HYDROLYSIS OF GLYCOSPHINGOLIPIDS BY BACTERIAL GLYCOSIDE HYDROLASES

Biochemical Aspects of Host–Microbial Interactions in the Gastrointestinal Tract

by

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I. Larson, G., Watsfeldt, P., Falk, P., Leffler, H. and Koprowski, H. (1987) Fecal excretion of intestinal glycosphingolipids by new-borns and young children. *FEBS Letters* **214,** 41-44

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V. Falk, P., Hoskins, L.C. Lindstedt, R., Svanborg, C. and Larson, G. Deantigenation of human erythrocytes by bacterial glycosidases - evidence for the non-involvement of medium-sized glycosphingolipids in the *Dolichos biflorus* lectin hemagglutination. Submitted manuscript

ABSTRACT

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Complex carbohydrates, and among them glycosphingolipids, are ubiquitous in the epithelial cell membranes of the gastrointestinal tract and high amounts are continuously being shed into the gut lumen. Subpopulations of bacteria of the human fecal flora are highly efficient in hydrolysis of gut mucin glycoproteins. Through the production of glycoside hydrolases bacteria can influence the chemical environment in their habitat. Utilisation of carbohydrates is of importance for intestinal ecological stability. This study investigated intestinal bacterial hydrolysis of glycosphingolipids *in vivo* and *in vitro*.

Glycosphingolipid hydrolysis was measured by analysis of the glycosylceramide residues remaining after various times of incubation with bacterial glycoside hydrolases using thin-layer chromatography with chemical and immunological detection.

The fecal excretion of glycosphingolipids was followed in a child from birth through the weaning period. The fucosylated blood group active fecal glycosphingolipids corresponded to the glycosphingolipids of intestinal epithelial cell membranes as long as breast milk was the only source of nutrients. As soon as solid food was introduced the fucosylated structures diminished and was replaced by lactosylceramide as the dominating glycolipid. This pattern was conserved up to two years of age.

Glycoside hydrolase mixtures from five strains of mucin degrading human fecal bacteria, of the *Ruminococcus* and *Bifidobacterium* genera, were tested for the ability to hydrolyse acid and non-acid glycosphingolipids and synthetic *para*-nitrophenyl (pnp) glycosides. All enzyme mixtures contained exo-β-galactosidases and exo-β-*N*-acetylglucosaminidases, and there was circumstancial evidence for the presence of endo-β-glycoside hydrolase activities. There was a clear preference for lactoseries type 1 and type 2 core chain based oligosaccharides, but the enzymes of *R. gnavus* strain VI-268 only hydrolysed *neo*lactotetraosylceramide (type 2 chain). Lactosylceramide was a dominating final product of glycosphingolipids solubilised in Triton X-100. However, enzyme activities and apparent specficities were critically dependent on type and amount of detergent. Thus, conversion of lactosylceramide to glucosylceramide was efficiently promoted in the presence of sodium bile salts in concentrations at or above their respective critical micellar concentrations.

The *R. torques* strains VIII-239 and IX-70 produced α-glycoside hydrolases releasing GalNAcα1- residues from mono- and difucosylated blood group A determinants, Forssman antigen, and from GalNAcα1-pnp. Weak α-*N*-acetylgalactosaminidases, active on monofucosyl A were found in *R. gnavus* VI-268 and *B. infantis* VIII-240. *R. gnavus* VI-268 was the only strain producing α-galactosidases selectively active on Galα1-3 residues. All five strains produced α-fucosidases hydrolysing Fucα1-2, Fucα1-3, and Fucα1-4 linkages in blood group H(Le^d), X, Y, Le^a, and Le^b glycolipids but not Fucα1-pnp. Neuraminidases releasing α2-3 and α2-8 linked sialic acids were produced by all strains.

The enzyme mixture of *R. torques* IX-70 and partially purified α-*N*-acetylgalactosaminidase fractions from this strain converted blood group A_1 erythrocytes to A_2 reactive cells, as defined by the loss of *Dolichos biflorus* agglutinability and a conserved agglutinability with anti-A antibodies. Only the unfractionated IX-70 mixture hydrolysed blood group A and H active glycosphingolipids with 6 to 12 sugars in the cell membrane.

Bacterial glycosphingolipid degradation could have an impact on intestinal ecological stability through the production of nutrients (monosaccharides), growth promoting factors (di- and oligosaccharides) and potential adhesion sites for intestinal bacteria (lactosylceramide).

