Kinetic studies of zero-trans influx performed on these liposomes before and after their treatment with trypsin under mild conditions demonstrate that about half of the transporter molecules are oriented properly. This system was used to estimate the involvement of the carbohydrate moiety of this transporter in glucose transport activity. For that purpose, proteoliposomes were treated either with N-glycanase (1), sialidase (2), sialidase + endo-beta-galactosidase (3) or with a pool of exo-glycosidases (4), including sialidase, fucosidase, N-acetylhexosaminidase, galactosidase and mannosidases. This latter treatment leads to the complete release of all the sugar residues, except the proximal N-acetylglucosamine. Kinetic measurements of zero-trans influx made on these treated-liposomes show a complete abolition of glucose transport activity after treatments (1) and (4), and only a partial decrease of activity after treatments (2) and (3). We concluded that the release of the carbohydrate moiety from the glucose transport glycoproteins leads to the loss of their transport activity, the loss of activity could not be related to a change in the peptide chain and the variations in the sialylation rate could be a factor modulating glucose transport activity.

5.13
SUBCELLULAR GLYCOSYLATION AND TRANSPORT OF GLYCOSPHINGOLIPIDS IN RAT LIVER

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Exogenous radiolabeled sphingolipids can be used as a tool for studying glycosylation *in vivo*, also at the subcellular level (1, 2). We injected rats with fatty acid labeled ceramide and with glucosyl- and lactosylceramide (GlcCer and LacCer, respectively) isotopically radiolabeled on the terminal sugar residue and homogeneous in their lipid moiety. Then, we studied the time courses of more glycosylated compounds (mainly gangliosides) obtained in the Golgi apparatus and in the plasma membrane fraction. Results indicate that gangliosides are not formed from exogenous ceramide but only from GlcCer and LacCer, and that a

precursor product relationship is apparent between individual gangliosides in both the fractions. Moreover, the ganglioside patterns obtained at the different time points are similar in the two fractions. Since we reported that glycosphingolipid glycosyltransferases are localized in different density membranes of the Golgi apparatus (3) a sub-Golgi processing and sorting of individual glycosphingolipid may occur. Being the fatty acid composition of rat liver gangliosides not homogeneous, we are now attempting to assess whether the fatty acid residue of the precursor affects the glycosylation pattern and can be considered as a putative sorting signal in ganglioside biosynthesis. For testing this hypothesis, we prepared radiolabeled LacCer containing either stearic acid or lignoceric acid. We plan to inject rats with different ratios of the two LacCer molecular species and verify whether or not it is modified in the different gangliosides obtained in the Golgi apparatus and in the plasma membrane fractions.

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5.14
RECYCLING OF EXOGENOUS GLYCOSPHINGOLIPIDS AFTER PARTIAL DE-GLYCOSYLATION

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A growing volume of results suggests that glycoconjugate degradation in the lysosomes does not always occur exhaustively. This indicates that catabolic fragments might escape further degradation and be metabolically re-processed. In the case of glycoproteins, a subcellular recycling has been described, which involves internalization, partial de-glycosylation and re-glycosylation (1). In the case of glycosphingolipids, we already found that catabolic sphingosine is recycled much more efficiently for sphingomyelin than for ganglioside biosynthesis (2). For studying the recycling of glucosylceramide (GlcCer), we injected rats with prepared glucose-labeled lactosylceramide (LacCer) and studied the distribution of the radioactive products metabolically obtained at various time points, in different liver subfractions. Results indicate that radioactive GlcCer was found first in the lysosomal fraction and then in the Golgi apparatus. Moreover, the amount of radioactive gangliosides in the Golgi apparatus and in the plasma membrane fractions was over twice that obtained after administration of galactose-labeled LacCer. These last results indicate that GlcCer formed during the lysosomal degradation of LacCer reaches the Golgi apparatus where it undergoes further glycosylations, serving as a substrate for ganglioside biosynthesis. In order to assess whether catabolic LacCer and ceramide undergo similar recycling and metabolic process, we prepared fatty acid-labeled galactosylceramide and a globotriaosylceramide labeled on the internal galactose. Results indicate that both compounds are efficiently taken up by liver cells and metabolized, giving rise to catabolic ceramide and LacCer, respectively. However, no radioactive ganglioside are formed.

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5.15
INTRACELLULAR PROCESSING OF SEMISYNTHETIC SINGLE-CHAIN GM1 DERIVATIVES IN PRIMARY CULTURES OF CEREBELLAR NEURONS

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The semisynthetic single-chain GM1 derivatives, carrying N-acetyl-sphingosine (LIGA4) or N-dichloroacetyl-sphingosine (LIGA20), were recently reported to exert a strong protection against glutamate-induced neuronal death in primary cultures of cerebellar granule cells. Elucidation of the molecular mechanism underlying the evoked effect requires knowledge of the metabolic fate of such molecules, in the same cultured cells. For this, LIGA4 and LIGA20 were made radioactive on the long chain base (LCB) moiety and administered to cerebellar granule cells in culture parallely to GM1 ganglioside. The metabolic fate was then investigated. Both LIGA4 and LIGA20 were extensively incorporated and actively metabolized by the cerebellar granule cells. The associated radioactivity after LIGA4 administration was lower than after GM1 administration. Conversely, the associated radioactivity after LIGA20 was significantly higher than the control. Both LIGA4 and LIGA20 underwent metabolization in either catabolic and anabolic directions. The formed compounds were the analogues of GM1 ganglioside, carrying the ceramide moiety of the administered precursor. Among catabolites after LIGA4 and LIGA20 administration, a relevant increase of N-acetyl-sphingosine as well of N-dichloroacetyl-sphingosine was observed, if referred to ceramide formation after GM1. With regard to anabolization, glycosilation of LIGA4 and LIGA20 to GD1a analogues occurred in a lesser extent than glycosilation of control GM1. In conclusion we demonstrated that the strongly bioactive single-chain derivatives of GM1 undergo a metabolic processing qualitatively similar to that of the parent GM1. This evidence allows to include them in the family of naturally-behaving molecules, at least from the metabolic point of view.

5.16
BIOCHEMICAL MECHANISMS OF PLATELET ACTIVATION BY CONCANAVALIN

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The lectin Concanavalin A (ConA) promotes cytoskeleton reorganization and full aggregation in human platelets. It has been demonstrated that ConA binds maximally to platelet membrane glycoprotein IIb–IIIa (GP IIb–IIIa) inducing patching/capping of the receptors and GP IIb–IIIa-cytoskeleton interactions. At present little is known of the mechanism underlying the coupling of ConA-receptors with activation pathways, therefore we have investigated this topic studying some early activation events in ConA-stimulated platelets. We have obtained evidence that ConA effectively behaves like an agonist for human platelets, inducing responses quantitatively similar to those obtained after thrombin treatment, with the difference that a slower time course was always observed. The lectin determines calcium influx in FURA 2 loaded platelets, moreover it appears to be able to promote calcium-release from intracellular storage sites in 1 mM EGTA-treated platelets. The effect of ConA on calcium movements does not involve TxA2 production, because similar results were obtained with aspirin-treated platelets. In addition, ConA induces phosphoinositide turnover and inositol phosphates increase, suggesting that phospholipase C (PLC) activation is a crucial event in ConA-activated platelets. Although actually the activation of PLC is thought to be triggered by G-protein, permeabilized platelets loaded with 500 uM GDPβS and then activated with ConA did not show any significant decrease of inositol phosphates production. These results suggest that the activation of PLC in ConA-activated platelets probably occurs via some different unknown mechanism, not involving GTP-binding transducers. Experiments performed in the presence of leupeptin or antipain showed a significant decrease in inositol phosphates produced in ConA-activated platelets, suggesting that calcium-proteases activation could be in some way involved in PLC regulation.

5.17
MITOGENIC RESPONSE OF CELLS TREATED WITH BACTERIAL SIALIDASE AND LACTOSYLCERAMIDE

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If the role for the cell surface glycoconjugate GM_3, is to regulate cellular proliferation through the modulation of growth factor-mediated signal transduction, a mechanism must exist to relieve the inhibition of this growth-stimulating system for the cells to progress through the cell cycle. We have investigated the correlation of cell density and growth inhibition with high levels of GM_3 and SAT-1 activity and are studying the metabolism of GM_3 and its *in situ* conversion to LacCer through the action of associated GM_3 sialidase activities (extracellular and membrane-associated). The preferential turnover of GM_3 in preconfluent fibroblasts by an extracellular (pH 6.5) sialidase to LacCer has led us to investigate the mitogenic response of LacCer and bacterial sialidase on both apparently normal GM03468A human fibroblasts and human epidermoid carcinoma cells (KB cells) in chemically defined-medium. Treatment of these cell lines with sialidase from *Clostridium perfringens* results in a 1.5 to 3-fold stimulation of cell growth as evidenced by increases in both cell density and $[^3H]$-thymidine incorporation. Similarly, incorporation of exogenous LacCer (100 μM) stimulates cell growth 3 to 4-fold in GM03468A cells. The data are supportive of our model for the regulation of cell growth through the cell cycle-dependent regulation of GM_3 sialidase and SAT-1 activities and the level of cell surface GM_3.

5.18
IDENTIFICATION OF GLYCOINOSITOL PHOSPHOLIPIDS IN RAT LIVER

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Glycoinositol phospholipids serve as membrane anchors for a number of extracellularly orientated proteins. In addition, a glycoinositol phospholipid(s) has been suggested to be a precursor for a putative insulin mediator. To date, the precursor glycoinositol phospholipid has not been structurally characterized. We used two approaches to identify free glycoinositol phospholipids from rat liver.

The first approach was to use $[^3H]$glucosamine to label metabolically lipids in H4IIE hepatoma cells or isolated hepatocytes, a procedure commonly used to label glucosamine-containing glycolipids. In both cell types, we were unable to find a glucosamine-containing, phosphatidylinositol phospholipase C(PIPLC)-sensitive lipid. The major deterrent to using this approach is the rapid metabolic conversion of glucosamine. After 1 hr. of incubation, 55% of the radiolabel in hepatoma lipid extracts and 85% in hepatocyte lipid extracts was present as galactosamine or neutral or anionic sugars. This point was underscored by the observation that after labeling hepatoma cells with $[^3H]$glucosamine for 24 hrs., the radiolabel in the only PIPLC-sensitive lipid was inositol.

The second approach was to take advantage of the unmodified amine group of the hexosamine in this group of lipids by radiomethylating lipid amines from rat liver plasma membranes. After sequential extraction of lipids with chloroform/methanol/conc.HCL (100: 50:1,v/v), chloroform/methanol (2:1,v/v), and finally methanol/pyridine/water (2:1:1,v/v), we identified at least two lipids by 1- and 2-dimensional thin layer chromatography in the final lipid extract which contained glucosamine and were cleaved by PIPLC. These lipids were also cleaved by a phospholipase D partially purified from bovine serum.

5.19
BIOSYNTHESIS OF THE MAMMALIAN GLYCOSYLPHOSPHATIDYLINOSITOL MEMBRANE ANCHOR

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Biosynthesis of the glycosylphosphatidylinositol (GPI) membrane anchor was investigated in murine T-lymphomas and their mutants, which are unable to express GPI-linked proteins on the cell surface. Glycolipids were labeled by growing the cells in the presence of $[^3H]$mannose or by incubation of microsomal fractions with UDP-$[^3H]$GlcNAc. GPI precursors were identified by extraction of labeled glycolipids, analysis by TLC, and treatment with nitrous acid, phospholipase C and D, and α-mannosidase. Glycans released by HNO_2 treatment were analyzed by HPLC. As a result of these studies, the principal features of the biosynthetic pathway were defined, and mutants were identified with defects at four different stages of GPI anchor assembly. In some cases, the defect apparently resulted from the inactivity of a biosynthetic enzyme, whereas in others specific donor molecules were probably unavailable for the elongation of the GPI core. In one particular class of mutants the defect was due to the inability to synthesize dolichol mannosyl phosphate (DPM). This defect was corrected by stable transfection of the yeast DPM synthase gene. These experiments demonstrated that DPM is the donor of the first mannose residue transferred to glucosamine-PI and suggest that it may also be the donor of the other mannose residues.

A comparison of the mammalian GPI biosynthetic pathway with that for the variable surface glycoprotein of trypanosomes, showed that while key features are conserved, there are also significant differences, especially regarding fatty acylation of the inositol residue.

5.20
GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORING INTERMEDIATES IN RABBIT RETICULOCYTE MEMBRANE

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The glycosylphosphatidylinositol (GPI)-anchored proteins comprise a new family of membrane proteins, in which protein C-termini are anchored to phosphatidylinositol (PI) in plasma membranes via a ethanolamine-PO_4-Man_3-$GlcNH_2$structure. Although some functions of GPI anchors as well as the signal for GPI-anchoring on peptides have become more clear recently, virtually nothing is known about the biosynthesis of the GPI-intermediates in mammals. In this report, we describe the GPI-intermediates produced by rabbit reticulocyte membranes (RRM).

Unlike the *Trypanosoma*, the intracellular pool of GPI-intermediates in mammalian cells appears to be large, so that incorporation of radioactive sugars into the intermediates is typically low. Exogenous, $GlcNH_2$-PI produced in *Trypanosoma* is also not further processed when introduced into RRM. To increase the incorporation, we modified our methods so that the reaction proceeded not in a membrane suspension, but in a semi-solid phase. Using this system, we identified radiolabeled products on TLC plates, which appear to correspond to GPI-intermediates (RRM GPI's). However, only 10–40% of them were cleaved by PI-PLC from *Bacillus thuringiensis* as well as by GPI-PLD

from human serum, while almost all of them were cleaved by commercial preparation of *Rodcoccus* endoglycoceramidase. Available commercial endoglycoceramidases are heavily contaminated with PLC activity. The detailed characterization of the oligosaccharides liberated from the RPM GPI's is in progress. Supported by HD 13563 and by The Pharmaceutical Laboratory of KIRIN BREWERY Co. Ltd.

S6. GLYCOLIPID RECEPTORS/RÉCEPTEURS DES GLYCOLIPIDES

6.1
GLYCOSPHINGOLIPID RECEPTORS

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Within the current progress of glycobiology (1) the glycosphingolipid has an interesting position among glycoconjugates (2). The glycolipid is not secreted (compare glycoproteins) and usually not shedded, making it strictly membrane-bound and ideal for cell-targeting by antibodies or microbes. Possible proximity of a receptor site to the membrane bilayer may be important for membrane penetration and for membrane modulation.

The location of the sphingolipid to the surface monolayer of the plasma membrane with a unique character of the lipophilic part ceramide is probably essential for the normal stability of the membrane (3). The structure of ceramide (mainly level of hydroxylation) may affect the accessibility of ceramide-close antigenic sites for antibodies (4) or receptor sites for bacteria (5), and a ceramide-close oligosaccharide may be restricted in its conformational freedom. This may have interesting consequences for the tropism of infections.

Oligosaccharides may be found preferentially in lipid- or protein-bound form and this may have an importance for microbial interactions with animal cells. Carbohydrate receptors for microbes are at present accessible for systematic processing by improved techniques of assaying and structural analysis (5). There is a growing medical interest in applications of receptor specificities in those cases where traditional therapy is insufficient. Structural knowledge of the receptor saccharide is the prerequisite for assay techniques to facilitate genetical cloning of receptor-binding proteins for crystallography and drug design. The lecture will discuss several microbial receptor specificities in relation to the techniques of characterization.

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6.2
1-DEOXY-1-PHOSPHATIDYLETHANOLAMINOLACTITOL TYPE NEOGLYCOLIPIDS SERVE AS ACCEPTORS FOR SIALYLTRANSFERASES FROM RAT LIVER GOLGI VESICLES

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Investigations on glycosphingolipid (GSL) metabolism often require rare glycolipids whose isolation from natural sources is difficult to achieve. Synthetic neoglycolipids with authentic carbohydrate moieties and 'artificial' lipid anchors could act as suitable substitutes. We synthesized, therefore, neoglycolipids of the 1-deoxy-1-phosphatidylethanolamino lactitol type (LacPEs) and used them as acceptors for sialyltransferases.

C_{12}- to C_{18}-ester PEs and dihexadecyl PE were attached to lactose by reductive amination. All LacPEs and their corresponding N-acetyl derivatives were characterized by fast atom bombardment mass spectrometry (FAB MS). The neoglycolipids were then used as acceptors in sialyltransferase assays. By analogy to the authentic substrate lactosylceramide (LacCer), which was sialylated to GM3 and GD3, the LacPE derivatives were converted to the corresponding mono- and disialylated compounds as demonstrated by FAB MS. Comparison of the reaction rates with those obtained with LacCer (set to 100%) showed:

a) Reaction rates for the LacPEs were in the range of 50 to 68%
b) N-Acetylation led to markedly higher activities (150 to 220%)
c) In both types of substances, LacPEs and N-acetyl-LacPEs, the highest rates were observed with the dihexadecyl compounds.

Additional sialyltransferase experiments with dihexadecyl NeuAcLacPE and it's N-acetyl derivative showed that these GM3 analogues were as efficient acceptors as the authentic substrate GM3 for synthesis of the corresponding $NeuAc_2$-compounds. Our results indicate that neoglycolipids of the LacPE type can be used instead of the authentic glycolipids in investigations on GSL metabolism. N-Acetylation enhances the acceptor quality and rises the possibility of introducing a label into the neoglycolipids. Ether neoglycolipids seem to be the best substitutes for natural glycolipids. Due to their resistance to hydrolysis they are probably suitable probes for cell culture studies.

6.3
DEAMINATED NEURAMINIC ACID(KDN)-CONTAINING GLYCOSPHINGOLIPIDS, KDN-GANGLIOSIDES: Their Structure and Function

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In 1986 we found a naturally-occurring novel sialic acid analogue (2-*k*eto-3-*d*eoxy-D-glycero-D-galacto-*n*ononic acid, KDN) as a minor component of poly-sialoglycoproteins (PSGP) first isolated from rainbow trout eggs. Recently, we have demonstrated the presence of KDN-rich glycoproteins (KDN-gp) in the vitelline envelope as a second member of naturally-occurring KDN-containing biopolymers [J.Biol.Chem.(1990) *265*, 21811–21819; A.Kanamori *et al.* (1991) Proc. XIth Int'l.Symp.on Glycoconj.,Toronto]. More recently, we have also found two distinct types of glycoproteins, poly(KDN)-gp and poly(Sia)-gp, in genital cavity (or ovarian) fluid of salmonid fish [S.Inoue *et al.*(1991) Proc.XIth Int'l. Symp.on Glycoconj.,Toronto]. (In 1989, a Russian group reported the presence of the KDN-residues in capsular polysaccharide of *Klebsiella Ozaenae* serotype K4 [Carbohydr.Res.(1989)*188*, 145–155]).

Most recently, we have unveiled the natural occurrence of KDN-containing glycosphingolipids (KDN-gangliosides) in trout sperm. In this paper we present the structure of a novel KDN-ganglioside that we have first isolated. From the data based on composition analysis, methylation analysis, negative ion FAB mass spectrometry, and one- and two-dimensional ^{1}H NMR spectroscopy, we have established the structure KDNα2-3Galβ1-4Glc β1-1 Cer for (KDN)G_{M3}. We suspect that more

sensitive assay procedures might demonstrate the occurrence of KDN-glycoconjugates in many additional cell types of a wider variety of animal species in future. In view of importance in a diverse range of biological functions exhibited by certain sialic acid and polysialic acid structures, the availability of various types of biologically relevant KDN-glycoconjugates will open up a new aspect in future glycobiology and glycoengineering research.

6.4
BINDING OF VEROCYTOTOXIN 1 TO ITS GLYCOSPHINGOLIPID RECEPTOR IS INFLUENCED BY DIFFERENCES IN RECEPTOR FATTY ACID CONTENT

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The terminal sugar residues of globotriosyl ceramide (galα1-4galβ1-4glc ceramide-Gb_3) are specifically recognized by the *E. coli* derived verotoxin (VT) which has been strongly implicated in the etiology of the renal pathology observed in the Hemolytic Uremic Syndrome. Gb_3 was separated from pooled human kidney and the fatty acid composition determined. Semisyntheic Gb_3's of increasing fatty acid chain length corresponding to the major species were prepared and compared for VT binding affinity by tlc overlay and a quantitative binding assay performed in the presence of cholesterol and lecithin. Our results indicate that within the natural range, fatty acid chain length has a small effect on VT binding affinity but that mixtures of Gb_3's containing different fatty acids can interact to provide a higher affinity toxin receptor than any of the individual component receptor species. Moreover Gb_3 fatty acid chain length was found to selectively change the exposure of the 6′ as opposed to the 3′ position of the terminal galactose moiety and can therefore selectively change carbohydrate conformation. Receptor function as assayed by tlc overlay, was not always found to correlate with binding in a lipid environment. Short chain fatty acid Gb_3 species could not function as VT receptors under these conditions.

6.5
POSSIBLE ARTIFACTS INDUCED BY POLYISOBUTYLMETHACRYLATE IN TLC GLYCOLIPID OVERLAY PROCEDURES

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Verotoxins (or Shiga-like toxins) are a family of closely related toxins elaborated by *E. coli.* At least three toxins have been described, VT1, VT2, and SLTII, in addition to Shiga toxin itself, and all bind to globotriosylceramide, Gb_3 (Gal α1-4Gal β1-4Glc-cer). Some discrepancies exist in the literature in regard to the binding of the toxins to Gb_4 (GalNAc β1-3Gal α1-4Gal β1-4 Glc-cer) as monitored by TLC overlay procedures. Polyisobutylmethacrylate, PIBM, is generally used in TLC overlay procedures to prevent silica loss and orient carbohydrate moieties for the binding of various ligands to glycolipids. We now report that pretreatment of chromatograms with PIBM causes artifactual binding of VT1 to Gb_4. We suggest that PIBM can alter the conformation of the glycolipid oligosaccharide and therefore caution is advised in analysis of ligand binding to glycolipids in the presence of this compound.

6.6
A NEW MONOCLONAL ANTIBODY DIRECTED TO NEUACα2-3LACTO*NEO*TETRAOSYLCERAMIDE AND ITS APPLICATION FOR DETECTION OF HUMAN GASTROINTESTINAL NEOPLASMAS AND THE INHIBITION OF THE RECEPTOR BINDING OF INFLUENZA VIRUS

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A new monoclonal antibody (NS24) directed to NeuAcα2-3lacto*neo*-tetraosylceramide [$1V^3$(NeuAc)nLc4Cer, NeuAcα2-3Galβ1-4Glc-NAcβ1- 3Galβ1-4Glc-Cer] was prepared by hybridoma technique. NS24 (IgM,$_3$K) reacted specifically to both naturally occurring and chemically synthesized IV^3 (NeuAc)nLc4Cer, whereas it has no reactivity to structurally related gangliosides, such as IV^6(NeuAc)nLc4Cer, IV^3(NeuGc)nLc4Cer, VI^3(NeuAc)nLc4Cer, $VIII^3$(NeuAc)VI^3(NeuAc)-IV^6kladoLc8Cer, GM4(NeuAc), GM3(NeuAc), GM3(NeuGc), GM1b(NeuAc), GD3(NeuAc). Synthetic IV^3(NeuAc)Lc4Cer and its asialo-derivative (Lc4Cer) carrying type I sugar chain also showed no reaction with NS24. One to 100 pmol of IV^3(NeuAc)nLc4Cer was detected dose-dependently by TLC/enzyme immunostaining procedure. Human gastric carcinomas showed positive reactions with NS24 immunochemically and histochemically.

IV^3(NeuAc)nLc4Cer exhibited significant specific receptor activity toward human and avian influenza viruses. The influenza virus (A/$_3$PR/8/34, H1N1) mediated hemolysis of the asialoerythrocytes reconstituted with IV^3(NeuAc)nLc4Cer was effectively inhibited by the pretreatment of the erythrocytes with NS24. Above results indicate that the membrane associated IV^3(NeuAc)nLc4Cer acts as a receptor molecule for influenza viruses.

6.7
ROTAVIRAL BINDING TO SYNTHETIC NEOGLYCOLIPID ANALOGS OF GA2: STUDIES TO DEFINE A MINIMAL EPITOPE

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The infection of intestinal epithelia by rotavirus is thought to involve cellular glycoconjugates in the initial stages of the process. *In vitro* studies have reported that rotaviruses specifically bind to GA1 and that coincubation with GA1 can reduce rotaviral infection (Willoughby *et al.*, J. Virol. 64, 4830, 1990). In testing an array of authentic glycolipids and mouse intestinal glycolipids, we have found that a simian derived strain of rotavirus, SA11, binds GA2 with an efficiency similar to GA1 in certain *in vitro* systems, although lactosylceramide or globoside are insufficient for viral binding. To delineate the epitope required for rotaviral binding, analogs of gangliotriose as well as gangliotriose were prepared synthetically. They were then fashioned into neoglycolipids using reductive amination with phosphatidyl ethanolamine dipalmitoyl in a process that significantly alters the reducing end sugar (Tang *et al.*, BBRC 132, 474, 1985).

Rotaviral binding to a dilution series of purified neoglycolipids was

assessed by TLC overlay and microtiter well adsorption assays with 125-I rotavirus as probe. The neoglycolipid constructed from gangliotriose (GalNAcβ1→4Galβ1→4Glc) bound rotavirus with an efficiency equal to native GA1 and/or GA2 in both assay systems, suggesting that ceramide *per se* and an intact glucose attached to it are not requisite for rotaviral binding. Furthermore, neoglycolipids constructed from GalNAcβ1→3-Galβ1→4Glc and GlcNAcβ1→4Galβ1→4Glc also supported rotaviral binding. However, variation tolerated in the terminal HexNAc was limited; substitution of β1→3 linked GlcNAc or β1→6 linked GalNAc for the terminal GalNAc abrogated binding. These *in vitro* data suggest that the disaccharide moiety of GalNAcβ1→(3,4)Galβ→(R), where R is a glycan or aglycone, contains an epitope sufficient for rotaviral binding.

6.8
GLYCOSPHINGOLIPIDS DURING MYOBLAST MEMBRANE FUSION: POSSIBLE ROLE OF CELL SURFACE GLYCOSYLTRANSFERASES

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We have previously reported a transient increase in total glycosphingolipid (GSL) biosynthesis during contact and membrane fusion of clonal myoblast cells, E63, while fusion-defective cells derived from the same parental line, fu-1 showed no such increase in synthesis (Cambron et al., *Glycocon. J.*, 5:303, 1988). Comparison of GSL synthetic enzymes in both cell lines revealed two notable differences: (1) an increase in lactosylceramide synthase (GalT-2) activity associated with E63 cells at the time of cell contact which was absent in fu-1 cells; and (2) GM3 synthase (SAT-1) activity increased two days earlier in fu-1 cells than in E63 cells (Cambron et al., *Glycocon. J.*, 6:446, 1989). Addition of an inhibitor of glucosylceramide synthesis (PDMP) to the growth media of E63 cells inhibited myoblast fusion which was reversed when the cells were treated concurrently with exogenous LacCer or GM3 before cell contact (Cambron et al., *Glycocon. J.*, 7:80, 1990).

It should be noted, however, that the glycosyltransferase assays employed whole cell homogenates which did not rule out the possibility of a cell surface glycosyltransferase being involved in myoblast differentiation. To investigate this, UDP-dialdehyde, which inhibits galactosyltransferase by forming a Schiff base with a lysine residue in the active site of the enzyme, was added to E63 cell cultures. In the presence of UDP-dialdehyde (250 μM) myotube formation was inhibited. Furthermore, intact cells treated with UDP-dialdehyde had significantly decreased activities ($p<0.05$) of GM2 synthase (GalNAcT-1) and GM3 synthase (SAT-1) without affecting the activities of either LacCer synthase (GalT-1) or $LcOse_3Cer$ synthase (GLcNAcT-1). The fact that UDP-dialdehyde did not suppress the activities of all the glycosyltransferases assayed argues against its entry into the cell and affecting enzymes in the Golgi apparatus. These results suggest cell surface glycosyltransferases may play a role in myoblast membrane fusion possibly serving as GSL-recognizing proteins.

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6.9
EFFECT OF CERAMIDE COMPOSITION ON THE BILAYER STRUCTURE OF CEREBROSIDE SULFATE AND MISCIBILITY WITH PHOSPHATIDYLCHOLINE

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Cerebroside sulfate (galactosylceramide I^3-sulfate) (CBS), a major acidic glycolipid of myelin, consists of a number of species with the fatty acid chain varying in length from 14–26 carbons, with and without a hydroxyl group at the α-carbon. The long fatty acid chain length species have an asymmetric hydrocarbon structure since the sphingosine chain is thought to penetrate into the bilayer by only 14 carbons. We have shown that these species by themselves can form highly organized bilayer structures in which the long fatty acid chain of lipid on one side of the bilayer interdigitates into the other side of the bilayer. In the mixed interdigitated structure the sphingosine chain is packed end to end with the sphingosine chain of another molecule and the fatty acid chains of each span the bilayer. In the partially interdigitated bilayer, the sphingosine chain of lipid on one side of the bilayer is packed end to end with the fatty acid chain of lipid on the other side of the bilayer. Intermolecular hydrogen bonding can occur only for the latter and thus it is the more stable arrangement, particularly if the charge on the sulfate is shielded. The fatty acid hydroxyl group contributes to the intermolecular hydrogen bonding network formed by the sugar and sphingosine hydroxyls and favors the partially interdigitated bilayer if the charge on the sulfate is shielded. We are interested in how these long chain length lipids can pack together with more symmetric chain length lipids, such as phosphatidylcholine (PC). The phase behavior of mixtures of semi-synthetic species of CBS containing palmitic acid (C16:0-CBS), lignoceric acid (C24:0-CBS), and α-hydroxy lignoceric acid (C24:0h-CBS) with PC in 0.1 M KCl was studied using differential scanning calorimetry. PC was more miscible with the short chain length C16:0-CBS than the long chain length C24 species. At low concentrations, C24:0-CBS is not very miscible with PC in the gel phase. At intermediate CBS concentrations the mixture contained CBS in its mixed interdigitated structure where hydrogen bonding cannot occur, while at high CBS concentrations, it was in its partially interdigitated structure where hydrogen bonding can occur. C24:0h-CBS was less miscible with PC than the non-hydroxy fatty acid species. Furthermore, the transition temperatures of the C24:0h-CBS/PC mixtures were higher than expected suggesting complex formation between the PC and the hydroxy fatty acid species of CBS.

6.10
THE ROLE OF THE CERAMIDE COMPOSITION OF GLYCOLIPIDS ON THEIR SURFACE EXPRESSION

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Glycosphingolipids are important membrane constituents involved in a wide variety of cell surface phenomena including interactions with numerous biological ligands. We have been examining the effect of the fatty acid chain length of the ceramide group of glycolipids on their accessibilty or exposure to external biological ligands in model membranes (liposomes). A spin membrane immunoassay has been employed to examine the effects of changes in the fatty acid chain length of the glycolipid cerebroside sulfate (galactosylceramide I^3-sulfate, *CBS*) have on its antigenic recognition. We found that in phosphatidylcholine (*PC*)/cholesterol liposomes increasing the fatty acid chain length of CBS markedly improved its recognition, but in sphingomyelin (*SM*)/cholesterol liposomes the fatty acid chain length suprisingly had no effect. Inhibition studies were undertaken to define the nature of the epitope recognized by the antibody. Using a variety of both liposome-bound and soluble inhibitors it was revealed that *CBS* was recognized by distinct sub-populations of antibodies in *PC* and *SM* liposomes respectively, indicating that the antigenic structure of *CBS* differed in these two phospholipid environments (Stewart and Boggs, 1990 *Biochemistry* ***29***, 3644–3653). The more general technique of galactose oxidase (*GO*)-NaB^3H_4 labeling is also being used to examine the

exposure of glycolipids in model membranes. Separation of the individual molecular species by reverse-phase HPLC allows the direct determination of the fatty acid composition of the labeled species and thus of the species which are accessible to *GO*. Initial studies have compared *GO* oxidation of galactosylceramide (*GC*) in tetrahydrofuran (*THF*) and in liposomes. In THF, *GC* is readily labeled and, as expected, none of the molecular species were selectively labeled. When *GC* was incorporated into PC liposomes, its accessibility to GO was greatly reduced. Studies of five semi-synthetic species of *GC* in liposomes, ranging in chain length from 16 to 26 carbons, show that the longer chain species are oxidized more than the shorter-chain species, confirming that an increase in fatty acid chain length causes increased exposure of the carbohydrate at the membrane surface.

6.11
Gg_4-AND Gb_4-SERIES NEUTRAL GLYCOSPHINGOLIPIDS OF NORMAL ADULT BRAIN

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Neutral glycosphingolipids of globo, ganglio and neolacto series are not predominant glycolipids of normal brain but do accumulate in the brain of some sphingolipidoses. Fetal human brain contains such neutral glycolipids as galactosyl/glucosylceramide (48%) lactosylceramide (17%), Gb_3Cer (3%), Gg_3Cer (9%) and traces of Gb_4Cer and Gg_4Cer. The last two glycolipids have not been chemically characterized. Two other neutral glycolipids have been characterized as nLc_4Cer (13%) and fuc-nLc_4Cer (10%) (Ishikawa *et al.*, 1987, J. Biochem. (Tokyo) *101*, 1369). The glycolipids of normal adult human brain are mainly galactosylceramide, sulfatide and gangliosides. The presence of GA1 or Gg_4Cer has been reported in mouse brain myelin and in natural killer (NK) cells but Gg_4Cer in normal brain and in NK cells has not been analytically confirmed (Kusunoki *et al.*, 1985, Brain Res. *334*, 117). We have recently purified and characterized Gg_4Cer from bovine brain by GC-MS and by specific stepwise exoglycosidase hydrolysis. Its presence in bovine and adult normal human brain is confirmed by immunostaining with anti asialo-GM1 antibody. In addition, a second neutral glycolipid of Gb-series has been partially purified from bovine brain and tentatively characterized as Gal-Gb_4Cer by GC-MS. Preliminary 500 MHz ^{1}H-NMR spectroscopy of the partially purified compound indicates the possibility of Gb-series structure. Immunostaining with MC-631 (SSEA-3) antibody (Developmental Studies Hybridoma Bank) strongly suggests the existence of a GalNAcβ1→3 Galα1→4 Gal linkage. Though Gal-Gb_4Cer is not detected in human brain, another neutral glycolipid of the Gb-series (Rf closer to nLc_4Cer and comigrates with Gb_4Cer) appears to be stained with MC-631 and is anticipated to be Gb_4Cer. Further chemical and immunochemical characterization is in progress.

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6.12
THE THYROTROPIN RECEPTOR GANGLIOSIDE IN A RAT THYROID CELL LINE (FRTL-5) IS A GD1a LACTONE

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We have reported the evidence that ganglioside is an integral part of the human TSH receptor (TSH-R). To investigate whether the TSH-R rat thyroid cell line (FRTL-5) TSH-R contains ganglioside we purified the TSH-R by affinity chromatography on wheat germ agglutinin and TSH columns from cells grown with ^{3}H-glucosamine and ^{3}H-galactose. Gangliosides were extracted by Folch partition from an affinity purified, single radioactive glycoprotein band of M ~70 kD in SDS-PAGE and were detected in the lower phase migrating at GM2. This ganglioside was also purified from unlabelled TSH receptor and was detected by binding of ^{125}I-Limax flavus agglutinin or the B subunit of cholera toxin after prolonged digestion with Vibrio cholerae neuraminidase on a TLC plate. Ganglioside did not bind Limulus polyphemus lectin or Cancer antenarius lectin which suggests it lacks O-acetylated sialic acids. Alkaline hydrolysis of the TSH-R ganglioside with sodium hydroxide resulted in mobility shift to the position of GD1a standard; in addition aquisition of the negative charge enabled the ganglioside to be extracted from the upper phase after Folch partition. Synthesis of lactones from GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b and GQ1b by 7 days exposure to glacial acetic acid shows that only GD1a is converted to lactone be migrating at GM2. This major band is devoid of negative charges and is extracted into the lower phase by Folch partition. This finding suggests that TSH-R ganglioside is a GD1a lactone which is strongly bound to the receptor and is neither dissociated nor de-lactonized by alkaline conditions of SDS-PAGE (pH ~8.2). It can also explain our previous observations that human TSH-R contains alkali labile ganglioside migrating below GM1 standard, similarly to GD1b lactone. These data suggest that TSH-R from different species may differ in their ganglioside component whereas the protein in the recently cloned dog, human and rat TSH-R is ~90% homologous.

6.13
EVIDENCE FOR MINUTE AMOUNTS IN HUMAN BLOOD GROUP B KIDNEYS OF A NOVEL BLOOD GROUP B HEPTAGLYCOSYLCERAMIDE BASED ON THE TYPE 4 CARBOHYDRATE CHAIN AND STUDIES OF ITS BIOSYNTHESIS IN VITRO.

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Blood group A glycolipid antigens have been found, based upon at least four different core saccharides (types 1 to 4). The biological significance of this structural polymorphism in not known, although the successful outcome of transplantations of blood group A_2 kidneys to blood group O individuals have been partly explained by the low expression of A type 3 and 4 chain glycolipid antigens in the A_2 kidneys (1). If graft rejection due to ABO incompatibility is, in any way, correlated to the expression of type 3 and 4 chain blood group glycolipids, it is of interest to identify possible blood group B structures based on these core saccharides. In a non-acid glycosphingolipid fraction isolated from human blood group B kidneys, minute amounts of a Galα1-3(Fucα1-2)Galβ-HexNAc-Galα1-4Galβ-Hex-Ceramide structure was identified by mass spectrometry, high-temperature gas chromatography-mass spectrometry, and by probing thin layer chromatograms with Galα1-4Gal specific *E.coli* and monoclonal anti-B antibodies. In contrast to the small amounts of this compound in blood group B kidneys, the blood group A kidneys have the type 4 chain A heptaglycosylceramide as the dominating blood group A glycolipid (2). No, or very low activity of the blood group B gene enzyme on the type 4 chain blood group H hexaglycosylceramide precursor was shown by biosynthetic experiments in vitro, which might explain the low expression of the type 4 chain blood group B heptaglycosylceramide in human blood group B kidneys.

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6.14
NON-ACID GLYCOSPHINGOLIPIDS IN DIFFERENT ORGANS OF A SEMI-INBRED PIG STRAIN

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The lack of donor organs is a major problem in human allotransplantation. Using organs from other species could solve this problem. The pig is for many reasons a suitable donor species (organ size, ethically acceptable, low virus transmission risk). The main reason for the hyperacute rejection of pig kidney perfused with human blood is naturally occuring human antibodies directed against pig tissue. Some of these antibodies have been shown to have carbohydrate specificity (1) A semi-inbred pig strain with a well defined antigenic phenotype has well known advantages as a donor compaired to ordinary pigs. The expression of non-acid glycosphingolipids was studied in a semi-inbred (with respect to the SLA-system) pig strain. Total non-acid fractions of glycosphingolipids were isolated from small intestine, kidney, liver, spleen, heart, and salivary gland. Further separation into total polar fractions (more than 5 sugars) was achieved by HPLC. Thin layer immunostaining with anti-H, anti-Y, anti-Le^a, and anti-Le^b antibodies showed a complex, organ-specific staining pattern. No reactivity was seen with anti-A or anti-X antibodies. Further characterization by high-temperature gas chromatography-mass spectrometry of oligosaccharides released from the intact glycosphingolipids by ceramidase cleavage, is in progress.

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6.15
BLOOD GROUP GLYCOSPHINGOLIPIDS OF PORCINE AORTA

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When organs are transplanted between discordant species the graft undergo hyperacute rejection. This is thought to depend on xenoreactive natural antibodies in the recipient, directed towards antigens located on endothelial cells of the transplanted organ. Human natural antibodies have been shown to recognize carbohydrate determinants on porcine endothelial cell membranes (1). In order to identify such possible determinants among glycosphingolipids, a total non-acid glycolipid fraction has been prepared from several porcine aortas (2). TLC on this fraction revealed two major bands migrating in the three- and four-sugar region, respectively. After further purification by HPLC, a polar glycolipid fraction was obtained, containing several components with more than four sugars in the carbohydrate chain. This fraction was analyzed by TLC-immunostaining and shown to have blood group H, A and Y reactivity. Several bands were stained using an antibody specific for blood group A, type 1 chain mono-and difucosylated structures. No staining was seen with antibodies specific for type 2, 3 or 4 chain A.

Further structural characterization by proton NMR spectroscopy and mass spectrometry is in progress. In addition, a primary cell culture of endothelial cells from porcine aorta has been established, to reveal the thin layer pattern of glycosphingolipids derived from pure endothelial cells.

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6.16
SEROLOGICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF ANTI-PP_1P^k (anti-Tj^a) ANTIBODIES IN BLOOD GROUP LITTLE p INDIVIDUALS: ANTI-A TYPE 4 ACTIVITY DUE TO INTERNAL BINDING

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Serum samples from 13 blood group little p individuals were tested in RIA technique for their IgG antibody subclass distribution against the P, P^k and P_1 antigens. There were no uniform subclass distribution pattern although all but one had IgG3 antihodies against all the P system antigens tested. Studies were performed passing anti-Tj^a serum sequentially through columns with synthetic sugars with P system antigenic structures coupled to silica beads (Synsorbs). The effect on agglutinin and indirect antiglobulin titers was determined after passage through Synsorbs with different P system antigens. Also the effect on IgM, IgG, IgA as well as IgG subclass antibody binding to P, P^k and P_1 antigens was determined by radioimmunoassay and chromatogram binding assay. Anti-PP_1P^k antibodies from a little p woman were shown to bind to placental antigens from the same individial as studied by chromatogram binding assay. This binding was eliminated by serum adsorption on to Synsorbs with P system carbohydrates. Anti-PP_1P^k antibodies were also shown to bind to extended structures in the globoseries i.e. globopentaosylceramide, globohexaosylceramide (globo-H) and globoheptaosylceramide (globo-A). This binding may be due to antibodies recognizing internal sequences in the carbohydrate chain.

6.17
THE GLYCOSPHINGOLIPID EXPRESSION IN SPONTANEOUSLY ABORTED FETUSES AND PLACENTA FROM BLOOD GROUP LITTLE-p WOMEN

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The P blood group system has three antigens all identified as glycosphingolipids, the p^k (globotriaosylceramide, $GbOse_3Cer$), the P (globoside, $GbOse_4Cer$) and the P_1 ($IV^4GalnLcOse_4Cer$) antigens. Individuals belonging to the little-p blood group system lack these three antigens on their red blood cells and have regular occuring antibodies against them. Little-p women are further characterized by a high rate of early spontaneous abortions if the father belongs to another group within the P-system. As IgG_3 antibodies from Rh immunizations are known to pass the placental barrier and affect the fetus the question arose whether the aborted fetus and/or the placenta in a blood group P incompatible pregnancy express the antigens of the P-system.

A 12 weeks old fetus and a 17 weeks old fetus + placenta were obtained from two little-p women after spontaneous abortions. The 17 weeks fetus was dissected into the intestine, liver, brain and residual tissue before analysis. Total non-acid glycosphingolipid fractions were prepared from the different tissues (total amount of glycolipids ranging from 2 mg to 7 mg, for the placenta 27.5 mg) and analyzed by thin-layer chromatography with immunostaining with monoclonal antibodies and Gal-Gal specific bacteria. In the 12 weeks fetus and the residual tissue of the 17 weeks fetus trace amounts of $GbOse_3Cer$ and small amounts of $GbOse_4Cer$ were identified while the intestine, liver and brain of the 17 weeks fetus lacked these compounds. In contrast the 17 weeks old

placenta contained large amounts of these structures. Blood group antibodies reacting with A type 1, 3, 3+4 and ALe^b determinants stained several bands in the intestine of the 17 weeks while the liver and brain fractions were negative. Structural studies of the glycolipid fractions by mass spectrometry are in progress. The fact that two fetuses were anatomically intact after the spontaneous abortions and since the placenta tissue contained large amounts of $GbOse_3Cer$ and $GbOse_4Cer$ while only small amounts of these antigens were present in the fetuses it is postulated that a primary destruction of the placenta is the cause of abortions in little-p women.

6.18
TWO NOVEL FUCOGLYCOSPHINGOLIPIDS FROM THE TESTES OF THE SEA SNAIL *TURBO CORNUTUS*

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Two novel glycosphingolipids (GL-A and GL-B) were isolated from the testes of the sea snail *Turbo cornutus* and their structures were determined using FAB/MS, ^{1}H-NMR, chemical analysis, methylation analysis and chemical degradation methods on both the intact and defucosylated lipids. The defucosylated lipids were obtained by treating the intact ones with trichloroacetic acid.

The structure of GL-A was determined to be 4-0-MeGalβ1-4(Fucα1-2Fucα1-3)Glc-NAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1-4Glcβ1-1 Ceramide and GL-B to be Galβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1-4Glcβ1-1 Ceramide. GL-B had galactose in place of the terminal 4-O-Me-Gal of GL-A. The structure of the ceramide moiety was almost the same in GL-A and GL-B. The long chain bases consisted of hexadecasphingenine (30%), heptadecasphingenine (18%), octadecasphingenine (25%) and docosasphingadienine (16%). The fatty acid was mainly palmitic acid.

The structural features of the sugar chains of these novel glycolipids are: the existence of 4 moles of fucose, linkage of fucose to glucosamine, and linkage of glucosamine to glucose.

The composition of the ceramide moiety of GL-A and GL-B was the same as that of glucosyl ceramide, which is the starting glycolipid in the biosynthesis of GL-A and GL-B. GL-B may be the precursor in the pathway of GL-A biosynthesis.

The fact that the Rf value of GL-B on TLC is slightly lower than that of GL-A may be due to the existence of galactose in the terminal sugar chain of GL-B instead of the O-Me-Gal of GL-A.

These two novel glycolipids were specific for the testes and not found in the ovary, muscle or viscera. They therefore may have important roles in the reproduction of *Turbo cornutus*.

6.19
SECRETION OF GLYCOSPHINGOLIPIDS BY THE HEP-G2 HUMAN HEPATOMA CELL LINE

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Glycosphingolipids (GSLs) are not only components of cell membranes but are also found in soluble form coupled to plasma lipoproteins (LPs). The origin of LP-associated GSLs and the mechanisms by which they transfer between LPs and cells are not well understood. Hep-G2 cells were examined as a possible model system for addressing these issues because they synthesize and secrete many plasma proteins, including LPs.

The GSL composition of Hep-G2 cells was determined by standard biochemical and immunological methods. These cells were found to synthesize only five major GSLs: glucosyl ceramide (CMH), lactosyl ceramide (CDH), ceramide trihexoside (CTH), lactosyl sulfatide, and ganglioside GM3. Several minor GSLs were also identified.

Hep-G2 cells were then cultured in serum-free media and the LP fractions (VLDL, LDL, and HDL) of the conditioned medium were isolated by preparative density gradient ultracentrifugation. Protein secretion linearly increased in the LP-containing and LP-deficient fractions over 48 hours. Over 90% of the total protein was found in the LP-deficient fraction but 90% of the cholesterol, triglycerides, and GSLs were found in the combined LP fractions.

To study the association of GSLs with secreted LPs in more detail, LP fractions were collected from cells metabolically labeled with ^{3}H-galactose. LP-associated alkali-stable Folch lower phase lipids were isolated, separated by thin-layer chromatography, and visualized using a radiochromatogram linear analyzer. CMH, CDH, and CTH were identified by this method. These three GSLs were predominantly found in the LDL and HDL fractions; very small amounts were associated with VLDL. CDH was the major labeled GSL found in each case and was also the major labeled GSL synthesized by Hep-G2 cells. The addition of fatty acid free bovine serum albumin or ethanol to serum-free medium did not affect the amount of LP-associated GSLs secreted by Hep-G2 cells. However, the addition of fetal calf serum did stimulate the production of LP-associated GSLs in a dose-dependent manner. The factor(s) in fetal calf serum and the mechanisms responsible for this effect are currently under study.

6.20
SIMIAN ROTAVIRUSES PREFERENTIALLY RECOGNIZE SIALYLATED GLYCOPROTEINS AND NEUTRAL GLYCOSPHINGOLIPIDS

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Rotaviruses are the major cause of severe gastroenteritis in infancy and early childhood. They are nonenveloped, double-stranded RNA viruses of the family *Reoviridae*. The pathogenic potential of many animal rotaviruses is determined by viral capsid protein interactions with sialic acids on the surface of host cells. In contrast, many human and some animal rotaviruses do not require sialic acid for *in vitro* replication. Regardless of sialic acid-dependency, many rotaviruses also specifically bind to the neutral glycosphingolipid, asialo-GM1.

We now report that binding to sialylated glycoproteins by simian rotaviruses is also dependent on the penultimate carbohydrate structures. In various solid phase binding assays, mucin-type sialoglycoproteins are bound more avidly than equimolar amounts of N-linked sialoglycoproteins. Sialylated glycosphingolipids do not support binding. In contrast, rotaviruses specifically bind the neutral gangliotetraosylceramide, but not the tetrasaccharide coupled to albumin. These and other observations suggest the presence of at least two virally-encoded attachment sites of different specificities (ie. mucin-type sialoglycoproteins vs. neutral glycosphingolipids), and may imply two temporally or mechanistically distinct mechanisms of infection by rotaviruses. This work has been supported by NIH Physician-Scientist Program Grant Award, DK01298–05, and by CA42486.

6.21
PIGLET INTESTINAL RECEPTORS OF LECTINS K99 AND K88

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Adhesion of enterotoxigenic *E. coli* to the epithelium of the small intestine is identified as an essential property for the initiation of colonization. In piglet, *E. coli* adhesion is mediated by K88 and K99 lectins which recognize glycoconjugates of host cells.

Cell surface characteristics are recognized as a fundamental feature in the susceptibility of the animal to infection. In this work, we studied the distribution of glycoconjugates, recognized by the three K88 lectin variants (ab, ac and ad). Glycoproteins isolated from brush borders were separated by SDS-PAGE. After blotting on nitrocellulose membrane, they were incubated with ^{14}C-labeled K88-positive *E. coli* cells. Binding patterns depended on the piglet phenotype and were found to reflect different carbohydrate specificities. In the Mr 40000–90000 range, five glycoproteins bound to K88ab which differ either by protein and/or glycan moieties. To study this variability, we propose an enzymatic approach. Peptide-*N*-glycosidase F, purified and analyzed in the laboratory, is one of the useful tools.

Evidence for the existence of adhesive and non-adhesive phenotypes of piglets was obtained based on adhesion susceptibility of K99 fimbriated *E. coli* to pig intestinal epithelium. Adhesion was related to the concentration of sialoglycolipids on epithelium and to the glycolylation of sialic acid-containing ganglioside GM3(NeuGc) and sialoparagloboside SPG(NeuGc).

Characterization of intestinal glycoconjugates will permit further understanding of the bacteria-host interactions and the variability observed in the bacterial colonization.

6.22
EIGHT IMMUNOTYPE LIPOOLIGOSACCHARIDES OF *NEISSERIA MENINGITIDIS* REACT WITH A MONOCLONAL ANTIBODY WHICH BINDS LACTO-N-NEOTETRAOSE

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Neisseria meningitidis can be serologically divided into at least 12 immunotypes based on its lipooligosaccharide (LOS) antigens. The LOS of *N. meningitidis* consists of oligosaccharides linked to lipid A through KDO. Lacto-N-neotetraose (LNnT, Galβ1-4GlcNAcβ1-3Galβ1-4Glc) has been reported to be at one of nonreducing ends of the branched oligosaccharides from the LOS of some meningococcal strains.

In the present study the reactivity of LOS from 12 known immunotypes with a mouse monoclonal antibody (anti-My-28) that had been characterized to be specific for LNnT, was investigated. Eight of the 12 LOS bound specifically with the antibody. Among the twelve LOS immunotypes, types 2, 3, 4, 5, 8, and 9 had strong binding; types 7 and 10 were intermediate; and types 1, 6, 11, and 12 were negative as measured by ELISA, immunodot and immunoblot assays. If an LOS showed multiple components by SDS-PAGE analysis, the large-size major component was the reactive component. The expression of the reactive epitope in the LOS was influenced by growth medium and the epitope could be masked by sialylation when *N. meningitidis* was grown in tryptic soy broth. The binding of the antibody to the LOS was inhibited best by LNnT, followed by N-acetyllactosamine; in contrast lacto-N-tetraose, Galβ1-3GlcNAcβ1-3Galβ1-4Glc, did not inhibit. These results indicate that the LNnT sequence is present in 8 of 12 LOS immunotypes. The presence of LNnT sequence, a structure found in a variety of human cells, in the LOS may play a role in the virulence of *N. meningitidis*.

6.23
GENETIC REGULATION OF GLYCOLIPID EXPRESSION BY *Gsl-7*

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The regulation of expression of carbohydrate structures in glycoconjugates is responsible for the production of remarkable number of carbohydrate structures. We have focused on genetic regulation for the expression of glycolipids and we have identified 5 autosomal mouse genes. In this paper, we report a newly identified gene named *Gsl-7*.

In erythrocytes, a strain of wild mice, *M. m. castaneus* Ttn, expressed two gangliosides which have not been found in laboratory strains of mice and other wild mice so far. Two gangliosides (X and Y) were purified and their structures were analyzed. The structures of X and Y are GM1 and GD1a, respectively, and both contain N-acetylneuraminic acid. On mating experiments, the expression of GM1(NeuAc) and GD1a(NeuAc) was demonstrated to be dominant over lacking their expression. The segregation of phenotypes in backcross mice demonstrated the involvement of a single autosomal gene. We have already reported that the expressions of GM2(NeuGc) and GM1(NeuGc) in erythrocytes are controlled by *Ggm-2* and *Ggm-1*, respectively. *Gsl-7* is genetically independent of *Ggm-2* and *Ggm-1*. These results indicate that different genes are involved in the systems which produce NeuAc and NeuGc or glycoconjugates containing NeuAc and NeuGc. Recently, we have been able to demonstrate the involvement of cytochrome b_5 for CMP-NeuAc hydroxylation and it becomes possible for us to identify genes required for the expression of NeuGc containing glycoconjugates. Therefore, the identification of *Gsl-7* is quite interesting from the viewpoint of genetic regulation of NeuGc containing glycoconjugates.

6.24
CHARACTERIZATION OF DISIALYLATED ASIALO GM1 (NeuGc-NeuGc-GA1) IN MOUSE THYMOMA

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Glycolipids are located in the plasma membrane of cells and are thought to play important roles in biological phenomena through the membrane. In the immune system, glycolipids in the membrane may play as recognition molecules in cell-matrix or cell-cell interactions. In the previous study, we analyzed mouse spleen gangliosides and reported the structures of monosialogangliosides including GM1b, GalNAc-GM1b and a new cholera toxin binding ganglioside: Gal-GalNAc-GM1b. Furthermore, the comparison of ganglioside patterns among several mouse tissues indicated that the biosynthetic pathway from asialo GM1 to Gal-GalNAc-GM1b is active only in immunological tissues such as the spleen and thymus.

Recently, we had a chance to analyze gangliosides of thymoma which spontaneously occurred in WHT/Ht mouse. The thymoma contained a ganglioside specific to normal mouse thymus and spleen. We purified this ganglioside from pooled thymomata which were collected by subcutaneous transplantation, and analyzed the structure with sugar-composition analysis, a permethylation study, enzymatic hydrolysis, negative-ion fast atom bombardment mass spectrometry, and ^{1}H-NMR spectroscopy. The structure was demonstrated to be NeuGcα2-8NeuGcα 2-3Galβ 1-3GalNAcβ 1-4Galβ 1-4Glcβ 1-1'Cer. Studies on the distribu-

tion of this ganglioside in thymocytes and T and B cells are now in progress. Bartoszewicz et al. had characterized a ganglioside of the same structure having NeuAc instead of NeuGc from AKR thymoma. In addition, we purified five disialogangliosides from WHT/Ht thymoma and characterized them as NeuAc-NeuGc-GA1, NeuGc-NeuAc-GA1, GD1a(NeuAc,NeuAc), GD1a(NeuAc,NeuGc) and GD1a(NeuGc,-NeuGc).

6.25
ACCUMULATION OF SULFOGLYCOLIPIDS IN HYPEROSMOSIS-RESISTANT CLONES DERIVED FROM MADIN-DARBY CANINE KIDNEY CELLS (MDCK)

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Recently we demonstrated that the concentration of sulfoglycolipids of several kidney cell lines depends on the osmolarity of the culture medium (1) suggesting a tied role of these acidic amphiphiles in adaptation to high osmolarity. During culture of MDCK in hyperosmotic media, we noticed marked inhibition of cell growth thereby enabling us to select clones resisitant to hyperosmosis.

Two clones (osmR-A and osmR-B) resistant to culture in hyperosmotic media, 700 and 800 mOsm/l, respectively, were selected from MDCK cells. When the above clones were cultured in the isosmotic medium (300 mOsm/l), the concentrations of SM4s (GalCer I^3-sulfate) and SM3 (LacCer II^3-sulfate) were 3.6- and 5.9-fold of the wild-type MDCK, respectively, in osmR-A, and 3.4- and 5.5-fold in osmR-B.

The turnover of sulfolipids in MDCK and osmR-A cells was assessed by culture with [^{35}S]sulfuric acid. The rate of incorporation into SM4s, SM3 of osmR-A was 2.9- and 6.6-fold, respectively, of those in MDCK. The hyperosmotic media stimulated incorporation into SM4s and SM3, respectively of MDCK and osmR-A after a lag time of 5 h. This increase of the incorporation into sulfolipids was inhibited by cycloheximide, suggesting that synthesis of proteins is responsible for the increase of label. The chase studies of the labeled lipid indicated that degradation of both sulfoglycolipids was accelerated in MDCK by hyperosmosis.

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6.26
A NOVEL GANGLIOTRIAOSYLCERAMIDE SULFATE FROM RAT KIDNEY

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Mammalian kidney had been believed to contain only two sulfoglycolipids, SM4s and SM3, until the discovery of the third sulfoglycolipid, Gg_3Cer II^3-sulfate (SM2a) (1), and two *bis*-sulfoglycolipids (SB2 and SB1a) belonging to *Ganglio*-series from rat kidney. Recently, SM2a was found to be synthesized in human endometrial cancer cell lines (2), and the accumulation of SB1a was observed in human hepatocellular carcinoma (3). In this study, we found a minor sulfoglycolipid (Ka) which migrated a little slower than SM2a on TLC.

Ka was purified by chloroform-methanol extraction, mild alkaline methanolysis, column chromatographies with DEAE-Sephadex and silica beads, and HPLC on latrobeads. From compositional analyses, ^{1}H-NMR, FT-IR and permethylation analyses, the complete structure of Ka was proposed to be HSO_3-3GalNAcβ-4Galβ-4Glcβ-1 Cer (SM2b) with the major ceramide composition of 24:0/t18:0. Multiple-relayed DQF-COSY and PH-DQF-COSY studies showed that the signals of H-2 to H-4 of GalNAc were shifted to the down field as compared to those of desulfated Ka or Gg_3Cer, consistent with an equatorial 3-sulfate and an equatorial H-4. Mass spectra were recorded on a triple quadrupole instrument with a cesium ion gun. Intense pseudomolecular ions as well as characteristic fragment ions which were cleaved sequentially at the glycosidic linkage were observed.

The yield of Ka was 50 pmol/g wet tissue, which was about 2% of that of SM2a. Rat kidneys contain 270 pmol of iGb_4Cer IV^3-sulfate and 70 pmol of Gb_4Cer IV^3-sulfate. Isoglobotetraosylceramide which may be the precursor of the biosynthesis of the former, is the species specific glycolipid of rat and expressed in some tumors. In contrast, *Ganglio*-series sulfoglycolipids seem to be ubiquitous among mammals.

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6.27
PRIMARY CULTURES OF NEURONS EXHIBIT AN EXTRALYSOSOMAL, PRESUMABLY PLASMA MEMBRANE-BOUND, SIALIDASE ACTIVITY ON GANGLIOSIDES

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Cerebellar granule cells differentiated in culture carry a sialidase activity, which, at least in part, is located in the lysosomes. The possibility was explored that a form of this enzyme resides at the cell surface and is active under the culture conditions. To this purpose cerebellar granule cells were fed for 4 hours at 37°C with disialoganglioside GD1a (10^{-6}M), ^{3}H labelled at the level of the sphingosine moiety, and the formation of radioactive metabolites was followed. Parallel experiments were run where the lysosomal inhibitor chloroquine (50 μM) was added 30 min prior to ganglioside treatment, or cells were kept at 4°C in order to prevent the internalization of the exogenous ganglioside. Under the control conditions labelled GM1, GM2, GM3, lactosylceramide, glucosylceramide, ceramide and sphingosine were recognized, indicating the occurrence of complete degradation of GD1a. When cells were incubated in the presence of chloroquine, or at 4°C, the only formed labelled compound was GM1. This indicates that desialosylation of GD1a occurred also when the lysosomal apparatus or the process of GD1a interalization were blocked. In addition, no degradation of GD1a occurred in the culture medium, due to possible release of sialidase. Similar results were obtained with ^{3}H labelled disialoganglioside GD1b, whereas no degradation of monosialoganglioside GM1 was observed in the presence of chloroquine, or at 4°C.It can be concluded that granule cells in culture are able to split sialic acid from gangliosides GD1a and GD1b, but not from GM1, by means of a non-lyosomal, non releasable, presumably plasma membrane-bound, sialidase.

6.28
GANGLIO-SERIES HISTO-BLOOD GROUP A- ACTIVE GLYCOLIPIDS: IN VITRO BIOSYNTHESIS, STRUCTURAL CHARACTERIZATION AND PRODUCTION OF MONOCLONAL ANTIBODIES DEFINING THESE COMPOUNDS

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Four structural variants of histo-blood group A antigens (type 1, 2, 3, and 4) have been isolated and characterized from human tissues.

Recently asialo ganglio-series A, AGA[1] ($IV^2FucIV\alpha GalNAcGg_4$), was identified from porcine erythrocytes, but this compound has not been found in human tissues. Using immunohistochemical staining, the H active ganglio-precurser HGM_1 (IV^2FucII^3NeuAc-Gg_4), can be found in some normal human tissue, and has been shown to be associated with small cell lung carcinomas. We describe the in-vitro biosynthesis and structural characterization of ganglio series A, AGA_1 and AGM_1 ($IV^2FucIV\alpha GalNAcII^3NeuAc$-$Gg_4$), as well as the generation of monoclonal antibodies defining these A variants. AGM_1 and AGA_1 were biosynthesized from HGM_1 and HGA_1 (IV^2Fuc-Gg_4) using purified histo-blood group A glycosyltransferase from human lung. Immunization of mice with either AGM_1 or AGA_1 adsorbed to Salmonella Minnesota resulted in the isolation of two hybridomas WH1, specific for AGM_1, and WH_2 specific for AGA_1. Using these antibodies AGM_1 was isolated from bovine erythrocytes and characterized by H-NMR, FAB, and Methylation analysis. A preliminary screen of glycolipid extracts of human tissues using thin-layer chromatography immunostaining did not show the presence of the ganglio series A variants. The ganglio A structures as well as the ganglio H precursors were identified in porcine and bovine tissues.

6.28
MYELIN GANGLIOSIDES OF HUMAN MOTOR AND SENSORY NERVES

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In the peripheral nervous system, motor and sensory nerves are functionally and anatomically distinct groups. Previously we reported that ceramide components of GD1a, GM1 and GD1b gangliosides of the sensory nerve differed apparently from those of the motor nerve, and that these gangliosides were enriched in the axonal fractions(1). The results suggest that the contrasted ceramide patterns of the gangliosides in the two nerves may be attributed to a distinct type of axons, *i.e.*, motor or sensory. However ganglioside compositions of the myelins have not been compared in the two nerves. In this report we studied the ganglioside and glycosphingolipid composition of myelin isolated from human motor and sensory nerves. The contents of GalCer, a marker glycolipid of peripheral nerve myelin, were almost comparable in the two nerves. Myelin fractions isolated from the nerves contained nLc_4Cer as minor glycosphingolipids. The predominant ganglioside in the human nerve myelins, both from motor and sensory nerves, was LM1 (NAα 2-$3nLc_4Cer$), as shown by previous papers (2,1). By TLC analyses and TLC immunostaining method using anti-GM1 antibody, it was revealed that the motor nerve myelin contained GM1 (about 15% of total gangliosides), but sensory nerve myelin, however, contained only a trace amount of GM1 (less than 5%). As for disialo-ganglioside fraction, the contents of GD1a was also differed between motor and sensory nerve myelins as well as GM1. Thus the difference of ganglioside compositions between human motor and sensory nerve myelins was demonstrated.

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6.30
THE FUCOSYLATED GANGLIOSIDE FUCGM1 MIMICS THE RECEPTOR FUNCTION OF GM1 GANGLIOSIDE FOR CHOLERA TOXIN

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GM1 ganglioside, a normal constituent of the plasma membranes, has been implicated as the receptor for the Cholera toxin (CT). However, even if other gangliosides have shown the ability to bind CT, with high affinity, the cellular responses following this interaction have been addressed only in the case of GM1 ganglioside. In recent years the presence of fucosylated gangliosides in tumoral cells has been the focus of increased research.

In this work we have studied the ability of the fucosylated ganglioside FucGM1 to mimic the receptor function of ganglioside GM1 for CT. For this purpose, rat glioma C6 cultured cells, lacking the receptor for CT, were enriched with FucGM1 and the responsiveness to CT addition was compared with that of cells enriched with GM1 ganglioside. FucGM1 was taken up by cells as rapidly and in the same extent as GM1 either in the serum stable or trypsin stable form of association, as shown by experiments using ^3H-labeled gangliosides. FucGM1 was as effective as GM1 to enhance the responsiveness of C6 rat glioma cells to CT-induced cAMP accumulation. Under conditions in which GM1 and FucGM1-treated cells bound comparable amounts of CT, the FucGM1-treated cells accumulated virtually the same amount of cyclic AMP as did the cells treated with GM1. The lag time preceding the CT-induced cAMP accumulation was similar in FucGM1 and GM1-treated cells. Moreover, at equal amounts of bound ganglioside cells enriched with FucGM1 were able to bind the same amount of ^{125}I-CT as did cells enriched with GM1. Isothermal titration calorimetry (ITC) experiments showed that the association constant of CT with FucGM1 or GM1 ganglioside were comparable (about 2×10^7 M^{-1} and 4×10^7 M^{-1}, respectively). Also the association constant of the isolated B subunit of CT to FucGM1 or GM1 ganglioside were comparable (about 4×10^7 M^{-1} and 7×10^7 M^{-1}, respectively).

6.31
NEW GANGLIOSIDES SPECIES IN CENTRAL NERVOUS SYSTEM

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Gangliosides, a family of glycosphingolipids containing sialic acids, are distributed on cell surface membranes and may play a leading role in the differentiation and development of the central nervous system (CNS). Monoclonal antibodies (MAbs) are expected to be useful in studies on ganglioside function in CNS. Even small quantities of uncharacterized gangliosides have been detected and isolated from adult bovine brain due to improved techniques including ion-exchange column chromatography of Q-Sepharose in combination with TLC/immunostaining with mAbs (M6704, M7104, 188C1, 493D4, NA-6, & KA-17)(1,2). From our results, the extremely minor gangliosides in adult brain are characterized as follows: They are

i) intensely expressed in the neural cells at embryonic stages and gradually decreased during development.
ii) major or dominant gangliosides in CNS of the lower vertebrate. GM1b and GD1α in frog brain; C-series gangliosides and acetylated polysialogangliosides in fish brain.
iii) expressed again in transformed cells. Therefore, they are useful marker for tumor diagnosis. C-series gangliosides are expressed in human melanomas, gliomas and neuroblastoma cells.
iv) cell surface marker for the subset of neurons.

The last point is very important when we consider the biological function of the extremely minor gangliosides. Quite recently, Whittaker *et al.* reported the occurrence of cholinergic-specific minor gangliosides in mammalian brain. We have been able to isolate the extremely minor

cholinergic-specific gangliosides from complex mixture of bovine brain ganglioside by Q-Sepharose. Characterization of their chemical structures are in progress.

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S7. IMMUNE MODULATION/IMMUNOMODULATION

7.1
THE ROLE OF ENZYMIC SIALIC ACID MODIFICATION IN IMMUNOLOGY AND BIOLOGICAL RECOGNITION

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The natural occurrence of more than 30 different sialic acids and their species- and tissue-specific distribution gives rise to the question of the biological function of these sugars (1). One of the basic properties of sialic acids is their masking of recognition sites of cells and molecules such as antigens or ligands for receptors (2). Especially sialylation of terminal galactose residues and desialylation of sialoglycoconjugates are tools for the regulation of cellular and molecular interactions and functions (3). These interactions may be disturbed by sialidases from pathogenic microorganisms or in cancer by a too low or too high degree of sialylation of cell surfaces. Another general but contrasting feature of the properties of sialic acids is their function as ligands for viruses, some other microorganisms and lectins.

The modification of Neu5Ac by various N- and O-substituents not only influences the physico-chemical behaviour of glycoconjugates, but also their biological and especially immunological properties (4). Sialic acids with N-glycoloyl and O-acetyl groups are expressed at various rates during differentiation or malignant transformation. The specific ligand function of 9-O-acetylated Neu5Ac for the binding of some viruses, e.g. influenza C virus, is most remarkable. Several enzyme systems are studied presently, which are involved in the O-acetylation, N-acetyl hydroxylation and O-methylation of sialic acids.

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7.2
GENTIOBIOSE HEPTAACETATE-DIPHTHERIA TOXOID CONJUGATE VACCINES PROTECT AGAINST DERMAL SHWARTZMAN REACTION AND GRAM-NEGATIVE SEPSIS

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We have synthesized aminoethyl (AE), aminopropyl (AP) and aminopentyl (APT) derivatives of gentiobiose heptaacetate (GH). These compounds (AEGH, APGH, APTGH) have been coupled to succinylated diphtheria toxoid (Suc.DT) to produce conjugate vaccines. These conjugates all bind to the anti-lipid A human monoclonal antibody A6(H4)C5 in an ELISA binding assay. Rabbits immunized with the conjugate vaccine Suc.DT-APGH in either Freund's complete adjuvant or aluminum hydroxide gel adjuvant produced antibody levels of 5,120 and 3,600 ELISA units respectively. These antibodies neutralized endotoxin in a Lemulus lysate neutralization assay. Protection against the dermal Shwartzman reaction was demonstrated by active immunization with this vaccine ($p < 0.05$). Protection against the dermal Shwartzman reaction by passive transfer of immune serum was not significant ($p < 0.10$).

We have also synthesized 6′-hydroxy(1,2,3,4,2′,3′,4′-hepta-*O*-acetyl) gentiobiose (6′-HGH) in the crystalline form. This compound has been coupled to Suc.DT by ester linkage to give the conjugate vaccine 6′-*O*-Suc.DT-GH. Rabbits immunized with this vaccine were protected against the dermal Shwartzman reaction caused by *E. coli* 0111:B4 LPS ($p < 0.05$). Rabbits were also protected by passive transfer of immune serum ($p = 0.004$). Immune rabbit serum when passively transferred protected mice (70%) against lethal challenge with *Ps. aeruginosa* strain 134 VA.

These results indicated that epitopes present in gentiobiose heptaacetate when properly presented were capable of inducing protective antibodies in rabbits against endotoxin.

7.3
AN INTESTINAL GALACTOSE-SPECIFIC LECTIN MEDIATES THE BINDING OF MURINE IgE TO MOUSE INTESTINAL EPITHELIAL CELLS

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A number of lactose-binding lectins have been recently identified in rat and mouse intestines. One of which corresponds to the C-terminal domain of IgE-binding proteins, originally identified in rat basophilic leukemia (RBL) cells and mouse 3T3 fibroblasts. In the present communication, we first describe the affinity purification of a rat intestinal lactose-specific lectin which binds murine IgE antibodies. This IgE-specific binding occurs likely via the immunoglobulin carbohydrate chains, because it is inhibited by lactose. This intestinal lectin molecule is also immunologically related to the previously described IgE-binding protein (εBP) isolated from RBL cells, since it is recognized by antibodies raised against recombinant εBP. The intestinal form of IgE-binding protein exhibits a molecular weight of 17,500 which is much lower than that of its RBL cell analogue (31,000). We then demonstrated by immunohistochemistry, the attachment of IgE to mouse intestinal epithelium, as well as the presence of a corresponding mouse intestinal IgE-binding protein. The carbohydrate-dependent nature of this attachment was established by demonstrating that IgE binding to mouse epithelium was specifically abolished by lactose (4 mM) and by a blood group A-active tetrasaccharide (0.2 mM), but not by mannose (10 mM). Finally, the association of IgE with the mouse intestinal epithelium was prevented by competition with the purified IgE-binding lectin isolated from rat intestine.

Although the physiological function of this intestinal protein is still unknown, the finding of IgE binding to a lectin in the intestinal epithelium suggests a novel mechanism for the regulation of IgE-mediated disorders, such as food allergy.

7.4
COOPERATIVE ACTION OF CARBOHYDRATE RECOGNIZING AND PEPTIDE RECOGNIZING RECEPTORS IN LECTINOPHAGOCYTOSIS BY LIVER MACROPHAGES

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Recently we found that the galactose-specific particle receptor of rat liver macrophages is identical with membrane-associated C-reactive protein (mCRP). We have now investigated the role of this lectin-like receptor activity in clearance of foreign particles from circulation.

As model particles we used silica dust, which is a known macrophage-toxic agent. The progressive killing of freshly isolated rat liver macrophages in incubation mixtures with silica particles and various additives was measured as a marker for uptake. Phagocytosis was also verified by electron microscopy.

Macrophages incubated with silica in Eagles medium at 37°C without further additives were indistinguishable from controls: they did neither take up the dust particles nor were they killed. When incubated in the presence of autologous or heterologous plasma, 80% of macrophages were killed within 60 min, this killing could be inhibited by addition of GalNAc as competing monosaccharide or by adding glycoproteins with terminal Gal-residues in μM concentrations. The amount of killing as well as the degree of inhibition was identical, when purified fibronectin was present instead of whole plasma.

Addition of the tetrapeptide RGDS, which represents the cell binding domain of fibronectin for its integrin receptor, inhibits the macrophage killing to the same extent as galactose.

A detailed analysis shows that interfering with one or the other receptor activity, disables the cell to mediate binding and endocytosis, thus showing that only cooperative action of two receptor activities leads to effective uptake.

7.5
DEANTIGENATION OF HUMAN ERYTHROCYTES BY BACTERIAL GLYCOSIDASES

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A subpopulation of the human fecal microbiota (*Ruminococcus* and *Bifidobacterium* spp) are unique in producing and excreting glycosidases hydrolysing complex fucosylated and sialylated carbohydrates in mucin glycoproteins and glycosphingolipids (GSLs; Fall, P. et al. (1990) *J. Biochem.* (Tokyo) **108**, 466–474). We have studied the immunological and structural effects of these enzymes on intact erythrocytes and their GSLs. Fresh human A_1 red blood cells were treated with the blood group degrading enzyme mixture of the *Ruminococcus torques* IX-70 strain (ATCC 35915), with final concentration of GalNAcα1-pnp activity of 42 mU/ml erythrocyte suspension (50% v/v), at 37°C with a constant glucose concentration at about 5 mM and a continuous gentle swirling up to 6 hours of incubation. With an iso-osmotic PBS-buffer containing 10 mg/ml of bovine serum albumin this treatment did not lyse nor affect the osmotic resistance of the cells. A_1 erythrocytes lost *Dolichos biflorus* (Ortho Diagnostic Inc.) lectin agglutinability within 30 min of incubation, showed a delayed appearance of *Ulex europaeus* lectin (DAKO-PATTS a/s) agglutinability, but remained A active by agglutination with BioClone anti-A monoclonal antibody (Ortho Diagnostic Inc.) during the 6 hours. Microscale preparation of GSLs and TLC-immunostaining with the lectins specified above showed hydrolysis of A and H active GSLs with 6–12 monosaccharides, but this at a slower rate than the loss of blood group A_1 (*D. biflorus*) agglutinability. The *D. biflorus* lectin was a good discriminator of A_1/A_2 subjects on lipid extracts in the TLC-overlay technique but showed a low affinity for the A type 3 and the A type 4 glycosphingolipid standards. Pretreatment of the cells with papain conserved the serological A_1 activity throughout the 6 h incubation but enhanced the rate of degradation of blood group active GSLs and also slightly increased the osmotic fragility of the cells. Higher concentration of the enzyme mixture (1 U/ml cell suspension) increased the degree of GSL degradation but did not abolish the blood group A agglutinability after 6 hours. Incubating A_1 red blood cells with an αGalNAc-ase preparation, purified from the *R. torques* IX-70 enzyme mixture, with αGalNAc1-pnp activity of 100 mU/ml cell suspension, did not affect blood group A active GSLs but abolished completely the *D. biflorus* agglutinability within 6 hours.

In conclusion, these data establish the hydrolysing effects of these bacterial glycosidases on cell surface glycoconjugates of intact cells and indicate that the *D. biflorus* agglutinability of A_1 erythrocytes is not dependent on medium-sized glycosphingolipids.

7.6
ASPERGILLUS GALACTOMANNAN

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Galactomannans (GM) represent one of the most widely distributed class of antigenic polysaccharides amongst zoopathogenic fungi. In the case of *Aspergillus fumigatus*, previous reports have demonstrated that GM-containing molecules are immunodominant antigens. It has been shown also that GM is present in serum and urine of immunocompromised patients with invasive aspergillosis. In spite of its antigenic properties and its diagnosis purpose, chemical characterization of the *Aspergillus* GM remain incomplete. GM can be chemically extracted from cell wall preparation of mycelium, from cytoplasmic material after total disruption of the mycelium or from culture filtrate. Because of its analogy with the *in vivo* situation, antigens secreted in the culture medium during active fungal growth were investigated.

Ethanol precipitate of young culture filtrates contains a mixture of proteins, galactosan and galactomannans. The largest GM molecule with a 20 kDa MW has been purified by successive hydrazine and nitrous acid treatments. Sugar linkages have been determined following methylation/acetylation of free OH on GM intact or submitted to acetolysis or light acid hydrolysis. GLC/MS and ^{13}CNMR data indicate that GM contains a core of branched mannan with α 1-2, 1-6 linkages and linear side chains with β 1-4 galactopyranose and β 1-5 galactofuranose linked to the α 1-2 mannose residues.

Monoclonal antibodies directed against GM are specific of the galactofuran side chain. Immunoblotting experiments demonstrated that in extracts produced *in vitro* and in the urine of patients with invasive aspergillosis, GM is a component of glycoproteins. Immuno-electron microscopy confirmed that galactofuran-containing antigens are secreted in the external medium but are also present in the wall and the cytoplasm of *A. fumigatus* mycelium.

7.7

IMMUNOLOGICAL PROPERTIES OF MONOCLONAL ANTIBODIES TO THE N-PROPIONYL DERIVATIVE OF GROUP B MENINGOCOCCAL POLYSACCHARIDE

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The serological and immunoprotective properties of monoclonal antibodies produced to the N-propionyl derivative of group B meningococcal polysaccharide (NPr-GBMP) have been investigated. Hybridomas were derived from mice immunized with a conjugate of tetanus toxoid and NPr-GBMP emulsified in Freund's complete adjuvant. Monoclonal antibodies specific to the N-propionyl epitope were selected on the basis of reactivity with a conjugate of bovine serum albumin and NPr-GBMP (BSA-NPr-GBMP) and lack of reactivity with BSA-NAc-GBMP in enzyme-linked immunosorbent assays. Ascites fluids produced for three hybridomas secreting IgG1, IgG2a and IgG2b isotypes were purified by affinity chromatography, standardized for protein content and used in bactericidal and immunoprotective studies. The bactericidal activities of the IgG1, IgG2 and IgG2b isotypes for meningococcal strain 80165 (B:2b) were > 500, 50, and 3 μg of protein per ml respectively. The IgG2b isotype was also most effective in passive protection experiments in mice. There was no bactericidal activity or immunoprotection against a heterologous strain 87147 (C:2b:P1.2) by the three isotypes.

The results support evidence for the presence of a unique intermolecular epitope on the cell surface of group B meningococci and further define the protective roles of anti-NPr antibody against group B meningococcal infection.

7.8

CARBOHYDRATE ANTIGEN SPECIFICITY OF PIG LYMPHOCYTOTOXIC IGM ANTIBODIES PRODUCED BY TWO EBV TRANSFORMED HUMAN B CELL LINES

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Hyperacute rejection is seen when organs are transplanted between discordant species and preformed IgM antibodies are thought to be the reason for it. After removal of human natural antibodies directed against pig renal tissue, hyperacute rejection could be prevented and secondary responses appeared to be limited and controlled by standard immunosuppression (1). Human natural antibodies have been shown to react with carbohydrate epitopes on pig endothelial cells (2). 350 Ig-producing, EBV-transformed human B cell lines, which were produced by UKTS as part of a programme designed to make anti-HLA antibodies, were screened for pig lymphocytotoxicity. Nine of the cell lines were lymphocytotoxic and two of these were characterized with regard to their carbohydrate specificity. Pig and non-pig, acid and non-acid glycolipid fractions were analyzed by thin layer chromatography followed by incubation with the two human anti-pig supernatants. Binding was seen in several acid fractions, but not to non-acid glycolipids or to blotted pig lymphocyte proteins. Staining corresponded to bands in the mixtures migrating as sulphatide I and II as well as sulphated gangliotetraosylceramide. The binding to sulphatide I was verified with a pure substance, but no reactivity was seen with cholesterol-3-sulphate. Further studies are in progress to assay the reactivity in an ELISA with pure substances and also in an ELISA using cultured pig endothelial cells as targets. Furthermore, inhibition studies with sulphated glycosaminoglycans are in progress.

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7.9

THE GLYCOSPHINGOLIPID EXPRESSION OF SMALL INTESTINE, STOMACH, KIDNEY AND PLASMA FROM A BLOOD GROUP A, LITTLE-p HUMAN INDIVIDUAL

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The blood group little-p phenotype is extremely rare. A high incidence of this phenotype has been found within a limited area in northern Sweden. They lack the P^k ($GbOse_3Cer$), the P ($GbOse_4Cer$) and the P_1 ($IV^4GalnLcOse_4Cer$) antigens on their red blood cells (1). Studies on the expression of these antigens in other tissues of little-p individuals have only occasionally been performed for obvious reasons, and so far only stomach (2, 3) has been studied. We have had the opportunity to obtain small intestine, stomach, kidney and plasma at autopsy from a little-p woman of blood group p, A_1 Le(a−b+) who died of pancreas cancer.

Total non-acid glycosphingolipid fractions were prepared from these tissues and compared with glycolipid fractions isolated earlier in our laboratory from P_1 or P_2, A_1 Le(a−b+) individuals. Initial studies with thin-layer chromatography analysis and immunostaining using anti-P, anti-P^k, anti-P_1 and different anti-A antibodies have been performed. Structural studies using proton-NMR and mass spectrometry are currently being undertaken.

The P^k- and P-antigens which are major components in these tissues from P_1- or P_2- individuals were absent in the little-p tissues. The P_1-antigen which is a minor antigen in P_1 tissues was also absent in the tissues of the little-p individual. The amounts of lactosylceramide and also more complex blood group compounds with 5 and more sugar residues were increased in the little-p tissues compared to the P_1 reference tissues. A very complex pattern of blood group A structures was revealed by the anti A antibodies. The globo-A ($V^3GalnV^2FucIV^3GalGbOse_4Cer$) antigens is a major component in P_1 or P_2 kidneys. No blood group A compound migrating as the globo-A reference compound could be found in the little-p kidney fraction as expected from the current view of biosynthesis of these antigens.

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7.10

A CATIONIC GLYCOPROTEIN PATTERN IN BACTERIAL INFECTIONS

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Patterns of highly glycosylated conjugates – probably glycoproteins – with mainly cationic isoelectric points, pH 6.5–9.5, were observed after isoelectric focusing and detection with an immunoassay specific for

digoxygenylated carbohydrate moieties of glycoconjugates in serum and cerebrospinal fluid of patients with various disorders. To our knowledge, these glycoproteins have not been described as regular serum proteins to date. These patterns were found among 7% of the patients studied (n = 400). Similar bands were not detectable in a reference group of 150 persons without clinical symptoms. The glycoprotein pattern was specific for each individual. It has been shown to differ from cationic immunoglobulin G and from cationic serum protein. By comparison with standard glycoproteins a carbohydrate content of 40 ± 10% is suggested. By lectin immunoassays it was shown that the pattern was caused by the same glycoprotein in each patient and that the oligosaccharides contain sialic acid. When studying well diagnosed patients it could be demonstrated that these glycoprotein patterns appear with acute bacterial infections.

7.11
STRUCTURAL BASIS OF HOST MIMICRY IN THE SURFACE LIPOOLIGOSACCHARIDES OF *NEISSERIA GONORRHOEAE* AND *HAEMOPHILUS DUCREYI*

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Neisseria gonorrhoeae and *Haemophilus ducreyi* are Gram-negative bacteria that cause sexually transmitted disease. Both *N. gonorrhoeae* and *H. ducreyi* primarily infect the mucosal surfaces of the human genital tract and result in gonorrhoea and chancroid, respectively. Gonococcal infection still remains high worldwide, with over 500,000 cases in the United States last year. In the absence of protective antibodies, gonococci can enter the bloodstream, survive and cause secondary infections. Genital ulcers (or chancroid) caused by *H. ducreyi* infection have recently been identified as an independent risk factor in the transmission of human immunodeficiency virus (HIV) and are cause for much concern as this disease is endemic to developing countries and has recently caused several small epidemics in North America and Europe. The virulence of mucosal pathogens is mediated by an array of surface antigens, including outer membrane proteins and glycolipids that could serve as potential vaccine targets. The surface glycolipids or lipooligosaccharides (LOS) consist of a membrane-associated lipid A attached to a much smaller glycan moiety than that found in Gram-negative enteric bacteria.

To determine the structures of these surface LOS, we have developed a comprehensive strategy using a combination of liquid secondary ion mass spectrometry, tandem mass spectrometry, methylation analysis, high pH anion exchange chromatography and NMR. We have determined the LOS structures of a set of pyocin-resistant isogenic mutants from the gonococcal strain 1291 and the first structure from a recent isolate of *H. ducreyi* (strain 35000). These LOS structures suggest a mechanism by which the bacteria can mimic two major groups of glycosphingolipids present in human tissue (paragloboside and P^k), and evade the host immune system as well as attach specific host cell surfaces.

7.12
CHARACTERIZATION OF CELL SURFACE LIPOOLIGOSACCHARIDES FROM A TYPE B AND A NONTYPABLE STRAIN OF *HAEMOPHILUS INFLUENZAE*

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Haemophilus influenzae type b (Hib), an encapsulated gram-negative pathogen, is well known as a leading cause of bacterial meningitis in children. Nontypable strains of *H. influenzae* (NTHI), which do not possess an immunogenic capsular polysaccharide, have only recently been recognised to cause serious middle ear infections in children and lower respiratory disease in adults. The pathogenicity of NTHI strains is mediated more directly by components of the bacterial outer membrane, including proteins and lipooligosaccharides (LOS).

We have investigated the cell surface LOS from NTHI strain 2019 using a combination of mass spectral techniques, methylation analysis, 1D and 2D NMR, and chemical methods. *O*-deacylation and dephosphorylation converted the amphipathic LOS molecule into a species suitable for direct analysis by liquid secondary ion mass spectrometry (LSIMS). Mild acid hydrolysis of the LOS liberated a biantennary hexasaccharide (M_r 1366) as the major oligosaccharide constituent present with a composition of Gal,Glc,Hep,PEA_2 and anhydro-KDO. Reducing terminal anhydro-KDO results from acid-catalyzed elimination of phosphate from C-4 of KDO during the hydrolysis. A terminal lactosyl moiety, Galβ1→4Glcβ1→, has also been identified and may represent a mechanism for host mimicry of tissue glycosphingolipids. The presence of this structure in *Haemophilus* LOS may be useful to the organism for evading the immune system and/or as a mechanism to form closer attachments to host mucosal surfaces. We also have evidence that this lactosyl epitope is extended to form larger oligosaccharides in the LOS of a type b strain of *H. influenzae*.