Key words: Glycosphingolipids, glycoside hydrolases, bacteria, gastrointestinal, ecology.

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ERRATA

Page 6	Figure 1	phospholipid (PL) is represented in the figure by the symbol 
Page 7,	line 11	"signalling" should be "signaling"
Page 11,	figure legend, line 7	"hedding" should be "shedding"
Page 12,	line 2	"utilize" should be "utilise"
Page 14,	line 15	"intestinal" should be "and intestinal"
Page 19,	line 11	"albuminconjugated" should be "albumin conjugated"
Page 21,	line 2	"continuous culture" should be "continuous flow culture"
	line 22	"1956" should be "1957"
	line 37	"milleu" should be "milieu"
Page 22,	line 19	"oligosacchride" should be "oligosaccharide"
	line 25	"struturally" should be "structurally"
Page 23,	line 14	"HPLC" should be "high performance liquid chromatography (HPLC)"
Page 24,	line 31	"equippment" should be "equipment"
Page 25,	line 2	"1991" should be "1989"
	line 8	"products part" should be "products"
Page 31,	line 32	"implies" should be "imply"
Page 34,	line 2	"environmemt" should be "environment"
	line 18	"concentrtions" should be "concentrations"
	line 21	"1981" should be "1984"
	line 25	"*Escerichia*" should be "*Escherichia*"
Page 37,	line 12	"enables" should be "enable"
Page 38,	line 1	"pathlogical" should be "pathological"
	line 12	"1986" should be "1988"
	line 16	"epithlium" should be "epithelium"
	line 29	"and has" should be "and it has"
Page 41,	line 21	"1981" should be "1984"
Page 42,	line 44	"1956" should be "1957"
Page 44,	lines 21–23	correct reference is "Freter, R., Brickner, H., Fekete, J., Vickerman, M.M. and Carey, K.E. (1983) Survival and implantation of *Escherichia coli* in the intestinal tract. *Infection and Immunity* 39, 686–703"
Page 46,	line 6	"1991" should be "1989"
	line 49	"75, 7520–7524" should be "72, 2520–2524"
Page 48,	line 12	year of publication: 1984
	line 33	"*Biochimica et Biophysica Acta*" should be "*Archives of Biochemistry and Biophysics*"
Page 49,	line 19	"in press" should be " 59, 1086–1092"
	line 30	"Cummings, J.H., MacFarlane, S." should be "Hay, S."
Page 53,	line 19	"1986" should be "1988"
Page 54,	line 40	"*Journal of Microbiology*" should be "*Journal of Clinical Microbiology*"

Paper IV

Page 16,	line 25	"a constituting" should be "a diet constituting"
Page 17,	line 8	"(45, 46)" should be "(47, 48)"

Paper V

Page 6,	line 21	" hydroxide for" should be "hydroxide in methanol for"
	line 24	"21" should be "20"

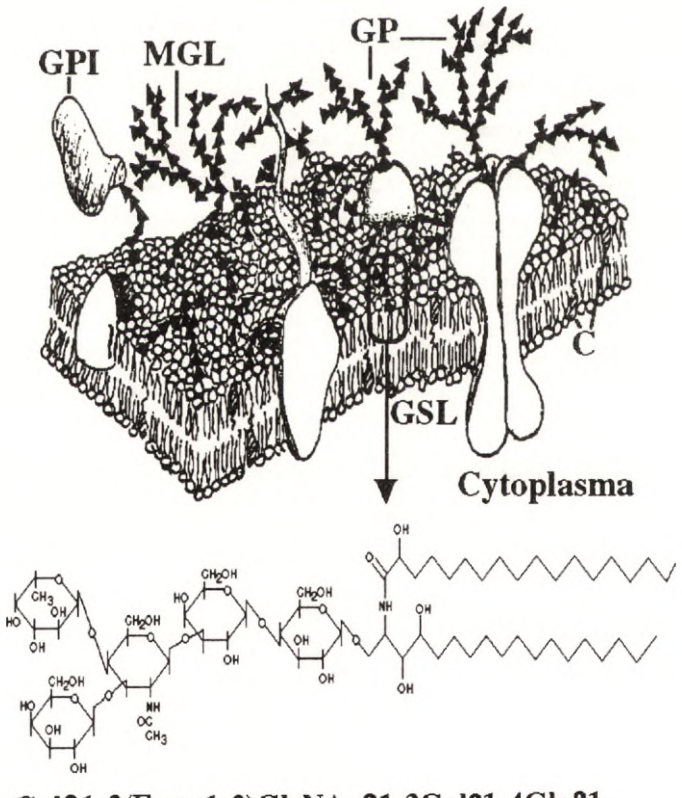
Minor spelling mistakes in the reference list have been omitted.