7.13
IMMUNOGENICITE DU DISIALOGANGLIOSIDE (GD_2) ET ANTICORPS MONOCLONAUX (IgG_3)

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Les disialogangliosides comptent parmi les glycosphingolipides acides les moins immunogènes. Par immunisation de souris A/J avec des cellules d'un neuroblastome humain LAN-1 ou d'un lymphome murin EL-4, trois familles d'anticorps monoclonaux (AcM) murins d'isotype IgG_3, ont été obtenus contre divers épitopes du disialoganglioside GD_2. Cette molécule retrouvée très faiblement et exclusivement dans le cerveau est un marqueur constant des tumeurs neuroectodermiques. La réactivité de ces AcM a été analysée par immunomarquage après chromatographie d'extraits gangliosidiques de mélanome et de neuroblastome sur couche mince (CCM) de silice. Quelques AcM se lient spécifiquement à GD_2 tandis que d'autres relativement plus nombreux reconnaissent en outre un ganglioside alkali-labile dont la migration en CCM avoisine celle du ganglioside GD_3 identifé par l'AcM R24. Une bande semblable, unique, alkali-labile est mise en évidence avec plusiers autres AcM. Dès lors, nous montrons qu'il s'agit d'un epitope associé au GD_2 qui se comporte de la même façon que du GD_2 acétylé chimiquement. La détermination structurale de ce nouveau ganglioside acétylé, le second découvert pas des AcM après le GD_3-9-*O*-acétylé, sera complétée par une étude en R.M.N. et par spectrométrie de masse. L'acétylation des gangliosides tumoraux pourrait contribuer selon nos observations à renforcer leur immunogénicité. Par ailleurs l'étude de la fixation de certains AcM murins (IgG_3) anti-GD_2 qui est de type coopératif révèle une double cinétique d'interaction avec l'antigène et d'association des anticorps entre eux. Des interactions Fab pourraient expliquer ce dernier phénomène et un pouvoir cytotoxique accru des IgG_3 murines. (Soutenu par contrat ARC 6022, FEGEFLUC et LNFLC-44.)

7.14
HUMAN GANGLIOSIDES: STRUCTURE AND SUPPRESSION OF T CELL RESPONSIVENESS

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Mechanisms underlying the cellular immunodeficiency frequently associated with cancer remain poorly understood. One possible cause is tumor cell shedding of immunosuppressive molecules, such as membrane gangliosides. Here we have tested the effects of an extensive series of individual human gangliosides on T cell function.

Ten gangliosides of two major biosynthetic pathways, many previously unstudied and each highly purified by HPLC, were compared for their ability to inhibit human T cell proliferative responses to tetanus toxoid. They are G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D2}, G_{D1a}, G_{T1b}, and G_{Q1b}. Strikingly, each ganglioside, from the simplest to the most complex, had potent inhibitory activity (ID_{90} = 1.5–10.7 μM). Even the most elemental ganglioside carbohydrate structure – one sialic acid linked to a monosaccharide (G_{M4}) – strongly inhibits T cell proliferative responses to tetanus toxoid (ID_{90} = 1.5 μM).

A complex interplay exists between oligosaccharide structure and immunosuppressive activity of these glycolipids, with sialic acid being critical to potent activity. Immunosuppression is *(i)* almost abolished by total desialylation (formolysis), *(ii)* reduced by partial alteration (lactone formation), *(iii* generally increased by higher numbers of sialic acid residues/molecule, and *(iv)* most potent in gangliosides containing a terminal sialic acid. Finally, overall oligosaccharide size also influences activity, as some neutral glycophingolipids retain measurable imunosuppressive activity.

These results were obtained in an assay system designed to reproduce the plasma-free interstitial fluid microenvironment surrounding tumor cells – conditions which were associated with highest ganglioside binding to PBMC and maximal inhibition of T cell responses. Since virtually complete inhibition occurs with the binding of relatively few ($\sim 10^7$) ganglioside molecules/cell, the results support the interesting hypothesis that in the tumour cell microenvironment, ganglioside molecules actively shed in high amounts from the surface of tumor cells may exert very potent immunoregulatory effects upon infiltrating host leukocytes, thereby aiding the proliferating tumor to escape host immune defences. (Supported by NCI grant CA42361.)

7.15
ISLET CELL GLYCOCONJUGATES: POTENTIAL TARGETS IN THE AUTOIMMUNITY OF TYPE I DIABETES MELLITUS

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Pancreatic islet cell glycoconjugates are putative target autoantigen(s) in Type I (insulin-dependent) diabetes mellitus (Diabetes 1985; 34:617). Towards characterisation of pathogenetically relevant islet cell autoantigens (ICAgs), we generated a novel series of murine monoclonal islet cell antibodies (mICAbs I-45, -51, -52 and -39) using human insulinoma homogenate as immunogen. Differentiation antigens recognised by these mICAbs displayed varied cytological distribution (panislet or peripheral only). mICAb I-45 reacted with all the endocrine cell subsets of the pancreatic islet, similar to the reactivity of islet cell autoantibody (hICAb) positive human sera of Type I diabetes. Preexposure to 100°C for 1 hour did not significantly alter the immunoreactivity of islet antigens recognised by hICAb and mICAb I-39, thus demonstrating the extraordinary heat stability of the corresponding epitopes; those recognised by mICAb I-45 were less heat stable. Immunoreactivity of I-45 epitope, like the hICAb epitope, was preserved on pre-treatment with pronase and trypsin, and destroyed by periodate oxidation. Islet cells share I-45 antigen/epitopes with other neuroendocrine cells viz. anterior pituitary, adrenal medulla, gut endocrine cells. A mICAb I-45 EL1SA for detection of the corresponding antigen has been developed.

While the autoantigenic hICAb epitope is evolutionarily well conserved and is islet restricted, mICAb I-45 epitope is neuroendocrine differentiation related. These findings do not rule out the possibility that, both the autoantigenic and monoclonal islet cell antibody recognised epitopes can be harboured on the same islet cell antigen molecule(s) (e.g. multiple carbohydrate determinants on identical protein backbone).

7.16
CONTROL OF T CELL RESPONSES TO CARTILAGE PROTEOGLYCANS

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Rheumatoid arthritis (RA) is a peripheral symmetrical destructive polyarthritis, frequently accompanied by multisystem involvement and more common in females. Part of the destruction may relate to immune responses to cartilage tissue antigens. To determine whether T cell responses to cartilage proteoglycans (PGs) are controlled by MHC genes, proliferation assays were used to compare primed popliteal lymph node responses in BALB and B10 congenic strains of mice.

There was a significant effect of MHC haplotype with responses by $H\text{-}2^k$ mice greater than or equal to $H\text{-}2^d$ and $H\text{-}2^d$ were greater than $H\text{-}2^b$.

Limiting dilution assays in BALB/c mice (the only strain susceptible to the model of PG-induced arthritis) showed that the proportion of PG-responsive T lymphocytes in female BALB/c mice was 45% lower than in male primed popliteal nodes. Human PG-responsive A^d-restricted T lymphotyctes responded well to murine costal cartilage PGs (A1 fraction). These results show that both MHC haplotype and sex may determine murine T cell responsiveness to PGs, and that PGs are autoantigenic for murine T lymphocytes. In humans, these mechanisms may be important in the pathogenesis of RA.

7.17
GBS-TOXIN; FROM NEONATAL PATHOGEN TO ANTI-TUMOR AGENT

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Group B streptococcus (GBS) isolated from human neonates diagnosed with sepsis and respiratory distress ('early onset disease'), produces a polysaccharide-exotoxin (GBS-Toxin) which, when infused in sheep, will induce similar pathophysiology as seen in the diseased neonate. Histological studies showed that GBS-Toxin induces a strong inflammatory response with pulmonary sequestration of granulocytes and extensive capillary endothelial damage. Elastase and lysosomal enzymes can be demonstrated in the lymph and plasma as a consequence of GBS-Toxin infusion. The presence of these enzymes is assumed to be related to the observed damage to the pulmonary vasculature. Immunohistochemistry showed GBS-Toxin bound specifically to the lung capillary endothelium.

The susceptibility to GBS in *humans* is limited in time to about four days postpartum, which suggested that the receptors for GBS-Toxin are embryonic in nature and present only on proliferating capillary endothelium. GBS is rarely a human pathogen after the newborn period.

We now report that the capillary vasculature obtained from human large, small and squamous cell carcinomas contains receptors for GBS-Toxin. Also, we found that GBS-Toxin when infused intravenously at picomole doses induces tumor necrosis and hemorrhage, in human tumors propagated in nude mice. Mice carrying human tumors grown to 0.3 ccm were infused i.v. with GBS-Toxin three times a week for three weeks. The actual size of necrosed tumors was reduced 50% at 2.5 and 25% at 0.25 picomole doses when compared to animals infused with dextran as a control. The induced hemorrhage, tumor necrosis, greatly reduced tumor growth rate, and lack of toxicity to mature vasculature and other tissue as a result of infusion of GBS-Toxin, suggests that GBS-Toxin can be utilized as an anti-tumor agent in human cancer therapy.

7.18
STRUCTURE-FUNCTION RELATION OF SULFATED XYLANS (SP54) IN INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTIVITY

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A sulfated polyxylan of plant origin and licensed as a heparin substitute (SP54, bene-Arzneimittel GMBH, Munich, FRG; MWtapp. 5000) inhibits the infection of T lymphoctes by HIV. It mimics the biological actions of the heparin/heparan sulfate (H/HS) GAGS and is used therapeutically in various disorders. SP54 is composed of 1,4-beta-linked xylopyranose monomers that are largely disulfated (DiSX). The xylan is inhomogeneous in size and in uniformity of sulfation; it contains 1 glucuronic acid in 10 sugars. Understanding the molecular basis of the multifunctional activity of such sulfated oligosaccharides (S-oligoS) could enhance their usefulness. In pursuit of this goal, SP54 was size-fractionated by open liquid chromatography of 10 mg on Biogel P10 (0.6 cm i.d. × 200 cm h; 0.5 M NH_4HCO_3 eluant, 3 ml/hr, 27 hr; standards: H, HS, S-beta cyclodextrin). A post-column assay was based on the hypochromism evoked in solutions of methylene blue or acridine orange by 5–10 μl aliquots of the fractions (detection 1 nmol DiSX). Recovery based on DiSX content of fractions from 5 preparations was 76%. Fractions were lyophilized 36 hours, removing eluant. Sixteen MWt. components, from 30000 to <1000 KDa were prepared by combining the dried peak fractions from 5 runs in distilled water (GDW) and relyophilizing. Dried components were weighed; recovery of SP54 based on the weights was 115%. S-oligoS were dissolved in 1 ml GDW and passed through a 0.22 μm filter (Millex-GV4) for assay of their anti-HIV activity *in vitro*. Quantitative analysis of DiSX-like content of purified components revealed a non-DiSX moiety(s) that comprised at least 25% of SP54; 86% of 2 components was composed of this unknown structure.

Twelve M.Wt. components of SP54 were assayed with comparison for *in vitro* inhibition of the killing of a T4-lymphocyte cell line (CEM-IW) by HIV (National Cancer Institute). Dose-response relations showed a biphasic dependence of antiviral activity on the MWtapp. Log of the 50% effective dose (log EC50, μg DiSX/ml) was −0.2 for components of MWtapp. 23, 16, and 17 KDa and −0.3 for those of MWtapp. 5–6 KDa. The log EC50 then rose sharply to 1.7 for components of MWtapp. between 5 and 2.5 KDa, except for the component at MWtapp. 3000 (log EC50 0.5). This decrease in EC50 indicates the presence of a more potent S-oligoS. Differences in EC50 were more pronounced when based on molecular molar concentration. Consideration of the data on potency vs mass or MWtapp. suggests the presence of at least two S-oligoS structures that have anti-HIV activity in SP54. One, contained in the high MWt. highly active components, appears to be about the size of an eicosaccharide; the other is smaller, possibly an octa- or nonomer.

7.19
GLYCANS ON THE ENVELOPE OF GLYCOPROTEIN GP120 FROM THE AIDS VIRUS: TARGET FOR IMMUNE THERAPY?

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The role of carbohydrates in the function of the human immunodeficiency virus (HIV) has been studied for several years. Since the realization that the glycogen antigens encoded by HIV induced antibodies in AIDS patients (1), the possibility of immune therapy through such approaches has been investigated. Oligosaccharide-mediated interactions of gp120 have been reported for both serum and macrophage-associated human lectins (2). Polyclonal antibodies raised against a yeast mannan successfully inhibited HIV infection in vitro (3). While the oligosaccharide structures on gp120 were shown to be of the high mannose, complex and hybrid types previously found in mammalian cells (4, 5), unusual carbohydrate structures have also been reported that could serve as possible targets for immune therapy (6).

To determine if antibodies are made against the glycans on gp120, studies are underway in which radiolabeled N-linked glycans from gp120 are incubated with polyclonal antisera from AIDS patients. If the glycan preparation is immunoreactive, subsequent studies will be the characterization of the glycans by hydrazinolysis, reducing end labeling and HPLC.

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7.20
SEQUENTIAL DESIALYLATION OF GLYCOPHORIN IN THE *IN VIVO* AGING OF ERYTHROCYTES

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The evidence for our glycophorin hypothesis for the aging and clearance of mammalian effete RBC has already been presented and substantiated by flow cytometric procedures (FCP). (Glycoconjugate J. 1988; 5:355–356). More recently, using FCP, we have been able to monitor the aging process of human RBC at the cellular level, suggesting the following sequence of events. a) RBC decrease with size due to shedding of vesicles. b) The smallest more spherical RBC appear to be more susceptible to physiological desialylation. c) The sialyl 2-6 N-acetylgalactosamine residues then are removed, followed by d) The sialyl 2-3 galactosyl residues to expose galactose β1-3 N-acetylgalactosaminyl residues which are recognized by macrophages (Aminoff, Blood Cells, 1988, *14*, 229–257). e) At this stage also it appears that the senescent RBC pick up auto-immune IgG & IgM.

This conclusion is based on the presumption that RBC cell size progressively decreases with *in vivo* age of RBC. This we have succeeded in demonstrating using the rat as the experimental animal model, and PKH26, a commercially available fluorescent probe used to label RBC. The cell size distribution of the labelled and unlabelled RBC were monitored in a FACScan flow cytometer over a period of 70 days. The number of fluorescent cells in circulation decreased with time and were almost completely eliminated at the end of the experiment. From the change in numbers of fluorescent RBC of a given cell size over a period

of time it was possible to determine the rate of turnover of the cells of a given size and of the population of cells as a whole. The rate is not constant, and we are in the process of determining the relationship of the biological to the chronological clock.

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7.21

GENERATION OF MURINE MONOCLONAL ANTIBODIES TO GANGLIO-SERIES GANGLIOSIDES

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Monoclonal antibodies (MAbs) against gangliosides have been shown to be powerful reagents not only in analyzing biological functions of gangliosides on cell surface membranes, but also in the diagnosis and treatment of cancer patients. It is, however, still difficult to routinely generate murine MAbs to gangliosides.

Recently, we have compared the immunogenicities of gangliosides in various mice strains and have found that they were different in mice strains and that C3H/HeN mice showed the highest antibody response to gangliosides among them. Based on these findings, we succeeded in generating murine MAbs to ganglio-series gangliosides by immunizing C3H/HeN mice instead of BALB/c or C57BL/6 with purified gangliosides. After each ganglioside attached to *Salmonella Minnesota* R595 was immunized in C3H/HeN mice several times, the spleen cells were fused with PAI (BALB/c-derived myeloma cells) by a routine procedure. We established hybridomas producing MAbs to gangliosides including GM4, GM3, GM3(NeuGc), GM1, GD3, GD3(NeuGc-NeuGc-), O-Ac-GD3, GD2, GD1b, GT1b, and GQ1b, in which sialic acid residues were N-acetylneuraminic acids unless otherwise noted. Several MAbs showed restricted binding specificities, detecting only the ganglioside that was used as immunogen. This is the first report on establishment of murine MAbs to GM3(NeuGc), GM1, GD3(NeuGc-NeuGc-), and GT1b. Studies on the reactivities of these MAbs with tumor cells are now in progress. We are also trying to establish MAbs to other gangliosides such as GM2, GM2(NeuGc), GM1(NeuGc), GD1a, and GT1a. This method may be generally applicable to the generation of murine MAbs to other glycolipids.

7.22

SYNTHESES AND BIOLOGICAL SIGNIFICANCE OF SOME SIALIC ACID GLYCOCONJUGATES

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Sialic acids on glycoproteins or glycolipids constitute receptors for the adherence of bacteria, toxins and viruses. In order to probe the binding interactions between the sialic acid ligands and their receptors, a series of model N-acetylneuraminic acid glycosides containing aglycons of different length and hydrophilicities were synthesized using Koenigs-Knorr and phase transfer catalysis methodologies. Proteins such as bovine serum albumin and tetanus toxoid were used as carriers for the N-acetyl-neuraminic acid conjugates. The immunogenicities of the protein conjugates were evaluated in rabbits. Specific antibodies of the IgG class were obtained. Water-soluble N-acetylneuraminic acid copolymers were also synthesized and were used as coating antigens in ELISA. Inhibition of binding between the rabbit IgG and the sialic acid copolymers with a number of sialic acid derivatives established the functional group requirements for the binding interactions.

Sialic acid, sialyl α-(2 $\rightarrow$ 3)- and α-(2 $\rightarrow$ 6)-lactose protein conjugates together with their corresponding copolymers were also prepared and evaluated as inhibitors of the hemagglutination of influenza A virus (H3N2) with chick red blood cells. Interestingly, the sialic acid copolymers with a spacer of 11 A were the best inhibitors of hemagglutination. The above conjugates were also shown to induce interferon in mice. A new strategy was therefore invoked as a potential therapy against viral infections. However, care should be taken to avoid multivalent sialic acid forms containing protein as carriers since these were immunogenic. The relative binding potency of the sialic acid copolymers with controlled valency has been evaluated. The results clearly demonstrated the beneficial binding cooperativity of multiple binding sites within the same molecules.

7.23

THE STRUCTURE OF A NEURAL SPECIFIC CARBOHYDRATE EPITOPE OF HORSERADISH PEROXIDASE (HRP) RECOGNIZED BY ANTI-HRP ANTISERUM

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Antiserum raised against horseradish peroxidase (HRP) specifically recognizes a neural specific carbohydrate antigen in *Drosophila* and other insects. Although it has been widely used as a neural specific probe, the biochemical nature of the epitope it recognizes is still unknown. Therefore, we determined the structure of the major HRP carbohydrate unit, which is recognized by anti-HRP antiserum and is thought to share the epitope with a neural specific carbohydrate antigen. The epitopic structure of this major oligosaccharide was also deduced.

HRP sugar chains released from HRP glycopeptides by almond glycopeptidase A digestion were subjected to pyridylamination. Pyridylamino (PA) oligosaccharides, which reacted with anti-HRP antiserum to the same extent as glycopeptides and oligosaccharides from HRP, were fractionated by reverse-phase HPLC. The major fraction, which comprised about 80% of the total sugars, reacted strongly with anti-HRP antiserum. The carbohydrate structure of this fraction was determined by sugar composition analysis by HPLC and 600-MHz ^{1}H-NMR spectroscopy as follows: Manα1$\rightarrow$6(Manα1$\rightarrow$3)(Xylβ1$\rightarrow$2)Manβ1$\rightarrow$4 GlcNAcβ1$\rightarrow$4(Fucα1$\rightarrow$3)PA-GlcNAc.

The reactivity of the antiserum with the modified derivatives of this major fraction was analysed to clarify the minimum structure required to react with the antiserum. The derivatives lacking α1$\rightarrow$6-linked mannose or α1$\rightarrow$3-linked fucose lost their reactivity, indicating their requirement in epitope reactivity. According to the molecular model of the oligosaccharide from pineapple stem bromelain which is similar to the major fraction from HRP (Bouwstra, J.B. *et al.* (1990) Eur. J. Biochem. **190**, 113), α1$\rightarrow$6-linked mannose and α1$\rightarrow$3-linked fucose appear to be located on the same side of the sugar chain. This position of these two sugar residues may contribute their recognition by the antibody.

Kurosaka, A. *et al.* (1991) J. Biol. Chem. in press.

7.24

ESSENTIAL STRUCTURE OF Tn ANTIGEN

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A murine monoclonal antibody (MLS 128), directed to Tn antigen, was obtained by immunizing mice with human colonic cancer cells (LS 180). MLS 128 bound to mucin glycopeptides from LS 180 cells and their asialoforms to the same extent as well as to ovine submaxillary mucin (OSM) and asialo OSM. To determine the epitopic structure for MLS 128, OSM was digested with various proteases and the digests were fractionated by immunoaffinity column chromatography and HPLC.

From the tryptic digest, a glycopeptide, GP-I, was separated from other glycopeptides by the immunoaffinity column. GP-I was strongly immunoreactive whereas other glycopeptides were poorly immunoreactive. On treatment with V8 protease, GP-I was converted to two glycopeptides, one with poor reactivity and the other with intermediate reactivity.

From the thermolysin digest, the smallest fragment, GP-II, was isolated, which was as strongly immunoreactive as GP-I. GP-II corresponded to a part of GP-I, its sequence being Leu-Ser*-Glu-Ser*-Thr*-Thr*-Gln-Leu-Pro-Gly, where asterisks denote amino acids to which an GalNAc residue is attached. Other anti-Tn monoclonal antibodies, NCC-LU-35 and CA 3239, showed essentially the same reactivity to these glycopeptides as MLS 128 did. The glycopeptides with poor immunoreactivity contained various GalNAc containing structures, such as GalNAc-Ser, GalNAc-Thr, GalNAc-Ser-(GalNAc)-Ser, GalNAc-Thr-(GalNAc)-Thr and GalNAc-Thr-(GalNAc)-Ser. These results indicate that a glycopeptide including a cluster structure, Ser*-Thr*-Thr*, is an essential part of the epitope recognized by anti-Tn antibodies.

Occurrence of other glycoproteins reactive with anti-Tn antibodies and a structure common to these glycoproteins will be discussed.

7.25

SPECIFICITY STUDIES OF MONOCLONAL ANTIBODIES AGAINST A MUCIN-TYPE GLYCOPROTEIN

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Mouse monoclonal IgM antibodies were prepared towards a mucin-type glycoprotein, epiglycanin which appears on the surface of the mouse mammary carcinoma cell line TA3-Ha. The specificities of 12 of the anti-epiglycanin antibodies were studied by an enzyme-linked competitive binding assay and by direct binding of antibodies to test substances immobilized on HPTLC-plates.

The antigenic activity of epiglycanin with respect to all the antibodies tested was reduced significantly when it was subjected to either periodate oxidation (10 mM $NaIO_4$, 30 min at 4°C), treatment with O-glycanase, TPCK-trypsin or pronase. Treatment with neuraminidase increased the antigenic activity of epiglycanin (to 140–280%) when tested with 9 of the 12 antibodies.

The antibodies cross-reacted less than 0.1% with bovine submaxillary mucin and 0.2–1.6% with intestinal mucin from germfree rats. The cross-reactivities of the corresponding asialo mucins were higher (2–50 times) for all antibodies. None of the antibodies showed detectable binding to a selection of GM, GD and GT gangliosides immobilized on HPTLC-plates. *Arachis hypogaea* lectin competed with the antibodies for binding to epiglycanin.

It could be concluded that the disaccharide Galβ-(1→3)-GalNAc, which is the most common disaccharide on epiglycanin forms an integral part of the epitope recognized by all the antibodies. The inhibitory activity of the disaccharide Galβ-(1→3)-GalNAc itself, however, was 10000 to 250000 times (by weight) less than that of epiglycanin for the antibodies tested. Therefore other, still poorly understood features of the macromolecular glycoprotein structure of epiglycanin are essential for high affinity (specific) binding of the family of closely related, but distinguishable antibodies.

7.26

EPITOPE SPECIFICITY OF HEMAGGLUTINATING ANTI-B Mabs DISCRIMINATING B, B-weak AND B-3 RBC. USE OF SYNTHETIC GLYCOCONJUGATES

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In order to identify molecular differences of subgroup B erythrocytes fine epitope specificity of 10 monoclonal antibodies (Mabs) was studied. Three methods were used: i) inhibition of Mabs binding to natural antigen by synthetic oligosaccharides (OS) and their water-soluble polyacrylamide conjugates, ii) direct Mabs binding to the series of OS-polyacrylamide conjugates (differing in carbohydrate epitope density), iii) direct Mabs binding to the affinity sorbents. All Mabs studied prefer trisaccharide B-determinant independently of their ability to discriminate serological subgroups of blood group B erythrocytes (B, B-weak, B-3); four subgroup agglutinating Mabs bind tetrasaccharide B (type 3) unfound in human RBC. We also observed unusual inhibiting activity of Fuc1-4GlcNAc and Fuc1-6GlcNAc disaccharides towards 8 of 10 anti-B Mabs, 3 anti-A Mabs studied were not inhibited by these disaccharides.

S8. CARBOHYDRATES IN CANCER AND DEVELOPMENT/HYDRATES DE CARBONE: CANCER ET DÉVELOPPEMENT

8.1

SERUM TEST OF HEPATOCELLULAR CARCINOMA

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Alterations in the carbohydrate structures of glycoproteins found in various tumors are considered to be the basis of abnormal social behaviors of tumor cells such as invasion into surrounding tissues and metastasis. Comparative studies of the sugar chains of glycoproteins produced by transformed *versus* normal cells therefore provide useful information for the diagnosis, prognosis as well as immunotherapy of tumors.

Study of the sugar chains of transferrin samples, purified from sera of patients with hepatocellular carcinoma and of healthy individuals, revealed that extensively altered glycosylation is commonly found in the hepatoma transferrins. The alteration is quite various, including the expression of highly branched sugar chains as well as Galβ1→4

(Fucα1→3)GlcNAcβ1→ and Neu5Acα2→3Galβ1→4GlcNAcβ1→ outer chains (1). Among the multiple alterations, we picked up the increase of fucosylated sugar chains for the development of serum test of hepatocellular carcinoma. These sugar chains interact with *Aleuria aurantia* lectin (AAL) (2). Successful large scale production of AAL in *E. coli* by genetic engineering opened a way to use this lectin as a reagent to detect fucosylated glycoproteins (3). Significantly higher proportion of transferrins in the sera of patients with hepatocellular carcinoma and cirrhosis bound to an AAL-Sepharose column than those of patients with hepatitis and healthy individuals. This result indicated that serum transferrin can be used as a good diagnostic marker of hepatocellular carcinoma.

(1) K. Yamashita *et al.*: *J. Biol. Chem.*, **264**, 2415 (1989); (2) K. Yamashita *et al.*: *J. Biol. Chem.*, **260**, 4688 (1985); (3) F. Fukumori *et al.*: *J. Biochem.*, **107**, 190 (1990)

8.2
FUNCTIONAL ROLE OF CELL SURFACE CARBOHYDRATES IN ONTOGENESIS AND ONCOGENESIS

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A functional role of cell surface glycoconjugates has been suggested by the remarkable changes closely associated with stages of embryogenesis, as well as oncogenic transformation and tumor progression. Our recent studies have been focused on two biological processes, as summarized below, related to steps of embryogenesis and degree of malignancy (e.g., metastatic potential) of tumor cells.

Involvement of CHOs in cell-cell and cell-matrix interactions related to specific cell adhesion and cell motility. Our observations on CHO-CHO interaction have been extended to cell spreading and motility, with various combinations of glycosphingolipids as a model. In addition Le^x-Le^x and GM3-Gg3, we have observed interactions between H-H, Le^y-H, $IV^3NeuAcnLc_4$-Gg3, GalCer-sulfatide, and GM3-Gb3 (weak). Not only adhesion, but also cell spreading and cell motility were promoted by Le^x-Le^x and GM3-Gg3 interaction, and the adhesive effect was synergistically enhanced by co-presence of fibronectin and laminin. This model system has been applied in studies of cell recognition during various stages of ontogenesis (e.g., compaction, implantation of embryo in endometrium), as well as tumor cell adhesion on endothelium, which initiates tumor cell metastasis.

Involvement of gangliosides and their catabolites in transmembrane signaling. Gangliosides (particularly GM3) and their catabolites (e.g., lyso-GM3, psychosine, N,N-dimethylsphingosine) are known to produce striking effects on a variety of membrane-associated protein kinases which play essential roles in transmembrane signaling, e.g., EGF and PDGF receptor kinases and protein kinase C. These studies have been extended to tumor cell-induced platelet aggregation, which is triggered by a kinase involved in phosphorylation of p40K. Since such aggregation is an essential step for initiation of metastasis, the observed strong inhibitory effect of ganglioside catabolites suggests a vital role of these compounds in regulation of tumor progression.

8.3
DIAGNOSTIC AND PROGNOSTIC SIGNIFICANCE FOR EARLY STAGE LUNG CANCER BY CYTOLOGICAL SPUTUM EXAMINATION WITH ANTI-CARBOHYDRATE MONOCLONAL ANTIBODIES

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Cytological examination of human sputum samples was performed with a panel of anti-carbohydrate monoclonal antibodies (MAbs). The MAbs used (and their epitopes) were SH-1 (Le^x), SH-2 (dimeric Le^x), SNH-3 (sialyl Le^x), AH-6 (Le^y), TKH-2 (sialyl-Tn), TKH-5 (fucosyl-GM1), TKH-6 (Tn) and CA3-F4 (Le a). MAbs AH-6, TKH-2, CA3-F4 and SH-1 defined antigens were highly expressed in a majority of paraffin-embedded human lung cancer tissues (30 squamous cell carcinomas, 30 adenocarcinomas, and 27 small cell carcinomas). The MAbs showing high expression in tissues were AH-6, TKH-2, SNH-3, SH-1 and CA3-F4. The sputum samples from cancer patients, as well as high-risk group subjects (at least 20 years of smoking history at one pack of cigarettes per day) were collected in Sacomano's solution, every morning for three days. After removal of mucus, cells were collected and smeared for immunohistological staining. Papanicolaou's staining was also performed for grading malignancies. 57 cases were found to have malignant cells, and 76 subjects were classified as borderline malignant. High ratios of positively-stained malignant cells were observed with AH-6, TKH-2, SNH-3, CA3-F4 and SH-1: 90%, 83%, 83%, 68% and 35%, respectively. It was comparatively easier to find malignant cells by immunostaining than by Papanicolaou's staining. The borderline cases were followed up for 18 months by repetitive cytological studies as well as chest x-ray examinations. Seven of those borderline individuals were found to have cancer during the follow-up period. Sputum samples from these individuals at least three months prior to the detection of the cancer showed positive staining with most of the MAbs. Subjects who had no positive staining in any of those MAbs remained cancer negative. These findings indicate that immunohistological studies of sputum samples with anti-carbohydrate MAbs could be useful for early diagnosis of lung cancer.

8.4
GANGLIOSIDE GM3 PROMOTES MEGAKARYOCYTIC DIFFERENTIATION AND PLATELET PRODUCTION

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Megakaryocytes (MgK) are nucleated bone marrow cells that give rise to platelets upon final maturation. Despite the recent accumulation of substantial information about platelet biochemistry and function, the molecular mechanism of MgK differentiation and platelet production remains unknown, because of the difficulty in collecting a sufficient number of MgK from bone marrow, or culturing them on a long-term basis. Recently human megakaryoblastic cell line CMK cells were established. In contrast to human MgK cell lines established so far, CMK cells are capable of differentiating into mature MgK cells, producing platelets *in vitro*.

Gangliosides of CMK are composed of sialosyllactosylceramides (GM3, GD3, GT3), sialosylparagloboside (SPG), and a branched lacto-series ganglioside, disialosyl I (DSI). Gangliosides of human platelets were composed almost exclusively of GM3; SPG and GD3 were minor ganglioside components. When CMK cells were induced to differentiate into mature MgK cells by TPA, a remarkable increase (3 times) of GM3 was observed.

When ganglioside GM3 was exogenously added to culture media, clear MgK differentiation of CMK cells was observed morphologically, in parallel with increased expression of GPIIb/IIIa and GPIb. Production of platelets in the differentiation-induced cells by GM3 was also observed by electron microscopy. These results strongly suggest that ganglioside GM3 plays an essential role in the promotion of MgK differentiation and platelet production.

8.5

SPECIFIC INTERACTION BETWEEN Le^y AND H AS A POSSIBLE BASIS FOR TROPHECTODERM-ENDOMETRIUM RECOGNITION DURING IMPLANTATION

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Endometrium "receptivity" to blastocyst attachment is associated with changes in the glycoprotein composition of the epithelium, the surface charge of the apical glycocalyx, and the profile of glycoproteins secreted into the luminal fluid. The early embryo also undergoes a complex program of glycosylation changes that prepare it for implantation. Previous studies by Lindenberg et al. (1) have implicated H type 1 chain, which is highly expressed on mouse endometrium, as a ligand for trophectoderm adhesion during implantation. Recognition molecules complementary to cell surface carbohydrates have been assumed to be proteins, however an alternative possibility has been proposed that recognition molecules for carbohydrates include carbohydrates: e.g., Le^x to Le^x (2) and GM3 to Gg3 (3). We now report a specific interaction between H structure and Le^y, a prominent differentiation antigen of mouse trophectoderm. Le^y glycolipid was prepared by enzymatic fucosylation of Le^x. Le^y liposomes labeled with ^{14}C-cholesterol were incubated with plastic wells coated with increasing quantities of various glycolipids. Le^y liposomes bound to both H type 1 and type 2 chain, but not to Le^y, Le^x, or paragloboside. In contrast, Le^x liposomes bound to Le^x, but not to Le^y or H. The strength of Le^y to H interaction was highly density dependent and was inhibited by EDTA. We hypothesize that Le^y structures on trophectoderm interact with H structures on uterine endometrium to initiate the implantation process. Carbohydrate-carbohydrate interactions may supplement other types of cell adhesion and provide an important mechanism for regulating cell recognition during development. This work was supported by NIH grant OIG CA42505 (SH) and by funds from the Biomembrane Institute (BF).

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8.6

IMMUNOHISTOCHEMICAL STUDY OF BLOOD GROUP ABO CARBOHYDRATES AND GLYCOSYLTRANSFERASES IN ORAL CARCINOMAS

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Oral carcinomas show a marked change of cell surface glycosylation resulting in loss of histo-blood group A carbohydrate antigens. In normal stratified epithelium of the oral mucosa, the distribution of histo-blood group ABO carbohydrate antigens and the corresponding A/B glycosyltransferase protein involved in the biosynthesis were found to coexpress. Thus, indicating that the stepwise build-up of carbohydrate antigens in this tissue may be directly gene regulated.

In order to gain insight into the basis of the biosynthetic regulation of aberrant glycosylation in carcinomas we have correlated the expression of the carbohydrate ABH antigens with that of the A/B glycosyltransferases in oral carcinomas by immunohistology. Monoclonal antibodies to the A carbohydrates and to the A/B transferase protein were used in indirect immunofluorescence assay of frozen acetone-fixed sections of oral squamous cell carcinomas including adjacent normal epithelium and occasionally dysplastic areas from 10 patients all belonging to blood group A.

In normal areas A carbohydrate and A/B glycosyltransferase antigens were distributed similarly, i.e. present in mature cell layers and absent from immature cell layers as previously shown. Generally, the A carbohydrate antigens were lost in dysplastic and carcinoma areas, and this was followed by a loss of A/B glycosyltransferase enzyme. In areas where expression of A carbohydrate antigens was found co-expression of the A/B glycosyltransferase enzyme was observed. These findings indicate that the deletion of the histo-blood group A carbohydrate antigen is a result of decreased expression of the glycosyltransferase protein.

8.7

STRUCTURAL CHARACTERIZATION, TISSUE DISTRIBUTION, AND P-BLOOD GROUP STATUS OF THE X2-GLYCOLIPID AND ITS EXTENDED FORMS

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The presence of a second P-antigen has been suggested since erythrocytes from a p-individual lacking pk(CTH) and P(gb4) antigens, showed agglutination with anti-P-antisera (Marcus et al. (1981). The recent identification of the X2 glycolipid could offer a possible structural basis for this P-like antigen activity in p individuals (Kannagi et al. 1982). Both the X2 and its sialylated form are present in very small quantities on human erythrocytes, but their tissue distribution is unknown. In order to clarify these issues, we isolated the compounds from human whole blood and raised a monoclonal antibody (TH2) with monospecificity for the GalNacβ1-3Galβ1-4GlcNAc epitope. Structural characterization of the X2- and sialosyl X2 glycolipids was obtained by positive ion fast atom bombardment–mass spectroscopy and methylation analysis, supplemented with proton nuclear magnetic resonance spectroscopy for X2, which confirms the structure GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer. X2, sialosyl-X2, and in many cases extended forms, were found in all of nine normal tissues tested, in leucocytes, and in a large number of tumours as well. X2 was identified by MoAb TH2 in glycolipid extracts of erythrocytes from a p-type person. Based on the previous observation that X2 exhibit P-like blood group activity the presence of X2 in the p blood could provide the basis for P-like activity in p individuals. Whether the X2 epitope is in fact genetically regulated by the P gene defined glycosyltransferase is yet unknown, but may be clarified using the MoAb TH2 on pk blood group erythrocytes.

8.8

ANTIMETABOLITE-INDUCED CHANGES IN CELLULAR GLYCOCONJUGATES

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Antimetabolites are able to interfere with nucleotide metabolism, thus inhibiting RNA/DNA synthesis, which can lead to cell death. An

additional mechanism of action of antimetabolites, in non-lethal concentrations, might be interference with the glycosylation of the cell-surface of target and non-target cells [1]. For a variety of cellular processes a correct glycosylation has been reported to be of great importance. In order to study possible changes in glycosylation induced by drugs a rapid screening method was developed. Using this method, large changes in metabolically incorporated [^{3}H]-labeled monosaccharides were found after exposure of L1210 murine leukemia cells to low concentrations of the antimetabolites arabinofuranosylcytosine (AraC), methotrexate (MTX), 5-fluorouracil (5FU), and 6-mercaptopurine (6MP), but not after 6-thioguanine (6TG) exposure. The incorporated radioactivity per 10^{6} cells, relative to the control, differed per antimetabolite, and was dependent on the monosaccharide used and the seeding cell density. These studies further substantiate the supposition that antimetabolites can induce changes in glycosylation.

Besides molecular changes, morphological changes were observed. Using DNA-flow cytometry, the cell-cycle appeared to be affected by 5FU, MTX (S-phase arrest), AraC and 6TG (G_2-arrest). Yet the large differences in incorporation of radioactivity observed for antimetabolites that caused the same type of cell-cycle arrest, indicate that cell cycle effects cannot be the only source of the induced changes in glycosylation. Another possible mechanism of action could be interference with nucleotide-sugar metabolism. However, there was no correlation between the type of nucleotide-analog used and the effects on radiolabeled monosaccharides incorporated via corresponding nucleotide-sugars. This observation excludes direct effects of the antimetabolites on their analog nucleotide-sugars.

[1] Peters, G.J., Pinedo, H.M., Ferwerda, W., De Graaf, T.W., and Van Dijk, W. Eur. J. Cancer, 26: 516–523, 1990.

8.9
PARALLEL CHANGES IN THE BLOOD LEVELS OF ABNORMALLY-FUCOSYLATED HAPTOGLOBIN AND ALPHA (1→3)-L-FUCOSYL TRANSFERASE IN RELATIONSHIP TO TUMOUR BURDEN

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A lot of evidence suggests that fucose metabolism is disturbed in cancer and that this change is related to the progression of the disease. A previous study has shown that there are high levels of abnormally-fucosylated forms of haptoglobin (FHp) in the blood of cancer patients which are related to tumour burden (Br. J. Cancer, 1987, *56*, 605). Furthermore, the expression of these molecules was associated with the amount of total protein-bound fucose in the blood but not the total haptoglobin. The cause of this change is unknown. In this study the blood levels of FHp and alpha (1→3)-L-fucosyl transferase (FT) have been compared in 46 serial specimens taken from 9 women with ovarian cancer and 6 women with breast cancer who were undergoing chemotherapy. The amount of FHp present was semi-quantitatively assessed by extracting sera with the fucose-binding lectin, *Lotus tetragonolobus*, and separating the extracted fucoproteins by SDS/PAGE. FHp was detected by silver staining or Western blotting. FT activity was measured by incubating sera with N-acetyl-2′-O-methyllactosamine and GDP-(^{14}C) fucose, and separating the labelled product by paper chromatography. In some specimens, the alpha-L-fucosidase activity was also measured using p-nitrophenyl-alpha-L-fucoside. The blood levels of FHp and FT increased serially in all women with progressive disease and decreased serially in all women who had a complete response to therapy or stable disease. The correlation between the changes in FHp and FT was very significant ($P < 0.0001$, χ^2 test). There was no correlation between FHp levels and blood fucosidase activity ($P > 0.05$). These results suggest that FHp is formed as a result of the increased FT activity, which adds extra fucose in a 1→3 position to external N-acetylglucosamine residues on the carbohydrate side-chains. A number of different sources (liver, bone marrow and tumour) could contribute to FT in the blood. Further work is required to establish the precise mechanisms involved.

8.10
PEANUT AGGLUTININ-BINDING GLYCOPROTEINS FROM HUMAN GASTRIC CARCINOMA: IDENTIFICATION OF THE CORE PROTEINS BY MOLECULAR CLONING

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Peanut agglutinin (PNA)-binding glycoproteins are expressed in many human cancer cells. The level of PNA-binding glycoproteins increases in sera of patients with various carcinomas (1). The use of PNA-binding glycoproteins or T antigen for immunological diagnosis and even treatment of malignancy has been proposed occasionally. To contribute to the above-mentioned project, we have been biochemically analyzing PNA-binding glycoproteins from human gastric cancer cells. The glycoproteins isolated by PNA-agarose affinity chromatography are heterogeneous, whose major components are molecular weights around 280,000 (2). Galβ1→3GalNAc linked to the core proteins has been identified as the PNA-binding site, and other glycans with tumor-associated carbohydrate markers are also present in the glycoproteins (3). The aim of the present investigation is to know the structure of the protein moiety.

PNA-binding glycoproteins isolated from Kato III human gastric cancer cells were deglycosylated by TFMS, and rabbit antisera were raised against the deglycosylated molecule. Rhamda gt11 expression library constructed from Kato III cells was screened by the antisera. The positive clones obtained could be classified to two groups.

One group of cDNA clones specified for proteins, which are identical or closely similar to polymorphic epithelial mucin (PEM) found in breast carcinoma. Structure of the clones belonging to this group was studied in detail in two cases. In one case partial cDNA structure of 2 Kb agreed completely with that of PEM, in the other case nucleotide differences were present, resulting in amino acid changes in tandem repeats.

The other group of cDNA clones specified for a new core protein. The predicted protein sequence was rich in VTT, VTS and NTT(S) sequences, while no obvious repeating motif was detected.

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8.11
CARBOHYDRATE PROFILES REVEALED BY *DOLICHOS BIFLORUS* AGGLUTININ AND A Le^x-RECOGNIZING MONOCLONAL ANTIBODY CORRELATE WITH METASTATIC POTENTIAL AND PROGNOSIS OF HUMAN LUNG CARCINOMAS

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The purpose of the present investigation is to find out carbohydrate markers whose mode of expression can be used to predict prognosis of patients with lung carcinomas.

Expression of two carbohydrate markers, namely, 4C9 antigen which is a Le^x antigen and the *Dolichos biflorus* agglutinin (DBA) binding site which is an N-acetylgalactosamine marker, was examined histochemically in tumors and adjacent non-tumorous tissues of 102 cases of human lung carcinomas. 4C9 antigen is defined by a rat monoclonal antibody, which inhibits cell-substratum adhesion of embryonal carcinoma cells (1). In non-tumorous tissues, the DBA binding site was more frequently expressed than 4C9 antigen, in adjacent bronchial epithelium in particular. As compared to adjacent non-tumorous tissues, the DBA-positive cases decreased in the cancerous portion.

Patients with tumors which expressed DBA binding sites but not 4C9 antigen [4C9(−), DBA(+)] showed lymph node metastasis and vascular metastasis less frequently and had less progressive stages than patients with tumors of other carbohydrate profiles, namely [4C9(+), DBA(+)] [4C9(−), DBA(−)] [4C9(+), DBA(−)].

With regard to recurrence of the tumor, there were fewer recurrences in patients with [4C9(−), DBA(+)] tumors. Especially when the tumors were curatively resected by operation, patients with [4C9(−), DBA(+)] tumors had no recurrence. Furthermore, when death due to unrelated diseases were excluded, all patients with [4C9(−), DBA(+)] tumors are alive, while in all other groups a significant number of patients died because of tumor regrowth. A statistically significant difference was found in survival rates between patients with [4C9(−), DBA(+)] tumors and those with tumors of other carbohydrate profiles ($p < 0.05$). Better prognosis of patients with [4C9(−), DBA(+)] tumors was observed in patients with disease stages both I and 3A, and the better prognosis was observed in patients both with blood group A antigen and without it.

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8.12

CONTROL OF *N*-GLYCOSYLATION IN NORMAL AND MALIGNANT CELLS: EFFECT OF ACTION OF BRANCHING *N*-ACETYLGLUCOSAMINYLTRANSFERASE III, IV AND V ON FURTHER OLIGOSACCHARIDE PROCESSING

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We have studied the effect of prior action of *N*-acetylglucosaminyltransferase (GlcNAc-Tase) III, IV and V on the activities of the elongating β4-galactosyltransferase (β4-Gal-Tase) and β3-GlcNAc-Tase, and the terminating α3-sialyltransferase (α3-NeuAc-Tase), α6-NeuAc-Tase and α3-Gal-Tase. Action of GlcNAc-Tase III yields bisected structures that are acted upon at diminished rates by the elongating as well as the terminating enzymes. GlcNAc-Tase IV catalyzes the formation of tri-antennary structures that are acted upon by the α6-NeuAc-Tase at a diminished rate, by the α3-NeuAc-Tase at an increased rate, whereas the activities of the α3-Gal-Tase and the β3-GlcNAc-Tase are essentially unchanged. Action of GlcNAc-Tase V yields tri'-antennary structures. The activities of β4-Gal-Tase, α3-NeuAc-Tase and α3-Gal-Tase with such structures are not significantly altered. However, the activity of α6-NeuAc-Tase is severely suppressed while that of β3-GlcNAc-Tase is strongly increased. Apart from the effect of particular GlcNAc residues on enzyme activities, these residues may also significantly influence the preference of the glycosyltransferases for certain branches (branch specificity).

As a result an altered expression level of either one of the branching GlcNAc-Tases causes a specific shift in direction wherein the oligosaccharides are further processed. Such specific shifts, that might be amplified by the altered expression of the elongating and terminating glycosyltransferases, seem to have occurred in a number of malignant cells. This will be illustrated for a case where N-*ras* proto-oncogene expression in NIH 3T3 cells causes a coordinated alteration of the activities of several glycosyltransferases.

8.13

LOCALIZATION OF β1,4GalNAc TRANSFERASE ACTIVITY IN GASTRIC MUCOSA AND ITS CANCER-ASSOCIATED ALTERATION

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A glycolipid NGM-1 detected commonly in Japanese normal gastric mucosa, but not in other organs or tissues, was previously determined to be the same structure as Cad blood group glycolipid (GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAcβ1-3 Galβ1-4Glc-Cer. J. Biol. Chem. 265:7880, 1990). NGM-1 was detected only in fundic mucosa of stomach, but not detected at all in pyloric or cancer tissue. The amount of G_{M2} which has the same terminal structure as NGM-1, was apparently increased in 70% of cancer cases, compared with the normal mucosa. To know the mechanism of the expression of NGM-1 glycolipid and G_{M2}, specimens of gastric fundic mucosa, pyloric mucosa, and gastric cancer from 6 cases were obtained, and β1,4-GalNAc transferase activity was measured using G_{M3} or sialylparagloboside (SPG) as substrate, which synthesize G_{M2} or NGM-1, respectively. Transfer to pyridylaminated-sialyllactose (PA-SL) and pyridylaminated-NeuAcnLc$_4$ (PA-SL4), which produce the carbohydrate moiety of G_{M2} and NGM-1, was also measured. Five of 6 specimens from fundic mucosa having NGM-1 showed fairly high activity of β1,4GalNAc transfer to SPG, PA-SL4, and PA-SL, while transfer to G_{M3} was relatively low. Trace GalNAc transferase activity to G_{M3} and PA-SL was detected in cancer as well as in normal mucosa, but the activity was not well related to the amount of G_{M2}.

Thus, the expression of NGM-1 in gastric fundic area was due to the high activity of β1,4GalNAc transferase. We could not always detect the increased activity of β1,4GalNAc transferase producing G_{M2} in cancer tissue. It seems that the enzymes producing G_{M2} and NGM-1 glycolipids are different ones or differently modulated in gastric fundic mucosa and cancer. In gastric cancer, the amount of G_{M2} is possibly determined by means more than the G_{M2} synthetase activity.

8.14

INCREASED α2,6 SIALYLTRANSFERASE ACTIVITY TOWARDS *N*-ACETYLLACTOSAMINE IN NUDE MICE XENOGRAFTS OF HUMAN COLON CANCER CELL LINES

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The activity of a α2,6 sialyltransferase acting on *N*-acetyllactosaminic sequences (α2,6 ST, E.C.2.4.99.1) had been previously found increased in 90% of human colon cancer specimens. The α2,6 ST activity expressed by established human colon cancer cell lines is usually much lower than that found in fresh colon cancer tissues, thus suggesting that the growth conditions may strongly influence the expression of this enzyme. In order to evaluate the importance of environmental factors on the sialyltransferase expression, the α2,6 ST activity of six human colon cancer cell lines grown in culture was compared with that of the corresponding nude

mice xenografts and of the cell lines derived from the xenografts. It was found that xenografts of COLO 205, HT-29, SW 620, SW 948 and SW 948 FL (a non-adherent subline of SW 948) cells express a α2,6 ST activity much higher than that of the *in vitro* grown cells. SW 48 cells grown either in culture or as xenograft are practically devoid of enzyme activity. All the xenograft-derived cell lines but HT-29, retained the increased α2,6 ST activity at least for the first six passages. Those derived from SW 948 xenografts showed an enrichment of round, non-adherent cells very similar to the subline SW 948 FL previously selected for the ability to grow in non-adherent conditions. This indicates that during *in vivo* growth a selection of these cells has occurred. Xenograft-derived cell lines show an increased binding of the fluorescent lectin from *Sambucus nigra* (SNA) thus indicating the presence of a higher number of α2,6-sialylated structures on cell membranes.

These data suggest that the enhancement of α2,6 ST activity in nude mice xenografts may arise either from a reversible phenotypic adaptation (e.g. HT-29 cells), or from a selection process (e.g. SW 948 cells). It is likely that high levels of α2,6 ST activity confer an advantage for the *in vivo* growth.

8.15
DEVELOPMENT OF ANTI-IDIOTYPIC MONOCLONAL ANTIBODY TO ANTI-SIALYL LE^a MONOCLONAL ANTIBODY AND ITS APPLICATION TO TUMOR VACCINE

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Many monoclonal antibodies (MoAbs) were developed by immunizing mice with anti-sialyl Le^a MoAb, KM231. Eight of them inhibited the binding of KM231 to sialyl Le^a antigen. Six out of the eight MoAbs reacted well with all the four anti-sialyl Le^a MoAbs examined including NS19-9, but not with anti-sialyl Le^x MoAb, anti-Le^a MoAb, anti-Le^x MoAb, anti-A, B, and H blood type MoAbs, anti-Forssman MoAb and many anti-protein antigens MoAbs. One of the anti-idiotypic MoAbs, KM468, which binding to KM231 was strongly inhibited by sialyl Le^a oligosaccharide, was thought to have the best mirror image and selected out for further studies.

When eight rats were immunized with anti-idiotype MoAb, KM468, with Freund's adjuvant, serum from one rat strongly and sera from some rats weakly reacted with sialyl Le^a antigen in ELISA. The sera also positively stained human gastric cancer cell lines which expressed sialyl Le^a antigen in immunofluorescence assay. These findings indicated that KM468 was able to induce anti-sialyl Le^a antibody in vivo.

We screened many mouse cell lines and found that two lymphoma cell lines expressed sialyl Le^a antigen as glycoprotein. Using one of the cell lines, the vaccine effect of KM468 was examined in syngeneic system. After immunization of ten mice with KM468 with Freund's adjuvant, the mouse lymphoma cell line was inoculated. The mice immunized with KM468 survived significantly longer than the mice immunized with a control mouse MoAb. Taken together, KM468 is a good candidate for a vaccine in active immunotherapy of human tumor which expresses sialyl Le^a antigen.

8.16
NEO-GANGLIOSIDES EXPRESSED IN HEPATOCELLULAR CARCINOMA IN AFRICANS

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Qualitative and quantitative changes in acidic glycosphingolipids have been demonstrated in all of 30 hepatocellular carcinomas occurring in southern African blacks. The changes were most striking in the monosialoganglioside fractions prepared from the tumours by solvent extraction, Folch partitioning and ion-exchange chromatography. Separation of monosialo-gangliosides by high performance thin layer chromatography (HPTLC) showed a significant accumulation of two gangliosides, sialyl α2-3 paragloboside and sialyl α2-6 paragloboside, and of lower prevalence, a ceramide heptasaccharide, none of which were detected in normal hepatic tissue.

The structural characterization, preparation of monoclonal antibodies against these gangliosides and their potential to be used in the radioimmunodetection and serological diagnosis of hepatocellular carcinoma (HCC) will be discussed.

8.17
RECOGNITION OF TYPE I CHAIN OLIGOSACCHARIDES AND LACTO-SERIES GLYCOLIPIDS BY AN ANTIBODY TO HUMAN SECRETORY COMPONENT

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Antibody 6C4 is a mouse IgM monoclonal antibody against human secretory component that also binds to some colon adenocarcinoma cell lines including HT29 (Woodward et al., J. Immunol. 133:2116–2125, 1984), LS-180 and SW1116 cells. Binding of the antibody is lost after treatment of free secretory component with peptide N-glycosidase F (Bakos et al., J. Immunol. 146:162–168, 1991) or periodate, suggesting that asparagine linked oligosaccharides contain the epitope recognized by this antibody. Inhibition of antibody binding to free secretory component by human milk oligosaccharides established that lacto-N-tetraose is the minimum structure recognized by the antibody, but larger oligosaccharides with terminal type I sequences bind with much higher affinity. The antibody also binds to type I oligosaccharide sequences substituted with Fucα1-4GlcNAc but not with Fucα1-2Gal. Milk oligosaccharides containing the Lewis Fucα1-4GlcNAc bind with higher affinity than those lacking fucose. However, free secretory component does not bind antibodies to Le^a or Le^b oligosaccharides, and the Le^a antibody does not inhibit 6C4 binding to free secretory component. Therefore, the epitope recognized by 6C4 on free secretory component is not an asparagine-linked Le^a oligosaccharide. The antibody binds to some purified lacto-series glycolipids including III^{4}αFuc-lactotetraosyl ceramide, lactotetraosyl ceramide, and to neutral glycolipids in human meconium and SW1116 and HT29 colon adenocarcinoma cell lines. By Western blotting, the antibody 6C4 binds to several proteins in colon adenocarcinoma cell extracts and binds to some purified human mucins.

8.18
BIOSYNTHESIS OF LIPID-LINKED OLIGOSACCHARIDES IN RAT LIVER AND ZAJDELA HEPATOMA

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In vitro biosynthesis of N-acetylglucosamine-, mannose and glucose-containing lipid-linked oligosaccharides from rat liver and Zajdela

hepatoma microsomal membranes was investigated. When microsomes from the above mentioned two types of cells were incubated with UDP-N-acetyl-^{14}C-glucosamine in the absence of exogenous Dol-P, about 3-fold higher rate of labelling of C/M(3:2) extracts was established in liver microsomes compared to that of Zajdela hepatoma. On the other hand the N-acetylglucosamine incorporation rate of the protein fraction of hepatoma cells was 3-fold higher in comparison with that of the C/M(3:2) extracts. The application of tunicamycin to the incubation medium pointed out that only 50% approx. of the incorporated monosaccharide derived from its lipid carrier. Those results gave us some reason to suggest that in hepatoma microsomes two distinct ways for the transfer of N-acetylglucosamine to the glycoprotein fraction exist. However, more detailed investigations would give us further information about this problem.

The higher incorporation rate of ^{14}C-mannose into the protein fraction compared to C/M(3:2) extracts of Zajdela hepatoma microsomes was also found. Those results raise the question whether it is possible for the process of elongation of the oligosaccharide chain initiated by the direct transfer of GlcNAc from UDP-GlcNAc onto protein molecules with mannose residues to occur.

8.19
BIOSYNTHESIS OF LIPID-LINKED OLIGOSACCHARIDES IN CHICKEN LIVER AND HEPATOMA Mc 29. FORMATION OF MANNOSE-CONTAINING DERIVATIVES

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We have previously shown the synthesis of dolichyl phosphate mannose in microsomal fractions of chicken liver and virus-induced hepatoma Mc 29. The effect of various detergents, divalent ions, incubation time etc. on the activity of GDP-mannose:dolichyl phosphate mannosyl transferase (EC 2.4.1.83) was investigated. The value of K_m for GDP-mannose of the enzyme activity was calculated to be 5.2×10^{-5}M for the liver and 6.6×10^{-5}M for hepatoma Mc 29 (1).

During preincubation of microsomes with unlabelled UDP GlcNAc and subsequent incubation with GDP-^{14}C-mannose in the absence of exogenous dol-P, lipid-linked tri- to undecasaccharides were discovered in $CHCl_3/CH_3OH$ (2:1 v/v) and $CHCl_3/CH_3OH/H_2O$ (1:1:0.3 v/v) extracts. The highest incorporation of the radioactive precursor into hepatoma microsomes was found in hepta- and undecasaccharides, whereas in chicken liver microsomes penta- and octasaccharides were detected.

The different steps for a regulation of the major lipid-linked oligosaccharide synthesis in both microsomal fractions were discussed.

1. *L. Stovkova*, R. Tosheva, V. Zhivkov. GDP-mannose: Dolichyl phosphate mannosyl transferase activity in microsomal fraction of chicken liver and hepatoma Mc 29. Gen. Comp. Pathol., 24, 1988, 43–51 (Bulg.)

8.20
ELEVATED ACTIVITY OF GLYCOLIPID SULFOTRANSFERASE IN HUMAN HEPATOMA SERUM

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Sulfotransferase, which catalyzes sulfation of the carbohydrate of galactosylceramide (GalCer) and is localized in the Golgi membrane of cells, was assayed for the activity in human serum. To do this, an organic solvent was added to the incubated reaction mixture containing GalCer as an acceptor and phospho-adenosine phospho(^{35}S)-sulfate as a donor of sulfate to dissociate the synthesized sulfolipid from serum protein(s). This was followed by isolation of the sulfolipid on an anion-exchange column. Through this procedure, human serum was found to contain sulfotransferase activity. The serum enzyme was activated by Mn^{++}. Km values of enzyme for GalCer and 'active sulfate' were 4.6 μM and 5.2 μM, respectively.

The sulfotransferase activity was assayed in sera of patients with several carcinomas. The activity was significantly elevated in 21 cases of 63 patients with hepatocellular carcinoma (mean ±S.E., 349 ± 32 pmol/ml/h, $n = 63$ $p < 0.001$) compared to healthy subjects (172 ± 12, $n = 85$). No significant elevation of the sulfotransferase level was observed in liver cirrhosis (219 ± 28, $n = 10$) and in hepatitis in which many of biochemical hepatoma markers increase concomitantly. Furthermore, the elevation of the sulfotransferase was independent of production of α-fetoprotein and of aminotransferase levels in hepatoma, providing complementary biochemical measurement for α-fetoprotein-negative hepatoma cases. Overall enzyme levels in patients with other carcinoma types appeared to be in normal range. The sulfotransferase levels (234 ± 21, $n = 32$, $p < 0.01$) in sera from patients with renal cell carcinoma, in whose involved tissues the enzyme activity markedly increased, were less than in hepatoma.

8.21
TUMOR ASSOCIATED ANTIGENS IN BONE AND SOFT TISSUE SARCOMAS

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In recent years, various carbohydrate tumor associated antigens are reported. Many of them are gangliosides, so the author tried to extract gangliosides from bone and soft tissue sarcomas; for the purpose of detection of tumor associated antigens of these sarcomas. Tumor tissues were obtained by surgical operation from 2 cases of human osteosarcoma, 3 cases of human chondrosarcoma, 1 case of human rhabdomyosarcoma and 6 cases of human malignant fibrous histiocytoma; and tumor tissues obtained from 5 cases of human benign chondroma were used as the normal control. In order to detect the tumor specific ganglioside, the author used enzyme-immunostaining of TLC plate. The gangliosides mixtures extracted from tumor tissues were developed on the TLC plate (Polygram Sil G, Marchery-Nagel) with the solvent, then the plate was treated with sialidase (Seikagaku K.K.) and the gangliosides on the plate were desialylated. The TLC plate was incubated with anti-GA2 rabbit monoclonal antibody (Iatron Co.) at 37°C for 2 hrs, then horseradish peroxidase conjugated anti-rabbit IgG (Cappel Lab.) was added and labeled. The enzymatic reaction was done with substrate of 4-chloro-1-naphthol. GM2 gangloside was detected. The mouse anti-GD2 monoclonal antibody was also used and GD2 ganglioside was detected. By these TLC analysis using enzyme-immunostaining method, it is confirmed that osteosarcomas and chondrosarcomas contained large amounts of GM2 and GD2; rhabdomyosarcoma and malignant fibrous histiocytomas contained large amounts of GM2 but not GD2; while benign chondromas contained no GM2 and GD2 at all. GM2 is not contained in normal tissue and recently GM2 is found in various cancers as the tumor associated antigen. So GM2 is decided as the tumor associated antigen of osteosarcoma, chondrosarcoma, rhabdomyosarcoma, and malignant fibrous histiocytoma.

8.22

GANGLIOSIDES WITH NeuAcα2-6Gal STRUCTURE IN HEPATOMA AND MECONIUM

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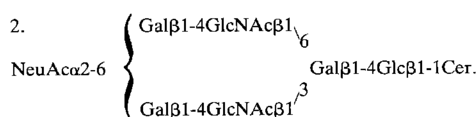
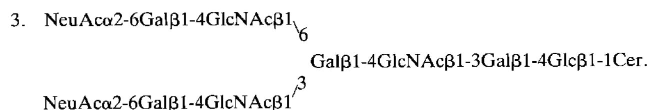
Gangliosides containing NeuAcα2-6Gal structure in human hepatoma have been studied and their structures were compared with those in meconium. Gangliosides were separated by column chromatographies with DEAE-Sephadex A-25 and silica beads. Three gangliosides containing NeuAcα2-6Gal structure were purified from hepatoma tissue. These gangliosides were structurally characterized by monoclonal antibodies, and mass spectrometry, proton NMR spectrometry, permethylation analyses and exoglycosidase treatments as follows.

1. NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer.

2.



3.



The compound 1 has the same structure as a characteristic ganglioside in meconium. The compound 2 was also found in monosialoganglioside fraction as a minor component in meconium. The third compound is a novel type of ganglioside having I type structure as core sequence. In meconium, presence of monosialoganglioside of compound 3 was confirmed.

Most of the gangliosides in hepatoma and meconium were found to contain neolacto-series structure as core sequence. The appearance of these gangliosides suggested activation of sialyltransferase involved in the formation of NeuAcα2-6Gal structure in hepatoma as well as in fetal tissue.

8.23

A NOVEL GLYCO-SIGNALING MECHANISM: SIALYL CHOLESTEROL-DEPENDENT NEURITOGENESIS IN A MOUSE NEUROBLASTOMA CELL LINE, NEURO2A, IS ASSOCIATED WITH ITS TRANSLOCATION INTO CELL NUCLEI

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The mechanism of neuritogenesis evoked by synthetic sialyl cholesterol (SC) was studied in a mouse neuroblastoma cell line, Neuro2a. After incorporation, SC was neither digested nor converted to other compounds until 24 hours, indicating that SC itself but not SC-derived compounds must be responsible for the neuritogenic activity. Incorporated SC increased the rates of Ca^{2+}-influx as well as -efflux, though the intracellular level of total Ca^{2+} and the IP_3 concentration did not change significantly. An excess amount of W-7 did not inhibit the SC-dependent neuritogenesis. These results suggest that SC neither utilize Ca^{2+}, nor IP_3, nor Ca^{2+}/CaM as a second messenger for neuritogenesis.

A large amount of incorporated SC was found in the nucleus (40%), besides in the granule (11–14%) and plasma membrane fractions (25%). Circular dichroism (CD) spectra revealed that the chromatin of SC treated cells had rather B-type structure in contrast to that of untreated cells, in which C-type structures was demonstrated. The transcription level of the chromatin from SC-treated cells was significantly higher than that of untreated cells. By adding SC to the chromatin of untreated cells, the CD spectra were converted from C- to B (or A)-type and the transcription level was also raised up in a concentration dependent manner. After adding SC to cells, the SC-dependent neuritogenesis started only after a definite lag-time. Furthermore, α-amanitin treated cells did not show the SC-dependent neuritogenesis. These results strongly suggest that nuclear SC changed the gene expression, and then the cells were promoted neuritogenesis.

Estradiol, cortisol and corticosterone could neither promote neuritogenesis nor be accumulated into nucleus so much as SC. SC must be carried to nucleus by different mechanism from that of steroid hormones.

8.24

EXPRESSION OF Le^a, SIALYLATED Le^a, AND SIALYLATED TYPE 1 BLOOD GROUP PRECURSORS IN GASTROINTESTINAL TISSUES

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We previously reported that Le^a, monosialosyl Le^a (CA19-9), and disialosyl Le^a (FH7) are often expressed in both normal and malignant human pancreatic and colonic tissues (*Cancer Res* 48:3834, 1988). Biosynthetic pathways for these antigens however, have not been elucidated. Recently, monoclonal antibodies (Mabs) for the possible precursors of these antigens have become available; i.e. Mab against lacto-N-tetraose (type 1 chain, LNT), Mab DUPAN-2 (which was recently discovered to recognize sialyllacto-N-tetraose a (LSTa)) and Mab against sialyllacto-N-tetraose b (LSTb). In this study we have used these Mabs to stain fixed specimens of gastrointestinal tissues by immunoperoxidase histochemistry. The table lists the number of positive cases with each Mab:

	LNT	*Le^a*	*DUPAN-2*	*LSTb*	*CA19-9*	*FH7*
Pancreas-normal	3/8	7/7	8/8	7/8	6/8	8/8
Pancreas-cancer	2/9	9/9	8/9	6/9	9/9	9/9
Colon-normal	2/14	4/14	0/14	3/14	1/14	8/11
Colon-cancer	0/17	8/15	1/17	0/16	14/17	12/15

In *pancreatic* tissues, both normal and malignant, type 1 precursor (LNT) was rarely exposed whereas LSTa and LSTb were commonly found. On a case-by-case basis, CA19-9 expression was similar to that of its likely precursor LSTa. LSTb expression was weaker than LSTa, and LSTb was expressed by more cells of normal pancreatic than malignant pancreatic tissues.

In the *colon* as in the pancreas, LNT was again rarely exposed, but in contrast to the pancreas, LSTa and LSTb expression was very rare in both normal and malignant colonic tissues.

Summary: 1) Type 1 chain precursor (LNT) is rarely exposed in pancreatic and colonic tissues; 2) LSTa and LSTb precursor substances are exposed more often in pancreatic than in colonic tissues. These reagents may be useful for studies dealing with organ-specific differences in blood group antigen biosynthesis.

8.25

MODULATION OF CELL SURFACE CARBOHYDRATES IN LECTIN-RESISTANT MELANOMA CELLS USING RETINOIC ACID

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Using a low-metastatic glycosylation defective variant (Wa4b1) of the B16 murine melanoma cells (F1) originally obtained by Tao and Burger[1], we have previously reported multiple differences between a wild type and a wheat germ agglutinin resistant clone[2]. Those differences include glycosylation variations associated with variation in enzymatic activity levels. Pattern of endogenous lectins, receptors for neurotransmitter coupled to adenylate cyclase, response to VIP and prostaglandines were also different in the Wa4b1 cells. Adhesion properties of the two variants were also estimated and suggested a correlation between the sialylation degree of cell surface receptors and biological properties.

Using retinoic acid, we were able to restore the parental glycosylation phenotype (cellular glycotype) increasing the sialyltransferase activities of Wa4b1 cells and decreasing the Galβ1-4GlcNAc fucosyltransferase one[3]. Expression of sialylated glycans was also closest to the parental pattern as estimated by HPAEC and HPLAC analysis. That the posttranslational modifications of N- and/or O-linked glycans may contribute to differences in the expression or activity of cell surface receptors or in their ability to activate corresponding metabolic pathway are suggested. The altered metastatic behaviour could be the result of one or several of these biochemical alterations. However, modulation of protein function by glycans could be a more accurate answer.

HPAEC: High Performance Anion Exchange Chromatography; HPLAC: High Performance Lectin Affinity Chromatography; VIP: Vasoactive Intestinal Peptide.

(1) Tao T. & Burger M. (1977) Nature, 270, 437–438. (2) Di Virgilio S. et al. submitted to Clin. Expl. Metastasis. (3) Prieels et al. (1983) Eur. J. Biochem. 130, 347–351.

8.26

INFLUENCE OF ARYL-N-ACETYL-α-GALACTOSAMINIDES ON THE EXPRESSION OF EPITECTIN BY H.EP.2 CELLS

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Epitectin is a mucin-type glycoprotein expressed on a wide range of human cancer cell lines but not on non-tumorigenic cell lines. Epitectin is detected on the surface of H.Ep.2 cells by the Ca2 monoclonal antibody. It is believed that the precursor protein of epitectin is expressed in both normal and malignant cells but that the difierence in the interaction of the Ca2 antibody is due to aberrant glycosylation. Previously we had demonstrated that over 95% of the saccharides in epitectin are derived from the core disaccharide Galβ1→3GalNAcα→ [R. Bardales *et al.* (1989) *J. Biol. Chem.* 264, 1980–1987]. In this study, we have examined the effect of aryl-N-acetyl-α-galactosaminides, a potential inhibitor of UDP-Gal:GalNAc-β1,3-galactosyltransferase [Kuan, S-F. *et al.* (1989) *J. Biol. Chem.* 19271–19277], on the synthesis and expression of epitectin by two malignant cell lines (H.Ep.2 and CGL 3) and one non-malignant cell line (CGL 1). Treatment with 4 mM of phenyl-N-acetyl-α-galactosaminide resulted in an 80% decrease in incorporation of $[^3H]$-$GlcNH_2$ while having no significant effect on the incorporation of $[^3H]$-Proline into the macromolecules of H.Ep.2 cells. The epitectin immunoprecipitated from the treated cells showed a doublet of apparent molecular weights 420 kDa and 460 kDa on SDS-PAGE compared to 350 kDa and 390 kDa for epitectin from untreated cells. The epitectin immunoprecipitated from treated H.Ep.2 cells had only 30% of $[^3H]GlcNH_2$-derived radioactivity compared to that from untreated cells. The oligosaccharide profiles of the epitectin from treated and untreated cells were compared by β-elimination followed by Bio-gel P2 chromatography. The radioactivity in tetra-, tri- and di-saccharides were reduced 4.6-fold in the epitectin from treated cells whereas that in N-acetylgalactosaminitol was increased 2.8-fold. As expected, these results show that the aryl-N-acetyl-α-galactosaminides are an effective inhibitor of elongation of O-linked GalNAc residues. (Supported by NIH grant CA38797).

8.27

POLYGLYCOSYLCERAMIDES ARE SYNTHESIZED BY HUMAN PANCREATIC TUMOR CELL LINES

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Numerous changes in the expression of glycosphingolipids have been detected during the transformation of normal cells to highly metastatic tumor cell types. One of the major changes that has been documented in human colorectal tumor cells is an increase in β-1,3 N-acetylglucosaminyltransferase resulting in a substantial induction of neolacto type glycolipid synthesis [Holmes, E.H., Hakomori, S. and Ostrander, G.K. (1987) J. Biol. Chem. *262*, 15649]. To determine if such a mechanism might also be occurring in other human tumor cell types, we have studied the glycosphingolipids synthesized by human pancreatic tumor cell line PANC-I. The sensitivity of the analyses was greatly increased by incubating cells with high specific activity tritiated sugar precursors to label glycolipids. A novel extraction procedure was also developed to optimize polyglycosylceramide isolation from small samples of cells. Upper layer $[^3H]$glycolipids derived by this procedure were digested with endoglycoceramidase to generate glycosphingolipid-derived $[^3H]$oligosaccharides. Gel filtration chromatography indicated that 20–30% of the $[^3H]$Gal or $[^3H]GlcNH_2$ labeled oligosaccharides ranged in size from 2 to over 6 Kd. Lactosaminoglycan type structure was confirmed by binding of 90% of the high molecular weight $[^3H]$oligosaccharides to wheat germ agglutinin-AffiGel. The results of methylation analysis of the $[^3H]$Gal labeled oligosaccharides also confirmed poly-N-acetyllactosamine type sequence. Several other structural features of the oligosaccharides were investigated using lectin affinity chromatraphy. These findings demonstrate that polyglycosylceramides represent a significant portion of the total glycolipids synthesized by PANC-1 cells. Preliminary results with other pancreatic tumor cell lines also indicate substantial polyglycosylceramide synthesis. It remains to be determined if this enhanced expression of polyglycosylceramides is normal for progenitor ductal epithelial cells or results from the induction of a specific glycosyltransferase as demonstrated for colorectal tumor cells. (Supported by the Edmondson Cancer Fund and the Medical College of Hampton Roads Foundation.)

8.28

ADRENAL-CARCINOMA TUMOR PROGRESSION AND PENULTIMATE CELL SURFACE OLIGOSACCHARIDES

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We have used three different specific sialidases and a highly sensitive high performance liquid chromatographic sialic acid assay to probe the cell surfaces of several metastatic adrenal carcinoma variants. There was no significant difference in the overall levels of cell surface or total cellular sialic acid among the three adrenal carcinoma variants. However using highly purified, linkage-specific sialyltransferase (STase) and

V. cholerae sialidase to probe the cell surface saccharide topography of specific penultimate oligosaccharides, we found that metastatic variants (F2 and F4) contain about 6-fold more penultimate Galβ1-4GlcNAc sialylation sites than a non-metastatic variant (HSR) when CMP[^{3}H]-NeuAc and the α2-6Galβ1-4GlcNAc STase were used to probe the adrenal carcinoma cell surfaces. The metastatic variants were found to contain 4.5-5 fold more Galβ1-3GalNAc sialylation sites than the non-metastatic variant when the α2-3Galβ1-3GalNAc STase was used as a cell surface probe. These results suggest that the penultimate cell surface sialo-oligosaccharide moieties are dramatically altered by progression to a malignant phenotype in these adrenal carcinoma tumors. They also support the notion that similar alterations in cell-surface oligosaccharides correlate with the metastatic phenotype of widely different tumor cell types. Supported by CA42486.

8.29

SIALYL-DIMERIC LEWIS-X: A CARBOHYDRATE ANTIGEN EXPRESSED IN ADVANCED COLON CARCINOMA WITH HIGH METASTATIC POTENTIAL

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The levels of sialyl-dimeric Lewis-X antigen (SLX) was previously compared in ~50 human colorectal carcinoma specimens by use of specific monoclonal antibodies. Higher levels of SLX was expressed in primary carcinomas at an advanced stage than at an early stage, and in metastases than corresponding primary tumors (1, 2). The observed difference was apparently due to difference in the proportion of cells expressing this antigen within a tumor (3). The antigenic molecules were unique mucins distinct from the MUC1 gene products that were also expressed in advanced colorectal carcinoma (3, 4).

We have retrospectively evaluated post-operative survival of colon carcinoma patients for their SLX levels in the primary tumors according to the percentage of stained cells by specific antibodies. Patients with high levels of SLX in their primary tumors (>30%) died due to recurrence and metastasis earlier than those with low levels of SLX (<30%).

To further elucidate the biological function of SLX, we have selected stable human colon carcinoma cell variants according to their differential expression of SLX, from the KM12C cell line, using FACS. The high SLX cells (KM12-HX) produced SLX associated with a M_r 900 kD mucin and >40 discrete glycoproteins (M_r 40–200 kD), whereas none of these components were observed in low SLX cells (KM12-LX). Northern blotting analysis with PCR products representing an α(1-3/4)-fucosyltransferase showed that transcripts for these enzymes was very low and did not significantly differ between KM12-HX and LX cells. KM12-HX cells strongly adhere to human umbilical cord endothelial cells treated with 20 ng/ml of TNF. The extent of adhesion of KM12-HX and LX cells to untreated endothelial cells was low. These results suggest that SLX mediates colon carcinoma cell adhesion *via* a cytokine induced adhesion molecule, likely to be ELAM-1, on endothelial cells.

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8.30

PARTIAL CHARACTERIZATION OF A FUCOSYLATED GLYCOPROTEIN ASSOCIATED WITH BREAST CANCER

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The fucosylated glycoprotein of Mr230000 identified by us in 70% of human breast carcinomas but no benign lesions (1), has been purified from the breast cancer cell line HS578T and partially characterized. The cell line HS578T which had previously been shown to express the glycoprotein, was grown in bulk on collagen coated microcarrier beads using a spinner culture system. An immunoaffinity column was prepared using a polyclonal antiserum previously raised against the pooled glycoprotein from primary human breast carcinomas. Medium from the cells was passed down the column and the glycoprotein of interest isolated as a broad peak by isocratic elution at pH 3.8. Deglycosylation of the glycoprotein by glycopeptidase F and resulting glycan fragments suggest an N-linked complex-type glycoprotein, distinct from the ubiquitous polymorphic epithelial mucins of the milk fat globule membrane.

A monoclonal antibody (TAG 230) has been raised by *in vitro* methods to the intact glycoprotein and this gives distinct focal cytoplasmic staining in many malignant specimens. Studies are in progress to determine the specificity of the monoclonal antibody in breast and to elucidate the protein core sequence of the glycoprotein.

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8.31

GANGLIOSIDES MODULATE PROTEIN KINASE C ACTIVITY IN PC12 PHEOCHROMOCYTOMA CELLS

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Gangliosides play a crucial role in many dynamic cellular processes, including the regulation of cell growth and proliferation. It is well known that addition of exogenous gangliosides to the tissue culture medium causes growth inhibition by extending the length of the G1 phase of cell cycle, and blocks cellular proliferation in the presence of growth factors. The expression of gangliosides is regulated during the cell cycle and oncogenic transformation. Despite many reports documenting the neuritogenic and neurotrophic effects of gangliosides, the underlying mechanisms remain obscure. One possible mechanism is that gangliosides can mediate these cellular processes by modulating protein use activities (1). In this study, we examined the effect of exogenous gangliosides on protein kinase C (PKC) activity in PC12 pheochromocytoma cells according to the procedure of Xia et al. (2). In comparing the PKC activities in PC12 cells and the two subcloned PC12D and PC12h cells, it was found that the activity of this enzyme was significantly elevated in the subclones, with the highest activity being in the PC12D cells. We found that gangliosides but not neutral glycolipid (LacCer and asialo-GM1), were potent inhibitors of PKC activity in the range of 25–100 μM, and that there was a direct correlation between the number of sialic acid residues and inhibitory potency, i e., polysialogangliosides such as GT1b and GD1a were more potent inhibitors than monosialylated species. We also found that fucosylated gangliosides which are abundant in PC12 cells were potent iniibitors of PKC activity. Gangliosides may participate in various cellular processes by regulating the PKC system.

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8.32
METASTATIC POTENTIAL OF TRANSFORMED RAT 3Y1 CELL LINES IS ASSOCIATED WITH A DECREASE IN OLIGOSACCHARIDE-SIALIDASE ACTIVITY

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Cell surface carbohydrates are known to undergo various neoplastic alterations. In particular, sialic acids have been proposed to be involved in cancer cell phenotype such as metastasis and invasiveness. To elucidate how change in sialic acids occurs in the expression of metastatic potential, we investigated sialidase and sialyltransferase activities in transformed rat fibroblast 3Y1 cell lines of different metastatic potential. 3Y1 cell was transfected with Rous sarcoma virus (SR-3Y1-2) and *v-fos* was transferred (fos-SR-3Y1-202) into SR-3Y1-2. SR-3Y1-2 is tumorgenic but low metastatic, and *v-fos* transfer enhances lung metastasis in relation to an increase in invasiveness.

In our previous studies on rat liver sialidase, we demonstrated that rat liver contains four types of sialidase differing in substrate specificity, in subcellular location and immunologically. They are located in lysosomal matrix, cytosol, lysosomal membrane and plasma membrane. They alter quantitatively in hepatocarcinogenesis. Based on those results, four types of sialidase were examined in the cell lines above. 3Y1 cell and its transformants exhibited only acidic sialidase activity toward 4MU-NeuAc and oligosaccharides in the particulate fraction. The sialidase seems to be a lysosomal matrix-type. The level was, however, low in the transformants when compared with control 3Y1 cells: the activities in SR-3Y1-2 and fos-SR-3Y1-202 were 40% and 10%, respectively, of that in control cell. Various lysosomal enzymes other than sialidase were hardly affected by the transformation. This indicates that marked decrease in the sialidase activity occurs in association with acquisition of metastatic potential.

We determined also the activity levels of sialyltransferases using glycoproteins and glycolipids as acceptor. The activity toward N-glycosylproteins was slightly but significantly elevated in the transformants. fos-SR-3Y1-202 had higher activity of the sialyltransferase than SR-3Y1-2. These results suggest that malignant transformation induces an increase in sialic acids bound to glycoproteins, and that the alteration occurs more profoundly in highly metastatic cells.

8.33
EXPRESSION OF α-3-FUCOSYLTRANSFERASES (α-3-FT) DURING NORMAL HUMAN EMBRYO-FOETAL DEVELOPMENT AND IN MALIGNANT SOLID TUMOURS AND CELL LINES

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The use of synthetic trisaccharides linked to an hydrophobic arm R = $(CH_2)_8COOCH_3$ as acceptors (Chembiomed), has allowed to distinguish 3 main patterns of α-3-FT activity in adult tissues. *Myeloid* type of α-3-FT, which use only H type 2 (αFuc1→2βGal1→4βGlcNAc-R); *Serum* type which use H type 2 as well as sialyl-LacNAc (αNeuAc2→3βGal1→4βGlcNAc-R); *Lewis* like or α-3/4-FT which can use, in addition, H type 1 (αFuc1→2βGal1→3βGlcNAc-R) (Mollicone et al. *Eur. J. Biochem.* **191**: 169, 1990).

The *myeloid* type is the first and the only α-3-FT found in most human tissues during the first 5 to 10 weeks of development. This embryonic stage is followed by a sequential and progressive appearance of the *serum* and *Lewis* like enzymes, in the digestive mucosa, during foetal and adult stages. In heart, muscle, lung or skin, the embryonic *myeloid* activity is replaced by a second wave of *serum* and *Lewis* like activities during foetal life, followed by a strong diminution or entire abolition of all α-3-FT activities in the adult. The liver express mainly *myeloid* activity at 5 weeks, followed by a progressive increase of the *serum* α-3-FT to reach equivalent amounts of activity on H type 2 and sialyl-LacNAc acceptors in the adult. The brain express only the *myeloid* type of α-3-FT at all stages.

Among 100 solid malignant tumours tested, all sarcomas expressed only *myeloid* α-3-FT, with an apparent Km corresponding to the *myeloid* type of α-3-FT, as in the embryonic period of development, while all adenocarcinomas expressed *serum* and/or *Lewis* like activities. The majority of human cell lines tested express only myeloid α-3-FT, others ($HepG_2$) have serum pattern of α-3-FT or express α-3/4-FT, in addition to a myeloid α-3-FT (HT29, ZR-75-1).

8.34
α1→3FUCOSYLTRANSFERASE AND SIALYLTRANSFERASES IN HUMAN MALIGNANT CELLS

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Human erythroleukemia (HEL) cells and neuroblastoma (CHP 134) cells express high activity of α1→3fucosyltransferase (1, 2). Even though there were several differences in the activities of the sialyltransferases, the most striking result was the extremely high α1→3fucosyltransferase activity in the HEL cells.

Depending on the substrate, an extract of CHP 134 cells transferred NeuAc from CMP[^{14}C]NeuAc to Galβ1→4GlcNAc (20 mM) only in α2→6 linkage, as detected by binding to immobilized SNA, specific for NeuAcα2→6Gal residues (3). On the other hand, when Galβ1→4Glc (40 mM) was used as a substrate, ^{14}C-product did not bind to immobilized SNA, suggesting that the [^{14}C]NeuAc transferred was in α2→3 linkage. With both substrates, the amount of [^{14}C]NeuAc transferred was similar (0.11 pmol/mg of cell protein). However, when an extract of HEL cells was examined for sialyltransferase activity, no activity was detected with Galβ1→4GlcNAc (20 mM) or Galβ1→4Glc (40 mM). When 40 mM Galβ1→4GlcNAc was added to the assay mixture, a transfer of [^{14}C]NeuAc (0.06 pmol/mg of cell protein) was detected suggesting that the sialyltransferases differ either in amount and/or V_{max}. The kinetic parameters of the purified enzymes will have to be determined in order to verify the differences.

Glycoproteins of HEL cells do not contain Fucα1→3GlcNAc (1) even in the presence of high activity of α1→3fucosyltransferase. In contrast, the activity of sialyltransferases appear reduced. Thus we hypothesize that α1→3fucosyltransferase is amplified in the HEL cells due to the arrest of maturation in these leukemic cells. Normal maturation would provide Fucα1→3GlcNAc on the cell surface and thus a potential ligand for LEC-CAMs (4, 5).

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8.35

ABERRANT GLYCOSYLATION IN α1-ACID GLYCOPROTEIN OF CANCER PATIENTS

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It is known that the quantitative changes in acute phase proteins correlate with occurence and degree of malignancy. In order to elucidate molecular basis of α1-acid glycoprotein (AGP) carbohydrates changes in human cancers we carried out comparative study of AGPs isolated from normal serum, breast and stomach cancer ascitic fluid. Normal serum AGP differed from cancer AGP by: molecular mass, pI, and affinity to ConA. By means of ConA chromatography AGPs were separated into non-bound and ConA-bound forms. Structures of N-glycans were determined by HPLC analysis of 7-amino-4-methylcoumarine derivatives (oligosaccharide map method) in combination with their sequential exoglycosidase digestion. Cancer AGPs differed from normal glycoprotein quantitatively but we did not find any unusual structure carbohydrate chains in aberrant molecules. The main features of cancer AGP are: i) per cent of ConA-binding form increase approximately from 10 to 30; ii) amount of agalacto-oligosaccharides in cancer ConA form become significant in contrast to trace amount in normal ConA form; iii) number of biantennary glycans doubled in cancer AGP. We found AGPs of both cancers studied are similar but not identical.

8.36

CORRELATION BETWEEN HIGH EXPRESSION OF PNA RECEPTORS AT THE SURFACE OF HUMAN MELANOMA CELLS AND THEIR SPONTANEOUS METASTATIC ABILITY

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Alterations in carbohydrate chains of cell surface glycoconjugates play a key role in regulation of cell proliferation, tumor progression and metastasis. To study the metastatic process in human tumor cells we have used clones and variants (CV) selected from one single human melanoma cell line by in vitro cloning techniques or by in vivo passages, and differing in their ability to give spontaneous lung metastasis in a recently described metastasis model using the immunosuppressed newborn rat. (Bailly et al., C.R. Acad. Sci., 304:111, 1987). Cell surface density of Peanut agglutinin (PNA) receptors were compared in high (HCV) and low (LCV) metastatic CV using flow cytometry analysis. Results showed that HCV expressed the highest levels of accessible terminal galβ1-3galNac whereas none or few terminal galβ1-3galNac were expressed in LCV. Some CV appeared constituted by two cellular subpopulations having high and low amounts of PNA receptors. Using EPICS Cell sorter, these two subpopulations were separated and injected in rats: high PNA binding cells gave higher lung metastasis than did the low PNA binding cells. In addition, PNA-digoxigenin/anti-digoxigenin-alcaline phosphatase staining of cell lysates blotted on nitrocellulose revealed different glycoprotein (GP) patterns which correlated with the metastatic phenotype, in accordance with FACS analysis. Whereas numerous GP could be detected in HCV, few if any GP are stained in LCV. Using lectin Maackia Amurensis Agglutinin (sialic acid 2-3 galactose) on blots, GP 150 appeared to contain sialic acid and terminal galactose in low metastatic cells, whereas it seemed galactosylated without sialylation in high metastatic cells. In contrast, GP 110-120 was sialylated without terminal galactose in low metastatic cells but enriched in terminal galactose and sialic acid in high metastatic cells. After neuraminidase treatment, similar PNA-binding patterns were observed in high and low metastatic cells. Our results suggest that the sialic acid masking of galβ1-3galNac sugar moities from the cell surface glycoproteins of malignant melanoma cells have effect on their spontaneous metastatic ability.

8.37

IMMUNOASSAY OF CIRCULATING GD2 GANGLIOSIDE SHED BY HUMAN RETINOBLASTOMA TUMORS

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Retinoblastoma is a rare tumor of the young child with an intraocular localisation that leads to some problems of diagnosis. In the aim of defining a biochemical marker that is still lacking for this disease, the gangliosides of fresh retinoblastoma tumors were analyzed. The ganglioside pattern was found to have GM3, GM2, GM1, GD3, GD2, GD1b and GT1b as major components. The occurrence of large amounts of GD2 in the tumor led us to search for an elevation of this ganglioside in the sera of tumor-bearing versus disease-free retinoblastoma patients. Highly specific antiGD2 monoclonal antibodies (MAbs of IgG3 and IgM isotypes) were produced from mice immunized with melanoma cells, and these antiGD2 MAbs were used for immunostaining on TLC plates followed by scanning densitometry for quantification. Only one false negative serum was found out of 20 patients, and in 14 sera, the level of GD2 was strictly consistent with the clinical diagnosis. The mean S.D. level for GD2 in sera of 9 tumor-bearing patients was 32 ± 20 ng/ml, and the normal value was at 7.8 ± 5.8 ng/ml serum. A two-years follow-up of patients showed that a successful treatment resulted in a rapid decrease of the serum level of GD2 down to the normal range, from where only the patients with relapses presented a further elevation of GD2. These results suggest that the level of serum GD2 could be valuable in both diagnosis and monitoring of human retinoblastoma.

8.38

IDENTIFICATION OF O-ACETYLATED SIALOGLYCOCONJUGATES BY INFLUENZA C VIRUS IN VARIOUS TISSUES

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O-acetylated sialic acids have been detected in different tissues. Acetylated gangliosides have been identified as tumor-associated antigens in some human melanoma cells. Their occurrence may also vary during brain development of different species. N-acetyl-9-O-acetyl neuraminic acid was shown to be the cellular receptor for influenza C virus. We show that influenza C virus can be used as a discriminating analytical probe to identify O-acetylated sialoglycoconjugates directly after western blotting of proteins and thin layer chromatography of lipids. The influenza C virus can detect the different forms of the two murine glycophorins which are known to be O-acetylated glycoproteins. The virus also can bind to O-acetylated gangliosides such as O-acetylated GT3. The esterase activity of the HEF protein of influenza C virus is used to unmask the sialic acid.

The specific binding of the HEF protein of the virus was then applied to analyse and identify the O-acetylated glycoconjugates in various malignant cell lines and in chicken brain during the embryonic development.

8.39

1-DEOXYMANNOJIRIMYCIN: INFLUENCE ON THE OLIGOSACCHARIDE PATTERN OF HIGH MANNOSE TYPE GLYCANS IN HEPATOCYTES AND HEPATOMA CELL LINES OF RAT

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A comparative study was undertaken in order to characterize the oligosaccharides released by endo-β-N-acetylglucosaminidase H (Endo H) from membrane glycoproteins of rat hepatocytes and three different Morris hepatoma cell lines (NA-MH 7777, HTC and MH_1C_1). The results show marked differences in the oligosaccharide pattern of the high mannosidic type carbohydrate chains between hepatocytes and hepatoma cells.

After metabolic labeling of the cells with (2-^{3}H)mannose in the absence as in the presence of 1 mM 1-deoxmannojirimycin, high mannose type oligosaccharides were released from delipidated membrane glycoproteins by enzymic digestion with Endo H and separated from protein by ultrafiltration. The carbohydrate chains were separated from detergent and salt and converted to their corresponding oligosaccharidalditols by reduction with sodium borohydride. Separation and sizing of the oligosaccharides was performed by HPLC on APS-2 Hypersil applying a decreasing gradient of acetonitrile in 0.015 M sodium phosphate buffer, pH 5.2. A mixture of glucose oligomers, fluorescence labeled by reductive amination with 2-amino-8-naphthol was used as internal standard.

Compared to hepatocytes the amount of the Endo H sensitive carbohydrate chains released from tumor cell lines was increased up to 5 fold. Moreover, a shift of the oligosaccharide pattern to higher mannosylated structures with $Man_8GlcNAcOH$ as the major component was observed in all hepatoma cell lines investigated, whereas in hepatocytes $Man_5GlcNAcOH$ was the predominant high mannose type structure.

By contrast, in the presence of Golgi mannosidase I inhibitor 1-deoxymannojirimycin no significant differences were observed in the distribution of high mannose type oligosaccharides between hepatocytes and hepatoma cells. Yet the ratio of radioactively labeled glycans sensitive to deglycosylation by Endo H was highly increased (>80%) in all cell lines investigated and $Man_{8-9}GlcNAcOH$ were the predominant structures.

8.40

GLYCOSYLTRANSFERASE CHANGES WITH DIFFERENTIATION OF CaCo-2 HUMAN COLONIC ADENOCARCINOMA CELLS

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The spontaneous differentiation of CaCo-2 human colonic adenocarcinoma cells is associated with a decrease in polylactosamino glycans (Youakim and Herscovics, Biochem. J. 247:299–306, 1987). To elucidate the biosynthetic mechanisms leading to these alterations we have compared glycosyltransferase activities that are involved in the synthesis of polylactosaminoglycans and of the N- and O-glycan structures that provide the framework for the attachment of these chains. CaCo-2 cells contain GlcNAc-transferases I to V involved in N-glycan branching; GlcNAc transferase II to V activities were significantly increased upon differentiation. The enzyme activities that are directly involved in the synthesis of linear polylactosaminoglycans, blood group I UDP-GlcNAc: Galβ-R β3-GlcNAc-transferase, and UDP-Gal:GlcNAc β4-Gal-transferase, were found at similar levels in undifferentiated and differentiated CaCo-2 cells. Since GlcNAc transferase III activity is known to inhibit further branching and galactosylation, these results suggest that its increased activity in differentiated CaCo-2 cells may be partly responsible for the decreased synthesis of fucosylated polylactosaminoglycans. Differentiated cells showed a two-fold increase in O-glycan core 2 UDP-GlcNAc: Galβ3GalNAcα-R (GlcNAc to GalNAc) β6-GlcNAc-transferase activity whereas O-glycan core 1 UDP-Gal: GalNAcα-R β3-Gal-transferase activity decreased. (This work was supported by grants from CCFF, MRC of Canada, Cancer Society, and Canadian Foundation for Ileitis and Colitis.)

8.41

INDUCTION OF A NOVEL GLYCOSIDASE ACTIVITY IN *RAS* TRANSFECTED RAT EMBRYO FIBROBLASTS BY TREATMENT WITH PLATELET ACTIVATING FACTOR

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Treatment of primary rat embryo cells (RECs) transfected with the human Ha-*ras* oncogene (REC/*ras*) with nanomolar concentrations of platelet activating factor (PAF) resulted in the induction of rare clones (REC/*ras*/PAF) which exhibited a novel glycosidase activity.

RECs, composed primarily of fibroblasts, were transfected with the human Ha-*ras* oncogene isolated from a EJ bladder carcinoma cell line and the *neomycin*-resistance gene using a replication-deficient DOEJS retrovirus driven by a MoMuLV long terminal repeat. Cells were selected in culture media containing G418. Following selection, REC/*ras* cells were treated with 4×10^{-13}M – 1×10^{-11}M PAF. 1×10^5 cells were then suspended in culture media in a 15 cm tissue culture plate over a 0.7 mm (0.6%) agarose layer. 1×10^4 REC/*ras*/PAF cells demonstrated an ability to penetrate the agarose base within 10 days and form monolayer colonies on the surface of the tissue culture dish. Untreated REC/*ras* cells and REC cells were incapable of forming colonies ($<3 \times 10^5$) within 35 days beneath the agarose base.

Agarose is a complex polysaccharide composed of repeating subunits of α(1→3)D-galactose β(1→4)3,6-anhydro-L- galactose. The conditioned media from REC/*ras*/PAF cells, REC/*ras* cells, and RECs was assayed for agarose degrading activity using a flourescently labelled agarose as a substrate. Supernatant removed from each of the three conditions was incubated with the labelled substrate for 16 hrs at 37°C. Low molecular weight degradation products were detected by their ability to diffuse through 0.6% unlabelled carrier agarose. Increased activity was detected in REC/*ras*/PAF conditioned media when compared to REC/*ras* and REC conditioned media. Further characterization of the activity is being conducted.

It is hypothesized that the physiological relevance of this novel glycosidase observed in REC/*ras*/PAF cells may represent induction of the ability to cleave the β(1→4) linkage between sugars in the glycosaminoglycan keratan sulfate and/or the β(1→4) bond between D-galactose and xylose in the trisaccharide link between glycosaminoglycans and core proteins in the extracellular matrix.

8.42

CMP-NEU5AC HYDROXYLASE DETERMINES THE WHEAT GERM AGGLUTININ-BINDING PHENOTYPE IN TWO MUTANTS OF THE LYMPHOMA CELL LINE MDAY-D2

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Glycosylation mutants of the highly metastatic murine lymphoreticular tumor cell lines, MDAY-D2, have previously been selected and partially characterized in an effort to determine whether specific classes of oligosaccharides contributed to tumor growth and metastasis. Class 2 mutants were selected for resistance to the toxic effects of WGA and the mutation was shown to be dominant in somatic cell hybrids made between mutant and MDAY-D2 cells. The observation that the mutant cells showed no change in sensitivity to lectins other than WGA suggested that sialic acid, a major cell surface ligand for WGA, may have been quantitatively or qualitatively altered in the mutant cells. HPLC separation of sialic acid residues released from cellular glycoconjugates metabolically labelled with [^{3}H]glucosamine in the class 2 mutant D33W25 cells revealed that the majority was Neu5Gc rather than the Neu5Ac form found in the MDAY-D2 cells[1]. The dominant character of the class 2 mutation suggested that a gene coding for CMP-Neu5Ac hydroxylase, the enzyme responsible for Neu5Gc biosynthesis, may have been activated in the cells.

We have addressed this question by further characterizing the interaction of WGA with the mutant and wild-type cell lines and correlating this with their sialic acid composition and level of CMP-Neu5Ac hydroxylase activity. The hydroxylase activity in the D33W25 mutant cells was approximately 60 fold higher than in wild-type tumor cells and exhibited kinetic properties identical to those of the same enzyme from mouse liver[2]. Growth rate experiments *in vivo* and *in vitro*, where the mutant cells grew more slowly at low cell densities in serum-free medium and also formed slower growing tumors in syngeneic mice, indicate that CMP-Neu5Ac hydroxylase expression may be associated with altered growth of the mutant cells.

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8.43
CHARACTERIZATION OF SIALYLTRANSFERASE AND SIALOGLYCOPROTEINS IN CHICKEN LIVER AND HEPATOMA MC-29

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The purpose of this study was to characterize microsomal sialyltransferase, their multiple forms and sialoglycoproteins in chicken liver and hepatoma induced by the leucosis virus strain Mc-29.

Kinetic investigations have revealed, that while there is no significant difference between the K_m values for liver and hepatoma enzymes, the value of V_{max} for liver sialyltransferase was two fold higher when compared to that of the hepatoma enzyme. It has been found that CMP is a competitive inhibitor of sialyltransferase from liver and hepatoma. The inhibitory constant for the hepatoma enzyme was lower than that of the same for the liver one.

The biosynthesis of sialoglycoproteins was studied by using double labelling in vivo with [3H]-leucine and N-acetyl-[14C]-mannosamine. The separation of sialoglycoproteins was achieved by chromatofocusing. It was observed that unlike the liver the hepatoma fractions localized in the alkaline part of the pH-gradient were with lower specific activity and with decreased rate of sialylation.

An attempt is made to discuss the relationship between the activity of sialyltransferase, their multiple forms and the different rate of sialylation of liver and hepatoma microsomal sialoglycoproteins.

8.44
SIALIC ACIDS IN RAT PROMEGAKARYOBLASTS (RPM) GROWN IN CULTURE

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The immortal rat bone-marrow derived cell line (RPM) is an analog of the promegakaryoblast which subsequently matures to form the megakaryoblast and mature megakaryocyte, before fragmenting in the pulmonary circulation to produce platelets. The sialic acid of human platelets is N-acetyl neuraminic acid (NANA) and is implicated in a number of functional events such as aggregation and adhesion. Platelet sialic acid cleaved by neuramidase is associated with loss of platelets from the circulation. Using pellicular resin anion-exchange column run at neutral pH with pulsed amperometric detection (DIONEX), following postcolumn addition of alkali a variety of sialic acids can be detected in the picomole range. The sialic acids from the immature platelet (RPM) has been analysed following mild acid hydrolysis and enzyme treatment. Interestingly a major peak corresponding to N-glycol neuraminic acid (NGNA) is observed, in front of a smaller peak corresponding to NANA, and if these two peaks are total sialic acid then 90% would be NGNA and 10% NANA. To prove that these results were not species specific a rat platelet rich plasma fraction was analysed and two corresponding peaks were observed, except this time 98% NANA, 2% NGNA.

It is postulated that NGNA provides a poor substrate for neuramidases and thus hinders degradation of sialoglycoconjugates. It is possible that NGNA in such comparatively large amounts provides a protective role in this immature stage of platelet development.

8.45
GLYCOPOLYPROTEINS WITH A SULFATED MULTIANTENNARY GLYCAN CHAIN IN EVERY REPEATING PEPTIDE UNIT FOUND AS COMPONENTS OF CORTICAL ALVEOLI: STRUCTURE AND FUNCTION

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Cortical vesicles are the Colgi-derived secretory vesicles found in the eggs of almost all the animals including mammals. One of the most striking response of the eggs to the stimulus of fertilizing sperm is the exocytosis of the cortical vesicles that store enzymes and materials, and transfer them to the extracellular space (perivitelline space) for regulation and protection of developing embryos. Our studies revealed that cortical vesicles of fish eggs (often called cortical alveoli) contain unique carbohydrate-rich 100–200 KDa glycoproteins that we named 'hyosophorin'. The most interesting feature of hyosophorin is that apohyosophorin is 'polyprotein', so that hyosophorin is made up of a tandem-repeat of identical 8 to 10 KDa glycopeptides. Within 10 min after egg activation, 100–200 KDa hyosophorin undergoes proteolytic fission to form the repeating units by specific protease (hyosophorinase) which also exists in cortical vesicles and is activated immediately after egg activation. The carbohydrate structures of hyosophorin are also unique and may probably be reflecting functional requirements. We have already established the structures of *O*-linked polysialylglycan chains ubiquitously found in polysialoglycoproteins (PSGP) present in the cortical alveoli of salmonid fish eggs. Carbohydrate moieties of hyosophorin obtained from fish other than *Salmonidae* have been identified to be large *N*-linked glycans. The structure of pentaantennary neutral

N-glycan chain from flounder hyosophorin has already been reported.

In this study, we report structures of penta- and tetraantennary poly-N-acetyl-lactosaminyl N-glycan chains of hyosophorin from 2 other species of fresh water-fish, *Plecoglossus altivelis* and *Tribolodon hakonensis*. In these fish, some glycan chains of hyosophorin are extensively sulfated at the GlcNAc and/or Gal residues and sulfation in addition to sialylation accounts for highly polyanionic nature of the hyosophorin molecules.

8.46
THE ROLE OF GLYCOPROTEINS IN GROWTH CONE COLLAPSE

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Guidance of axon growth cones during embryonic development involves both adhesion and repulsion. We are characterising the role of axon-repelling glycoconjugates during the segmental outgrowth of peripheral nerve axons in the somite mesoderm of chick embryos. By affinity chromatography on PNA-agarose and jacalin-agarose we have isolated from posterior half-somite a glycoprotein fraction (M_r 48K and 55K) which causes the collapse of dorsal root ganglion growth cones when applied *in vitro*. Treatment of the isolated material with neuraminidase and O-glycanase abolishes collapse activity, suggesting the O-linked carbohydrate residues may be involved in the initiation of growth cone collapse.

A similar activity is also detectable in normal adult chicken and rat grey matter. This activity may be removed by affinity chromatography on immobilised PNA, or by the use of hydroxyapatite. Polyclonal antibodies directed against the somite-derived material also cross-react with a 48K component in detergent extracts of normal adult chicken brain. Finally, collapse activity can be induced in cultured rat cortical astrocytes by treatment with interleukin-1 and basic fibroblast growth factor. We suggest that the presence of growth cone inhibitory glycoproteins in the grey matter may contribute to the failure of regeneration of the injured higher vertebrate CNS.

8.47
DEVELOPMENTAL ABNORMALITIES IN TRANSGENIC MICE EXPRESSING A VIRAL ENZYME SPECIFIC FOR 9-O-ACETYLATED SIALIC ACIDS

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Mutations in many steps of glycosylation are easily obtained in tissue culture cells but do not show remarkable phenotypes *in vitro*, suggesting that many oligosaccharides are not required for the growth and maintenance of single cell types in a simple environment. On the other hand, glycosylation mutants in intact higher animals are very rare. One explanation is that many types of glycosylation play crucial roles during embryogenesis, and that mutations in the intact animal result in lethal aberrations. The developmentally regulated expression of many carbohydrate structures supports this hypothesis, as do their re-expression as oncofetal antigens in cancer. To address this, it is necessary to selectively interrupt or alter glycosylation in the intact developing embryo. However, this must be done in a way that avoids an early embryonic lethal event.

The hydroxyl groups of sialic acids can be O-acetylated at different positions, significantly altering the size, hydrophobicity, net charge and enzymatic susceptibility of the parent compound. O-acetyl esters at the 9-position of sialic acids show developmental regulation, tissue-specific expression, and regional distribution in a variety of systems, and sometimes re-appear as oncofetal antigens. We therefore sought to selectively abrogate expression of these esters during embryogenesis. The Influenza C virus hemagglutinin-esterase specifically recognizes 9-O-acetylated sialic acids, and is synthesized as a type-I membrane-bound polypeptide. Under physiological conditions, it functions only as a highly specific sialate:9-O-acetylesterase. Such viral 'receptor destroying enzymes' are known to eliminate receptors on cell surfaces on which they are expressed. We reasoned that if the Influenza C esterase were expressed in cells of an intact developing embryo, it should destroy 9-O-acetyl esters of sialic acids on the same cell, and perhaps on adjacent cells.

DNA constructs encoding the Influenza C esterase were microinjected into fertilized mouse eggs. Constructs driven by the metallothionein promoter gave a markedly decreased yield of transgenic and non transgenic mice. Observation of such embryos *in vitro* showed an arrest in development at the two-cell stage. A similar construct driven by the phenylethanolamine N-methyltransferase promoter did not cause this block. Mice transgenic for this construct showed selective expression of the protein in the expected tissues, the retina and the adrenal gland. These two organs also showed variable abnormalities in organization, while all other tissues examined appeared normal. The expressed enzyme had destroyed 9-acetyl-G_{D3}, a predicted target structure. Thus, 9-O-acetylated sialic acids may play an role in the development of mouse embryos, both at the two-cell stage and in certain differentiated tissues. In principle, this approach can be generalized to any situation where a highly specific carbohydrate-degrading enzyme can be expressed as a cell surface protein.

8.48
TERMINAL GLYCOSYLATION CONVERSION FROM SIALYLATION TO FUCOSYLATION DURING POSTNATAL DEVELOPMENT OF RAT SMALL INTESTINAL EPITHELIAL CELLS: DEMONSTRATION WITH LECTIN-GOLD TECHNIQUES

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Post-embedding lectin-gold cytochemistry was employed to investigate the distribution of sialic acid and fucose residues in rat small intestinal epithelial cells during postnatal development. During the suckling phase (postnatal day 1) the apical and basolateral plasma membranes of epithelial cells, as well as goblet cell mucus was intensely stained with the sialic acid-specific *Sasbucus nigra* L. lectin I-gold complex (SNL I-g). By the weaning period (postnatal day 23), the entire villus contained both SNL I-g-positive and negative cells. In adult small intestine, the plasma membranes of all epithelial cells were unreactive with SNL I-g; however, abundant staining was detectable in goblet cell mucus, cells of the lamina propria, and smooth muscle cells. Similar results were obtained with the *Limax flavus* lectin, another sialic acid-specific lectin. Single and double incubations with lectins recognizing other terminal sugars, such as galactose and N-acetylgalactosamine, demonstrated that the developmentally-related loss of lectin-binding sites was restricted to sialic acid residues.

The distribution of fucose residues as detected by a *Ulex europaeus* lectin I-gold complex (UEL I-g) was virtually opposite that of sialic acid. At postnatal day 1, staining was restricted to goblet cell mucus, whereas by postnatal day 23, a portion of epithelial cells displayed UEL I-g

binding sites along the apical and basolateral plasma membranes. In adult animals, the apical and basolateral plasma membranes of all epithelial cells, as well as goblet cell mucus were stained with UEL I-g.

These results support biochemical data demonstrating a shift from sialylation to fucosylation of intestinal microvillar glycoconjugates during the weaning phase of postnatal rat development. Moreover, the results indicate that rather than a general decrease in cellular sialylation, specific individual cells at all positions along the crypt-to-villus axis become devoid of lectin-detectable sialic acid.

8.49
SEX-RELATED DIFFERENCES IN N-LINKED GLYCOSYLATION OF MEPRIN-A, A MEMBRANE-BOUND PROTEINASE

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Meprin-a, a plasma membrane metalloendopeptidase is selectively expressed in the kidney, intestine and salivary glands of rodents. The purified enzyme is a glycoprotein of 350K comprised of four subunits (85K each) linked by disulfide bridges, zinc and calcium. Previous studies with inbred strains of mice expressing high- and low-meprin-a activity and F1 hybrids of these strains indicated that high-meprin-a activity is an autosomal dominant trait; the sex of the parents or progeny is without influence on the activity of the protease in the kidney. However, immunohistochemical studies with polyclonal antibodies prepared against meprin-a from high activity adult male mice indicated that there was decreased immunoreactivity in female mice. Western blots of kidney membrane proteins from C57BL/6 mice, a high-meprin-a activity strain, indicated that the female form of meprin-a had a decreased electrophoretic mobility relative to the male form; this difference could be eliminated by treatment of kidney preparations with endoglycosidase F. Studies with purified preparations of meprin-a from male mice indicated that the enzyme contains oligosaccharides that react with Con-A, PHA-E_4, PHA-L_4 and RCA-I. Quantitative densitometry of blots indicated that meprin-a reacts similarly with Con-A and RCA-I in male and female mice. However, the binding of PHA-L_4 and PHA-E_4 was 2- to 4-fold more intense for male than female forms of the enzyme indicating that there are differences in glycosylation. Meprin-a in juvenile male and female mice displayed similar immunohistochemical staining in kidney slices, as well as similar electrophoretic mobilities and binding with all the lectins tested. Thus, the data indicate that it is only after puberty, that meprin-a differs in the sexes. Previous studies had revealed that administration of 17-β-estradiol to gonadectomized adult mice decreased the immunoreactivity of meprin in kidney slices and its electrophoretic mobility. Thus, these studies indicate that estrogens affect N-linked glycosylation of meprin-a. (NIH Grant #DK19691).

8.50
A PROGENITOR T CELL SPECIFIC GANGLIOSIDE THAT DISAPPEARS DURING DIFFERENTIATION

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Progenitor of T (pro-T) cells are present in bone marrow and migrate to thymus to eventually differentiate into mature T cells. However, little is understood about pro-T cells and the mechanism by which they recognize and enter the thymus. In this study pro-T cell lines were established by the hybridoma method to clarify the mechanism. BM216B9, one of the hybridomas produced by fusion of nude mouse bone marrow cells with thymoma BW5147, had the phenotype for immature T cells such as Thy-1^+, $CD3^-$, $CD4^-$, $CD8^-$, T cell receptor (TCR) $\alpha\beta^-$, TCR$\gamma\delta^-$, expressed mRNA of non-rearranged TCRγ gene, and homed to the thymus following *in vivo* transfer. This cell line thus appears quite likely to derive from a pro-T cell.

Attention was first directed to the surface expression of the glycosphingolipids of the pro-T cell hybridoma, being possibly closely associated with the regulation of cell differentiation and intercellular recognition. A pro-T cell specific ganglioside with a unique structure was found present in BM216B9 but no longer present in the thymus and peripheral T cells. By chemical analysis and ^{1}H-NMR spectroscopy a novel saccharide sequence of the ganglioside was found to be NeuAcα-Galβ-Galβ-Galα-Galβ-Glcβ-Cer. This ganglioside may possibly function as a marker component for pro-T cells during T cell ontogeny.

8.51
GLYCOSYLATION CHANGES IN HCG AND FREE α MOLECULES AS A FUNCTION OF GESTATIONAL AGE

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We investigated hCG and free α molecules from early and late pregnancy for changes in glycosylation. Five volunteers provided 24 h urine samples throughout pregnancy. First trimester samples (7–12 wk) from each volunteer were compared to their own third trimester samples (28–32 wk). Urine samples were precipitated with acetone and subjected to gel filtration to separate hCG from free α. HCG and free α molecules from early and late pregnancy were subjected to lectin affinity chromatography on ConA-Sepharose and *Lens culinaris*-agarose. ConA fractions were categorized as unbound (eluted with buffer) weakly bound (eluted with 10 mM α-methylglucose), and tightly bound (eluted with 500 mM α-methylmannose. *Lens culinaris* fractions were categorized as unbound (eluted with buffer) or bound (eluted with 500 mM α-methylmannose). All five pregnancies displayed similar lectin binding patterns. HCG was found to bind tightly to ConA in both early and late pregnancy. In contrast, differences were observed in the ConA fractions of free α-molecules obtained from early versus late pregnancy. In all five pregnancies, a substantial decrease in the ConA tightly bound fraction was observed in late pregnancy relative to early pregnancy. The decrease in tightly bound forms of free α was accompanied by a concomitant increase in ConA unbound forms. Additionally, in all five pregnancies, there was striking increase in fucosylation in the third trimester as evidenced by increased amounts of free α and hCG that bound to *Lens culinaris*. The changes observed in the lectin binding patterns of free α and hCG in third trimester pregnancy indicate a shift toward synthesis of more highly branched and fucosylated oligosaccharides in late pregnancy.

8.52
ALTERATION IN GALACTOSYLTRANSFERASE LEVEL OF PC12 CELLS ASSOCIATED WITH NEURONAL DIFFERENTIATION

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PC12 is a clonal cell line established from a rat pheochromocytoma (1). Exposure to nerve growth factor (NGF) causes PC12 cells to cease proliferation, extend neurites and undergo several biochemical changes.

Accumulation of the unique globo-series glycolipids which are characterized by the repetitive Galα1-3 linkage are reported on PC12 cells (2). Previously, we reported that the polar components of neutral glycosphingolipids of PC12 cells greatly decreased during the early stage of neuronal differentiation (3). In this study, we have examined the enzymatic basis for changes in neutral glycosphingolipid expression of PC12 cells during neuronal differentiation.

UDP-galactose: globotriaosylceramide α-galactosyltransferase of PC12 cells was studied on effects of detergent, metal requirements, optimal pH, substrate specificity; and the reaction products were determined. When PC12 and its subclones, PC12D, PC12h and MR31 (ras transfected PC12), were differentiated by exposure to either NGF, cAMP or staurosporine, galactosyltransferase activity was found to decrease in all the cell lines. These results suggest that changes in the expression of neutral glycosphingolipids are due to those in the galactosyltransferase activity.

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(2) T. Ariga, et al. (1989) J. Biol. Chem. *264*, 1516–1521

(3) H. Kojima, et al. (1988) 3rd Rinsyo-ken Int. Confer. (Abst.) 138–139

8.53

N-ACETYLGLUCOSAMINYL-, GALACTOSYL- AND SIALYLTRANSFERASE ACTIVITIES IN MICROSOMES FROM PIG EMBRYONIC AND ADULT LIVER

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The activities of three terminal enzymes for synthesis of N-glyco-proteins – N-acetylglucosaminyl-, galactosyl- and sialyltransferases towards different acceptors in liver microsomes from 55-day-old pig embryos and sows were studied. The activity of N-acetylglucosaminyl-transferase to ovalbumin is 2.5 times higher in microsomes from embryonic liver as compared to the adult one. The results indicate that the galactosyltransferase activity towards ovomucoid, N-acetylglucosamine and glucose is 5, 7 and 2-fold, respectively higher in liver microsomes from pig embryos. Evidence is presented for the existence of two galactosyltransferases – β1-4 and β1-3-galactosyltransferases in microsomes from pig embryonic and adult liver. The ratio of β4/β3-galactosyltransferases in embryonic liver is 11 and in adult liver – 3. It was found that the activity of sialyltransferase towards asialofetuin and lactose is 3 times higher in embryonic liver as compared to the adult liver. The data indicate the presence of sialyl-(2→3)-lactose and sialyl-(2→6) lactose in liver from 55-day-old pig embryos and the 2→3 to 2→6 isomer ratio is 1.24.

8.54

CD77 ANTIGEN OF GERMINAL CENTRE B CELLS ENTERING APOPTOSIS

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Gb3, a glycolipid antigen, first reported as Burkitt's Lymphoma associated Antigen (BLA), is also expressed on a subset of germinal centre tonsilar B lymphocytes. Recently this molecule was recognized as a new B cell differentiation antigen and now defines the CD77 cluster.

Here we report an extensive phenotypic and functional characterization of the tonsilar CD77(+) B lymphocytes. These cells have a low buoyant density and are thus purified using a Percoll gradient. They express various B cell antigens such as CD19, CD20, CD21, CD22 and CD40. They are positive for surface IgM and negative for surface IgD but they are negative for the classical activation antigens CD23 (low affinity Fc IgE receptor), CD25 (IL2 alpha chain receptor) and CD71 (transferrin receptor). Proliferation and protein synthesis of CD77(+) cells was measured after stimulation with a range of mitogens and interleukins. None of the agents tested is able to induce proliferation and protein synthesis with the exception of a combination of rIL-4 plus anti-CD40 antibody. When examined in electron microscopy, CD77(+) B lymphocytes show a morphology similar to that of cells undergoing the programmed cell death called apoptosis (chromatin condensation, nuclear fragmentation, membrane blebbing). As shown by direct examination of DNA, these CD77(+) cells are indeed in the process of apoptosis. Treatment of the CD77(+) cells by rIL4 and anti-CD40 antibody prevents apoptosis.

In germinal centres B lymphocytes undergo selection on the basis of their ability to receive a positive signal from antigen. This process arises by hypermutation, acting selectively on rearranged immunoglobulin variable-region genes. Since the CD77(+) lymphocytes are localized in germinal centres, it will be of great interest to investigate the role of Gb3/CD77 in the selection process of cells with high affinity for antigen. The role of the Gb3/CD77 as a possible receptor for a signal molecule inducing apoptosis is currently under investigation.

8.55

MODIFICATION OF GLYCOSPHINGOLIPID BIOSYNTHESIS DURING B LYMPHOCYTE DIFFERENTIATION

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The neutral glycolipid globotriaosylceramide (Gb3) was recently recognized as a new B cell differentiation antigen and now defines the CD77 antigen. We have previously shown that the CD77 antigen is specifically expressed on Burkitt's lymphoma cells and on a subset of germinal centre tonsilar B cells. In order to test if other glycolipid could constitute differentiation markers of the B cell lineage, monoclonal antibodies directed against glycolipids of the globo, ganglio and lacto-series were used for immunofluorescence studies on B cell lines. Two glycolipids seemed specifically expressed on certain B cell lines: globoside was strongly positive on 6/8 LCL and on low buoyant density fraction of tonsillar B lymphocytes. Although preliminary, these results suggest that globoside could be considered as a new marker for mature/activated B lymphocytes. GM2 ganglioside recognized by 10.11 monoclonal antibody was only detected on the 2 myeloma cell lines. Further studies with a larger panel of normal cells will determine if GM2 represent a new B cell antigen specific of plasma cell stage.

Glycolipid biosynthesis was investigated at various stages of B cell differentiation. Glycolipids were extracted from 19 different B cell lines and from normal lymphocytes. The pre-B cell lines expressed lactoseries type II chain-based glycolipids and GM3 ganglioside. Upon differentiation, the lactoseries synthesis was shut down whereas compounds of the globoseries appeared: resting lymphocytes and lymphoblastoïd cell lines (LCL) expressed GM3, Gb3 and Gb4. At a latter stage of B cell differentiation, biosynthesis of the ganglioseries was extended and myeloma cells expressed GM2. We then measured the activities of the enzymes involved in the glycosylation process. The α-galactosyltransferase (transforming LacCer in Gb3) and the sialyltransferase (transforming LacCer in GM3) activities, were compared in all the B cell lines. Further studies are in progress to test the expression of the other enzymes.

8.56
THE EXPRESSION OF FUCOSYL–LACTOSYLCERAMIDE-DERIVED, GLOBO-SERIES GLYCOLIPIDS IN NEURONS OF THE DEVELOPING RAT OLFACTORY SYSTEM

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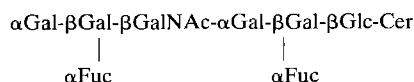
Two monoclonal antibodies CC1 and CC2 were produced which react with unique glycolipids in the olfactory system of rodents. The CC1 and CC2 reactive glycolipids are virtually restricted to the olfactory system and are not readily detectable in other regions of the nervous system. CC1 antibodies react with standard globotetraosylceramide. In the olfactory system of rats, little if any globoside is present, however, a CC1 immunoreactive glycolipid is detected which chromatographs slightly lower than globoside. CC2 antibodies react with an α-galactosyl- α-fucosyl derivative of galactosyl-globoside which is also fucosylated on the internal β-galactose. A tentative structure of the CC2 immunoreactive glycolipid is:



CC2 antibodies also react with 38 and 55 kDa glycoproteins. The CC1 immunoreactive glycolipid is likely to be the GalNAc-terminal, penta-glycosylceramide precursor of CC2.

Both CC1 and CC2 antibodies react with selective groups of cells found uniquely within the olfactory system of rats. CC1 immunoreactivity in embryos and in early postnatal rats is restricted to neurons in the central region of the vomeronasal organ (VNO, a subset of olfactory neurons). CC1 is also expressed on a subset of vomeronasal axons and in the rostral half of the accessory olfactory bulb (AOB), which is the target site of these axons. CC1 antigens thus define a topographical map of central (VNO) neurons to rostral targets in the vomeronasal system. CC2 reacts with a subset of neurons in dorso-medial regions of the main olfactory epithelium, their axons and their termination sites in the dorso-medial regions of the main olfactory bulb. It is thus likely that CC2 antigens are involved in axon guidance mechanisms for dorso-medial neurons in the epithelium to dorso-medial target sites in the bulb. Likewise CC1 antigens likely participate in guiding central VNO axons to rostral AOB targets.

8.57
GLYCOSYLATION IN ENTEROCYTE-LIKE CELLS DURING DIFFERENTIATION TRIGGERING CULTURE CONDITIONS

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One prominent influence on glycan processing is the cell differentiation. The concomitant biochemical events are under intensive study. Published data suggest that the amount of high mannose structures attached to glycoproteins varies with the differentiation in HT-29 cells.

In order to investigate the glycan processing of cells conditioned with different media we estimated the glycosylation pattern of the colon carcinoma HT-29 and Caco-2 cell lines. The cells were cultured in the presence of suramin or absence of glucose-conditions which are commonly used to induce cell differentiation. We measured the binding of glycoconjugates of cell homogenates or culture supernatants to Galanthus nivalis Agglutinin (GNA) and Concanavalin A (ConA) by quantitating their inhibition of lectin-binding to an immobilized mannosylated glycoprotein. The developmental stage of these enterocyte-like cells was characterized by the activity of differentiation dependent cellular enzymes (sucrase, alkaline phosphatase).

The data of quadruplicate measurements show that the GNA-binding activity (equivalent to the amount of terminal mannose residues) increases in cells and even more in the supernatant shortly after priming the delineated culture conditions. This effect is not seen for the ConA-binding activity. The present data indicate that changes of glycan pattern in both cell lines can be monitored by the use of the competitive lectin-binding inhibition assay. Glycoconjugates with terminal mannose residues but comparably low ConA-binding are secreted or shedded into the culture supernatant after induction of differentiation.

8.58
HISTOCHEMICAL ANALYSIS OF CONTACTINHIBIN, A PLASMAMEMBRANE GLYCOPROTEIN INVOLVED IN CONTACT-DEPENDENT INHIBITION OF GROWTH

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Contactinhibin, a plasmamembrane glycoprotein isolated from human diploid fibroblasts, is involved in the contact-dependent inhibition of growth in a reversible and non toxic manner.

We have raised monospecific antibodies against contactinhibin, which release confluent cultures from contact-inhibition: proliferation rate, measured by ^{3}H-Thymidine incorporation during DNA-synthesis, is increased 2.5-fold in presence of 10 μg/ml anti-contactinhibin antibodies compared to control cultures treated with antibodies isolated from preimmune serum.

By western-blot analysis contactinhibin could be proved in many human and animal tissues and organs. Tissue sections of different mouse organs, immunologically stained with anti-contactinhibin antibodies show different distribution of contactinhibin. Sections of different developmental stages of the frog Xenopus laevis indicate that contactinhibin appears at a very early stage during embryogenesis.

8.59
IDENTIFICATION OF THE RECEPTOR FOR CONTACTINHIBIN

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The growth of non-transformed diploid mammalian cells in vitro is characterized by a cell density dependent regulation mechanism. It has been shown that specific cell-contacts via plasma membrane proteins are of major importance in the growth regulation of normal cells. Our studies gave evidence for the central role of the integral plasma membrane glycoprotein Contactinhibin in the contact-dependent regulation of cell growth. Its growth inhibitory activity was found to be connected with the glycosidically linked oligosaccharide chains (see 1 and contribution of Wieser et al. this volume). Since transformed cells are not contact inhibited, but still express Contactinhibin in biologically active form, we focussed on the identification of a receptor specific for this glycoprotein.

First studies using biotinylated Contactinhibin and FITC-labelled avidin showed specific binding to cultured human fibroblasts. The following data have been obtained by a novel aggregation assay: the binding of Contactinhibin to the postulated receptor can specifically be inhibited by anti-Contactinhibin-antibodies and by solubilized plasma

membrane proteins; strongly reduced binding has been observed to plasma membrane proteins obtained from virus transformed or tumor promoter treated fibroblasts, suggesting a defective or absent Contactinhibin-binding to these cells. The attempt to directly identify the receptor by cross-linking experiments showed a membrane protein with a molecular weight of 90 kDa.

The Contactinhibin-binding fraction has been isolated from membrane protein extract by preparative SDS-PAGE and electroelution. It was proved for functional activity by the aggregation assay after renaturation. The active fraction consists of a single 90 kDa protein with charge microheterogenity and isoelectric points between pH 5 and 5.5.

Reference: (1) Wieser, R.J.; Schütz, S.; Tschank, G.; Thomas, H.; Dienes, H.-P. and Oesch, F.: Isolation and Characterization of a 60–70 kDa Plasma Membrane Glycoprotein Involved in the Contact-dependent Inhibition of Growth. In: J. Cell Biol., Vol 111 (No. 6, Pt 1), Dec. 1990 pp 2681–2692.

8.60

THE N-GLYCANS OF CONTACTINHIBIN ARE RESPONSIBLE FOR ITS ACTIVITY IN THE CONTACT-DEPENDENT INHIBITION OF GROWTH

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Contactinhibin is a 60–70 kDa plasma membrane glycoprotein involved in the contact-dependent inhibition of growth of non-transformed cells recently isolated from human diploid fibroblasts (Wieser et al. (1990) J. Cell Biol. 111: 2681). The addition of 50 ng/ml of contactinhibin in immobilized form to sparsely seeded fibroblasts resulted in a reversible 70–80% inhibition of growth. Interestingly, contactinhibin added in 'soluble' form had only marginal effects on the growth behaviour. Culturing confluent human fibroblasts in the presence of *anti*-contactinhibin-antibodies led to a release from contact-dependent inhibition of growth, resulting in enhanced saturation density, criss-cross growth pattern and formation of foci. Removal of contactinhibin from a plasma membrane extract resulted in a complete loss of the growth-inhibitory activity of the extract, indicating that contactinhibin at least in human fibroblasts is the only compound responsible for the contact-dependent inhibition of growth. Treatment of contactinhibin with glycopeptidase F resulted in the reduction of the apparent molecular weight by appr. 22 kDa altogether with a complete loss of the growth-inhibitory activity. Loss of biological activity was also observed after treatment of contactinhibin with β-galactosidase, although terminal β-glycosidically linked galactose is not sufficient for growth-inhibitory activity. This is shown by the fact that asialo-fetuin or asialo-acid-glycoprotein are without effect on the growth of human fibroblasts. These data indicate that the N-glycans of contactinhibin are responsible for the growth-inhibitory activity. This is in agreement with the data obtained studying the receptor for contactinhibin, which show that the N-glycans of contactinhibin are the ligands for the contactinhibin-receptor (see Müller G. et al., this volume).

Transformed cells are refractory to the activity of contactinhibin, although still expressing contactinhibin in active form. On the other hand, it has been shown that the binding of contactinhibin to its receptor is strongly reduced in transformed cells, suggesting that aberrant growth might result from impaired function of the contactinhibin-receptor.

8.61

INTERACTION OF THE CYTOSKELETON WITH PLASMALEMMA GLYCOCONJUGATES IN THE DEVELOPING NEURONS

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Topography of carbohydrate determinants, localized in the neuron plasma membrane glycocalix, is known to be immediately connected with processes of cell differentiation, recognition and development.

The lectin (WGA, HPL, RCA) -colloidal gold complexes were used to mark cytochemically the corresponding glycoconjugates in plasmalemma of dissociated spinal neurons (the monolayer culture) of the mouse. In tests preparations, colchicine or cytochalasine were added to incubation medium of 5- and 15-d-old preparations to modify some cytoskeleton elements. An electron microscopic study was carried out with the use of quantitative treatment of the micrograms (plane random profiles of the observed neuron fragments).

Numerical parameters (spectra of intervals, sizes, marker density etc.) of the marker arrangement along the membrane profiles were obtained. A developed statistical stereological approach allowed to reconstruct averaged 'images' adequately illustrating two-dimensional topography of the marked glycoconjugates on the neuron membrane surface observed under various experimental situations.

The data obtained permit to evaluate a contribution of the cytoskeleton elements into cooperative properties of membrane elements associated with the surface glycoconjugates. An analysis of these quantitative and 'image' data could be considered as a glycoconjugate surface pattern of the studied membranes.

8.62

MODIFICATION OF CHICKEN SEROTRANSFERRIN GLYCOSYLATION DURING EMBRYOGENESIS AND HEPATOCYTE CELL CULTURE

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Previous studies have shown that glycoprotein glycans from cancer cell lines in culture display striking structural modifications. However, these changes could be due, not only to the malignant transformation but also to the cell culture conditions. In order to detect the effects of cell culture, chicken serotransferrin has been choosen as a model. In fact, transferrin represents the major protein synthesized and secreted by chicken embryo hepatocytes cultured in a serum free minimum medium. Hepatocytes were isolated by perfusing livers from 16 day-old chicken embryos and cultured up to 3 days in calf serum free medium. The culture medium, as well as the sera of chicken embryos and from adult chicken, were submitted to immuno-aff inity chromatography on a rabbit anti-chicken transferrin IgG column. The oligosaccharides released from the isolated transferrins by hydrazinolysis were analyzed, after N-reacetylation, by 400 MHz proton-NMR spectroscopy. The obtained results are as follows:

1 – The unique glycan of each transferrin was found to possess the basic structure of a biantennary glycan of the N-acetyllactosaminic type.

2 – In the adult chicken serum transferrin, this basic structure is α-2,6-disialylated, whereas, in embryo transferrin, the glycan is not sialylated but possesses an intersecting GlcNAc residue. In embryo cell culture medium, the basic structure is monosialylated (α-1, 3-antenna) or disialylated and α-1,6-fucosylated on the terminal GlcNAc residue.

In conclusion, embryogenesis is characterized by a lack in sialylation and an activation of the N-acetylglucosaminyltransferase III. In addition, the cell culture modifies the glycosylation by activating sialyl- and fucosyltransferases. Consequently, the results concerning the glycosylation of proteins must be carefully interpreted if obtained using cell cultures.

8.63
CONTROL OF THE EXPRESSION OF THE X AND SIALYL-X DETERMINANTS ON MYELOID CELLS DURING MATURATION

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The X determinant (Galβ1-4[Fucα1-3]GlcNAc) is strongly expressed on myeloid cells from the promyelocyte stage onwards but expression on leukaemic myeloblasts is frequently negative despite high levels of α-3-fucosyltransferase (3-FT). Earlier studies from this laboratory showed that the 3-FT purified from leukaemic blasts differed from the enzyme purified from mature cells in its much lower capacity to transfer fucose to sialylated acceptors. Studies on the expression of X and sialyl-X (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc) on normal neutrophils, leukaemic myeloblasts and HL6O cells have now been carried out together with an examination of the activities in these cells of 3-FT, α3′- and α-6′-sialyltransferases (STs).

Much higher levels of 3-FT were detectable in blasts than in neutrophils with substrates containing terminal Galβ1-4GlcNAc structures but, in contrast to the enzyme in mature neutrophils, which reacted equally well with 3′-sialylated and non-sialylated acceptors, the transferase in blasts showed a marked preference for non-sialylated acceptors. 6′-ST activity was strong in blasts but was not detectable in mature neutrophils. A much lower level of 3′-ST was present in both blasts and neutrophils. DMSO induced maturation of HL60 cells was associated with a fall in both 6′-ST and 3-FT activities, a change in the substrate specificity of 3-FT towards that found in mature cells and by increased cell surface expression of sialyl-X. These results suggest that 1) the reduced expression of X in myeloblasts is related to the strong 6′-ST which utilises the precursor substrate at the expense of the 3-FT and 2) failure of expression of sialyl-X results from the fact that any NeuAcα2-3Galβ1-4GlcNAc structures formed by the 3′-ST fail to act as substrates for the 3-FT expressed in the cells at an early stage of maturation.

8.64
GALACTOSYLTRANSFERASE ACTIVITY IN NORMAL AND RHEUMATOID B-LYMPHOCYTES

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β1,4-Galactosyltransferase (EC 2.1.4.38) is an enzyme of oligosaccharide biosynthesis. Interest in the enzyme with respect to B-lymphocytes is due to a proposed defect in the biosynthesis of the oligosaccharides of IgG in a number of diseased states, particularly rheumatoid arthritis.

Initial studies on an assay were carried out on bovine milk galactosyltransferase with ovalbumin as acceptor. Time courses were based on incorporation of ^{3}H-galactose into phosphotungstic acid precipitable material with subsequent pelleting and washing of the precipitate. This method was successfully used to assay Triton extracts of the lymphoblastic B-cell line, IM-9. No incorporation into endogenous acceptor was detected. The assay was modified so that free oligosaccharides from hen ovalbumin and IgG could be galactosylated *in vitro*, with IM-9 and bovine milk galactosyltransferase. It was possible to analyse the products by Bio-Gel P4 gel filtration chromatography.

Using the assay technique described above, initial results have been obtained on oligoclonal pools of EBV-transformed B-lymphocytes derived from peripheral blood of normal and rheumatoid subjects as well as synoviocytes of rheumatoid subjects. In assays to date, there is no indication of a significant difference in the levels of galactosyltransferase activity between normal and rheumatoid EBV-transformed B-lymphocytes. Freshly isolated B-lymphocytes, purified with anti-CD19 immunomagnetic particles, have also been assayed. The study is being pursued on transformed and non-transformed cells to investigate whether differential galactosyltransferase expression occurs and whether there is a form of galactosyltransferase that does not interact with α-lactalbumin.

8.65
EFFECT OF GLYCATION AND OXYGEN FREE RADICALS ON COLLAGEN CROSS-LINKING

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Age-related changes in the properties of long-lived glycoconjugates, such as collagen, correlate with increases in cross-linking. In addition to an enzyme-catalyzed mechanism, collagen cross-linking can also proceed through a non-enzymatic pathway. The acceleration of collagen changes in diabetes mellitus is believed to be due to an enhancement of the non-enzymatic pathway which is influenced by glucose concentration. The process starts with a non-enzymatic modification (glycation) of ϵ-amino groups of lysine and hydroxylysine to yield Schiff bases which undergo Amadori rearrangement followed by dehydration and rearrangements (Maillard reaction) to yield covalent inter-molecular cross-links. To investigate this phenomenon we utilized radioactive collagen type I isolated from rabbit corneas radiolabeled *in vivo* by injection of ^{14}C-proline into the anterior chamber of the eye. Incubation of collagen (1 mg/ml) in the presence of glucose (100 mM) and $CuSO_4$ (10 μM) for 72 h at 37° resulted in the formation of collagen aggregates that were insoluble in 8 M urea. Both glucose and $CuSO_4$ were necessary for the reaction. Collagen aggregation was inhibited by addition of catalase, as well as by the chelating agent diethylenetriaminepentaacetic acid, supporting a mechanism involving H_2O_2 and transition metal ions. H_2O_2 may originate through dismutation of superoxide generated during autoxidation of glucose and Amadori products. In turn, H_2O_2 reacts with transition metal ions to produce hydroxyl radicals. $CuSO_4$ was effective at concentrations as low as 2.5 μM. Glucose was effective at physiological concentration (5 mM) suggesting that this mechanism may play a role in collagen changes associated with diabetes and with normal aging. Comparative studies showed that $FeCl_3$ was less effective than $CuSO_4$, and that ribose was more effective than glucose. The latter observation is consistent with the higher percentage of the non-cyclic carbonyl form of sugars in aqueous solutions of pentoses versus hexoses. After a 4 h incubation with ribose (100 mM) and $CuSO_4$ (10 μM), 50% of the collagen was insoluble in 8 M urea, whereas in the presence of glucose a 2–3 day incubation period was required. The urea insoluble material obtained after a 4 h incubation of collagen with ribose and $CuSO_4$ undergoes further changes upon longer incubation. Gel electrophoresis of the CNBr peptides from collagen aggregates obtained at 4 h showed only slight evidence of stable cross-links. Although very little additional changes were observed after a 1 day incubation, CNBr peptides from a 3 day incubation were extensively cross-linked. The possible role of oxygen radicals in the changes observed in the urea insoluble collagen aggregates is currently being investigated. Supported in part by the Presbyterian Health Foundation and Research to Prevent Blindness, Inc.

8.66
GLYCOPROTEIN METABOLISM IN NEURONAL CEROID LIPOFUSCINOSIS FIBROBLASTS

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Neuronal Ceroid Lipofuscinosis (NCL) is a group of inherited neurodegenerative diseases of children and rarely adults which occurs with an incidence estimated at 1:25,000 live births. Of the 3 childhood forms defined clinically by age of onset and course, the infantile (onset 6 months) is the most severe. Affected individuals typically exhibit seizures, dementia, loss of vision, and severe motor deterioration. The defect in NCL is not known but abnormalities in glycoconjugate metabolism have been observed in brain, skin, and cultured fibroblasts using biochemical and histochemical methods (Am. J. Med. Gen. Suppl. 5 (1988) Proc. Intl. Conf. on Ceroid Lipofuscinosis and pp. 27–46). To investigate abnormalities in the metabolism of glycoproteins, NCL fibroblast lines (3 infantile form and 2 juvenile form) were compared to 3 control lines. Cells were cultured in the presence of [^{3}H]GlcN and [^{3}H]Man, and [^{35}S]Met and after 66 hours cell monolayers were lysed with SDS (0.2%) in the presence of protease inhibitors, heated (100°C for 10 min), and clarified by centrifugation (10,000 × g for 20 min). The molecular weights (M_r) of radiolabelled components of the lysate supernatants were compared by SDS-PAGE under reducing conditions. Infantile NCL had ≈25% of the radioactivity of both sugars and methionine in the region of M_r = 120–140 kDa compared to controls; this suggested that 3 or 4 glycoproteins were greatly reduced in this region in the infantile NCL. The radioactivity derived from [^{3}H]GlcN and [^{3}H]Man in the infantile NCL and control was released by PNGase F, demonstrating that these sugars were incorporated into constituents of N-linked oligosaccharides. In contrast, the juvenile NCL was similar to control in the M_r = 120–140 kDa region. The reduction of the M_r = 120–140 kDa glycoproteins in the infantile NCL may be due to a lack of synthesis or increased degradation of the defective components. Since the infantile and juvenile forms of NCL differed in the components of M_r = 12O–140 kDa, this may be helpful in the diagnosis of these forms of NCL. This research was funded by NYSOMRDD and NIH Grant 23717–03.

8.67
LASER DESORPTION AND ELECTROSPRAY MASS SPECTROMETRY IN THE ANALYSIS OF THE SCRAPIE PRION PROTEIN

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The scrapie prion protein (PrP^{Sc}) has been implicated in the development of a number of animal and human neurodegenerative diseases. It has a phosphoinositol glycolipid anchor at the C-terminus and the majority of molecules are doubly glycosylated at Asn-181 and 197. It is encoded by a single-copy gene that also encodes a normal cellular protein (PrP^C). Detailed analysis of these proteins by enzyme digestion, HPLC separation and characterization of the resulting peptides by amino acid analysis, Edman sequencing and LSIMS has so far failed to reveal any differences between them and the structures predicted from the gene sequence. However, there is a danger that labile groups may be lost in the various steps involved in such degradative studies. In order to assure that the global structural nature of this complex glycoprotein glycolipid glycan is established completely, we have used matrix-assisted laser desorption (LD) and electrospray (ESP) to study the intact proteins and larger peptides to reveal post-translational modifications that introduce significant mass differences. Proteinase K removes approximately 65 amino acids from the N-terminus of PrP^{Sc} to leave PrP 27–30, a smaller protein that retains the N-linked oligosaccharides and the glycolipid anchor. For example, the laser desorption spectrum reveals three broad unresolved humps which can be attributed to the heterogeneity of both the oligosaccharides and the anchor glycan and the presence of some unglycosylated material. A 75-amino acid residue containing the first glycosylation site was obtained from digestion with Endoproteinase Lys-C and HPLC separation. The LD spectrum is still poorly mass resolved but shows sufficient structure to give mass assignments for glycopeptides with 4–5 different oligosaccharides. By contrast, electrospray provides much better mass resolution and gives molecular masses for at least 28 different glycopeptides in the range 10256–11388 Da. After deglycosylation with PNGase F, ESP gives the predicted mass of 8606 Da, which confirms that the heterogeneity is entirely due to the oligosaccharides.

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8.68
5′-β-GALACTOSYLATION OF NUCLEOSIDES 1-β-D-ARABINOFURANOSYLCYTOSINE (ARAC) AND 1-β-D-2-DEOXYRIBOFURANOSYLCYTOSINE

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Future cancer chemotherapy may benefit from tissue insertion of genes capable of enhancing either the sensitivity of cancer cells to cytotoxic drugs or the resistance of normal cells to the same or different drugs (1). The ultimate goal of the current study is to explore the possibility that the E. coli β-galactosidase (lacZ) gene may serve both these purposes by enabling cells to remove a masking galactose from a cytotoxic drug (β-cytosine arabinoside) or from an antagonist of this drug (2-deoxycytidine). To permit a test of this possibility, we investigated chemical synthesis of 5′-β-galactosylated AraC and 2-deoxycytidine.

Glycosylation of the primary hydroxyl in nucleosides is surprisingly difficult and under common reaction conditions (using an ester protecting group at C-2) an orthoester is formed (2). Low yields of 5′-glycosides of 2-deoxy-nucleosides such as thymidine were obtained using Bredereck conditions (2). Since an interaction between the primary hydroxyl and the heterocyclic base is probably responsible for the decreased reactivity of the hydroxyl, we have investigated acid glycosylation conditions that should overcome these interactions. Thus, the desired galactosides are formed using galactosyl trichloroacetimidate activated by boron trifluoride (3) as glycosyl donors but they are accompanied by 5′-acetates, the products of acetyl transfer from the galactosylation reagent. The peracetates of the desired compounds were obtained pure by chromatography on Sephadex LH 20 and were deprotected by base methanolysis.

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8.69

CHARACTERIZATION OF MONOCLONAL ANTIBODY-DEFINED GLYCOLIPID ANTIGENS WHICH CORRELATE WITH THE EXPERIMENTAL METASTATIC ABILITY OF KHT FIBROSARCOMA CELLS

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We have examined the binding and functional activity of monoclonal antibody (MAb) SG-1 that was raised by immunization against embryonal carcinoma cells and screened using KHT fibrosarcoma cells. Quantitative absorption, binding, and *in situ* immunochemical staining assays indicate that the MAb SG-1-defined epitopes are expressed preferentially by the highly metastatic KHT35-L1 cells relative to the weakly metastatic parental cells. Furthermore, there was a significant correlation ($p < 0.05$) between the expression of MAb SG-1-defined antigen on the cells and their metastatic ability. Binding of MAb SG-1 to antigen was inhibited by specific sulfated glycans including cerebroside sulfate (brain sulfatide), and fucoidan, but not by heparan, chondroitin, keratan, or dextran (5 kD) sulfates.

The differential expression of this antigen in KHT35-L1 vs. KHTp cells was primarily detected in the chloroform/methanol extracts of these cells. The initial characterization of the antigens was done on chloroform/methanol extracts from KHT35-L1 cells. The extracts were subjected to reversed-phase chromatography on a C18 column using methanol-acetonitrile (1:1) mobile phase. The separation was monitored by MAb SG-1. The antigen-containing fractions were rechromatographed using methanol acetonitrile (4:1). The purified antigens were investigated by Fast Atom Bombardment Mass Spectrometry. The structural analysis of these glycolipid antigens will be discussed.

8.70

UTILIZATION OF THOMSEN-FRIEDENREICH ANTIGEN FOR POPULATION SCREENING FOR COLORECTAL CANCER

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Colorectal cancer is the second most common cause of tumour deaths in both men and women. One approach to reduce mortality from this disease is to diagnose the tumour in its localized, asymptotic stage, when it is still amenable to cure. It has been shown that colorectal neoplasms secrete a glycoprotein containing the disaccharide D-Galp (β1-3)-D-GalpNAc (α1-Ser/Thr) (= T-antigen) that is not present in normal colonic mucosa. It has been reported (1) that the T-antigen can be detected in rectal mucin by a simple test, based on biochemical analysis of a filter disc smeared with mucin obtained during a routine digital rectal examination. To produce a clinically useful screening test, a modification of the mucin test has been developed in our laboratory. It has a sensitivity approaching 100% for cancer, and 91% for adenomatous polyps. The test is based on oxidation of OH-6 of gal and galNAc with galactose oxidase and the development of colour reaction on treatment of the product with Schiff reagent. The intensity of colour depends, in addition to the quantity of saccharides, on the length of reaction with galactose oxidase and with Schiff reagent. The background coloration which often obscures the true result of the test critically depends on the purity of the reagent, of water used, and the material from which the supporting filters are made. The major problem in optimization test conditions is that it is impossible to obtain enough material from human subjects and thus standarized conditions have been worked out using asialo fetuin and synthetic model substances.

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8.71

CHARACTERIZATION OF A M_r 120000 MEMBRANE GLYCOPROTEIN PURIFIED FROM MORRIS HEPATOMA 7777

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A M_r 120000 glycoprotein (gp 120) was purified to homogeneity from Morris Hepatoma 7777 plasma membranes by use of ConA-affinity chromatography and semipreparative SDS-electrophoresis. Antibodies raised against this purified protein recognized gp 120 monospecifically as evidenced by 2-dimensional gel electrophoresis followed by immunoblot-detection. Expression of gp 120 was not restricted to malignant cells but was also found in hepatocytes. The polypeptides from both cell types displayed similar molecular properties: a M_r of 120000, which subsequently shifted to 110000 on treatment with sialidase and to 97000 after complete removal of N-glycans with PNGase F indicating a glycosylation with 6-7 N-linked carbohydrate chains. In pulse-chase experiments biosynthesis and flow-kinetics of gp 120 were studied: gp 120 was transported from the endoplasmic reticulum to the Golgi apparatus within 30 minutes as indirectly assessed by its acquisition of endo H-resistance, thus resembling other cell surface proteins. Transport to the cell surface was examined by surface labelling of accessible glycoproteins using a cleavable biotin derivative (NHS-SS biotin). Gp 120 was detectable on the cell surface after 1 h of chase. Interestingly, gp 120 was degraded in a glycosylation-dependent manner: gp 120 with complex-type N-glycans had the longest half life, whereas 1-Deoxymannojirimycin induced variants with high mannose glycans was degraded more rapidly. Non-glycosylated gp 120 synthesized in the presence of tunicamycin had the shortest half life. Gp 120 was exclusively expressed on Morris hepatoma 7777 cell clones of the adherent phenotype (A, N→A) but was absent in adhesion-defective cell lines (N, A→N) indicating gp 120 might be involved in cell adhesion.

8.72

UNCOVERED Galβ-3GalNAc-α-*O*-Ser/Thr (T) AND ITS IMMEDIATE PRECURSOR EPITOPE (EP) Tn ARE PIVOTAL IN CARCINOMA (CA) PATHOGENESIS, DIAGNOSIS AND PROGNOSIS. HUMAN T (Tn) ANTIGEN (Ag) IS EFFECTIVE AS CA VACCINE

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T & Tn EPs are immediate precursors of human histo-blood group N & M immunodominant structures. T & Tn occur in epithelial & blood cell

membranes *occluded* from the immune system of persons without CA; hence, everyone has anti-CA-T & -Tn antibodies (Abs) due to intestinal flora. T & Tn are found in 85% of primary CAs & in corresponding metastases excised up to >6 yrs. Differentiated CAs have orderly T expression; Tn predominates in anaplastic CAs.-CA-T & -Tn are adhesion-motility molecules. Adhesion is specifically & concentration-dependently inhibited by T & Tn EPs. – *Immune responses*: *Humoral*: At initial visit, anti-T assays detected 96% of 26 incipient clinical pTis & $T_1N_0M_0$ CAs, and 89.5% of 195 St. I-IV CAs; 90% of 146 non-CA persons were negative. Anti-T is carcinolytic. – *Cellular*: delayed skin reaction to healthy O RBC-derived T(Tn) Ags detected 85.5% of 41 pTis & $T_1N_0M_0$ and also 399 St. I-IV CA patients (pts). All p: < 0.0001. – Of 32 subjects reacting repeatedly + in our tests but – on biopsy/X-ray, 23 (72%) have so far developed biopsy-verified CA at the originally suspected site, months to 10 yrs later. Probability of developing + bioptic CA within 9 yrs in pts with + anti-T tests but – biopsy is >90%. Anti-T tests are more reliable in early CA detection than any other tests.

SURVIVAL STATISTICS [*U.S. NCI; PDQ, 1990*]: breast CA 5 yr post-surg., post-standard treatment: St. IV 10%; St. III 41%. At 10 yrs, survival additionally decreases ~50%.

Therapeutic efficacy, post-standard therapy, of *active specific T Ag vaccination* (*ASTI*): *5 yr survival* breast CA pts: St. IV: 5/5 without other therapy were 100% clinically well, 4 NED, 1 HO with metastases, stable >3 yrs by ASTI alone. – St. III: 6/6 (100%), 4 NED, 2 HO. – *10 yr survival*: as of 01.1991 all NED St. IV pts are 13.5 to 15.8 yrs post-op; 1, HO, has not yet reached 10 yrs. – St. III: 3 NED >10 yrs; 1 now at 6.3 yrs; 2 with >12,000 Rad & intermittent chemotherapy died <7 yrs post-op. – St. II: 4/5 NED after 10 yrs, 1 HO clinical status (100%) [NCI statistic: 54% survival].

Probability that the combined ASTI results are due to chance: 5 yrs, $p < 10^{-7}$; 10 yrs, $p < 10^{-8}$. (Aid: NCI CA 22540 & CA 19083; H.M. Bligh Cancer Fund.)

S9. GLYCOSAMINOGLYCANS/GLYCOSAMINOGLYCANNES

9.1
MODULATION OF SACCHARIDE STRUCTURE IN THE BIOSYNTHESIS OF HEPARIN AND HEPARAN SULFATE

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The biosynthesis of heparin (and heparin sulfate) is initiated by the formation of polysaccharide chains, largely composed of alternating GlcA and GlcNAc units, that occur in proteoglycan structures. Subsequent modification reactions involve N-deacetylation/N-sulfation of GlcN units, C5 epimerization of GlcA to IdoA units and O-sulfation at various positions [Lindahl *et al.* (1986) Trend Biochem. Sci. **11**, 221–225). Then structural complexity of the final products, heparin or heparan sulfate, is due to the fact that the various reactions are incomplete; thus, at each reaction step a fraction of the potential target units escape modification. The mechanisms by which certain residues are selected for modification are only partially understood, but depend to a large extent on the substrate specificites on the enzymes involved in the process. Recent results of enzyme purification (from mouse mastocytoma tissue) point to the importance of specific protein interactions in the regulation of polymer modification. These findings will be discussed in relation to the generation of two types of saccharide sequences that have been implicated in the inhibition of blood coagulation and inhibition of angiogenesis, respectively.

9.2
UNIQUE DERMATAN SULFATE PROTEOGLYCANS FROM THE HYPERTROPHIC SCAR OF A BURN PATIENT WITH EHLERS-DANLOS SYNDROME

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Hypertrophic scar (HSc) is an abnormally healed skin that extends beyond the confines of the original wound. It is characterized by excessive deposition of dermal collagen in bundles of whorl-like nodules. Proteoglycans (PGs), conjugates of species-specific core proteins and glyosaminoglycans (GAGs) side chains, have been shown to play an important role in the rate and organization of collagen fibrillogenisis. Studies in our laboratory demonstrated HSc to contain elevated amounts of PGs in comparison to normal skin, and changes in their structure and distribution (Garg et al., Biomed. Res. 10, 197–207, 1989; Collagen Rel. Res., 8, 295–313, 1988). Thus alterations in collagen organization in burn scar tissues may result from modifications in PC structure and distribution.

Ehlers-Danlos syndrome (EDS) is a disease of collagen synthesis, which has recently been ascribed to a molecular defect in PGs. Thus, in hopes of clarifying the role of PGs in collagen organization, small PGs from burn HSc from a patient with EDS were isolated and characterized as follows. The HSc was extracted with 4M Gdm.cl and PGs were isolated by DEAE-cellulose chromatography. Fractionation of PGs with differential ethanol precipitation gave two types of PGs; one rich in glucuronic acid (PG_{GlcA}) and a second, rich in iduronic acid (PG_{IdoA}). Mr values of the GAG side chains of these PGs were 27 and 24.8 KDa, respectively. Testicular hyaluronidase treatment of these PGs gave different sizes for the largest oligosaccharide. The GAG chains of $PG_{Glc}A$ and $PG_{Ido}A$ were composed of GlcA-GalNAC (84%) and IdoA-GalNAC (95%) dissaccharide units, respectively. Chondroitinase by ABC digestion of both PGs gave a single core protein of similar Mr value. Work aimed at identifying the regulatory mechanism for expression of the various PGs is currently underway.

This work was supported by a grant from Shriners Hospital for Crippled Children.

9.3
CHEMICAL PROPERTIES OF RAT SKIN GLYCOSAMINOGLYCANS

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Dermatan sulfate (DS45) of seven-weeks-old albino rat skin was analyzed by Fourier transform, proton nuclear magnetic resonance spectroscopy in one dimension at 500 MHz and 60°C. Integration values of resonance intensities of the H-1 and acetamido methyl protons of D-GalNAc were employed to estimate the percentage N-acetylation of

this sugar (M.O. Longas, 1990, *Anal. Biochem.*, 187:355–358). Uronic acid was quantified, by using integration values of H-1 signals of L-IdUA and D-GlcUA. Ratios of uronic acid to hexosamine were calculated by the same technique. All rat skin glycosaminoglycans (GAG) were assayed for sulfate by infrared spectroscopy (M.O. Longas & and K.O. Breitweiser, 1991, *Anal. Biochem.*, 188:193–196). The data showed N-acetylation of $GalNH_2$ In DS45 to be 63% (mol/mol) in the control (group I), 70% in the animals irradiated with ultraviolet (UV) light (group II), and about 65% in those whose diets contained 2,000 IU of vitamin E/kg of daily food and were also exposed to UV light (group III). IdUA of DS45 was around 32% (mol/mol) of total uronic acid, except in group III which had a 44%. Uronic acid/hexosamine ratios were: 2.5:1, 2.4:1 and 1.6:1 in groups I, II and III, respectively. Sulfate of DS18 was about 17% (w/w) in all groups studied. Chondroitin 4-sulfate of group I had 28% sulfate which increased to 44% in group II and dropped to 26% in group III. DS45 was resolved into two species, DS45a and DS45b, by electrophoresis. In DS45a of groups I, II and III, sulfate was 24%, 30% and 21% (w/w) respectively. DS45b of the control had a 21% sulfate which rose to 35% and 28% in groups II and III, respectively. The data indicated sulfate to be the GAG component most affected by UV light irradiation of the skin. These effects were reversed, except in DS45b, by dietary vitamin E.

9.4
NOVEL FUCOSE-RICH GLYCOSAMINOGLYCANS FROM SQUID INK

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The amounts and features of glycosaminoglycans (GAGs) in six tissues of four kinds of squid, *Todarodes pacificus*, *Nototodaus sloani*, *Illex argentinus* and *Ommastrephes bartrami*, were examined by one-, two-dimensional cellulose acetate membrane electrophoresis and sequential enzyme digestion using GAG saccharidases. These results showed that squid ink contained novel type of GAGs, which were not digested by hyaluronidase, chondroitinase ACII, chondroitinase ABC and other GAGs saccharidases.

Then, three fucose-rich GAGs, M-1, M-2 and M-3, were isolated from the ink of squid, *Illex argentinus*, by the method of actinase digestion, DEAE Sephadex A-50 ion exchange and Sephacryl S-300 gel chromatographies. M-1, M-2 and M-3 gave a single band migrating slower than hyaluronic acid on the cellulose acetate membrane electrophoresis, and were composed from equal molar ratios of Fuc:GlcU:GalNAc (1:1:1). Molecular weight of M-1, M-2, and M-3 were determined by Sephacryl S-300 gel chromatography to be 50,000, 50,000–80,000, and 80,000, respectively.

M-1, M-2 and M-3 were not able to be digested with α-L-fucosidase, hyaluronidase, chondroitinase ACII and chondroitinase ABC. By the mild acid treatment of M-2 with 0.1 N H_2SO_4 at 80°C for 12 h, M-2 was quantitatively changed into two subunits with molecular weight of 1,800 and 1,300 with the release of trace amount of Fuc. On the basis of 400 MHz ^{1}H-NMR spectra of M-1, M-2, M-3, and their carboxy-reduced derivatives, it was suggested that the existence of α-linkage of GlcU (M-series H-1; 5.219 ppm, carboxy-reduced derivatives H-1; 5.266 ppm), α-linkage of Fuc(M-series H-1; 5.289 ppm, carboxy-reduced derivatives H-1; 5.299 ppm), and β-linkage of GalNAc(M-series H-1; 4.561 ppm, carboxy-reduced derivatives H-1; 5.581 ppm).

These results indicated that Fuc-containing GAGs are distinct from any known GAGs.

9.5
GLYCOSYL TRANSFERASE REACTIONS IN THE BIOSYNTHESIS OF HEPARIN

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The biosynthesis of heparin (and heparan sulfate) is initiated by the formation of polysaccharide chains, essentially composed of alternating GlcA and GlcNAc units, that occur in proteoglycan structures. Subsequent modification reactions involve N-deacetylation N-sulfation of GlcN units, C5 epimerization of GlcA to IdoA units and O-sulfation at various positions (Lindahl *et al.* (1986) Trends Biochem. Sci. **11**, 221–225).

The GlcA and GlcNAc transferases catalyzing the polymerization reaction were solubilized (1% Triton X-100) from a mouse mastocytoma microsomal fraction, and used to study the transfer of monosaccharide units from the corresponding UDP-sugars to exogenous oligosaccharide acceptors. Oligosaccharides of the appropriate structure were obtained by partial N-deacetylation (hydrazinolysis) of *E. coli* K5 capsular polysaccharide, which was then subjected to deaminative cleavage by HNO_2. The resulting GlcNAc acceptors were digested with β-D-glucuronidase to yield GlcA acceptors.

The K_M of the GlcA transferase for a trideca acceptor (~600 μM) was about 100-fold greater than that of the GlcNAc transferase for a hexadeca acceptor (~4 μM). However, contrary to the GlcA transferase, the GlcNAc transferase was strongly dependent on the molecular size of the oligosaccharide substrate, the GlcNAc acceptor ability decreasing with decreasing chain length. The GlcA transferase showed appreciable activity in the absence of added divalent metal ions but required Mg^{2+} and Ca^{2+} for optimal activity. The GlcNAc transferase showed an absolute requirement for Mn^{2+} ions.

Use of partially N-sulfated oligosaccharides as substrates showed that GlcNAc transfer was essentially unaffected by the N-substituent ($-NSO_3$ or -NAc) patterns of the oligosaccharide acceptors. In contrast, the GlcA transferase showed a marked preference for acceptor molecules with a nonreducing terminal GlcNAc-GlcA-$GlcNSO_3$-sequence. These findings suggest that the chain elongation and N-deacetylation/N-sulfation reactions are coupled, and may explain the stimulatory effect of concurrent sulfation on chain elongation observed in previous experiments with intact microsomal fraction (Lidholt *et al.* (1989) Biochem. J. **261**, 999–1007).

9.6
PARTIAL PURIFICATION OF O-SULFOTRANSFERASES INVOLVED IN THE BIOSYNTHESIS OF HEPARIN

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The biosynthesis of heparin (and heparan sulfate) is initiated by the formation of polysaccharide chains, essentially composed of alternating GlcA and GlcNAc units, that occur in proteoglycan structures. Subsequent modification reactions involve N-deacetylation/N-sulfation of GlcN units, C5 epimerization of GlcA to IdoA units and O-sulfation at various positions (Lindahl *et al.* (1986) Trends Biochem. Sci. **11**, 221–225). This report is concerned with the purification of O-sulfotransferases from mouse mastocytoma tissue. Enzyme activity was assayed using 3′-phosphoadenosine-5′-phospho[^{35}S]sulfate (PAPS) as sulfate donor and N/O-desulfated, re-N-sulfated heparin as O-sulfate acceptor.

Tumors were homogenized in the presence of 1% Triton-X100, and the solubilized proteins were fractionated by consecutive chromatographies on columns of heparin-Sepharose, Blue Sepharose, 3′,5′-ADP-

agarose, and DEAE-Sephacel. About 22% of the enzyme activity in the initial tumor extract was recovered in the final fraction, with an apparent >100,000-fold increase in specific activity. This material catalyzed sulfate transfer to C2 of IdoA and to C6 of GlcN units, i.e. the major O-sulfotransferase reactions in heparin biosynthesis.

The activity of the purified preparation emerged as a distinct, symmetrical peak with a K_{av} of ~0.30 on Sephacryl S-300 chromatography (in the presence of 0.1% deoxycholate), suggesting a M_r >100,000. Analysis of this material by SDS-PAGE yielded silver-staining products in the M_r range of 20–30,000. These observations point to the possibility that the native O-sulfotransferases occur as an enzyme complex composed of two or more polypeptides.

9.7
PURIFICATION AND PROPERTIES OF THREE NOVEL CHONDROITINASES

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A bacterium newly isolated from soil, *Flavobacterium* sp. Hp102, produced three kinds of galactosaminoglycan degrading enzymes designated chondroitinase (Chase)s AC III, C and B II. The enzymes were extracted from the cells cultivated with chondroitin sulfate (CS) as an inducer, and isolated using hydroxyapatite, Sulfate-Cellulofine and Sephacryl S-200 columns.

The purified enzymes showed maximum activities at pH 6 to 7 (AC III) and 8 to 9 (C, B II). They were most active at 45°C (AC III) and 50°C (C, B II). Chase AC III was activated by Ca^{2+} and Ba^{2+} and inhibited by Zn^{2+} while Chase B II was strongly activated by Ca^{2+} and inhibited by Ba^{2+}, Co^{2+} and Zn^{2+}.

Chase AC III acted on hyaluronic acid (HA) producing unsaturated di-, tetra- and hexasaccharides. It also acted on various types of CS and produced ΔDi-OS, ΔDi-4S and ΔDi-6S, but no ΔDi-diS. Unlike previous Chases AC (*F. heparinum*, *A. aurescens*), this enzyme seems not to be able to cleave the linkage to disulfated disaccharides.

Chase C acted on CS producing two kinds of disaccharides, ΔDi-6S and ΔDi-diS_D. The disaccharide units possessing C4-sulfated N-acetylgalactosamine such as ΔDi-4S, ΔDi-diS_E and ΔDi-diS_B were still remained in oligosaccharides of the digests. It acted on chondroitin producing mainly ΔTetra- and ΔHexasaccharides, and never acted on HA.

Chase B II acted on only dermatan sulfate (DS) producing ΔDi-, ΔTetra- and higher oligosaccharides. The disaccharides produced with Chase B II were ΔDi-4S and ΔDi-diS_B. Dermatan (chemically desulfated DS) was resistant to this enzyme. These substrate specificities resembles apparently those of Chase B from *F. heparinum*.

These three enzymes might be useful for processing the galactosaminoglycan if they are used selectively with previous chondroitinases.

9.8
ENDOCYTOSIS OF SMALL DERMATAN SULPHATE PROTEOGLYCAN II (DECORIN)

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Decorin is efficiently internalized by fibroblasts and osteosarcoma cells by receptor-mediated endocytosis. This process, in which decorin core protein-binding proteins of 51 and 26 kDa have been implicated, is followed by intralysosomal degradation of the proteoglycan.

Both, endocytosis of decorin and binding to the 51 and 26 kDa proteins can be inhibited by heparin. Data on the inhibition of endocytosis suggest a partially competitive inhibition mechanism, which is compatible with the existence of two binding sites on the endocytosis receptor, one for decorin core protein and one for heparin. Binding of heparin to the 51 and 26 kDa proteins could be demonstrated directly by Western blotting and by affinity chromatography on immobilized heparin.

In spite of the high affinity binding of heparin to the putative decorin endocytosis receptor (K_D about 5 nM) heparin itself is only poorly internalized and degraded by fibroblasts. In contrast to decorin, binding of heparin to the 51 and 26 kDa proteins is insensitive to acidic pH, thus presumably preventing its dissociation from the receptor in the endosome. Instead of being delivered to the lysosome, internalized heparin is recycled to the cell surface.

In contrast to fibroblasts, human keratinocytes only barely endocytose decorin. Whereas total cell extracts contain similar amounts of the 51 and 26 kDa proteins as osteosarcoma cells, endosomal fractions are almost devoid of them. Keratinocytes synthesize and secrete predominantly heparan sulphate proteoglycans which inhibit binding of decorin to the 51 and 26 kDa proteins. In the presence of heparinase, the efficiency of decorin uptake by keratinocytes is significantly increased. It is therefore suggested that binding of cell surface associated heparan sulphate proteoglycan alters the intracellular routing of the receptor.

9.9
HEPARAN SULFATE GLYCOSAMINOGLYCANS FROM CHINESE HAMSTER OVARY CELLS ARE CLEAVED BY A HEPARANASE

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Evidence suggests that Chinese hamster ovary (CHO) cells contain a heparanase, since newly synthesized heparan sulfate glycosaminoglycan chains are large, but the steady-state chains isolated from the cells are both large and small. To determine whether the small heparan sulfate chains were derived from the large species, CHO cells were pulsed with $^{35}SO_4$ for one hour to label the glycosaminoglycan chains, chased in media containing unlabeled sulfate for 0 to 12 hours, and the ^{35}S-heparan sulfate glycosaminoglycans in the cells were isolated, and analyzed for size. Initially, all the heparan sulfate was large, but with increasing chase time the chains became smaller, indicating that the glycosaminoglycans were being cleaved by a probable heparanase. The glycosaminoglycans must be inside the cell to be cleaved, since trypsin accessible or cell surface heparan sulfate remained the same size through out the chase period.

An assay for the putative CHO heparanase was developed by separating small heparan sulfate chains (enzyme product) from large chains (substrate) by precipitation with cetylpyridinium chloride (CPC). When large CHO ^{35}S-heparan sulfate chains were incubated with homogenates prepared from CHO cells, ^{35}S-counts were released into the CPC supernatant, indicating that the chains had been cleaved. The heparanase in CHO cells appears to be enclosed in some type of vesicle, since enzyme activity was slightly elevated when detergent was added to the cell homogenate, and activity could be released into the supernatant when the cell membranes were broken and the homogenate centrifuged at 15,000 × g. The optimal pH for the CHO heparanase activity is pH 5.8–6.0, which is similar to the optimal pH of heparanases in rat liver, platelets and placenta. Thus, it appears that the heparanase activity measured in CHO cells is similar to the enzymes described in other tissues.

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9.10

SPONTANEOUS AND TGFβ1 INDUCED ACTIVATION/TRANSFORMATION OF RAT LIVER PERISINUSOIDAL LIPOCYTES IS ASSOCIATED WITH AN ENHANCED EXPRESSION OF BIGLYCAN AND DECORIN

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Perisinusoidal lipocytes (PL) were shown to produce significant amounts of several extracellular matrix components and are suggested to play a central role in liver fibrogenesis. In toxic liver injury but also in culture on uncoated plastic materials PL loose their differentiated phenotype and transform within 2 weeks into highly activated myofibroblast-like cells (MFBIC). Using highly purified PL in monolayer we show in the present study that (i) PI synthesize and secrete chondroitin- and dermatan-sulfate rich proteoglycans (PG), (ii) TGFβI and cell transformation induce the PG synthesis and (iii) the newly synthesized PGs were identified as decorin and biglycan.

PL were prepared from normal rat livers by the pronase-collagenase method and cultured in Dulbecco's modified Eagles medium with 0.5%–10% fetal calf serum. Intracellular, extracellular and membrane bound fractions of proteoglycans were measured in PL at different time intervals after seeding and in MFBIC (after passage). Labeled ([^{35}S]sulfate) glycosaminoglycans (GAG) were isolated by binding to DEAE-Sephacel, eluted, washed and measured in a β-counter. Specific GAGs were measured after enzymatic and chemical degradation of total PG. PGs and PG-core proteins (chondroitinase ABC) were determined by fluorography ([^{3}H]leucin) after SDS-PAGE.

Cell surface- and medium-GAG synthesis increase gradually in primary cultured PL whereas intracellular GAG levels decrease. Compared to PL MFBIC synthesize and secrete about 19-fold more GAG than untransformed PL. By determination of the GAG subfractions [chondroitin-sulfate (CS), dermatan-sulfate (DS) and heparan-sulfate (HS)] it was shown that predominantly the synthesis of CS and DS increases during transformation (CS about 20-fold, DS about 8-fold, HS about 3-fold). The enhanced GAG synthesis during culture was stimulated further by TGFβ1. The majority of the newly synthesized PGs were identified as decorin and biglycan having PG-core proteins of 52 kD and 48 kD. In summary our results raise the possibility that an enhanced extracellular matrix synthesis by PL in vivo requires cell activation and is under control of TGFβ.

9.11

SEPARATION AND QUANTITATION OF CHONDROITINASE DIGESTION PRODUCTS BY HPLC

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The use of high pressure liquid chromatography (HPLC) for the identification and quantitation of unsaturated disaccharides produced by bacterial chondroitinases is a powerful tool for the identification and structural analysis of glycosaminoglycans. In this report we describe a system capable of resolving all of the known unsaturated disaccharides derived from the chondroitin sulfates, dermatan sulfate, and hyaluronic acid. This system is superior to others in that the non-sulfated and mono-, di-, and tri-sulfated disaccharides can be separated with good resolution in approximately 40 minutes in an isocratic solvent. The system employs an amino-cyano silica gel column (Whatman Partisil 5 PAC, 25 cm) and is eluted with an isocratic solvent consisting of 48% (v/v) acetonitrile, 14% (v/v) methanol, and 38% (v/v) aqueous buffer. This buffer contains 0.5 M Tris HCl, 0.1 M boric acid, 23.4 mM sulfuric acid, pH 8.0. U.V. absorption is monitored at 232 nm and for most disaccharides as little as 150 ng can be reliably determined. The addition of boric acid to the eluent is essential for good resolution of all components and the addition of low concentrations of sulfuric acid is used to control the elution times of various components. The system was applied to the analysis of glycosaminoglycan standards and excellent agreement with previous compositional analyses was obtained. This work was supported by funds from the N.Y.S. Office of Mental Retardation and Developmental Disabilities and by N.I.H. Grant NS 23717–03.

9.12

GLYCOSAMINOGLYCAN AND SUGAR ANALYSES OF NORMAL AND ALZHEIMER DISEASE BRAIN SECTIONS

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The brains of patients with Alzheimer Disease are characterized by the accumulation of neurofibrillary tangles and amyloid plaques. Using specific antibodies, it was observed that both heparan sulfate and dermatan sulfate proteoglycans accumulate very early in neurofibrillary tangle and plaque deposition (Snow et. al., Am. J. Path. 135: 1253–1270, 1990; Snow et. al., J. Cell Biology 111:267A, 1990). In this work proteoglycans were prepared from frontal cortex brain sections by homogenization in a buffered detergent, clarified by centrifugation and the resultant supernatant fractionated by step gradient ion exchange chromatography on DEAE Sephacel (Klinger et. al., J. Biol. Chem. 260:4082–4090, 1985).

The 0.26 M, 0.40 M, and 1.0 M NaCl fractions were analyzed by appropriate HPLC methods for 1) neutral sugars produced by 2N TFA (4 hrs, 100°C) hydrolysis and 2) disaccharides of various glycosaminoglycans after digestion with chondroitinase ABC, chondroitinase AC I, chondroitinase AC II, and heparitinase plus heparinase.

Analyses of neutral sugars showed relatively small changes in the total amount in the 0.26 M, 0.4 M, and 1.0 M fractions of the normal and Alzheimer brain when normalized to protein content. However, fucose was elevated, both quantitatively and relative to the other sugars present in all fractions from Alzheimer brain compared to control brains.

No GAGs were detected in the 0.26 M NaCl fraction of either Alzheimer or normal brain. However significant changes were observed in the 0.4 M and 1.0 M NaCl fractions with respect to both the absolute amount of dermatan sulfate present relative to chondroitin sulfate and in the structure of a putative hybrid of the dermatan sulfate and chondroitin sulfate. These same fractions also contained less heparan sulfate in the Alzheimer brain than in normal brains.

This data suggests than an error in processing of both oligosaccharide and glycosaminoglycan metabolism exists in Alzheimer Disease brains.

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9.13

REGIOSELECTIVITY IN THE SULFATION OF SOME CHEMICALLY-MODIFIED HEPARINS, AND OBSERVATIONS ON THEIR CATION-BINDING CHARACTERISTICS

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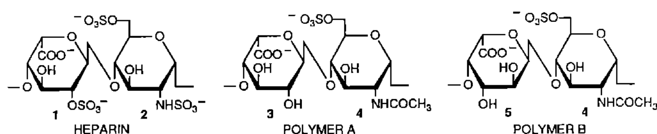
Two modified forms of heparin, polymer A and B, have been prepared,

one containing residues of non-sulfated α-L-iduronic acid (**3**) and the other residues of α-L-galacturonic acid (**5**) in place of the normal 2-sulfate (**1**), and also an *N*-acetyl group (residue **4**) in place of *N*-sulfate (residue **2**). These polymers were subjected to sulfation (SO_3-TMA in DMF) under varying conditions. Examination of the products by NMR spectroscopy showed that A was selectively sulfated initially at position-0 of residue **3**, and that slower substitution occured at position-0 of **4**. By contrast, OH-2 and -3 of residue **3** in dermatan sulfate are equally reactive. Polymer B also exhibited low regioselectivity, as sulfation occured with equal facility at positions-2 and -3 of **5** and -3 of **4**. The sulfation products showed no significant anti Xa activity.

Based on the paramagnetic effects of Cu^{2+} and chemical shift displacements induced by Ca^{2+}, NMR spectroscopy has been used to compare cation-binding properties of A and B with those of heparin. Polymer A formed a complex with Cu^{2+} at a level of $<10^{-3}$ mol per dimeric unit of the polymer, as does heparin, whereas in contrast to the latter and to a modification comprised of residue **2** and **3**, it did not bind Ca^{2+}. Polymer B exhibited no significant binding with either cation.



9.14
PROTEOHEPARAN SULFATE SYNTHESIZED BY HUMAN METASTATIC MELANOMA CELLS

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For a better understanding the alteration of cell microenvironment with malignancy, biosynthetically labeled proteoglycans (PG) were characterized and their metabolic fate was studied in the cultures of highly metastatic A 2058 cell line and of its HT 168 variant with a reduced capacity to form metastases in liver.