CONTENTS

1. INTRODUCTION

1.1 Glycosphingolipids - Principal Structure and Biological Implications

Glycosphingolipids are the glycosides of *N*-acylsphingosines (ceramides) (Hakomori 1983). They are primarily found in the outer leaflet of the plasma membrane bilayer (Figure 1). Recent studies have shown that glycosphingolipids are localised in intracellular membranes as well (Symington et al. 1987). This structurally highly variable group of compounds, with glycosidic chains of 1 up to 60 monosaccharides, have been isolated from a large repertoire of tissues and species (Hakomori 1983). They are considered to be present in all eucaryotic cells and show a high degree of species, tissue and developmental variations. The synthesis of glycosphingolipids from ceramides are genetically controlled (Watkins 1986; Oriol et al. 1986) through the production of specific glycosyl transferases (Paulson & Colley 1989) adding activated sugars in a step-by-step elongation process (Watanabe & Hakomori 1976; Watkins 1986; Pohlentz et al. 1988).



Galβ1-3(Fucα1-2)GlcNAcβ1-3Galβ1-4Glcβ1-

Figure 1. Schematic illustration of a part of a plasma cell membrane with different types of glycoconjugates. GP, glycoprotein; MGL, megaloglycolipid; GPI, glycophosphoinositol linked protein. Glycosphingolipids (GSL) are represented by a pentaglycosylceramide carrying the blood group Le^a determinant linked to ceramide part with trihydroxysphingosine (phytosphingosine) and a h18:0 fatty acid. PL, phospholipid; C, cholesterol. (Cell membrane redrawn and modified from Hakomori 1986).

In analogy with the specific synthesis, there are also specific glycoside hydrolases responsible for the degradation of glycosphingolipids (Wan Ho et al. 1977; Cabezas et al. 1983; Conzelmann & Sandhoff 1987) in eucaryotic cells. These acid lysosomal enzymes are crucial for the physiological processing of lipid bound oligosaccharides. The lipid storage diseases are caused by inherited defects in the lysosomal glycoside hydrolases.

Glycosphingolipids are carriers of histo-blood group antigens (Clausen & Hakomori 1989) (Table 1). Apart from stabilising the lipid bilayer matrix (Yamakawa & Nagai 1978) recent studies indicate several functions of glycosphingolipids of eucaryotic cells (reviewed by Hakomori 1990). It has for instance been suggested that they play a role in transmembrane signalling processes through the sphingosine residue of their ceramide parts (Hannun & Bell 1989). Glycosphingolipids have been suggested to participate in specific cell-cell interactions in embryogenesis (Fenderson et al. 1984; Bird & Kimber 1984; Fenderson et al. 1990), between tumor cells (Kojima & Hakomori 1989), as well as in inflammatory response (Phillips et al. 1990; Walz et al. 1990), Cell-bacterial interactions (Larson 1989; section 1.7) have also been associated with glycosphingolipids.

Table 1. Histo-blood group antigenic determinants of the ABH(O) and Lewis types

Antigen	Structure
A	GalNAcα1-3(Fucα1-2)Galβ1-
B	Galα1-3(Fucα1-2)Galβ1-
H (O)	Fucα1-2Galβ1-
Le^d	Fucα1-2Galβ1-3GlcNAcβ1-
Le^a	Galβ1-3(Fucα1-4)GlcNAcβ1-
Le^b	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-
Le^x (X)	Galβ1-4(Fucα1-3)GlcNAcβ1-
Le^y (Y)	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-
ALe^b	GalNAcα1-3(Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-
BLe^b	Galα1-3(Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-
ALe^y	GalNAcα1-3(Fucα1-2)Galβ1-4(Fucα1-3)GlcNAcβ1-
BLe^y	Galα1-3(Fucα1-2)Galβ1-4(Fucα1-3)GlcNAcβ1-