At equilibrium state of labeling, at identical cell densities and on cell number basis, the A 2058 cells shed higher amount of proteoheparan sulfate (PHS) and less proteochondroitin sulfate (PCS) into the medium, than the low metastatic counterpart. Trypsin released PHS (~350 KDa) from the pericellular compartment. It contained HS chains (~40 KDa) build up from large (6 KDa) N-acetylated, non sulfated domains and tetra- and disaccharide repeats separated by N-sulfated glucosamine residues. The high ratio of glucuronate to iduronate (8:2) is in agreement with the low N-sulfation. The oligosaccharide patterns obtained on deaminative cleavage of HS did not change with the metastatic capacity. In the trypsinized cell residue HT 168 cells accumulated three times higher amount of HS and HS fragments than the A 2058 cells.

After long term pulse and chase, pericellular PCS was quantitatively recovered in the medium and part of PHS was internalized and degraded by both cells. The HT 168 cells internalized three times higher amount of PHS than the A 2058 cells.

The HS chains obtained on reductive alkaline hydrolysis from the extracellular PHS did not bind fibronectin, laminin or type I collagen fibers nevertheless they could be retained on bFGF-Sepharose affinity column. A reduced capacity to metabolise PHS can be a reason of the increased release of this PG from the cells and this altered metabolism its turn could be involved in the accumulation of HS observed in human melanoma tumors. HS fulfilling the structural requirements to bind FGF may have a function in the control of growth factor activity in the environment of melanoma cells.

9.15
GLYCOSAMINOGLYCANS AND PROTEOGLYCANS FROM DIFFERENT AREAS OF THE BOVINE TEMPOROMANDIBULAR JOINT DISC

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The articular disc of the temporomandibular joint is a pliable, oval, plate-shaped structure, often described as fibrocartilage, which facilitates movement of the mandible during mastication. Because it is subjected to both tensile and compressive forces, it is an interesting connective tissue in which to study the relationship between biochemical composition and mechanical stress. We have previously shown (Nakano, T. and Scott, P.G., Matrix 9:277–283, 1989; Scott, P.G., Nakano, T., Dodd, C.M. Pringle, G.A. and Kuc, I.M., Matrix 9:284–292, 1989) that the disc contains both large ('cartilage-type') chondroitin-sulphate proteoglycans which aggregate with hyaluronic acid and small dermatan sulphate proteoglycans of the DSPG-II (decorin) class.

In the present study we found major differences in composition between tissue from the periphery of the disc and that from the interior. The latter was higher in total uronic acid and contained more chondroitin sulphate and less hyaluronic acid. Comparative immunochemical analyses suggested a higher proportion of keratan sulphate in the inner regions of the disc. By chromatography on Octyl-Sepharose™ the dermatan sulphate proteoglycans could be resolved into two fractions, corresponding to DSPG-I (biglycan) and DSPG-II (decorin), with the latter predominating. The proportion of DSPG-I was higher in the inner tissue and dermatan sulphate chains isolated from these proteoglycans were longer than those from the corresponding proteoglycans isolated from the peripheral tissue. Although the significance of this latter observation is unclear, the other differences are consistent with adaptation of the inner portions of the disc to withstand predominantly compressive forces, while the periphery is presumably subjected to mainly tensile stress.

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9.16
EVIDENCE FOR IMMORTALIZATION INDUCED ALTERATIONS IN THE EXPRESSION OF PROTEOGLYCANS IN E1A-TRANSFECTED RAT FETAL INTESTINAL EPITHELIAL CELLS IN CULTURE

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In this study, comparison was made of the ^{35}S-labeled proteoglycans (PGs) isolated from rat fetal intestinal epithelial cells which were maintained in primary culture (control cells) or were immortalized by transfection with the early E1A region of type 2 adenovirus (SLC-11 cells). The immortalized SLC-11 cells were characterized by the formation of continuous monolayers of closely adherent cells, contact inhibition at confluency, the absence of growth in soft agar or in nude mice, and limited chromosomal rearrangement. Both cell-associated and secreted PGs were analyzed in control and SLC-11 cells. Analysis of the distribution of radiolabeled macromolecules revealed that immortalized cells secreted larger amounts of PGs. Hydrophobic affinity chromatography on octyl-Sepharose of cell-associated PGs indicated a greater proportion of membrane-intercalated PGs in SLC-11 cells than in control cells. PGs isolated from immortalized cells were of smaller hydrodynamic size (determined by chromatography on Sepharose CL-4B) than

those from control cells. However, in control and SLC-11 cells, the Kav values of cell-associated PGs were higher than those for secreted PGs. The glycosaminoglycans (GAGs) carried by the PGs were then isolated by treatment with alkaline borohydride and characterized by their differential susceptibility to GAG degrading enzymes: SLC-11 cells produced cell-associated and secreted heparan sulfate (HS) and chondroitin sulfate (CS) with shorter polysaccharide chain length (determined by chromatography on Sepharose Cl-6B), and with lower degree of sulfation (analyzed by ion-exchange chromatography), than those from control cells. In conclusion, our results demonstrate that E1A-immortalization of rat fetal intestinal epithelial cells markedly alters the expression of PGs with special concern to hydrophobic properties, hydrodynamic size and structural characteristics of their GAG side chains.

9.17
AFFINITY INTERACTION OF CARTILAGE PROTEOGLYCANS WITH HYALURONIC ACID COVALENTLY LINKED TO AGAROSE

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Since the demonstration of the specific interaction between hyaluronic acid (HA) and cartilage proteoglycans (PGs), a large number of interactions have been described for the PGs and glycosaminoglycans (GAGs), both among themselves and with other components of connective tissues including both the soluble and insoluble matrix components, basement membranes and cell surfaces. It was also shown by the present authors that PGs interact non-specifically with insoluble matrices and surfaces (BBRC **167**, 81–88 and Anal. Biochem. **191**, 50–57). These interactions function in the organization of the connective tissue matrix components and there is increasing evidence that they function in the regulation of cellular differentiation and cell function.

In order to study the affinity interaction of HA with the PGs it was first necessary to define experimental systems where the non-specific interactions are eliminated or minimized and to develop a system where the degree of association or interaction between the macromolecules could be precisely measured. We found in our earlier studies a variety of buffers in which the non-specific interactions are minimized or eliminated including the 0.05 M sodium acetate buffer containing 0.5% (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at pH 5.8. In order to study quantitatively the affinity interactions we have used the newly available HA-agarose gels (XL Chemicals; RRI, Edwards, Ontario, Canada) which contain different amounts of covalently bound HA (0–6000 g of HA/g of moist gel cake). The studies were performed by adding labeled PG to HA-Agarose in the above buffer followed by gentle mixing at room temperature on a rotator. After various mixing times the gels were allowed to settle and the supernatant was sampled for radioactivity determination. There was no binding of the PC in HA-Agarose-0 (HA-A-0) (0 μg of HA) over 24 hours but with HA-A-I (25 μg of HA/g of moist gel cake) the radioactivity in the supernatant was reduced to 68% after 2 h of mixing and remained constant over the next 24 h. With higher ligand concentrations binding of the PC by the gel was both faster and more complete so that with HA-A-3 (300 μg of HA/g of gel) only 36% of the activity remained in the supernatant after 2 hours and with HA-A-4–HA-A-6 binding was complete after 10–30 min of mixing. In order to show the reversible nature of this interaction various anionic glycoconjugates were added to the reaction mixtures containing the bound PG and it was shown that excess cold PG, HA and heparin produced solubilization of the bound PG. However, dermatan sulfate and chondroitin sulfate were much less effective.

9.18
ISOLATION AND CHARACTERIZATION OF CHONDROITIN SULFATE LINKAGE OLIGOSACCHARIDES

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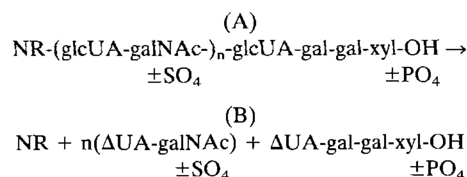
Cultures of rat chondrosarcoma chondrocytes were labeled for 20 h with [^{3}H]glucose. Radiolabeled proteoglycans (PGs) from the medium compartment were purified using dissociative CsCl isopycnic centrifugation (D1 fraction, $\rho > 1.5$ g/ml). PGs were treated with alkaline borohydride and the released chondroitin sulfate (CS) chains separated from O-linked oligosaccharides and N-linked glycopeptides by chromatography on Superose 6. These CS chains (structure A below) were exhaustively digested with chondroitinase AC II to eliminate all the disaccharides in the chain from the linkage region, leaving tetrasaccharides with unsaturated uronic acid at the nonreducing end (structure B below):

NR = nonreducing end sugars; glcUA = glucuronic acid;
galNAc = N-acetylgalactosamine; gal = galactose; xyl-OH = xylitol;
ΔUA = unsaturated uronic acid



The tetrasaccharides were separated from the smaller digestion products by chromatography on Bio-Gel P-4, and then resolved into 2 peaks (B1 and B2) by elution on CarboPac PA1 with a 0.1–1.0 M sodium acetate gradient in 0.1 M NaOH. After treatment with alkaline phosphatase, the more tightly bound peak B2 (1.8% of the ^{3}H in the PG; B2/B1 = 3.5) then eluted in the same position as peak B1. Thus, ~78% of the CS linkage oligosaccharides were phosphorylated. Monosaccharide analysis of peaks B1, B2 and phosphatase-treated B2 yielded gal:xyl-OH ratios of 2.1, 4.2 and 2.1 respectively, indicative of the poor recovery of xyl-OH after acid hydrolysis of its 2-phosphorylated form (1). Mercuric acetate treatment (2) removed the ΔUA from peaks B1 and B2 and the oligosaccharide products, gal-gal-xyl-OH (±PO_4, respectively), were isolated by chromatography on TSK G2500. Subsequent digestion of these mercuric acetate-treated linkage oligosaccharides with β-galactosidase (bovine testes) followed by monosaccharide analysis revealed that both gal residues were removed from the reducing end xyl-OH (±PO_4). These experiments provide methods for removing all of the sugars in CS chains from the reducing terminal xylose and for identifying key intermediate structures derived from the linkage region. These methods are currently being used on intact PGs to isolate xylosylated core protein for further experimentation.

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9.19
HEPATOCYTE MUTANTS DEFICIENT IN O-SULFATION OF HEPARAN SULFATE PROTEOGLYCAN (HSPG) IMPAIR CELL GROWTH OF RLC

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In the studies of metabolism of heparan sulfate proteoglycan (HSPG) in

a rat liver cell line (RLC), it has been found that the cellular HSPG undergoes changes in both sulfate density and heparan sulfate (HS) structure when the cell growth phase changes. This work was undertaken to further study the correlation between HS structure and cell growth. RLC mutants were generated by chemical mutagenesis with ethyl methane sulfate (EMS) and clones were isolated by replica plating on polyester cloth and autoradiographic screening of the colonies for reduced [S-35] sulfate incorporation. The mutants were examined for alterations in metabolism and structure of HSPG and for changes in cell growth behavior. Two mutants, M3 and M6, incorporated the same amount of [H-3] glucosamine into their HS but much reduced levels of [S-35] sulfation as compared to wild type RLC. The sructural analyses of HS revealed that the HS from both mutants had slightly decreased N-sulfation whereas the O-sulfation was reduced to 1/5 to 1/50 of that in the wild type, depending on the type of sulfation. The enzymatic basis accounting for the deficiency of O-sulfation in the mutants has been under investigation. Coordinated with the reduction in O-sulfation, both mutants exhibited longer lag times after plating and higher cell densities after reaching confluence. The growth rate could be partially restored when mutants were fed with wild type HSPG in a chemically defined medium. It is suggested that the O-sulfation may play some role in the regulation of RLC cell growth.

9.20
CARTILAGE FIBROMODULIN IS VARIABLY SUBSTITUTED WITH POLYLACTOSAMINE AND KERATAN SULFATE

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Fibromodulin is a glycoprotein present in the extracellular matrix of a number of connective tissues, including articular cartilage. Its biological function appears to be based on its ability to bind to collagen type I and II during fibrillogenesis, influencing the rate of fibril formation and the resulting fibril thickness. Therefore its interaction with collagen in the extracellular matrix is important for the construction and maintenance of a highly organized collagenous network, which provides mechanical stablity for the tissue and might also influence the shape and arrangements of cells. The fibromodulin core protein consists of globular domains at both the C- and N-termini, which are separated by a series of leucine rich repeat sequences. There are four N-linked glycosylation sites within the leucine rich region. We have isolated each of these sites from bovine articular cartilage fibromodulin, following trypsinization, ion exchange chromatography and HPLC. We have shown that the N-linked oligosaccharides at each of the four glycosylation sites are substituted with extended N-acetyllactosamine sequences. A portion of the polylactosamine chains were also identified in a sulfated form as keratan sulfate. The extent of polylactosamine/keratan sulfate substitution was found to be highest in fibromodulin isolated from epiphyseal cartilage, which contained 35.8 moles of glucosamine and 26.9 moles of galactose per mole of core protein. On the other hand, fibromodulin isolated from mature bovine articular cartilage contained only 17.0 moles glucosamine and 11.4 moles of galactose per mole of core protein. Mannose content for epiphyseal and articular fibromodulin was 11.4 and 12.3 moles per mole core protein respectively. Western blots of GdmHCl extracts of epiphyseal and articular cartilage using a fibromodulin specific antiserum indicated a larger and more heterogenous molecular weight range for epiphyseal fibromodulin (60,000–120,000) when compared to articular fibromodulin, which migrated as a tighter band between 50,000 and 70,000. The age differences were apparently a result of biosynthetic changes, since epiphyseal and mature chondrocytes in culture synthesised fibromodulin characteristic of the tissue source. Further this differential glycosylation of fibromodulin was seen when cells were maintained in ascorbate supplemented culture medium. This would suggest that the interactive properties of fibromodulin with collagen may be controlled by the type of glycosylation of the core protein. In this regard it is interesting that polylactosamine substitution on N-linked oligosaccharides on other collagen binding proteins such as fibronectin and a cell surface glycoprotein PA2 were reported to markedly influence binding of these molecules to collagen.

9.21
THE NOVEL SULFATED GALACTOSE STRUCTURES IN THE CARBOHYDRATE-PROTEIN LINKAGE REGION OF CHONDROITIN 6-SULFATE PROTEOGLYCANS OF SHARK CARTILAGE

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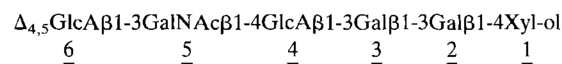
Thirteen different hexasaccharide alditols were isolated from the carbohydrate-protein linkage region of shark cartilage chondroitin 6-sulfate proteoglycans after exhaustive protease digestion, β-elimination and chondroitinase ABC digestion. Their structures were determined by enzymatic digestions in conjunction with HPLC and by 1D and 2D ^{1}H-NMR spectroscopy. They share the conventional common backbone structure:



$\Delta_{4,5}$GlcAβ1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl-ol
6 5 4 3 2 1

One compound has no sulfate or phosphate. Two of the monosulfated compounds have an *O*-sulfate on C-6 or on C-4 of the GalNAc. The third monosulfated compound has an *O*-sulfate on C-6 of Gal-2. The two phosphorylated compounds have an *O*-phosphate on C-2 of the Xyl-ol, and one of them has in addition sulfate on C-6 of the GalNAc. Two disulfated compounds have an *O*-sulfate on C-6 of Gal-2 attached to Xyl-ol in combination with an *O*-sulfate on C-4 or on C-6 of the GalNAc. The third disulfated compound has *O*-sulfate on C-6 of Gal-2, and also on C-6 of Gal-3. Two of the trisulfated compounds have the same disulfated Gal-Gal core with in addition sulfate on C-6 or C-4 of the GalNAc. The other two trisulfated compounds have *O*-sulfate on C-6 of Gal-2, and on C-4 of Gal-3 together with an *O*-sulfate on C-6 or C-4 of the GalNAc.

Previously we demonstrated Gal(4-*O*-sulfate)β1-3Galβ1-4Xyl in the linkage region of chondroitin 4-sulfate from tumorous (1) and normal cartilage (2), but not in heparin (2). In the present study hitherto unrecognized disulfated structures, Gal(6-*O*-sulfate)β1-3Gal(6-*O*-sulfate) and Gal(4-*O*-sulfate)β1-3Gal(6-*O*-sulfate), have been discovered. It has become evident that there are distinct structural differences in the linkage region of various proteoglycans. The sulfated Gal structures may play important role(s) in biosynthesis such as the sorting of galactosaminoglycans and glucosaminoglycans. A part of this work has been presented (3).

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9.22
BINDING OF A BASIC DYE TO GLYCOSAMINOGLYCANS

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Some cationic dyes are known to show metachromasy in the presence of polyanions. Metachromasy is characterized by hypso- and hypochromic shifts in absorption spectra and induction of Cotton effects in circular dichroism spectra. To elucidate the binding mode of dye molecules to polyanionic chain, mixed solutions of toluidine blue (TB) and glycosaminoglycans (GAG) were examined spectrophotometrically. Ultrafiltration was employed to separate metachromatic complexes from free dye or polysacchasides.

TB and GAG were mixed very slowly in an ice bath. The mixture was filtered under mild pressure through Nuclepore membrane, through which either the dye or the polysaccharide could pass without significant absorption when filtered separately. The mixed solution of excess GAG, however, most dye molecules were retained with equivalent amount of GAG, and some polymer were filtered through with small amount of dye. Metachromatic complexes may form large aggregates probably by the interaction between dye molecules. TB bound preferentially to the GAG which already bound some dye molecules. In other words, dye bound to polysaccharide concertedly, probably by hydrophobic interaction between dye molecules.

Saturating amount of TB to a GAG could be determined from the amount retained on a filter membrane. As expected from their chemical compositions, chondroitin sulfates bound two TB molecules per disaccharide repeat. Hyaluronic acid also bound two dye molecules per disaccharide, even though it has only one ionic group. The difference spectrum between original mixture and filtrate was quite similar to that of sulfated GAGs. Extra dye molecules might be hold on the polymer chain through the interaction to the molecule already bound by electrostatic force to the GAG.

9.23
GOLGI SUBFRACTIONATION AND LOCALIZATION OF ENZYMES INVOLVED IN THE SYNTHESIS OF CHONDROITIN SULFATE

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Although extensive information has been obtained in recent years regarding the sub-cellular sites for the synthesis of the oligosaccharide portions of glycoproteins, similar information concerning the sub-cellular sites for synthesis of the glycosaminoglycan portion of proteoglycans such as proteochondroitin sulfate is limited. Thus, chondroitin polymerization has been localized to Golgi, but not defined within Golgi subfractions. Even less is known regarding the sub-cellular localization of the Gal-Gal-Xyl linkage oligosaccharide. In order to obtain such information, membranes from chick embryo epiphyseal cartilage were fractionated by equilibrium sucrose density gradient centrifugation and assayed for galactosyl xylose transferase, chondroitin polymerization and sulfation, as well as the marker enzymes glucose 6-phosphatase, NADH cytochrome c reductase, galactosyl ovalbumin transferase and sialyl transferase. All the Golgi complex activities were found in densities lighter than the sucrose concentration (51%) where the peak of the glucose 6-phosphatase ER marker appeared. The peak of the NADH cytochrome c reductase appeared at 48% sucrose in between glucose 6-phosphatase and galactosyl ovalbumin transferase consistent with its reported *cis* location. Galactosyl ovalbumin transferase and sialyl transferase were distributed identically in two peaks with maximum activities at 44% and 32% sucrose concentrations consistent with *medial* and *trans* location of these enzymes. The order of distribution of chondroitin sulfate synthesis from dense to light membranes correlated with the established sequence of events for its synthesis. The linkage region enzyme viz; galactosyl xylose transferase distributed with NADH cytochrome c reductase in an earlier and heavier *cis* compartment. Chondroitin polymerization and sulfation had a dual distribution similar to the galactosyl ovalbumin transferase and sialyl transferase in later and lighter *medial* and *trans* compartments. The galactosyl xylose transferase had a distribution distinctly different from that of the galactosyl ovalbumin transferase indicating that these distinct enzymes showed no cross reactivity with their respective acceptor substrates. The dual distribution of chondroitin sulfate synthesis was consistent with our previous demonstration of the two nascent proteochondroitin populations produced by microsomal preparations from the same source. The results indicated separate sub-cellular locations for synthesis of the two forms. Thus the initiation of the oligosaccharide linkage appears to be an early *cis* Golgi event, while the chondroitin polymerization and sulfation take place together in later *medial* and *trans* Golgi compartments.

9.24
PROTEOGLYCANS SYNTHESIZED BY RAT PERITUBULAR CELLS IN CULTURE

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In the mammalian testis, the boundary tissue of the seminiferous tubule consists of mesenchymal cells (peritubular cells) separated by a basal lamina from adjacent epithelial cells (Sertoli cells) and germinal cells within the tubule. Peritubular cells and Sertoli cells act conjointly in the formation and deposition of the extracellular matrix (E.C.M).

The purpose of this work was to characterize the proteoglycans (PG) synthesized by highly purified peritubular cells. Confluent rat testicular peritubular cells were incubated for 48 h in the presence of (^{35}S)-sulfate. Culture medium (M) was collected and cell layer was sequentially extracted with: 1°) PBS containing 100 μg/ml heparin (pericellular extract, H); 2°) 0,2% Triton X-100 at neutral pH (membrane and intracellular extract, T); 3°) 4 M urea, 1% Triton X-100 (extracellular matrix extract, U). (^{35}S)-proteoglycans (PG) from medium and cellular extracts were purified by anion exchange chromatography, followed by gel filtration chromatography on Superose 6; the nature of glycosaminoglycan chains was determined by enzymatic and chemical treatment. (^{35}-S) macromolecules from medium exclusively consisted of proteochondroitin with a Superose 6 Kav 0, 12. In H extract, two types of PG were detected: a first type was a proteoheparan eluted in the column void volume, a second type (Superose 6 included, Kav = 0, 10) was enriched in heparan sulfate and contained a minor proportion of chondroitin sulfate (=30%). In Triton X-100 solubilized extract (T), three populations of (^{35}S)-molecules were separated: two proteoheparans with Kav 0, 0 and 0, 10 respectively, and, in addition, a small proteoheparan with little or no core protein and low molecular weight oligosaccharides derived from heparan sulfate chains. Urea extract contained a large Superose 6 excluded proteoheparan, with heparan sulfate chains (Kav = 0, 16 on Superose 6) larger in size than heparan chains detected in proteoglycans of other extracts (Kav 0, 16 and 0, 25 on Superose 6, respectively). Interaction chromatography on octyl-Sepharose and intercalation into liposomes showed that over 60% of Superose 6 excluded proteoheparan from Triton extract was hydrophobe. A significant proportion (54%) of the larger proteoheparan (Kav = 0, 0) from heparin extract was retained on octyl-Sepharose, whereas only minor proportion ($<$10%) of PG isolated from M and U extracts exhibited hydrophobic properties.

Our results indicate that peritubular cells are able to synthesize different forms of PG according to their physico-chemical parameters.

These PG may play a role in the hemato-testis barrier formation and in the interactions with neighbouring Sertoli cells.

9.25

PHORBOL ESTER-INDUCED DECREASE IN GLYCOCONJUGATE SYNTHESIS IN DIFFERENTIATED Caco-2 CELLS

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The human colon cancer cell line Caco-2 cultured *in vitro* displays enterocytic differentiation which was shown to be a growth related event; the typical enterocytic differentiation undergone by Caco-2 cells is characterized by morphological, functional and enzymatic differentiation. Once differentiated, Caco-2 cells exhibit a marked increase in glycosaminoglycan synthesis: the specific activity of glycosaminoglycans which amounted to 2724 ± 150 cpm/μg uronic acid/10^6 cells in non-differentiated cells (day 5 of the culture) rose up to 3750 ± 210 cpm/μg uronic acid/10^6 cells in differentiated cells (day 15 of the culture). This increase in ^{35}S-sulfate incorporation in glycosaminoglycans was markedly reduced by 4-β phorbol 12β-myristate, 13α-acetate (100 ng/ml), a phorbol ester activating protein kinase C (PKC) by promoting its translocation from cytosol to membrane. Similarly, the differentiation-induced increase in glycosaminoglycan sulfation was inhibited (31 ± 5%) by 1,2 dioctanoyl glycerol, a physiological activator of PKC, whereas it remained unchanged in response to 4α-phorbol 12,13 didecanoate (100 ng/ml), an inactive phorbol ester. These results led us to suspect a role for PKC in the regulation of glycosaminoglycan synthesis. To test this possibility, we investigated whether PKC activity is altered during the differentiation process. The total cellular activity of PKC as well as its subcellular distribution were examined from day 5 to day 15. Although the total cellular activity of PKC (23 pmol/min/10^6 cells) did not exhibit any modification whatever the day studied (day 5, 6, 9 and 15), the sub-cellular distribution of the enzyme was profoundly altered: membrane-bound PKC, which represented 55 ± 6% of the total activity of the enzyme in non-differentiated cells, represented only 12 ± 4% in the differentiated ones. Reciprocally, cytosolic PKC exhibited a parallel increase from 45 ± 6% to 88 ± 4% of total PKC activity. Thus the spontaneous differentiation of Caco-2 cells is associated with an increase in glycosaminoglycan sulfation which is concomitant with a redistribution of PKC from membrane to cytosol.

Taken together, our results suggest a subtle regulation of glycoconjugate synthesis in Caco-2 cells, which is probably achieved through a change in the ratio of the active/inactive forms of PKC.

9.26

NEGATIVE AND POSITIVE CIS-ACTING REGULATORY ELEMENTS WITHIN THE 5′ FLANKING REGION OF THE GENE WHICH ENCODES THE SERINE-GLYCINE RICH PROTEOGLYCAN PEPTIDE CORE OF HEMATOPOIETIC CELLS

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The gene that encodes a novel serine-glycine rich proteoglycan peptide core (SG-PG) is selectively expressed by hematopoietic cells that store negatively charged proteoglycans in cytoplasmic granules ionically bound to numerous positively-charged proteins. Using deletion-analysis, various lengths of the 5′ flanking region of the mouse SG-PG gene that all extend upstream of residue +24 were ligated to plasmid DNA that contains a human growth hormone (hGH) reporter gene to identify cis-acting elements that regulate constitutive transcription of the gene. Rat basophilic leukemia-1 (RBL-1) cells, mouse myelomonocytic WEHI-3 cells, rat-1 fibroblasts, and mouse 3T3 fibroblasts were then transfected with the resulting chimeric constructs to compare the regulation of this gene in hematopoietic cells and fibroblasts. A negative regulatory element was located between residues −250 to −118, and a positive regulatory element was located between residues −118 to −81. The negative regulatory element was dominantly active in fibroblasts which do not express SG-PG, whereas the positive regulatory element was dominantly active in hematopoietic cells which express SG-PG. Because a classical regulatory element such as a TATA box is not present immediately 5′ of the transcription-initiation site of the mouse gene, three 504 bp constructs were prepared that had a mutated residue −28, −30, and −38. Based on the amount of hGH produced by cells transfected with the native and mutated chimeric constructs, the proximal element within this atypical promoter resides between residues −40 and −20. As assessed by gel-shift-mobility analyses, the nuclei of RBL-1 cells contain a number of trans-acting factors that interact with the positive and negative cis-acting regulatory elements of the SG-PG gene. Further, some of these RBL-1 cell-derived trans-acting factors appear to be different from those present in the nuclei of rat-1 fibroblasts. These studies indicate that transcription of the SG-PG gene is regulated constitutively by both positive and negative cis-acting elements located 5′ of an atypical promoter.

9.27

BIOCHEMICAL CHARACTERIZATION OF CHONDROITIN SULFATE PROTEOGLYCANS OF BRAIN IDENTIFIED WITH MONOCLONAL ANTIBODIES

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Monoclonal antibodies to five chondroitin sulfate proteoglycans (CSPGs) of rat brain were used for studies on their immunocytochemical localization during cerebellar development, and for the isolation and biochemical characterization of three of the proteoglycans. One of the CSPGs (designated 1D1) consists of a major component with an average molecular size of 300 kDa in 7-day brain, containing a 245 kDa core glycoprotein and an average of three 22 kDa chondroitin sulfate chains. A 1D1 proteoglycan of ≈180 kDa with a 150 kDa core glycoprotein is also present at 7 days, and by 2–3 weeks postnatal this becomes the major species. The concentration of 1D1 decreases during development, from 20% of the total CSPG protein at 7 days postnatal to 6% in adult brain. There is also a developmental change from 20% chondroitin 6-sulfate at 7 days to >97% chondroitin 4-sulfate in adult brain and an increase in chondroitin sulfate chain size from 22 to 32 kDa, together with the appearance in adult brain of the novel *O*-glycosidic mannose-linked oligosaccharides which we have previously described. 3-Sulfated HNK-1 carbohydrate epitopes are present at both ages. The 1D1 proteoglycan contains an associated 45 kDa link protein, and aggregates to a significant extent with hyaluronic acid. Peptides were prepared from two proteoglycans by CNBr and protease treatment for microsequencing and the design of oligonucleotide PCR primers. Data from the 1D1 proteoglycan demonstrated the presence of EGF-like sequences, and 67–74% identity over 39 amino acids with the C-terminal portion (including the 24 C-terminal amino acids of the lectin like domain) of fibroblast and cartilage proteoglycans. Also isolated by immunoaffinity chromatography were the 3H1 chondroitin/keratan sulfate proteoglycan and the 3F8 chondroitin sulfate proteoglycan, both of which have average

molecular sizes of ≈500 kDa. During development there is a large increase in the concentration of 3H1 and in its proportion of keratan sulfate, accompanied by a significant decrease in the branching and/or sulfation of the keratan sulfate chains. The 3F8 proteoglycan contains an average of four 28 kDa CS chains, and also shows developmental changes in sulfation (from ≈30% chondroitin 6-sulfate at 7 days postnatal to >96% chondroitin 4-sulfate in adult brain, and the disappearance of HNK-1 epitopes).

S10. O-GLYCANS/O-GLYCANNES

10.1
LEUKOSIALIN, A MAJOR *O*-GLYCAN CONTAINING SIALOGLYCOPROTEIN DEFINING LEUKOCYTE DIFFERENTIATION AND MALIGNANCY

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Leukosialin is a major sialoglycoprotein on leukocytes and appears to be involved in various immune reactions; the addition of a monoclonal antibody specific to this molecule induces T-cell proliferation, activation of monocytes, and increased activity of NK cells. These activations are apparently mediated by phosphorylation of leukosialin catalyzed by protein kinase C (*J. Biol. Chem. 264*, 18824). Since leukosialin contains a large number (~70) of *O*-glycans and one *N*-glycan, this glycoprotein serves as an ideal model for studying *O*-glycosylation in temporal relation to *N*-glycosylation. Our work indicates that leukosialin produced in T-cell leukemia cell line Jurkat contains only GalNAcα→Ser/Thr structure. By using this system, we found that initiation of *O*-glycan synthesis takes place in the *cis*-Golgi (*J. Biol. Chem., 265*, 9264). By using K562 cells that synthesize NeuNAcα2→3Galβ1(NeuNAcα2→6) GalNAc on leukosialin, we found that this addition of Galnac to apoprotein is immediately followed by the addition of Gal to GalNAc (*Eur. J. Biochem, 183*, 123).

Addition of β1→6GlcNAc to GalNAc is another key step in *O*-glycosylation. We found that T-cell activation is associated with the conversion of NeuNAcα2→3Galβ1→3(NeuNAcα2→6) GalNAc to NeuNAcα2→3Galβ1→3(NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAc in *O*-glycans attached to leukosialin (*J. Biol. Chem. 263* 15146). Similarly, we found recently that T-acute lymphocytic leukemia (Saitoh et al. (1991) *Blood*, in press) and Wiskott-Aldrich syndrome (Piller et. al. (1991) *J. Exp. Med.*, submitted) are characterized by the appearance of this hexasaccharide, caused by the increase of β1→6*N*-acetylglucosaminyltransferase. The same hexasaccharides are also present in thymocyte leukosialin. These results strongly suggest that the appearance of β1→6*N*-acetylglucosaminyltransferase is associated with acquiring the immature phenotype or conversion from quiescent to actively growing cells. Our studies now are focused on characterizing the key enzyme, β1→6*N*-acetylglucosaminyltransferase that may be defining differentiation and malignancy of leukocytes. (Supported by CA33895).

10.2
CLEAVAGE OF THE O-GLYCOSYLATED CELL-SURFACE GLYCOPROTEINS CD34, CD43, CD44, AND CD45 BY A NOVEL GLYCOPROTEASE FROM *PASTEURELLA HAEMOLYTICA*

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We have assessed the ability of a neutral metallo-protease secreted by the bovine fibrinous pneumonia pathogen *Pasteurella haemolytica* strain A1 to cleave cell-surface glycoproteins expressed on the primitive human myeloid/T-lymphoid cell-line KG1a. Since our previous studies using glycophorin A have indicated that the substrate specificity of this enzyme may be uniquely restricted to the cleavage of O-sialoglycoproteins, we have designated this activity, P.h. glycoprotease. In this study, SDS-PAGE analysis of immunoprecipitations performed on lysates of KG1a cells, have shown that the O-glycosylated cell surface antigens CD7, CD34, CD43 (leukosialin), CD44 (hyaluronic acid receptor), and CD45 (leucocyte common antigen), are all cleaved by the glycoprotease. Although these glycoprotease-sensitive structures contain N-linked glycans, they are all additionally glycosylated with O-linked carbohydrates, which are especially abundant on CD34 and CD43. However, the glycoproteins CD18/11[a,b,c] (leucocyte integrins), CD71 (transferrin receptor), HLA class I, and 8A3 antigens, which contain N-linked glycans but no O-sialo-glycans, were resistant to the action of the enzyme. Preliminary studies using fluorescence microscopy and a variety of antibodies to different epitopes of the P.h. glycoprotease-sensitive structures indicate that this enzyme may have widespread applications in epitope-mapping studies, and represents a novel tool with which to study structure/function relationships for O-sialo-glycosylated cell-surface proteins.

10.3
A GLYCOPROTEASE OF *PASTEURELLA HAEMOLYTICA* SPECIFIC FOR O-SIALOGLYCOPROTEINS: CLONING, NUCLEOTIDE SEQUENCE AND EXPRESSION OF THE GLYCOPROTEASE GENE

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A neutral metallo-protease, with marked specificity for O-sialoglycoproteins, has been isolated from culture supernatants of *Pasteurella haemolytica* A1, a Gram-negative bacterium associated with bovine fibrinous pneumonia. The enzyme cleaves human erythrocyte glycophorin A, which is O-glycosylated, but does not cleave N-glycosylated proteins or non-glycosylated proteins. Glycophorin A was cleaved when present *in situ* in erythrocyte ghost plasma membranes, and when it was free in solution. The glycoprotease did not hydrolyze glycophorin A from which sialate residues had been removed by neuraminidase treatment. An immobilized preparation of the enzyme cleaved glycophorin A at several positions, with a major site of cleavage being at Arg31-Asp32. The enzyme has been purified to homogeneity by HPLC. The glycoprotease is inhibited by EDTA, citrate and ascorbate. The enzyme is non-toxic to a variety of eucaryotic cells in culture, from which it cleaves cell-surface O-sialoglycoproteins but not N-glycoproteins. From a DNA library of the *P. haemolytica genome*, two identical 3.2 kbp plasmid pBR322 inserts were found that code for the glycoprotease. One of these clones was transformed into *E. coli* CSR603 and gave rise to expression of the glycoprotease. The recombinant enzyme was visible on SDS-PAGE gels as a 35 kD band, and the native

product was able to cleave ^{125}-glycophorin A. The gene was sequenced and shows an open reading frame of 975 nucleotides, which encodes 325 amino acids with a predicted M_r of 35.2 kD. These estimates are in agreement with the size of the expressed protein. A Zn^{2+}-binding region can be seen in the protein sequence, but no homology with other known bacterial or eucaryotic proteolytic enzymes can be detected at the DNA or protein level. The glycoprotease gene is 76% homologous with an open reading frame of the *E. coli* genome, the *orfx* gene, whose function is unknown but which is associated with the macromolecular synthesis operon. (N.S.E.R.C. Canada).

10.4
EVIDENCE FOR IN VITRO MICROSOMAL GLYCOSYLATION OF MUCIN PEPTIDE

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Although most mucin carbohydrates are added to nascent mucin peptides within the Golgi there are conflicting views as to whether the initial step (GalNAc addition to ser/thr) may be initiated in intestinal cells at an earlier stage of biosynthesis (in the RER). Our goals were to use in vitro translations of rat intestinal poly A+ RNA to establish (a) the size of the nascent mucin peptide and (b) to detect evidence for microsomal glycosylation of mucin peptide (GalNAc addition).

In vitro translations (IVT) were carried out using rabbit reticulocyte lysates, [^{3}H] thr/ser/pro and poly A+ or total RNA from epithelial scrapings of rat small intestine. Using a polyclonal antibody to the highly deglycosylated mucin glycopeptides, we were able to immunoprecipitate a specific product of Mr >25OkDa as detected by SDS/PAGE followed by fluorography. Amino acid analyses of pooled products from several IVT experiments revealed a composition typical of mature mucin peptide. In the presence of microsomes, IVT reactions gave rise to an increased quantity of mucin product having a slower electrophoretic mobility, suggesting that glycosylation had occured. Amino sugar analyses of the product revealed trace amounts of GlcNAc and ~7% of mature mucin GalNAc. Since detection of ^{3}H-labelled product on gels required 12 weeks of exposure time, a more rapid technique was employed to confirm the incorporation of radiolabelled amino acids or sugar into the newly synthesized mucin peptide. Radioactivity from precursor UDP[^{14}C] GalNAc was incorporated into specific mucin immuno-precipitates in the presence, but not in the absence of microsomes. ^{3}H-thr incorporation was also enhanced (~2 fold) in the presence of UDP-GalNAc and microsomes, providing they were added during the initial 10 min of translation reactions. Protease protection experiments confirmed that the mucin peptide was within the microsomes.

In conclusion (1) the primary in vitro translation product of mucin from rat intestinal mRNA is very large (>250 kDa) (2) the mucin peptide can undergo initial O-glycosylation (i.e GalNAc addition) cotranslationally in the microsomes and (3) microsomal glycosylation appears to enhance mucin peptide synthesis. Thus the in vitro translation system employed in this study may prove to be valuable in future studies of the regulation of the early steps of glycosylation and peptide synthesis of intestinal mucins. Financial support from CCFF and MRC.

10.5
IN SITU DETECTION OF mRNA FOR THE PUTATIVE 'LINK' COMPONENT OF INTESTINAL MUCIN

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Purified intestinal mucins contain a 118 kDa putative 'link' glycopeptide which is released by thiol reducing agents and is thought to play a role in mucin polymerization (*Biochem J.* **243**, 1987, **261**, 1989). In order to further our understanding of the nature of this protein, sequences of the N-terminus of rat intact 118 kDa 'link' glycopeptide and one of its CNBr fragments were used to generate two degenerate primers. PCR amplification was carried out using rat small intestinal cDNA as a template. A specific 1.2 Kb product encoding 374 amino acids (AA) gave an AA composition similar to that of the intact 118 kDa 'link' glycopeptide. The deduced AA sequence was unique, although it showed some homology (particularly conservation of cysteines) with the **D** domain of human von Willebrand factor (HvWF). A 500 nt *Hind* III fragment (3' end of the 1.2 Kb PCR product) was then used to screen a rat intestinal λ ZAP II library. Sequence determination of clones (RL 13, RL 14 and RL 15) revealed that the 374 AA domain was followed by two tandem repeats, (a total of 54 AA) which exhibited homology (mainly cysteine distribution) with the **B** domain of HvWF. *In situ* hybridization showed that the gene was expressed in goblet cells but not in endothelial cells of rat intestine. Northern blots of rat intestinal and colonic RNA gave a single band at ~9 Kb, suggesting that the 118 kDa is a portion of a much larger protein. Further sequencing and identification of the gene are in progress.

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10.6
P. CEPACIA BINDING TO MUCINS AND TO BUCCAL EPITHELIAL CELLS

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P.cepacia (*PC*) is an opportunistic pathogen which has been found to colonize the lungs of 15 to 18% of the patients with cystic fibrosis (CF) at our hospital. In some cases, *PC* infection is associated with rapid clinical deterioration culminating in death. The first step of pathogenesis may be the adherence of this pathogen to host respiratory tract surfaces. We have measured the binding of ^{3}H-labelled *PC* to purified mucins and to buccal epithelial cells (BEC) to investigate potential early sites of bacterial attachment. *PC* isolates (53) obtained from 22 patients with CF were tested under *in vitro* (microtiter binding assay) for their ability to bind to normal human intestinal mucin (IM) and CF respiratory mucin (CF-RM). 38 of the isolates (from 18 patients) bound in a specific, saturable and reversible fashion to both mucins. The most avid binding was exhibited by heavily piliated isolates obtained from patients with clinically advanced disease. Receptors on mucin molecules appeared to be short oligosaccharides enriched in GlcNAc (eg. GlcNAcβ6[GlcNAcβ3-Galβ3]GalNAc). Two heavily piliated *PC* isolates which bound avidly to mucin (*PC* 1 and *PC* 2) as well as two poorly piliated isolates which did not bind to mucin (*PC* 3 and *PC* 4) were next tested for their capacity to bind to buccal epithelial cells (BEC) from CF patients and normal subjects. All 4 *P.C.* isolates bound to both CF and normal BEC's in a saturable fashion with increasing bacterial concentration. The association constant K, was the same (10^{-10} ml/BEC) for all 4 isolates. GalNAc (20mM) inhibited the BEC binding of *PC* 1 and *PC* 2 by 36%, and *PC* 3 and *PC* 4 by 61%. Purified mucins (both IM and CF-RM) inhibited *PC* 1 and *PC* 2 binding to BEC (50 to 60%), but did not inhibit *PC* 3 and *PC* 4 binding to BEC. Bacterial overlay assays performed on BEC homogenates showed a major BEC binding component at Mr 60 kDa. TLC overlay assays performed on standard glycolipids (from Dr. C. Lingwood, Dept. of Microbiology) showed binding of isolates to asialo-GM1.

Therefore two types of *P.cepacia* from CF patients have been disting-

uished: (a) heavily piliated isolates from patients with advanced disease. These isolates bound to mucin carbohydrates and to BEC by separate adhesins. Mucins partially inhibited binding to BEC. (b) poorly piliated isolates which bound to BEC but not to mucins. BEC receptors for both (a) and (b) may include a 60 kDa(glyco?) protein and/or asialo-GM1.

(Financial support obtained from the Canadian Cystic Fibrosis Foundation).

10.7
COMPARATIVE STUDY OF *PSEUDOMONAS AERUGINOSA* ADHESION TO SALIVARY MUCIN GLYCOPEPTIDES FROM NORMAL AND CYSTIC FIBROSIS SUBJECTS

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Pseudomonas aeruginosa is the most common pathogen involved in cystic fibrosis (CF) respiratory infection. The mechanism of colonization is not yet elucidated but some previous studies have reported differences in the adhesion of *P. aeruginosa* to respiratory mucins from patients suffering from chronic bronchitis and from cystic fibrosis (1). However, these differences might originate, to a certain extent, from differences in the degradation of human respiratory mucins related to the important infection process usually encountered in CF (2). Since saliva does not seem to be infected in CF patients, it might represent a more appropriate tool to compare the adhesion of *P. aeruginosa* to CF and non CF mucin glycopeptides.

In the present study, we collected the total salivas from one CF patient, from 2 groups of CF patients, from one healthy individual and from 3 groups of normal subjects. Salivary mucin glycopeptides were obtained by fractionation of proteolyzed salivas using two successive steps of gel-chromatography. The adhesion of the piliated and non piliated strains of non mucoid *P. aeruginosa* 1244 on the salivary glycopeptides was studied using a microtiter plate adhesion assay (1).

We observed that: (i) the concentration of non-dialyzable material was two fold higher in CF salivas than in non CF salivas; (ii) the yields of the glycopeptide fractions were higher in CF secretions than in the normal salivas; (iii), on an average, the CF glycopeptides had a higher adhesion capacity for the piliated strain of *P. aeruginosa* than did the non CF glycopeptides, (iv) the non piliated strain of *P. aeruginosa* gave similar results, suggesting that the adhesion of *P. aeruginosa* to salivary glycopeptides was mediated mainly by a non pilus adhesin.

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10.8
MEMBRANE ANCHORED MUCINS IN NORMAL AND CANCER CELLS

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Mucus glycoproteins (mucins) have been implicated in cell surface changes associated with development and disease, notably malignant transformation and metastasis.

Cell surface molecules have been described recently which are linked to the membrane via a unique glycosyl-phosphatidyl inositol membrane anchor. We report here the detection of mucins which are linked to human colonic mucosal cell membranes in this way. These molecules can be separated using their segregation into the detergent layer of Triton X114 containing buffers under conditions where two phases are formed. They can be cleaved within the unique membrane anchor structure by the action of phosphatidyl inositol specific phospholipase C enzymes (PIPLC) or nitrous acid to yield products which segregate in the aqueous phase of Triton X114 buffers indicating the release of the diacyl glycerol 'tail'.

Metabolic labelling of the mucins with D-glucosamine and of the membrane anchor with ethanolamine was carried out in organ and cell culture and the membrane products analysed. Material released from the octyl-glucoside extracted membranes by PIPLC showed bands at 400–600kDa high molecular weight aggregates greater than 10^6Da on 3% SDS-PAGE and migrated at a density of 1.3–1.35 on CsCl density gradients.

Human colon cells at different stages of malignant transformation expressed membrane anchored mucins which varied with the stage of transformation.

10.9
CHROMATOGRAPHIC MAPPING OF SULFATED OLIGOSACCHARIDES FROM RAT GASTROINTESTINAL MUCINS

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The carbohydrate portion of mucin generally consists of many different neutral and acidic oligosaccharide chains. For the analysis of these oligosaccharide chains, a mapping method by gel-permeation and amino-plate thin-layer chromatography conducted together was developed. In gel-permeation chromatography using a Toyopearl HW40S column with 0.1M pyridine acetate, pH 5.0 as the solvent, both neutral and acidic oligosaccharides were eluted according to molecular weight. Each fraction from a Toyopearl HW40S column was further analyzed by amino-plate thin-layer chromatography. The sites of oligosaccharides on the thin-layer plate could be determined according to size, anionic charge and sugar composition and thus mapped on a thin-layer plate.

In this manner, the regional distribution of mucin-derived sulfated oligosaccharide along the rat gastrointestinal tract was determined. Mucins were extracted from rat gastrointestinal tissues incubated with [^{35}S]sulfate *in vitro*, and then purified. Radiolabeled sulfated oligosaccharides obtained from the mucin by alkaline-borohydride treatment were mapped on the thin-layer plate as described above. In the chromatogram, sulfated oligosaccharides from the corpus and antrum each showed a different pattern, this being in agreement with our previous data. Region-specific chromatograms of sulfated oligosaccharides were also obtained for small and large intestinal mucins. The results indicate sulfated oligosaccharides of mucin to be distributed region-specifically along the gastrointestinal tract.

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10.10
NEW DEVELOPMENTS IN MASS SPECTROMETRY: SOLVING THE NEXT GENERATION OF STRUCTURAL PROBLEMS IN GLYCOBIOLOGY

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High field magnetic double focussing mass spectrometry has played a major role in biopolymer analysis in the 15 years since we introduced it

(1), and the VG Analytical ZAB range of high-field instruments (e.g. the ZAB 2SE which has a mass range of 15,000 dalton at high sensitivity) have been especially widely used in protein and carbohydrate MS laboratories throughout the world for sequencing studies, and for defining post-translational modifications. Despite the many successes achieved with high field instrumentation, there are many projects in the field of glycobiology that demand even greater sensitivity than that afforded by the ZAB 2SE. In 1987 we proposed a new instrument incorporating wide angle multichannel array focal plane detection with high field, high resolution capability for the ultrasensitive high mass analysis of biopolymers by fast atom bombardment (FAB) and field desorption (FD). A two sector version of the ZAB 2SE, the ZAB-FPD, has now been constructed for our laboratory. It incorporates one wide angle (1.5:1) array for high sensitivity, low resolution survey analysis up to 15 kilodaltons, and a second array (1.05:1) for narrow angle (5% window) high resolution analysis. The instrument retains the off-axis multiplier detection of the ZAB 2SE together with its Linked Scanning/CAD sequencing capabilities.

Data will be presented demonstrating the operation and power of this new instrumentation in FAB and FD analyses on polysaccharides and glycoconjugates including studies of cellulase, nematode glycoprotein antigens, glycosaminoglycans and O-linked GlcNAc glycoproteins. The sensitivities achievable with the ZAB-FPD, and the range of structural problems that can be addressed using this sophisticated sector instrumentation will be described. Further we will report our experience with the electrospray mass spectrometer and compare the potential in the glycobiology field of this exciting new instrumentation with that of the ZAB-FPD.

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10.11

IDENTIFICATION OF O-GlcNAc ATTACHMENT SITES IN TRANSCRIPTION FACTORS

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Several years ago a novel type of glycosylation, in which N-acetylglucosamine is O-glycosidically attached to proteins in the nuclear and cytoplasmic compartments, was described (1). O-GlcNAc occurs on, among others, nuclear pore proteins, chromatin proteins, transcription factors and cytoskeletal proteins. The functions of O-GlcNAc remain enigmatic but there is experimental evidence to suggest that it may play a vital role in critical cellular processes such as regulation of gene transcription and nucleocytoplasmic transport. A better understanding of the role of O-GlcNAc awaits rigorous structural studies, including sequencing glycosylated peptides, determining site occupancy and establishing whether the O-GlcNAc becomes modified in any way. We are addressing these structural features using Fast Atom Bombardment Mass Spectrometry (FAB-MS).

Our studies have focussed on the transcription factor Serum Release Factor (SRF), (obtained from an insect cell line and also HeLa cells). The methods we have employed are based on a strategy which we have optimised using synthetic O-GlcNAc-containing glycopeptides as model compounds. In this paper we will describe this novel strategy and demonstrate its applicability to sub-namomolar quantities of glycoproteins. Glycosylated hepta- to deca-peptides from SRF were produced by sequential digestion with cyanogen bromide, trypsin and proline specific enzyme. Four glycopeptides, each containing a single GlcNAc at sub-stoichiometric levels, were revealed by FAB-MS analyses. After labelling with ^{3}H-Gal/Gal transferase sequencing was carried out by a combination of enzymic and Edman digestion and FAB-MS characterisation in order to locate the precise sites of glycosylation.

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10.12

BOVINE LENS α–CRYSTALLINE SUBUNITS ARE MODIFIED BY O-LINKED GlcNAc

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N-acetylglucosamine glycosidically linked through a serine or threonlne hydroxyl (O-GlcNAc) is a recently identified form of intracellular glycosylation unique to proteins exposed to the nuclear and cytoplasmic compartments (for review see **Annu. Rev. Biochem. 58**:841). We report that all four major subunits of bovine lens α-crystallin are modified with O-GlcNAc. The relative abundance of α-crystallins in eye lens and the unusual capacity of this organ to retain crystallins synthesized throughout the life span of the animal make this tissue ideally suited for extensive structural and functional studies of O-GlcNAc. An in vitro enzymatic assay which transfers [^{3}H]Galactose to terminal GlcNAc moieties labels proteins in bovine lens extract which can be immunoprecipitated with polyclonal antisera to α-crystallin and which migrate on two dimensional IEF and NEPHGE polyacrylamlde gels with molecular weights and pIs consistent with those reported for the A1, A2, B1 and B2 α-crystallin subunits. We have also detected this modification on α-crystallins from the lenses of two avian species, *rhea* and *starling*. O-linkage of the saccharide is demonstrated by its sensitivity to base catalyzed β-elimination and its resistance to peptide: N-glycosidase F treatment. Both Biogel P4 and high voltage paper chromatography of the β-elimination products confirm the saccharide structure. Preliminary stoichiometric determinatlons based on anion exchange chromatography of acid hydrolyzed carbohydrate indicate the presence of 0.5–0.8 mole GlcNAc per mole αA crystallin and 0.3 mole GlcNAc per mole αB crystallin, although significant levels of hexosaminidases detectable in the lens extracts may have contributed to an underestimation of the stoichiometry. Reversed phase HPLC of α-crystallin tryptic peptides and SDS/PAGE of CNBr cleavage products indicate the presence of multiple sites of O-GlcNAc attachment. Sequence analysis and mass spectrometry of the major radiolabeled tryptic peptide reveal that GlcNAc is attached to the A subunit at serine 162. Work is currently underway to further characterize the attachment sites and stoichiometries of O-GlcNAc on the α-crystallin subunits and to investigate the presence of this modification on proteins homologous to the crystallins, particularly the mammalian small heat shock proteins. Supported by HD13563.

10.13

PURIFICATION AND INITIAL CHARACTERIZATION OF A CYTOPLASMIC AND NEUTRAL N-ACETYLGLUCOSAMINIDASE FROM RAT SPLEEN THAT HAS SELECTIVE ACTIVITY AGAINST O-GlcNAc

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Glycosylation of nuclear and cytoplasmic proteins by O-linked N-acetylglucosaminide (O-GlcNAc) monosaccharides, has been described in eukaryotes ranging from yeast to man. We have suggested that this O-GlcNAc glycosylation and deglycosylation might serve a regulatory function analogous to phosphorylation and dephosphorylation by protein kinases and phosphatases, respectively, and we have shown that the apparent levels of O-GlcNAc on lymphocyte proteins change rapidly upon lymphocyte activation (**Kelly Kearse and Gerald Hart, PNAS in press**). Recently, we have identified a cytoplasmic, neutral N-acetylglucosaminidase which can readily cleave O-GlcNAc from enzymically glycosylated synthetic peptides. This enzyme has been partially purified to 25,000-fold by ammonium sulfate precipitation, DEAE-Cellulose, concanavalin A-sepharose, phenyl sepharose, hydroxylapatite, sepharose 4B, blue A agarose, and p-aminophenyl-N-acetyl-1-Thio-β-D-glucosaminide sepharose 4B affinity chromatography. The molecular weight is approximately 224 Kd as analyzed by sepharose 4B gel filtration. The enzyme displays strong activity against p-nitrophenyl-N-acetyl-β-D-glucosaminide, but not toward to p-nitrophenyl-N-acetyl-β-D-galactosaminide. The neutral hexosaminidase has pH optima at about 6.0, and it does not require divalent ion for its activity. It is inhibited by glucosamine, N-acetylglucosamine, and Tris, but is not inhibited by galactosamine or N-Acetyl-galactosamine. The properties of the neutral hexosaminidase are distinct from lysosomal hexosaminidases which have both glucoaminidase and galactosaminidase activity. The purified N-acetylglucosaminidase also has stronger activity against O-GlcNAc-synthetic glycopeptide than hexosaminidase from *Diplococcus pneumoniae*. These studies suggest that this neutral N-acetylglucosaminidase might be involved in the regulated removal of O-GlcNAc from important intracellular proteins. Supported by NIH Grant HD-13563.

10.14
THE C-TERMINAL DOMAIN OF RNA POLYMERASE II IS MODIFIED BY O-LINKED GlcNAc

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The linkage of N-acetylglucosamine to protein via the hydroxyl of a serine or threonine is a post-translational modification that is found on the nuclear and cytosolically-exposed faces of many intracellular proteins in all eukaryotes (for review, see **Ann. Rev. Biochem.**, *58*:841). These include nuclear pore components, endoplasmic reticular membrane residents, cytoskeletal proteins, viral proteins, and numerous chromatin proteins, including, but not restricted to, transcription factors. Interestingly, this modification is found on RNA polymerase II-specific transcription factors, but it has not been found on the polymerase I or III-specific factors that have been examined.

The largest subunit of RNA polymerase II from eukaryotes is distinguished by the presence of a heptapeptide repeat (consensus sequence YSPTSPS) in its C-terminal domain (CTD) that is repeated 52 times in mammalian Pol II (**PNAS**, *82*:7934). The similarity of this sequence with a peptide used in our lab as an *in vitro* substrate for O-GlcNAc transferase suggested that Pol II itself may be modified by O-GlcNAc. We have found that RNA polymerase II does indeed contain O-GlcNAc, and that it is only detectable on polymerase containing intact CTD, suggesting it is localized to the C-terminal repeat. In addition, the phosphorylated form of Pol II, which is the major form of the enzyme in actively dividing cultured cells (**J. Biol. Chem.**, *261*:14219), does not appear to contain detectable O-GlcNAc. These data suggest that the O-GlcNAc modification of Pol II and its phosphorylation may be mutually exclusive, indicating that O-GlcNAc may be involved in the regulation of RNA polymerase activity *in vivo*. Supported by HD13563 and The March of Dimes Predoctoral Fellowship Program.

10.15
GLYCOSYLATION IN THE CYTOPLASM AND THE NUCLEUS: PURIFICATION AND CHARACTERIZATION OF THE O-GLCNAC TRANSFERASE

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O-linked N-acetylglucosamine (O-GlcNAc) is a form of protein glycosylation in which single GlcNAc moieties are attached in O-linkage to the hydroxyl side chains of either serine or threonine residues. Unlike glycoproteins modified by the well characterized N- and O-linked glycans, proteins bearing O-GlcNAc occur mainly in the cytoplasm and the nucleus. Recent evidence from our laboratory suggests that this post-translational modification may serve a regulatory function (Kearse, K. and Hart, G.W. (1991) *Proc. Natl. Acad. Sci.* in press). We have identified an enzymatic activity in the cytoplasmic portion of the cell which adds O-GlcNAc moieties to peptides and proteins, a UDP-GlcNAc: peptide N-acetylglucosaminyltransferase (O-GlcNAc transferase, Haltiwanger, R.S., Holt, G.D., and Hart, G.W. (1990) *J. Biol. Chem.* **265**: 2563–2568). Using hydrophobic interaction, immobilized dye, ion-exchange and affinity chromatography, we have purified this activity over 20,000-fold from rat liver cytosol. The enzyme has an apparent molecular weight of 115,000 on SDS-PAGE and appears to migrate as a dimer on gel filtration under non-denaturing conditions. We are currently characterizing the enzyme and beginning to determine the minimal peptide sequence requirements for activity. Supported by NIH HD13563.

10.16
O-LINKED CARBOHYDRATE EPITOPES OF HIV ARE TARGETS FOR NEUTRALIZING ANTIBODIES

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The envelope glycoproteins of HIV are heavily glycosylated, and the general core structure of the N-linked glycans have been described in detail. Lectins and monoclonal antibodies binding to N-linked glycans of HIV block infection in vitro. Less attention has been paid to O-linked oligosaccharides on gp120.

To identify carbohydrate structures exclusively expressed by HIV and HIV infected cells, we have searched for simple O-linked glycans bound to serine or threonine residues, which are only very restrictively expressed in the healthy adult. Using monoclonal antibody (MAb) TKH2 specific for NeuAc-GalNAc-Ser/Thr (Sialosyl-Tn), the major envelope glycoprotein of HIV, gp120, was precipitated from an ^{125}I-lysate of $HTLV_{111}B$ propagated in human lymphocytic cells. To examine whether antibodies against simple O-linked epitopes of the HIV envelope could interfere with infection, a standard in vitro infectivity inhibition assay was employed. Briefly, 25 $TCID_{50}$ HIV was incubated with MAb and used to infect lymphocytic cells (MT-4). After 4 days of culture, infection was evaluated by measuring HIV antigen production by ELISA. MAbs TKH2 and B72.3 specific for sialosyl-Tn and MAbs 1E3 and TKH6 specific for GalNAc-Ser/Thr (Tn) inhibited infection of several HIV-1 isolates in a concentration dependent manner. One HIV-1 isolate and an HIV-2 isolate were not neutralized. Specificity of the anti-

Tn mediated neutralization of HIV was demonstrated by the ability of synthetic GalNAc-Serine to block this neutralization.

MAbs defining simple, O-linked epitopes on the HIV envelope thus inhibit a broad range of HIV isolates in vitro, which suggests that HIV vaccine constructions may benefit from inclusion of such epitopes.

10.17

INDUCTION OF NEUTRALIZING CARBOHYDRATE EPITOPE (Tn ANTIGEN) OF HIV SPECIFIED GLYCOPROTEIN IS INDEPENDENT OF NON-STRUCTURAL VIRAL PROTEINS

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We have shown that gp120, produced in various human cell lines infected with human immunodeficiency virus type 1 (HIV-1), contains simple, O-linked glycans (1). These glycans were not expressed in the uninfected cells. The objective of this paper was to investigate if the induction of Tn antigen in HIV-1 infected cells was signalled by the peptide sequence of gp120 or if engagement of HIV-specified regulatory factors and other non-structural proteins was necessary.

A recombinant vaccinia vector, expressing the HIV *env* gene product under the 11K promotor was used as a source for production of HIV glycoproteins, without involving expression of other HIV glycoproteins. The presence of Tn antigens on the surface of infected cell surfaces was measured by the ability of a monoclonal anti-Tn antibody to block gp41/gp120 dependent cell fusion between infected cells and subsequently added CD4-expressing cells.

Both HIV-infected and vector-infected cells expressed HIV *env* gene products and gave rise to syncytia formation in the assay system. The fusion activity of both HIV-infected and vector-infected cells was blocked by addition of monoclonal antibodies, directed against gp120 and Tn. These results show that formation of Tn glycan of gp120 is dependent on structural features of gp120 without any involvment of HIV regulatory factors.

1 Hansen, J-E.S. et al. J Virol 64: 2833–2840, 1990.

10.18

TISSUE PLASMINOGEN ACTIVATOR HAS AN O-LINKED FUCOSE ATTACHED TO THR-61 IN THE EPIDERMAL GROWTH FACTOR DOMAIN

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An unusual type of glycosylation has been observed for tissue plasminogen activator (t-PA), in which the monosaccharide fucose is glycosidically linked to Thr-61 within the epidermal growth factor domain of t-PA. The presence of attached fucose was demonstrated by carbohydrate analysis and mass spectrometry of tryptic and chymotryptic peptides that contain this site. The O-linked nature of the linkage and the specific point of attachment were demonstrated by an alkaline elimination experiment, which resulted in destruction of Thr-61. The susceptibility of the fucose residue to α-fucosidase indicated that it had the anomeric configuration. Fucosylation of Thr-61 was observed in t-PA isolated from the Bowes melanoma cell line as well as in human t-PA produced by recombinant expression in Chinese hamster ovary or human embryonic kidney cells. Fucosylation of the homologous residue in pro-urokinase has also been reported recently by others (Kentzer *et al.*, *Biochem. Biophys. Res. Commun. 171*, 401–406). Our results indicate that this novel type of glycosylation may be common to many of the epidermal growth factor domains found in coagulation and fibrinolytic proteins and, therefore, suggest that the modification may have functional significance.

10.19

GLC-PHOSPHOTRANSFERASE ACTIVITY IS MODULATED BY CYTOPLASMIC Ca^{++} IN ANIMAL CELLS AND BY HEAT SHOCK IN YEAST

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UDP-glucose: glycoprotein glucose-1-phosphotransferase (Glc-phosphotransferase) catalyzes the transfer of αGlc-1-P from UDP-Glc to O-linked mannose residues on acceptor glycoproteins. This enzyme is membrane-associated and oriented with its catalytic site facing the cytoplasm. The sole detectable acceptor in both mammalian cells and Paramecium tetraurelia is a cytoplasmic glycoprotein of 63 kDa. In Paramecium αGlc-1-P appears to be removed from this acceptor and then rapidly replaced as cytoplasmic Ca^{++} is increased following a secretory stimulus (Satir *et al.* (1990) *J. Cell Biol. 111*:901–907). A similar sensitivity has now been found in mammalian cells. Rat pheochromocytoma (PC12) cells were permeabilized with digitonin and assayed for Glc-phosphotransferase activity using the ^{35}S β-phosphorothioate analogue of UDP-Glc. Conditions that activate the regulated secretory pathway (40μM Ca^{++} and 1mM ATP) in these permeabilized cells resulted in the selective addition of phosphodiester-linked glucose to the cytoplasmic 63 kDa acceptor. In the absence of μM levels of Ca^{++} no labeling was detected, even though both Mn^{++} and Mg^{++} were present. In experiments with intact PC12 cells and synaptosomes that were briefly labeled with (^{14}C)Glc, activation of a voltage-dependent Ca^{++} channel or the presence of a Ca^{++} ionophore led to a greatly increased labeling of pgp63. Biochemical analyses demonstrated that the addition of (^{14}C)Glc was through a phosphodiester. Thus, in contrast to glycosylation reactions within the endoplasmic reticulum and Golgi, this cytoplasmic glycosylation event appears to be rapidly regulated, suggesting that glycosylation might modulate a cytoplasmic protein's function. In Saccharomyces cerevisiae a 62 kDa acceptor is again the only protein labeled in homogenates incubated with ($\beta^{35}S$)UDP-Glc. Preparations from log phase yeast exhibited no 62 kDa labeling; as the cells moved into stationary phase the labeling dramatically increased. A similar increase in labeling was seen in log phase cells that were heat-shocked at 39° for as little as 15 min. This increase required protein synthesis.

10.20

ISOLATION AND CHARACTERIZATION OF SERINE-RICH AND THREONINE-RICH GASTRIC MUCUS GLYCOPEPTIDES

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Stanley *et al.* reported that porcine gastric mucus glycoprotein (PGM) consist of heterogenous subpopulations differed distinctly from the polydispersity of the same molecules of different size (1).

In this study, three components from purified PGM prepared by extensive pepsin digestion were found to be separated from each other by CsCl density gradient centrifugation. A comparative study of these components was conducted as follows: Highest density fraction and two lower density fractions separated by CsCl density gradient centrifugation

were designated VHD, HD and LD, respectively. The low density fractions, HD and LD appeared quite similar or identical to each other. However VHD differed completely in various respects from these two, as follows: 1) VHD but not HD and LD exhibited strong alcian blue binding activity. 2) 59% of VHD and less than 0.7% of HD and LD bound to the DEAE-Toyopearl column equilibrated with 0.2 M NaCl. 3) VHD eluted from Sephacryl S-400 column as a lower molecular weight subunit. 4) One third of sialic acid present in VHD was N-glycolylneuraminic acid and the remainder N-acetylneuraminic acid. 5) Its carbohydrate composition was the same as that of typical PGM with slightly higher fucose content. 6) The amino acid composition showed the highest Ser/Thr ratio, 1.4, compared to 0.62, 0.46, and 0.52 for HD, LD and purified PGM, respectively. Furthermore, the Ser/Thr ratio of the DEAE binding fraction from VHD was 2.2. 7) Oligosaccharide released from VHD by alkaline degradation was larger than that from HD or LD.

(1) R.A. Stanley *et al.* BBA *760* (1983) 262–269.

10.21
NEW TYPE OF ENDO-GLYCOSIDASE, ENDO-α-N-ACETYLGALACTOSAMINIDASE-S, FROM *STREPTOMYCES* SP. OH-11242 CULTURE MEDIUM

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A new type of endo-α-N-acetylgalactosaminidase from a culture medium of *Streptomyces* sp. OH-11242 (endo-GalNAc-ase-S) was reported previously. The crude enzyme was capable of liberating not only Gal β1–3GalNAc but also other larger oligosaccharides from porcine gastric mucus glycoprotein by hydrolysis of O-glycosidic linkage between GalNAc and Ser(Thr) (1,2). The partial purification of endo-GalNAc-ase-S and a comparison of endo-GalNAc-ase-S with endo-α-N-acetyl-galactosaminidase from *Alcaligenes* sp. (endo-GalNAc-ase-A) (3) were carried out. The crude enzyme of endo-GalNAc-ase-S was prepared as an 80% ammonium sulfate precipitate from the concentrated culture medium. The enzyme was partially purified by gel chromatofocusing and subsequent DEAE-Toyopearl chromatography. The endo-enzyme was eluted around pI 4.9 on a gel chromatofocusing column and around 0.23 M NaCl on a DEAE-Toyopearl column. In the enzyme fraction thus obtained, no exo-glycosidases were present. The endo-GalNAc-ase-S hydrolysed the O-glycosidic linkage between GalNAc and Ser(Thr) in asialofetuin, liberating both di- and tetra-saccharides (Gal β1–3(Gal β1–4GlcNAc β1–6)GalNAc). Asialofetuin was incubated with endo-GalNAc-ase-A, resulting only in the liberation of the oligosaccharide, Gal β1–3GalNAc.

(1) H. Iwase *et al.* Biochem. Biophys. Res. Comm., *151*, 422–428 (1988).
(2) H. Iwase *et al.* Proceedings of the Xth Int'l. Symposium on Glycoconjugate, Abstr. # 217 (1989).
(3) J.-Q. Fan *et al.* Agric. Biol. Chem., *52*, 1715–1723 (1988).

10.22
HETEROGENEITY OF MUCUS GLYCOPROTEINS FROM PORCINE TRACHEA

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Respiratory tract mucus glycoproteins (mucins) are secreted by at least two cell types, the goblet cells interspersed among the ciliated cells in the epithelial surface, and the mucous cells in the submucosal glands. To identify differences in the nature of mucins of these different origins, we have isolated such macromolecules from the epithelial layer and the submucosal tissue of porcine trachea by isopycnic density-gradient centrifugation in caesium chloride. "Subunits" were obtained after treatment with DTT, and constituent oligosaccharide clusters were isolated as high-M_r glycopeptides by subsequent proteolytic digestion. Both "subunits" and high-M_r glycopeptides were examined with gel chromatography. After density-gradient centrifugation, at least two populations of whole mucins from the epithelial layer (SH and SL), and one from the submucosal tissue (DL) were identified. DL and SL had the same buoyant density although their "subunits" differed in size as determined by gel chromatography. Proteolytic digestion of SL-"subunits" gave rise to one population of glycopeptides, whereas such treatment of the DL-"subunits" revealed two glycopeptide species. This suggests that there are at least two populations of airway mucins, probably originating from different secretory cells, with different length of their oligosaccharide clusters.

10.23
VISCOELASTICITY OF MUCIN GLYCOPROTEIN SOLUTIONS AND GELS

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Mucin glycoproteins are the primary viscoelastic component of mucus secretions. The viscoelastic properties of ovine submaxillary mucin (OSM) and human tracheobronchial mucin (HTBM) are studied in a dissociative solvent as a function of molecular weight and concentration in order to correlate molecular structure with physical properties. OSM has simple disaccharide side chains whereas the oligosaccharides of HTBM are longer, branched and sulfated.

The dynamic storage and loss moduli, $G'(\omega)$ and $G''(\omega)$, and complex viscosity $\eta^*(\omega)$, were obtained by dynamic frequency sweeps between 0.1–100 rad/s. Lower molecular weight fractions of OSM show predominantly dissipative behavior, $G'(\omega)<G''(\omega)$, throughout this frequency range and the complex viscosities are Newtonian. The highest molecular weight fraction shows shear thinning behavior above 10 rad/s and enhanced elasticity at high frequencies, $G'(\omega)>G''(\omega)$, typical of an entanglement network. At comparable degrees of molecular interpenetration, HTBM forms weak viscoelastic gels, $G'(\omega) = G''(\omega)$, which may be due to oligosaccharide interdigitation. The complex viscosity exhibits a power law dependence on frequency. Thus, mucins with longer side chains exhibit better gel-forming capability, larger elastic moduli, and viscosities that are more shear sensitive than mucins with shorter side chains at comparable molecular weight and concentration. Rheological behavior is discussed in terms of polymer solution theories and gelation theory.

10.24
CHARACTERIZATION OF TWO HIGH-M_r GLYCOPEPTIDES DERIVED FROM THE LARGE 'INSOLUBLE' MUCINS OF RAT SMALL AND LARGE INTESTINE

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The major mucins from the small and large intestine were isolated as guanidinium chloride insoluble material. They were solubilized by reduction of the disulphide bonds followed by trypic cleavage. The obtained high-M_r glycopeptides were separated into two populations by gel chromatography on Sephacryl S-500. The elution profiles from the small and large intestine were identical.

Electron microscopy of the two glycopeptides from the small intestine showed relatively homogeneous populations of linear structures with an average length of 194 nm for the larger (called A) and 96 nm for the smaller (called B). Light scattering measurements gave a relative molecular mass of 518 kDa for the larger and 261 kDa for the smaller species.

The oligosaccharides of the glycopeptides from the small and large intestine were released and separated into neutral, sialic acid-containing, and sulphate-containing oligosaccharides. About 60% of the oligosaccharides were neutral and 14% sulphate-containing in the small intestine, whereas 28% were neutral and 67% sulphate-containing in the large intestine. The neutral and the sialic-acid containing oligosaccharides were analyzed by high-temperature gas chromatography-mass spectrometry which showed that glycopeptides A and B from the small intestine were identical and different from those from the large intestine. The results show that the two different oligosaccharide clusters in the major mucins from both the small and large intestine are similar in size but differ in glycosylation.

10.25
ISOLATION AND PARTIAL CHARACTERIZATION OF BOVINE BONE SIALOPROTEIN II

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Bovine diaphyses powder was extracted with 4M guanidine hydrochloride (GdHCl) in the presence of protease inhibitors. The second demineralizing extraction, 4M GdHCl, 0.25M EDTA and protease inhibitors, contained bone sialoprotein II (BSP II) and other noncollagenous proteins. Both bone sialoprotein I (BSP I, osteopontin, secreted phosphoprotein I (SPP I)) and BSP II co-eluted from a DEAE-Sephacel, pH 6.0 anion exchange column at 0.6M sodium acetate. The two sialoproteins are then separated by a combination of DEAE-Sephacel, pH 4.0/FPLC Mono Q, pH 7.4 anion exchange chromatography. SPP I is eluted at a lower salt (NaCl) concentration than BSP II. Also, the utilization of a serotonin affinity column is being studied as an alternative method for the isolation of the sialoproteins. A variety of modified sialic acids exist in nature. The heterogeneity of BSP II elution profiles may be a result of different modified sialic acids. The detection and quantitation of the sialic acids of BSP II will be investigated by Fast-atom Bombardment Mass-Spectrometry (FAB-MS). Supported by Medical Research Council of Canada and a Arthritis Society Studentship to MAS.

10.26
INFLUENCE OF DEGLYCOSYLATION OF HUMAN SALIVARY MUCINS ON THEIR ADSORPTION TO HYDROXYAPATITE

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Native human salivary mucins readily adsorb onto hydroxyapatite (HAP) (Nieuw Amerongen et al., J.Biol. Buccale *17*, (1989) 85–92), and protect tooth enamel against an acidic attack (Caries Res. *21*, (1987) 297–309). Mucins, preadsorbed onto the HAP-surface, can be desorbed by subsequent incubation with the egg yolk protein phosvitin, but not by incubation with the phosphocompound phytate.

Due to bacterial glycosidase activity in saliva, part of the mucin population *in vivo* will loose its terminal carbohydrate residues. Therefore, in the present investigation we have studied the effect of partial deglycosylation of isolated salivary mucins on their adsorption onto hydroxyapatite in the absence or presence of a phosphocompound.

The mucins were deglycosylated by acidic hydrolysis in HCl. Adsorption was performed *in vitro* to 10 mg of HAP with 400 μg/ml mucin and increasing amounts of a phosphocompound up to 1000 μg/ml.

Preadsorbed asialo- as well as asialo-afuco-mucins could not be desorbed from HAP by phosvitin and only slightly by phytate. Likewise, the adsorption of asialo- and asialo-afuco-mucins to HAP preincubated with one of the phosphocompounds was also better than that of native mucin. Essentially the same was observed when partially deglycosylated mucins were simultaneously incubated with one of the phosphocompounds.

These data indicate that *in vitro* partially deglycosylated human salivary mucins can compete successfully for the hydroxyapatite surface with both phytate and phosvitin even better than the native mucins.

10.27
CHARACTERIZATION OF HUMAN SALIVARY MUCINS USING MONOCLONAL ANTIBODIES

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Mucins present in human saliva originate from different (sero)mucous glands, among which the submandibular (SM), sublingual (SL), palatinal (Pal) and labial (Lab) glands. In human whole saliva (HWS) two different mucin populations are present: high molecular weight mucins (Mol. wt>1×10^6) and low molecular weight mucins (Mol. wt2–3×10^5) (Tabak et al., J. Oral Pathol. *11*, (1982) 1–17). It is not known whether the high MW mucins from the various salivary glands are identical or not with respect to their polypeptide- and/or carbohydrate chains, and hence whether heterogeneity is present among the high MW mucin population in whole saliva or not.

In the present investigation, the high MW mucins of HWS as well as of SM-, SL-, Pal- and Lab-saliva have been analysed in ELISA's using four monoclonal antibodies (Mabs) recognising different epitopes on HWS mucins: Mabs E9 and 19–9 directed to (different) sialic acid-containing epitopes, Mab B11 recognising a fucose-containing epitope and Mab F2 directed to an internal oligosaccharide sequence. It appeared that within one individual the high MW mucins of the various glandular salivas can be distinguished immunochemically: high MW mucins from Pal-saliva virtually exclusively immunoreacted with Mab F2, whereas Lab and (to a lesser extent) SL-mucins were predominantly recognised by E9 and 19–9, and relatively less by F2 and B11. The relative immune-reactivity of the various Mabs towards the high MW mucins in SM-saliva descended in the order: B-11>19–9>E9>F2. In addition, CsCl density ultra-centrifugation analysis indicated that palatinal mucins displayed a distinctly higher buoyant density than SL- and SM-mucins.

These results indicate that the high molecular weight mucin population in human whole saliva consists of several immuno- and physicochemically different species.

10.28

BOVINE SUBMAXILLARY MUCIN CORE PROTEINS

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We are interested in elucidating the primary subunit structure of bovine submaxillary mucin (BSM). In our previous studies we cloned and sequenced a 2.0 kb cDNA clone encoding for a 59 kDa bovine submaxillary gland protein containing two distinct domains. [Bhargava *et al.* (1990) PNAS **87**, 6798–6802]. The continuing studies are aimed at directly characterizing the BSM core protein and understanding its relationship to the cloned 59 kDa protein. Deglycosylation of BSM with trifluoromethane sulfonic acid at 2O°C for 3h gave a product which had about 7.5 percent of the original hexosamines but no detectable sialic acid or neutral sugars. Amino terminal analysis of the product failed to reveal any dansyl α-amino acids indicating that the NH_2-terminal is blocked and that no peptide bond cleavage had occurred during deglycosylation. A major problem in characterizing the core protein of mucins is the difficulty in detecting or visualizing it after operations such as gel filtration and SDS-PAGE. We are using both *in vitro* and *in vivo* radiolabeling of the core protein to overcome this problem. Previously we have radiolabeled deglycosylated BSM by amidation of carboxyl groups with [^{14}C] ethanolamine. In the present study the deglycosylated BSM was treated with galactose oxidase followed by sodium borotritide and the [^{3}H]GalNAc-labeled product purified by affinity chromatography on *Vicia Villosa* agglutinin-agarose. Gel filtration analysis and SDS-PAGE/fluorography revealed polydisperse material having molecular weights in the range of 25 kDa to 44 kDa. The deglycosylated BSM was subjected to gel electrophoresis under varying conditions and visualized by immunoblotting using anti-deglycosylated BSM antisera or staining with alcian blue/silver nitrate. The results confirmed the polydisperse nature of the core protein with molecular weights similar to that of the [^{3}H]GalNAc-labeled product. Analytical isoelectric focusing followed by immunoblotting revealed the presence of at least five discrete components with pI in the range of 5.2 to 7.4. In parallel studies, we have prepared BSM metabolically labeled with [^{14}C]glucosamine and/or [^{3}H]Proline from explant cultures of bovine submaxillary glands. Deglycosylation of the metabolically labeled BSM would provide additional information on the core proteins. (Supported by NIH grant HL–42651).

10.29

STIMULATION AND CHARACTERIZATION OF MACROMOLECULAR SECRETIONS FROM HT29–C1.16E GOBLET CELL LINE

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The HT29-C1.16E colon carcinoma cell line (C. Augeron, C. L. Laboisse, Cancer Res. (1984) *44* 3961–3968) reproducibly differentiates, after reaching confluency, into a population of mucus secreting goblet cells. Intracellular and secreted [^{3}H]-glucosamine labelled glycoprotein components were characterized by Sephacryl S-1000 column chromatography. Both intracellular and secreted fractions contain very high molecular weight labelled macromolecules (Mw>10^7) which appear as a sharp peak in the void volume of the column. These high molecular weight components comprise approximately 1/3 of the total incorporated label.

Experiments were designed to determine whether the mucin secretion could be stimulated with different types of secretagogues. The cells were grown on Vitrogen-coated Millicell-CM filters. After transepithelial conductance reached less than 10mS/cm^2, the cells were labeled with [^{3}H] glucosamine for 24 hours. The labeled cells were stimulated with ATP, Foskolin or Carbachol, and the apical sectretions were analyzed by gel filtration chromatography as described above. These analyses show that all three secretagogues stimulate the secretion of high molecular weight mucin. (Supported by NIH CF core center DK 2765I, and NIH DK 39918 to T.G, NIH DK 39658 to U.H and A.F.L.M. to C.L.L.)

10.30

EVIDENCE FOR SECRETION OF HIGH MOLECULAR WEIGHT MUCINS BY CANINE TRACHEAL CELLS IN PRIMARY CULTURE: EFFECTS OF SELECT SECRETAGOGUES ON MUCIN SECRETION

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The purpose of this investigation was to provide evidence for the secretion of high molecular weight mucins, CTM-A and CTM-B [Biochem. J. (1991, in press)], in primary culture of canine tracheal epithelial (CTE) cells. CTE cells were isolated from tracheas of mongrel dogs by pronase treatment. Primary cultures of the epithelial cells were established using ICN cellagen inserts in DMEM/F-12 medium supplimented with growth factors and could be maintained upto 23 days. The evidence for the mucin secretion in culture medium and their localization in the cells was established by i) positive immunocytochemical staining using specific antibodies developed against purified native as well as deglycosylated CTM-A and CTM-B, ii) comparison of the amino acid composition of mucin purified from canine tracheal pouch secretions and those purified from the culture medium and iii) western blot analysis using specific polyclonal antibodies directed against deglycosylated CTM-A and CTM-B. Immunoaffinity purified secreted labeled glycoconjugates were resistant to hyaluronidase treatment. The effects of cyclic AMP (1 x 10^{-5}M), dibutyryl cyclic AMP (1 x 10^{-5}M), 8-bromocyclic AMP (1 x 10^{-5}M) and prostaglandin E_1(1 x 10^{-6}M) on mucin secretion by CTE cells were also investigated. Secretion of mucins, by cultured CTE cells in culture was considerably more enhanced by 8-bromocyclic AMP than that observed for other secretagogues used in this study. Supported, in part, by NIH grant HL 41200.

10.31

POLYPEPTIDE CHAIN LOCALIZATION OF THE *O*-LINKED OLIGOSACCHARIDES OF BOVINE FETUIN

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Fetuin is a major glycoprotein of fetal calf serum whose carbohydrate structures have been investigated over the past 30 years. The cDNA sequence has recently been reported and a high degree (>70%) of homology with human α_2HS glycoprotein was observed (Dziegielewska et al., J. Biol. Chem. 265, 4353–4357); however, its function remains unknown. Although approximately 30 oligosaccharides have been described for bovine fetuin, their locations and proportions at individual glycosylation sites have not been completely determined. In this study, we report the assignment of *O*-linked oligosaccharides to their respective Thr and Ser sites. *O*-linked glycopeptides were prepared from either

Pronase or tryptic digests of reduced and pyridylethylated bovine fetuin (Gibco, Spiro method; Lot No. 14P7696). Jacalin-agarose chromatography was used to recover *O*-linked glycopeptides from proteolytic digests. The glycopeptides were further purified using reversed-phase chromatography. These purified glycopeptides were then analyzed using liquid secondary ion mass spectrometry (LSIMS) and tandem mass spectrometry. From the mass spectra (LSIMS and CID analysis) and Edman sequencing, we found that residues 253 (Ser), 262 (Thr), 264(Ser) and 278(Ser) were glycosylated with at least one Hex-HexNAc unit. Previous studies reported the presence of only three *O*-linked glycosylation sites (Spiro and Bhoyroo, J. Biol. Chem. (1974) 249, 5704–5717). Serine 264 was found to be variably glycosylated. The relative proportions of the sialylated and neutral species were determined using high-pH anion-exchange chromatography with pulsed amperometric detection of the β-eliminated oligosaccharides.

Mass spectrometry was supported by NIH grant RR01614 and NSF grant DIR 8700766.

10.32
STRUCTURAL ANALYSIS OF THE OLIGOSACCHARIDES OF OVARIAN CYST FLUID MUCINS FROM A PATIENT WITH BLOOD-GROUP B.

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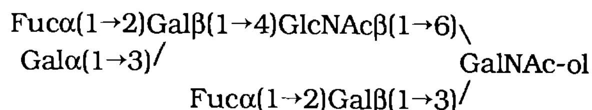
The oligosaccharide chains of the ovarian cyst mucins [OCM) from a patient (P.H.) with blood-group B have been examined. Alkaline borohydride reductive cleavage was applied to the pronase-digest of the OCM to generate oligosaccharide-alditols. These were purified by gel filtration (Bio-Gel P6), ion-exchange chromatography (DEAE Tris-acryl), and HPLC [Dynamax-NH_2). Here we report on the detailed investigation of the HPLC-purified smaller-size neutral oligosaccharide-alditols.

By a combination of monosaccharide composition analysis, fast-atom-bombardment mass-spectrometry (FAB-MS) and 500-MHz ^{1}H-NMR spectroscopy, over thirty structures were identified, ten of which are previously unreported for mucin oligosaccharides. In keeping with the blood-group B status of the patient, many structures were found to contain the Fucα(1→2)[Galα(1→3)]Galβ(1→•) moiety. An example of one such structure is:



Of those HPLC fractions investigated, many contained a mixture of two or three components. Nevertheless, their structures could be determined by 500-MHz ^{1}H-NMR spectroscopy and FAB-MS when at least one component had been identified previously. [This research is supported by NIH grants RO1-HL-38213 and P41-RR-05351.]

10.33
FOLLICULAR PHASE CERVICAL MUCUS GLYCOPROTEIN OF BONNET MONKEY

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Cervical mucus is a hydrophilic, gel-like, dynamic epithelial secretion of columnar cells and plays a key role in mammalian reproduction. The mucus exhibits distinct differences in biophysical, physiological and chemical properties during the menstrual cycle, and these changes are accompanied by alterations in carbohydrate composition and structure. The bonnet monkey, whose menstrual cycle is very similar to that of man and which produces copious amounts of mucus, was used as a model for this study.

The crude cervical mucus, obtained from the same secretor-type monkeys at the follicular phase of the menstrual cycle, was purified by gel-chromatography on Bio-Gel P-200 and Sepharose 2B. The major glycoprotein from Sepharose 2B chromatography was further fractionated on DEAE-cellulose into two main glycoproteins. In addition to amino acids, both glycoproteins contained fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, sialic acid and sulfate groups. The glycoprotein of the major fraction in SDS-PAGE was free of contaminating proteins and exhibited a diffused band. Reductive B-elimination of the DEAE-cellulose purified glycoprotein released oligosaccharide chains. Acidic oligosaccharides were purified by gel filtration and by h.p.l.c. These oligosaccharides, characterized using sequential enzyme treatments and by chemical methods, were found to have a general structure similar to that of periovulatory phase oligosaccharides with minor but definite structural variations.

Estrogenic cervical mucus helps fertilization whereas gestagenic mucus restricts fertilization. Relationship between structure and function of variant glycoproteins using glycoprotein antibodies shall be discussed.

10.34
PRESENCE OF N-GLYCANS ON THE PEPTIDE PRECURSORS OF HUMAN RESPIRATORY MUCINS

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Several data are in favor of the occurence of N-glycans among the multiple O-linked carbohydrate chains of human respiratory mucins: low amounts of mannose have been found in the carbohydrate composition of bronchial mucins (1) and potential sites of N-glycosylation have also been observed in the peptide sequence of human respiratory mucins (2). Recently the biosynthesis of mutiple mucin precursors with different molecular sizes was demonstrated by pulse-labeling of human bronchial mucosa explants (3) and by cell free translation of bronchial mucosa mRNA (4).

To find evidence for the presence of N-glycans on the peptide precursors of human respiratory mucins, explants of human airways were pulse-labeled for 5 min with [^{3}H] threonine, [^{3}H] glucosamine or [^{3}H] mannose. The products, which immunoprecipitated with an antiserum directed against the apomucin peptide, migrated in SDS-PAGE as broad smears. The labeling of these smears was markedly higher with [^{3}H] threonine, but a similar distribution of the radiolabel was observed with [^{3}H] glucosamine or [^{3}H] mannose.

The labeled precursors were bound to immobilized *Concanavalin A* and *Helix pomatia agglutinin,* indicating the presence of some mannose and N-acetylgalactosamine residues. After treatment with endo-β-N-acetylglucosaminidase H, mucin precursors did not react anymore with *Concanavalin A,* suggesting the presence of oligomannosidic chains.

Therefore some mannose and N-acetylgalactosamine residues are incorporated in human respiratory apomucins after 5 min but the precise subcellular localization of the initiation of O-glycosylation (in the RER or the Golgi apparatus) is still an open question.

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10.35
NOVEL NEUTRAL OLIGOSACCHARIDE-ALDITOLS FROM HUMAN RESPIRATORY-MUCUS GLYCOPROTEINS

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In previous studies, 59 low-molecular-mass neutral and monosialylated oligosaccharide-alditols were isolated and characterized from the mucins of a patient with blood group O suffering from bronchiectasis [1–3]. These glycans possessed less than 8 sugar residues. A new series of neutral oligosaccharide-alditols with 7 to 10 sugar residues have been isolated and characterized from the mucins of the same patient.

These glycans were purified by HPLC, using subsequently two silica columns, one bonded with aminopropyl-groups and the other with octadecyl-groups and afforded 46 fractions. The structure of 23 oligosaccharide-alditols have been characterized by employing 500-MHz ^{1}H-NMR spectroscopy, in conjunction with FAB-MS, methylation analysis and sugar analysis.

In some oligosaccharide new structural elements were found that were not present in the low-molecular-mass oligosaccharide-alditols:

(i) 6 structures present a 3, 6 disubstituted galactose residue.

(ii) The periphery of these glycans is characterized by the presence of H, X, Y, Lewis a determinants but also Lewis b determinant that was not observed previously in human bronchial mucins.

(iii) 6 oligosaccharide-alditols present an "internal" H determinant:

(i.e Galβ(1→3 or 4)GlcNAcβ(1→3)[Fucα(1→2)]Galβ(1→4)GlcNAc).

These 23 oligosaccharide-alditols, increases the number of different structures isolated from the bronchial mucins of a single patient to 82 [4–5].

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10.36
SULFATED OLIGOSACCHARIDES FROM RESPIRATORY MUCINS OF A PATIENT SUFFERING FROM CYSTIC FIBROSIS

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Previous studies have shown that respiratory mucins secreted by patients suffering from cystic fibrosis (CF) were highly sulfated [1,2] and it has been reported that "mucins" synthesized by cultured CF nasal epithelial cells were more sulfated than "mucins" produced by normal cells [3].

In order to further investigate the sulfation of respiratory mucins in CF, we have started a structural comparison of sulfated carbohydrate chains from respiratory mucins secreted by CF patients and by patients suffering from other chronic bronchial diseases.

Human respiratory mucin glycopeptides were isolated from the sputum of a CF patient. They were treated with alkaline-borohydride. Fractionation by ion-exchange chromatography yielded several fractions containing sulfate. One of these fractions containing a mixture of small sulfated oligosaccharides was subsequently submitted to gel-filtration and high performance liquid chromatography. The structure of the sulfated oligosaccharide-alditols was determined using 400 MHz ^{1}H-NMR and FAB mass spectrometry. They have a type 2 core and the sulfate is 3-linked to a terminal galactose residue.

Mawhinney *et al* [4] have described small linear sulfated oligosaccharides in respiratory mucins from CF patients with core 1 or 3 and sulfate 6-linked to galactose. It will be interesting to compare the distribution of the different sulfated oligosaccharides (6-sulfated *vs* 3-sulfated) in mucins from patients suffering from CF and from other bronchial disorders and to define their role in the bacterial colonization of the airways in CF patients.

References: [1] Lamblin *et al* 1977, Mod. Probl. Paediat. *19* 153–164; [2] Boat *et al* 1977, Mod. Probl. Paediat. *19* 141–152; [3] Cheng *et al* 1989, J. Clin. Invest *84* 68–72; [4] Mawhinney *et al* 1987, J. Biol. Chem. *262*, 2994–3001.

10.37
GENETIC CONSERVATION OF MUCIN CODING SEQUENCES

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Lung Mucin is a complex glycoprotein of respiratory tract secretions and its abnormal levels have been correlated with its role in cystic fibrosis and asthma. This macromolecule has binding domains for passive clearance of both protein and lipid and may also function as a ligand (via its saccharides) for cell surface receptors of microorganisms. To understand the structure and regulation of expression of canine mucin, we constructed a cDNA library (using canine tracheobronchial mRNA), in the bacteriophage vector lambda gt11 and screened with apo-mucin antisera as well as oligonucleotides as probes. One of the recombinants, MVM-AB5-114–2, was characterized in detail and its 1.8 kb Eco RI insert was used as a probe to identify mucin coding sequence analogs in a wide spectrum of organisms using Southern hybridization. The most significant results obtained are as follows: (i) mucin analogs are present in a variety of organisms like human, cow, rabbit, mouse, bovine, monkey, and chicken suggesting that mucin is a genetically conserved protein; (ii) the canine mucine gene sequence diverged in human, monkey, rat, mouse, cow, rabbit, chicken, and yeast by 13.5, 20.0, 23.5, 25.5, 22.5, 21.5, 28.5, and 29.5% respectively in their mucin counterparts (based on the hybrid melting profile); (iii) the hybridization bands show different restriction patterns in different organisms showing the existence of RFLP; (iv) in the small intestine, mucin cDNA identifies mainly 2.5 kb mRNA and the message is polydisperse in nature; (v) since the size of the canine genomic DNA hybridized is much higher than the mRNA of mucin, it suggests that mucin, like other eukaryotic genes, contains introns; and lastly, (vi) mucin cDNA is rich in threonine as judged by nucleotide sequencing. Based on the above results we propose that mucins are a genetically conserved protein family and can be used in understanding molecular evolution. Supported by grant HL 42332–03 from the National Institute of Health.

10.38

COMPLETE ^{1}H and ^{13}C RESONANCE ASSIGNMENTS FOR THIRTEEN SIALYLATED OLIGOSACCHARIDE-ALDITOL

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400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR spectra of thirteen sialylated oligosaccharide-alditols isolated from hen ovomucin and swallow nest (collocalia mucin) were studied. The resonance assignments were made by combining multiple-relayed coherence-transfer chemical-shift-correlated 2D experiments. The complete analyses of the ^{1}H and ^{13}C spectra of the thirteen compounds was performed, particularly those isolated from collocalia mucin, such as:

```
Gal(α1-4)Gal(ß1-4)Gal(ß1-4)GlcNAc(ß1-6)
                                        \
                                         GalNAc(α1-3)GalNAc-ol
                                        /
                  NeuAc(α2-3)Gal(ß1-3)
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10.39

STRUCTURE OF O-GLYCOSIDICALLY LINKED OLIGOSACCHARIDES ISOLATED FROM MUCINS SECRETED BY THE HUMAN COLONIC GOBLET CELL LINE C1. 16E

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The human colonic CI.16E cell line (1) is a stably differentiated derivative of the HT29 cell line. In confluent cultures, CI.16E cells form homogeneous monolayers of typical goblet cells secreting mucins at their apical surface as a visco-elastic gel (1). The aim of this work was to determine the structure of the oligosaccharide side chains of mucins secreted in culture. To this end C1.16E cells were cultured to confluency in 25 cm^{2} plastic flasks. Mucus secretion was stimulated in serum-free culture medium over a 45 min incubation period. Secretory mucins were purified from contaminating proteins by CsCl gradient centrifugation.

Oligosaccharide chains were released from 12.5 mg of mucins by alkaline borohydride treatment. The oligosaccharide-alditols were purified by gel filtration and fractionated by anion exchange chromatography (AX-10) and partition chromatography on an alkylamine column (2). The structural determination of glycan chains shows:

- a low heterogeneity by comparison with the O-glycans described by Podolsky (3) from human colonic mucins
- the absence of neutral and fucosylated oligosaccharides
- the presence of sialic acid essentially conjugated by (α2–3) linkages.

This is the first determination of the structure of oligosaccharide chains of mucins secreted by a cultured human colonic cell line.

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10.40

HETEROGENEITY OF MUCIN GLYCOPEPTIDES FROM NON-DISEASED AND DISEASED HUMAN AIRWAY

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Many respiratory diseases, e.g. chronic bronchitis, are characterised by mucus hypersecretion and changes in the histochemical staining of secretory cells, however, the cellular events underlying these changes are not well known. To assess whether airways disease could result in alterations in the structure of mucus glycoproteins (mucins), we have studied the nature of the glycosylation of mucins in secretions from diseased and non-diseased human subjects. Mucus samples aspirated from the tracheas of 10 non-diseased human individuals and sputum collected from 5 patients with chronic bronchitis. High-M_r glycopeptides were then prepared from whole mucins isolated by isopycnic density-centrifugation and examined by ion-exchange h.p.l.c. (0–0.25M $LiClO_4$ gradient) followed by slot-blotting and histochemical staining. This revealed a heterogeneous distribution of high-M_r glycopeptides in mucins from both the diseased and non-diseased subjects. All samples contained at least two populations of glycopeptides one less acidic species which became stained with periodic acid/Schiffs reagent and one which eluted later and was more heavily sulphated as shown by staining with high-iron diamine. In subpopulations of mucins with a higher buoyant density a much later eluting species, which was also heavily sulphated, was present. There was no consistent difference between the elution patterns observed for glycopeptides from the non-diseased and chronic bronchitic secretions. The differences observed may therefore be due to individual variation rather than as a result of the disease process.

10.41

STRUCTURE AND PROPERTIES OF RAT GASTRIC MUCUS GLYCOPROTEINS

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The gastric mucosa has the ability to resist hydrochloric acid and pepsin and to recover after damage. One of the protective factors is mucus secreted from the surface epithelial cells. The mucus glycoproteins provide the polymer matrix of mucus and their structure and macromolecular behaviour are important for understanding how they determine the properties of the gel. The molecule consists of a central protein core (20%) substituted with tightly packed 'clusters' of oligosaccharides (80%). These regions are interspersed with 'naked' stretches of protein.

We have purified rat gastric mucus glycoproteins with density-gradient centrifugation in CsC1/4M-guanidinium chloride and showed that they are large (M_r approx, 10x10^{6}, laser light-scattering) macromolecules that can be reduced into 'subunits' by cleavage of disulphide bounds and further fragmented into high-M_r glycopeptides (T-domains) by trypsin digestion. Two populations of T-domains with different size were revealed by gel h.p.l.c and the cognate whole mucins were partially separated with density-gradient centrifugation in CsC1/0.2M-guanidinium chloride. Both T-domain populations showed a pronounced charge heterogenity when subjected to ion-exchange h.p.l.c on Mono-Q.

10.42

INITIATION OF O-GLYCOSYLATION OF PROTEINS

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Comparison of some 120 O-glycosylation sites in mammalian proteins do

not reveal any primary sequence similarity which may be recognized by the polypeptide: α1-0-GalNAc transferase. Our studies on a number of human secretory proteins suggest that a structural motif is recognized by the enzyme initiating O-glycosylation, since all proteins secreted from different recombinant host cell lines analyzed so far bear O-glycans at the same amino acid that is occupied by carbohydrate in their natural counterpart. In all cases>90% of O-glycans have the Galβ1–3GalNAc core structure with one or two NeuAc (BHK, CHO, Ltk, Hela and C 127 cells; C 127 cells synthesize a small proportion of sulfated O-linked structures).

By site directed mutagenesis of several cDNA's coding for human glycoproteins we could show that O-glycosylation of a given protein at a specific site is *specific* for Ser or Thr, irrespective of the host cell line used.

An "improved" O-glycosylation site (..G-G-A-P-T-P-P-G-G...) when introduced at different locations of model proteins (EPO, IL-2) could be shown to be quantitatively O-glycosylated in all cell lines tested whereas the wild-type glycosylation sites of 1L-2 and EPO are recognized by GalNAc-transferase only at their natural positions.

In vitro glycosylation studies, using protein or peptide substrates reveal that initiation of O-glycosylation occurs also at sites which are not recognized intracellularly. Studies using expression of mutant proteins bearing O- as well as N-glycosylation sites indicate that initiation of O-glycosylation (GalNAc transfer in BHK cells) occurs *after* N-glycans become resistant to treatment with Endo H.

Computer modelling of proteins/peptides containing natural as well as genetically engineered (artificial) O-glycosylation acceptor sequences will be presented.

10.43
PURIFICATION OF MUCIN β6-N-ACETYLGLUCOSAMINYLTRANSFERASE TO APPARENT HOMOGENEITY AND CHARACTERIZATION OF ITS ENZYMIC PROPERTIES.

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UDP-GlcNAc:Galβ3GalNAc (GlcNAc to GalNAc) β6 N-acetylglucosaminyltransferase is responsible for the synthesis of the branched structures of ser/thr-GalNAc-linked oligosaccharides. This enzyme activity is enriched in mucin-secreting tissue and has been proposed as a differentiation marker of hematopoitic cells (Fukuda, CIBA Found. Symp. 145:257, 1989). The objective of this study was to purify and characterize this enzyme.

The enzyme was solubilized from the Golgi membranes of bovine tracheal epithelium with 1% Triton X-100 containing 2 mM Galβ3GalNAcαBzl and bound to UDP-hexanolamine-Actigel ALD superflow column in the presence of 1 mM Galβ3GalNAcαBzl. SDS-PAGE analysis of the enzyme eluted from the affinity column with 0.5 mM UDP-GlcNAc showed a single band at about 69 Kd. The intensity of this band follows enzyme activity across the peak. The enzyme was purified 134,000 fold with 1.3% yield and a specific activity of 70 μm/min per mg protein. Initial velocity and product inhibition studies suggest an ordered sequential mechanism, in which UDP-GlcNAc binds first and UDP leaves last. Acceptor competition studies employing Galβ3GalNAcβBzl, GlcNAcβ3GalNAcβPNP, and GlcNAcβ3GalβMe as the acceptor substrates demonstrated that this enzyme catalyzes the formation of branched β6 N-acetylglucosaminide structure with these acceptors.

In conclusion, we have purified the β6-N-acetylglucosaminyltransferase to apparent homogeneity. This enzyme does not have a strict specificity for acceptor substrates and is capable of forming all branched β6 N-acetylglucosaminide linkages in glycoproteins, which contain the following precursor structures, GlcNAc/Galβ3GalNAcα/βR or GlcNAcβ3GalβR.

10.44
O-GLYCAN CORE 2 UDP-GlcNAc: Galβ1–3GalNAc-R β6-GlcNAc-TRANSFERASE FROM AML CELLS DIFFERS SIGNIFICANTLY IN SPECIFICITY FROM THE ENZYME IN MUCIN SECRETING CELLS

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The enzyme synthesizing O-glycan core 2 [GlcNAcβ1–6(Galβ1–3)GalNAc-R], UDP-GlcNAc: Galβ1–3GalNAc-R β6-GlcNAc-transferase (core 2 β6-Gn-T) is increased in activity in granulocytes from patients with chronic myelogenous leukaemia and in leukocytes from patients with acute myeloblastic leukaemia. The enzyme appears to be regulated during the process of differentiation. To develop an inhibitor for this activity, we synthesized a number of substrate analogues and tested the substrate specificity of the enzyme. The results using a number of synthetic derivatives will be presented. The pig gastric and rat colonic mucosal enzyme activity is accompanied by activities that synthesize core 4 [GlcNAcβ1–6(GlcNAcβ1–3)GalNAc-R]and blood group 1 [GlcNAcβ1–6(GlcNAcβ1–3)Galβ-R] branches; these two activities are absent from leukaemic cells. Core 2 β6-Gn-T has an absolute requirement for the 6-hydroxyl of the Gal residue of Galβ1–3GalNAcα-benzyl substrate. Omission of the 3– or 4– hydroxyl of Gal significantly reduces the activity. This suggests that upon elongation of core 1 [Galβ1–3GalNAcα-R] the pathway to core 2 is blocked. We observed significant kinetic differences towards various substrate derivatives between the enzymes from leukaemic cells and mucin secreting tissue. The results suggest the existence of at least two different core 2 β6–Gn–T species. (This work was supported by grants from the CCFF, MRC and NCI of Canada and NATO.)

10.45
CONTROL OF O-GLYCAN SYNTHESIS: SPECIFICITY OF O-GLYCAN CORE 1 UDP-Gal: GalNAcα-R β3-Gal-TRANSFERASE FROM RAT LIVER

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The specificity of glycosyltransferases is a major control factor in the biosynthesis of O-glycans. O-glycan Core 1 UDP-Gal:GalNAcα–R β3-galactosyltransferase was purified from rat liver by chromatography on CM-Sephadex, Affi-Gel Blue, Sephadex G 200 and 5-Hg-UDP-GlcNAc-thiopropyl-Sepharose. Series of GalNAcα-R derivatives were chemically synthesized.

The enzyme is sensitive to changes in the R-group of the GalNAcα-R acceptor substrate and is strongly stimulated when the R-group is a peptide or an aromatic group. Removal of the 3- or 4-hydroxyl or substitution of the 3-hydroxyl of GalNAc abolishes the activity. Removal or substitution by GlcNAc of the 6-hydroxyl of GalNAc reduces activity

only slightly and 6-deoxy-GalNAcα-benzyl acted as a competitive substrate. This indicates that the enzyme needs the 3- and 4-hydroxyl but not the 6-hydroxyl as an essential requirement for binding and activity. The results of specificity studies are used to design a specific inhibitor. In the usual biosynthetic pathway, Gal is added first in β-linkage to the 3-hydroxyl of GalNAc to form core 1 Galβ1–3GalNAc-R. We have demonstrated, that the enzyme can act on GlcNAcβ1–6GalNAc–Bn to synthesize O-glycan core 2 GlcNAcβ1–6(Galβ1–3)GalNAcα-benzyl. This unusual pathway may be of importance in tissues containing UDP-GlcNAc:GalNAc-R β6-GlcNAc-transferase. (This work was supported by CCFF, MRC of Canada, Deutsche Forschungs-gemeinschaft and NATO grants.)

10.46
DEFECTIVE CELL SURFACE EXPRESSION OF BLAST-2 (CD23) IN EBV-B CELLS WITH ABERRANT O-LINKED OLIGOSACCHARIDES

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The Wiskott-Aldrich syndrome (WAS) is an X-linked, recessive, immunodeficiency in which B cells fail to respond to certain mitogens. Epstein-Barr virus-immortalized B (EBV-B) cells from these patients have very little UDP-GlcNAc:Galβ3-GalNAc-R (GlcNAc to GalNAc) β-6-N-acetylglucosaminyltransferase activity (0.4 nmol/mg/h) relative to normal EBV-B cell lines (2.4 nmol/mg/h). The WAS EBV-B cell lines also have a higher CMP-SA:SAα3Galβ3GalNAc-R (SA to GalNAc) α6-sialyltransferase II activity (2.0 nmol/mg/h) than the normal EBV-B cell lines (0.1 nmol/mg/h). Concomitant to this change in the activities of these two glycosyltransferases, the WAS EBV-B cell lines have a dramatic reduction in the amount of polylactosamine on their O-linked oligosaccharide structures.

In response to T cell-derived interleukin-4, B cells show an increase in the cell-surface expression of Blast-2 (CD23) as well as an increase in the soluble form of Blast-2 which acts as an autocrine B cell growth factor. EBV-B cells express a large amount of Blast-2 on their cell surface and their growth in serum-free medium is dependent upon soluble Blast-2 in the medium. WAS EBV-B cells have longer doubling times in serum-free medium than normal EBV-B cells and this difference is more pronounced at lower cell densities. We examined Blast-2 expression in WAS and normal EBV-B cells and observed significantly less cell surface Blast-2 on WAS EBV-B cells than on normal EBV-B cells in spite of the fact that WAS EBV-B cells produce similar amounts of Blast-2 protein in a 15 minute labelling period. Further work is in progress to determine whether the reduction in cell surface expression of Blast-2 on WAS EBV-B cells is related to aberrant glycosylation in these cells.

10.47
PEPTIDE SPECIFICITY OF UDP-N-ACETYLGALACTOSAMINE: PROTEIN α-N-ACETYL-GALACTOSAMINYLTRANSFERASE FROM PIG TRACHEAL EPITHELIUM

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Pig tracheal epithelium contains a highly active UDP-GalNAc:proteinα-N-acetylgalactosaminyltransferase similar in properties and specificities to the enzyme obtained from other sources; porcine & bovine submaxillary glands, bovine colostrum, BW5147 mouse lymphoma & BHK cells.

The enzyme is membrane bound and was released from microsomal preparations of pig tracheal epithelium by extraction with Triton-X-100. It specifically catalysed the transfer of N-acetylgalactosaminyl residues from UDP-N-acetyl [^{3}H]-D-galactosamine to the core proteins of de-glycosylated bovine submaxillary mucin, the C-terminal region of SV40 viral protein, and bovine brain myelin basic protein. The octapeptide of identical sequence to residues 96–103 of myelin basic protein (VTPRTPPP) was also an excellent substrate.

Tritiated product from the enzyme reaction with VTPRTPPP was cleaved with trypsin. This yielded two fragments both of which were similarly [^{3}H]-labelled. Treatment of each of the fragments with α-N-acetylgalactosaminidase released the radiolabel from the peptide. This showed that both threonines in VTPRTPPP were similarly glycosylated, probably in the α-configuration.

Peptides containing the TPPP sequence required a peptide length $\geqslant 5$ for high acceptor activity. Highest activity of the enzyme was found with peptide substrates containing L-threonyl residues rather than seryl or L-allo-threonyl residues in the equivalent positions.

S11 CELL ADHESION/ADHÉSION DE CELLULES

11.1
CARBOHYDRATE-MEDIATED GAMETE ADHESION DURING FERTILIZATION IN MAMMALS

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For both plants and animals, the ability of male and female gametes of the same species to recognize one another is a ubiquitous and essential feature of the fertilization process. Among mammals, the unfertilized egg extracellular coat, or *zona pellucida* (ZP), is the site of "sperm receptors" that, together with complementary "egg-binding proteins" located on the sperm surface, support species-specific gamete adhesion during the course of fertilization (Wassarman, 1987, 1990).

The mouse sperm receptor, called mZP3, is a ZP glycoprotein (83,000 Mr) that consists of a 44,000 Mr polypeptide (402 amino acids), 3 or 4 complex-type N-linked oligosaccharides, and an undetermined number of O-linked oligosaccharides. Solely as a consequence of its oligosaccharides, mZP3 is quite acidic and exhibits considerable heterogeneity on SDS-PAGE. There are more than a billion copies of mZP3 in the ZP. Each sperm binds to as many as tens-of-thousands of copies of mZP3 at the ZP surface. Binding to mZP3 causes sperm to undergo a form of exocytosis, called the "acrosome reaction," that enables bound sperm to penetrate through the ZP and to reach, and then fuse with, egg plasma membrane.

The sperm receptor function of mZP3 is attributable to certain of the glycoprotein's O-linked oligosaccharides. These O-linked oligosaccharides have an apparent Mr of 3,900, have a galactose residue at their

nonreducing terminus (α-linkage) that is essential for sperm receptor function, and, at least some of these oligosaccharides, are located on the carboxy-terminal half of the mZP3 polypeptide. Certain cell lines, stably transfected with the mZP3 gene, synthesize and secrete functional sperm receptors, suggesting that the glycoprotein is properly glycosylated. These cell lines, together with other experimental approaches, are being used currently in our laboratory to investigate O-linked glycosylation of glycoproteins in general.

Wassarman, P.M., *Science* 235, 553–560, 1987; *Development 108*, 1–17, 1990.

11.2
ACIDIC GLYCANS AS NOVEL MEDIATORS OF CELL ADHESION

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Cell recognition and adhesion in the marine sponge *M. prolifera* is mediated via two acidic glycans, Mr 6,000 (G-6) and Mr 200,000 (G-200), from a sponge proteoglycan-like molecule. These acidic glycan adhesion molecules (AGAMs) function via a conceptually new mechanism of highly polyvalent low affinity carbohydrate-carbohydrate and carbohydrate-protein interactions. This is in contrast to cell adhesion molecules of the immunoglobulin, integrin and lectin gene superfamilies which operate through monovalent or tetravalent, moderate affinity protein-protein or protein-carbohydrate bindings. Using immunological, biochemical and chemical studies it was shown that the G-6 and G-200 polysaccharides belong to a novel class of N-linked acidic glycans which contain glucuronic acid, fucose, and mannose. Thus, these AGAMs have some properties of glycosaminoglycans and N-linked polysaccharides.

Monoclonal antibodies which inhibit cell adhesion in sponges, named Block 1 and 2, recognize G-6 and G-200 respectively. Immunofluorescence microscopy with Block 1 and 2 showed that AGAM epitopes similar to those from G-6 and G-200 are also present in sea urchin embryos, mice embryos and normal and malignant human cells. In sea urchin embryos both AGAM 1 and AGAM 2 epitopes appeared in morula stage and their content increased during development to gastrula stage. AGAM 1 was localized mainly in cell-cell contacts whereas AGAM 2 epitope in hyaline layer and blastocoel extracellular matrix. Total polysaccharides were isolated from different stages of development and immunoblotting analyses of separated glycans showed that two large acidic glycans carry AGAM 1 and 2 epitopes. The functional studies using Block 1 and 2 revealed that both antibodies were capable of inhibiting reaggregation of dissociated sea urchin blastula cells. Furthermore, cell adhesion was strongly promoted by the sponge proteoglycan carrying multiple repeats of the AGAM epitopes, thus revealing that these novel carbohydrate structures mediate cell aggregation via polyvalent interactions also in the sea urchin embryos.

In 16 days mouse embryos AGAM 1 and 2 were present in different parts of the brain, in the stomach and spinal cord. In adult humans AGAM 2 was detected in cell-cell contact of normal goblet cells whereas AGAM 1 stained extracellular matrix of human colon carcinomas. Activated human platelets express both AGAM 1 and 2 and a subpopulation of the human monocytic cell line Mono-Mac 6 is positive for AGAM 1. Functional studies will establish whether AGAMS in mice and humans also mediate cell adhesion. Taken together these results indicate that the new class of acidic glycans functions as novel adhesion molecules in the animal kingdom. Control of the spatial and temporal expression of different AGAM isoforms may contribute to the regulation of cell recognition and adhesion during morphogenesis and metastasis.

11.3
FIBRONECTIN FROM TUBEROUS SCLEROSIS CELLS CARRIES A SULPHATED GLUCURONIC ACID STRUCTURE ON A 140kDa PROTEOLYTIC FRAGMENT

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Fibronectin is a dimeric glycoprotein of the extracellular matrix. This protein contains several distinct binding domains which mediate its interaction with cells and with other extracellular matrix components. Collagen and cell binding domains are known to contain N-glycans. We present evidence that fibronectin produced by cultures of fibroblasts from skin and skin lesions of patients with tuberous sclerosis contains a carbohydrate structure unusual in fibronectin. This structure, which is recognized by HNK-1 antibody, contains sulphated glucuronic acid and is usually associated with glycolipids and neural cell adhesion molecules. It is believed to be involved in cell-cell and cell-substratum interactions during neural development.

We have observed this HNK-1 positive epitope to be associated with a 140kDa fragment of fibronectin (derived by thermolysin proteolysis) which probably contains the cell binding domain of this protein. In cultures of cells from skin and skin lesions of patients with tuberous sclerosis this fibronectin-linked epitope is associated primarily with unusually large neuron-like cells.

These observations may be important indicators of the aetiology of tuberous sclerosis.

11.4
IMMUNOCHEMICAL PROBES FOR KDN AND OLIGO/POLY(KDN) STRUCTURES IN GLYCOCONJUGATES

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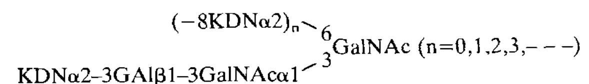
The naturally occurring sialic acid residues had been confined to derivatives of *N*-acylneuraminic acids until our recent finding of the deaminated neuraminic acid (KDN; 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) residue in polysialoglycoproteins (PSGP) of salmonid fish eggs. More recently, we have reported isolation of glycoproteins containing more than 50% (w/w) KDN but no *N*-acylneuraminic acids from the vitelline envelope of rainbow trout eggs as well as from the ovarian fluid of ovulating fish, and named them KDN-gp-VE and KDN-gp-OF. The chemical structures of *O*-linked oligosaccharide units of KDN-gp-VE have been elucidated as:



Our findings represent the first example showing the occurrence of oligo/poly (KDN) chains in glycoconjugate molecules.

In this study we have searched for utility of anti-(KDN-gp-VE)antiserum raised in rabbit, designated RM (identified as IgG). (1) We examined the specificity of RM and found that RM binds strongly to KDN-gp-VE, and the binding is completely inhibited by oligo(KDN). RM also reacted strongly with KDN-ganglioside, (KDN)G_{M3}, recently isolated from rainbow trout sperm. These and other data indicate that RM can be regarded as specific reagent for recognition of KDNα2–3Gal and α–2,8–linked oligo/poly(KDN) groups. (2) RM was used to survey the localization of KDN-gp in the sites other than egg surface and ovarian fluid. RM reacted with the serum of pre-ovulating female trout but not with those of post-ovulating trout or male trout fish. These

findings, though still preliminary, indicate that KDN-gp is synthesized not in the ovary but in the other organ (most likely liver) under the regulation of relevant hormones and transported via blood stream to the ovary and partly onto the vitelline envelope stage-specifically immediately prior to ovulation. The biosynthetic and functional relationships between KDN-gp-OF and KDN-gp-VE remain to be solved. KDN-gp-VE has been demonstrated to localize in the second layer of VE by the electron microscopic observation of the immunostained section.

11.5

STRUCTURE AND FUNCTION OF POLY(KDN)-GP AND POLY(SIA)-GP: Two Distinct Types of Glycoproteins Isolated from the Vitelline Envelope and Ovarian Fluid of Salmonid Fish

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All vertebrate eggs are surrounded by extracellular coats generally called the vitelline envelope (VE: the zona pellucida or chorion). Carbohydrate is a functional component of VE and play the regulatory and protective roles including species-specific recognition during fertilization and the early embryonic development. Recently, we reported isolation of a $\sim 10^3$ KDa deaminated neuraminic acid-rich glycoprotein (KDN-gp; renamed as poly(KDN)-gp) from both VE and the ovarian fluid (OF: a viscous fluid surrounding ovulated eggs) of rainbow trout. The most unusual feature of KDN-gp is that it contains about 50%(w/w) of KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) and no acylneuraminic acid. Poly(KDN)-gps have been found to have a number of *O*-linked glycan chains: KDNα2–3Galβ1–3GalNAcα1–3[(–8KDNα2)$_n$–6]GalNAcα1–Thr(Ser).

More recently we found that VE and OF of some species of salmonid fish contain poly(NeuGc)-gp in place of poly(KDN)-gp. Poly(NeuGc)-gp contains about 50%(w/w) NeuGc together with a small amount of KDN, and has the trisaccharide core structure, Galβ1–3GalNAcα1–3GalNAcα1–Thr(Ser), identical with that of poly(KDN)-gp.

Now our interests are centered at the function and biosynthesis of these two types of glycoproteins, poly(KDN)-gp and poly(Sia)-gp. Both poly(KDN)-gp and poly(Sia)-gp were found to show avidity towards homologous sperm. Our preliminary data suggest that poly(KDN)-gp and poly(NeuGc)-gp are synthesized in the liver of pre-ovulating female fish under the influence of hormone and transported through blood stream to the ova. The structural, functional, and biogenetic relationships between poly(KDN)-gp and poly(NeuGc)-gp, deposited on VE and accumulated in OF, and the structural change, if any, of these glycoproteins after fertilization are most intriguing subjects which remain to be clarified in future.

11.6

ENHANCED PHOSPHORYLATION OF HYALURONECTIN, AN ADHESIVE PROTEIN IN TRANSFORMED CELLS

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The extracellular matrix in most tissues contain several distinct molecules that may influence tumor cell adhesion. Amongst the GAGs present in ECM, hyaluronate (HA) is known to influence cell differentiation, tumor invasion. In order to clarify HA cell surface interaction, we purified for the first time a 68 kDa glycoprotein which binds specifically with HA amongst GAGs. Amino acid composition confirms that it is distinct from other HA binding protein e.g., link protein and fibronectin. In order to identify its important role in matrix, hyaluronectin is shown to interact with FN, laminin and collagen suggesting its role in structural organization. In order to study its physiological role, we confirmed hyaluronectin as cell surface secretory and adhesive protein since its coating on the plates promotes the cell attachment. The adhesive property of hyaluronectin and its role in tumor formation is further confirmed by measuring its level in skin tumor as well as in polyoma transformed fibro-blast cells. Further, we have confirmed its autophosphorylation nature and enhancement of its rate of phosphorylation in transformed cells. The exogenous addition of a hyaluronectin alone and along with HA to the cells stimulates in vivo protein phosphorylation. Simultaneously a significant increased phosphorylation of hyaluronection is observed as shown by immunoprecipitation. The possible role of hyaluronectin as oncoprotein will be discussed.

11.7

STUDIES ON STRUCTURAL REQUIREMENTS AND FUNCTIONAL IMPLICATIONS OF THE L2/HNK-1 CARBOHYDRATE EPITOPE IN NEURAL CELL RECOGNITION.

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The N-linked sulfated L2/HNK-1 carbohydrate epitope has been implicated in the adhesion of early postnatal cerebellar cells to laminin as well as in neurite outgrowth and cell migration (Künemund et al., J. Cell Biol., 213–223, 1988). Here we investigate the L2/HNK-1 dependent adhesion of postnatal cerebellar neurons and embryonic astrocytes from total brain to laminin in more detail. In order to understand the structural requirements of the L2/HNK-1 epitope for its biological function, we also studied the properties of different synthetic glycosphingolipids (Nakano et al., Tetrahedron Lett. 31, 1597–1600, 1990) in TLC overlays, ELISA and microexplant assays.

The results can be summarized as follows:

(i) The sulfated glucuronic acid of the L2/HNK-1 epitope carrying glycolipid is necessary, but not sufficient for antibody binding or for inhibition of cell adhesion, cell migration and growth of neuritic and astrocytic processes as observed in *in vitro* assays.

(ii) The adhesion of astrocytes and neurons to laminin is not only inhibited by the synthetic or natural L2/HNK-1 glycolipid, but also by Fab fragments of an L2 monoclonal antibody and, when cells are cultivated in the presence of castanospermine or swainsonine. The binding properties of the L2/HNK-1 epitope to laminin is compared to those of heparin and sulfatide.

11.8

N-LINKED NEUTRAL SUGAR CHAINS OF PORCINE ZONA PELLUCIDA GLYCOPROTEINS

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The zona pellucida (ZP) is an extracellular coat surrounding mammalian eggs, and plays important roles in fertilization. Several lines of evidence

suggest that the sugar moieties of glycoproteins constructing the ZP may function as sperm receptors. (1, 2) However, no detailed structural analysis of the sugar moieties of ZP glycoproteins has been reported.

ZP glycoproteins were isolated from porcine ovaries as described previously (3), and *N*-linked sugar chains were released by hydrazinolysis. By paper electrophoresis, the oligosaccharides were separated into neutral and acidic fractions in a percent molar ratio of 33:67. The neutral oligosaccharides were fractionated by a serial lectin column chromatography and Bio-Gel P-4 column chromatography, and their detailed analysis was performed. Almost all of the oligosaccharides were of complex-type with a fucosylated trimannosyl core. However, several variations were found in their outer chain moieties. First, biantennary, 2,4- and 2,6-branched triantennary, and tetra-antennary oligosaccharides were included in an approximate molar ratio of 4:2:1:1. Second, 26% of these oligosaccharides contained *N*-acetyllactosamine repeating units in their outer chain moieties. Thirdly, exposed *N*-acetylglucosamine residues were included in non-repeating and repeating outer chains of 39 per cent of the total neutral oligosaccharides. These structural information will help us to find a clue to the understanding of the molecular mechanism of mammalian fertilization.

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11.9
THE SUGAR CHAINS OF HUMAN SERUM VITRONECTIN

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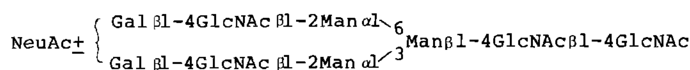
Vitronectin, also termed as S-protein and serum spreading factor, is one of major multifunctional glycoproteins present in mammalian plasma and serum. In a previous report, we purified six animal vitronectins and found that marked diversity in vitronectins is in their glycosylation[1], which may affect their stability against the proteolysis. The entire primary structure and functional domains of human serum vitronectin (hVN) have been elucidated. However, the carbohydrate moiety of vitronectin has not yet been characterized although three asparagine residues are predicted to link with sugar chains.

Sugar composition of purified hVN was found to be GlcN, Man, Gal, NeuAc and no GalN. Binding studies with peroxidase-labeled lectins revealed that hVN contained complex-type, sialylated *N*-linked sugar chains. *N*-linked oligosaccharides were released from hVN by almond glycopeptidase digestion after desialylation. The reducing ends of the oligosaccharides were labeled with fluorescent reagent, 2-aminopyridine, by reductive amination. Upon HPLC on ODS-silica column, pyridylamino oligosaccharides were separated into 7 fractions. The each fraction was further separated by HPLC on amide-silica column and 13 fractions were finally obtained. The structure of each oligosaccharide thus isolated was analyzed by a two-dimensional mapping method using a combination of sequential exoglycosidases digestion and the two kinds of HPLC.

Mono-, bi- and tri-antennary lactosamine type sugar chains were present on hvN and the structure of the major oligosaccharide was determined to be:



1) H. Kitagaki-Ogawa et al. Biochim. Biophys. Acta 1033 (1990) 49–56.

11.10
GLYCOSAMINOGLYCANS CONJUGATED TO PHOSPHATIDYLETHANOLAMINE (NEOPROTEOGLYCANS) INHIBIT HEPATOCYTE ATTACHMENT TO COLLAGEN, FIBRONECTIN AND LAMININ SUBSTRATES

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Our previous results suggested an importance of the interaction of hepatocytes with extracellular matrix (ECM) proteins in growth stimulation of hepatocyte in response to partial hepatectomy [1] and a possible involvement of proteoglycans in growth suppression [2]. In order to examine whether glycosaminoglycans (GAGs) (or proteoglycans) in ECM affect the interaction of hepatocytes with the other ECM components, we studied the effect on hepatocyte attachment of GAGs fixed on the culture surface together with some ECM proteins. Microtiter plates were coated with collagens, fibronectin and laminin, then with phosphatidylethanolamine dipalmitoyl (PE)-conjugates of: CS, shark cartilage chondroitin sulfate; DS, porcine skin dermatan sulfate; HS, porcine kidney heparan sulfate; heparan sulfate from mouse EHS sarcoma; heparin from porcine small intestine; cockscomb hyaruronic acid. Primary rat hepatocytes were seeded onto the plates and were tested in a short term assay for attachment. The attachment to fibronectin and laminin substrates was inhibited most effectively by CS-PE (1μg/ ml for 50% inhibition). HS-PE and DS-PE were also inhibitory but less effective. The attachment to collagen type I, III, IV and V substrates was inhibited by HS-PE as efficiently as by CS-PE (0·5–4μg/ ml for 50% inhibition). DS-PE was also inhibitory but less effective. The other GAG-PEs had little effect on the attachment to these substrates. EGF-dependent DNA synthesis of primary hepatocytes on collagen type I substrates was slightly but significantly inhibited by pretreating the substrates with 3–10μg/ml CS-PE. These results support the idea that GAG moiety of some proteoglycans in ECM may play a role in keeping hepatocytes quiescent in normal tissue.

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2) Otsu, K. *et al.* (1989) Cell Differentiation Develop. 27, S83.

11.11
INFLUENCE OF SIALIC ACID O-ACETYLATION OF MOUSE ERYTHROCYTE GLYCOCONJUGATES ON MALARIA INFECTION

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For a number of *Plasmodium falciparum* strains, the invasion of human erythrocytes has been shown to be dependent on the presence of N-acetyl-neuraminic acid residues on the red blood cells (RBC) [1]. Although the erythrocytes of other mammals are also highly sialylated, they are not infected or only to a much lower degree by these *P. falciparum* strains. To elucidate whether this may be due to the type of sialic acids, the RBC of several mouse strains were analyzed, and the results correlated with the degree of infection by two different *P. falciparum* strains. Three different sialic acid species, N-acetyl-, N-glycoloyl-, and N-acetyl-9-O-acetylneuraminic acid were found on mouse RBC. The ratio of these sialic acids varies considerably among the mouse strains. Infection with *P. falciparum* merozoites was best for

those RBC with the highest relative amount of Neu5Ac, whereas high percentages of Neu5,9Ac_2 significantly reduced parasitemia. A similar effect was observed for binding of the malaria-specific erythrocyte-binding antigen [2]. Invasion and binding were increased after treatment of the erythrocytes with influenza C virus that has a specific sialate 9-O-acetylesterase [3]. This shows that the function of sialic acid as receptor is reduced by 9-O-acetyl groups, which is in contrast to the generation of such a receptor function by these ester groups for e.g. influenza C virus [3].

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[2] D. Camus and T.J. Hadley (1985) Science 230, 553–556
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11.12
STRUCTURE-FUNCTION STUDIES ON THE CARBOHYDRATE LIGANDS FOR ELAM-1

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The acute inflammatory response requires that circulating leukocytes bind to and penetrate the vascular wall to access the site of injury. Several receptors have been implicated in this interaction, including a family of carbohydrate binding proteins, the LEC-CAMs. We have identified and characterized endogenous carbohydrate ligands for one of these receptors, LECAM-2 (ELAM-1). Radiolabeled COS cells transfected with a plasmid containing the cDNA for LECAM-2 were used as probes to screen glycoconjugates extracted from human leukocytes, synthetic glycoconjugates or enzymatically or chemically altered carbohydrates. COS cells expressing LECAM-2 adhered to a subset of sialylated glycolipids resolved on TLC plates or adsorbed on PVC microtiter wells in a calcium dependent manner. Structural analysis of purified compounds indicate that LECAM-2 recognizes terminally sialylated lactosyl ceramides with a variable number of N-acetyllactosamine repeats and at least one fucosylated N-acetylglucosamine residue. Data from direct binding and inhibition studies indicate that both sialic acid and fucose residues are required for binding. The linkage of the sialyl and fucosyl residues are critical for recognition, although some changes can be made in the N-acetyllactosamine backbone. Removal of C-8 and C-9 from the sialic acid had little effect on recognition, while derivatives of the carboxylic acid had reduced activity. These and other data will be used to define required structural features of the ligands.

11.13
5'-NUCLEOTIDASE AND INTEGRIN: THEIR RELATIONSHIP WITH EXTRACELLULAR MATRIX GLYCOPROTEINS DURING THE CHICKEN STRIATED MUSCLE DEVELOPMENT

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The ecto-enzyme-5'-nucleotidase isolated from chicken gizzard has been previously shown to be a potent ligand of two extracellular matrix glycoproteins, fibronectin and laminin respectively. Furthermore *in vitro* studies have revealed that 5'-nucleotidase was involved in the spreading of various mesenchymal derived cells, including myoblasts, on laminin substrate. In the present work we show that 5'-nucleotidase codistributes with laminin during the chicken striated muscle development. Whereas ecto-5'-nucleotidase is faintly detectable on cells surrounded by a matrix expressing a high level of fibronectin. This pattern of distribution distinguishes 5'-nucleotidase from the chicken integrin, which recognizes both laminin and fibronectin, expressed as well in muscular tissue as in connective tissue. In addition, the specific activity of striated muscular ecto-5'-nucleotidase increases significantly from early stages of development to the adult. At each age considered this specific activity corresponds to a 80 KDa enzyme which is inhibited by α,β-methylene adenosine diphosphate or by a monoclonal antibody directed against the smooth muscle forme of the enzyme. In adult muscle, 5'-nucleotidase displays a heterogenous distribution. The immunolabelling was mainly observed in two specialized structures, mimicking the focal contacts observed in culture cells, i.e., plasmalemma associated with costameres in the I band of muscle myofibers and with myotendinous junctions, respectively.

11.14
DEFECT IN FIBRONECTIN RECEPTOR FUNCTIONS DURING RAT HEPATOCYTES TUMORIZATION ARE RELATED TO ALTERATIONS IN GLYCOSYLATION

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The integrins are heterodimers consisting of noncovalently associated α and β subunits. They mediate both cell-substratum and cell-cell adhesion. Integrins are modified by post-translational events (phosphorylation and glycosylation). The role of these modifications in the integrins function are largely unknown. Therefore, the biosynthesis of the fibronectin receptor (FNR) was followed in normal and tumor rat hepatocytes. FNR was isolated in hepatocytes: it is composed of an α–155 kDa and a β–115 kDa subunits (Johansson et al., 1987). Our study demonstrated that the α and the β subunits are synthesized in the tumor cells, but the post-traductional maturation of the β chain was modified in these cells. Polyclonal antibodies directed against the β-chain recognized proteins of Mr 100 and 115 kDa in hepatocyte lysate, while proteins of Mr 100 and 130 kDa were detected in the tumor cell lysate. Pulse-chase experiments in hepatocytes showed that the 100 kDa protein was progressively converted into the typical mature 115 kDa protein, and only this later was found at the surface of the cells. N-glycanase treatment of this mature form decreased its molecular weight to 82 kD. In contrast, in the tumor cells two different fates were observed for the precursor. A part of the 100 kDa protein remained Endo H sensitive all along the chase period and was surprisingly expressed at the surface of the cell, while the other part was converted very slowly into the 130 kDa protein. The molecular weight of this mature form recovered at the surface of the tumor cells was decreased to 105–110 kD after N-glycanase digestion, indicating that beside N-glycosylation other post-translational modifications were present on the β–130 kD subunit. Furthermore these post-translational changes appeared to perturb the biological functions of the FNR expressed in the tumor cells (adhesion process, fibronectin binding).

11.15
SIALOADHESIN, A MOUSE MACROPHAGE RECEPTOR INVOLVED IN CELLULAR INTERACTIONS, RECOGNISES SPECIFIC CELL SURFACE SIALOGLYCOCONJUGATES

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Sialoadhesin is a sialic acid specific receptor expressed on stromal macrophage subpopulations in the mouse. High levels of expression were found on macrophages in bone marrow, lymph nodes, and spleen[1]. Evidence for the involvement of sialoadhesin in the development of myelomonocytic cells in the bone marrow came from the specific distribution of this receptor at the contact sites between progenitors of these cells and resident bone marrow macrophages[2]. In addition, the distribution of sialoadhesin expression in lymph nodes also suggests a possible role of this receptor in lymphocyte trafficking[1].

Sialoadhesin mediated binding required sialic acid but was Ca^{2+}-independent. To further investigate the specificity of this receptor, we studied the interaction of sialoadhesin, either expressed on macrophages or purified, with derivatised human erythrocytes, glycoproteins, and glycolipids. On derivatised erythrocytes, sialoadhesin exhibited a clear preference for NeuAcα2→3Galβ1→3GalNAc, suggesting that on native human erythrocytes, the receptor recognised this structure present in O-linked oligosaccharides on glycoproteins. This conclusion was supported by the findings (a) that human glycophorin was a potent inhibitor of haemagglutination and (b) that sialoadhesin bound specifically to glycophorins on Western blots of erythrocyte membranes. The specificity of sialoadhesin towards glycolipids was studied using either gangliosides coated on asialo-erythrocytes or separated on TLC plates. In both systems, sialoadhesin recognised, in rank order: $G_{T1b}>G_{D1a}>G_{M3}>G_{Q1b}>G_{M2}>G_{D1b}>G_{D3}$. G_{M1} was not bound.

In conclusion, sialoadhesin specifically recognises the oligosaccharide sequence NeuAcα2→3Galβ1→3GalNAc in either sialoglycoproteins or gangliosides. These findings imply that specific sialoglycoconjugates carrying this structure may be involved in cellular interactions between macrophages and haematopoietic cells in the bone marrow or between macrophages and lymphocytes in lymph nodes.

[1]Crocker, P.R. and Gordon, S. (1989) *J. Exp. Med.* **169**, 1333–1346
[2]Crocker, P.R., Werb, Z., Gordon, S. and Bainton, D.F. (1990) Blood **76**, 1131–1138

11.16
THE EFFECT ON NEUTROPHIL ADHESION OF ANTIBODIES AGAINST SURFACE GLYCOPROTEINS WHICH EXPRESS CD15 (3-FUCOSYL-N-ACETYLLACTOSAMINE)

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The carbohydrate antigen, CD15, 3-fucosyl N-acetyllactosamine has recently been shown to be the ligand for endothelial lectins, ELAM-1 and GMP140. NCA-160, a carcinoembryonic antigen (CEA) related glycoprotein is the major carrier of CD15 which is also expressed on the common beta chain of the LFA-1/CR3/gp150,95 family of adhesion promoting leucocyte integrins. We have shown that rabbit IgG antibodies directed against CEA which cross-react with neutrophil non-specific cross-reacting antigens (NCAs) increase the adhesion of neutrophils to plastic. $F(ab')_2$ and Fab' antibody fragments as well as a monoclonal antibody recognising the same antigen have the same effect. The monoclonal antibody MC2, specific for CD15, both inhibits (at saturating concentration) and augments adhesion (at sub-saturation levels). Anti-CEA and MC2 antibodies cause the homotypic adhesion of neutrophils demonstrable by light microscope and flow cytometry. Anti-CR3 beta chain monoclonal antibodies inhibit both adhesion to plastic and homotypic adhesion. Flow cytometry was also used to study the surface expression of these glycoproteins, and it was shown that CR3, NCA and CD15 are upregulated in response to stimulation by the chemotactic peptide f-met-leu-phe. It was also shown that in adhesion assays the adherent PMN express more NCA and CD15 than non-adherent cells.

11.17
LAMININ CARBOHYDRATES ARE IMPLICATED IN CELL SIGNALLING

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Laminin, a glycosylated basement membrane protein, is known to promote cell adhesion, growth, migration, differentiation and neurite outgrowth. Recent studies suggest that laminin carbohydrates may participate in such cellular responses. We have found that whereas unglycosylated laminin is able to fully support cell adhesion it did not promote subsequent spreading of mouse melanoma cells or neurite outgrowth of rat pheochromocytoma cells. We have now examined whether those cellular responses could be restored.

We report that when a mixture of unglycosylated and glycosylated laminin was used to study the spreading of B16 F1 mouse melanoma cells, some cells begin to spread when at least 30% glycosylated laminin is available while 65% glycosylated laminin is required for the majority of the cells to spread. Cell spreading also occurred on unglycosylated laminin when a pronase digest of glycosylated laminin was added to the culture medium; a digest of unglycosylated laminin did not restore spreading. Purified laminins containing immature oligosaccharides were prepared using either swainsonine or castanospermine. When these laminins were individually used to study neurite outgrowth, we found that the most immature oligosaccharides were as effective as the most mature forms; the intermediate forms were only partially active. Similar results were found for cell spreading.

These composite results establish that cells attached to laminin must recognize the carbohydrates of that glycoprotein in order to progress further in their biological responses. We had previously shown that absence of the glycosyl groups did not affect laminin conformation; the current data support that observation and show that cellular responses can be restored by laminin glycopeptides. An excess of glycopeptides, compared to intact, glycosylated laminin, was required to obtain the responses. Use of the glycosyl inhibitors to generate immature laminin carbohydrates has provided some insight into which oligosaccharide determinants are being recognized by the responding cells. We should now be able to determine the minimal structural determinants required for response as well as elucidate the cellular responding elements themselves.

S12 LECTINS/LECTINES

12.1
LEUKOCYTE-ENDOTHELIAL CELL ADHESION MOLECULES: CELL SURFACE LECTINS OF EL_4 LYMPHOMA CLONES

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Molecules involved in leukocyte/endothelial cell interactions (1) include cell surface lectins called **LECAM** (2). **Cell surface lectins** were identified on mouse lymphocytes (3) by using **fluoresceinyl neoglycoproteins.** Recently, one cell surface **lectin** on leukocytes: **LECAM-1** (or mouse MEL-14 antigen) has been shown to contain a calcium dependant sugar domain (4). Two cell surface lectins on endothelial cells were also shown to contain a calcium dependant sugar-binding domain **LECAM-2** (or ELAM-1) (1); **LECAM-3** (or CD62, GMP 140, PADGEM) (2) which recognize **fucosylated oligosaccharides** namely **sialyl-Lewis**^{x} and **Lewis**^{x}, respectively.

Here we report the identification of a **6-phosphogalactose specific lectins** on mouse EL_4 **thymoma cells and a subline producing IL_2 (EL_4-IL_2)** (5) and the **modulation of its expression by cytokines.** EL_4 cells do not bind significantly any of the ten **neoglycoproteins** tested while 10% of **EL_4-IL_2 cells** binds mainly two neoglycoproteins, one containing α-L-**fucose (Fuc-BSA)**, the other **6-phosphogalactose (6PGal-BSA).**

Upon **stimulation** by phorbol myristate acetate (PMA), during 4 days, more than **70% of IL_4-IL_2 cells bind these two neoglycoproteins.** EL_4 cells very poorly adhere on to lymph node derived **endothelial cell lines** in agreement with (6), while a **large proportion of EL_4-IL_2 cells efficiently adhere to the same monolayer.**

1. Springer T.A. 1990, Nature **346**, 425–434. 2. Brandley *et al.*, 1990, Cell **63**, 861–863. 3. Kieda *et al.*, 1979, FEBS Letters **99**, 329–332. 4. Siegelman *et al.*, 1989, Science **243**, 1165–1172; Lasky *et al.*, 1989, Cell **56**, 1045–1055. 5. Farrar *et al.*, 1980, J. Immunol. **125**, 2555–2558. 6. Bargatze *et al.*, 1987, J. Exp. Med. **166**, 1125–1131.

12.2
LEUKOCYTE-ENDOTHELIAL CELL ADHESION MOLECULES : CELL SURFACE LECTINS OF AN IMMORTALIZED HIGH ENDOTHELIAL VEINULE DERIVED CELL LINE

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Molecules involved in leukocyte/endothelial cell interactions (1) include cell surface lectins called LECAM (2). Cell surface lectins were identified on mouse lymphocytes (3) by using fluoresceinyl neoglycoproteins.

Recently, one cell surface **lectin** on leukocytes LECAM-1 (or mouse MEL-14 antigen) has been shown to contain a calcium dependant sugar-binding domain (4). Two cell surface lectins on endothelial cells were also shown to contain a calcium dependant sugar-binding domain **LECAM-2** (or ELAM-1) (5); **LECAM-3** (or CD62, GMP 140, PADGEM) (6) which recognize **fucosylated oligosaccharides** namely **sialyl-Lewis**^{x} and **Lewis**^{x}, respectively.

Here we report the characterization of an immortalized mouse **peripheral lymph node endothelial cell line.** Endothelial cells, harvested from mice treated according to Ager, 1987, were transfected with SV40 large T antigen containing plasmid. Selected clones express: SV40 large T antigen, Factor VIII, angiotensin converting enzyme and grow efficiently with a characteristic epitheloid morphology. Selected clones are labelled with anti LECAM-2 monoclonal antibody and bind **neoglycoproteins** bearing α-L-**fucose**.

The expression of the fucose-binding protein is up regulated by GVH activated lymphocytes, conditioned medium, IL_1, **IL_4 and IL_6**.

These cell lines growing in monolayers allow efficient binding of lymph node **lymphocytes** as well as **lymphoma cells.**

1. Springer T.A. 1990, Nature **346**, *425–434.* 2. Brandley *et al.*, 1990, Cell **63**, 861–863. 3. Kieda *et al.*, 1979, FEBS Letters **99**, 329–332. 4. Siegelman *et al.*, 1989, Science **243**, 1165–1172; Lasky *et al.*, 1989, Cell **56**, 1045–1055. 5. Bevilacqua *et al.*, 1989, Science **243**, 1160–1165. 6. Johnston *et al.*, 1989, Cell **56**, 1033–1044. 7. Ager A. 1987, J. Cell Sci. **87**, 133–144.

12.3
CARBOHYDRATE BINDING SPECIFICITY OF *Tetracarpidium conophorum* LECTIN

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A galactose-specific lectin TCA (*Tetracarpidium conophorum* agglutinin) has been isolated from seeds of the Nigerian walnut. The lectin is a dimer of M_r = 34,000 subunits. The N-terminal sequence of the TCA subunit is E-L-K-R-I-V-G-P-N-G-L-X-M-D-V and shows homology with the carbohydrate-binding B chains of *Ricinus communis* I, II and with elderberry bark lectin II.

Hapten inhibition of binding to asialofetuin and affinity chromatography of standard oligosaccharides and glycopeptides on TCA Sepharose shows that terminal galactose residues are essential for binding while linkages to asparagine, the chitobiose unit or core fucosylation are not involved. Milk oligosaccharides containing a terminal Galβ1→4GlcNAc sequence bind with higher affinity than those containing a Galβ1→3GlcNAc sequence. Substitution of lactosamine with sialic acid or fucose abolishes the binding affinity. Complex-type bi-antennary glycans bind more strongly than mono-antennary hybrid type glycans (eluted at 20° with 100 mM and 10 mM lactose respectively). For the strong binding of bi-antennary glycans, the presentation of the lactosamine end groups on C2 of Manα1→3 and Manα1→6 is important, since branched milk oligosaccharide sequences carrying lactosamine residues on C3 and C6 of Galβ1→4 and bi-antennary hybrid type glycans containing lactosamine on C2 and C4 of Manα1→3 do not show such strong affinity. Tri-antennary glycans carrying lactosamine sequences on C2 and C4 of Manα1→3 bind even more strongly and are eluted from TCA Sepharose with 100 mM lactose at 37°. Tri-antennary glycans containing lactosamine at C2 and C6 of Manα1→6 and tetra-antennary glycans are bound more weakly and are eluted with 10 mM lactose at 20°C.

Therefore, TCA complements L-PHA and DSA in binding specificity and is a valuable additional tool for fractionation of complex-type glycans.

12.4
EFFECTS OF ALTERED SECRETION OF AN ENDOGENOUS LECTIN IN CYSTIC FIBROSIS LUNG

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Immunostaining of human heparin lectin (HHL) in normal, cystic fibrosis (CF) and chronic bronchiectatic (CB) lungs, revealed that HHL was found at the mucosal surface of upper airways, within cells of the

underlying secretory glands of the upper airways and within the type II pneumocytes of the lung. In CF lung sections lectin over-secretion is seen at the ciliated surfaces of the airways, where staining of the apical surface of the ciliated cells is seen, and in the secretory glands of the upper airways, however in CB excess secretion is only seen at the level of the mucosal surfaces of the upper airways. Lectin purified from lungs obtained at postmortem from two CF patients appeared identical to lectin from control tissue with respect to its purification, molecular weight, hapten specificity and immunological reactivity. Lectin was seen to specifically bind the exopolysaccharide, alginate, produced by *Pseudomonas aeruginosa,* however no specificity was demonstrated for the alginate derived from isolates of the same patient.

The effect of the apparent oversecretion of lectin in the CF lung was studied in an effort to determine if this may contribute to the environment which facilitates chronic *Pseudomonas* infections. Lectin added to a human lung fibroblast cell line (WI-38) showed a concentration dependent cytotoxicity which was inhibitable by heparin. Also, in ^{3}H-Thymidine incorporation studies HHL displayed a biphasic response curve, being mitogenic to WI-38 but not 3T3 cells at low concentrations (0.2 μg/ml) and inhibitory to growth at higher concentrations (>0.8 μg/ml). Again this activity was heparin inhibitable. HHL was also seen to have a biphasic response with respect to the oxidative burst produced by polymorphonuclear cells (PMNs). At 0.2 μg/ml the release of superoxide was stimulated, while at 2.5 μg/ml the release was inhibited. PMNs were found to be viable at these lectin concentrations. These studies indicate that an endogenous lectin is oversecreted in CF lungs and as such may directly effect cells of the lung and those of the immune system. It is conceivable that such changes, expecially those to the immune system, may facilitate the chronic lungs infections seen in CF.

12.5
NOVEL OLIGOMANNOSE-SPECIFIC LECTINS: ISOLATION, CHARACTERISATION AND EFFECTS ON HIV-INFECTIVITY AND VIRUS-INDUCED CELL FUSION

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Eighteen lectins, mainly from West African and South American seeds, have been identified, characterised and tested for their effects on HIV-infectivity and syncytium formation. Most of the 13 mannose-binding lectins were inhibitory to varying degrees whereas the eight galactose-binding lectins were ineffective.

The novel lectins were purified and their subunit composition was determined by SDS-PAGE. The carbohydrate-binding properties of the lectins were elucidated by (i) inhibition of haemagglutination by sugars; (ii) Ouchterlony double diffusion of lectins against glycoproteins; (iii) adsorption of glycoproteins onto ELISA plates and detection by biotinylated lectins using the streptavidin-peroxidase system; (iv) Western blotting of glycoproteins and detection by biotinylated lectins and (v) lectin-Sepharose affinity chromatography. MBA (*Machaerium biovulatum* agglutinin) and MLA (*M.lunatus* agglutinin), bound ovalbumin and precipitated a dextran-containing α1,2 bonds, while BMA (*Bowringia mildbraedii* agglutinin) is specific for high mannose structures especially Man_9 and certain Man_8 and Man_7 isomers. VRA (*Vigna racemosa* agglutinin) recognises fucosylated Man_3-hybrid glycopeptides.

HIV-1 was pre-treated with lectin followed by continual presence of lectin during the 5 day culture with C8166 cells. Syncytium formation, production of progeny virus, viral p24 production, cell viability and cytotoxicity were monitored. The most effective lectin was MBA which prevented syncytium formation at 4 μg/ml and blocked completely virus production and maintained high cell viability at less than 1 μg/ml. A lesser, although still significant protection, was obtained with MLA, BMA, GNA, LNA (*Lablab niger* agglutinin) and DLA (*Dolichos lablab*). All these lectins are more protective than Con A. The inhibitory lectins appear to exert their effects by binding to HIV virions. Experiments are under way to identify viral carbohydrate structures which are important in determining HIV infectivity.

12.6
LECTINS IN MICROBIOLOGICAL DIAGNOSTICUMS

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Carbohydrate-binding center of lectins with strong specifity is the main reason to use lectins for analysis, identification and typing of microorganisms in practice. If a microbe has unique carbohydrate structure it is possible to develop simple, cheap and standard diagnostic tool, for example: *Streptococci, Neisseria.* Review: Pistole 1981, Doyle 1984, Kalinin 1989, Slifkin 1990.

However, the absence of such carbohydrate structure in bacteria, viruses or protozoa makes the creation of the tool rather doubtful. Only differences between strains were shown for *Bacillus, Leishmania, Trypanosoma* and some other. In such case it seems necessary to carry out the structural investigation of carbohydrate of cell wall or, as more suitable alternative, we suggest the evaluation of inhibitory activities of mono- and poly- saccharides in traditional serological reactions: indirect method for determination of contribution of carbohydrates to predominant antigenic determinants. Attempts to decide these problems by using accidental pairs of lectin-microbe would be unsuccessful: *Legionella* – Doyle et al 1982, *Pseudomonas* – Doyle et al 1984. In using lectins for above purposes other difficulties are connected with the transfer of results of lectin agglutination test in diagnostic systems which are using lectins marked enzyme, biotin, FITC.

Other very perspective approach is using as markers of microbes in diagnostic aims the lectins or lectin-like substances on surface of microorganisms by employing of carbohydrates or neoglycoconjugates with artifical vesicles.

Problems of practical utilization of lectins in microbiological diagnostic are discussed.

12.7
PROLIFERATION-DEPENDENT EXPRESSION AND LOCALIZATION OF CARBOHYDRATE BINDING PROTEIN 35

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Carbohydrate Binding Protein 35 (CBP35, M_r 35,000) was isolated from mouse 3T3 fibroblasts on the basis of its binding to galactose-containing glycoconjugates. Amino acid sequence analysis and immunolocalization studies have suggested that CBP35 is a component of ribonucleoprotein complexes in the cytoplasm and the nucleus. Quiescent cultures of 3T3 cells exhibited a low level of CBP35; the polypeptide was almost exclusively in the phosphorylated form (pI 8.2) and was located predominantly in the cytoplasm. The addition of serum to these cells increased the expression of CBP35, in terms of elevated transcription rate of the gene, increased accumulation of the mRNA, and increased amount of the protein. These proliferating cells had an increased level of the phosphorylated polypeptide, both in the cytosol and the nucleus. More striking, however, was the dramatic increase in the level of the un-

modified form (pI 8.7), which was confined to the nucleus. We have also compared the expression and localization of CBP35 in human fibroblasts of different replicative capacities: young (passage 11), intermediate (passage 19), and old (passage 33) SL66 cells, and fibroblasts derived from a patient with Werner's syndrome. The levels of CBP35 mRNA and protein in young SL66 cells behaved similarly to those of mouse 3T3 cells. In contrast, older SL66 fibroblasts failed to exhibit the correlation between the level of CBP35 and the proliferation state of the culture. The levels of CBP35 mRNA, as well as protein, remained high (no down regulation) in serum-starved cells, and serum addition resulted in a decrease rather than the expected increase in CBP35 expression. The unphosphorylated form of the CBP35 polypeptide was not observed in cultures of high-passage SL66 cells. These results establish that the expression of CBP35 becomes altered as human fibroblasts acquire reduced replicative capacities.

12.8
EVIDENCE FOR CARBOHYDRATE RECOGNITION IN THE SPLICING OF MESSENGER RNA PRECURSORS IN A CELL FREE ASSAY

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Using a cell-free assay for the splicing of intervening sequences from mRNA precursors, we have accumulated several lines of evidence to indicate that carbohydrate recognition by nuclear lectin(s) may play a role in the processing of RNA. The assay system includes: (a) a soluble extract prepared from the nuclei of HeLa cells (splicing extract); (b) an RNA (pre-mRNA) transcribed from a DNA template containing the first and second exons, separated by an intron, of the β-chain of human hemoglobin; and (c) a denaturing gel system capable of delineating, among the components of the reaction mixture, the pre-mRNA, the spliced mRNA product, and intermediates of the splicing reaction. When included in the assay, lactose inhibited the splicing reaction in a dose-dependent fashion. The RNA remained exclusively in the precursor form; no intermediates were observed. Similar results were also obtained with mannose. In contrast, glucose and myo-inositol failed to yield any inhibitory effect. A mouse monoclonal antibody (4F4) directed against proteins C_1 and C_2 of the heterogeneous nuclear ribonucleoprotein complex (hnRNP)and a monoclonal antibody reactive against a M_r 70,000 polypeptide found on the small nuclear RNP U1 both inhibited splicing, consistent with their effects observed by others. More strikingly, a rat monoclonal antibody reactive against Carbohydrate Binding Protein 35 (CBP35) also inhibited splicing. CBP35 is a lactose/galactose-specific lectin identified in the cytoplasm and nucleus of cells, predominantly in the form of a ribonucleoprotein complex. These results suggest that lectin-carbohydrate interaction(s) may play a role in the assembly or function of the spliceosome and provoke new views on the binding activities and physiological significance of an intracellular lectin.

12.9
THE ROLE OF ASPARAGINE-LINKED OLIGOSACCHARIDES TERMINATING IN SIALYLACTOSAMINE AS RECEPTORS FOR PERTUSSIS TOXIN BINDING

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Pertussis toxin (PT), the etiological agent of whooping cough, is an important virulence factor produced by the organism *Bordetella pertussis.* PT contains lectin-like binding activity that allows the toxin to recognize asparagine-linked oligosaccharide structures terminating in sialylactosamine. Previous work from our laboratory has suggested that glycoconjugates terminating in α(2–6) linked Neu5Ac are important for high affinity PT binding.

In order to further examine the α(2–6) specificity we have recently completed an investigation in which we have compared the binding characteristics of PT with two plant lectins that have differential specificities for α(2–6) and α(2–3)-linked Neu5Ac. In this study we demonstrated that PT's binding specificity was very similar to that of the lectin from *Sambucus nigra* which has a strict specificity for α(2–6) Neu5Ac Gal sequences while the lectin from *Maackia amurensis* which recognizes α(2–3)-linked Neu5Ac did not bind in a similar manner.

Studies recently completed with the copper binding protein, ceruloplasmin, demonstrated that upon treatment with the enzyme PGNase F which removes all asparagine-linked oligosaccharides, PT binding was abolished. The evidence that we have gathered indicate that all the necessary information for PT binding is found in the oligosaccharide sequences themselves and that the protein serves only as a scaffolding to which multiple oligosaccharides are attached.

With this information in hand we have focused our attention on the use of native oligosaccharides to study PT binding. Preliminary experiments with α(2–6) and α(2–3) sialylactose isolated from bovine colostrum have shown that α(2–6) sialylactose can inhibit PT binding at millimolar concentrations in binding inhibition experiments while α(2–3) sialylactose does not inhibit. Furthermore, PT has been shown to bind with high affinity (micromolar range) to purified biantennary oligosaccharide structures which terminate in α(2–6) Neu5Ac Gal sequences. Important evidence that we have gathered indicates the possibility of an extended oligosaccharide binding site in PT that can recognize both the reducing and non-reducing ends of complex N-linked oligosaccharides.

12.10
IMMUNOHISTOCHEMICAL LOCALIZATION OF GLYCOSYL RECEPTORS IN RAT LIVER, SPLEEN AND LYMPH NODES WITH THE USE OF NEOGLYCOPROTEINS

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Galactose and mannose specific receptors of liver cells and macrophages (Mø) in spleen and lymph nodes participate in the clearance of glycoproteins. Neoglycoproteins, particularly useful because of their uniform physical properties and their homogeneous sugar composition, have been used in vivo (125I- or gold labelled) to characterize the specific hepatic and extrahepatic clearance of mammalian glycoproteins and in vitro (biotin- or gold labelled) to detect endogenous sugar receptors.

In the present study we developed an immunohistochemical method to detect neoglycoproteins after their in vitro binding or in vivo uptake for the demonstration and characterization of glycosyl receptors in rat liver cells and in Mø of spleen and lymph nodes. Neoglycoproteins (Gal-23-BSA, Gal-34-BSA, GalNAc-28-BSA, Man-45-BSA, Fuc-28-BSA and GlcNAc-28-BSA) were prepared as described earlier (Lee et al. Biochemistry 15:3956, 1976). A double staining procedure with monoclonal antibodies against Mø and endothelial cells was developed for the phenotypic characterization of neoglycoprotein binding cells.

Results with the in vitro procedure indicated that the neoglycoprotein binding was $Ca2^{+}$ dependent. The galactose binding in liver was neuraminidase sensitive and could be inhibited by galactose derivatives. Mannose and fucose binding could not be inhibited. Mø binding in

spleen and lymph node was only confined to Man-BSA, Fuc-BSA, GlcNAc-BSA and could be inhibited by mannan.

Results with the double staining procedure indicated that a) hepatocytes bind Gal-23-BSA, Gal-34-BSA and GalNAc-28-BSA; b) Kupffer cells bind Gal-34-BSA, GalNAc-28-BSA, Man-45-BSA and Fuc-28-BSA; c) endothelial cells bind Man-45-BSA and Fuc-28-BSA; d) marginal zone Mø and marginal metallophils in spleen and medullary sinus Mø and outer cortex Mø in lymph nodes bind Man-45-BSA, Fuc-28-BSA and GlcNAc-28-BSA.

12.11
LECTIN-CARBOHYDRATE INTERACTIONS AND INFECTIVITY OF HIV-1

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We have examined whether mannosyl-specific lectins intervene in recombinant gp160 (rgp160) binding to the cell membrane and to modified susceptibility to HIV-1 infection of monocytic cells as compared with lymphoid cells. The effects of mannosyl-specific lectins, ConA, LCA, PSA and VFA i) on ^{125}Irgp160 binding to CEM, U937 cells and to monocyte-derived-macrophages ii) on the interaction of viral gp120 and soluble CD4 (sCD4) and iii) on HIV-1 infectivity for monocytic cells as compared with lymphoid cells were investigated. sCD4 did not interact with a ConA-Sepharose affinity matrix and HIV-1 preincubated with buffer or with ConA bound to sCD4 in a similar manner. When preincubated with rgp160 or the cells, the lectins significantly enhanced rgp160 binding to the cells in a dose-dependent, carbohydrate specific, and CD4 independent manner. Despite lectin-mediated enhanced binding of rgp160, ConA neutralized HIV-1 infectivity for monocytic as well as for lymphoid cells. These results demonstrate that mannosyl-specific lectins i) induce CD4-independent bridge formation between *env* glycoprotein and CD4$^+$ cells, ii) do not inhibit gp120-CD4 interactions – which demonstrates that gp120 mannosyl residues are not involved – iii) neutralize HIV-1 infectivity for monocytic as compared with lymphoid cells. Therefore mannosyl-specific lectins behave like neutralizing antibodies that do not interfere with CD4 binding of gp120 but with post-binding events.

12.12
IDURONATE CONTAINING GLYCOSAMINOGLYCAN BINDING SITES OF MALIGNANT CELLS

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The physiological effect of the glycosaminoglycans (GAG) are due to their ability to interact with extracellular matrix components, cell membranes and growth factors. Binding sites for GAGs were observed on the surface of different types of normal and malignant cells. GAGs especially heparin and heparan sulfate, are implied in the regulation of cell growth and cell adhesion. GAG protein interactions should be involved in the control of a variety of events in the metastatic cascade. The distribution and the structure of the cell associated GAGs in strongly and weakly metastatic cells is different in most model systems. Strongly metastatic cells exhibit lower iduronate levels in their heparan sulfate and a decreased dermatan sulfate content than their weakly metastatic analogues. Non tumorigenic myoblasts and weakly metastatic rat rhabdomyosarcoma (RMS) cells expressed, bound and internalized higher amounts of iduronic acid containing glycosaminoglycans (heparan sulfate, dermatan sulfate) and accumulated more GAG-binding proteins on their surface then their highly metastatic counterparts. A negative correlation was found between the inhibitory effect of heparin on the cell growth in vitro and the metastatic potential of rhabdomyosarcoma cells. The recognition of extracellular GAGs was evidenced by isolating the GAG binding proteins from RMS cells by affinity chromatography. The main 19 kDa GAG binding protein was purified till homogeneity by exclusion and ion exchange chromatography. The affinity of this protein for the GAGs increased with their iduronate content. It was immunologically distinct from aFGF or bFGF. The membranes of the highly metatstatic variants contained about 2 times higher amount of 19 kDa protein than the membranes of the highly metastatic RMS0 cells. This finding is in agreement with the decresed receptor mediated binding of the iduronate containing exogenous or endogenous GAGs with the increase of the metastatic capacity of the cells. The decrease of the GAG binding sites diminish the capacity of malignant cells to respond matrix controled growth regulation signals.

12.13
ALTERATION OF THE STRUCTURE AND FUNCTION OF CONGLUTININ BY ENDOGENOUS PROTEASE(S)

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Conglutinin (Kg) is a unique bovine plasma protein which mediates the agglutination of the sensitized erythrocyte-solid phase iC3b complex (conglutination), and characterized as a lectin specific for N-acetylglucosamine (1,2). Kg has been isolated from heat-inactivated bovine serum by using its selective adsorption to zymosan or to immobilized mannan (1) in the presence of calcium ion. In this study we found that without prior heat treatment Kg underwent limited proteolysis during the process of isolation with the complete loss of the conglutination activity while retaining its sugar binding activity.

Upon purification by Sepharose 4B-mannan affinity chromatography, essentially no Kg was isolated from the serum without prior heat inactivation. This loss of Kg was, however, accompanied with the appearance of a new lectin activity in the washings of the 1st affinity chromatography (designated as Kg*). The subunit size of Kg* (38kDa) was a little smaller than that of Kg (45kDa). The molecular size of the intact protein also reduced from 1000kDa (Kg) to 600kDa (Kg*). Immunoblot analysis revealed that Kg* and Kg reacted equally with rabbit anti-Kg IgG. Despite the difference in the NH_2-terminal amino acid sequences of Kg and Kg*, those (20 amino acids) of the peptides (24kDa) obtained after collagenase digestion of Kg and Kg* were identical suggesting that the removal of the NH_2-terminal portion of Kg produced Kg*. Being consistent with this hypothesis, incubation of ^{125}I-Kg with the eluate of the first affinity chromatography of serum without heat-inactivation converted ^{125}I-Kg to ^{125}I-Kg*. This conversion of Kg to Kg* was inhibited by the presence of APMSF (plasma serine protease inhibitor). Kg* had neither conglutination activity nor binding activity to EAiC3b, although it retained the original sugar binding specificity. These results indicated that the NH_2-terminal deletion of Kg subunit caused some conformational change of Kg, resulting in the alteration of its conglutination activity.

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12.14
MICROCALORIMETRIC STUDIES OF WHEAT GERM AGGLUTININ BINDING OF CHITO-OLIGOSACCHARIDES

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Interactions of wheat germ agglutinin with β-(1,4)-linked GlcNAc oligomers up to pentamer were studied by isothermal titration microcalorimetry at pH 4.5 and 7.0. The values of $\triangle H$, $\triangle G$, and $\triangle S$ were obtained by titration at a single temperature (25°C). The Ka values obtained by us varied from 0.4×10^3 M^{-1} for the monosaccharide to 19×10^3 M^{-1} for the pentasaccharide. These values are in general agreement with previous reports. The enthalpy and entropy values were negative. For the monosaccharide, $\triangle H$ was −7 kcal/mol, and increased in magnitude up to −18 kcal/mol for the pentasaccharide. These results indicate that the ligand binding by wheat germ agglutinin is enthalpically driven, and suggest the importance of hydrogen-bonding and van der Waals' forces in the binding process.

The magnitude of $\triangle H$ and $\triangle G$ values increased as the number of GlcNAc residue increased up to 3, beyond which the increase is insignificant. This is in agreement with a linear arrangement of three subsites for binding GlcNAc residues, as has been suggested. The $\triangle G$ contributions at the three subsites were −3.54, −1.57, and −0.52 kcal/mol, respectively.

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12.15
PURIFICATION AND PARTIAL CHARACTERIZATION OF A NOVEL LECTIN, SNA-III, FROM ELDER (SAMBUCUS NIGRA L.) FRUITS

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A previously unknown hemagglutinin, named SNA-III, has been purified from the fruit of *Sambucus nigra* by anion exchange chromatography and affinity chromatography on immobilized asialofetuin. SNA-III occurs mainly as a monomeric glycoprotein, but tends to form di- and oligomeric aggregates. This aggregation seems to mediate the multivalent interaction leading to agglutination. SDS/polyacrylamide gel electrophoresis revealed two major polypeptides with apparent molecular masses of 32 000 and 33 000, respectively. Binding to concanavalin A and treatment with peptide:*N*-glycosidase F demonstrated the presence of *N*-glycosidically linked oligosaccharides. The electrophoretic heterogeneity observed is neither a result of *N*-terminal proteolysis nor due to the number of attached *N*-linked oligosaccharides.

Whereas elder bark agglutinin I (SNA-I) is highly specific for terminal α(2–6) linked sialic acid residues, SNA-III displays a high affinity for oligosaccharides containing exposed *N*-acetylgalactosamine and galactose residues. Different *N*-terminal sequences and the amino acid composition distinguish the fruit lectin from elder bark agglutinin II (SNA-II) which shows a similar carbohydrate specificity. However, SNA-III exhibits a 40-fold higher affinity for asialofetuin than for asialo-α_1-acid glycoprotein or asialotransferrin, respectively.

The marked specificity for oligosaccharide structures resembling the basic unit of *O*-linked glycans emphasize potential application in glycoconjugate research.

12.16
THE ROLES OF POLY-N-ACETYLLACTOSAMINE CHAINS AND ANIMAL LECTINS IN ADHESIVE INTERACTIONS BETWEEN CELLS AND LAMININ

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Animal cells synthesize a variety of carbohydrate binding pro-teins or lectins and one of the most widely distributed is a class having a molecular weight in the range of 14 kD, designated L-14. We have investigated the carbohydrate binding specificity of L-14 from several sources and determined that it interacts weakly with a variety of β-galactosides, but binds most strongly to β-galactosides contained within the repeating disaccharide unit $[\text{-3Gal}\beta\text{1–4GlcNAc}\beta\text{1-}]_n$ or poly-N-acetyllactosamine sequence. As one probe for these sequences we have used tomato lectin, which binds with high affinity to linear poly-N-acetyllactosamine chains. Our studies have revealed that poly-N-acetyllactosamine chains are expressed primarily in selected animal cell glycoproteins, such as the lysosome-associated membrane proteins LAMP-1 and LAMP-2 and laminin.

To examine the biological functions of poly-N-acetyllactosamine sequences and L-14, we investigated the expression and function of L-14 in the mouse teratocarcinoma cell line F9 and its differentiated derivatives. L-14 was purified from F9 cells and shown to be immunologically cross-reactive with antibodies to L-14 from calf heart tissue. Using the polymerase chain reaction, F9 cells were found to contain mRNA for L-14 and a cDNA transcript of 400 bp was identified, which is of the predicted size. Immunoprecipitation studies revealed that F9 cells also synthesize LAMPs and that they are the primary carriers of poly-N-acetyllactosamine sequences in these cells.

Using a solid-phase binding assay, we found that L-14 can mediate the Ca^{++}-independent adhesion or interaction of F9 cells to laminin. Similar results were obtained using F9 cells induced to irreversibly differentiated into parietal endoderm upon treatment with 10^{-7} M all trans retinoic acid. Adhesion of cells to laminin mediated by L-14 was found to depend on expressed poly-N-acetyllactosamine sequences. These results demonstrate that poly-N-acetyllactosamine chains on cell surfaces interact with animal lectins and suggest that such interactions might be influential on cell adhesion and interactions with basement membrane components.

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12.17
ENGINEERING AND USE OF BIOTINYLATED BACTERIAL LECTINS: C-bio-K99 AND N-bio-K88

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Enterotoxigenic *Escherichia coli* strains express fimbrial K99 and K88 lectins, involved in the attachement to the intestine of the newborn lamb, calf and piglet.

K99 lectin preferentially binds to NeuGc-glycoconjugates. The three immunogenic variants of K88 lectin recognize asialoglycoproteins, containing GalNAc and GlcNAc- which seem to play an important role in the binding. Carboxylic groups of the K88 and amino-groups of the K99 subunits are implicated in the binding activity, therefore two labelling methods were used:

1-biotinylation of K99 lectin was achieved by activation of the carboxylic groups and action of biocytin hydrazide (BCHZ). Optimal yield was obtained when the BCHZ/K99 ratio was 250. Biotinylated K99

(C-bio-K99) specifically detect, at least 100 ng of the N-glycolyl neuraminyl lactosyl ceramide.

2-Biotinylation of the three K88 lectins was carried out via amino-groups using biotin amidocaproyl hydroxysuccinimide ester (BACHS). A BACHS/K88 ratio of 2 was sufficient to biotinylate the K88 without altering the binding activity.

Biotinylated lectins, C-bio-K99 and N-bio-K88, will be useful tools for detection of the glycoconjugates implicated in the adhesion of enterotoxigenic *E. coli* to host intestinal mucus and epithelial cells. In addition to their use for the establishement of the phenotype of domestic animals for their sensitivity or resistance to the bacterial adhesion, they can be used in column affinity, overlay assay for isolation and detection of glycoconjugates issued from all origins.

12.18
PARTIAL BIOCHEMICAL IDENTIFICATION OF CARBOHYDRATE-BINDING PROTEINS ISOLATED FROM NUCLEI OF THE PROMYELOCYTIC TUMORAL CELL LINE HL60

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Carbohydrate-binding molecules have been previously localized in mammalian cell nuclei by using fluoresceinylated neoglycoproteins (1.2) The results presented here deal with the partial biochemical characterization of glucose-binding proteins extracted from HL60 nuclei and isolated by affinity chromatography.

The glucose-binding proteins have molecular masses ranging from Mr 10 000 to Mr 120 000. The low molecular masses proteins Mr from 10 000 to 28 000 were found to be acidic. Some proteins Mr from 30 000 to 36 000 were basic whereas others of Mr from 36 000 to 67 000 were neutral. Some of them had Mr and pI similar to that is known for nuclear hnRNPs. Different control experiments, including a method allowing the detection of nuclear carbohydrate-binding proteins (NCBPs) on polyacrylamide electrophoresis gels, confirmed the elution specificity.

In the aim to know whether these nuclear lectins possess only specific binding sites for glucose or whether they are able to bind to an other kind of sugar, affinity chromatography using columns containing immobilized mannose or galactose was also performed.

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12.19
THERMODYNAMIC AND CONFORMATIONAL ANALYSIS OF LIGAND BINDING TO WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS*) ACIDIC AGGLUTININ REVEALS ITS SPECIFICITY FOR THE TERMINALLY FUCOSYLATED H-ANTIGEN

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Thermodynamic studies of ligand binding to Winged Bean Acidic agglutinin, together with conformational analysis, brings to light some remarkable features of its interaction with fucosylated oliogosaccharides. The lectin clearly shows a preference for monofucosylated sugars with fucose linked α1–2 to galactose, inspite of L-fucose being non-inhibitory. 2′-fucosyllactose along with its N-acetylated derivative Fucα1-2Galβ1–4GlcNAc were found to be the most complementary ligand followed by H-disaccharide. LNF-I, which represents type I chain, is not accomodated as well in the combining site of the lectin as is the case with the type III saccharide. Difucosylated sugars and monofucosylated derivatives with L-fucose in α1–3 linkage with glucose in type II structures are not tolerated. Substitution of L-fucose in α1–3 or α1–4 to glucose as in LNF II and LNF III sterically prevents the access of these sugars to the binding site. Conformational analysis shows that the lectin interacts with these sugars from all three surfaces designated as α, β and γ unlike the fucose binding lectins from *Ulex* and *Evonymus* seeds which bind to their complementary saccharides from one side alone. Moreover, the thermodynamics of binding with 2′-fucosyllactose shows a positive entropy change accompanying the binding process, and unusual features observed for the first time in lectin-sugar interactions. Its unique ability to distinguish terminally monofucosylated sugars from the difucosylated compounds combined with its ability to recognize type II structures better than type I confers upon the lectin a unique position as a biochemical tool in the characterization of glycoconjugates.

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12.20
OLIGOSACCHARIDE RECOGNITION BY THE ADHESIVE SERUM PROTEINS CONGLUTININ, MANNAN-BINDING PROTEIN AND AMYLOID P.

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This communication will be concerned with a comparison of the carbohydrate binding specificities of two types of adhesive proteins that occur in serum. The first, bovine conglutinin and human mannan-binding protein, are members of a superfamily of calcium-dependent carbohydrate binding proteins (C-type lectins), and have affinities for distinct components of the complement system. The second, human amyloid P protein, occurs as an integral component of all types of amyloid deposits, and is a member of a family of proteins known as pentraxins. Calcium-dependent binding of this protein to certain glycosaminoglycans, pyruvated galactose residues and to certain polysaccharides containing mannose residues reported by others has raised the possibility that it too may be a lectin. The carbohydrate binding specificity of all three proteins has been investigated by direct binding assays with immobilised glycolipids and with neoglycolipids derived from N-linked oligosaccharides released from glycoproteins. Earlier observations (1-3) on the subtle differences in recognition of mannose, N-acetylglucosamine and fucose terminating oligosaccharides by conglutinin and mannan-binding protein have been extended, and two novel carbohydrate ligands for amyloid P protein have been identified.

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12.21
COAGGREGATION-MEDIATING POLYSACCHARIDE FROM *STREPTOCOCCUS GORDONII* 38:

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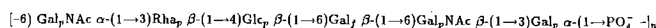
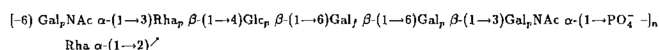
The receptor polysaccharides of oral *Streptococci* such as *S. oralis* 34 and *S. mitis* J22, although immunologically different, act as receptors for the fimbrial lectins of *Actinomyces viscosus* T14V. As a result these strains participate with lactose sensitive coaggregation with A. viscosus T14V. High field nmr spectroscopy reveals structures of these polysaccharides as

S. oralis 34:



S. mitis J22:

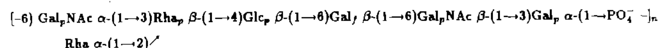


The GalNAc β-(1→3)Gal and Gal β-(1→3) GalNAc moeities of these structures were proposed to be the site of lectin binding based on the potent activity of these disaccharides to inhibit coaggregation. Antigenic determinants of these polymers are appear to be different from the lectin binding site and presumably involves structural motifs in the non-reducing end of the repeating unit.

S. gordonii 38 also participates in lactose sensitive coaggregation with *A. viscosus* T14V and has a receptor polysaccharide which is strongly cross reactive with anitbody raised against *S. mitis* J22 but does not react with antisera against *S. oralis* 34. We report the structure of the polysaccharide, which is composed of a heptasaccharide repeating unit [two units each of Gal, GalNAc, Rha and a unit of Glc and Phosphate] polymerized through phosphodiester bonds.

48% HF hydrolysis of the polysaccharide and subsequent analysis by HPAE chromatography with electrochemical detection gave a heptasaccharide as the major component as well as a pentasaccharide GalNAc α-(1→3)[Rha α-(1→2)] Rha β-(1→4)Glc β-(1→6)Gal and a disaccharide GalNAc β-(1→3)Gal, which were previously isolated from *S. mitis* J22 and *S. oralis* 34 polysaccharide respectively.

Complete ^{1}H and ^{13}C NMR assignments on the intact polysaccharide yielded the covalent structure of a heptasaccharide repeating unit, which is a hybrid structure made of the two structural domains from *S. mitis* J22 and *S. oralis* 34 polysaccharides as



12.22
PURIFICATION AND CARBOHYDRATE BINDING SPECIFICITIES OF TWO LECTINS FROM TRICHOSANTHES JAPONICA

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Recent studies revealed that lectins recognize not only peripheral monosaccharides but also their glycosidic linkage and their tri-dimensional conformation containing *N,N'*-diacetylchitobiose. In this paper, we report about the properties of two lectins purified from the rhizome of *Trichosanthes japonica*. These two lectins were purified by affinity chromatographies of human α_1-antitrypsin- and porcine submaxillary mucin-Sepharose 4B, and named TJA-I and TJA-II, respectively. Their physicochemical properties were summarized as follows.

Properties	TJA-I	TJA-II
Molecular weight	70 kD	64 kD
Subunits	38 kD and 32 kD	33 kD and 31 kD
pI	5.45, 5.55	5.80, 5.95, 6.05

The carbohydrate binding specificites of TJA-I and TJA-II were investigated by analyzing the behaviors of tritium labeled oligosaccharides on the respective lectin-Sepharose columns.

TJA-I basically interacts with Galβ1→4GlcNAc group. Substitution of their terminal galactose residues by Neu5Acα2→6 or HSO_3^-→6 enhanced their affinity to the lectin. By contraries, substitution at C-2 or C-3 position of their terminal galactose residue with other sugars deprives their affinity to the TJA-1.

TJA-II also interacts weakly with β-galactose residue. When the hydroxyl group at C-2 position of their terminal galactose residues were substituted with α-fucosyl residue or *N*-acetyl group, they enhanced affinity to the lectin. While, substitution at C-6 or C-3 position of their terminal galactose residues diminished their affinity to TJA-II.

Furthermore, ^{1}H-NMR studies of lectin-carbohydrate interaction were performed to confirm whether both lectins recognize β-galactosyl residues. When β-methylgalactoside was mixed with TJA-I and TJA-II, respectively, the ^{1}H-NMR signal changes of β-methylgalactoside were obserbed that H-4 was markedly broad, although H-1 and OCH_3 were rather sharp in both lectins. The remarkable line broadening of H-4 suggests that hydrogen at C-4 position of β-galactoside at least has contact with the binding sites of TJA-I and TJA-II.

12.23
PURIFICATION AND MOLECULAR CLONING OF A LECTIN SPECIFIC FOR GALACTOSE/N-ACETYL-GALACTOSAMINE FROM TUMORICIDAL MACROPHAGE

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A lectin specific for galactose/N-acetyl-galactosamine (Gal/GalNAc) was purified from murine peritoneal exudate macrophages which had been induced with an antitumor streptococcal preparation, OK-432. The lectin was detected on the surface of OK-432-elicited and thioglycolate-elicited macrophages, but it was not detected on resident macrophages. The binding of tumor cells to macrophages was inhibited by the addition of the purified lectin to the binding mixture. This binding of the macrophages to P-815 cells was also inhibited on preincubation of the macrophages with a neoglycoprotein (Gal-BSA) which was a specific inhibitor for the macrophage lectin. Furthermore, the tumoricidal activity of the activated macrophages was inhibited by the addition of the anti-macrophage lectin antiserum. These results suggest that the binding of activated macrophages to tumor cells through the Gal/GalNAc-specific macrophage lectin is an important part of the tumor cell killing mechanism.

In order to characterize this lectin further, the primary structure of the lectin has been deduced from its cDNA sequence. The macrophage lectin cDNA encoded a protein consisting 304 amino acid residues (Mr. 34600) with a single transmembrane domain. The extracellular domain was found to contain two potential N-glycosylation sites and three leucine zipper-like domains. The sequence was highly homologous with

those of hepatic lectins especially in its transmembrane domain and carbohydrate recognition domain (CRD). A 1.6 kb mRNA coding this lectin was detected in OK-432-elicited and thioglycolate-elicited macrophages, but not in resident macrophages.

Soluble recombinant macrophage lectin was obtained by expression of the macrophage lectin cDNA without the region encoded transmembrane domain in Escherichia coli. Sugar binding specificity of the recombinant lectin was completely same as that of the purified lectin from OK-432-elicited macrophages.

The binding molecules for this lectin on tumor cells is now under investigation.

12.24
EXPRESSION OF *BAUHINIA PURPUREA* AGGLUTININ cDNA AND ITS MUTANTS

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Bauhinia purpurea agglutinin (BPA) is one of legume lectins purified from the seeds of *Bauhinia purpurea alba*. BPA is specific for galactose and lactose and binds preferentially Galβ1–3GalNAc. We have cloned a cDNA cording BPA from the cDNA library constructed from poly A^+ RNA from *Bauhinia purpurea* seeds. We have already reported the purification and characterization of a carbohydrate-binding peptide from BPA. This peptide consists of 9 amino acids and its amino acid sequence is Asp-Thr-Trp-Pro-Asn-Thr-Glu-Trp-Ser. This peptide is a part of metal-binding site and its amino acid sequence is highly conserved among legume lectins.

We constructed cDNAs cording BPA whose carbohydrate-binding peptide sequence was changed to those of lentil lectin (mannose-binding lectin) or *Ulex europeus* lectin I (fucose-binding lectin) by using polymerase chain reaction. These PCR generated DNAs were inserted between the Nde *I* and Bam H*I* sites of pET-3c to give plasmids pET-3c/BPA mutant. The constructed plasmids were introduced into *E. coli* and recombinant BPA mutants were expressed in the cells. PCR generated DNAs were also inserted between the Nhe *I* and Xho *I site of pMAM-neo* to give plasmids pMAM-*neo*/BPA mutant. The constructed plasmids were introduced into NIH3T3 cells and recombinant BPA mutants were also expressed. We will report the sugar-binding specificity of recombinant BPA mutants compared to that of BPA.

12.25
THE PRIMARY STRUCTURE OF *LABURNUM ALPINUM* ANTI-H(O) LECTIN

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The anti-H(O) hemagglutinating activity in extracts from the seeds of *Laburnum alpinum* was first discovered by Renkonen (1) and further confirmed by Morgan and Watkins (2). The hemagglutination-inhibition studies on crude extracts from the seeds revealed a specificity towards di-N-acetylchitobiosyl residues (3–5). In a previous paper (6) we have shown the specific purification and characterization of two kinds of the *Laburnum alpinum* lectin, a di-N-acetylchitobiose-binding lectin (LAA-I) and a new type lectin which is inhibited by lactose or galactose (LAA-II). We have also previously determined the primary structures of the *Lotus tetragonolobus* (LTA) (7) and two types of the *Ulex europeus* (UEA-I and II) (8) anti-H(O) lectins, and compared them with those of several lectins. Extensive homologies were found among them. Here we show the determination of the complete amino acid sequence of LAA-I by use of a protein sequencer. After digestion with endoproteinases of Lys-C and Asp-N of the lectin, the resulting peptides were purified by reversed phase high performance liquid chromatography and subjected to the sequence analysis. The complete primary structure of this lectin was compared with those of 14 lectins already determined, including those of LTA and UEA-I and II which we have determined. Among these lectins extensive homologies, especially between LAA-I and UEA-II (both are di-N-acetylchitobiose-binding lectins), were also found.

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12.26
THE *N*-TERMINAL AMINO ACID SEQUENCES AND SUGAR CHAINS OF A MANNOSE-SPECIFIC AND SUGAR SPECIFICALLY AGGREGATABLE LECTIN FROM THE BARK OF *SOPHORA JAPONICA*

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The reactivity of plant lectins toward plant glycoproteins must be important in the survey of their functions. A novel Man-specific lectin of *Sophora japonica* bark (B-SJA-II) composed of four subunits (13.2–19.4 kDa) was found to aggregate to form precipitates in the absence of a specific sugar and become soluble by addition of the sugar. (1)

The four subunits of B-SJA-II were classified into two groups with *N*-terminal sequences. The *N*-terminal sequences of the first group exhibit 50 % homology to those of other *S. japonica* lectins and correspond to the sequences initiating at position 123 and 1 in Con A and favin β-chain, respectively. The *N*-terminal sequences of the second group exhibit 62 % homology to that of Con A and correspond to the sequence initiating at position 112 in favin β-chain. However, they differ completely from those of other *S. japonica* lectins including the first group. All subunits bound to HRP-glycoproteins which have *N*-linked sugar chains indicating that all subunits of B-SJA-II have sugar recognition sites and contribute to sugar specific aggregation.

Three of them contained GlcN, Man, Fuc and Xyl and bound to HRP-Con A, -LCA, -pea and -favin. *N*-linked oligosaccharides released by almond glycopeptidase digestion were labeled with 2-aminopyridine and analyzed by two-dimensional mapping method. The structure of major oligosaccharide was the typical core structure in plant glycoproteins: Xyl β1–2 (Man α1–6) (Man α1–3) Man β1–4GlcNAc β1–4 (Fuc α1–3)GlcNAc.

The results suggest that B-SJA-II originates from the common gene of legume lectin, but has evolved tissue-specifically in possessing a unique sugar-binding specificity in the *S. japonica* lectin family to recognize its own sugar chains and thus becomes exhibiting a sugar-specific self aggregation.

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12.27

THE MANNOSE BINDING PROTEIN AND CONGULUTININ IN BOVINE SERUM HAVE A ANTIVIRAL ACTIVITY AGAINST INFLUENZA VIRUS

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Normal horse and guinea pig sera contain α_2-macroglobulin which inhibits the infectivity and hemagglutinating activity of influenza virus of the H2 and the H3 subtypes. In this study, the nature and presence of inhibitors of influenza A virus in bovine serum was investigated. The characters of inhibitor of bovine serum is different from that of horse serum. This inhibitor activity is Ca-dependent, resistant to both neuraminidase and periodate. This character is similar to inhibitor, but it is stable against heat treatment (56, 30min).

To investigate the nature of β inhibitor, we isolated the mannose binding protein(MBP) and conglutinin from bovine serum. The purified both MBP and conglutinin have a neutralizing activity and inhibition of hemagglutinating activity. This activity were inhibited by D-mannose and N-acetylglucosamine, N-acetylglucosamine respectively. And these activity were not destroyed by neuraminidase and periodate treatment.

These data indicate that β-like inhibitor of MBP and conglutinin in bovine serum that inhibit hemagglutination and neutralize the virus infectivity to block the attachment to the receptor-binding site by binding to carbohydrate site at the HA.

12.28

MICROHETEROGENEITY OF RAT LIVER ARYLSULFATASES A AND B BY LECTIN AFFINITY IMMUNOELECTROPHORESIS

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Extract of rat liver lysosomal fraction was used in crossed immunoelectrophoresis with lectin in the first dimentional gel. Specific rabbit antisera enabled detection of arylsulfatases A and B in crude extract. Nine lectins were used: *concanavalin A* (Con A), *Lens culinaris* (LCA), *Wheat germ* (WGA), *Helix pomatia* (HPA), *Phytohaemagglutinin* (PHA), *Soybean* (SBA), *Phytolacca americana* (PAL), *Dolichos biflorus* DBA) and *Lotus tetragonolobus* LTA) agglutinins. Appearance of several peaks or the retardation of the peak observed in the control, suggested the interaction between lectin and glycan.

Three retarded peaks can be observed in arylsulfatase A and two retarded in arylsulfatase B in electrophoresis with Con A, suggesting the existence of high-mannose, hybrid and bi-antennary complex types glycans or the presence of one of them but in different number depending on the variant. The absence of the tri-, tetra-antennary complex type glycans and the absence of bi-antennary complex type glycans containing bisecting GlcNAc was demonstrated by the lack of reaction with PHA. Two retarded peaks was observed in arylsulfatase A reaction with LCA resulting from the presence of different number of bi-antennary complex type fucosylated glycans what can be confirmated by the reaction with LTL. Arylsulfatase B reacted only weakly with LCA and LTL. Retardation of migration of both enzymes caused by WGA, negative reaction with PHA and positive reaction with Con A may suggest the presence of hybrid type glycans. HPA, SBA and DBA – GalNAc specific lectins were tested and the retardation of migration was only observed in case of DPA for both arylsulfatases. This result needs very careful interpretation and further specificity determination of these lectins. The results obtained do not answer the question concerning the number of glycans on the molecule of studied enzymes.

12.29

α1,3-FUCOSYLATION OF OLIGO-N-ACETYLLACTOSAMINOGLYCANS REDUCES THE AFFINITY FOR WHEAT GERM AGGLUTININ

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The relative affinities of several α1,3-fucosylated and nonfucosylated oligo-N-acetyllactosaminoglycans for immobilized WGA were studied using a chromatographic technique. The experiments showed that α1,3-fucosylation of the GlcNAc unit in Galβ1–4GlcNAcβ1–6Galβ1–4Glcol results in a dramatic loss of the high WGA-affinity of the tetrasaccharide alditol. Similar changes were observed when the hexasaccharide alditol Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4Glcol, or the hexasaccharide Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4Glc, were compared with derivatives which were α1,3-fucosylated in the GlcNAc unit of the 1->6 branches. However, α1,3-fucosylation of the GlcNAc unit of the 1->3 branch of the hexasaccharide alditol Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4Glcol had a rather small effect, showing that the 1->3 branch is of minor importance in the WGA binding. These results are compatible with the early work of Allen et al. [1], in which they showed that methylation of the C-3 hydroxyl group of GlcNAc methyl glycosides strongly reduces the WGA affinity.

In marked contrast, α1,2-fucosylation of oligo-N-acetyllactosaminoglycans did not reduce the WGA-affinity. This implies that WGA-chromatography can be used to differentiate between α1,2- and α1,3-fucosylated poly-N-acetyllactosaminoglycan backbones in a non-destructive manner.

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12.30

THE EFFECT OF AMINO ACID SUBSTITUTION AT POSITION 128 OF RECOMBINANT PEA LECTIN

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The cDNA for the lectin from the common garden pea (*Pisum sativum*) was previously expressed in and purified, in a functional form, from *E. coli.* (J. Biol. Chem. 261, 6141-6144, 1986). This system was developed to examine the role of certain amino acids postulated to be important in binding fucosylated glycopeptides of the N-linked class. Following deletion of the 17 N-terminal amino acid residues in our original construct, via site-directed mutagenesis, a variety of substitutions were made at position 128, which is Trp in the wild-type molecule. We were unsuccessful at establishing conditions which would allow refolding and carbohydrate binding and thus affinity purification of mutant lectins with Val or Ala at position 128. Lectins with Cys and Phe could be recovered, although with very low yields, and these proteins, PL3-C128 and PL3-F128 respectively, were further characterized. FAB-MS analysis of tryptic fragments was done to confirm that the protein produced was consistent with that predicted from the DNA sequence. The N-terminus of some of the PL3-F128 has a mass difference consistent with acetylation. The fragment containing amino acid 128 is as expected for PL3-F128 but contains an unidentified modification leading to a new tryptic site in the PL3-C128. The C-terminus loses six residues in 80% of PL3-C128. The mutant proteins are very similar in relative molecular mass

as assessed by SDS-PAGE. In addition the CD curves of the two mutants are identical to the wild-type recombinant and natural (nPL) lectins in the far UV range, but show an altered spectrum in the near-UV range, as would be expected upon the loss of a Trp. The amount of each lectin needed to agglutinate human red blood cells varied with the temperature at which the assay was done, due to a reversible instability of the mutants observed with increasing temperatures. The lectins are ranked nPL≤PL3-W128<PL3-F128<PL3-C128 with nPL being the most effective at agglutination over the widest range of temperatures (4°C-48°C). The differences between the lectins in terms of the amount needed for agglutination increased as the temperature increased. The effects of fucosylated and afucosylated glycopeptides in inhibition of agglutination by these lectins are under investigation.

12.31
LECTINS FOR INVESTIGATION OF GLYCOPROTEINS. A REVIEW

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The data on using of lectins (mainly, lectin sorbents) for isolation, separation and/or oligosaccharide structure analysis of both 250 enzymes and/or receptors are reviewed. Examples of lectin sorbents application for separation of glycoproteins (extracellular lectins and/or hydrolases and their forms) from bacterial, fungal, plant and mammalian sources are demonstrated. It is suggested, that enzymes bound to external or internal cell structures (plasma and nuclear membranes, lysosomes, endoplasmic reticulum, Golgi system, ribosomes etc.) may serve as targets for endogenic or exogenic lectins in vivo.

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12.32
CILIATED CELL MEMBRANE SUGAR RECEPTORS OF RABBIT TRACHEAL EPITHELIUM IN CULTURE

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This study was designated to investigate lectins and sugar receptors in outgrowth cultures from rabbit tracheal explants. On this model, previous studies from the laboratory have shown that during the first days of culture, tracheal epithelial cells presented many morphological and ultrastructural similarities with the normal tracheal epithelium: The cultures contained basal cells and epithelial polarized cells that exhibited tight junctions and desmosomes (1). Ciliated cells stay functional during the whole culture time (1).

Using different lectins (Wheat germ agglutinin, Canavalia ensiformis, *Arachis hypogaea*, *Ulex europaeus I* and *Bandeiraea simplicifolia BSI-B_4*), we were able to observe that during their migration to constitute the outgrowth, the cells retained their affinity for the different lectins. Moreover, ciliated cells were observed to keep an active system of syaliltransferases after being treated with neuraminidase as shown by decrease of the peanut agglutinin lectin (PNA) binding. A result which could be of interest since syalidase activity have been demonstrated in respiratory airways (2,3).

These reasons lead us to focus our study on the ciliated cells within this model and to investigate the membrane lectins of these cells. Fluoresceinylated neoglycoproteins (4) were used to analyzed the ciliated cell sugar receptors. Our data indicate that ciliated cells in our conditions of culture could be a good model to further study sugar receptors and their susceptibility to pharmaco-toxicological effects of various molecules.

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12.33
CRYSTALLOGRAPHIC STUDIES ON THE LENTIL LECTIN AND DOUBLE MANGANESE DERIVATIVES OF CONCANAVALIN A

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The legume lectins are a large family of homologous proteins capable of recognizing specific polysaccharide cell determinants. Although some of them have been studied extensively, their true role is still obscure and the residues involved in carbohydrate recognition have been identified with certainty only in a limited number of cases.

We obtained crystals from the lentil (Lens culinaris) lectin in the monoclinic space group $P2_1$ with unit cell dimensions a = 58.0 Å, b = 56.0 Å, c = 82.0 Å and β = 104.4°. Data extending to a resolution of 2.4 Å were collected on a FAST area detector.

In order to study the structure-function relationship in the legume lectins, we prepared metal substituted forms of concanavalin A and the lentil lectin. The metal binding properties of these lectins are well studied in solution and should now be interpreted in terms of the three dimensional structure of their metal binding regions and of conformational differences induced by substituting the native manganese and calcium by other transition metals. A preliminary 2.3 Å resolution data set of a double manganese substituted concanavalin A has already been recorded. The crystals had the same space group (I222) as the native protein but diffract to a much higher resolution.

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12.34
SEPARATION AND QUANTIFICATION OF HUMAN α1 AND α2 CHAINS OF TYPE VI COLLAGEN BY TWO-DIMENSIONAL GEL ELECTROPHORESIS AND CONCANAVALIN A STAINING

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Two major concanavalin A - binding glycoproteins were isolated from 5 M guanidinium chloride extracts of human uterine leiomyoma by two-dimensional gel electrophoresis. The glycoproteins appeared as elongated spots of M_r 140,000 indicating variations of their isoelectric points from 5 to 6. These glycoproteins were also present in normal and fibrotic skin. They contained hydroxyproline and hydroxylysine, formed disulfide-bonded components of high molecular mass sensitive to collagenase treatment after reduction. Collagenase treatment generated peptides corresponding in size to those of the non collagenous domains

of type VI collagen. Antisera raised against these purified glycoproteins reacted with either pepsin-derived α1(VI) or pepsin-derived α2(VI) chains but not with α3(VI) chain of human type VI collagen.

These results indicate that the glycoproteins represent the integral α1 and α2 chains of type VI collagen. The globular domains of α1(VI) and α2(VI) chains remaining after collagenase treatment appeared on two-dimensional gel electrophoresis as elongated spots suggesting that the non collagenous portions determine the well known microheterogeneity of the molecule. The differences in isoelectric points between and within α chains may facilitate the formation of microfibrillar network.

12.35

ISOLATION AND CHARACTERIZATION OF A NOVEL I5 kD β-GALACTOSIDE BINDING LECTIN FROM HUMAN SPLEEN

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We have carried out studies to identify and characterize β-galactoside binding lectins other than galaptin from human spleen (1). In the present study we concentrated on one such polpeptide of I5 kD molecular weight. Lactose elutable polypeptides from a spleen extract were purified on lactose-Sepharose column. The polypeptides were reductively alkylated and electrophoressed on SDS-PAGE followed by blotting onto PVDF membrane and staining with Coommassie blue. The stained polypeptide band was subjected to sequencing using an ABI liquid pulse phase sequencer. Additionally, chemical cleavage of the polypeptide at methionine residues was performed by treating the PVDF membrane with CNBr. The results of the sequencing indicated a blocked N-terminal whereas two distinct signals were obtained for CNBr cleavaged peptide; proline and valine were the leading amino acids. OPA blocking of the sequence starting with valine was performed to identify the complete sequence of the two fragments generated by CNBr cleavage. The sequence obtained for the CNBr peptides is as follows: Peptide 1, PFDLGFLVQSS; Peptide 2, VNGILFVQYF. The homology search of this sequence to protein data bank yielded no sequence homology indicating it to be a unique protein. Supported by CA42564(HJA).

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12.36

ISOLATION OF A MELIBIOSE BINDING LECTIN FROM HUMAN SPLEEN

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A melibiose (Gal α1–6 Glc) binding lectin was isolated from human spleen by serial affinity chromatography of lactosyl-Sepharose, mannosyl-Sepharose and melibiose-Sepharose equilibrated with Tris-HC1 buffer containing Ca^{2+}, Mg^{2+}, and Triton X-100, pH 7.3. The protein obtained from melibiose-Sepharose was repurified by chromatography on a fresh melibiose-Sepharose column. The purified lectin agglutinated trypsinized rabbit erythrocytes. The hemagglutination was inhibited by melibiose, but not by lactose. The lectin did not appear to recognize Galα1–3 Gal residue as it was not retarded in laminin-Sepharose column. On SDS-PAGE electrophoresis it showed a major polypeptide at 53 kD and two minor polypeptides at 30 kD and 29 kD. All three polypeptides were present in buffy coat cells as revealed by western blot analyses with anti buffy coat serum. B-142 Lymphoblastoid cells were labeled with [^{35}S]-methionine/[^{3}3H]-leucine and melibiose binding protein was isolated as detected on SDS-PAGE followed by fluorography. None of the polpeptides of melibiose binding protein reacted with anti-[human galaptin] serum, anti-[C-reactive protein] serum, anti-[amyloid P component] serum and anti-[epidermal keratin] serum when assayed by western blot analyses. But anti-[human core specific lectin] serum reacted very weakly with 53 k D polypeptide. 53 kD Polypeptide did not react with either anti-[murine CBP 35] serum or anti-[rat lung 29] serum. Western blot analysis of spleen extract with rabbit anti-[53 kD polypeptide] serum showed a single band at 53 kD. Supported by CA42564 (HJA).

12.37

CONCEPTS OF SUBGROUPINC GAL, GalNAc AND Galβ1→3 (4) GlcNAcβ1→ SPECIFICITIES OF APPLIED LECTINS

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Based on the binding properties studied with glycan by inhibition and affinity assays, Gal, GalNAc and Galβ1→3 (4) GalNAc specificities of applied lectins have been divided into six classes (subgroup) according to their specificity for the disaccharide as all or part of the determinants and GalNAcα1→Ser(Thr) of the peptide chain. Abbreviation of the following lectin determinants can also be used to classify these lectins. (1) **F**, GalNAcα1→3GalNAc-*Dolichos biflorus*; *Helix pomatia* and *Wistaria floribunda*; (2) **A**, GalNAcα1→3Gal; $\mathbf{A}_f$, fucosylated **A**, as GalNAcα1→3 [LFucα1→2]Gal-*Griffonia Simplicifolia* A_4, *Glycine max* agglutinin and Lima bean; (3) **Tn**, GalNAcα1→*O* to Ser/Thr of the protein core-*Vicia villosa* B_4 and *Salvia sclarea*; (4) **T**, Galβ1→3GalNAcα1→*O* to Ser/Thr determinant-Peanut and *Bauhinia purpurea alba*; (5) **I/II**, Galβ1→3(4)GalNAcβ1→ determinants-*Ricinus communis* agglutinin (RCA_1) *Datura stramonium* and *Wheat germ*; (6) **B**, Galα1→3Gal-*Griffonia* (Bandeiraea) *simplicifolia* B_4. Many of them demonstrate dual or multiple specificities such as *Maclura pomifera* is specific for **T and Tn** residues; *Bauhinia purpurea alba*, for both **T and I/II** and *Wistaria floribunda for* **A, F, Tn, and I/II**. Grouping of lectins will aid the selection of lectins for carbohydrate residue studies as well as for the interpretation of the distribution and the properties of carbohydrate chain residues on the cell surface.

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12.38

STRUCTURAL STUDIES OF A NEW SOLUBLE GALACTOSIDE-BINDING LECTIN FROM RAT INTESTINE

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Soluble S-Lac lectins are proteins that can be extracted without

detergents and have an affinity for lactose. The sequence, carbohydrate binding specificity and other functional properties have been determined for two, L-14 and L-29. Both lectins have affinity for laminin. L-29 has been identified both as a surface protein of macrophages (Mac-2) and as a nuclear protein (CBP-35) However, there appear to be many other members of the S-Lac lectin family. Here we describe a new S-Lac lectin in rat intestine, called RI-H.

The amino acid seguence of RI-H was determined by tandem mass spectrometry and Edman degradation. A cDNA encoding RI-H was cloned by using a PCR cloning method and sequenced. The sequence is homologous to other S-Lac lectins such as L-14 and L-29 but it also has unique features. RI-H is 37% identical to the C-terminal part of rat L-29 and is 23% identical to rat L-14. However, RI-H is clearly distinct from these lectins since 10 of 30 amino acids shared by L-29 and L-14 are different in RI-H. RI-H also differs from L-14 and L-29 in its relative affinity for certain glycoconjugates.

12.39
LECTIN BINDING SPECIFICITY AND BINDING CHARACTERISTICS OF HUMAN C-REACTIVE PROTEIN

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C-Reactive protein (CRP) is a member of the Pentraxin family and is composed of five identical polypeptide subunits. Although CRP is the major acute phase protein and has been shown to have lectin-like binding activity towards pneumococcal C-polysaccharide and galactans, and also to bind phosphorylcholine and various polycations, its physiologic function is still not sufficiently understood.

Here we report experiments designed to further characterize the binding properties of CRP. Using highly purified human CRP and a new method incorporating the ELISA technique it could be shown that binding of CRP to IgA, IgM, IgG, fibronectin and fibrinogen requires immobilization of both CRP and the ligand. CRP binding to these glycoproteins exhibited specificity for galactosyl residues in that binding was decreased by both sialylation and degalactosylation. Comparing various mono-, di- and trisaccharides as competitive inhibitors, only β-D-Gal(1–3)-D-GalNAc and β-D-Gal(1–4)β-D-Gal(1–4)-D-GlcNAc had significant inhibitory power at a concentration of 8 mMol/l. Monosaccharides and lactose had no inhibitory effect. Binding activity of CRP was pH-dependent with an optimum at pH 5 to 6 and was reduced by 90% when pH was shifted from 6 to the physiologic pH value of 7.4. It is therefore suggested that CRP exhibits lectin activity towards galactosyl groups preferentially in a mildly acidic environment as present at sites of inflammation.

12.40
STERIC FACTORS CONTROL THE RATE OF PROTEIN-SUGAR RECOGNITIONS

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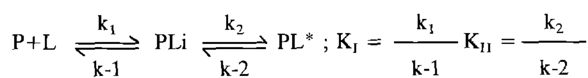
The association rate constants (k_I) for recognition of sugars (L) by lectins (P) has generally been observed to be several orders of magnitude slower than the diffusion controlled reactions. Hence these reactions are presumed to proceed through formation of an intermediate, PLi. This intermediate is assumed to be formed within the diffusion limits. Its isomerization forms the final complex PL* as depicted below (1-3).



$$P+L \underset{k-1}{\overset{k_1}{\rightleftharpoons}} PLi \underset{k-2}{\overset{k_2}{\rightleftharpoons}} PL^* \; ; K_I = \frac{k_1}{k-1} \; K_{II} = \frac{k_2}{k-2}$$

Despite these considerations the agreement between kinetically determined values of association constant and the amplitude of the changes in the intensity of ligand fluorescence or absorbance viz $\triangle F$ or $\triangle A$ are similar to those obtained in equilibrium titrations and the linearity of k_{app} vs [P] plot have been qualitatively consistent with a single step binding mechanism. Yet these recognitions have been explained to proceed through PLi to PL*. Failure to observe PLi has been explained by an unobservable signal change for the formation of PLi. Lack of hyperbolic appearance of the plots of k_{app} vs protein concentration has been suggested as due to a low ka value for its formation and as a consequence significant quantities of it do not accumulate viz PLi is converted to PL* or dissociates back to P & L, soon it is formed. In contrast to these presumed mechanisms our recent kinetic studies on Jacalin & Winged Bean Basic lectin clearly show that k_I for these recognitions are lower due to unfavourable steric factors & ligands that display greater complementarity in the juxtaposition of interacting moieties are recognized at a faster rate, as expected from this theory, as compared to those which do not show optimal juxtaposition of reacting groups.

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S13. CARBOHYDRATE INTERACTIONS IN NON-MAMMALIAN SYSTEMS/INTERACTIONS DES HYDRATES DE CARBONE DANS LES SYSTÈMES NON MAMMELIENS

13.1
CELL SURFACE CARBOHYDRATES OF *RHIZOBIUM*, STUDIES ON THEIR CHEMISTRY AND FUNCTION

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Chemical analyses of the capsular and lipopolysaccharides of *Rhizobium* reveal that the surfaces of these organisms are a complex mosaic of carbohydrates structures, the exact nature of which is a function of several external parameters. Any given species appears to be capable of the elaboration of structurally distinct surface carbohydrates, the exact nature of which is determined by host factors, pH, carbon source or by genetic manipilation such as transposon-induced mutations.

The lipopolysaccharides of *Rhizobium* are characterized by unusual lipid A structures both at the carbohydrate and fatty-acyl levels. There is a very high degree of conservation of the fatty acid components of the lipid A moieties between the species. There is also a very high degree of conservation of one R-core component between some of the species.

There is a strong relationship between capsular polysaccharide structure and host range as well as a strong dependence of nodulation and nitro fixation ability on the presence of an intact lipopolysaccharide structure.

13.2
STRUCTURE AND FUNCTION OF GLYCOSYLATED PHOSPHATIDYLINOSITOLS IN *LEISHMANIA*

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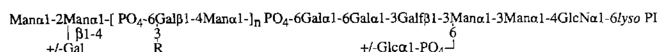
Glycosylated phosphatidylinositols (GPIs) are the major glycoconjugates of parasitic protozoa belonging to the genus *Leishmania.* Three distinct classes of GPI have been identified; (1) the GPI anchors of surface glycoproteins, (2) the polydisperse lipophosphoglycans (LPGs) and (3) the family of low molecular weight glycoinositol phospholipids (GIPLs). Studies on the GPI anchor of the major surface glycoprotein of *L. major* indicate that the protein-linked GPI glycolipids of *Leishmania* are structurally similar to the those found in Trypanosomes and in mammalian cells in containing the sequence: ethanolamine-PO_4-6Manα1–2Manα:1–6 Manα1–4GlcNα1–6PI. By contrast the LPGs and some of the GIPLs contain a number of novel structures and have no clearly defined analogues in higher eukaryotes. These molecules coat the cell surface and appear to be essential for parasite infectivity and survival. Their structures have been determined by chemical and enzymatic modifications, one and two dimensional 500mHz ^{1}H-NMR and fast atom bombardment-mass spectrometry. Analysis of the LPGs from three species of *Leishmania* indicates tnat these molecules have the consensus structures shown below. In addition to displaying species-specific features, the LPGs appear to be structurally modified as the parasites undergo developmental changes from noninfective, dividing forms to infective, nondividing forms. The possible role of these modifications in determining parasite infectivity will be discussed.



where R = H in *L. donovani*; H or Glcβ1– in *L. mexicana* and; H, $[Gal\beta 1\text{–}3]_{0\text{–}3}$Galβ1– or Ara_p1–2$[Gal\beta 1\text{–}3]_{0\text{–}2}$Galβ1– in *L. major*.n (average number of repeats) = 10–30.

We have also characterized the primary sequences of the GIPLs from *L. major* and *L. donovani.* Surface- and metabolic-labelling experiments indicate that these glycolipids are expressed predominantly at the cell surface, although some GIPL species may act as intracellular precursors to LPG. While the GIPL profiles of the promastigote (insect) stage were markedly different in the two species, similar GIPLs were present in both amastigote stages, which reside in the phagolysosome of mammalian macrophages. The amastigote GIPLs are structurally similar to the protein anchors, but lacked terminal ethanolaminephosphate. We propose that these surface GIPLs may be involved in amastigote invasion of macrophages either by direct interaction with macrophage receptors or indirectly after being recognized by the mannose-binding protein in mammalian serum.

13.3
LIPID STRUCTURE AND BIOSYNTHESIS OF THE GLYCOSYLPHOSPHATIDYLINOSITOL PROTEIN ANCHOR FROM A TRYPANOSOME PROCYCLIC MEMBRANE GLYCOPROTEIN

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The insect (procyclic) stage of the life cycle of the African trypanosome, *T. brucei*, expresses a stage specific major GPI-anchored glycoprotein, PARP. The anchor is insensitive to the action of PI-PLC, suggesting that it contains an acyl-inositol[1]. We have recently described the structure of a PI-PLC resistant lipid GPI, PP1, which is seen only in the procyclic stage, and not in the bloodstream form, and have suggested that this lipid is the precursor of the PARP GPI-anchor[2]. Inhibition of GPI biosynthesis with an amino sugar results in the coordinate shut-down of PP1 production and addition of GPI to PARP[3].

We have now shown that the PI moiety of the PARP GP1-anchor is in fact identical to that in PP1, by metabolic-labelling experiments, and GCMS, i.e. a palmitate residue is esterified to the inositol, and a stearate is present at the sn-1 position of the glycerol. These data strengthen our proposal that PP1 is the GP1-anchor precursor for PARP. Furthermore, we demonstrate that both fatty acids are derived from a metabolically large pool of precursors, suggesting the involvement of a phospholipid, rather than acyl-CoA, as the acyl donor, a mechanism similar to that reported previously for the acylation of lipoproteins in bacteria[4]. We propose that these observations may be of general validity as a number of other GP1-anchored proteins have been reported to contain acylated inositol[5].

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13.4
THE ROLE OF CARBOHYDRATE SIDECHAINS IN THE SECRETION OF PLANT GLYCOPROTEINS[1]

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Most plant extracellular proteins have N-linked oligosaccharide chains of the high mannose and/or complex type. We have recently shown a relationship of N-linked glycosylation and the secretion of glycoproteins in carrot and sycamore suspension cultured cells. Effectively, when treated with tunicamycin (TM) these cells do not accumulate newly synthesized glycoproteins in their extracellular compartment. The use of glycan processing inhibitors such as castonospermine (CAST), deoxymannojirimycin (DMM), or swainsonine (SWAIN) causes the secretion of glycoproteins with high-mannose or incompletely matured N-linked glycans. These results indicate that the presence of carbohydrate sidechains is necessary for plant glycoprotein secretion, irrespective of their oligosaccharide structure.

When treated with a mixture of CAST and DMM, sycamore cells secrete glycoproteins having mostly of Glc_3 Man_9 $(GlcNac)_2$ structure. We have studied the targeting and rate of secretion of these glycoproteins with N-linked precursor type oligosaccharide chains. We have taken advantage of the high susceptibility of these glycoproteins to digestion by endoglucosaminidase H (endoH) to produce deglycosylated extracellular glycoproteins, which is not possible through *in vivo* treatment of cells with glycosylation inhibitors. The stability of these glycoproteins, deglycosylated with endoH, was compared to that of control secreted glycoproteins upon incubation in sycamore cell culture medium. The results are discussed in this paper in respect to the apparent inhibition of glycoprotein secretion observed when plant cells are treated with glycosylation inhibitors.

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13.5
GLYCOCONJUGATES AND THE VITELLINE COAT

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It is well-known that the glycoprotein coat (vitelline coat or zona pellucida) which surrounds the egg in many invertebrate and vertebrate species plays a key role in the fertilization process. Indeed it bears the receptors which specifically bind sperm plasma membrane components thus triggering in these cells the cascade of events leading to the fusion of the gametes. However, both the structure of this coat and the organization of the receptors in it are largely unknown.

In this research we studied the vitelline coat of the egg of a mollusk bivalve particularly interesting as it is highly polarized; in fact, the sperm receptors are localized in a restricted area of the vegetal pole. By SDS-PAGE we found that the vitelline coat is made up of two main glycoproteins with an apparent MW of 230 kD and 180 kD which are differently solubilized using different extraction methods while the use of non-denaturating (native) conditions revealed that both of them take part in the formation of oligomeric structures. In order to analyze the composition of the saccharide chains of the two glycoproteins as well as their localization in the vitelline coat we utilized different lectins while their polypeptide chains were studied after removal of the glycosidic moieties with TFMS or endoglycosidases. Interestingly, the receptors area is the only region of the vitelline coat containing fucosyl residues and only the 230 kD glycoprotein has oligosaccharides bearing this sugar residue. We also raised polyclonal and monoclonal antibodies against these glycoproteins to study their structure and their organization in the vitelline coat.

13.6
SYNTHETIC AND NATURAL NON-SIALIC ACID-CONTAINING COMPOUNDS AS SUBSTRATES FOR INFLUENZA C VIRUS *O*-ACETYLESTERASE (EC 3.1.1.53)

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The strain of virus that we used was C/Johannesburg/1/66 (kindly provided by G. Herrler) and selected for its relatively good growth capacity. The virions were grown, harvested, concentrated and purified (1). The high degree of purity of the final influenza C virus preparation was checked (1). The pH optimum of the enzyme was around 7.6, and its activity was well maintained in the pH range from 7.0 to 8.5 (all these tests being performed with 4-nitrophenyl acetate as substrate). Remarkable differences were found in the values of both K_m and V_{max} with the synthetic substrates 4-nitrophenyl acetate, 2-nitrophenyl acetate, 4-methylumbelliferyl acetate, 1-naphthyl acetate and fluorescein diacetate. The use of 4-nitrophenyl acetate, 4-methylumbelliferyl acetate or 1-naphthyl acetate as substrate seems to be convenient for routine work, but it is better to carry out the measurements in parallel with those on bovine submandibular gland mucin. It was found that 4-acetoxybenzoic acid, as well as the methyl ester of 2-acetoxybenzoic acid, but not 2-acetoxybenzoic acid itself, are cleaved by this enzyme. Triacetin, di-*O*-acetyl-adenosine, tri-*O*-acetyladenosine, and di-O-acetyl-N-acetyladenosine phosphate, hitherto unreported as substrates for this viral esterase, are hydrolysed at a different rate by this enzyme. *We conclude that the O-acetylesterase from influenza C virus has a broad specificity towards both synthetic and natural non-sialic acid-containing substrates.* However, this broad specificity should not be generalized; thus, linear acyclic *O*-acetyl-containing compounds (*O*-acetylserine and its methyl ester) and cyclic compounds such as acetylsalicylic and *O*-acetylmandelic acids, are not cleaved by this enzyme. Nevertheless, the corresponding methyl esters are hydrolysed by the enzyme. *This broad specificity towards very different O-acetyl-containing compounds,* although with very different efficiencies, *seems to be a property which could be related to the infective capacity of influenza C virus.* Finally, the possibility that this enzyme might facilitate or enable the action of sialidases (2) could also be deduced from our work.

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13.7
THE EFFECT OF GLYCOSIDASE INHIBITION ON THE N-GLYCANS OF HIV GP120 EXPRESSED IN LEPIDOPTERAN CELLS

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The major envelope glycoprotein of HIV-1, gp 120, is extensively glycosylated, with approximately half its molecular mass composed of N-linked oligosaccharide. In the vertebrate system, considerable diversity of glycan structure has been reported (1) and modification of glycosylation events using specific glucosidase trimming inhibitors, results in reduced viral envelope protein mediated syncytium formation (2). In this study we have expressed gp 120 in insect cells using baculovirus recombinants and sequenced the N-glycan components. Although our analysis is consistent with the known data regarding a lack of complex glycan synthesis in insect cells (3), gp 120 contains a major, novel fucosylated oligosaccharide with the following structure: Manα1,6 (Manα1,3) Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc. Our results further indicate the presence in lepidopteran cells of a novel form of fucosyltransferase capable of generating these structures. Insect cells grown in the presence of a glucosidase I specific inhibitor, express gp 120 molecules which contain high molecular weight, incompletely processed N-glycans, but which retain their ability to bind to the cellular receptor CD4.

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13.8
N-LINKED GLYCOPROTEIN BIOSYNTHESES IN *DROSOPHILA MELANOGASTER*

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N-linked glycoprotein biosynthesis has been studied extensively in mammalian cell culture system where cell mutants have been used to great effect to study glycoprotein biosynthesis and the biological roles of surface membrane carbohydrates.

It is of considerable interest to extend such studies in genetically accessible invertebrate systems, in particular Drosophila where we have the capacity to use genetic analysis to study the function of these molecules during development of the organism. Such knowledge may provide valuable insights into the biosynthetic control of surface carbohydrate structure and the roles of cell surface glycoproteins in cellular behavior.

In this poster, we will present: 1) The assays developed for *in vitro* measurement of two glycosidase activities in late steps of N-linked glycoprotein synthesis pathway. 2) Preliminary data on the profile of carbohydrate moiety of lectin-reactive glycoprotein isolated by affinity chromatography. The results indicate that the biosynthesis of N-linked glycoprotein in *D. melanogaster* is similar to that of the relatively well characterized pathway in vertebrates. 3) Genetic studies of a tunicamycin resistant mutant TMR1C17. This gene has been mapped to the right arm of the chromosome 3 between *ra* and *ca* loci, we have begun to generate deficiencies in that region which will enable us to pin down its location. A mosaic experiment has also been carried out for functional assay of the N-linked glycoproteins in this system.

13.9

THE SPECIFICITY OF AN ANTIBODY AGAINST THE *N*-GLYCAN OF AN INSECT GLYCO-PROTEIN, PHOSPHOLIPASE A_2

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Insect cells are gaining increasing attention as expression systems for recombinant glycoproteins. The possible generation of determinants which are immunogenic for the recipient organism must be taken into account. Recent data, however, suggest the presence of antibodies directed against the *N*-glycan moiety of an insect glycoprotein, phospholipase A_2 (PLA_2, E.C. 3.1.1.4.) from honey bee venom, in sera of bee sting-allergic patients (1).

Rabbit polyclonal IgG antibodies against this glycoprotein reacted with a neoglycoprotein containing PLA_2-*N*-glycan. Crossreaction of the antibodies was observed with the *N*-glycans from several plant glycoproteins (pineapple stem bromelain, horseradish peroxidase). The antibody-carbohydrate interaction was studied in inhibition experiments using plant glycopeptides as inhibitors and, conversely, antisera against plant glyco-proteins which recognize carbohydrate epitopes on PLA_2. Although it was suggested that the crossreaction of antibodies directed against plant glycoproteins with PLA_2 is due to the presence of xylose (2), the glycan of PLA_2 does not contain this structural element (L. März et al., in preparation).

We propose that it is not the addition of xylose but rather the mode of fucosylation which mediates the development of the immunogenic determinants described.

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13.10

THE MOLECULAR BASIS FOR ANTIGENIC SPECIFICITY IN THE CORE STRUCTURE OF LIPOPOLYSACCHARIDES FROM *CAMPYLOBACTER JEJUNI*

Campylobacter jejuni, a common cause of human enteritis, affords lipopolysaccharides [LPS] as heat-stable antigens upon whose interactions with homologous antisera a serotyping system for 50 serovariants is based. Most LPS are of low M_r and devoid of O chains as shown by gel electrophoresis [SDS-PAGE], and structural investigations have been undertaken to ascertain the molecular basis for serological differentiation. The polysaccharides liberated on cleavage of the ketosidic linkage to lipid A from LPS of low M_r from selected serotypes have been subjected to detailed structural investigations by methylation linkage analysis in conjunction with FAB-MS of the permethylated glycans and derivatives. The results shows:- (1) that several of the *C. jejuni* LPS are unusual among those from human pathogens in containing terminal residues of Neu5Ac; (2) that the short polysaccharide chains contain no repetitive structures typical of O antigen chains; and (3) that despite similarities in overall composition, the core regions consist of a very limited inner region of invariable structure with sufficient variations in sequences of sugar residues and linkage types in the outer core for the LPS to account for serotypical differences. Neuraminic acid residues attached to core regions in structural units similar to those found mammalian cell surface glycolipids and glycoproteins represent potential virulence factors.

13.11

STRUCTURE OF O ANTIGEN CHAINS IN LIPOPOLYSACCHARIDES FROM *CAMPYLOBACTER JEJUNI* SEROTYPES O:23 AND O:36

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Campylobacter jejuni, a common cause of human enteritis, afford lipopolysaccharides [LPS] as heat-stable antigens upon whose interactions with homologous antisera a serotyping system for 50 serovariants is based. Whereas many LPS are of low M_r and devoid of O chains as shown by gel electrophoresis [SDS-PAGE], some of a high M_r and studies have been undertaken to determine the molecular basis for the serological cross reaction between serotypes 0:23 and 0:36. The polysaccharides from the LPS of the two serotypes have been liberated on cleavage of the ketosidic linkage to lipid A. Methylation linkage analysis in conjunction with FAB-MS of the permethylated glycans and specific chemical degradations to give oligosaccharide derivatives coupled with NMR studies have been used to define the repetitive structures in extended O chains. Four glycans of closely related structure have been characterized and shown to contain trisaccharide repeating units with identically linked β-D-Glc*p*NAc and α-D-Gal*p* residues and a third similarly linked heptose residue but of variable type in the general structure [–3β-D-Glc*p*NAc 1–>3 α-D-Gal*p* 1–>2 α-Hep*p* 1–]. The heptose sugars have been characterized as 6-deoxy-*altro*-Hep, 6-deoxy-3-*O*-methyl-*altro*-Hep, D-*glycero*-D-*altro*-Hep (or its enantiomer), and 3-*O*-methyl-D-*glycero*-D-*altro*-Hep (or its enantiomer).

13.12

EVIDENCE FOR A DIRECT N-GLYCOSYLATION OF ENDOGENOUS MICROSOMAL PROTEINS IN ASCARIDIA GALLI

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Some studies of late have demonstrated a direct N-glycosylation of protein acceptors from nucleotide sugars in different organisms.

Using UDP [^{14}C] N-acetylglucosamine and microsomes from A.galli as enzyme source and protein acceptor in an in vitro system we investigated the incorporation of the radioactive marker in the microsomal proteins. For studing the possibility for a direct N-glycosylation of

microsomal proteins in A.galli the 'main' pathway was blocked by tunicamycin. Under these conditions we found a considerable amount of radioactivity incorporated in the microsomal protein (3.3 pmol [^{14}C] GlcNAc incorporated in mg protein per 30 min.), which indicates existence of possibility for a direct N-glycosylation of proteins in A.galli microsomes.

Preliminary attempts with 5 lectins for characterizing the carbohydrate structure of glycoproteins synthesized under above conditions showed that the incorporated radioactivity is divided in structures corresponding to three- and tetraantennary complex type and high mannose type oligosaccharides.

13.13
STRUCTURE OF THE GLYGAN CHAIN FROM THE SURFACE LAYER GLYCOPROTEIN OF *CLOSTRIDIUM THERMOHYDROSULFURICUM* L77–66.

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Regularly arranged surface layers (S-layers) are ubiquitously distributed among archaebacteria and eubacteria. Many archaebacteria and several members of the *Bacillaceae* family have glycoproteins as constituent subunits of the S-layer lattices[1–3]. *Clostridium thermohydrosulfuricum* L77–66 is covered by a hexagonal S-layer lattice with a molecular mass of ca. 140,000 per subunit. Upon proteolytic degradation of this material a single high molecular weight glycopeptide fraction was obtained by gelfiltration on Bio-Gel P-30. By composition analysis, methylation, periodate oxidation and a combination of homonuclear and ^{1}H-detected heteronuclear shift-correlated nuclear magnetic resonance experiments, it was concluded that the polysaccharide portion of the glycopeptide has the following structure:

```
→3)-α-GalpNAc-(1→3)-α-GalpNAc-(1→
                        4
                        ↑
                        1
                     β-Manp
                        2
                        ↑
                        1
                    α-GlcpNAc
```

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13.14
GALACTOPLIPIDS OF A TAPEWORM, *METROLIASTHES COTURNIX*

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The major sphingoglycolipids of a cestode, *Metroliasthes coturnix*, living as parasite in quail intestine were galactolipids as follows:

monogalactosylceramide,

galactosyl-α-1–4-galactosylceramide.

galactosyl-β-1–6-galactosyl-β-1–6-galactosylceramide

galactosyl-β-1–6-galactosyl-β-1–6-galactosyl-β-1–6-galactosylceramide.

Their uniqueness also reside in their ceramide moiety mainly composed of C18 and C20 sphinganine or 4-D-hydroxysphinganine which is N-acylated with normal and hydroxy saturated acids of extraordinarily long chain as represented by the most abundant C26 acids (ranged C16 to C30).

The structure was confirmed by fast atom bombardment mass spectrometry after their chromatographic separation into each molecular species using reversed phase high performance liquid chromatography. A LC-FABMASS system enabled us on-line analysis of chromatographic peaks introduced into a ri-FAB apparatus in mass spectrometer. Using this analytical method, the presence of a minor glycolipid, fucosyl-dihexosylceramide was also suggested.

13.15
COMPLETE STRUCTURE OF *VIBRIO VULNIFICUS* CAPSULAR POLYSACCHARIDE

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Virulence of *V. vulnificus (Vv)* has been strongly associated with encapsulation and the presence of an opaque colony morphology. *Vv* capsular polysaccharide was purified from a whole cell, PBS-extracted preparation of the opaque, virulent *Vv* strain MO6–24/O by centrifugation, enzymatic digestion, and phenol chloroform extraction. Carbohydrate analysis by acid hydrolysis followed by HPAE chromatography with electrochemical detection gives peaks corresponding to quinovosamine, galactosamine uronic acid and a disaccharide containing $GalNH_2A$ α-(1→3)$QuiNH_2$ by comparison with standards derived from the lipopolysaccharide of *Pseudomonas aeruginosa* IID 1009 (ATCC 27585). Two-dimensional ^{1}H NMR spectroscopy as well as ^{1}H-detected ^{13}C correlation (HMQC) shows four sugars per repeating subunit including three residues of 6-deoxy, 2-acetamido hexose having the α-glucopyranose configuration (QuiNAc). The remaining residue has the α-galactopyranose configuration, with 2-acetamido function consistent with GalNAcA, which can be converted to GalNAc upon carboxyl reduction of polysaccharide. Complete resonance assignments (^{1}H & ^{13}C) with linkage assignments by ^{1}H detected ^{13}C heteronuclear multiple bond correlation spectra (HMBC) and NOESY spectra showed a repeating unit with a branched structure.

$$[\rightarrow 3)\ Qui_pNAc\ \alpha\text{-}(1\rightarrow 3)Gal_pNAcA\ \ \alpha\text{-}(1\rightarrow 3)Qui_pNAc\ \ \alpha\text{-}(1\rightarrow]_n$$
$$Qui_pNAc\ \ \alpha\text{-}(1\rightarrow 4)\nearrow$$

Periodate oxidation of polysaccharide a gave linear polymer with a trisaccharide repeating unit.

13.16
STRUCTURE OF GLYCOPHOSPHOSPHINGOLIPIDS OF *TRITRICHOMONAS FOETUS* AND *TRICHOMONAS VAGINALIS*: NOVEL INOSITOL-PHOSPHOSPHINGO LIPIDS

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Trichomoniasis is an infection of the genitourinary tract caused by the

flagellated protozoan, *Trichomonas vaginalis* in humans and by *Tritrichomonas foetus* in cattle. We have used metabolic labeling, chromatography, high performance tandem mass spectrometry, and NMR to elucidate the structures of glycophosphosphingolipids of *T. foetus* and *T. vaginalis*. Two of the acidic lipid fractions (obtained from DEAE Sephadex A-25 followed by HPTLC and HPLC chromatography), designated TF_1 and TF_2 from *T. foetus* and TV_1 and TV_2 from *T. vaginalis* could be metabolically labeled with [^{3}H] myo-inositol and [^{32}P] orthophosphate. [^{3}H] Fucose and [^{14}C] ethanolamine were preferentially incorporated into the TF_1 fraction. Each of the lipids from *T. foetus* and *T. vaginalis* contain ceramides. TF_2 and TV_2 contain inositol-P-ceramides. TV_1 contains inositol diphosphate, linked to the ceramide and to the ethanolamine. TF_1 contains three closely related components, in each of which fucose is linked to inositol diphosphate with one of the phosphates linked to the ceramide moiety, the other phosphate being either free or linked to ethanolamine or *N*-acetylethanolamine. TF_1 and TV_1 appear to belong to a novel class of glycophosphosphingolipids which share some structural similarities to the glycosylphosphatidylinositol anchors of eukaryotic proteins. Investigating the role of glycosylated-phosphatidyl inositol anchor membrane antigens is fundamental to understanding the host-parasite relationship.

13.17

BINDING AND ACTIVATION OF WALL-BOUND ENZYMES OF *TRICHODERMA REESEI*: STRUCTURE AND PROPERTIES OF THE RESPONSIBLE CELL WALL COMPONENT

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Application of *Trichoderma* – cellulases for cellulose saccharification is hampered by limiting amounts of β-glucosidase. In contrast to the other cellulase compounds (cello-biohydrolases and endoglucanases), this enzyme predominantly is secreted into the cell wall. We isolated a complex heteroglycan from purified cell walls as well as from culture supernatants of *Trichoderma reesei*, which reassociates with purified β-glucosidase *in vitro* and increases its enzymatic activity twofold. We assume that this glycan represents the β-glucosidase-binding cell wall component. Other wall bound enzymes from *T. reesei* like β-xylosidase and N-acetyl-glucosaminidase are also stimulated by the purified glycan.

The structure of the *T. reesei* glycan) from cell walls and from the supernatant was determined by ^{1}H–, ^{13}C-NMR and two-dimensional techniques and GC/MS after methylation. Less complex fragments were obtained by mineral acid hydrolysis and allowed the eludication of the complete structure of this functional cell wall component. Glycans from both sources consist of a mannan backbone [α-Manp- (1,6)] with two types of branching: A single mannose residue [α-Manp-((1,2)] and a trisaccharide containing a terminal glucuronic acid [α-GlcAp-(1,2)-β-Galf-(1,6)-β-Galf-(1,2). In the glycan isolated from cell walls this trisaccharide is elongated to a high extent by an additional terminal glucose [α-Glcp-(1,4)] linked to the uronic acid.

A corresponding polysaccharide is also present in the culture filtrates of *Aspergillus niser*. A purified glycan from this source also stimulates the activities of both β-glucosidase and β-xylosidase from *Trichoderma reesei*, and a compareable effect was observed by yeast mannan. This "heterologous activation" suggests a general mechanism for activation and probably binding of wall proteins and gives rise for investigations of the composition and structure of the "anchor glycan" of *Trichoderma*.

13.18

CROSSED-IMMUNOELECTROPHORETIC ANALYSIS OF HETEROGENEOUS CARBOHYDRATE DETERMINANTS OF *LEISHMANIA*

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Leishmanial promastigotes generate glycoconjugates that they secrete to their environment. These are glycolipids, glycoproteins and phosphoglycans bearing antigenic determinants that confer specific serotypes on them and the parasites producing them. Three leishmanial serotypes have been recorded: A, B and AB, which separate into subserotypes according to reactivities with specific rabbit anti-leishmanial polyclonal antibodies (α-1.Pab).

Crossed-immunoelectrophoresis (CIE), using mouse anti-leishmanial monoclonal antibodies (α-1.Mab) with specific reactivities to either A or B epitopes, permitted analysis of mixed AB serotypes to determine if both types of epitope are borne on the same glycoconjugate molecule or segregated on separate ones. Three formulations of CIE were used. Purified A, B and AB secreted glycocongugates were run into: 1, anti-A or Bα-1.Pab, after filtration through either A or B antigen to check reactions of identity; 2, anti-ABα-1.Pab after filtration through either anti-A or Bα-1.Pab; 3, anti-A or Bα-1.Pab after filtration through anti-A or Bα-1.Mab (WIC79.3 and CA7AE respectively).

After filtration through antigen, the AB conjugate showed identity with A and B marker conjugates, confirming the presence of both epitopes in the mixed conjugate. After filtration through either anti-A or anti-B Pab, the AB conjugate separated into 3 components: 1 precipitated with anti-A and 2 with anti-B. After filtration through anti-A Pab, the A and AB conjugates displayed, identically, two A components precipitating in the anti-A Mab gel. No reactions occurred in the adjacent anti-A Pab gel. After filtration through anti-B Mab, the B and AB conjugates were precipitated, but not identically. No reaction occurred in the adjacent anti-B Pab gel. The anti-B Mab had high affinity for pure B conjugate, but lower affinity for the B component of the AB conjugate. Clear separation of the A epitopes was seen in the anti-A Pab gel with high homology.

CIE confirmed mixed carbohydrate antigens and proved, definitively, that A and B epitopes are separate and not on the same molecule in secreted glycoconjugate.

13.19

SYNTHESIS OF ARYL GLYCOSIDES AND THEIR *VIR* INDUCING ACTIVITIES ON *AGROBACTERIUM TUMEFACIENS* STRAINS

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Phenolic derivatives as acetosyringone, acetovanillone or coniferyl alcohol were previously shown to induce the *vir* genes of *Agrobacterium tumefaciens*. Aryl β-glycosides have been synthesized by coupling acetovanillone, acetosyringone, syringaldehyde, syringic acid and coniferyl alcohol with D-glucose, D-galactose, maltose and L-fucose in a two steps reaction. Peracetylated glycosyl halides and phenolates in aqueous acetone afforded the acetylated β-glycosides which were deacetylated. The *vir* inducing activities of these above glycosides were determined on two different agro-bacteria strains, one harboring the nopaline Ti plasmid pTiC58 and *vir*E::*LacZ* fusion plasmid, and the second one harboring the octopine Ti plasmid pTiA6 and *vir*E::*LacZ* fusion plasmid. The *vir* inducing activities of these aryl β-glycosides and their

parent phenols were tested in 1-5000 μM concentration range. The octopine strain is easier inducible than the nopaline strain. All tested glycosides are less effective *vir* inducers than free acetosyringone. In the concentration range used, acetosyringone showed an optimum concentration for induction at 300 μM in contrast with the aryl β-glycosides at 5000 μM in the order: Maltose < D-Glc < D-Gal < L-Fuc < acetosyringone.

13.20
LINEAR CHITIN AS A RESEARCH TOOL

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Mechanistically valid characterization of cellulases became possible with the advent of Avicel. Valid mechanistic studies of chitinases have now also become feasible through newly available Linear Chitin (US Patent #4,958,011) which retains the native ultra-structure of chitins in covalently stabilized form. Difficulties with "chitin analogs" for enzyme characterization have been legion, whether the substrate consisted of "swollen chitin," "soluble chitin," or "reconstituted chitin." Unlike these, Linear Chitin as the substrate for specific chitinases gives the following data: 1. extremely rapid product generation that is strictly linear with time; 2. no interference from lysozymes during the assay period; 3. valid kinetic data permitting reliable mechanistic inferences for specific chitinases; 4. graphic demonstration of mechanism of induction of microbial chitinases via specific receptors to which chitin must firmly attach before these adaptive enzymes are generated; 5. means for experimentally testing the validity of a model for eucaryotic exochitinase activity; 6. means for experimentally testing the internal structure of chitin fibrils; 7. ability to draw inferences for cellulases from chitinase data, and vice versa. Other important gaps in our knowledge, such as whether or not insectivorous birds utilize chitin as an energy source and the role of chitin in global nitrogen balance, can now also be filled in. (Supported, once upon a time, by NIH and NSF, and by Boston College).

13.21
CHARACTERISATION OF NEMATODE GLYCOPROTEINS: THE MAJOR *O*-GLYCANS OF *TOXOCARA* EXCRETORY-SECRETORY ANTIGENS ARE *O*-METHYLATED TRISACCHARIDES

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Toxocara canis is a common intestinal nematode parasite of dogs with a typical ascarid life cycle. In paratenic hosts, the infective larvae remain developmentally arrested and migrate through tissues, resulting in ocular toxocariasis or visceral larva migrans in human infections. When maintained in long term serum-free culture, the larvae released large quantities of excretory-secretory (ES) antigens, defined at 32, 55, 70, 120 and 400 kDa and shown to be substantially glycosylated with highly immunogenic carbohydrate epitopes. In work aimed at identifying *Toxocara*-specific epitopes for diagnostic applications and potential vaccine development, we have initiated detailed structural studies of these ES antigens based on Fast Atom Bombardment-Mass Spectrometry (FAB-MS), gas chromatography and electron impact-mass spectrometry. We showed that the major *O*-glycans of the ES glycoproteins, as released by reductive elimination, are two, approximately equi-abundant, naturally *O*-methylated trisaccharides: 2-*O*-Me-Fuc*p*(α1→2)-4-*O*-Me-Gal*p*(β1→3)-GalNAcitol, and its counterpart lacking the *O*-Me group on the Gal residue. Anomeric configurations were defined using a novel chromium trioxide oxidation/FAB-MS strategy which we have recently developed, enabling us to achieve full characterisation at μg level with minimal purification. We are currently localizing these specific epitopes to individual ES glycoproteins as well as probing their glycosylation sites. Work has also been extended to ES antigens from related ascarids including *T. cati* and *Ascaris suum,* both of which exhibit cross-reactivity with monoclonals raised against the *T. canis* ES antigens. Preliminary data indicates that the ES products of *T. cati* may differ from those of *T. canis* only in the degree of *O*-methylation.

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13.22
STRUCTURAL DIFFERENCES OF GALACTOMANNANS FROM *ASPERGILLUS FUMIGATUS*

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Galactomannans, the main antigenic substances of **Aspergillus fumigatus**, were obtained from hyphae of two different strains of this fungus by alkaline extraction. C-13 NMR spectra of alkali-soluble galactomannans of **A. fumigatus** indicated the presence of certain structural features similar to those found in the water-soluble poly-saccharide fractions, which differed, however, for samples obtained from different strains of this fungus. Signals at δ c 109.5 and 108.5 ppm corresponding to the anomeric carbons of the galactose units, respectively, β-D-Gal**f**(1–6)-α-D-Man**p** and D-Gal**f**(1–5)-β-D-Gal**f** structures, were present in the spectrum of the galactomannan of one strain. These signals were much diminished in the spectrum obtained for the other **A. fumigatus** galactomannan. In the latter, the α-D-Man**p**(1–2)-α-D-Man**p** common structure of fungal polysaccharides predominated, as indicated by prominent signals at δ c 103.7 and 102.2 ppm. A signal at δ c 103.7 is consistent with the presence of non-reducing end-units of α-D-mannopyranosyl units, possibly enhanced as a result of the reduced substitution of the side chains with terminal β-Gal**f** units.

These findings support the occurrence of different structures in the galactomannans from different strains and could explain why a complete inhibition of binding of the human antibody to the fungal galactomannan was not always obtained, using the β-Gal**f**-(1–5)-β-Gal**f**$)_2$ tetrasaccharide.

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13.23
O-GLYCOSYLATED 43 kDA GLYCOPROTEIN FROM PLASMODIUM FALCIPARUM

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Malaria is a widespread disease caused by the protozoan parasite P. falciparum. This is the most virulent of the species causing malaria and it is responsible for considerable mortality and high morbidity. The presence of carbohydrates in the surface glycoprotein antigens of P. falciparum, particularly merozoites, have been described and these

compounds are known to be of biological and immunological significance. More recently glycoprotein surface antigens of 43–46 kDa have been described that are antigenically distinct from the 195 kDa glycoprotein and inhibit the invasion of erythrocytes by merozoites in vitro.

In the present investigation, the P. falciparum cells cultured in asynchronous mode were radiolabeled with [^{3}H]-GlcN, [^{3}H]-Gal and [^{3}H]-Man separately as well as in admixture. The enriched, infected erythrocytes were lysed with NP-40 in isotonic TRIS buffer containing EDTA. The labeled glycoproteins in the supernatant were separated by SDS-PAGE and the 43 kDa band labeled was excised from the gel and treated with (a) 4M HCL and (b) 50 mM NaOH containing $NaBH_4$-NaB^3H_4. Acid hydrolysis of the glycoprotein showed the presence of GlcN, GalN, Gal and Man. Beta-elimination-reduction of the glycoprotein(s) in the gel and subsequent acid hydrolysis showed the presence of GlcNAc-ol and traces of GalNAc-ol, GlcAc and Gal. Treatment of the glycoprotein with exoglycosidases showed the presence of terminal GlcNAc and Gal. These experiments suggest that the 43 kDa glycoprotein, like the 195 kDa glycoprotein, is an O-glycosylated glycoprotein containing GlcNAc in O-linkage to peptide. It is being ascertained whether the Man, GlcNAc and Gal are only components of oligosaccharide chains of the glycoprotein or are present also in the glycan moiety of the putative anchor region of the glycoprotein.

13.24
CHARACTERIZATION OF NOVEL O-LINKED OLIGOSACCHARIDES FROM THE CELLULASE COMPLEX OF *Bacteroides cellulosolvens*

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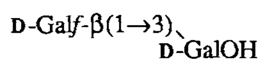
Bacteroides cellulosolvens, an anaerobic mesophilic bacterium, is claimed to express cellulose-degrading capacity equal to that of *Clostridium thermoccellum*. The cellulases are also organized in a multicomponent cellulosome-like complex (1). The interaction with the α-Gal-specific B_4 lectin from *Griffonia simplicifolia* and other properties shared by the cellulase systems of both bacteria prompted an investigation of the structures of the carbohydrate moiety. Information is presented with respect to primary structures of O-linked carbohydrate chains present in the cellulase complex. Alkaline borohydride treatment of the cellulosome-like entity of *B. cellulosolvens* yielded oligosaccharide-alditols, which were isolated via gel permeation chromatography and HPLC. The structures of the compounds were determined by monosaccharide analysis, methylation analysis, GLC-MS, FAB-MS, and 1D and 2D (COSY, HOHAHA, ROESY) 500-MHz^{1}H-NMR spectroscopy, and turned out to be:



and partial elements of this compound. The results indicated a remarkable partial similarity of the oligosaccharide moiety of the cellulase complexes of *B. cellulosolvens* and *C. thermocellum* (2,3), two evolutionarily and physiologically diverse bacterial species.

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13.25
CHARACTERIZATION OF LINKAGE REGIONS AND LIPID INTERMEDIATES OF EUBACTERIAL S-LAYER GLYCOPROTEINS

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Crystalline bacterial surface layers (S-layers), composed of protein or glycoprotein species, are found as outermost cell envelope component in almost every taxonomic group of walled eubacteria and archaebacteria. With the discovery of covalently linked carbohydrates on thermophilic *Bacillaceae* we have demonstrated that not only archaebacteria but also eubacteria are able to synthesize glycosylated proteins. The glycan structures of these eubacterial glycoproteins were analyzed by composition analyses and nuclear magnetic resonance experiments.

Currently we are studying the carbohydrate-protein linkage regions of selected glycopeptides which were obtained by pronase digestion of highly purified S-layer glycoproteins of *Bacillaceae*. Preliminary data obtained from glycopeptides of different *Clostridium thermohydrosulfuricum* strains indicated the presence of O-glycosidic linkages.

Detailed studies on halophilic and methanogenic archaebacteria have revealed that both dolichol and undecaprenol are involved as lipid carrier in the biosynthesis of different glycopeptides of their S-layer glycoproteins. We have now characterized the lipid intermediates involved in the biosynthesis of the S-layer glycoproteins of *C. thermohydrosulfuricum*. Recent data indicate that rhamnose and mannose, the constituent sugars of the glycan chains of two *C. thermohydrosulfuricum* strains, were, together with phosphate, found lipid bound to dolichol.

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13.26
STRUCTURAL STUDIES OF TWO SURFACE POLYSACCHARIDES FROM THE CAPSULE OF *BACTEROIDES FRAGILIS* NCTC 9343.

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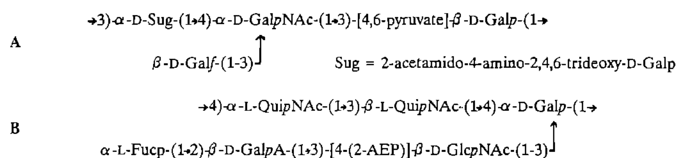
Bacteroides fragilis is the most common isolate from clinical cases of intra-abdominal sepsis and anaerobic bacteremia. Recently it has been shown that the capsule of *B. fragilis* NCTC 9343 is comprised of two distinct, surface expressed polysaccharides (A and B). They primarily exist in an aggregate form and can be separated and purified by anion-exchange chromatography and mild acid treatment.

The structure of A and B has been investigated mainly by nmr spectroscopy. The constituent monosaccharides and substituents were determined from the chemical shifts and coupling patterns from their respective spin-system. The connectivities and absolute configurations were deduced with help of long-range ^{1}H-^{13}C correlated spectroscopy (HMBC), 2D-N.O.e. and chemical shift analysis. HMBC and ^{1}H-^{31}P correlated spectroscopy gave the positions of the substituents. Sugar

analysis and determination of absolute configuration with GC-MS confirmed the results from the nmr investigation. Polysaccharide A consist of a tetrasaccharide repeating unit with a 4,6-pyruvic acetal as a substituent. Polysaccharide B consist of a hexasaccharide repeating unit with a 2-aminoethyl phosphonate (2-AEP) as substituent.



These results define the existence of two structurally different, surface exposed polysaccharides that constitute the capsule of *B. fragilis*. The aggregation is probably due to ionic interactions between the two polysaccharides. A complex capsule containing two distinct polysaccharides has previously not been described for other encapsulated human pathogenic bacteria.

13.27
N-LINKED OLIGOSACCHARIDE CHAINS OF α-GALACTOSIDASE FROM *ASPERGILLUS NIGER*

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Various kinds of glycoidases have been isolated from the culture of *A. niger*[1], and some of them have been demonstrated to be glycoproteins. An α-galactosidase (α-galactoside galactohydrolase [EC3.2.1. 22]) from *A. niger* has also been reported to be a glycoprotein[2]). In order to investigate the molecular structure of carbohydrate moiety, we isolated the carbohydrate chains from the purified α-galactosidase and analyzed the structure of them.

An α-galactosidase was purified from a commercial *A. niger* culture broth, Transglucosidase Amano, by duplicate chromatographies on Sephacryl S-200. The enzyme had a molecular weight of approximately 60,000 and was well stained by the method using Con A – peroxidase complex. To study the structure of the carbohydrate moiety of this enzyme, the glycoprotein was treated with *N*-glycosidase F. The released *N*-linked oligosaccharides were isolated by succesive chromatographies on Biogel P-4 and anion exchange resin, and analyzed by ^{1}H-NMR. The structure of the oligosaccharides was determined as oligomannoside type.

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13.28
IN CARROTS, THE COMPLEX N-LINKED OLIGOSACCHARIDES OF INTRACELLULAR GLYCOPROTEINS ARE INDISTINGUISHABLE FROM THE COMPLEX N-LINKED OLIGOSACCHARIDES OF EXTRACELLULAR GLYCOPROTEINS

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The N-linked glycans from the 52/54 kd medium protein and cell wall β-fructosidase, two glycoproteins secreted by carrot suspension culture cells, were characterized. Both proteins have one high mannose-type glycan similar to those from yeast and animal glycoproteins. In addition, the 52/54 kd medium protein has three complex-type and cell wall β-fructosidase two complex-type glycans per polypeptide. The complex-type glycans isolated from individual glycosylation sites are fairly large and very heterogeneous. The smallest of these glycans has the structure (Xyl)Man_3(Fuc) $(GlcNAc)_2$Asn whereas the larger ones carry additional sugars like terminal N-acetylglucosamine and possibly rhamnose and arabinose in the case of the 52/54 kd medium protein and only arabinose in the case of cell wall β-fructosidase. These terminal sugars are linked to the β-mannose residues of the glycan cores. The 52/54 kd medium protein is secreted with large and homogeneous complex glycans, their heterogeneity originates from slow processing after secretion. The complex glycans from cell wall β-fructosidase are processed before the enzyme is integrated into the cell wall.

Soluble glycoproteins were isolated from purified carrot protoplasts and their N-linked glycans analyzed. Their complex-type structures are fairly large and very heterogeneous. A comparison of the complex glycans of intracellular carrot glycoproteins with those from extracellular carrot glycoproteins shows that they are indistinguishable.

13.29
STRUCTURAL FEATURES OF GLYCOPROTEIN OLIGOSACCHARIDES FROM SNAKE VENOM

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The carbohydrate nature of the glycoproteins present in the venoms of cobras and most other snakes is virtually unknown. In the present study, we have analyzed the venom glycoproteins from several species of snakes. Affinity staining of Western blots with lectins indicated that almost all the glycoproteins from the venoms of five species of cobra that we have studied contain terminal α-galactose residues with a negligible proportion of sialic acid. In contrast, the glycoproteins from cobra plasma and tissues such as brain, heart, lung, liver, muscle and kidney contain little or no α-galactosyl residues. However, these glycoproteins appear to contain both 2,3- and 2,6-linked sialic acid. Carbohydrate composition and structural analyses of glycoproteins from the venom of one of the cobra species viz *Naja naja kaouthia* indicated that the majority of the cobra venom glycoproteins contain mainly asparagine-linked oligosaccharides with relatively little or no *O*-linked saccharides. Furthermore these glycoproteins also contain a high proportion of fucose. The unusual feature of these venom glycoproteins is the presence of an α-galactosylated Lewis X antigenic structure, Galα1–3Galβ1–4(Fucα1–3)GlcNAcβR, which is analogous to sialyl Lewis X antigens present in glycolipids and glycoproteins of neutrophils and some tumor cells. A 72 kDa glycoprotein (>50% carbohydrate) isolated from the venom of *Naja naja kaouthia*, appears to contain predominantly poly-*N*-acetyllactosaminyl structures which are also substituted with fucose and terminated with α-galactosyl residues. This is the only glycoprotein in the venom of this cobra that contains poly-*N*-acetyllactosaminyl structures.

To determine whether the above unusual glycosylation pattern of venom glycoproteins is restricted to cobra or is common to all other snakes, we have studied the nature of glycoprotein oligosaccharides in the venoms of representative species of snakes other than cobras. None of the glycoproteins from the venoms of these snakes contain α-galactose residues. However, these glycoproteins do contain sialic acid. For

example, *Bungarus fasciatus* and *Ophiophagus hannah* contain both 2,3- and 2,6-linked terminal sialic acid while *Bitis arietans arietans* and *Agkistrodon piscivorus piscivorus* contain exclusively 2,3-linked sialic acid. Interestingly the venom glycoproteins of *Crotalus adamanteus* contain neither sialic acid nor α-galactose residues.

13.30
STRUCTURAL ELUCIDATION OF THE CORE OLIGOSACCHARIDE OF *AEROMONAS HYDROPHILA* BY THE COMBINATION OF FAB MASS SPECTROMETRY AND METHYLATION ANALYSIS

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The Gram-negative bacterium *Aeromonas hydrophila* is a significant cause of mortality in Salmonid fishes, particularly in aquaculture ventures; this poses a serious economic problem. Since pathogenicity may be mediated by carbohydrates, the oligosaccharide structures of different chemotypes are of considerable interest. The core oligosaccharide of the capsular polysaccharide of Chemotype III has been analyzed by positive and negative fast atom bombardment mass spectrometry (FAB-MS) and the molecular weight has been determined to be 1630. From the fragmentation pattern in FAB-MS and MW information, it has been concluded that the structure of the core oligosaccharide is $(Hep)_3$ $(Hex)_3$ $(HexNH_2)$ (deoxyHexNHAc) (KDO).

FAB-MS of the permethylated derivative of the core oligosaccharide exhibits fragment ions of A^+ type (1) and confirms the sugar composition. The sequence information, obtained from these fragment ions, will be described. The information on linkages among monosaccharide units was obtained from methylation analysis using EI-MS (electron-impact mass spectrometry) and CI-MS (chemical-ionization mass spectrometry). How the combination of various chemical and mass spectrometric techniques led to the determination of the core oligosaccharide structure will be discussed.

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13.31
PRIMARY STRUCTURE, STRUCTURE-FUNCTION & EVOLUTIONARY RELATIONSHIP: OF A LOW MOLECULAR WEIGHT XYLANASE (LMX) FROM *CHAINIA SP.*

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Two cellulase free xylanases, with Mr 26,000 and 4,000 were isolated in the ratio 20:80 respectively, from Sclerotial Actinomycete *Chainia sp.* which are potentially important in paper and pulp industry for selective hydrolysis of hemicelluloses (1). The complete amino acid sequence of this xylanase (LMX) as determined with semiautomated solid phase sequencer developed in our laboratory (2) utilizing the chemistry of DABITC/PITC double coupling method (3), is reported here. LMX is only 40 amino acids long thus making it the smallest enzyme reported so far. Edman degradation was performed upto residue 34. Ala-Thr-Thr & Ile were the residues at N-terminus. Residue 31 was Asn & 32 was Gly. Hence, hydroxylamine cleavage at this Asn-Gly site was performed in order to complete the sequence and to get the information about C-terminus peptide. The C-terminus peptide is made up of nine residues with C-terminus residue being glutamic acid. LMX sequence is rich in Thr and Gly. That the sequence thus obtained is a complete representation of the primary structure of LMX is borne out by the following: Its amino acid composition determined by sequence with Mr of 4330 Da. matches with its chemically determined composition; composition of the C-terminus peptide is in accordance with its sequence. The sequence thus determined differs considerably from that reported earlier (4). We did not find any Cys in LMX sequence which invalidates the claim regarding an essential cysteine residue in the activity of LMX (5). This enzyme consists essentially of β-pleated sheet and β turn with no α-helix. Interestingly enough, LMX shows considerable homology with *Bacillus pumilus* xylanase which throws considerable light on its structure-function and evolutionary relationships.

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S14 STRUCTURAL ANALYSIS/ANALYSE DE STRUCTURE

14.1
REGULATION OF POLYLACTOSAMINE-GLYCOCONJUGATE BIOSYNTHESIS

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Blood group active (ABH) and polylactosamine (PolyLAm) antigens contain carbohydrate structures which are found as glycoproteins and glycosphinglipids. These antigens are present on erythrocytes as well as on cell surfaces of varities of tumor tissues including human adenocarcinoma and colon carcinoma. Cell surface glycoconjugates of normal and tumor cells have been shown to carry specific biological messages for cell to cell recognition factors as well as for various toxin, antibody and hormone receptors. In recent years, we have been involved in the biosynthesis *in vitro* of nLcOse4Cer (Galβ1–4GlcNAcβ1–3Galβ1–4Glc-Cer; nLc4), the core glycolipid for all ABH and PolyLAm antigens in two different tumor tissues of human and mouse origin and also in 11-day-old embryonic chicken brain (11d ECB). The galactosyltransferase GalT-4, which catalyzes the biosynthesis *in vitro* of nLc4 from LcOse3Cer (GlcNAcβ1–3Galβ1–4Glc-Cer, Lc3) have been purified from both mouse lymphoma (106,000-fold) and human colon carcinoma (40,900-fold), respectively. It has been reported recently that glycosyltransferases can be classified on the basis of their substrate recognition site requirements. The anion-specific inhibitor DIDS (4, 4′-Diisothiocyanato-2,2′-stilbenedisulfonic acid) was tested as a potential inhibitor of GalT-4. Inhibition (50%) of highly purified GalT-4 activities from P-1798, Colo-205 and 11d ECB was observed between 0.05–1.25 μM DIDS concentration. Most purified GalT-4 fractions from both these tumors show evidence that the enzyme is independent of the hydropho-

bic part of the substrate, Lc3 (natural) and acetyl-Lc3, where fatty acyl groups of ceramides are C_{22} and C_2 respectively. The glycoprotein acceptor, asialo, agalacto-αglycoprotein also behaves as potential acceptor for the purified enzyme from mouse lymphoma with comparable V_{max} and K_m values. These results indicate that the highly purified mouse lymphoma GalT-4 has a specificity for the carbohydrate moiety of the substrate and is indifferent towards the hydrophobic part (ceramide or protein) of the acceptor glycolipid or glycoprotein. This GalT-4 is also involved in the biosynthesis of polylactosamine-containing glycoconjugates of both glycosphingolipid and glycoprotein origin. (Supported by: NIH Grants NS18005 and CA 14764).

14.2
SUGAR ANALYSIS OF SIALOGLYCOCONJUGATES AFTER METHANOLYSIS BY HPLC WITH PULSED AMPEROMETRIC DETECTION

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High-performance anion-exchange chromatography with pulsed amperometric detection (PAD) is a recently developed, sensitive method, which can be used for carbohydrate analysis at a subnanomolar level. However, when studying the monosaccharide composition of a glycoconjugate, the sample must undergo acid hydrolysis, and different conditions have to be used for releasing different sugar residues. If one wants to analyze hexoses, hexosamines and sialic acid from the same sample, methanolysis would be a better alternative.

Methyl glycosides of monosaccharides are usually quantified by gas-liquid chromatography as trimethylsilyl derivatives. They can also be analyzed by high-performance reverse phase chromatography, and, in this case, no derivatization is necessary. We have used a reverse phase column (Dextro-Pak, Waters) and PAD for analyzing the monosaccharide composition of glycolipids and sialoglycoproteins. The procedure is simple and rapid: methyl glycosides are eluted from the column with water, and post-column alkali is then added for electrochemical detection. As compared to methods involving refractive index detection, this method is more sensitive, and gradient elution with water/acetonitrile can be performed.

As a difference to procedures employing acid hydrolysis, methods based on methanolysis give several derivatives for each monosaccharide (α- and β-pyranosides and furanosides, and a small amount of free sugars). However, the majority of methyl glycosides are well resolved on a Dextro-Pak column, and when the peaks overlap, quantification is still possible by using subsidiary peaks or simultaneous equations.

14.3
A STRATEGY FOR THE N-GLYCAN MAPPING BY HPAE-PAD

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The structure analysis of complex carbohydrates requires expertise and infrastructure for methylation analysis/GC-MS, FAB-MS, and high resolution ^{1}H-NMR spectroscopy. Therefore, an alternative appears attractive that would allow to reduce these highly costly and time-consuming demands.

We have evaluated the high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (1) with respect to its suitability to establish a carbohydrate 2D-mapping database that would enable structure analysis by mere comparison of retention times. The HPAE-PAD allows oligosaccharide separation and detection without requiring prior derivatization and appears therefore superior to the existing two-dimensional mapping technique (2) that needs introduction of a fluorescent label.

In order to initiate the desired database, approximately 60 N-glycans were isolated from purified glycoproteins according to known procedures (N-glycanase digest, gel filtration, FPLC/Mono Q, HPLC/Lichrosorb-NH_2, desalting), and their structures were determined by methylation analysis, FAB-MS, and 600 MHz ^{1}H-NMR spectroscopy.

The mapping was performed, using a Dionex Bio-LC system equipped with a CarboPac PA-1 column (4.6 × 250 mm) and a AG-6 guard column, working at a flow rate 1 ml/min at ambient temperature. The pulse potentials and durations were 0.05V/300ms, 0.60V/120ms, −0.60V/60ms at 300 nA full scale, requiring 0.1 − 0.2 μg of N-glycan per injection for reliable measurements. The retention times of distinct N-glycans, prepared and measured at different days, were shown to be highly reproducible, with a degree of variation of ~ 0.5%. Thus, the HPAE-PAD method fulfills the analytical requirements with respect to accuracy, precision, reproducibility, and sensitivity.

(1) Townsend et al. (1989) Methods Enzymol. 179, 65
(2) Tomiya et al. (1988) Anal. Biochem. 171, 73

14.4
STRUCTURES OF N-GLYCOSIDES OF FETUINS FROM DIFFERENT SPECIES

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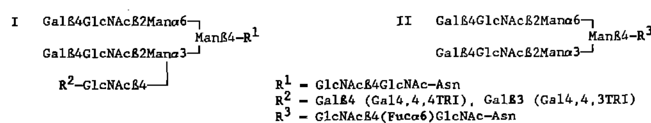
Fetuin is a fetal glycoprotein, expression of which is considered to be regulated developmentally. N-glycosides of fetuins from cow, sheep, and pig were liberated with glycopeptidase F and their structures were examined with high-pH anion exchange chromatography (Anal. Biochem. **189**: 151 [1990]). Additionally the asialo oligosaccharides were derivatized with 2-aminopyridine and chromatographed on Shim-pack CLC-ODS and TSK Amide-80 HPLC column (Anal. Biochem. **171**: 73 [1988]). Commercial bovine fetuin preparations have been shown to contain comparable amounts of "Gal4,4,3TRI (triantennary)" complex type structure as well as "Gal4,4,4TRI" (Biochemistry **25**: 5716 [1986], see below I).

Two preparations of bovine fetuin, each from a single calf, showed only quantitative difference in the ratio of Gal4,4,4TRI and Gal4,4,3TRI and the degree of sialylation. The N-glycosides of ovine fetuin also had both Gal4,4,4TRI and Gal4,4,3TRI structure, in the ratio similar to that of bovine fetuin. However, the major N-glycoside of porcine fetuin was of the biantennary complex type, fucosylated at the reducing terminal GlcNAc (see below II). This oligosaccharide is negligible in bovine and ovine fetuins. In view of the close homology of the peptide sequences between porcine fetuin and other fetuins (ca. 75%), abundance of this biantennary oligosaccharide is unexpected.



14.5
CHARACTERIZATION OF N-LINKED GLYCOPROTEIN OLIGOSACCHARIDES BY FLUORESCENCE-ASSISTED CARBOHYDRATE ELECTROPHORESIS (FACE)*

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The relative mobilities of various N-linked glycoprotein oligosaccharides reductively aminated to the charged fluorophore 8-aminonaphthalene -1,3,6-trisulfonic acid (ANTS) was determined following electrophoresis on polyacrylamide slab gels. Each ANTS-coupled oligosaccharide (ANTS-OS) was assigned a mobility expressed in terms of glucose equivalents, which was conveniently determined by reference to a homologous series of similarly derivitized maltooligosaccharides also included on each gel. High mannose type structures (oligomannose 5 through oligomannose 9) were completely resolved, and extraordinary resolution of various bi-, tri-, and tetraantennary complex -type structures was also achieved. Application of these method to the analysis of the N-linked structures of bovine fetuin released by treatment with PNGase F will also be described.

* FACE is a registered trademark of Glyko, Inc.

14.6
GLYCOLIPID SEQUENCE ANALYSIS USING FLUORESCENCE ASSISTED CARBOHYDRATE ELECTROPHORESIS (FACE)*

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A glycolipid of unknown structure purified from chronic myelogenous lymphocytes was subjected to various enzymatic digestions, and the resulting oligosaccharides were reductively aminated to the charged fluorophore 8-aminonaphthalene -1,3,6-trisulfonic acid (ANTS). Separation of the resulting ANTS-coupled oligosaccharides (ANTS-OS) on polyacrylamide slab gels provided a readily interpretable banding pattern which suggested the following linear structure for the glycolipid:

NANA-Gal-GlcNAc-Gal-GlcNAc-Gal-Glc-Cer

Digests were performed on as little as 300 pmoles, with 100 pmoles routinely loaded onto a gel. Specific linkage information was inferred from the known specificity of the enzymes, and the results illustrate the potential of FACE-based methods for oligosaccharide sequence analysis.

* FACE is a registered trademark of Glyko, Inc.

14.7
BLOTTING, ELUTION AND ANALYSIS OF DERIVITIZED OLIGOSACCHARIDES SEPARATED BY FLUORESCENCE ASSISTED CARBOHYDRATE ELECTROPHORESIS (FACE)*.

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Oligosaccharides were covalently modified at the reducing terminal sugar with 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) and electrophoretically separated (P. Jackson, Biochem. J. **270**, 705-713, 1990). ANTS-derivitized oligosaccharides (ANTS-OS) rapidly and efficiently electroblotted onto positively charged nylon and polyvinylidene difluoride (PVDF) membranes. Membrane bound ANTS-OS were washed with water to remove electrophoresis buffers and eluted with 2 M NaCl. The rate of release was affected by pH. Microdialysis with 500 M.W.C.O. membranes was used to remove salt from the eluted samples. The carbohydrate composition of defined ANTS-OS was determined following hydrolysis and quantified using a Dionex/pulsed electrochemical detection system. Compositional data could also be obtained from washed PVDF membrane pieces after direct hydrolysis of the bound ANTS-OS. Liquid secondary ion mass spectra of ANTS-OS were acquired by a Finnigan MAT 900 or by a Finnigan MAT TSQ-70 mass spectrometer, using a cesium ion gun with triethanolamine as matrix. These results suggest that the extraordinary resolution of FACE can be coupled with traditional carbohydrate analytical methods to identify structures of interest.

* FACE is a registered trademark of Glyko, Inc.

14.8
A METHOD FOR DETERMINATION OF CONSTANT AND VARIABLE GLYCANS ON GLYCOPROTEINS BY A TWO-SITE LECTINOENZYMATIC ASSAY

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Mucins and other glycoproteins have several glycans per molecule, enabling simultaneous binding of two different glycan detecting ligands, lectins or antibodies, to one single glycoprotein molecule.

A sandwich titer-tray method with one lectin as capture ligand (WGA or PNA) and one HRP-labelled lectin (WGA,PNA,DBA) as labelling ligand was developed, in order to quantitate specific changes in the variable terminal glycan part of glycoproteins.

As standard calibrating glycoprotein, bovine submaxillary gland asialomucin was chosen due to its positive reaction in all assays. Standard curves obtained with this glycoprotein served as a reference against which the examined crude human colorectal mucin (CHCM) was calibrated at known sample concentrations.

Validation of the assay: The intra-assay variation ranged from 2.4% (WGA-DBA) to 7.5% (WGA-PNA). The day-to-day variation during one week ranged from 3.9% (PNA-WGA), at low concentrations, to 20.8% (PNA-WGA) at high concentrations. Analytical recovery ranged from 94-112% for standard asialomucin, and from 90 to 108% for human crude mucin. Addition of low to high samples gave parallel curves in both WGA-PNA and PNA-WGA assays. Monosaccharide inhibition studies of labelled lectins revealed the binding specificity, and showed asialomucin standard to have an inhibition profile identical with CHCM. Neuraminidase treatment of CHCM increased the extinction in the WGA-PNA assay, whereas the WGA-DBA assay was unreactive. SDS-PAGE and W-Blotting was used (+/− neuraminidase) to characterize the different molecules reacting in the titer-tray assay. Clinical results: The assay was applied to >100 samples of CHCM and the WGA-PNA combination identified carcinomas with a specificity of 87% and sensitivity of 85%.

14.9
THE MAJOR OLIGOSACCHARIDE OF BOVINE α-LACTALBUMIN CARRIES TERMINAL β-LINKED GALNAC

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In the presence of α-lactalbumin (α-LA), milk UDP-galactose:*N*-acetyl-

D-glucosamine-β4-galactosyltransferase transfers galactose from UDP-Gal to glucose, synthesizing lactose. α-LA, the protein modifier of the galactosyltransferase, occurs in glycosylated and non glycosylated forms. Although the non-glycosylated form of bovine α-LA has been studied extensively and the primary structure is known, the oligosaccharides on the glycosylated variant have not been studied. In the present study it has been shown that only one of the two potential *N*-glycosylation sites, Asn[45], is glycosylated. The oligosaccharides have been cleaved using PNGase F and shown to contain GalNAc in addition to GlcNAc, Man and Fuc; there is very little Gal or sialic acid. Experiments with hexosaminidase A and hexosaminidase B have shown that GalNAc is the terminal sugar, is β-linked to GlcNAc and is not sulphated. The oligosaccharides have been separated by HPLC. ^{1}H-NMR techniques (1-D and 2-D:COSY, RECSY, TOCSY AND NOESY) at 500 MHz in combination with GC/MS and methylation analysis have been used to show that the structure of the major oligosaccharide is:

```
GalNAc-β1,4-GlcNAc-β1,2-Man
                            \ α1,6
                             Man-β1,4-GlcNAc-β1,4-GlcNAc-α,β
                            / α1,3                  |
GalNAc-β1,4-GlcNAc-β1,2-Man                        α1,6
                                                    Fuc
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Thus bovine α-LA is unique in that the major *N*-linked oligosaccharide carries terminal β-linked GalNAc that is not sulphated.