1.2 Man and microbes

The rationales for and consequences of the co-existance of man and microbes (microorganisms) have intrigued scientists over the last 150 years. Antoine van Leeuwenhoek opened the door to the world of microbes in the late 17th century through his development of the microscope. This new technique enabled him to demonstrate the presence of microbes in our environment and also in the human body, e.g. the oral cavity. In the mid 18th century Lazzaro Spallanzani proved the microbes to be a hitherto unknown form of life with the ability of being distributed by air and multiplying through cell division, an at that time extremely radical perspective especially to be put foreward by a catholic priest. He was also the first to postulate that microbes could live and multiply without the presence of oxygen. In spite of the early reports from pioneers like van Leeuwenhoek and Spallanzani it was not until in the middle of the last century that scientists started investigating the effects of microbes on humans and other mammals. Louis Pasteur established the connection between infectious diseases and microbes and made important contributions to the development of vaccines. Robert Koch isolated the anthrax bacillus and tuberculosis pathogen and developed methods for culturing and studying microbes. He also postulated the classical criteria for the causative relationship between microorganisms and disease, i.e. i) regular isolation of the microorganism from cases of illness, ii) the microorganism must be isolated and grown in pure culture *in vitro*, iii) disease must occur upon inoculation of such a pure culture and iv) the microorganism must be isolated from such an experimentally induced disease. In 1909 Paul Ehrlich synthesised the first antimicrobial drug, an arsenic derivative known as compound 606 or Salvarsan, successfully used in the treatment of syphilis. The field of microbiology exploded and the scientists became heroes as the causes of many of the deadly epidemics that regularly harassed the world were unravelled and cures to many of them could be offered (De Kruif 1926).

Even though research initially focused on pathogens and diseases, scientists also started considering the importance of host-microbial interactions in health. Thus Pasteur and others postulated that the presence of microbes was necessary for human life (Schottelius 1902) (a statement that, although not completely undisputable, still have a lot of bearing today). Elie Metchnikoff claimed that the composition of the intestinal flora was crucial for the wellbeing of the host and stressed the importance of bacteria-bacterial interactions (Metchnikoff 1908; 1910). Another scientist that early took an interest in the normal intestinal flora was Theodor Escherich who studied the fecal bacterial contents of neonates and breast-fed infants (Escherich 1885). He stated that his studies were based on "the conviction that the accurate knowledge of these conditions is essential, for the

understanding of not only the physiology of digestion, in which the decomposition processes in the intestines are still an unknown quantity, but also the pathology and therapy of microbial intestinal diseases".

The hundred years that have passed have proven Escherich's statement to be altogether accurate. Numerous studies over the last 40 years have established the impact of the normal bacterial flora on the infectious defence of the host (reviewed by van der Waaij, 1989). An ever intensified use of more and more potent antibiotics have illustrated the consequences of manipulating the resident ecological systems and led to a shift in the panorama of infectious diseases. As most of the classical, and previously often fatal infections, now can be cured, many of the infections that offers a therapeutic problem in the Western world today are caused by resistant minor subpopulations of the indigenous flora. These bacteria starts to multiply as the normally stable and down regulating ecological systems are disrupted as a result of antibiotic therapy (Tillotson & Finland 1969; Boriello & Larson 1981; Wilson et al. 1981). Multiresistant strains are especially frequent within hospitals and primarily in intensive care units (van der Waaij 1989; Hersh et al. 1965; Meyerowitz et al. 1971) probably due to an extensive use of broad spectrum antibiotics. Broad spectrum anitbiotics are often used in combinations with cytotoxic and immunosuppressive drugs, and total parenteral nutrition in the treatment of severely diseased patients with i.e. malignancies, organ transplants and prematurities. This will make the host even more vulnerable to opportunistic infections primarily from the intestinal flora (Meyers & Atkinson 1983; Tutschka 1988).

A vital part of the therapy of these conditions is the reconstitution of the indigenous intestinal flora (Heidt et al. 1981). A protective role of the resident intestinal bacteria is evident, excluding and down-regulating potential pathogens from the intestinal habitats of mammals (Tannock 1987; van der Waaij 1989). However, the molecular bases for the bacteria-bacterial (Mahe et al. 1986; Yurdusev et al. 1989) and host-bacterial interactions are largely unknown. It is generally thought that competition between bacterial strains for space and nutrients is of major importance. The chemical environment, as governed by the microbial interactions with the host and metabolism of associated bacteria is also a possible regulating factor (Savage 1977; Midtvedt 1986).