14.10

BOVINE MILK EPITHELIAL GLYCOPROTEIN IV (CD36) CONTAINS NOVEL SIALYLATED TRI- AND TETRAANTENNARY COMPLEX-TYPE SUGAR CHAINS HAVING A β-*N*-ACETYLGALACTOSAMINE LINKED TO AN *N*-ACETYLGLUCOSAMINE RESIDUE

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GP IV (CD36) is a integral membrane glycoprotein of bovine milk fat globule membrane (MFGM) derived primarily from mammary secretory epithelial cells during lactation. The Asn-linked sugar chains were released from GP IV as oligosaccharides by hydrazinolysis. These sugar chains were converted to radioactive oligosaccharides by reduction with NaB^3H_4 and separated into neutral and acidic fractions by paper electrophoresis. Ninety five percent of the acidic oligosaccharides was converted to neutral ones by sialidase digestion. The neutral and sialidase-treated acidic oligosaccharides were fractionated by Bio-Gel P-4 column chromatography in combination with serial lectin columns immobilized with AAL (*Aleuria aurantia* lectin), WFA (*Wistaria floribunda* agglutinin), DSA (*Datura stramonium* agglutinin) and RCA (*Ricinus communis* agglutinin). WFA is known to bind oligosaccharides terminating with *N*-acetylgalactosamine at their non-reducing termini. Structural studies of oligosaccharide in each fraction by sequential exoglycosidase digestion as well as the binding specificities of lectins revealed that the oligosaccharides in the WFA-bound fraction (28% of the whole sugar chains) are of bi-, tri- and tetraantennary complex-type and hybrid-type, whose outer branch(s) is the GalNAcβ1→4GlcNAcβ1→ group. The glycosidic linkages of these oligosaccharides are currently investigated by methylation analysis. The outer chains in sulfated form were reported to occur in biantennary complex-type and hybrid-type sugar chains of pituitary glycohormones from several mammals (1). However, most of the acidic oligosaccharides with the GalNAcβ1→4GlcNAcβ1→ outer chain of bovine milk GP IV are sialylated but not sulfated. Whether terminal *N*-acetylgalactosaminylation is limited to GP IV of MFGM or not is under investigation.

(1) Green, E. D. and Baenziger, J. U. (1988) J. Biol. Chem., **263**, 25–35.

14.11

SIMPLE AND ECONOMICAL APPARATUS FOR GAS-PHASE HYDRAZINOLYSIS: RELEASE OF N-GLYCANS AND GPI ANCHORS

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Hydrazinolysis is one of the major methods used to cleave N-linked oligosaccharides from glycopeptides or glycoproteins. Previous work has relied on hydrazinolysis carried out in the liquid phase (1), but a recent report (2) has described the use of hydrazinolysis in the gas phase for releasing N-glycans, and an apparatus for carrying out gas-phase hydrazinolysis has become commercially available (3). We have developed an experimental protocol for gas-phase hydrazinolysis involving the simple adaptation of readily available and relatively inexpensive equipment used for amino-acid analysis.

The apparatus and protocol for gas-phase hydrazinolysis was evaluated using the glycoprotein transferrin. The method was found to be much more rapid than the commonly used liquid-phase protocols, and recoveries of the carbohydrate chain are comparable to those achieved using liquid hydrazine. Integrity of the released N-glycans was assessed by FAB-MS analysis of the re-N-acetylated, desialylated, permethylated derivative – an $M+H^+$ molecular ion was observed at m/z 2047, which corresponds to the expected bi-antennary structure. Work is in progress to optimize and simplify the protocol, and to fully evaluate the method using a variety of other glycoproteins with known oligosaccharide structures.

We have also shown that it is possible to release glycosyl phosphatidylinositol (GPI) anchors from proteins by liquid-phase hydrazinolysis. FAB-MS analysis of the N-acetylated, permethylated product showed that the anchor is released free of amino-acids while retaining the ethanolamine-phosphate residue. The effectiveness of gas-phase hydrazinolysis in releasing GPI anchors is being examined.

(1) Takasaki, S., T. Mizuochi, & A. Kobata (1972) *Meth. Enzymol.* 83, 263.

(2) Kamei, M., T. Murata, M. Goto, F. Koyama, & T. Yamaguchi in *Proceedings of the XVth International Carbohydrate Symposium*, Aug. 12–17, 1990, Yokohama, Japan.

(3) Honen Corporation, Chiyoda-ku, Tokyo 100, Japan.

14.12

GEL AND CAPILLARY ELECTROPHORESIS BASED OLIGOSACCHARIDE SEQUENCING

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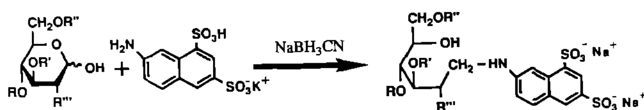
The carbohydrate components of glycoprotein play many important biological roles. Therefore, information about glycoprotein sugar side-chain composition, and more importantly their sequence is required to fully understand and establish structure-function relationships. Gel electrophoresis has become a routine, versatile and powerful tool for sequencing of nucleic acids. This approach has not been useful for sequencing of glycoprotein-derived oligosaccharides due to their absence of fixed charge.This paper describes a new method for sequencing

oligosaccharides on gels using charged, fluorescent conjugates. Oligosaccharides were conjugated with 7-amino-1,3,naphthalene disulfonic acid, a fluorescent and negatively charged compound, by reductive amination. These fluorescent charged derivatives facilitated the sequencing of neutral oligosaccharides by polyacrylamide gel electrophoresis (PAGE). The sugar-conjugates were purified by preparative gradient PAGE followed by semi-dry transfer to positively charged nylon membranes and elution with sodium chloride. The structure of the conjugates were established by f.a.b.-m.s. and 2D n.m.r. Recovered fluorescent-oligosaccharide derivatives were treated sequentially with exoglycosidases and with endoglycosidases. Analysis of the PAGE banding pattern provided sequence information. Capillary zone electrophoresis (CZE) was also used to resolve these fluorescent oligosaccharide conjugates. Detection during CZE analysis using fluorescence detection permits the quantitation of pg amounts of conjugate. CZE has also been used in sequencing studies and may represent an easily automated method for oligosaccharide sequencing. These methods may be useful for sequencing oligosaccharides that are chemically or enzymatically released from glycoproteins, glycolipids and proteoglycans.



14.13

GLYCAN COMPOSITION AT THE FIVE GLYCOSYLATION SITES IN HUMAN α_1-ACID GLYCOPROTEIN (OMD) IN HEALTH AND DISEASE

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Standard HPLC chromatograms have been developed to determine the relative percentages of each of the three complex asialo-glycan forms at each of the five glycosylation sites of OMD. The glycoprotein was cleaved with endoproteinase Glu-C and the desialylated glycopeptides for each site resolved by HPLC. Peak assignments were made by positive-ion FAB MS, with further confirmation from enzymatic and acid hydrolysis for fucosylated antennae, con A affinity chromatography for biantennary, and correlation of numbers of sugar units/antenna with k'.

The standard chromatograms were used to analyze the distribution of glycoforms present at each glycosylation site in 1) the OMD molecular variants resulting from lectin affinity chromatography on con A, and 2) the shifts in the glycoforms as a function of disease. For 1), the most significant results were that con A retained OMD (R), uniquely, is capable of possessing bi-antennary at all five sites, while the unretained variant contains no bi-antennary. For R and a weakly retained fraction, site II is 100% bi-antennary. In addition, the two gene products of OMD present in the preparation were differentially glycosylated. For 2), the methodology was applied to 20-50 nmoles of immunoaffinity purified OMD extracted from each of 17 different diseased sera, with repeats. In all cases, the trend was for total tetra-antennary to increase. Between diseases, the general patterns of the extent of glycan processing at each site appeared to be random, although 95% of the site III samples showed an increase in tetra-antennary. Within a disease, the trends at each site appeared disease specific. Some diseases were devoid of, or had severely depressed levels of bi-antennary at some sites, while the total level changed little. This casts doubt on the practice of using total bi-antennary content, as measured by con A reactivity, as an indicator or prognosticator of disease.

14.14

ANALYSIS AND PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES, DNA RESTRICTION FRAGMENTS, PCR PRODUCTS AND PLASMIDS BY HPLC

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The analysis and purification of synthetic oligonucleotides, DNA fragments generated from restriction endonuclease digestion or the polymerase chain reaction (PCR), and plasmids are routinely performed in many laboratories using a variety of techniques including gel electrophoresis and ultracentrifugation. Although successful, these techniques are both time-consuming and labor-intensive. Recently, the development of high performance chemistries has extended the use of High Performance Liquid Chromatography (HPLC) to the analysis and purification of these various nucleic acid species. As described, HPLC separations are rapid (e.g. 30 – 60 min.) and result in quantitative recovery of biologically active material suitable for subsequent use. In addition, high sensitivity detection with direct quantitation is made possible via on-line U.V. 260nm monitoring eliminating the use of ethidium bromide, a potent mutagen. As such, these quick and efficient methods will allow investigators to accelerate the pace of experimentation while offering significant advantages over other separation and analysis methodologies.

14.15

ANALYSIS OF THE OLIGOSACCHARIDE AND PEPTIDE PORTIONS OF GLYCOPEPTIDES BY CAPILLARY ELECTROPHORESIS, HPLC AND PEPTIDE SEQUENCING

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Recent advances in capillary electrophoresis (CE), HPLC and peptide sequencing are providing researchers with orthogonal separation techniques which now permit the resolution of previously difficult to separate biomolecules. It is well known that glycopeptides are complicated molecules due to the microheterogeneity of both the oligosaccharide and peptide portions. Hence, total characterization of a glycopeptide should involve the analysis of the carbohydrate moiety and the peptide backbone to which it is attached.

Presented in this paper is a method, which in principle, can be generally applicable, for the large scale purification and characterization of glycopeptides employing preparative reversed phase HPLC, CE, and peptide sequencing. Glycopeptides purified from a large scale separation of trypsin digested, reduced and alkylated fetuin were assessed for purity by employing high resolution reversed phase chromatography using various mobile phase modifiers on Delta-Pak™ C18 and C4 columns, amino acid analysis using Pico.Tag™ and CE which permits the separation of the various glycoforms attached to a single glycosylation site. After treatment of the isolated glycopeptide with N-Glycanase®, the released oligosaccharides were separated on a high performance anion exchange column employing pulsed amperometric detection. Finally, the glycopeptide fraction was sequenced using a ProSequencer™ 6625 to reveal two peptides differing only by a terminal lysine. These results demonstrate how orthogonal, complementary techniques are employed to resolve very closely related compounds.

14.16

STRUCTURAL ANALYSIS OF EARLY BIOSYNTHETIC INTERMEDIATES OF PROTEIN *N*-GLYCOSYLATION USING HIGH-PH ANION-EXCHANGE CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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It is generally accepted that *N*-glycosylation is initiated by the *en bloc* transfer of a common $Glc_3Man_9GlcNAc_2$ oligosaccharide to AsnXxxSer-(Thr,Cys) sequons during protein translation. In yeast, an α-mannosidase removes a single, branch-specific residue to form the only Man_8GN_2-branch isomer found in yeast (Byrd et al., J. Biol. Chem. 257, 14657). An α(1→6) mannosyl transferase adds a residue to either the Man_8GN_2 or the archetypical Man_9GN_2 (to form a $Man_{10}GN_2$, *in vitro*) (Romero and Herscovics, J. Biol. Chem. 264, 1946). The precise branch location of the initial Man added in yeast has been difficult to ascertain. Based on ^{1}H-NMR spectroscopy, α(1→2)-specific exoglycosidase digestions and methylation analysis, two locations for the new Man residue were plausible. Recently, using liquid secondary ion mass spectrometry (LSIMS) and an α1→2-specific mannosidase, a novel yeast Man_9 structure from glycosylation mutants was reported in which the Man was added to the α(1→3) linked Man (Residue **5** in Byrd et al.) instead of to the α1→6-linked Man of the uppermost branch (Residue **6**) (Hernández et al., J. Biol. Chem. 264, 11849). In this study, we investigated the global occurrence of the new Man_9 structure using high-pH anion-exchange chromatography (HPAEC) followed by LSIMS and tandem mass spectrometry.

Isobaric branch and linkage isomers were separated using HPAEC with in-line desalting e.g. archetypical Man_9GN_1 oligosaccharides were separated from yeast Man_9GN_1 oligosaccharides by approximately 5 min. The purified oligosaccharides (1 – 5 nmols) were then derivatized with *n*-octyl-p-aminobenzoate and mass spectra were recorded with 300–500 pmols. The total Endo-H-released Man_9GN_1 pool (after BioGel P-4 chromatography) from yeast cells (FH_4C) contained significant amounts of both types of Man_9GN_1 structures while only yeast-type Man_9GN_1 oligosaccharides were found on invertase expressed in either the natural host (*Saccharomyces cerevisiae* or in *Pichia pastoris*. Man_9GN_1 isolated from bovine thyroglobulin, human IgM and soybean agglutinin co-eluted during HPAEC and showed mass spectra which were consistent with the archetypical structure. HPAEC in combination with LSIMS and tandem MS represents a significant advance for determining the branching configuration of *N*-linked oligosaccharides.

Mass spectrometry was supported by NIH grant RR01614 and NSF grant DIR 8700766.

14.17

N-LINKED GLYCOSYLATION SITES IN PORCINE FIBRINOGEN

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Fibrinogen is a highly soluble, multi-subunit plasma glycoprotein, having the structure (Aα, Bβ and $\gamma)_2$. When the fibrinopeptides A and B are excised proteolytically by thrombin, the (α, β and $\gamma)_2$ chains spontaneously polymerise into a fibrin clot. The structures and location of the carbohydrate moieties have been determined for human fibrinogen although their role in clotting is controversial. Studies on human and bovine fibrinogens have shown that their carbohydrate structures are N-linked sialylated non-fucosylated biantennary complex. The carbohydrate structures we have isolated from porcine fibrinogen differ from those found in human and bovine. Consistent with our earlier demonstration of α1–6 fucosyltransferase activity in pig liver, we find that porcine fibrinogen carbohydrates are core fucosylated.

SDS PAGE of peptide-N-glycosidase F treated porcine fibrinogen revealed that the α chains were devoid of carbohydrate, as is the case for human and bovine fibrinogen. Porcine fibrinogen has an apparent mol. wt. of 405,000 and the two Bβ and γ chains each have one glycosylation site giving a total of four N-linked carbohydrates per intact molecule. Pronase glycopeptides were isolated and purified to homogeneity by a combination of gel filtration and ion exchange chromatography followed by HPLC DEAE 5PW and reverse phase C_{18} HPLC. Most glycopeptides had additional amino acids attached to the Asn.

Analysis of the 500 MHz proton NMR spectra identified the major (approx. 70%) structure to the N-linked monosialylated fucosylated biantennary complex, (GS+F). Identification of the amino acid adjacent to the Asn of the glycopeptides made tentative chain assignment possible. The Glu-Asn-Lys-Thr glycosylation sequence in the γ chain was found to have mainly GS+F. Disialylated (SS+F) fucosylated biantennary glycopeptides (approx. 23%) located predominantly to the sequence Glu-Asn-Arg consistent with their location on the Bβ chain. A small amount of the biantennary Glu-Asn-glycopeptide could be attributed to either site. Amino acid analysis and 2-D 500 NMR (COSY and HOHAHA) were done to confirm the amino acid sequences of the glycopeptides. The minor (<8%) neutral fraction was not further investigated.

14.18

PREPARATION OF N-ACETYLNEURAMINIC ACID AND ITS DERIVATIVES FROM EGG-YOLK

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N-acetylneuraminic acid (Neu5Ac) and its derivatives in the form of oligosaccharides, glycoproteins and glycolipids have shown to be involved in many biological processes and their applications in food and pharmaceutical industries are increasing. Previously, we reported the large-scale preparation of Neu5Ac from chalaza of hen egg[1]. In the present study, an economical process of Neu5Ac and its derivatives preparation from delipidated egg yolk has been investigated.

The delipidated hen egg yolk powder containing 0.35% Neu5Ac (total sugars 1.5% w/w), was suspended in water and mixed. It was acidified to pH 1.4 with 6N H_2SO_4 and heated at 80°C for 1h. After cooling, saturated $Ba(OH)_2$ solution was added till ph 5.0 and filtered. The electrodialysis was carried out till 150 μS/cm. The material obtained was applied on a column of Dowex 1×8 (200–400 mesh). The column was washed with water and eluted with a linear gradient of former acid from 0 to 2N. The eluates containing Neu5Ac were evaporated to dryness at 40°C under reduced pressure. The residue obtained was decolorized with activated charcoal powder and then lyophilized. The Neu5Ac contents were measured by thiobarbituric acid (TBA) method. The purity of Neu5Ac obtained was >90%.

For the preparation of Neu5Ac-conjugates, water-soluble and water-insoluble fractions of delipidated egg yolk were separated. The water-

soluble fraction (10% of total sialic acid contents) was treated with ethanol and further purified.

The water-insoluble fraction of egg delipidated fraction was treated with industrial preparation of protease (porcine pancreas) at 45°C and ph 8.0. The enzyme-treated solution was filtered. The filtrate containing Neu5Ac-conjugates was chromatographically purified and evaporated at 40°C under reduced pressure.

1. L.R. Juneja et al. Proceedings of XVth International Carbohydrate Symposium, Yokohama, Japan, (August 12–17, 1990), p. 423.

14.19
CHEMICAL AND ENZYMATIC STUDIES ON HUMAN CHORIONIC GONADOTROPHIN

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HCG is a glycoprotein hormone of the placenta. It consists of two noncovalently linked subunits, α and β. Our studies have centered on the development of a method for the convenient isolation, separation, and quantitation of the N- and O-linked oligosaccharide present in hCG. In addition, a strategy has been developed for the isolation and purification of glycopeptides from the hCG-α subunit which can then be used to investigate if glycosylation is site specific in hCG-α.

The structural investigation of the carbohydrate chains was based on cleavage of the N-linked chains with PNGase-F followed by alkaline borohydride treatment of the remaining O-glycoprotein material. The released oligosaccharides were isolated by HPLC and their structures determined by ^{1}H-NMR spectroscopy.

The three major N-linked oligosaccharides were shown to consist of monoantennary with α2→3-linked sialic acid, disialylated with α2→3-linked sialic acid, and disialylated with α2→3-linked sialic acid and a core α1→6 fucose. In addition, there was evidence for some α2→6 linked sialic acid along with tri- and tri'-antennary oligosaccharides. The O-linked structures were tri-, tetra- and hexosaccharides.

The glycoprotein was further investigated to determine if glycosylation is site specific in hCG-α. This was achieved by digesting the desialylated hCG-α subunit with endoproteinase Lys-C. The resulting mixture of peptides and glycopeptides was purified by reverse phase HPLC and the structures of both the peptide and glycopeptide fractions were determined by amino acid analysis.

Analysis of the isolated glycopeptides following treatment with endo Lys-C, using both reverse-phase and amino bonded HPLC, indicated that glycosylation is not fully site specific in hCG-α.

14.20
CARBOHYDRATE VARIANTS OF HUMAN CHORIOGONADOTROPIN AND ITS SUBUNIT EXPRESSED IN BACULOVIRUS EXPRESSION SYSTEM

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Human choriogonadotropin (hCG) is a heterodimer of α and β subunits with two N-linked complex-type carbohydrates on each and four mucin type O-linked oligosaccharides on the carboxyterminus of the β subunit only. Using recombinant DNA methodology, four carbohydrate variants of hCGβ and hCG, rhCGβ, DelhCGβ (hCGβ C-terminus), rhCG and SelhCG (selenomethionyl hCG) were efficiently expressed in the baculovirus expression system and purified by immunoaffinity chromatography.

RhCGβ and DelhCGβ, the latter prepared by deleting 31 amino acid residues from the carboxyterminus of hCGβ via the oligonucleotide-directed mutagenesis, had apparent M.W.s of 33k and 27.5k on SDS-PAGE under nonreducing conditions. The studies with endoglycosidase digestion, lectin binding and carbohydrate analysis indicated that rhCGβ contained two high-mannose type N-linked carbohydrates with 6–7 mannoses per chain and 3–4 O-linked simple disaccharides, while DelhCGβ had virtually no O-linked glycosylation. These carbohydrate modifications of the β-subunit seemed not to alter its immunopotency or its ability to combine with hCGα. The reconstituted hormones were found to be similar to hCG in receptor binding and in its ability to stimulate cAMP and steroidogenesis.

RhCG, prepared by coinfection of insect cells with recombinant viruses containing hCGα and hCGβ cDNAs, and SelhCG with 84% methionine substitution by selenomethionine as indicated by amino acid analysis had apparent M.W.s of 38k on SDS-PAGE under nonreducing conditions. The carbohydrate analysis of rhCG and SelhCG showing the presence of 2.1, 3.3, 7.38, 4.2 and 27.8 residues of Fuc, GalNAC, GlcNAC, Gal and Man respectively per mole of the hormone was consistent with the presence of 4 N-linked high-mannose type carbohydrates and 4 O-linked simple carbohydrates. Despite the altered glycosylation and/or selenomethionine substitution, rhCG and SelhCG demonstrated close similarity to the native urinary hCG in receptor binding, immunopotency and in its ability to stimulate cAMP and steroidogenesis. Thus, the modification of the carbohydrates to high-mannose type and/or the selenomethionine substitution in both subunits had no detectable effect on the proper folding and the biological functions of hCG.

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14.21
DETECTION OF SULFATE IN OLIGOSACCHARIDES BY NEURAL NETWORK ANALYSIS OF FOURIER-TRANSFORM INFRARED SPECTRA

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Both *N*-linked and *O*-linked carbohydrate side chains of glycoproteins may bear sulfate ester groups. The first problem encountered when attempting to characterize the structures and possible functions of such sulfated oligosaccharide chains is detecting the presence of sulfate. To date, available methods (with the exception of ^{35}S-radiolabeling) typically involve hydrolysis and subsequent indirect detection of free sulfate by colorimetric methods with sensitivity in the μg range. Such destructive approaches with limited sensitivity are inappropriate when only small amounts of biologically important samples are available.

Thus, we set out to develop a non-destructive, sensitive and fast assay for the detection of sulfate in oligosaccharides. We utilize Fourier-transform infrared (FT-IR) spectroscopy to monitor samples (*e.g.*, fractions from a chromatography run) for the presence of sulfate; the spectra obtained are then analyzed via a neural network [1]. To date, FT-IR microscopy has been used to obtain spectra of dried sample depositions. These spectra are then presented to the input layer of an artificial neural network, trained previously by an iterative feed forward-back propagation procedure to identify the presence or absence of sulfate in monosaccharides and in well-defined mixtures of sulfated monosaccharides and non-sulfated oligosaccharides of known structure. As little as 1 to 2 nanomoles of oligosaccharide (in 1 μl aqueous solution) were sufficient for analysis. Our method recognizes sulfate in the oligosaccharides in a reliable, fast and nondestructive fashion. We are exploring the feasibility of gaining, in addition to detection of sulfate,

structural information on the identity of the sulfated glycosyl residue(s) from the FT-IR spectra as analyzed by the neural network.

[1] B. Meyer *et al.* (1991) *Science*, in press.

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14.22

N-GLYCOSYLATION SITE MAPPING OF GLYCOPROTEINS BY SERIAL LECTIN AFFINITY CHROMATOGRAPHY, FAB MASS SPECTROMETRY AND ^{1}H-NMR SPECTROSCOPY

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A general and sensitive method has been developed for identifying the attachment sites of *N*-linked oligosaccharides in glycoproteins, and for determining the structures of the oligosaccharides at each specific site. We will illustrate our strategy for human serotransferrin (STF). STF was first *S*-carboxymethylated and digested with trypsin. Glycopeptides in the tryptic digest were then separated by serial lectin affinity chromatography. One fraction of glycopeptides bound to Concanavalin A (ConA), and was eluted with 10mM α-methylglucoside. The glycopeptides which did not bind to ConA bound to *Sambucus nigra* agglutinin (SNA) and were eluted with 100 mM lactose. The ConA$^+$ and the SNA$^+$ glycopeptide fractions were individually digested with *N*-glycanase. One part of the digest of each fraction was analyzed by fast-atom-bombardment mass spectrometry (FAB-MS) to identify the peptide sequence(s), *i.e.*, the glycosylation site(s). The other part was used to isolate the oligosaccharide by the corresponding lectin affinity chromatography (*i.e.*, ConA and SNA, respectively). The structures of thus isolated oligosaccharides were characterized by ^{1}H-NMR spectroscopy. It was shown that the oligosaccharides in the ConA$^+$ fraction have bisialyl, diantennary, *N*-acetyllactosamine type structures, and that the SNA$^+$ fraction contains trisialyl, triantennary structures (*cf.* [1]). Both types of oligosaccharides were found to be evenly distributed over the two *N*-glycosylation sites of STF.

Our method is a considerable improvement over existing methods in that the structures of the oligosaccharides are characterized by NMR spectroscopy and FAB-MS is applied on a much less complicated mixture of glycopeptide-derived peptides than, for example, in [2]. Since the glycopeptides are fractionated by lectin affinity chromatography according to the structural types of their oligosaccharides, fairly homogeneous oligosaccharides are analyzed by NMR, leading to unambiguous results of *N*-glycosylation site mapping. Our method, therefore, has great potential for studying various multi-*N*-glycosylated proteins.

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14.23

CHARACTERIZATION OF THE OLIGOSACCHARIDE STRUCTURES ON RECOMBINANT PRORENIN EXPRESSED IN CHINESE HAMSTER OVARY CELLS

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Prorenin was isolated by immunoprecipitation from the culture medium of Chinese hamster ovary cells transfected with a human prorenin cDNA. The N-linked oligosaccharide structures on the *in vivo* (^{3}H)-mannose labeled, purified protein were characterized using a combination of serial lectin affinity chromatography, HPLC, ion exchange chromatography, size exclusion chromatography and digestion with specific glycosidases. Approximately 61% of the structures on the molecule are complex type, the majority of which are biantennary. All complex type structures are core fucosylated. Terminal sialic acids are linked α2–3 and the degree of sialylation of the bi- and triantennary structures varies between non and fully sialylated; no tetraantennary structure contain more than three sialic acid residues. Recombinant prorenin contains 4% hybrid type structures, all of which carry a terminal sialic acid residue. The remaining 35% of the structures on the molecule are high-mannose type, the majority of which contain 5, 6 or 7 mannose residues. Interestingly, approximately 6% of the high-mannose type structures appears to be phosphorylated, as judged by their susceptibility to digestion with alkaline phosphatase; evidence for phosphorylation was also found for a portion of the hybrid structures.

Compositional analysis of an unlabeled preparation of the protein produced results in good agreement with the structural characterization and was consistent with the presence of approximately 1.4 oligosaccharide units per molecule.

14.24

A NOVEL N-GLYCOSYL LINKAGE UNIT IN MAMMALIAN GLYCOPROTEINS

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β-Glc-Asn (β-Glucosylasparagine) has been identified as a linkage unit in the cell surface glycoprotein of the extreme halophilic archaebacterium H.halobium (1). An α-Glc-Asn linkage has been described to occur in mammals (2).

We have synthesized the compounds α and β-Glc-Asn, linked them to a carrier and obtained polyclonal antibodies specific for the α- and β-anomers, respectively. These antibodies were used to screen mammalian tissues for the occurrence of these linkage units. After O-deglycosylation with hydrogenfluoride, anti-β-Glc-Asn positive proteins and no α-Glc-Asn positive protein could be detected in Western blots so far. One of these proteins was further characterized. It turned out to be laminin, and its content of the linkage unit β-Glc-Asn was established by isolation of a tryptic peptide that showed immunocrossreactivity after O-deglycosylation. Presently we are trying to chemically prove this finding by the identification of the phenylthiohydantoin-derivative of Glc-Asn after Edman degradation.

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14.25

CHARACTERIZATION OF GANGLIOSIDE 9-O-ACETYL-GD$_3$ FROM BOVINE CHEESE WHEY AND HUMAN MILK

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A ganglioside isolated from bovine butter milk has been identified as 9-O-acetyl-GD$_3$, one of human melanoma antigen, by TLC-immunostaining with a mouse monoclonal antibody JONES (1). We alose prepared 100 mg of 9-O-acetyl-GD$_3$ from bovine cheese whey powder. The cheese whey was first hydrolyzed with a protease; then ganglioside

were concentrated through the ultrafiltration. 9-O-acetyl-GD_3 was purified with a series of column chromatography. The purified fraction migrated in between GM_3 and GD_3 on TLC, whereas its mild alkali treatment altered the migration to the same position with that of GD_3. Additionally NMR spectometry and methylation analyses with or without O-acetyl protection demonstrated that the purified fraction was identical to 9-O-acetyl-GD_3.

We prepared mouse monoclonal antibodies to 9-O-acetyl-GD_3; they were found to be IgM and IgG_3. While the antibodies did not react with either GM_3, GD_3, or LacCer, highly specfic affinity was shown toward 9-O-acetyl-GD_3. With this monoclonal antibody, as well as biochemical analyses such as TLC and mild alkali hydrolysis, we obtained clear evidence that human milk from healthy mother contained 9-O-acetyl-GD_3. The occurrence of 9-O-acetyl-GD_3 not only in melanoma cells but also in healthy bovine and human milk implies its significant role on the cell multiplication.

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14.26
NON-SELECTIVE RELEASE AND RECOVERY OF UNREDUCED INTACT O-GLYCANS FROM GLYCOPROTEINS, MUCINS, AND CERTAIN CLASSES OF GLYCOLIPIDS

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While chemical and enzymatic methods are available for the release of intact unreduced N-glycans from glycoproteins, similar methods for the release of unreduced O-glycans are not yet available. We have investigated the use of anhydrous hydrazine to cleave O-glycans from a variety of glycoconjugates, and in particular glycoproteins. Conditions have been defined which allow the release in high yield (>90%) of intact unreduced O-glycans, as judged by a variety of techniques including 600 MHz^{1}H-NMR, mass spectrometry and enzymatic analysis of the radio-labelled alditol derivatives. The conditions so defined do not lead to any detectable 'peeling' of the Galβ1→3GalNAc linkage, and are furthermore generally applicable to glycoproteins, mucins and certain glycolipids. This chemical reaction, together with a defined chromatographic procedure should significantly assist the recovery, labelling, and analysis of O-glycans from both very small and large amounts of starting material.

14.27
GLYCOPEPTIDE MAPPING OF SERUM AND CELL SURFACE GLYCOPROTEINS

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The current strategies for analysing site-specific glycosylation patterns (e.g. refs 1–3 and reviewed in 4) have mainly been carried out on relatively large amounts of glycoprotein having only a small percentage of carbohydrate. Other glycoproteins such as those from the cell surface can be highly glycosylated and available in only limited amounts. In order to be able to analyse these glycoproteins, we have perfected methods for microscale chemistry, HPLC, peptide analysis and liquid secondary ion mass spectrometry (LSIMS).

RP-HPLC with UV detection has been used to fractionate 200pmole proteolytic digests of glycoprotein according to size and hydrophobicity. The majority of peptides, which are of molecular weight 400-3000, were detected by LSIMS of the whole digest or the RP-HPLC fractions. Glycopeptides were not detected at this sensitivity due to their relative hydrophilicity leading to low ionisation and to their large mass (usually greater than 3000). Peptide N-terminal analysis of RP-HPLC fractions from a protease digest was therefore used to give a more complete assignment of peptide sequences and to detect glycopeptides at the same sensitivity as peptides. Glycopeptide fractions were identified by sensitive monosaccharide analysis using high performance anion exchange chromatography with pulsed electrochemical detection (HPAE-PED) from which O- and N-glycosylation type could be distinguished. Fractionation of enzyme-released oligosaccharides by HPAE-PED gave further information on oligosaccharide structure at pmole sensitivity.

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14.28
MASS SPECTROMETRIC STRATEGIES FOR *IN SITU* STRUCTURE DETERMINATION OF OLIGOSACCHARIDE LIGANDS

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Neoglycolipids, derived from N- and O-linked chains of glycoproteins by conjugation to phosphatidylethanolamine dipalmitoate (PPEADP) are proving to be powerful probes in carbohydrate recognition studies (1). We have reported on the application of their excellent ionization properties in liquid secondary ion mass spectrometry (LSIMS) as part of an analytical strategy to provide information on saccharide composition, sequence and branching at sensitivities of 5–500pmol of oligosaccharide (2). The high sensitivity enables structural information to be obtained directly from the thin layer chromatogram surface (3,4) even after completion of ligand binding experiments. The derivatives also give sensitive product ion spectra which permit characterisation of monosaccharide sequences of components in mixed samples and TLC bands. In addition these informative spectra obtained by B/E linked scanning reflect monosaccharide linkage in isomeric molecules with, for example, the distinction of type 1 from type 2 chains and of the antigenic structures Le^a and SSEA-1. The combination of these MS approaches should greatly assist in determining the involvement of specific oligosaccharide structures of glycoproteins in macromolecular interactions.

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14.29

STRUCTURAL CHARACTERIZATION OF GLYCOPROTEIN GLYCOTYPES AND GLYCOFORMS BY ELECTROSPRAY IONIZATION-MASS SPECTROMETRY

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Carbohydrate chains attached to proteins modulate the chemical and biological properties of the resultant conjugate in numerous ways. These glycan chains have been shown to be of three major types, (glycotypes), high mannose, complex, and/or hybrid. Adding to greater microheterogenity, each glycotype may have a number of glycoforms, (e.g., Man_nGN_2, n ± 5 residues). In the manufacture of these products by recombinant technology, alterations in the culture-growing conditions have led to alterations in the glycotype with consequent variations in biological activity. This observation has considerable bearing on product viability and concerns of quality control. Thus, batch-to-batch monitoring of glycosylated products, in a cost-effective manner, is a challenging and necessary obligation that biotech firms are preparing to undertake. We have considered this problem of glycoprotein microheterogeneity by proteolysis, glycopeptide isolation and profiling these fractions using ESI-MS. With the assistance of numerical and computational analysis, this has provided elements of glycan abundance, glycotype, and glycoform for each of the constituents present. Chemical alteration of glycan structures folowed by ESI-MS has been investigated as a supporting strategy for structural confirmation. In a most recent study, the N-linked glycoforms have been resolved in a glycopeptide carrying two different glycotypes.

14.30

ANALYSIS OF GLYCOCONJUGATES BY PLASMA DESORPTION, ELECTROSPRAY AND LASER DESORPTION MASS SPECTROMETRY

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Molecular weight determination of proteins and peptides at the low picomole level by plasma desorption mass spectrometry (PDMS) is now a routine procedure. However, glycoconjugates and underivatized carbohydrates have until recently imposed a major problem. At the best, nanomole amounts were required to obtain reasonable ion yields.

In glycoconjugate structure determination it is important to obtain the molecular weight information of the underivatized sample prior to either chemical or enzymatic treatment as well as structure information about the carbohydrates.

In PDMS addition of dithiotreitol as a modifier to the nitro-cellulose target allows recording of spectra dominated by molecular ions of carbohydrates and glycopeptides at the picomole level. Confirmative structural information of carbohydrates can be obtained by acetylation of the carbohydrate moiety.

Recently, it was reported that glycoconjugates could be analyzed by two new mass spectrometric methods, laser desorption (LDMS) and electrospray mass spectrometry (ESMS). These methods are now available in our laboratory. The present state of art of PDMS for glycoconjugates analysis will be compared with results obtained by LDMS and ESMS.

14.31

MATRIX-ASSISTED LASER DESORPTION TIME-OF-FLIGHT MASS SPECTROMETRY AND LSIMS TANDEM MASS SPECTROMETRY APPLIED TO GLYCOCONJUGATE AND CARBOHYDRATE STRUCTURAL DETERMINATIONS

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Over the past few years, the molecular weight limit for mass spectral analysis has been raised dramatically, through the introduction of matrix-assisted laser desorption (maLD) by Hillenkamp *et al.* (1), and of electrospray ionization by Fenn *et al.* (2). Although the use of electrospray has spread more quickly because it may easily be adapted to most quadrupole analyzers, the maLD technique coupled with a time-of-flight analyzer has some advantages for the analysis of glycoconjugates and enzymes because of its better tolerance for predominantly carbohydrate materials, its greater sensitivity, and its higher demonstrated mass range (above 300 kD). We have begun to explore the use of maLD-TOF mass spectrometry for structural problems related to glycoconjugates and now employ this approach as a complement to methods based on liquid secondary ionization (LSIMS) high performance tandem mass spectrometry (MS/MS).

The molecular weights of intact glycoproteins may be determined by maLD-TOF MS with an accuracy of about 0.01%. Re-examination of the protein after enzymatic cleavage of specific sugars or of entire carbohydrate chains provides a measure of carbohydrate content and an indication of the degree of heterogeneity due to the carbohydrate moieties. In the mass range common to maLD and LSIMS, the laser desorption technique has the advantage of higher sensitivity, requiring only pmol (or smaller) amounts of material. In this range, however, the LSIMS tandem instrument offers the potential for sequence analysis in addition to molecular weight determination. It is therefore useful to employ both techniques, maLD to follow the isolation procedures and LSIMS/MS for examination of structural details.

Proteins and glycoproteins have been the initial subjects of our maLD-TOF investigations, because early workers in the field had demonstrated the utility of the approach for these classes (1,3). We have extended these studies to also include glycolipids and various oligosaccharides, and will present a summary of the results.

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14.32

LOW ENERGY COLLISION ACTIVATED DISSOCIATION TANDEM MASS SPECTROMETRY OF GLYCOLIPIDS

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To elucidate the various functions of glycolipids, characterization of the chemical structure of the glycolipids is a fundamental requirements. However, in many cases, the content of the glycolipids is low and the purification is difficult and time-consuming. To overcome these problems, direct coupling of chromatography and mass spectrometry, TLC/MS (1) and HPLC/MS (2), was reported. Tandem mass spectrometry (MS/MS) is another approach for this problem. By using fast atom bombardment (FAB) and collision activated dissociation (CAD), MS/MS spectra were simplified by selecting a single molecular ion or fragment ion in the analysis of mixtures, and abundant fragment ions were observed. The spectra were also free from the back ground signals from the liquid matrix. The low energy (tens electronvolts) CAD MS/MS spectra of glycolipids in daughter scan mode, the fragment ions with

ceramide moieties due to the sequential cleavage at the glycosidic bonds were observed, but the fragment ion due to the cleavage at the sugar ring was not detected. In parent scan mode, it gave the clear information about the ceramide portions and besides the fragment ions due to the cleavage at glycosidic bonds, the fragment ions due to the cleavage at the sugar ring were also observed. These results indicate the usefulness of low energy CAD MS/MS for the analysis of glycolipid structure.

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14.33

ANALYSIS OF GLYCOSPHINGOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING LASER LIGHT-SCATTERING DETECTION

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The application of high-performance liquid chromatography (HPLC) to the analysis of glycosphingolipids (GSLs) has been limited by the absence of an adequate UV chromophore in these membrane biomolecules. Thus, previous investigators have had to rely either on derivatization (e.g. perbenzoylation) before separation or on chromogenic reaction on TLC plates after separation in order to achieve adequate GSL detection. We now report that GSLs may be quantitatively analyzed without derivatization or post-HPLC TLC through the use of laser light-scattering (LLS) detection, a method pioneered by J.M. Charlesworth (*Anal. Chem. 50*, 1414–1420, 1978). In this method all non-volatile solutes are detected on the basis of mass. The solvent is evaporated after nebulization in a heated column producing finely divided solute particles which pass through a laser light-beam. Light scattered from the particles is detected by a photomultiplier placed at 120° to the laser beam. The signal which varies directly with the mass of solute particles is amplified and recorded.

In a typical analysis 1 mg of mixed GSL is injected on a preparative Dynamax 60A (TM) column (25cm × 10mm, 8mμ silica) and eluted using a binary gradient (chloroform: methanol 95:05 to 75:25 over 80 min, then 75:25 for 100 min) mixed and pumped by a Schimadzu LC-610 HPLC system. The eluate is passed through the LLS detector (Varex ELSD) at a flow rate of 5 mL/min and a heater temperature of 120°. For neutral GSLs extracted from human blood platelets (Koerner et al. *Blood 74*, 274–284, 1989) the following analysis was obtained (R_t in min. and of total mass): GlcCer 21.6 and 1.3%; LacCer 43.8 and 57.6%; Gb3Cer 60.7 and 9.1%; and Gb4Cer 75.3 and 32.0%. Similar analyses have also been carried out on human erythrocytes and leukocytes and murine liver cells, for both neutral and acidic GSLs, with equivalent resolution and sensitivity. We find the lower limit of sensitivity of GSL detection to be 50 ng and the reproducibility to be ±2%. This new method of LLS detection should make HPLC analysis of GSLs convenient for the first time.

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14.34

A NEW METHOD FOR DETECTION OF 9-O-ACETYL-N-ACETYLNEURAMINIC ACID ON IMMOBILIZED GLYCOCONJUGATES USING INFLUENZA C VIRUS

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The receptor determinant for influenza C virus has been identified as 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac_2) and the receptor destroying enzyme of the virus was found to be a specific sialate 9-O-acetylesterase (1,2). The binding site for Neu5,9Ac_2 (hemagglutinin) and the acetylesterase activity are located together with a fusion activity on a single viral glycoprotein (3). To study the receptor specifity of influenza C virus in more detail, different sialoglycoconjugates were immobilized on microtiter plates. These compounds were incubated with influenza C virus at 4°C to avoid release of O-acetyl groups by the esterase, and bound virus was detected with a fluorigenic esterase substrate. Influenza C virus bound to bovine brain gangliosides, bovine submandibular gland mucin or rat serum, all known to contain Neu5,9Ac_2. Glycoconjugates which contain other sialic acids than Neu5,9Ac_2 did not show receptor activity. Examples are fetuin and human α_1-acid glycoprotein (N-acetylneuraminic acid), equine submandibular gland mucin (50–70% 4-O-acetyl-N-acetylneuraminic acid) and porcine submandibular gland mucin (ca. 80% N-glycoloylneuraminic acid). These results suggest that influenza C virus has a high specifity for Neu5,9Ac_2. Other commonly used methods for the analysis of sialic acids require their release from glycosidic linkage by either enzymatic or chemical hydrolysis. Problems generally observed with these methods include incomplete release of sialic acids and destruction of acetyl esters. The new assay presented is a rapid, very sensitive and specific test for the presence and quantification of Neu5, 9Ac_2 on glycoconjugates without these disadvantages.

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14.35

MICRO HPLC/FAB/MS FOR THE STRUCTURAL CHARACTERIZATION OF GLYCOSPHINGOLIPIDS

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We have established a micro method involving a micro high-performance liquid chromotography-fast atom bombardment mass spectrometry (micro HPLC/FAB/MS) for the structural characterization of neutral glycosphingolipids and monosialogangliosides. The micro HPLC composes of a micro silica gel column (0.3 mm i.d. × 100 mm, Aquasil-SS-B, Senshu Scientific, Co.) and a HPLC apparatus (120A, Applied Biosystems) working at a flow rate of 6 μ l/min. A mixture containing 160 ng each of five neutral glycosphingolipids (GlcCer, LacCer, Gb_3Cer, Gb_4Cer and $IV^3\alpha$ GalNac-Gb_4Cer) and a mixture containing 160 ng each of three monosialogangliodsides [GM3(NeuAc), GM2(NeuAc) and GM1(NeuAc)] were injected into the micro HPLC with programmed elution with isopropanol-n-hexane-water with or without ammonium hydroxide. As a matrix, tiethanolamine was added at a concentration of 0.9% (w/v) to HPLC solvents.

In mass chromatograms of the neutral glycosphingolipids and monosialogangliosides, peaks selected as to the pseudo-molecular ions ($[M-H]^-$) of the major molecular species were clearly separated. Mass spectra of individual glycosphingolipids were successfully obtained without any significant interference by glycosphingolipids eluted before or after them. These mass spectra were of much better quality than those obtained with purified glycosphingolipids on FAB/MS with a direct inlet system in terms of the successful detection of pseudo-molecular ions and fragment ions, and the signal to noise ratio. Thus, the characterization of the glycosphingolipids was achieved with small amounts of materials, 160 ng each, and even in mixtures.

We are now applying this method for the characterization of glycosphingolipids prepared from isolated homogeneous populations of cells.

14.36
THE EFFECT OF MANNOSAMINE ON THE FORMATION OF LIPID-LINKED OLIGOSACCHARIDES IN MDCK CELLS.

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Madin – Darby Canine Kidney (MDCK) cells normally accumulate lipid-linked oligosaccharides (LLO) having mostly $Glc_{1-3}Man_9(GlcNAc)_2$ oligosaccharides. However, when these cells are incubated in the presence of mannosamine (MN) and labeled with [2–^{3}H]mannose, they accumulate various LLOs that have smaller-sized, high-mannose oligosaccharides which exhibit unusual behavior. The formation and size of these LLOs was dependent on the concentration of MN, and on the length of the incubation. Thus, these high-mannose oligosaccharides bound weakly to columns of Con A-Sepharose, and some of the oligosaccharides also bound to cation exchange columns (Dowex 50 H^+). Thus, at 10 μM or higher concentrations of MN, 70–90% of the oligosaccharides were eluted from Con A with 10 mM α-methylglucoside, whereas in control cells only 10% could be eluted with α-methylglucoside. In addition, 20–40% of the oligosaccharides produced in MN bound to Dowex-50 H^+ columns, whereas none of the control oligosaccharides had a positive charge. Interestingly enough, these abnormal oligosaccharides were still transferred from LLO to protein in MN-treated cells.

In order to characterize the MN-induced oligosaccharides, they were first separated into neutral and basic fractions on a cation-exchange resin. The neutral oligosaccharides sized on the Biogel P–4 column as a series from $Hexose_3$ $(GlcNac)_2$ to $Hexose_{10}(GlcNAc)_2$, whereas the basic oligosaccharides appeared to be much smaller in size, ranging from $Hexose_2$ to $Hexose_4$ $(GlcNac)_2$. Each of these oligosaccharides was susceptible to α-mannosidase digestion, and with the exception of the $Hexose_2$ structure each was susceptible to Endo H. When each of the oligosaccharides was subjected to N-acetylation and rechromatographed on the Biogel P–4 column, its position was shifted to that of a larger-sized oligosaccharide. In addition, the basic oligosaccharides exhibited anomalous behavior on these columns depending on the pH of the solvent. The data indicate that MN is incorporated into these LLOs and thereby causes marked alterations in the oligosaccharide structure. (This study was supported by NIH Research Grant HL17783).

14.37
LARGE SCALE PREPARATION OF URINARY DISACCHARIDES

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Urinary oligosaccharides represent metabolites originating from both biosynthesis and degradation of different tissue macromolecules. Low molecular weight compounds up to the size of tetra-pentasaccharides may also derive from the food via an intestinal absorption. The presence of some oligosaccharides is strictly regulated by the ABO, secretor and Lewis status of the individual, whereas others are excreted apparently independent of any known genetic system. Finally certain oligosaccharides are present only in pathologic urine and excreted as a result of enzymatic defects affecting the degradation of glycoconjugates. The study of urinary oligosaccharides has been an excellent way to obtain indirect structural information on various plasma and tissue glycoconjugates. Components that occur in minute amounts in the tissue are concentrated and therefore more easily detected in the urine. This is illustrated by the recent finding of a unique glycosylation in coagulation factors VII and IX (1). The disaccharide, Xylα1–3Glc and the trisaccharide Xylα1–3Xylα1–3Glc, were both identified as characteristic glycosides linked to serine 53 and 52 in the EGF domain of factors VII and IX. The disaccharide Xylα1–3Glc was discovered in 1973 as a normal component in human urine (2). The origin of the oligosaccharide was unknown at that time. Additional urinary disaccharides with unknown origin have been described, e.g. Fucα1–2Glc and Fucα1-*myo*inositol. With a goal to collect further information on the physiological role of various structural elements in urine, to produce immunogens, affinity matrices etc. it was necessary to develop an efficient large scale preparation system. In the present report the deionized disaccharide fraction obtained after gel chromatography (Sephadex G-15) of 77 liters of pooled human blood group B urine was further fractionated by sequential separation on Bio-Gel P-2 and Fractogel TSK HW 40. By the use of an automated recirculation system, with up to 14 cycles, eight disaccharides were purified to more than 95% purity. One of the structures (Fucα1-3Glc) has previously not been described as a free oligosaccharide in urine but represents a well known structural element in several blood group related oligosaccharides in both human milk and urine.

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14.38
NEW METHODS FOR STRUCTURAL ANALYSIS OF HIGH MANNOSE GLYCOPROTEIN OLIGOSACCHARIDES

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High mannose oligosaccharides are common constituents of glycoproteins. Even though mannose and N-acetylglucosamine are the only monosaccharides found in these oligosaccharides the number of structures and isomers is considerable. High mannose structures can be released from the protein by chemical or enzymatic methods. Two new methods based on mass spectrometry fast atom bombardment (FAB-MS) and electron ionization (EI) will be presented.

1. High mannose oligosaccharides are released by endo-β-N-acetyl-hexosaminidase, purified and separated.
2. Treatment of glycopeptides or intact glycoproteins with trifluoroacetolysis releases high mannose structures. The released oligosaccharides are after this treatment specifically degraded from the reducing terminal yielding oligosaccharide with a reducing terminal mannose (1).

Oligosaccharides obtained from method 1 and 2 are subjected to structural analysis using periodate oxidation in combination with FAB-MS and EI-MS (2). From mass spectra binding positions of all glycosidic linkages including branched residues can be deduced.

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14.39
STRUCTURES OF THE COMPLEX GLYCANS FOUND ON THE β-SUBUNIT OF NA^+, K^+-ATPASE.

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The integral membrane enzyme Na^+,K^+-ATPase (NKA) is composed of

an α-subunit which possesses the catalytic domains and ligand binding sites, and a heavily glycosylated β-subunit. The function of the β-subunit in the NKA already in place in the cell membrane is not known. Enzymatic evidence indicated that the β-subunit contains three N-linked complex carbohydrate chains. Whether these three glycosylation sites contain complex bi, tri and tetraantennary glycan chains was previously unknown.

This work describes the fractionation of glycopeptides, generated from the β-subunit of lamb kidney NKA, using the lectins concanavalin A and wheat germ agglutinin. The lectin isolated glycopeptides were purified on reverse phase HPLC. Identification of the glycosylation sites was accomplished by amino acid analysis and N-terminal sequencing of the purified glycopeptides. All three glycosylation sites were isolated and characterized. Glycosylation site I (asn 157) was identified to include residues 150-169, site II (asn 192) included residues 182-202 and site III (asn 264) residues 253-272. The glycoforms within an individual glycosylation site were isolated by conservative peak cutting on reverse phase HPLC columns, and the total structure is being identified by Fast Atom Bombardment/ Mass Spectrometry.

The β-subunit of NKA was found to contain complex bi, tri and tetraantennary glycan chains. However, only glycosylation site III (asn 264) contains a significant amount of the biantennary glycan form. Glycosylation sites I and II, as well as the remainder of site III were more highly processed and therefore only the tri/tetra glycan forms were detected. The major glycan form found at sites I and II appeared to be tetraantennary. The data also suggested that glycosylation site II also contained a small percentage of the tetraantennary + fucose glycoform.

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14.40

SYNTHESIS OF A SERIES OF NEUTRAL TRISACCHARIDES HAVING IDENTICAL A→B LINKAGES AND DIFFERENT B→C LINKAGES FOR COLLISION MASS SPECTROMETRY STUDIES OF LINKAGE POSITION

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Disaccharides D-Glucopyranosyl (B1→X)-D-Glucopyranose and Glucopyranosyl (al→X) where X = 2,3,4 and 6 were obtained from commercial or academic sources. D-Galactopyranosyl (B1→4)-Glucopyranose, UDP-D-Galactose-D-Galatopyranosyl (B1→4)-transferase was used to transfer B-D-Galactose to the 4 position of the non-reducing terminal glucose, providing low yields of a set of oligosaccharides identical in the Gal (B1→4) Glucosyl non-reducing terminal disaccharide and differing in the Glu (B1→X) Glucose reducing terminal disaccharide. Linkages of terminal hexose or methyl pentose can be discerned by fast-atom-bombardment. Low energy collision-induced-dissociation tandem mass spectometry (FAB-MS-CID-MS) (J. Am. Chem. Soc. Vol. 110, pp. 6931–39, 1988) which is greatly improved by use of lithium or ammonia cationization or by permethylation (Laine, et al., manuscript submitted). Molecular modelling was used to rationalize fragmentation patterns. Compounds resulting from our above-described enzyme synthesis will be used for models to determine whether linkage position can be determined in neutral oligosaccharides and their permethyl and other proton replacement derivatives by (FAB-MS-CID-MS) without the usual lengthy methylation linkage analysis. (Supported, in part by NIH grant #RIDK33755B to RAL).

S15. ENZYMOLOGY/ENZYMOLOGIE

15.1

Manβ1–4GlcNAcβ-OMe, Glcβ1–4GlcNAcβ-OMe, GlcNAcβ1–4GlcNAcβ-OMe and GalNAcβ1–4GlcNAcβ-OMe are substrates for rat liver Galβ1–4GlcNAc α2–6sialyltransferase

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It has been shown that the urine of patients with β-mannosidosis contains, apart from the major storage product Manβ1–4GlcNAc, the unusual sialotrisaccharide NeuAcα2–6Manβ1–4GlcNAc [1]. This compound is possibly formed by action of a sialyltransferase on Manβ1–4GlcNAc, since in vitro incubation of Manβ1–4GlcNAc with CMP-NeuAc in the presence of rat liver Galβ1–4GlcNAc α2–6sialyltransferase resulted in the formation of NeuAcα2–6Manβ1–4GlcNAc [2]. These results prompted us to investigate the activity of rat liver Galβ1–4GlcNAc α2–6sialyltransferase on substrates which contain β1–4 linked terminal monosaccharides other than Gal.

Four different substrates containing the Hex(NAc)β1–4GlcNAc sequence were synthesized, namely, Manβ1–4GlcNAcβ-OMe, Glcβ1–4GlcNAcβ-OMe, GlcNAcβ1–4GlcNAcβ-OMe, and GalNAcβ1–4GlcNAcβ-OMe. In vitro incubations of these substrates with CMP-NeuAc and rat liver Galβ1–4GlcNAc α2–6-sialyltransferase under standard conditions, resulted in each case in the formation of a product, in which the terminal monosaccharide bears a NeuAc residue in α2–6 linkage, but in various yields. The characterisation of the substrates and products was carried out mainly with ^{1}H-NMR spectroscopy.

The possibility of transferring NeuAc to Man, Glc, GlcNAc and GalNAc instead of to Gal suggests that the axial/equatorial positions of the hydroxyl groups at C-2 and C-4, and the presence of a hydroxyl group at C-2 of the terminal monosaccharide are not absolutely critical for the effectivity of the α2–6sialyltransferase action.

Present investigations are directed to analyse the effect of the non-reducing end residue of the Hex(NAc)β1–4GlcNAcβ-OMe sequence on the kinetics of the transfer of NeuAc.

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15.2

IDENTIFICATION OF A NOVEL UDP-Gal:GalNAcβ1–4GlcNAc-R β1–3-GALACTOSYLTRANSFERASE IN CONNECTIVE TISSUE OF THE SNAIL *Lymnaea stagnalis*

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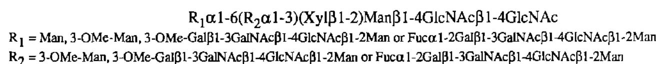
One of the most conspicious cell types in the connective tissue (CT) of gastropods and bivalves is the pore cell. It has been suggested that pore

cells of the snail *Lymnaea stagnalis* synthesize and store hemocyanin, a high-molecular-mass copper-containing oxygen transport protein, which is freely dissolved in the hemolymph. The hemocyanin is a glycoprotein with a carbohydrate content of 3% (w/w), and in previous studies the primary structures of the hemocyanin glycans have been established, affording a series of novel N-linked oligosaccharides [J.A. van Kuik, R.P. Sijbesma, J.P. Kamerling, J.F.G. Vliegenthart and E.J. Wood, Eur. J. Biochem. 160 (1986) 621–625/169 (1987) 399–411]:



As part of our program on the structural analysis, organic synthesis, biosynthesis, and conformational analysis of xylose-containing N-linked carbohydrate chains, substrate-specificity data will be presented for a novel galactosyltransferase from the CT of *L. stagnalis*, involved in the biosynthesis of the antennae of the hemocyanin glycan. A snail CT homogenate was shown to transfer Gal from UDF-Gal in β1–3 linkage to terminal GalNAc of GalNAcβ1–4GlcNAc, GalNAcβ1–4GlcNAcβ1-OMe, and GalNAcβ1–4GlcNAcβ1–2Manα1-$O(CH_2)_8COOMe$. The formed products were purified by HPLC and identified by 500 MHz ^1H-NMR spectroscopy. On the basis of this observation the novel activity has been called UDP-Gal:GalNAcβ1–4GlcNAc-R β1-3-galactosyltransferase. The CT homogenate was inactive towards GlcNAc, GalNAcβ1–3Galα1-OMe, GalNAcα1-OC_6H_5, GalNAcα1-OSM, Galβ1–4Glc, and Galβ1–4GlcNAc. The same galactosyltransferase activity was also observed in an albumen gland preparation of the snail, which contained in addition a high activity of a novel N-acetylgalactosaminyltransferase, another enzyme, essential for the biosynthesis of hemocyanin oligosaccharides.

15.3
PROPERTIES AND SUBSTRATE SPECIFICITY OF PURIFIED UDP-GlcNAc: Manα3R β2-GlcNAc-TRANSFERASE I FROM RAT LIVER

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UDP-GlcNAc: Manα3R β2-GlcNAc-transferase I (Gn-T I) is the key enzyme in the synthesis of complex and hybrid N-glycans. We have purified the enzyme from rat liver 25 000-fold. The molecular weight was 42, 000 as determined by SDS-PAGE. The V_{max} for the purified enzyme with Man_5-glycopeptide substrate was over 9 μmol/min/mg. Rat liver Gn-T I had a similar molecular weight and substrate specificity, and showed similar response to Triton X-100, buffer pH and divalent metal ions as the enzyme from rabbit liver (Nishikawa et al., JBC263:8270–8281, 1988).

A series of synthetic derivatives of the Manα6(Manα3)Manβ-R acceptor were synthesized and tested as substrates to determine the structural requirements for Gn-T I activity. An unsubstituted equatorial 4-hydroxyl on the β-linked mannose of Manα6(Manα3)Manβ-R was found to be essential. Elimination of the 4-hydroxyl of the α3-linked mannose of the substrate interferes with binding to the enzyme as it increases the K_m 20-fold. Modifications on the α6-linked mannose or on the core structure affect mainly K_m and to a lesser degree the V_{max}. The results of specificity and inhibition studies with analogues of Manα6(Manα3)Manβ-R will be presented. (This work was supported by grants from the CCFF, MRC of Canada and NATO.)

15.4
PURIFICATION AND CHARACTERIZATION OF RAT KIDNEY *N*-ACETYLGLUCOSAMINYLTRANSFERASE-V

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Transformation of cells by various tumor viruses and specific oncogenes alters the expression of cell surface oligosaccharides found on both glycoproteins and glycolipids. In one well-studied case, the N-linked oligosaccharides of BHK fibroblasts transformed with either Rous sarcoma or polyoma virus differ from those of non-transformed parental cells in that they show a significant increase in the structure [GlcNAcβ(1,6)Mana(1,6)Man], known as the β(1,6) branch. This increase in the virally transformed cells correlates with a significant increase in the specific activity of the Golgi enzyme, *N*-acetylglucosaminyltransferase V (GlcNAc-T V, EC 2.4.1.155), the enzyme responsible for synthesis of the β(1,6) branch. Studies have documented in several cell types a correlation between decreased expression of this branch, decreased activity of GlcNAc-T V, and decreased metastatic potential. By contrast, increased expression of the β(1,6) branch and GlcNAc-T V has been reported for many human breast tumor biopsies. In order to determine the molecular mechanism of the specific regulation of GlcNAc-T V expression after oncogenic transformation and to be able to test directly the hypothesis that changes in β(1,6) branch expression regulate tumorigenicity or metastatic potential, we have focused on isolating a cDNA encoding the enzyme. Pursuing the classic approach of purification and amino acid sequencing, we have purified GlcNAc-T V about 400,000-fold in 26% yield to a specific activity of 1.2 μmol/(mg*min) from a Triton X-100 extract of rat kidney acetone powder. Purification of GlcNAc-T V was achieved by sequential affinity column chromatography on UDP-hexanolamine and on a synthetic oligosaccharide inhibitor (specific for the enzyme) conjugated to BSA. Silver-stained SDS-PAGE of the purified enzyme revealed two major bands at apparent molecular weights of 69 and 75 kDa. The optimal ranges of pH and Triton X-100 concentrations for assay of enzyme activity were 6.5–7.0 and 1.0–1.5%, respectively. The cations, $MnCl_2$, $CaCl_2$, and $MgCl_2$, were each found to have a negligible (<10%) effect on activity. Moreover, the enzyme was fully active in the presence of 20 mM EDTA. Enzyme activity was stablized and enhanced by the addition of 20% glycerol, 0.5 mg/ml BSA, and 0.2 M NaCl. The K_m of the purified enzyme towards a synthetic trisaccharide acceptor was 138 μM, similar to that previously reported for this acceptor using a crude enzyme from transformed BHK cells. We are presently analyzing the amino acid sequence of the purified enzyme and generating an antibody specific to it. This work was supported by NCI CA35377.

15.5
DEOXYGENATED ACCEPTOR ANALOGS AS INHIBITORS OF *N*-ACETYLGLUCOSAMINYLTRANSFERASE V

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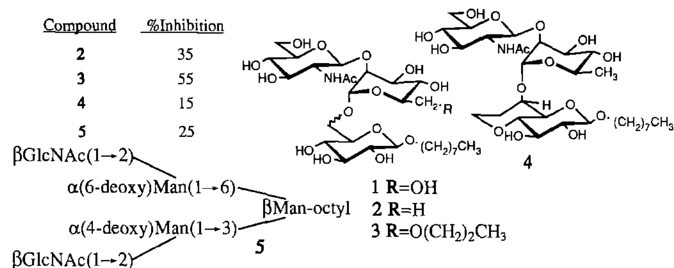
N-Acetylglucosaminyltransferase V (GlcNAcT-V) catalyses the transfer of βGlcNAc from sugar-nucleotide donor UDP-GlcNAc to the 6'-OH of the central αMan unit of the synthetic trisaccharide acceptor 1 (Km≈30μM). As suggested by previous work (JBC (1990) *265*:6759), a conformationally restricted analog (**4**) and other 6'-deoxy analogs (**2,3** and **5**) were found to be inhibitors of the partially purified enzyme from hamster kidney. Some typical preliminary data are shown below. Enzyme extract was prepared essentially as described (JACS (1991)*113*:216). Assays were performed at 20μM concentration of acceptor **1** and 40μM concentrations of inhibitor, using ^3H-UDP-

GlcNAc and reisolating radiolabelled tetrasaccharide product on C:18 SepPak cartridges (Glycoconj. J. (1988) *5*:716; JBC (1990)*265*:6759).



15.6
EVALUATION OF DEOXYGENATED OLIGOSACCHARIDE ANALOGS AS SPECIFIC INHIBITORS OF GLYCOSYLTRANSFERASES

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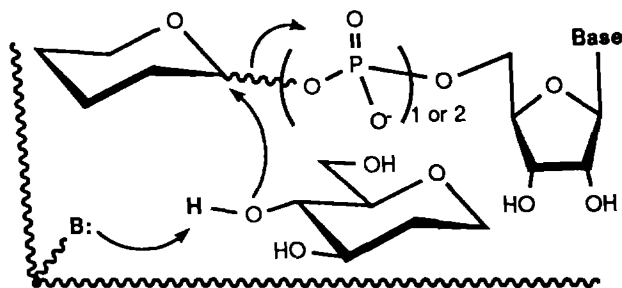
The glycosyltransferases controlling the biosynthesis of complex cell-surface carbohydrates transfer sugar residues from sugar nucleotides to specific hydroxyl groups of acceptor oligosaccharides. These enzymes represent prime targets for the design of glycosylation inhibitors with the potential to specifically alter the structures of cell-surface glycoconjugates. With the aim of producing such inhibitors, synthetic oligosaccharide substrates were defined for 10 different glycosyltransferases and the kinetic parameter Km was measured for each of these substrates and found to vary within the range 0.07–1.3 mM. The glycosyltransferases investigated were the α(1→2, both human serum and pig submaxillary), α(1→3) α(1→3/4) and α(1→4)-fucosyltransferases, β(1→4)-galactosyltransferase, β(1→6)-N-acetylglucosaminyltransferase-V, β(1→6)-mucin N-acetylglucosaminytransferase ("core-2" transferase), β(1→3)-N-acetylglucosaminyltransferase ("i" or "extension" transferase) and α(2→3)-sialytransferase from rat liver, all of which transfer sugar residues with inversion of configuration at their anomeric centers. For each of these 10 enzymes, acceptor analogs were next prepared where the active hydroxyl group undergoing glycosylation was chemically removed and replaced by hydrogen. Only 5 of the 10 resulting deoxyoligosaccharides inhibited their target enzymes (enzymes 1,2,5,7 and 8) and their Ki's (competitive where determined) remained in the general range of the corresponding acceptor Km's.

For the remaining 5 enzymes absolutely no inhibition was observed, even at exceedingly high concentrations of deoxygenated-acceptor analog. For these latter enzymes it is proposed that the active hydroxyl groups are involved in critical hydrogenbond donor interactions with basic groups on the enzymes which remove the developing proton during the glycosyl-transfer reaction. [Supported by grants from the NIH: RO1 CA45363–01A1 (MBT) and RO1 CA 5054201 (OH) and the NSERC (MMP)].



15.7
BIOSYNTHESIS OF BLOOD GROUP I AND i ANTIGENS IN RAT TISSUES: IDENTIFICATION OF β1–3 AND β1–6 N-ACETYLGLUCOSAMINYLTRANSFERASES

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The blood group antigens I and i are well-defined carbohydrate structures which are found in glycoproteins and glycolipids. They are well characterized on the surfaces and in contain mucins. The carbohydrate structures associated with the i antigen are linear chains with N-acetyllactosamine units attached to each other in β1–3 linkage. The I antigenic determinants are branched lactosaminoglycans with one or several branch points 3 and 6 positions of a galactose.

The β1–3 and β1–6 N-acetylglucosminyltransferases (GnT) which synthesize blood group i and I antigens, respectively were identified in rat tissues, using pyridylaminated lacto-N-neotetraose (Galβ1–4GlcNAc β1–3Galβ1–4Glc-PA) as an acceptor. Studies on the substrate specificity of β1–3 and β1–6GnTs indicated that the preferred substrate for β1–3GnT was general structure Galβ1–4GlcNAc-OR and for β1–6GnT, Galβ1–4GlcNAc β1–3Gal-OR. This is the first report that the β1–6GnT can act on an internal Gal residue of lacto-N-neotetraose. The products of both transferases reactions were separated on HPLC and identified by ^{1}H-NMR as GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA and Galβ1–4GlcNAcβ1–3(GlcNAc β1–6)Galβ1–4Glc-PA, respectively. The kinetic experiments were carried out using crude enzyme extracts from rat serum for β1–3 GnT and from rat intestine for β1–6 GnT. β1–3GnT has a pH optimum of 7.5 and essentially required Mn^{2+} for optimal activity. β1–6GnT has a pH optimum at 7.0 and does not require Mn^{2+}. Both transferase activities were significantly changed in various rat hepatomas.

15.8
THE IMPORTANCE OF THE CARBOHYDRATE CHAINS OF Gal β1–4 GlcNAc α2–6 SIALYLTRANSFERASE FOR ENZYME ACTIVITY.

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Gal β1–4 GlcNAc α2–6 Sialyltransferase (ST) is a rat liver Golgi enzyme responsible for the addition of terminal sialic acid residues to carbohydrate chains of glycoproteins. The enzyme is synthesized in the rough endoplasmic reticulum and passes to the Gologi complex where it expresses its catalytic activity. The enzyme contains three potential sites for glycosylation (Weinstein *et al.* 1987, J. Biol Chem **262**: 17735–17743) and it has been shown to be a glycoprotein. In this study the importance of the carbohydrate chains for catalytic activity was studied. Treatment of native ST with N-glycanase^{TM} resulted in loss of about 80% of enzyme activity after 6 hours of incubation; controls incubated in the absence of N-glycanase showed little loss of activity under the same conditions. It was found that the presence of up to 4% methanol or ethanol was essential for efficient removal of carbohydrate chains from native ST. Immunoblot analysis of N-glycanase treated ST showed three bands. One corresponding to the native enzyme with M_r about 43,000, a second corresponded to a completely deglycosylated form of the enzyme with M_r about 40,000 and a third band was intermediate between the two. The completely deglycosylated ST was the main form of the enzyme found after treatment of the native or denatured enzyme with N-glycanase for 18 hours. The results show that catalytic activity of ST is dependent on the presence of the carbohydrate chains which presumably influence the conformation of the enzyme. The work also suggests that

the presence of the carbohydrate chains of the correct structure on ST may influence the expression of the catalytic activity of the enzyme in the Golgi complex.

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15.9
REGULATION OF MOUSE KIDNEY GALACTOSYLGLOBOSIDE GALACTOSYLTRANSFERASE

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During our studies on the biosynthesis of galactosylgloboside we have discovered a putative endogenous inhibitor of the galactosyltransferase that synthesizes this glycolipid antigen. Galactosylgloboside is a member of the family of antigens that are extended globoseries structures expressed in a stage specific manner during development, hence called stage specific embryonic antigens (SSEA). There are increased levels of SSEA-3 and SSEA-1 globoseries glycolipids in male vs female DBA/2 and C57BL/6 mouse kidneys, respectively. We have shown that the observed sexual differences in the levels of SSEA-1 and SSEA-3 glycolipids are due, at least in part, to the differences in the activity of UDP:galactose globoside galactosyltransferase. Some of the kinetic properties suggest similarity of the enzyme in both sexes and others indicate differences. In both male and female Mn+2 was necessary for expression of the enzyme activity. CHAPS and sodium cholate activated the enzyme activity. Maximal enzyme activity in the presence of sodium cholate and CHAPS was obtained at 0.1% and 0.5% respectively. However, the extent of activation was greater in cholate than in CHAPS. In 0.1% sodium cholate the enzyme activity increased by approximately 70% in female microsomes and only 6% in male microsomes in comparison to the activity obtained in the presence of 0.05% sodium cholate. For the activity in female microsomes, the Km (globoside) in the presence of 0.1% cholate decreased to the value obtained for the male microsomal activity. This differential activation suggested the presence of a modulator in the female microsomes. The presence of a putative modulator in female kidney fractions was assayed with male kidney microsomes as a source of activity of globoside GalTr. The enzyme activity was inhibited 30 to 50 percent by the addition of either the 12,000 xg pellet, 100,000 xg supernatant or the microsomes from female kidney. This inhibitor activity was stable to heating at 55°C for 1 hour. Treatment of membranes containing the inhibitory activity with pronase for 4h at 37°C destroyed the inhibitory activity. Experimental studies on solubilization of the membranes in sodium cholate suggest complex formation between the detergent and the inhibitor molecules. Further characterization of the inhibitory activity is in progress. Supported by NIH grants HD05515 and NS15037.

15.10
THE ROLE OF HEPARIN BINDING SERUM PROTEIN(S) IN THE STABILISATION OF SIALYLTRANSFERASE RELEASED FROM RAT INTESTINAL SLICES

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Rat intestinal slices on incubation released soluble sialyltransferase (ST) into the medium, which was susceptible to proteolytic degradation. Heat inactivated horse serum (HHS) or α-1 proteinase inhibitor when added to incubations, due to their antiproteolytic action, stabilised the ST released.

Addition of increasing concentrations of heparin to HHS incubations, caused a decrease in medium ST activity. Like HHS the heparin binding fraction (HBF) of HHS, purified by heparin agarose affinity chromatography was able to stabilise medium ST. The HBF from horse serum further purified by gel filtration HPLC gave two fractions A and B. Fraction B on SDS-PAGE showed one major band and when used in incubations resulted in the stabilisation of medium ST activity. Fraction A showed multiple bands on SDS-PAGE and did not have an effect on medium ST. HBF and fraction B from HHS exhibited the ability to inhibit the proteolytic activity of trypsin. HBF isolated from human plasma and rat serum was also able to stabilize medium ST and showed antitryptic capacity. When heat inactivated serum obtained from rats injected with turpentine was used in incubations, there was an increase in medium ST activity compared to when serum from control rats was used. Serum from turpentine treated rats also showed an increased ability to inhibit trypsin.

These results suggest that serum contains one or more protease inhibitors which are inactivated by heparin and which protect the ST from proteolytic degradation.

15.11
A SOLUBLE FACTOR PRESENT IN RAT COLON ACTIVATES SIALYL AND FUCOSYL TRANSFERASE

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A soluble factor present in rat colon increased the activity of Golgi membrane sialyltransferase, fucosyltransferase and pure rat liver Galβ1–4GlcNAc α2,6 sialyltransferase (α2–6ST) by about 4–6 fold. This increase in activity was dose dependent. The factor had no effect on galactosyltransferase and N-acetylglucosaminyltransferase. The stimulation of α2–6ST activity using different acceptors, and by using a variety of assay techniques was highly reproducible. The soluble factor when chromatographed by gel-filtration separated into 3 peaks (peak 1 MW approx 670 K, peak 2 MW approx 80–20 K and peak 3 MW approx <1300). Peaks 1 and 2 retained the stimulatory activity, although the activity was considerably less when compared to the intact factor. The factor did not lose its activity on freezing/thawing or by extraction with chloroform, but on heating its activity could be completely destroyed. The factor lowered the K_m for CMP-NANA from 0.236 mM to 0.136mM but increased the V_{max} of the Golgi membrane sialyltransferase. Among the various tissues tested for the presence of such factors capable of stimulating α2–6ST activity, the colon was found to contain the highest activity. The presence of such factor(s) in colon and possibly in other tissues may serve as regulators of these enzymes. (Supported by the Medical Research Council and the Heart & Stroke Foundations of Canada).

15.12
ENZYMATIC *IN VITRO* SYNTHESIS OF THE BRANCHED PENTASACCHARIDES GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4GlcNAc/Glc AND THE ISOMERIC Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc/Glc

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Galactosylation of GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (1) and

GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4Glc (**7**), carried out with UDP-galactose and bovine milk β1,4-galactosyltransferase (EC 2.4.1.22), proceeded in a branch specific manner, preferentially at the 1–>6 branches. Partial reactions yielded pentasaccharide fractions containing mainly GlcNAcβ1–3(Galβ1–4GlcNacβ1–6)Galβ1–4GlcNAc (**2**), and almost pure GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4Glc (**8**), respectively; the fully galactosylated hexasaccharide products were also formed. Paper chromatography allowed partial separation of the isomeric pentasaccharides **2** and Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**3**), enabling us to purify both isomers. Partial degalactosylation of the hexasaccharide Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4Glc (**10**) with *E. coli* β-galactosidase (EC 3.2.1.23) proceeded also in a branch specific manner, favoring the 1–>6 branch, and yielding almost pure pentasaccharide Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4Glc (**9**); the fully degalactosylated tetrasaccharide product was also formed. The branch specific cleavage of (**10**) was remarkable in view of the random hydrolysis observed with the closely related hexasaccharide Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4GlcNAc (**4**) (Renkonen et al., Biochem. Cell Biol. 68: 1032–1036, 1990). The pentasaccharides **2**, **3**, **8** and **9**, all of which contain two non-identical distal branches, are valuable primers for enzymatic *in vitro* synthesis of oligo-N-acetyllactosaminoglycans.

15.13

HUMAN SERUM β-(1–>3)-N-ACETYL-D-GLUCOSAMINYLTRANSFERASE ELONGATES BOTH BRANCHES OF BI-ANTENNARY BACKBONES OF OLIGO-N-ACETYLLACTOSAMINOGLYCANS

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Partial reactions catalyzed by a β-(1–>3)-N-acetyl-D-glucosaminyltransferase (EC 2.4.1.149), known to be present in human serum, were studied using bi-antennary "backbone" saccharides of oligo-N-acetyllactosamine type as acceptors. Incubation of the radiolabeled blood-group I-active hexasaccharide βGal->4βGlcNAc->3(βGal->4βGlcNAc->6)βGal->4GlcNAc (**1**) and UDP-GlcNAc with serum gave first a transient (1:1)-mixture of two isomeric heptasaccharides βGlcNAc->3βGal->4βGlcNAc->3(βGal->4βGlcNAc->6)βGal->4GlcNAc (**2**) and βGal->4βGlcNAc->3(βGlcNAc->3βGal->4βGlcNAc->6)βGal->4GlcNAc (**3**). This shows that both branches of **1** reacted equally well. The two heptasaccharides reacted further in the incubation mixture to form the radiolabeled octasaccharide βGlcNAc->3βGal->4βGlcNAc->3(βGlcNAc->3βGal->4βGlcNAc->6)βGal->4GlcNAc (**4**); during this second reaction the composition of the heptasaccharide mixture remained unchanged, indicating that **2** and **3** reacted at approximately equal rates. The heptasaccharides **2** and **3** could not be separated from each other, but they could be detected, identified and quantitated by stepwise enzymatic degradations. Partial β-(1–>3)-N-acetyl-D-glucosaminylation reactions carried out with another acceptor, the branched pentasaccharide βGal->4βGlcNAc->3(βGal->4βGlcNAc->6)Gal (**11**) revealed that it, too, reacted equally well at both branches. Moreover, the initially formed hexasaccharides βGlcNAc->3βGal->4βGlcNAc->3(βGal->4βGlcNAc->6)Gal (**12**) and βGal->4βGlcNAc->3(βGlcNAc->3βGal->4βGlcNAc->6)Gal (**13**) were converted to the heptasaccharide βGlcNAc->3βGal->4βGlcNAc->3(βGlcNAc->3βGal->4βGlcNAc-6)Gal (**14**) at nearly equal rates. Radiolabeled tetrasaccharides by βGlcNAc->3(βGal->4βGlcNAc->6)Gal (**17**) and βGal->4βGlcNAc->3(βGlcNAc->6)Gal (**18**) were N-acetyl-D-glucosaminylated almost completely by UDP-GlcNAc in serum-catalyzed reactions. Put together, our data suggest that, intrinsically, the (1–>6)-branches of oligo-N-acetyllactosaminoglycan backbones can be elongated as well as the (1–>3)-branches. This makes it possible to construct a large number of different oligo-N-acetyllactosaminoglycans of branched nature by enzymatic *in vitro* synthesis.

15.14

HETEROGENEITY OF BETA-1,4 GALACTOSYLTRANSFERASE AND BETA-GALACTOSIDASE FROM AN EXPERIMENTAL MOUSE KIDNEY TUMOR

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Neoplastic transformations are accompanied by changes in metabolic activities which involve namely modifications of glycoconjugates. In order to clarify this problem, we have studied the properties of some enzymes which participate to the galactose pathway in a kidney tumor produced after injection of a C 57 mouse with lymphoma cells (RL 12 NP). The normal kidney or the tumor were homogenized in 0.15 M NaC1. After centrifugation, the supernatant was discarded and the pellet was homogenized in the presence of Triton X-100. The activity of the Beta-1,4 galactosyltransferase in the tumor corresponded to 200 % of that present in normal kidney. This latter enzyme can be resolved into five isoforms by chromatofocusing. Three of these isoforms were present in the tumor extract. For the Beta-galactosidase activity, we have also measured a dramatic increase in the tumor extract (334 %) using 4-methylumbelliferyl-galactoside as substrate. The chromatofocusing patterns of the enzyme activity showed that the same isoforms were present in both extracts. However, the relative proportions of the isoforms were significantly different.

These results demonstrate quantitative and qualitative differences between enzymes from a tumor and from the normal organ and suggest that these methods may be useful in characterizing renal neoplasia.

This work is supported by a SMB-GALEPHAR -ULB grant.

15.15

PURIFICATION AND PARTIAL CHARACTERIZATION OF A UDP-GALACTOSE: GLUCOSYLCERAMIDE B1–4 GALACTOSYLTRANSFERASE (GALT-2) FROM HUMAN KIDNEY AND CULTURED HUMAN PROXIMAL TUBULAR (PT) CELLS

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A membrane-bound galactosyltransferase; GalT-2 was purified 60 fold from the Golgi-rich preparation of human kidney. The techniques employed were: subcellular fractionation, detergent extraction, Sepharose Q column chromatography, lactalbumin column chromatography and octylsepharose column chromatography.

Sodium dodecylsulfate electrophoresis under reducing conditions revealed two protein bands with apparent Mr of 60,000 and 58,000. The activity of GalT-2 from kidney and cultured PT cells resolved in the same area upon gel electrophoresis. Western immunoblot assays revealed that the IgG against GalT-2 recognized both the kidney and cultured proximal tubular cell GalT-2. (Supported by NIH grant RO-1-DK-31722 to S.C.)

15.16
THE USE OF HUMAN MILK FUCOSYLTRANSFERASE IN THE SYNTHESIS OF TRIMERIC X DETERMINANTS

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We have studied the α3-fucosylation of a chemically synthesized trimer of N-acetyllactosamine, triLacNAcTAPE. When fucose residues are added to this structure poly X-determinants, (Galβ1→4[Fucα1→3]GlcNAcβ1→3)$_n$, are formed. Glycolipids with di- or trimeric X-determinants have been found to accumulate in lung, gastric, liver and colonic cancers. Fucosyltransferases from these tumor cells appear to have a distinct order in which they catalyze the attachment of fucosyl residues to polylactosaminoceramides. We have studied the order of fucosylation of triLacNAc-TAPE with a partially purified α3– fucosyltransferase from human milk. The enzyme preparation used contains the α3-(X) and the α3/4-(Lewis) fucosyltransferase copurified from human milk by SP-Sephadex chromatography (250-fold purification). With this preparation we synthsized trifucosyltriLacNAc-TAPE in almost quantitative yield. The structure of the product was confirmed by 400 MHz ^{1}H NMR. The time course of incorporation of [^{14}C]Fuc into triLacNAc-TAPE was monitored by HPLC using a Spherisorb-NH$_2$ column by which the different intermediates were separated. Formation of reaction products corresponding to mono-, di- and trifucosyltriLacNAc-TAPE with increasing incubation time was observed. The location of the Fuc residues on these intermediates was determined by digestion with endo-β-galactosidase (EC 3.2.1.103). This enzyme hydrolyses the β1,4-linkage between Gal and GlcNAc. However if fucose is attached to the GlcNAc residue the linkage is resistent to endo-β-galactosidase treatment. Analysis of fragments obtained after digestion indicated the original position of the fucosyl residues. It appears that there is a highly preferred order in which the fucosyl residues are attached to triLacNAc-TAPE. In the major pathway the first two Fuc residues are transferred with equal preference to the inner and middle GlcNAc, while the third Fuc is attached to the terminal GlcNAc residue.

15.17
A NOVEL QUANTITATIVE ASSAY METHOD FOR α(1→3)-L-FUCOSYLTRANSFERASE ACTIVITY IN HUMAN SERA

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A novel method has been developed to quantitatively assay α-(1→3)-L-fucosyltransferase activity in human sera by applying a sandwich-type radioimmunoassay. H type 2 trisaccharide (Fucα1→2Galβ1→GlcNAcβ) covalently attached to BSA as an acceptor and GDP-Fuc as a sugar donor were used, and incubated with human sera and buffer. The resulting product, Y tetrasaccharide (Fucα1→2Galβ1→4[Fucα1→3)-GlcNAcβ-BSA), was captured by an anti-BSA antibody coated bead.

After washing, the bead was incubated with ^{125}I-labeled anti-Y antibody and washed again. Radioactivity remaining on the bead was measured by γ-counter. This method provides a simple assay to determine the serum α(1→3)-L-fucosyltransferase activity quantitatively by using the partially purified enzyme as a calibrator. Inter and intra-assay CVs of the present method were <3% and <2%, respectively and the tests for the dilution linearity of the sample diluted with zero standard and for the analytical recovery of the standard enzymes in samples were also found to be satisfactory.

Using the present assay method the enzyme activity in sera from patients with benign and malignant gastric disorder and healthy subjects were measured. In gastric cancer, the detection by ratio was apparently higher than that of carcinoembryonic antigen (CEA) measured in the same samples, especially in the early stage, and no correlation between α(1→3)-L-fucosyltransferase activity and CEA levels was observed. It is concluded that the present method seems to be excellent for determination of the serum α(1→3)-L-fucosyltransferase activity, and may be a useful tool for the detection of early gastric cancer.

15.18
MEASUREMENT OF THREE KEY GLYCOSYLTRANSFERASE ACTIVITIES IN HUMAN LEUKOCYTE CELL EXTRACTS WITH AN ENZYME-LINKED IMMUNOSORBANT ASSAY

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Two distinct families of glycosphingolipids have been identified as antigens associated with different types of human leukemias. The globo family is expressed by chronic lymphoid leukemia cells, whereas the neolacto family is expressed by chronic myeloid leukemia cells. Both families are expressed by all acute leukemia cells. The level of expression of these antigens is partially regulated by the activities of specific glycosyltransferases. Therefore, an assay was developed in which glycosyltransferase activity could be specifically and conveniently measured. After a preparation containing the enzyme reacts with an immobilized glycosphingolipid substrate, product identification and quantification is accomplished with an enzyme-linked immunosorbent assay (ELISA), which utilizes monoclonal antibodies and an avidin-biotin alkaline phosphatase complex for detection.

This assay has been used to measure the activities of the three enzymes involved in the first committed steps of the biosynthesis of the two families of leukocyte antigens. The enzymes are: (neolacto series) UDP-GlcNAc:galactose β(1,3)N-acetylglucosaminyltransferase and UDP-Gal:N-acetylglucosamine β(1,4)-galactosyltransferase; (globo series) UDP-Gal:galactose α(1,4)-galactosyltransferase. The monoclonal antibodies used for detection of each product were: TE5 (GlcNAcβ1–3Gal), 1B2 (Galβ1–4GlcNAc), and Anti-P^k (Galα1–4Gal), respectively. For several different human leukocyte cell lines, which represent various types of leukemia, the activities of the three enzymes are measured and compared with antigen expression levels. Making this correlation is the first step in the determination of how the expression of leukocyte antigens is regulated.

15.19
IDENTIFICATION OF A UDP-GLUCOSE-BINDING POLYPEPTIDE IN A β-GLUCAN SYNTHASE COMPLEX FROM *Lolium multiflorum* USING A NOVEL PHOTOREACTIVE ANALOGUE OF UDP-GLUCOSE

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A new photoreactive pyrimidine analogue of UDP-Glc, 5[3-(*p*-

azidosalicylamide)]allyl-UDP-Glc, was synthesized in a three-step reaction sequence involving mercuration of UDP-Glc, alkylation of 5-Hg-UDP-Glc and acylation of 5-(3-amino)allyl-UDP-Glc and characterized by chemical and spectroscopic analysis. The compound inhibits and, upon UV-irradiation, inactivates a β-glucan synthase from *Lolium multiflorum* endosperm cells. The synthase has an apparent K_i of 16 μM for the analogue compared with an apparent K_m of 435 μM for UDP-Glc, the natural substrate. The corresponding UDP derivative has an apparent K_i of 6.4 12M. Kinetic studies using both the UDP-Glc and UDP analogues and their iodinated forms (5-IASA-UDP-Glc and 5-IASA-UDP, apparent K_i 6.6 μM and 1.0 μM respectively) indicate that the increase in affinity relates to their increased hydrophobicity. The UDP-Glc analogue was iodinated with $Na^{125}I$ to give a radiolabelled, photoreactive compound and used in photoaffinity labelling of UDP-Glc pyrophosphorylase, UDP-Glc dehydrogenase and several putative UDP-Glc-binding proteins from membrane preparations from *L.multiflorum.* The radiolabelled analogue photolabels a 31 kDa polypeptide in the CHAPS extract. This is one of the four major polypeptides in a β-glucan synthase complex immunoprecipitated with a monoclonal antibody from the CHAPS-extract (Meikle, P.J. *et al.*, this meeting). The photolabelling of this polypeptide is strictly UV-dependent, is blocked by UDP-Glc and UDP and reaches saturation at above 300 12M analogue. These results indicate that the 31 kDa polypeptide in the β-glucan synthase complex bears a UDP-Glc-binding site and is involved in the catalysis of β-glucan synthesis.

15.20
DETERGENT SOLUBILIZATION OF A β-GLUCAN SYNTHASE FROM *Lolium multiflorum* AND ITS PURIFICATION USING MONOCLONAL ANTIBODIES

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Membrane bound β-glucan synthase from *Lolium multiflorum* endosperm cells has been solubilized by both non-ionic and zwitterionic detergents. A complex relationship exists between the ratio of (1→3)–, (1→4)– and (1→3,1→4)-β-glucan products of the solubilized enzyme, the cations present and the concentration of the uridine 5′-diphosphoglucose substrate. Monoclonal antibodies directed against the β-glucan synthase complex were generated by immunisation of mice with an unfractionated microsomal preparation. Hybridoma cell lines were screened using a combination of indirect ELISA followed by an enzyme-capture assay. The purified monoclonal antibodies were used with Pansorbin (stabilized protein A-bearing *Staphylococcal* cells) to immunoprecipitate an active β-glucan synthase complex which had been solubilized from a microsomal preparation with 0.6% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (HAPS). SDS-PAGE analysis of the immunoprecipitated synthase complex revealed four major polypeptides of apparent m 30, 31, 54 and 58 kDa together with several minor components. The immunoprecipitated β-glucan synthase complex was capable of synthesizing both (1→3)- and (1→4)-β-glucans in approximately equal amounts with 10 μM uridine 5'-diphosphoglucose, 7 mM $MgCl_2$ and 2 mM EGTA. The 31 kDa polypeptide in the purified preparation has been photolabelled with a uridine 5'-diphosphoglucose analogue (Ng *et al.*, 1991, this meeting).

15.21
ON THE SPECIFICITY OF DOLICHOL KINASE TOWARDS ISOPRENOID ALCOHOLS OF DIFFERENT CHAIN LENGTH AND THEIR POSSIBLE INVOLVEMENT IN GLYCOSYLATION PROCESSES

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In order to explore the biosynthesis of asparagine-linked glycoproteins the function of all intermediates involved have to be investigated. We concentrated on the specificity of dolichol kinase in rat liver microsomes. CTP-dependent phosphorylation of dolichols and polyprenols ranging from 11 up to 32 isoprene residues was tested. Obtained results point out that dolichols of these chain lengths are phosphorylated. Kinase seems not to make any significant discrimination between administrated dolichols. That experiment was performed at low detergent concentration necessary to introduce the substrates to the reaction mixture. Increased concentration of Nonidet P-40, destroying natural environment of the membrane, had little effect on kinase affinity to dolichols. It only slightly lowered the level of phosphorylation. Polyprenols introduced to the incubation mixture also underwent phosphorylation. Mild acid hydrolysis proved that resulting compounds were indeed phosphate esters of polyprenols. At low concentration of detergent kinase prefers dolichol over polyprenol. Solubilization of the enzyme during incubation by increasing the Nonidet P-40 concentration stimulates phosphorylation of polyprenols. Experiments with prenol-32 and dolichol-32 phosphorylation confirm the preference of polyprenols versus dolichols at higher detergent concentration. Obtained results support the conclusion that dolichol kinase has rather low specificity.

The investigation of the activity of glycosyl transferases towards long chain dolichyl and prenyl phosphates showed that they can serve as lipid acceptors of mannose, glucose and N-acetylglucosamine.

15.22
BIOSYNTHESIS OF THE CORTICAL ALVEOLAR-DERIVED POLYSIALOGLYCOPROTEINS IN SALMONID FISH EGGS

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Recently, we have isolated and characterized naturally occurring unique glycoproteins, i.e. α-2,8-linked polysialyl group-containing glycoproteins (PSGP), which are localized in cortical alveoli of the unfertilized eggs. We have established unambiguously their complete chemical structure. Such details of chemical characterization of fish egg PSGP raised the question of how these molecules are synthesized. We set out to investigate the mechanism of biosynthesis of PSGP, and completed our studies on molecular cloning and characterization of cDNAs encoding rainbow trout apo-PSGP[J.Biol.Chem.(1988)*263*, 17678–17684] and also on organization and primary sequence of the multiple genes [J.Mol. Biol.(199O)*107*, 61–67].

We are now in a position to put forward a few testable hypotheses about biosynthesis of the oligo/polysialylglycan chains present in PSGP. Based on the capacity to transfer [^{14}C]NeuAc onto a series of exogenous acceptors including asialo-PSGP and PSGP, two distinct acceptor specificities have been identified: (1) CMP-NeuAc(or NeuGc): α-N-acetylgalactoside α-2,6-sialyltransferase (in cytosolic form) which catalyzes the initial transfer reaction of NeuAc and NeuGc onto the proximal GalNAc residues in asialo-PSGP; (2) cortical alveolar-derived CMP-

NeuAc(or NeuGc):α-sialyl α-2,8-sialyltransferase which is responsible for formation of α-2,8-linked oligo/polysialic acid chains. These observations strongly indicate that the enzyme which is relegated to the elongation of oligo/polysialic acid chains is located within the cortical alveoli and both *in vivo* level of CMP-NeuGc as well as the intrinsic property of this enzyme control the average length of oligo/polysialyl chains in PSGP.

15.23
PARTIAL PURIFICATION OF GLYCOSYL TRANSFERASES, CATALYSING THE BIOSYNTHESIS OF *SALMONELLA ANATUM* AND *S. KENTUCKY* BIOSYNTHESIS, THROUGH GEL PERMEATION HPLC

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Pathway of the biosynthesis of O-specific polysaccharides of *Salmonella anatum* and *S.kentucky* includes intermediate formation of polyprenyl diphosphate *1* and *2*, respectively.

DMan(β1–4)LRha(α1–3)DGal(α)-PP-Pre (*1*)

DMan(α1–2)DMan(α1–3)DGal(α)-PP-Pre (*2*)

High performance gel permeation chromatography Superose 12, in the presence of Polytergent SL–305F, was shown to be fast and efficient method for separation and partial purification of glycosyltransferases, which participate in the biosynthesis process. Activity of the enzymes in the fractions was measured with the use of the corresponding polyprenyl phosphates or polyprenyl diphosphate sugars and the radioactive nucleotide sugars.

30-Fold purification of *S.anatum* galactosyl phosphate transferase was achieved with separation of the enzyme from the rhamnosyltransferase (purified 70-fold) and the mannosyltransferase (purified 4-fold). In the case of *S.kentucky* the activity of the galactosyl phosphate transferase was found to be eluted in three different peaks (degree of purification was 6–10), an efficient separation of two different mannosyltransferases was achieved.

The resulting partially purified glycosyltransferase preparations were rather stable and may be successfully used for chemical-enzymic synthesis of polyprenyl diphosphate oligosaccharides. Particularly, the modified derivatives of 2, which contain residue of D-talose instead of ManI or ManII, were obtained using the a preparations of the mannosyltransferases I and II, with synthetic polyprenyl diphosphate galactose, GDP-D-mannose or GDP-talose as starting materials.

15.24
STUDIES ON THE GLUCOSYLATION OF N-LINKED OLIGOSACCHARIDES IN GLYCOPROTEIN BIOSYNTHESIS

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The dolichol-P-glucose: $Man_9GlcNAc_2$-PP-dolichol glucosyltransferase involved in glycoprotein biosynthesis catalyses the transfer of glucose from dolichol-P-glucose (donor substrate) to $Man_9GlcNAc_2$-PP-dolichol (acceptor substrate) to form $Glc_1Man_9GlcNAc_2$-PP-dolichol as product. Acceptor substrate was prepared by incubating porcine pancreas microsomes with UDP-GlcNAc, GDP-Man and a $CHCl_3$: CH_3OH:H_2O (10:10:3) extract of porcine pancreas. Lipid linked oligosaccharides containing at least five mannose residues were isolated by $CHCl_3$:CH_3OH:H_2O extraction and purified by ion exchange and gel filtration chromatography. This was followed by incubation with dolichol-P-mannose. The resultant lipid-linked oligosaccharides when subjected to mild acid hydrolysis and subsequent Bio-Gel P4 chromatography eluted as $Man_9GlcNAc_2$ as the major component and $Man_{7-8}GlcNAc_2$ and $Man_5GlcNAc_2$ as relatively minor components. This preparation of substrate served as an efficient acceptor of glucose from dolichol-P-glucose.

The glucosyltransferase from the intimal membrane of porcine aorta was solubilized with 0.5% NP-40 at a protein: detergent ratio cf 2:1. For purification of the enzyme, a combination of ammonium sulfate precipitation, ion exchange chromatography and hydroxyapatite column chromatography have given best results. The partially purified enzyme has a pH optimum of 6.5, exhibits a requirement for divalent metal ions, and is quite specific for dolichol-P-glucose as donor substrate and $Man_5GlcNac_2$-PP-dolichol or $Man_9GlcNAc_2$-PP-dolichol as acceptor substrate. Furthermore, enzyme activity appeared to be stimulated by phosphatidyl serine and is inhibited by dolichol-phosphate, dolichol and other isoprene compounds.

In order to study comparative rates of glucosylation of $Man_5GlcNAc_2$-PP-dolichol and $Man_9GlcNac_2$-PP-dolichol, homogenous populations of each of these species were obtained by digestion of $Glc_3Man_9GlcNAc_2$-PP-dolichol (obtained from MDCK cells) with exoglycosidases. Both serve as acceptors of glucose in vitro. Currently, using B421 cells (a CHO cell line that is deficient in dolichol-P-mannose synthase activity) we are studying the glucosylation of $Man_5GlcNAc_2$-PP-dolichol in vivo, in the presence of glycoprotein processing inhibitors. (Supported by NIH grant NL 17783)

15.25
PURIFICATION, PROPERTIES, PHOSPHOLIPID DEPENDENCE, AND PHOTOAFFINITY LABELING OF β-MANNOSYL TRANSFERASE FROM PIG AORTA

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The β-mannosyl transferase that catalyzes the synthesis of Man-β-GlcNAc-GlcNAc pyrophosphoryl-dolichol from GDP-mannose and dolichylpyrophosphoryl-GlcNAc-GlcNAc was solubilized from microsomes of pig aorta using 1% Triton X100 and was purified about 500 fold by chromatography on DEAE-cellulose, hydroxylapatite, gel fitration and GDP-affinity column. The dolichyl-PP-GlcNAc-GlcNAc substrate used for this enzyme was isolated from $CHCl_3$: CH_3OH: H_2O (10:10:3) extract of pig liver and was purified by saponification, chromatography on DEAE-cellulose (acetate) and LH-20 columns. Chromatography of the enzyme preparation on a DEAE-cellulose column clearly separated β-mannosyl transferase and dolichyl-P-mannose synthase activities. The product of the enzyme was identified as Man-β-GlcNAc-GlcNAc-PP-dolichol when examined by thin layer chromatography. The oligosaccharide portion of the product released on mild acid hydrolysis was characterized as a trisaccharide on Biogel P-4 column and was susceptible to β-mannosidase digestions. The enzyme had a pH optimum of about 7.0 and showed an absolute requirement for $Mg2^+$, with optimal activity at 5–8 mM. The enzyme required either detergent or phospholipids for maximum activity and the effects of these two were not additive. Among the various phospholipids tested, phosphatidylcholine showed maximum stimulation. The phosphatidylcholines with 18 or 20 carbon unsaturated fatty acids were most effective whereas phosphatidylcholines with saturated fatty acids were not effective. The enzyme was inhibited by dolichyl phosphate and also by compounds such as p-chloromercuribenzoate and N-ethylmaleimide that affect sulfhydryl groups. The enzyme was competitively inhibited by guanosine containing nucleotides such as GDP-glucose, GTP, GDP and GMP. A photoaffinity

analogue of GDP, $8N_3GDP$ was used to detect the catalytic subunit of the enzyme. After UV-irradiation of the enzyme with $[\alpha\text{-}^{32}P]8N_3GDP$, a major photoinserted polypeptide of 46 kDa was detected on SDS gels by autoradiography. This polypeptide is being used for antibody production to confirm its identity as the catalytic subunit of the enzyme. This research was supported by NIH grant HL-17783.

15.26

PURIFICATION, PHOTOAFFINITY LABELING AND PROPERTY COMPARISON OF GLC-P-DOL SYNTHASE FROM PIG AORTA AND MUNG BEAN SEEDLINGS

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Glucosyl phosphoryldolichol (Glc-P-Dol) is the glucosyl donor in the biosynthesis of $Glc_3Man_9GlcNAc_2$-pyrophosphoryl-dolichol, the oligosaccharide portion of which is transferred to protein during the synthesis of N-linked glycoproteins. Glc-P-Dol Synthase (GPDS), which catalyzes the formation of Glc-P-Dol from UDP-Glc and dolichol-P, was purified over 250-fold from pig aorta and 750-fold from mung bean seedlings. Both enzymes were purified by a combination of TX-100 solubilization, DEAE-cellulose, hydroxylapatite, and affinity chromatography procedures. This enzyme had not been previously purified from a plant source, so comparison to the mammalian enzyme was of interest. The catalytic subunits of GPDS from both sources were determined by photoaffinity labeling with the UDP-Glc analog, $[^{32}P]$5-azido-UDP-glucose ($5N_3$UPD-Glc). A 37 kD polypeptide from aorta and a 39 kD polypeptide from mung beans were identified by autoradiography after SDS-PAGE separation.

Photolabeling of the GPDS subunits with $[^{32}P]5N_3$UDP-Glc was saturable and could be protected by UDP-Glc, but not other NDP-sugars. The K_m of UDP-Glc for aorta GPDS was 5 μM and 27 μM for mung bean GPDS. Similarly, half-maximal photoinsertion with $[^{32}P]5N_3$UDP-Glc was 7 μM for aorta GPDS and 23 μM for mung bean GPDS. Both enzymes had similar broad pH optima between 6.0–7.0, and required Mg^{++} as a co-factor. In the presence of EDTA, but without Mg^{++}, activity for both enzymes was abolished and photolabeling of the mung bean GPDS was weakly protected. However, EDTA completely inhibits $5N_3$UDP-Glc photolabeling of the aorta GPDS. This indicates that Mg^{++} is required for UDP-Glc binding and subsequent catalysis in aorta GPDS, while Mg^{++} appears to be only required for catalysis in mung bean GPDS.

Identification of these GPDS subunits by photoaffinity labeling has allowed for electrolelution of the corresponding protein band from SDS-gels, resulting in an apparent homogeneous preparation for use in antibody production. This work was supported by grants from the National Institites of Health, HL-08238 to RD, and DK-21800, HL-17783 to AE.

15.27

PROTEIN *N*-GLYCOSYLATION IN INSECT CELL LINES

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Insect cells are increasingly being used as expression systems for recombinant glycoproteins. We have therefore set out to explore their glycosylation potential.

Extracts from membrane pellets of cultured Mb 0503 (*Mamestra brassicae*), Sf 9 (*Spodoptera frugiperda*) and Bm-N (*Bombyx mori*) cells were separated by SDS/polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. Lectins from *Maackia amurensis* (MAA), specific for α(2–3)bound sialic acid and from *Sambucus nigra* (SNA-I), which recognizes α(2–6)bound sialic acid residues, reacted with many proteins from all three cell lines. Binding of the sialic acid-specific lectins decreased after treating blots with peptide:*N*-glycosidase F or neuraminidase (*C. perfringens; A. ureafaciens*). On the other hand, binding to the galactose-specific lectin RCA_{120} increased after neuraminidase treatment. These results indicate that sialylated complex-type N-glycans are present in the cell lines tested.

Using the appropriate ^{3}H-labelled sugar nucleotides and acceptor substrates we could demonstrate the presence of N-acetylglucosaminyl-, galactosyl- and fucosyltransferase activities. However, no direct evidence could be obtained so far for the presence of sialyltransferase activity.

In an insect tissue, honeybee (*Apis mellifica*) venom glands, we were be able to identify a novel α(1–3)fucosyltransferase, which converts a monofucosylated *N*-glycan acceptor substrate to a difucosylated product with both fucoses linked to the asparagine-bound GlcNAc (Staudacher et al., submitted).

15.28

RAPID AND SIMULTANEOUS DETERMINATION OF SUGAR INCORPORATION INTO GLYCOSPHINGOLIPIDS AND GLYCOPROTEINS

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Glycosylation inhibitors are useful tools for studying the involvement of the carbohydrate moieties in the biological activities of glycoconjugates. Here, we present a rapid procedure for assessing such inhibitors by simultaneously measuring the incorporation of ^{14}C-galactose into lipidic fractions (mainly glycosphingolipids (GSLs)) and glycoproteins as well as the incorporation of H-leucine into proteins. After metabolic labelling for 24 hours in 96-well plates in presence or absence of a test substance, cells are collected on glass fiber filters by washing with PBS and 5% TCA. The filters are then extracted with chloroform/methanol (2:1), extracts and filters dried and counted. In order to compensate for cytotoxicity of potential inhibitors, the galactose incorporation is normalised relative to protein synthesis (^{3}H-leucine) and specific values for glycolipid and glycoprotein are derived.

Using EL-4 cells and the glucocerebroside synthetase inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, 5μM of the isomeric mixture) [Vunnam R.R. and Radin N.S. (1980) Chem. Phys. Lipids, *26*, 265] the specific glycolipid synthesis was reduced to 50%, whereas specific glycosylation of proteins was not disturbed; protein synthesis was 90%. With 0.5 μM tunicamycin, the corresponding values were 130%, 40%, and 40%. The inhibitory effect for a series of homologs of PDMP depended pronouncedly on the N-acyl chain length. By contrast, these compounds were roughly equipotent by enzymatic *in vitro* assays.

15.29

PURIFICATION OF N-ACETYLGLUCOSAMINE 1-PHOSPHOTRANSFERASE FROM HUMAN LYMPHOBLASTS USING AFFINITY CHROMATOGRAPY

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N-acetylglucosamine 1-phosphotransferase transfers N-acetylgluco-

samine 1-phosphate to selected high mannose oligosaccharide chains on lysosomal enzymes. Removal of N-acetylglucosamine exposes the resulting mannose 6-phosphate, a recognition marker responsible for targeting of these enzymes to lysosomal organelles. A deficiency of the enzyme activity results in two childhood autosomal recessive neurodegenerative disorders, I-cell disease and pseudo-Hurler polydystrophy. Previously the human lymphoblast enzyme had been partially purified by us through chromatographic procedures employing lentil lectin, DEAE-Sephacel and S-400 gel filtration. In the present study, the enzyme has been further purified using affinity chromatography involving either uteroferrin or lysosomal enzymes immobilized to hydrazine-activated agarose. These columns bind up to 85% of the partially purified enzyme, and subsequent elution revealed several bands on SDS-PAGE. The appearance of these bands could be attributed either to the presence of dissimilar subunits comprising the N-acetylglucosamine 1-phosphotransferase or to non-specific binding between positively charged affinity ligands and negatively charged proteins present in the partially purified preparation applied to the column. Current experiments are focusing on resolving these possibilities and purifying the enzyme to apparent homogeneity. (Suppoprted by NIH-NS12138)

15.30

PARTICIPATION OF CYTOCHROME b_5 IN CMP-N-ACETYLNEURAMINIC ACID HYDROXYLATION IN MOUSE LIVER CYTOSOL

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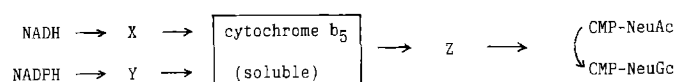
N-Glycolylneuraminic acid (NeuGc) residue in glycoconjugates is an epitope of Hanganutziu-Deicher heterophile antigen which has been found in several human cancer tissues. In order to elucidate the expression mechanism of NeuGc, we have studied CMP-N-acetylneuraminic acid hydroxylase, which converts CMP-N-acetylneuraminic acid (CMP-NeuAc) to CMP-NeuGc.

The enzyme activity in mouse liver was determined by a newly developed HPLC method using non-radioactive CMP-NeuAc as a substrate. The activity was detected in the cytosol fraction but not in the microsomal fraction. Either NADH or NADPH was used as an electron donor by the CMP-NeuAc hydroxylase, but NADH was much more efficiently used than NADPH. An antibody against cytochrome b_5 markedly reduced the CMP-NeuAc hydroxylase activity when added to incubation mixture containing either NADH or NADPH as an electron donor. These data led us to postulate the following electron transport system, which is involved in the CMP-NeuAc hydroxylation in mouse liver cytosol:



where X, Y, and Z are components supposedly involved.

Recently, we could separate two components required for enzyme activity and confirmed that one component can be replaced with soluble cytochrome b_5 purified horse erythrocyte lysate.

15.31

CMP-N-ACETYL NEURAMINIC ACID HYDROXYLASE ACTIVITY REQUIRES AT LEAST 2 SUBCOMPONENTS

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Recombinant human glycoproteins expressed in Chinese hamster ovary cells differ from their human counterparts by the presence of N-glycolylneuraminic acid in their sialylated carbohydrate chains. In normal adult humans the conversion of CMP-Neu5Ac into CMP-Neu5Gc does not take place, due to the absence of expression of CMP-N-acetylneuraminic acid hydroxylase. In order to characterize the enzyme we have focused on the purification of the protein responsible for the hydroxylase activity in mouse livers. Hydroxylase activity is clearly detectable in the high-speed supernatant of homogenized livers, but is greatly reduced, or vanishes completely if regular purification procedures are employed to enrich the enzyme. Absorption of hydroxylase activity containing solutions to a Mono-Q ion-exchange medium at pH 5.2, followed by elution using an increasing salt gradient, does not allow recovery of activity in any of the individual collected fractions. However, the combination of two different individual fractions, K1 which does not bind the ion-exchange matrix, and K2 which was eluted at about 100 mM NaCl, lead to restoration of activity. Both fractions could be dialyzed extensively without loosing their ability to restore activity upon combination. The experimental results suggest that the 2 components are dissimilar. The two fractions were further investigated by gelpermeation chromatography. The inactive fraction K2 was fractionated over a Superose-12 column, and individual fractions combined with the K1 fraction. A mass of about 30 kD was calculated for the proteins contained in the fraction yielding hydroxylase activity. A similar experiment was performed with the K1 fraction, yielding a mass of about 20 kD.

15.32

INFLUENCE OF PROTEIN CONCENTRATION, IONIC STRENGTH AND DETERGENTS ON THE ACTIVITY OF CMP-N-ACETYLNEURAMINIC ACID HYDROXYLASE: POSSIBLE RELEVANCE TO ENZYMIC FUNCTION

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Recent work from this group has unequivocally established that the biosynthesis of N-glycoloylneuraminic acid occurs by the hydroxylation of CMP-N-acetylneuraminic acid [1,2]. The influence of several factors on the activity of CMP-Neu5Ac hydroxylase in high-speed supernatants of mouse liver was investigated. In diluted supernatants, the hydroxylase exhibited anomalously low specific activities. The enzyme was strongly inhibited by increasing ionic strength. About 50% inhibition was observed with 20mM KC1, NaC1, NH_4C1, Na_2SO_4, Na_2HPO_4 or $MgC1_2$, respectively. In contrast, the following non-ionic detergents (10mM): Nonidet P-40, Triton X-100, octyl glucoside and decyl glucoside, gave rise to significant (two- to fourfold) increases in activity. Since more than one protein is involved in generating the enzyme activity (see abstract: P. Schneckenburger et al.), increased ionic strength may disrupt ionic interactions between these components. The anomalous behaviour of the enzyme in diluted extracts is consistent with this suggestion. Non-ionic detergents might, therefore, be influencing other, possibly hydrophobic, interactions or could be mimicking a more hydrophobic environment *in vivo*; for example association with intracellular membranes.

1. Shaw, L. & Schauer, R. (1988) Biol. Chem Hoppe-Seyler **369**, 477–486
2. Shaw, L. & Schauer, R. (1989) Biochem. J. **263**, 355–363

15.33
STUDIES ON THE PURIFICATION OF CMP-N-ACETYLNEURAMINIC ACID HYDROXYLASE FROM MOUSE LIVER

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The hydroxylation of N-acetylneuraminic acid to N-glycoloylneuraminic acid is catalysed by CMP-Neu5Ac hydroxylase (EC 1.14.99.18) [1,2]. This enzyme is a monooxygenase requiring both NADH and Fe-ions as cofactors and is probably cytosolic. The high specific activity of the hydroxylase in supernatants of mouse liver made this tissue a good potential starting material for the isolation of this enzyme. Fractionation of high-speed supernatants on Cibacron Blue-3GA agarose columns revealed no detectable activity in either the non-bound fraction or in fractions eluted from the columns with NaCl or NADH. However, a portion of the hydroxylase activity could be recovered by combining these two fractions. Similarly, chromatography of mouse liver supernatants on Q-Sepharose gave rise to two inactive fractions eluting at 200mM and 300mM NaCl which, when mixed, yielded a significant increase in hydroxylase activity. The results clearly demonstrate that CMP-Neu5Ac hydroxylase is an enzyme system consisting of at least two chromatographically separable components. By analogy with other monooxygenases, the component eluting from blue agarose with NaCl or NADH could possess an NADH-reductase activity. The suggested involvement of cytochrome b5 in catalysis [3] raises the possibility of the participation of cytomchrome b5 reductase. The sensitivity of this enzyme system to increased ionic strength and dilution (see abstract: L. Shaw et al.) may result from disruption of the catalytic interactions between these components.

1. Shaw, L. & Schauer, R. (1988) Biol. Chem. Hoppe-Seyler **369**, 477–486
2. Shaw, L. & Schauer, R. (1989) Biochem. J. **263**, 355–363
3. Kozutsumi, Y., Kawano, T., Yamakawa, T. & Suzuki, A. (1990) J. Biochem. **108**, 704–706.

15.34
PARTIAL PURIFICATION AND CHARACTERIZATION OF A SIALIDASE FROM *TRYPANOSOMA BRUCEI*

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A membrane-bound sialidase (EC 3.2.1.18) was found in procyclic *Trypanosoma brucei*. The mammalian stage bloodstream form, however, showed no sialidase activity. After osmotic lysis at 0°C and solubilization with Triton CF-54, the sialidase was enriched 2000-fold by gelfiltration and ion-exchange chromatography. In addition, release of the sialidase activity was tested with various proteases and phospholipases. The molecular weight, as determined by HPLC-gelfiltration, is 67 kDa. The sialidase is active over a broad pH- and temperature range with optima at pH 6.9 and 35°C, respectively. No loss of activity was observed after four freeze-thaw cycles. *Trypanosoma brucei* sialidase activity is inhibited by a wide range of metal ions, N-(4-nitrophenyl)-oxamic acid and 2-deoxy-2,3-didehydro-N-acetylneuraminic acid. N-(4-nitrophenyl)oxamic acid was found to be a much more potent inhibitor than Neu5Ac2en. α-D-N-Acetylneuraminic acid shows no inhibitory effect. The sialidase activity is activated by di- and tricarboxylic acids, but inhibited by chloride. The following compounds were tested as substrate and are given in the order of decreasing relative hydrolysis rate: de-O-acetylated bovine submandibular gland mucin (100%), Neu5Ac-α(2–3)-lactose (79%), native bovine submandibular gland mucin (75%), Neu5Ac-α(2–6)-lactose (39.5%), fetuin (37%), Collocalia mucin (25%), gangliosides from bovine brain (23%) and equine submandibular gland mucin (9%). More detailed kinetic analyses were performed with MU-Neu5Ac (K_M 0.16mM, V_{max} 3.5mU), Neu5Ac-α(2–3)-lactose (K_M 0.08mM, V_{max} 1.44mU) and Neu5Ac-α(2–6)-lactose (K_M 0.124mM, V_{max} 0.48mU).

To our knowledge this is the first sialidase from African trypanosomes studied in detail.

15.35
STUDIES ON SUBSTRATE SPECIFICITY OF GLYCOASPARAGINASE

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Glycoasparaginase (EC 3.5.1.26) is a lysosomal enzyme that cleaves the N-glycosidic linkage between asparagine and N-acetylglucosamine in asparaginylglycopeptides. We have recently purified and characterized glycoasparaginase from human leukocytes (1). We now report on our studies of the substrate specificity of the enzyme studied by using different, structurally definied asparaginyglycopeptides.

The purified enzyme (specific activity 2.2 U/mg protein) had a Km of 110 μM and a Vmax of 34 $\mu M \times l^{-1}$ with N4-(*B*-N-acetylglucosaminyl)-L-asparagine (aspartylglucosamine) as substrate. The enzyme was capable to hydrolyze the N-glycosidic of high mannose-type and complex-type glycopeptides as well as asparaginylglycopeptides lacking the di-N-acetylchitobiose moiety with comparable rates to aspartylglucosamine to afford aspartate and intact oligosaccharides. However, the enzyme was completely inactive against aspartylglucosamine if either the α-amino or the α-carboxyl group of the asparagine moiety was chemically modified.

The results indicate that glycoasparaginase recognizes its substrates through the free α-amino and α-carboxyl groups of the asparagine moiety to produce intact carbohydrate chains and aspartate. The capability of the enzyme to cleave aspartate from asparaginylglycopeptides which contain N-glycans of various sizes and structures indicates that the enzyme may have a significant role in catabolism of asparaginylglycopeptides which lack the di-N-acetylchitobiose moiety or which are not for other reasons efficiently catabolized by endo-*B*-N-acetylglucosaminidase.

1) Kaartinen, V., Williams, J.C., Tomich, J., Yates III, J., Hood, L.E., and Mononen, I. (1991) J. Biol Chem. in press.

15.36
AFFINITY LABELING OF GLYCOASPARAGINASE WITH 5-DIAZO-4-OXO-L-NORVALINE

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Human leukocyte glycoasparaginase was characterized as a 88 kDa heterotetrameric enzyme containing two light chains of 19 kDa and two heavy chains of 25 kDa. Incubation of glycoasparaginase in the presence of 5-diazo-4-oxo-L-norvaline (DONV) resulted in the irreversible inactivation of its enzymatic activity. At all concentrations of the compound, the inactivation of the enzyme was pseudo-first-order with time. Since the natural substrate of glycoasparaginase, aspartylglucosamine, competetively protected against this inactivation, it was con-

cluded that DONV binds to the active site or close to it. Incubation of glycoasparaginase in the presence of the radioactive inhibitor, [5-^{14}C] DONV, indicated that radioactive label was incorporated to the light chain of the enzyme. Cyanogen bromide cleavage and chymotrypsin digestion were used to generate peptides of the light chain, and the radioactivity was demonstrated to be incorporated within a few amino acids at the amino terminal end of the polypeptide by Edman degradation. The amino acid residue binding the reacting group of DONV was characterized by mass spectrometry (1).

(1) Kaartinen V, Williams JC, Tomich J, Yates III JR, Hood LE, and Mononen I. (1991) J Biol Chem, in press.

15.37

PURIFICATION AND STRUCTURE OF 1-ASPARTAMIDO-β-N-ACETYGLUCOSAMINE AMIDOHYDROLASE FROM HUMAN LIVER

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We have recently diagnosed aspartylglucosaminuria (AGU) in four members of a Canadian family. AGU is a lysosomal storage disease in which asparagine-linked glyco-peptides accumulate to particularly high concentrations in liver, spleen and thyroid of affected individuals. A lesser accumulation of these glycopeptides is seen in the kidney and brain, and they are also excreted in the urine.

The altered metabolism in AGU results from a deficiency of the enzyme aspartylglucosaminidase (1-aspartamido-β-N-acetylglucosamine amidohydrolase) which hydrolyses the asparagine to N-acetylglucosamine linkages of glycoproteins and glycopeptides.

We have used human liver as a source of material for the purification of aspartyl-glucosaminidase. The enzyme has been purified to homogeneity using heat treatment, ammonium sulfate fractionation, and chromatography on Con A Sepharose, DEAE-Sepharose, SP-Sephadex, hydroxylapatite, DEAE-cellulose and Sephadex G-100.

Enzyme activity was followed by measuring colorimetrically the N-acetylglucosamine released from aspartylglucosamine at 65°C. The purified enzyme protein ran at a "native" molecular weight of 56 K in 12% SDS-PAGE gels, and the enzyme activity could be quantitatively recovered at this M_r by using gel slices as enzyme source in the assay. After denaturation by boiling in SDS the 56 K protein was lost with the corresponding appearance of polypeptides lacking enzyme activity at 24.6, 18.4 and 17.4K.

Treatment of heat denatured enzyme with N-glycosidase F resulted in the following reductions in molecular weight; 24.6 to 23 K and 18.4 and 17.4 to 15.8 K. These studies indicate that human liver aspartylglucosaminidase is composed of two non-identical polypeptides each of which is glycosylated. We are presently generating (i) N-terminal sequence, and (ii) antibodies to each of the two polypeptides.

15.38

SUBSTRATE SPECIFICITY AND PROPERTIES OF MAN$_9$-MANNOSIDASE FROM PIG LIVER

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A processing α–1,2-mannosidase (Man$_9$-mannosidase) has been purified in our laboratory from pig liver crude microsome by a combination of CM-cellulose-, DEAE-Sephacel-, Con A-Sepharose- and affinity chromotography on immobilized N-5-carboxypentyl-1-dMM. The isolated enzyme has a molecular mass of 49 kDa when determined by SDS-PAGE under reducing conditions. Immunological studies revealed that the 49 kDa protein is a catalytically active fragment generated by non-specific proteolysis of a 65 kDa protein during purification. The intact 65 kDa enzyme is a transmembrane protein containing a cytosolic polypeptide tail of at least 5 kDa size (Biochem. J. (1989) 264, 347–355). By electron microscopy using the immuno-gold-technique, labelling was detectable over parts of the ER and transient vesicles but not, however, in the Golgi fraction indicating that Man$_9$-mannosidase may be an ER-resident enzyme (with J. Roth, Zürich).

Man$_9$-mannosidase has a pH optimum close to 6.0, requires divalent cations for activity, is not active against nitrophenyl- or methylumbelliferyl-α-mannosides but cleaves specifically three of the four α1,2-mannosidic linkages in free Man$_9$-GlcNac$_2$ (M$_9$) and Man$_9$-GlcNac$_2$-glycopeptides (M$_9$-GP); the relative rates for the degradation of these substrates to M$_8$, M$_7$, M$_6$ and M$_5$ intermediates, were found to be 1.0: 0.8: 1.7: 0.03 and 1.0: 0.8: 1.0: 0.03, respectively. The low cleavage rate for the fourth α1,2-mannose residue increased significantly when the terminal GlcNAc residue in Man$_9$-GlcNAc$_2$ is reduced to the amino alcohol or when Man$_9$-GlcNAc-ol is used strongly suggesting that an 'intact' acetal conformation for the chitobiose core (only possible in Man$_9$-GlcNAc$_2$ or Man$_9$-GlcNAc$_2$-glycopeptides) may be a controlling factor for Man$_9$-mannosidase specificity in vivo. Our results from kinetic studies with Man$_{8-5}$-GlcNAc$_2$-glycopeptides of known oligosaccharide structure, indicate that the α1,2-mannosidic linkage which is not cleaved by Man$_9$-mannosidase, appears to be the one which is hydrolysed in vivo by the Kornfeld ER-α1,2-mannosidase. This observation, together with the recent identification of other trimming α1,2-mannosidases (Golgi-mannosidase (Touster); endo-mannosidase (Spiro); α1,2-mannosidase (Schutzbach)) suggests a branching of the processing sequence at the Man$_9$-stage, the biological significance of which could be to tag different N-glycoproteins with specific oligosaccharide signals for different pathways andd estinations.

15.39

MANNO-VALIDAMINE, A PSEUDO-AMINOSUGAR THAT INHIBITS α-MANNOSIDASES IN N–LINKED OLIGOSACCHARIDE PROCESSING

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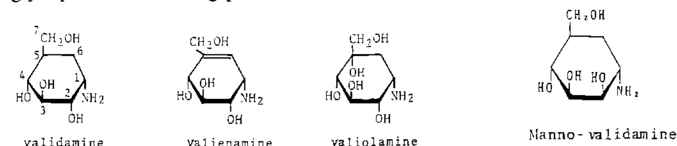
Pseudo-aminosugars, validamine, valienamine and valiolamine, the glucose analogs in which the ring oxigen is replaced with a carbon atom, were isolated from the fermentation broth of Streptomyces hygroscopicus subsp. limoneus. These show potent competitive inhibition on mammalian intestinal α-glucosidases, such as maltase, sucrase, isomaltase and glucoamylase, and also on rat liver glucosidase I and II in the glycoprotein trimming process.



Manno-validamine, a mannose analog was semi-synthesized by the configurational inversion of validamine. 3-O-Acetyl-4,7-O-benzylidene-2-O-(p-tolylsulfonyl)-N- benzyloxycarbonylvalidamine was prepared by the selective sulfonation, in known manner. Reaction of the 2-sulfonate derivative with an excess sodium benzoate in DMF at 140°C gave the 2-epi derivative. The derivative was hydrolyzed with hydrochloric acid and then treated with 10% Ba(OH)$_2$ at reflux temperature gave the free base, manno-validamine.

The inhibitory effect of manno-validamine on rat liver α-mannosidases was elucidated by comparing with swainsonine and deoxymannojirimycin. Manno-validamine showed competitive inhibition on the endoplasmic reticulum, Golgi I, II and soluble α-mannosidases, respectively. There are significant differences in the inhibitory specificity between manno-validamine, swainsonine and deoxymannojirimycin.

15.40
THE SUBSTRATE SPECIFICITY OF MAMMALIAN LYSOSOMAL α-D-MANNOSIDASES IN RELATION TO α-MANNOSIDOSIS.

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Because species differences in the structures of oligosaccharides accumulated by humans, cattle, and cats with α-mannosidosis might reflect differences in the substrate specificities of the respective lysosomal α-mannosidases, these enzymes were partially purified and employed to digest oligosaccharides corresponding to the oligomannosyl parts of complex, hybrid and high mannose glycans.

The incubation products were identified by HPLC with reference compounds of defined structure, and by acetolysis. For all classes of substrates, the lysosomal α-mannosidases displayed a high degree of *in vitro* specificity. Thus usually one, or at most two, mannose residues were always preferentially cleaved so that the degradative process proceeded down a well defined pathway.

The results of this study, together with those of others employing mannosidosis fibroblasts and swainsonine, allow conclusions to be drawn regarding the severity of α-mannosidase deficiency and nature of residual α-mannosidase activity in humans, cattle, and cats. In human α-mannosidosis, the pattern of oligosaccharide storage is compatible with a severe or complete deficiency of major lysosomal α-mannosidase, and the presence of a minor α-mannosidase specific for α-(1→6) linkages, together with the lysosomal endo-β-N-acetylglucosaminidase. In bovine α-mannosidosis, the structures and relative concentrations of oligosaccharides are those expected for a partial deficiency of lysosomal α-mannosidase, so that they correspond to intermediates in the normal catabolic pathway. In feline α-mannosidosis, the pattern of oligosaccharide storage indicates that the deficiency of normal lysosomal α-mannosidase activity is more severe than in the cattle, and the accumulated oligosaccharides primarily represent intact oligomannosyl moieties of *N*-linked glycans rather than products of residual α-mannosidase activity.

15.41
A NOVEL HUMAN LYSOSOMAL α-MANNOSIDASE ACTIVE ON THE CORE OF COMPLEX GLYCANS

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Normal human fibroblasts and fibroblasts from a patient with α-mannosidosis were grown in the presence or absence of 100 μM swainsonine for 7 days. Stored oligosaccharides were isolated from harvested cells, analysed by HPLC, and characterized by permethylation. Manα1→3Manβ1→4GlcNAc, and Manα1→2Manα1→3Manβ1→4-GlcNAc comprised >80% of the total oligosaccharides accumulating in untreated mannosidosis cells; these are identical to the major components previously identified from α-mannosidosis urine. However, after 7 days of swainsonine treatment, Manα1→6[Manα1→3]Manβ1→4GlcNAc was the major $Man_3GlcNAc$ isomer present. No mannose-containing oligosaccharides were detected in control fibroblasts in the absence of swainsonine but, in its presence, oligosaccharides containing 3 to 9 mannose residues accumulated. Manα1→6[Manα1→3]Manβ1→4GlcNAc and Manα1→6[Manα1→3]Manα1→6[Manα1→3]Manβ1→4GlcNAc were the major components (67%). Surprisingly, Manα1→3Manβ1→4GlcNAc was only observed in swainsonine-treated control cells during the recovery period after removal of swainsonine, whereas it was the major oligosaccharide (60%) in untreated mannosidosis fibroblasts. Purified lysosomal α-mannosidase from human spleen readily cleaved branched $Man_3GlcNAc$ to yield Manα1→6Manβ1→4GlcNAc; further degradation to Manβ1→4GlcNAc was much slower. Since α-mannosidosis fibroblasts have a genetic deficiency of the lysosomal α-mannosidase coded on chromosome 19, these studies suggest the presence of a second lysosomal α-mannosidase activity, which is unaffected in genetic α-mannosidosis but is inhibited by swainsonine. This enzyme is responsible for cleaving the α-(1→6)-linked mannose residue from branched $Man_3GlcNAc$ to form Manα1→3Manβ1→4GlcNAc.

To confirm this hypothesis, post-Con A fractions from α-mannosidosis and control fibroblasts were incubated at pH 4.0 with the putative substrate Manα1→6[Manα1→3]Manβ1→4GlcNAc, obtained from feline α-mannosidosis liver by Endo D treatment. Using enzyme from control fibroblasts, the major product was Manα1→6Manβ1→4GlcNAc, while enzyme from mannosidosis fibroblasts gave Manα1→3Manβ1→4GlcNAc as the sole product. This confirmed the presence of an α-mannosidase activity unaffected by the disease. This activity did not cleave p-nitrophenyl α-mannoside, or $Man_9GlcNAc$, or Manα1→6[Manα1→2Man-α1→2Manα1→3]Manβ1→4GlcNAc. As anticipated from the cell culture studies, the ability of the isolated enzyme to hydrolyse Manα1→6-[Manα1→3]Manβ1→4GlcNAc was completely inhibited by 10 μM swainsonine.

15.42
ISOLATION AND CHARACTERIZATION OF α-L-FUCOSIDASES FROM TWO BACTERIAL SPECIES

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α-L-Fucosidase has been isolated from a number of prokaryotic and eukaryotic sources. The substrate specificity of the enzyme differs depending on its origin. The enzymes from mammalian tissues and marine gastropods have extremely broad substrate specificity, but the enzymes from microorganisms and almond emulsin have narrow specificity. An enzyme with limited substrate specificity is needed when the biological functions and structures of glycoconjugates are to be identified. We looked for a microbial fucosidase that hydrolyzes a fluorescence-labeled pyridylamino oligosaccharide containing α-linked fucose residues. Screening was done with a medium that contained L-fucose as the sole carbon source.

Two species of bacteria, *Corynebacterium* sp. FS-0077 and *Streptomyces* sp. 142, isolated from soil samples were found to produce fucosidases that have different substrate specificities. Various pyridylamino oligosaccharides were used as substrates. The *Corynebacterium* enzyme specifically cleaved Fucα1–2Gal linkages as well as *p*-nitrophenyl α-L-fucoside, with a pH optimum of 6.5–7.0. The *Streptomyces* enzyme hydrolyzed terminal α1–3 and at α1–4 fucosidic linkages in the oligosaccharides with a pH optimum of 6.0, but this enzyme did

not hydrolyze pnitrophenyl α-L-fucoside. The *Streptomyces* enzyme also released L-fucose from an α1-acid glycoprotein in the presence of ammonium sulfate.

15.43
ENZYMATIC SYNTHESIS OF N-ACETYLLACTOSAMINE AND ITS RELATED COMPOUNDS BY GLYCOSIDASE

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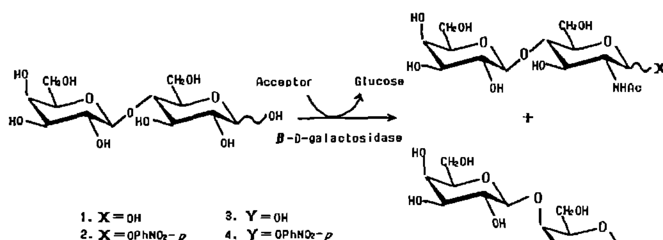
N-Acetyllactosamine **1** is known as a representative disaccharide which is itself important as a core component in oligosacharide moieties of glycoproteins and glycolipids. Owing to the growing need for substantial amounts of **1** as a biomaterial, we have developed a practical route for the synthesis of **1**, utilizing the transglycosylation reaction through β-D-galactosidase from *Bacillus circulans.* The enzyme induced preponderant β-D-galactopyranosyl transfer from lactose to the secondary (OH-4) over to the primary hydroxyl group of N-acetylglucosamine. **1** was thus readily synthesized in a gram scale and isolated conveniently by chromatography on a Carbon-Celite column.

Further utilization of the transglycosylation by *B. circulans* β-D-galactosidase led to the syntheses of β-(1–4)-linked disaccharides and trisaccharide containing a 2-amino-2-deoxy-D-hexose. The derivatives of GlcNAc and GalNAc were used as acceptors for the preparation of the following sugars: β-D-GlcNAc-OPhNo$_2$-p, D-GalNAc, β-D-GalNAc-OPhNO$_2$-p, and β-D-GlcNAc-(1–4)-D-Man. The β-D-galactosidase-catalyzed reactions was efficient enough to allow us to perform the one pot preparation of **2, 3, 4** and β-D-Gal-(1–4)-β-D-GlcNAc- (1–4)-D-Man by the use of lactose.



15.44
ENHANCING EFFECTS OF BILE SALTS ON THE DEGRADATION OF GLYCOSPHINGOLIPIDS BY BACTERIAL GLYCOSIDASES

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Different concentrations of ionic and non-ionic detergents were examined for optimization of the *in vitro* degradations of intestinal glycosphingolipids by α- and β-glycosidases from human fecal bacteria of *Ruminococcus* and *Bifidobacterium* genera. In 5 mM Triton X-100 the enzymes hydrolyzed glycosphingolipids with lactoseries type 1 and 2 chains essentially to lactosylceramide (LacCer; Falk, P. et al (1990) *J. Biochem.* (Tokyo) **108**, 466–474). In 5 mM sodium di- and tri-hydroxy bile salts lactosylceramide was degraded to glucosylceramide (GlcCer) in varying extent by enzymes from all five strains. The minimal bile salt concentrations for optimal 1,4-β-galactosidase activities varied between 1 and 20 mM, i.e. close to or above the critical micellar concentrations (cmc). Dihydroxy bile salts were the most efficient in promoting conversion of LacCer to GlcCer at concentrations below 10 mM and conjugation with a taurine residue did not markedly lower the GlcCer yield. The optimal detergent concentrations for hydrolyses of the *p*-nitrophenyl (pnp) glycosides Galβ1-pnp and GalNAcα1-pnp were approximately 0.05 mM for Triton X-100 and 0.5 mM for sodium taurodeoxycholate, i.e. clearly below their reported cmc values. With sodium ursodeoxycholate maximal pnp release was observed in 10–20 mM concentrations.

Galabiosylceramide, globotria- and globotetraosylceramides, not degraded in the Triton X-100 micelles, were also resistant to hydrolysis using the sodium bile salts as detergents. In contrast, lactotetraosylceramide and *iso*globotriaosylceramide were degraded by enzymes from a *R. gnavus* strain and gangliotetraosylceramide by enzymes from a *B. bifidum* and a *B. infantis* strain using bile salt detergents. All strains but *R. gnavus* released terminal GalNAc from *para*-Forssman but not from the globotetraosylceramide or Forssman structures using 5 mM sodium deoxycholate as detergent. GM1 desialylation by two *R. torques* strains, and the *R. gnavus* and *B. bifidum* strains were enhanced under identical conditions. We conclude that the observed effects on glycosphingolipid hydrolyses reflects variations in the micellar presentation of the substrates. In addition, detergents seem to have a direct stimulating effect on the glycosidases, however at concentrations 10–100 times below the one used in our glycolipid degradations. These results, with optimized bile salt concentrations, further support our previous observations that these five fecal bacterial strains produce enzymes with selected specificities towards glycosphingolipid core chains of the lactoseries type 1 and 2.

15.45
PURIFICATION AND COMPARISON OF THE SPECIFICITIES AND RELATED PROPERTIES OF α-L-FUCOSIDASE FROM HUMAN TUMOR, EARTHWORM AND LEECH

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α-L-Fucosidase was purified from human ovarian tumor (OT) earthworm (EW) and leech through fucosylamine-sepharose affinity chromatography followed by gel filtration on a Sephacryl S-200 column. The purified enzymes had specific activities in the range 10–18 μmol/min/mg protein.

α-L-Fucosidase activities with various synthetic natural substrates were percentwise compared towards Fucα1→2Galβ1→4Glc. Several significant substrate affinity differences were found among the different species of α-L-fucosidase. OT large species (OTLS) exhibited >3-fold activity with Fucα1→4GlcNAcβ1→3Galβ1→ OCH$_3$ as compared to OT small species (OTSS) (282% to 87%); EW and leech enzymes respectively were 127–166% and 92% active with this substrate. Further, EWLS was 50% more active than EWSS (124% to 82%) towards Fucα1→2Galβ1→4GlcNAc. Comparatively, the leech enzyme was only 57% active with this substrate. Of all the fucosidases, OTLS exhibited the highest activity towards substrates terminating in α1→3, α1→4 or α1→6 fucosylated GlcNAc (α1→4 linkage: OTLS 282%; 0TSS 87%; EW 127–166%; leech 92%; α1→3 linkage: 0TLS 58%; 0TSS 21%; EW 25–37%; leech 31%; α1→6 linkage: OTLS 30%; OTSS 13%; EW 10–13%; leech 15%). When Fuc was linked to penultimate GlcNAc, the ability of α-L-fucosidases to hydrolyze Fuc was found to decrease tremendously (OTLS 282% decreased to 34%; 0TSS 87% to 13%; EWLS 166% to 13%; EW 130% to 15%; leech 92% to 13%). All fucosidases showed less activity (23–49%) towards Fuc α1→2Gal as compared to Fucα1→2-Galβ1→4Glc.

Other important observations regarding pNP-glycoside specificity were made. For example, when Fuc was α-linked to the C-3 or C-6 instead of the C-2 hydroxyl of Gal in pNP-β-D-galactoside, the ability to cleave Fuc decreased tremendously (5–10%) except for the EW enzymes, which were able to act on the α1→6 linkage (32–36%).
Supported by NCI grant CA35329.

15.46
6-ACETAMIDO-6-DEOXYCASTANOSPERMINE (MDL 102,373): A NOVEL AND POTENT INHIBITOR OF MAMMALIAN β-N-ACETYLGLUCOSAMINIDASES

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β-Hexosaminidase is a lysosomal enzyme that catalyzes the removal of terminal β-linked N-acetylglucosamine residues from glycoproteins, glycolipids and glycosaminoglycans. Enhanced activity of β-N-acetyglucosaminidase was reported in tissues and fluids from cancer patients suggesting potential usefulness of inhibitors of this enzyme in cancer chemotherapy. We have recently reported snythesis of MDL 102,373 as a potential inhibitor of mammalian β-N-acetyglucosaminidase. The objective of this study was to evaluate this compound for activity and specificity against a number of glycosidases. Glycosidases were assayed using the appropriate p-nitrophenyl glycosides. MDL 102,373 was found to be a potent inhibitor of β-N-acetyglucosaminidases from jack bean (IC_{50}=1.6 μM), human placenta (IC_{50}=0.5 μM), bovine kidney (IC_{50}=1.5 μM), porcine placenta (IC_{50}= 0.4 μM) and bovine epididymis (IC_{50}=0.7 μM). The specificity was demonstrated by lack of inhibition by the compound against α- and β-mannosidase, α- and β-glucosidase, α-fucosidase and β-galactosidase up to 500 μM against each of these enzymes tested. These data demonstrate that MDL 102,373 is a potent and specific inhibitor of mammalian β-N-acetyglucosaminidases.

15.47
MAPPING OF THE SUBSTRATE BINDING DOMAIN OF MICROBIAL SIALIDASES

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The substrate binding region of several microbial sialidases has been characterized using a photoaffinity labeling reagent. These studies were prompted by our recent discovery that the well known sialidase inhibitor, 2,3 dehydro-N-acetylneuraminic acid (Neu5Ac2en) could be modified by the introduction of aryl azides in place of the terminal hyroxyl moiety without affecting recognition and binding by mammalian sialidases (1–3). The enzymes from *C. perfingens*, *S. typhimurium* LT 2, and *V. cholerae* were photolyzed with, 5-acetamido-2,6 anhydro,2,3,5,9-tetradeoxy-9-(^{125}I–2'-hydroxyl-4'-azido-benzamido)-**D**-*glycero*-**D**-*galacto*-nonuloso-2-enoic acid (4). The labeling was highly specific for the substrate binding domain. The evidence for this is based on the following: (i) protection from labeling was observed when Neu5Ac2en was included in the photolysis mixture, (ii) compounds which contained structural and chemical features similar to Neu5Ac2en but which were not competitive inhibitors of the enzymes did not provide protection from labeling, (iii) dark controls and prephotolyzing the reaction mixture did not result in protein labeling and finally, (iv) single cyanogen bromide fragments containing a labeled residue were isolated from the photolyzed proteins. Amino terminal sequence combined with Chou-Fasman analysis identified the labeled CNBr fragment from the Salmonella sialidase as having β sheet characteristics (5). Based on tertiary structural analogy to the influenza sialidase, this peptide lies within the active site cleft. Molecular modeling of the minimal energy conformation (maximal extension) of the inhibitor indicates that the aryl nitrene would be positioned approximately 14 angstroms from the anomeric center of the sialic acid moiety at C-2. This suggests that the label could be within 4 to 5 amino acid residues of the active site.

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5. Hoyer, L. Vimr, E.R. manuscript in preparation

15.48
2-α-(N'-DANSYL-4'-AMINOPHENYLTHIO)-9-O-ACETYL-N-ACETYLNEURAMINIC ACID: A NEW SPECIFIC AND HIGHLY SENSITIVE SUBSTRATE IN SIALATE-O-ACETYL-ESTERASE-ASSAY

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Sialate-O-acetyl-esterases catalyze specifically the hydrolysis of acetyl groups at the 9- and/or 4-position of sialic acids and thereby initiate the catabolism of sialoglycoconjugates. The estimation of sialate-O-acetyl-esterase activity has been carried out up to now by using free or glycosidically bound O-acetylated sialic acids or 4-methylumbelliferyl acetate. The disadvantages of these substrates are either their low sensitivity for detection or their low specificity.

We have synthesized a specific and sensitively detectable sialate-O-acetyl-esterase substrate. Using phase transfer-catalyzed glycosidation we prepared the N-acetylneuraminic acid-α-aminophenyl-thioketosides. This α-thioketoside is an excellent molecule for introducing a fluorescent substituent. On the other hand thioketosides are stable against hydrolysis of sialidases and therefore are suitable for examinations of esterases in tissues or in preparations containing sialidases. By coupling the free amino group of the α-aminophenylthio-ketoside with dansylchloride we synthesized a fluorescent neuraminic acid derivative which allows quantitative determinations down to picomolar amounts. Regioselective O-acetylation with trime-thylorthoacetate resulted in the corresponding 9-O-acetyl-derivative.

We tested the highly specific and also highly sensitively detectable substrate with sialate-O-acetyl-esterase from bovine brain. The esterase accepted the substrate and hydrolyzed the acetyl group at the 9-O-position of the fluorescent N-acetylneuraminic acid derivative. We could easily separate O-acetylated and de-O-acetylated derivatives by HPLC and quantificate the hydrolysis products by fluorimetric detection. This assay will be used to estimate a possible alteration in the esterase activity on lymphocytes of malignoma patients, as a different distribution on N- and O-acetylneuraminic acids on T-cells in cases of carcinoma has been observed by us.

15.49
CHARACTERIZATION OF SIALIDASE AND β-GALACTOSIDASE FROM BOVINE LIVER

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Sialidase is widely distributed in living organisms and participates in the

metabolism of sialoglycoconiugates, but it is not characterized well because of its lability. Previous studies of a sialidase from human placenta demonstrated that the enzyme is co-purified with β-galactosidase (β-gal) activity. In this study, we tried to purify a sialidase from bovine liver and studied the relationship between sialidase and β-gal.

A sialidase was purified 6,500-fold from bovine liver by the procedures involving affinity chromatographies on Con A-Sepharose and p-aminophenyl thio-β-D-galactoside-CH-Sepharose, and gel filtration of HPLC. The enzyme was apparently co-purified with a portion of β-gal like a human placental sialidase. Although we observed significant activation of sialidase activity during the purification of human placental sialidase, bovine enzyme could not activated at all. The purified enzyme liberated sialic acid residues from sialooligosaccharides, sialogycoproteins and gangliosides. On SDS-polyacrylamide gel electrophoresis in the presence of dithiothreitol, the purified enzyme gave four protein bands with the molecular weights of 65KDa, 48KDa, 32KDa and 20KDa. Of these protein components, 65KDa, 32KDa and 20KDa proteins were also observed in β-gal multimer from bovine liver and brain. To investigate the relationship between bovine hepatic sialidase and β-gal, we prepared an antiserum raised against 32KDa protein from bovine brain β-gal multimer. The antiserum was specifically recognized 32kDa protein on immunoblot and could precipitated the enzyme activities sialidase and β-gal multimer, but not β-gal monomer on immunoprecipitation experiments. These results seem to suggest that 32kDa protein observed in β-gal multimer is a sub-component essential for the molecular assembly of both sialidase and β-gal.

S16. ORGANIC SYNTHESIS/SYNTHÈSE ORGANIQUE

16.1
A CONCISE β-N-ACETYLGLUCOSAMINIDATION OF ALCOHOLS AND OLIGOSACCHARIDES, AND ITS APPLICATION FOR THE SYNTHESIS OF AMINO CTH GLYCOSPHINGOLOPID

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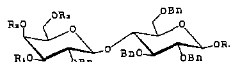
Although various methods of β-N-acetylglucosaminidation are known (1), they require some modifications at C-1 or C-2. Now, we would report a concise β-N-acetylglucosaminidation using peracetyl-β-D-glucosamine (**1**) and PPTS(pyridinium p-toluenesulphonate) or PTF-(pyridinium triflate) as catalysts.

First, reaction of **1** with several alcohols were carried out by heating at 70–80°C overnight in the presence of PPTS in 1,2-dichoroethane, afording the corresponding β-glycoside exclusively. Results: with benzyl alcohol, yield=89%; *p*-methoxybenzyl alcohol, 90%; allyl alcohol, 72%; cyclohexanol, 82%, respectively. The α-anomer of **1** also reacted with benzyl alcohol to give the β-glycoside although the reaction rate was very slow.

Secondly, for the synthesis of oligosaccharide, attachment of N-acetylglucosamine residue to three benzyl lactose derivatives (**2, 3** and **4**) as glycosyl acceptors was examined under the same condition above, except for **3** using PTF instead of PPTS. The glycosyl acceptors and the trisaccharides (**5, 6** and **7**) obtained in the respective reactions are shown below.



Yield
2:R_1=Bn, R_2=R_3=H, R_4=Bn **5**:R_1=Bn, R_2=H, R_3= β-AcGlcNAc, R_4=Bn, 50%(79%*)
3:R_1=R_2=H, R_3=R_4=Bn **6**:R_1= β-AcGlcNAc, R_2=H, R_3=R_4=Bn, 73%
4:R_1=H, R_2=R_3=R_4=Bn **7**:R_1= β-AcGlcNAc, R_2=R_3=R_4=Bn, 44%
8:R_1=H, R_2=R_3=Bn, R_4=3-OBn-Cer *conversion yield

Thirdly, the same reaction was applied for the synthesis of Amino CTH glycosphingolipid (GlcNAc β1–3Gal β1–4Glc β1–1-Cer). Glycosyl acceptor(**8**) was chemically derived with several steps from natural NG-hematoside which was isolated from horse erythrocytes. These results and the applicability of the above β-N-acetylglucosaminidation will be discussed.

1.a) H.M.Flowers, Methods in Enzymology, **138**(Part E), 357-404(1987)
b) P.J.Garegg and A.A.Lindberg, Carbohydrate Chemistry edited by J.F.Kennedy, 500-559(1988).

16.2
SYNTHESIS OF 2,7-ANHYDRO-*N*-ACETYLNEURAMINIC ACID

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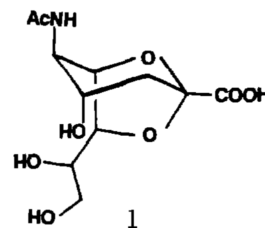
Sialic acid contaning intramolecular glycoside, 2,7-anhydro-*N*-acetylneuraminic acid (1), was isolated in a free form from cerumen of the wet type.(a) As part of program on the glycosylation of sialic acid, we now discuss the first synthesis of 1, starting from already prepared methyl 5-acetamido-3,5-dideoxy-8,9-isopropylidene-β-D-*glycero*-D-*galacto*-2-nonuropyranosonate (2).(b)

Benzoylation of 2 using benzoic anhydride in pyridine gave regioselectively 4-*O*-benzoyl derivative (3) of 2, mp151–153°C. The treatment of 3 with *S,S'*-bis(1-phenyl-1*H*-tetrazol-5-yl) dithiocarbonate (c) in MeCN in presence of DMAP gave *S*-glycoside, methyl [1-phenyl-1*H*-tetrazol-5-yl 5-acetamido-4-benzoyl-3,5-dideoxy-8,9-isopropylidene-β-D-*glycero*-D-*galacto*-2-nonulopyranosid]onate (4) in 75% yield, mp177–178°C.

Intramolecular glycosylation of 4 was performed in CH_2Cl_2 in presence of AgOTf-$(MeCN)_2PdCl_2$, and MS 4A to give the full protected 1 (5) in 45% yield, a white powder, and 2-deoxy derivative of 3 in 40% yield. In the deprotection of 5, isopropylidene and benzoyl group were removed by the treatment of 80% AcOH-H_20 and 2% MeONa-MeOH, respectively, to give methyl 2,7-anhydro-*N*-acetylneuraminate mono-hydorate (6) in 90% yield, mp95–96°C. Subsequent treatment of 6 with 0.5*N* NaOH gave 2,7-anhydro-*N*-acetylneuraminic acid (1) in 95% yield, a white powder, CD(MeOH) [θ]+3550(213nm), [θ]–350(235nm), which was identified as 1 by comparing its FAB-MS, GC-MS, and TLC behavior with those of an authentic sample of natural 1. The stereostructure of 6 was determind by X-ray crystal analysis. This evidence indicates that the pyranose ring have a $5C_2$(D) conformation.

Ref. (a) M. Suzuki, A. Suzuki, T. Yamakawa, and E. Matsunaga, *J. Biochem.*, 97, 509 (1985). (b) H. Ogura, K. Furuhata, S. Sato, K. Anazawa, M. Itho, and Y. Shitori, *Carbohydr. Res.*, 167, 77 (1987). (c) K. Takeda, K. Tsuboyama, K. Torii, K. Furuhata, N. Sato, and H. Ogura, *Carbohydr. Res.*, 203, 57 (1990).



1

16.3

THIOPHENYL GLYCOSIDE FOR THE SYNTHESIS OF HIGHER OLIGOSACCHARIDES CONTAINING Galβ1→4GlcNAc, Fucα1→3GlcNAc AND Galβ1→4(Fucα1→3)GlcNAc AS TERMINAL UNITS

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Phenyl 6-O-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside upon treatment with 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-fluoride provided phenyl 4-O-(2,3,4,6-tetra-O-acetyl-β-D-galacto-pyranosyl) -6-O-benzyl -2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**1**). Further treatment with methyl 2,3,4-tri-O-benzyl-1-thio-β-L-fucopyranoside in the presence of $CuBr_2$-Bu_4NBr afforded a key glycosyl donor, phenyl 4-0-[(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-3-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)]-6-O-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**2**). Additionally, 1,3,6-tri-O-acetyl-2-deoxy-2-phthalimido-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galacto-pyranosyl)-α/β-glucopyranose on treatment with (phenylthio) trimethyl silane and trimethylsilyl triflate gave phenyl 3,6-di-O-acetyl-2-deoxy-2-phthalimido-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galacto-pyranopsyl)-1-thio-β-D-glucopyranoside (**3**). These reagents were utilized for the synthesis of oligosaccharides containing either Galβ1→4GlcNAc or Galβ1→4(Fucα1→3)GlcNAc as terminal units. Several schemes will be presented. It is an interesting observation that the methylthio glycosyl donor can react in the presence of suitably protected thiophenyl acceptors to provide the desired oligosaccharide donors for further employment. In conclusion, thiophenyl glycosyl donors can be employed without conversion to fluoride or bromide for stereoselective Gal β-linked oligosaccharide syntheses.

These investigations were supported by grant No. CA35329 awarded by the National Cancer Institute and by grant No. CH419 awarded by the American Cancer Society.

16.4

PHENYL 2,4,6-TRI-O-ACETYL-3-O-BENZYL-1-THIO-α/β-D-GALACTOPYRANOSIDE AS A NOVEL GLYCOSYLATING AGENT FOR THE SYNTHESIS OF 2-O-SUBSTITUTED GAL-β-D-LINKED OLIGOSACCHARIDES

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In recent years a growing interest has developed in the study of the α-L-fucosyltransferase activities involved in the biosynthesis of blood-group active substances as well as in the process of premalignant transformation. It has been suggested that α-L-fucosyl-transferases are responsible for accumulation of the highly fucosylated lactosamine compounds that have been found to be associated with various human cancers. Therefore, the availability of compounds capable of acting as acceptors for single enzyme species, even in the presence of other, related enzymes is of particular importance. For this reason we have synthesized three compounds, 2′-O-methyl lactosamine (**2**), 2′-O-sulfo lactosamine (**3**), and 2′-O-fucosyl lactosamine (**4**) which can be utilized as specific acceptors for a single fucosyltransferase activity. We have developed a novel glycosylating reagent for the synthesis of the above mentioned compounds, namely, phenyl 2,4,6-tri-O-acetyl-3-O-benzyl-1-thio-α/β-D-galactopyranoside (**1**) Compound **1** was reacted with benzyl 2-acetamido-2-deoxy-3,6-di-O-benzyl-α-D-glucopyranoside using NIS/TFOH as the catalyst to afford an intermediate disaccharide which after de-O-acetylation, followed by acetal formation provided benzyl 2-acetamido-2-deoxy-4-O-(3-O-benzyl-4,6-O-benzylidene-β-D-galacto-pyranosyl)-3,6-di-O-benzyl-α-D-glucopyranoside as key intermediate for the preparation of compounds **2, 3** and **4**. Similarly, this reagent can be utilized for the synthesis of 2-O-substituted Galβ1→3 linked oligosaccharides. In conclusion, thiophenyl glycosyl donors can be employed without conversion to fluoride or bromide for stereoselective Gal β-linked oligosaccharide syntheses.

Supported by NCI Grant #CA35329 and ACS Grant #CH419.

16.5

DESIGN, SYNTHESES, AND PROPERTIES OF NOVEL SYNTHETIC GLYCOCONJUGATES

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Synthetic glycoconjugates having pendant sugar residues are of great interest not only as simplified models of the biopolymers bearing oligosaccharides but as artificial glycoconjugates from the point of view of biochemistry and medical science.

Our recent attention has been mainly focused on design and facile syntheses of novel types of synthetic glycoconjugates as glycoprotein models containing the appropriate spacer function in order to evaluate and utilize the unique molecular recognition nature of oligosaccharide chains more efficiently (1,2).

The present paper deals with the availability of the spacer-armed polymerizable glycosides with high reactivity and wide applicability. A facile procedure for the preparation of new models having pendant N,N′-diacetylchitobiose [β-D-GlcpNAc-(1→4)-β-D-GlcpNAc], N-acetyllactosamine [β-D-Galp-(1→4)-β-D-GlcpNAc], and the related oligosaccharide sequences was established on the basis of radical copolymerization of the n-pentenylated derivatives with acrylamide. Adhesion behavior of rat hepatocytes on these affinity matrices was also preliminarily examined.

(1) S. I. Nishimura et al., *Macromolecules* **1990,** *23,* 4182.
(2) S. I. Nishimura et al., *Macromolecules,* submitted.

16.6

USE OF GLYCOSYLAMINES IN A REVERSIBLE DERIVATIZATION OF OLIGOSACCHARIDES WITH THE 9-FLUORENYLMETHYLOXYCARBONYL GROUP, AND HPLC SEPARATION OF THE DERIVATIVES

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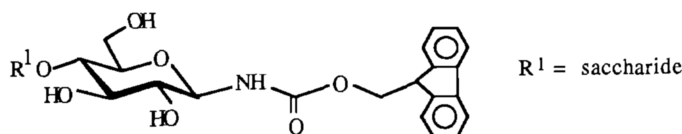
Analytical and preparative fractionation of complex oligosaccharide mixtures is often complicated by the lack of chromophores on the oligosaccharides, and also, more importantly, by the fact that each oligosaccharide gives rise to two peaks in the HPLC-chromatograms (α and β anomers). Therefore, various chemical derivatization procedures have been developed. We have reported [1] reductive amination of oligosaccharides with p-trifluoroacetamidoaniline as a reversible derivatization procedure. We now report another reversible derivatization procedure for oligosaccharides, using milder conditions.

Mixtures of human milk oligosaccharides (neutral or acidic pools) were transformed into the glycosylamines by treatment with saturated sodium hydrogen carbonate [2]. The glycosylamines were then derivatized with 9-fluorenylmetyloxycarbonyl chloride [3]. The so obtained FMOC-derivatives were separated by straight phase HPLC using

chloroform:methanol:aquoeus ammonia as mobile phase. By changing the concentration of ammonia in the mobile phase, the retention times could be varied. After separation the free oligosaccharides were recovered by treatment with first aquoeus ammonia, then aqueous acetic acid.



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1. E.Kallin, H. Lönn and T.Norberg, Glycoconjugate J., 5, 145–150 (1988).
2. K.Kochetkov, Carbohydrate Res., 146, C1-C5 (1986).
3. L.Carpino and G.Han, J.Org.Chem., 37, 3404–3409 (1972).

16.7
SYSTEMATIC SYNTHESIS OF LACTO- AND NEOLACTO-SERIES GANGLIOSIDES TOWARD THE ELUCIDATION OF THEIR STRUCTURE-FUNCTION RELATIONSHIPS

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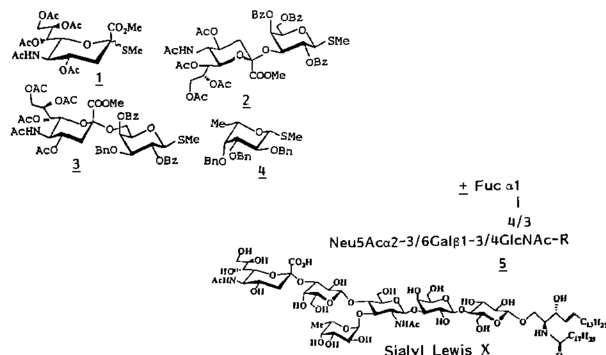
The lacto- and neolacto-series gangliosides, which are intimately related to the blood group glycolipids, are often detected as the tumor associated antigens. The monoclonal antibodies specific to those carbohydrate epitopes are being utilized as novel tools for the diagnosis and therapy of cancer. Recently, it has also been found that the endothelial-leukocyte adhesion molecule-1 (ELAM-1) recognizes a carbohydrate ligand containing the sialyl Lewis X*structure. We describe here the systematic synthesis of a variety of lacto- and neolacto-series gangliosides, including their derivatives and analogs, for the elucidation of structure-function relationships.

We have developed a facile, regio- and stereo-selective α-glycoside synthesis of sialic acid (Neu5Ac) by use of the methyl 2-thioglycoside of Neu5Ac (*1*) and thiophilic promotors (DMTST, NIS) in acetonitrile. Thus obtained Neu5Acα(2–3)Gal and Neu5Acα(2–6)-Gal disaccharide donors (*2, 3*) were successfully coupled with the suitably protected mono-, di- and trisaccharide acceptors, with or without α-L-fucosyl residue, to give a variety of fucosylated and non-fucosylated ganglioside epitopes (*5*).



* A. Hasegawa et al., Carbohydr. Res., *209* (1991).

16.8
SYNTHESIS OF BIOMEDICAL POLYMER HAVING BLOOD-GROUP ANTIGENIC OLIGOSACCHARIDE CHAIN

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It has been reported that blood-group antigen oligosaccharide chains play an important role in vivo. It is well-known that human blood-group antigen has A, B, or H type oligosaccharide chains at the end of glycoproteins and glycolipids according to the blood group. We ateempted to prepare a new polymeric material having biocompatibility by polymerization of the vinyl monomer with chemically synthesized H type oligosaccharide.

Glycosylation of 5-methoxycarbonylpentyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-glucopyranoside with 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-galactopyranosyl bromide by the use of $Hg(CN)_2$ as catalyst gave disaccharide derivative. The disaccharide derivative was deacetylated and then fucosylated with 2,3,4-tri-O-benzyl-α-L-fucopyranosyl bromide to give trisaccharide (H hapten) derivative. After ester-amide-exchange reaction of trisaccharide derivative and 2-aminoethanol, double bond was introduced to the end of the aglycon portion by the reaction with acryloyl chloride. The obtained monomer was 5-(2-acryloyloxyethylcarbamoyl)pentyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-[3,4,6-tri-O-benzyl-2-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-β-D- galactopyranosyl]-β-D-glucopyranoside, which was Fucα1–2Galβ1–3GlcNAcβ1–O-$(CH_2)_5$CONH $(CH_2)_2$OCOCH=CH_2 protected with benzyl and benzylidene groups.

H hapten containing monomer was copolymerized with acrylamide or methylacrylate. Copolymerization of the sugar monomer with acrylamide was carried out in dimethylformamide with AIBN as initiator, and copolymerization with methyl acrylate was carried out in benzene with the same initiator. The copolymer composition was calculated by ^{1}H NMR spectroscopy and was controlled by the monomer composition in feed. Mole fraction of H hapten containing monomeric unit in the copolymer was in the range of 0.005 to 0.54. Deprotection of the obtained copolymers and the recognition of the polymeric material by GalNAc transferase were also atempted.

16.9
REACTIONS AT THE ANOMERIC C-2 CENTER OF 1-METHYLENE SUGARS AND HEPTULOSES

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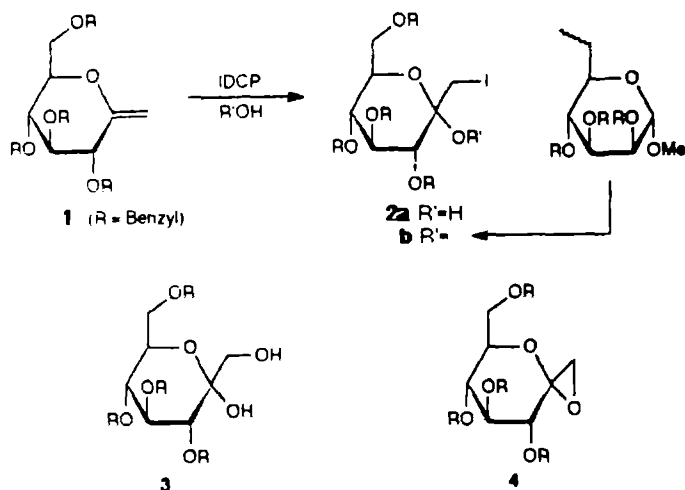
As part of a programme directed towards the synthesis of bisubstrate analog inhibitors of UDP-glucuronosyltransferase and other glycosyltransferases, we report here on the results of the iodonium ion mediated addition reaction to the methylene moiety of readily available 2,6-anhydro-3,4,5,7-tetra-O-benzyl-1-deoxy-D-gluco-hept-1-enitol (**1**) and on the results of glycosylation reactions at the anomeric center of heptuloses.

In the presence of water or an alcohol (e.g. methyl 2,3,4-tri-O-benzyl-α-D-mannopyranoside), reaction of **1** with iodonium *sym*-dicollidine perchlorate (IDCP) afforded the respective α-1-iodoheptulosides (**2**) in good yield (65-95%). The presence of a participating group (e.g. O-benzoyl) at C-3 gave rise, after a iodonium ion promoted reaction of **1** with the same mannopyranoside, to the formation of α- and β-heptulosides. Glycolation of **1** with N-methyl-morpholine-N-oxide and

catalytic osmium tetroxide resulted in the formation of the corresponding diol **3** (yield: 96%). Acetylation of **3**, followed by treatment with $TiBr_4$, gave a labile glycosyl bromide, which could be used in a coupling reaction with the above mentioned mannopyranoside in the presence of $Hg(CN)_2$ to afford an α-linked dimer (yield: 60%). In addition, glycosylation reactions with the corresponding glycosyl fluoride, using $Zr(Cp)_2Cl_2$ and $AgBF_4$ as catalysts, also afforded exclusively α-glycosides. Finally, addition of Amberlite 400 IRA (OH-form) to a solution of 1-iodoheptulose **2a** in methanol gave the anomerically pure spiroepoxide **4** (yield: 96%). Opening of the epoxide function may give access to a wide array of interesting compounds.



16.10
IODONIUM-ION ASSISTED STEREOSPECIFIC GLYCOSIDATION: SYNTHESIS OF OLIGOSACCHARIDES CONTAINING α(1–4)-LINKED L-FUCOPYRANOSYL UNITS

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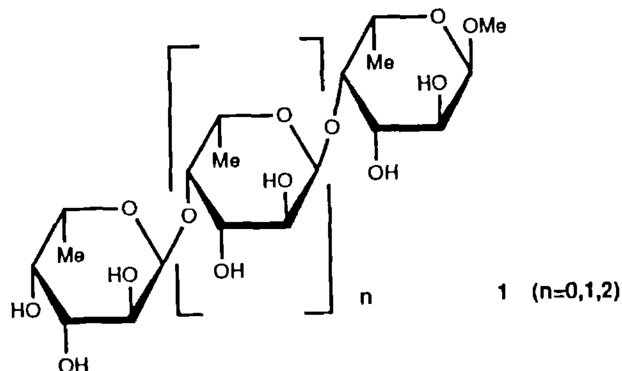
Contamination of stored grain, fruits and vegetables by mould species belonging to the order of *Mucorales*, some members of which may also cause mucormycosis in humans, is responsible for a considerable amount of food spoilage. Some years ago, Notermans *et al.* proposed a method in which the immunochemical properties of the extracellular polysaccharides (EPS) of the *Mucorales* species could be used for their detection. Preliminairy structural studies on the *Mucorales* EPS revealed the presence of D-glucuronic acid, D-mannopyranoside and L-fucopyranoside residues. In addition, it was found *inter alia* that the α(1–4) linked L-fucopyranoside dimer **1** (**n=O**) interfered in an enzyme linked immunosorbent assay (ELISA) with antibodies raised against *Mucorales* EPS. In order to assess the possible role of the L-fucose residues in the immunodominant part of the *Mucorales* EPS, longer oligosaccharides of α(1–4)-linked L-fucopyranosides **1** (**n=0,1,2**) were synthesized. Recently, we reported that iodonium dicollidine perchlorate (IDCP) mediated glycosidation of glycosyl acceptors with ethyl 1-thioglycosyl donors, having a non participating ether group at C-2, proceeds in a high yield and with the predominant formation of 1,2-*cis* linkages. According to this procedure the fully protected tetramer could be obtained by extending the terminal glycosyl acceptor methyl 2,3-di-O-benzyl-α-L-fucopyranoside three times with the non-terminal glycosyl donor ethyl 4-O-acetyl-2,3-di-O-benzyl-1-thio-β-L-fucopyranoside and intermittent removal of the C-4 acetyl group in the intermediate fully protected dimer and trimer. The exclusive formation of 1,2-*cis* linked oligomers could be explained by through-bond interactions exerted by the electron-withdrawing C-4 acyl group in the glycosyl donor.



16.11
SYNTHESIS OF A TETRAMERIC UNIT FROM MYCOBACTERIUM AVIUM VIA AN IODONIUM PROMOTED GLYCOSIDATION APPROACH

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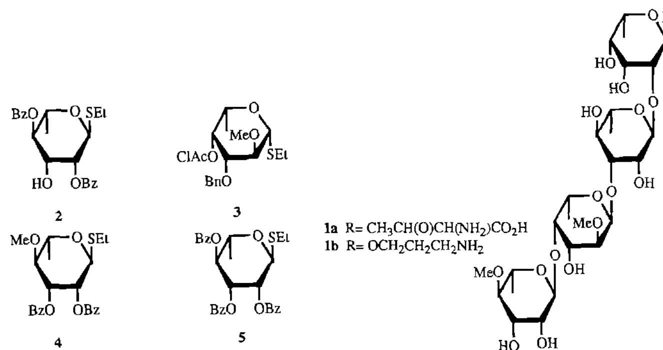
A large number of patients with acquired immune deficiency syndrome develop infections due to serotype 4 of the M. avium complex. It has been determined that the immunizing epitope of M. avium resides in the polysaccharide component of the surface-located glycopeptidolipid antigens and may induce cell-mediated immunologic responses.

The structure of the haptenic oligosaccharide of serotype 4 of M. avium was established as 4-O-Me-L-Rhap-(α1–4)-2-O-Me-L-Fucp-(α1–3)-L-Rhap-(α1–2)-6-deoxy-L-Tal and is attached to the hydroxyl function of D-allo-threonine (**1a**).

With the objective to develop diagnostica for identification of mycobacterial infections in AIDS patients, fragment**1b** was synthesized starting from properly protected rhamnose and fucose ethylthioglycosides (i.e. eompound **2.5**). An iodonium promoted glycosidation procedure was used for the introduction of the glycosidic linkages.



16.12
GLYCOSIDASE-CATALYSED SYNTHESIS OF DISACCHARIDE GLYCOSIDES

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Glycosidases have several advantages as catalysts compared with glycosyltransferases: they are present in relatively high concentrations in

biological material and are easy to prepare; moreover, the substrates are easily available.

Several of the disaccharide sequences present in glycoconjugates have been synthesised with glycosidases as catalysts (K. Nilsson, Trends in Biotechnology, 256–264, 1988; K. Nilsson, Carbohydr. Res. volumes 167, 188 and 203). However, yields of syntheses have generally been in the range 10–50%, which is lower than yields reported with glycosyl-transferases (30–95%).

In this paper the influence of various parameters on yield will be discussed. It was found that the yields of glycosidase-catalysed syntheses can be considerably improved employing facile methods. Thus, we obtained up to 90% yield of disaccharide formation in some of the reactions.

16.13 SYNTHESIS OF 1,2-*TRANS* LINKED GLYCOSYL PHOSPHATES *VIA* IODONIUM ION MEDIATED CONDENSATION OF ETHYL 1-THIO GLYCOSIDES WITH DIBENZYL PHOSPHATE

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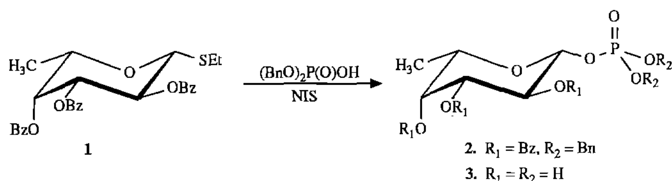
Phosphate esters attached to the anomeric centre of carbohydrates are frequently present as components of bacterial cell walls and are important intermediates in enzymatic glycosyl transfer processes. For instance, β-L-fucopyranosyl phosphate **3** is an key-intermediate in the preparation of guanosine 5′-(β-L-fucopyranosyl)-diphosphate (GDP-fucose).

As part of a programme directed to the activation of ethyl 1-thioglycosides with iodonium ions[1,2], we now report that acylated ethyl 1-thioglycosides can be readily converted into glycosyl phosphates by *N*-iodo-succinimide mediated reaction with dibenzyl phosphate.



1) G.H. Veeneman and J.H. Van Boom, *Tetrahedron Lett.*, **31**, 275 (1990).

2) G.H. Veeneman, S.H. Van Leeuwen and J.H. Van Boom, Tetrahedron Lett., **31**, 1331 (1990).