The most classical example of superinfections caused by an endogenous opportunistic species is perhaps the severe colitis caused by *Clostridium difficile* and its toxins (pseudomembranous colitis; PMC). Carriage of this bacteria is, for unknown reasons, frequent in infants (14%) as compared to adults (3%). The lack of association between colonisation and disease in children is unclear, but a neutralising effect on toxins A and B have been demonstrated for human colostrum (Kim et al 1984). The typical *C. difficile* pseudomembranous colitis occurs almost exclusively during or after antibiotic treatment and is associated

with an overgrowth of the bacteria. Almost any type of antibiotic can induce PMC and it has also been associated to antitumor chemotherapy (Lyerly et al. 1988). Current therapy for PMC includes antibiotics, primarily vancomycin and metronidazole, which is suboptimal since both these drugs also can trigger the disease. However, successful attempts to treat relapsing PMC and re-establishing the indigenous flora have been reported using local administration of total fecal flora (Schwan et al. 1984), non-toxigenic *C. difficile* strains (Seal et al. 1987), human lactobacillus GG strains (Gorbach et al. 1987) and *Streptococcus faecium* (Öhman et al. 1991).

1.3 The Human Gastrointestinal Tract - Digestive and Barrier Functions

The gastrointestinal tract is responsible for the primary processing and uptake of nutrients in the human body. This is normally achieved through the combined effects of extreme ventricular acidity and pepsinogen secretion, secretion of bile and pancreatic juice and mucosal associated hydrolytic enzymes for proteins, lipids and carbohydrates. Absorbtion occurs selectively at the epithelial cell surfaces of the small and large intestine (Guyton 1986). Gastrointestinal motility also plays an important role in these processes by mixing the solid food particles with the intestinal secretions and by transporting the nutrients to the respective parts of the intestines where digestion and absorption takes place. Peristalsis also removes the non-absorbed waste materials from the intestines. Two main cell types, in addition to neuroendocrine and lymphoid cells, are found in the intestinal epithelium, i.e. enterocytes and goblet cells. The enterocytes have specific digestive functions for carbohydrates (Semenza 1976), peptides, and to a limited extent also lipids. These functions are exerted by specific plasma membrane associated hydrolases. Absorbtion of water, ions, carbohydrates, amino acids and lipids is also performed by these cells. In addition they are responsible for the re-assembly of triglycerides and formation of chylomicrons after absorbtion of glycerol and fatty acids over the apical cell membranes, and for the uptake of nutrients to lymph and blood across the basal membranes for further transport to the tissues of the body. As will be evident later (section 1.4) these cells are rich in protein and lipid linked complex carbohydrates (Keränen 1975; Siddiqui et al. 1978, Björk et al. 1987; Holgersson et al. 1988; Finne et al. 1989). Goblet cells are continuously secreting mucus that covers the entire epithelial surface of the gastrointestinal tract. Mucus is a thick secretion composed mainly of water, electrolytes and a highly variable mixture of complex glycoproteins (Podolsky 1985; Capon et al. 1989; Hounsell et al. 1989). The characteristics of mucus makes it both an excellent lubricant and a barrier for the gut epithelium.

The gastrointestinal epithelium is the largest human surface facing the environment (Figure 2). This means that the gastrointestinal tract, at the same

time as being an efficient absorbtive organ, also has to function as a barrier excluding potentially toxic agents from the human body and preventing the establishment of invasive pathogenic microbes. The mechanisms by which the alimentary tract contributes to the exclusion of pathogens are of both mechanical, biochemical and immunological origin (Perman 1989; Walker & Owen 1990; Mellander et al. 1985; Brandtzaeg et al. 1987). However, although endogenous factors like peristalsis, mucus coat, bile acids, secretory IgA and phagocytes are important for the composition of the intestinal microbiota, the host is also dependent on the cooperation of the resident bacteria of the gut for an efficient local infectious defence.

Even though both digestive and absorbtive functions of the intestines appears already in early fetal life (Grand et al. 1976) the newborn infant is highly sensitive to provocations with diets other than breast milk or specially adapted formula and to potentially pathogenic microorganisms.

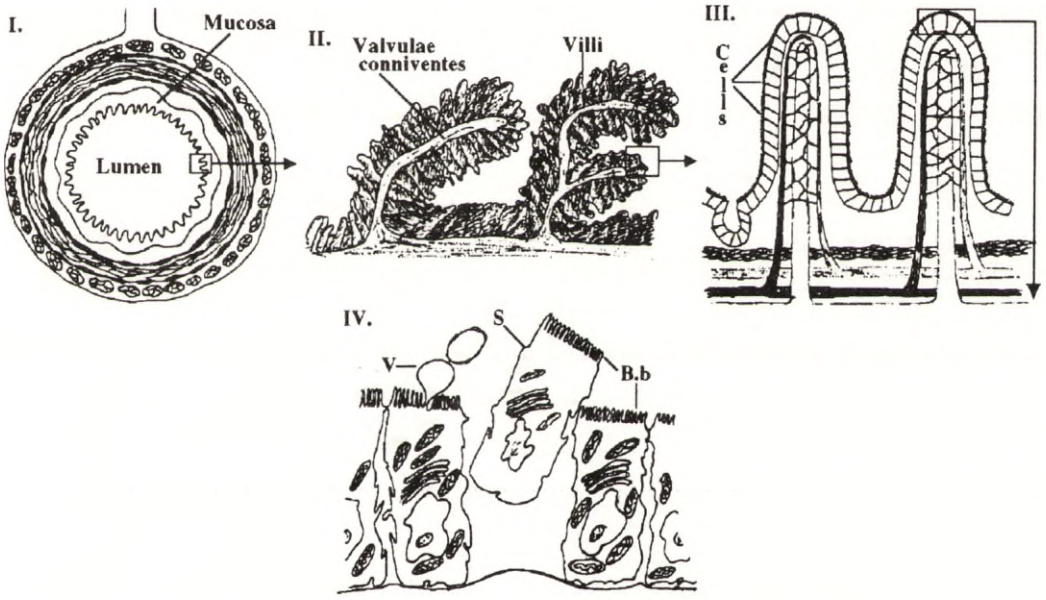


Figure 2. Schematic illustration of how the morphology of the small intestine leads to an extreme surface enlargement. The foldings of the intestinal mucosa (I) are covered with villi (II) on which the cells of the intestinal epithelium grow (III). The brush border (B.b) of the enterocytes apical plasma membranes (IV) constitutes the last and most potent step of the surface amplification. Goblet cells and mucus layer have been omitted from the picture. Picture IV also examplify different ways that membrane bound glycoconjugates can enter the intestinal lumen. S, hedding of entire cells at the villus tip due to a continuous regeneration of the epithelium from stem cells in the crypt region inbetween the villi. V, micro-vesiculation from the apical cell membrane. (Pictures redrawn and modified from; I, Ganong 1986; II, Guyton 1986; III, Vander et al. 1980; IV, Ross & Reith 1985)

1.4 Gastrointestinal Glycoconjugates with Emphasis on Glycosphingolipids

Since there are considerable variations among bacteria in their abilities to utilize carbohydrates as energy source and attachment sites (see sections 1.7 and 1.8), differences in oligosaccharide composition might have an impact on the regional composition of the intestinal microbiota.

The alimentary tract is a rich source of various forms of carbohydrates. In the cell membrane of the enterocytes oligosaccharides are associated with proteins (Finne et al. 1989) and lipids (Keränen 1975; Siddiqui et al. 1978; Björk et al. 1987; Holgersson et al 1988). A continuous sloughing of up to 55 million epithelial cells per minute (Croft & Cotton 1973) and a shedding of apical membrane vesicles, as demonstrated in rabbit caecum (Hill 1985), implies that endogenous membrane bound glycoconjugates are abundant in the lumen of the intestinal tract. Apart from cellbound glycoconjugates the mucus coat, covering the entire epithelial cell lining, contributes to the endogenous intestinal carbohydrates (Podolsky 1985; Capon et al. 1989; Hounsell et al. 1989). A summary of different glycoconjugates carrying histo-blood group antigenic determinants is shown in Table 2.

Extensive studies of intestinal glycosphingolipids of both humans and other mammals (reviewed by Hansson 1988) have illustrated regional differences as well as developmentally associated changes in carbohydrate expression in the gastrointestinal tract. Studies in the rat have shown distinct differences in lipid bound oligosaccharide composition between cells of epithelial and stromal origin in both small (Breimer et al. 1982a; Ångström et al. 1982a) and large (Hansson et al. 1984a) intestine. Blood group active fucolipids and gangliosides with *N*-glycoloylneuraminic acid were confined mainly to the epithelial compartments whereas globoseries glycosphingolipids and gangliosides with *N*-acetylneuraminic acids were exclusively of nonepithelial origin. A cellular polarisation of fucosylated glycosphingolipids and gangliosides with *N*-glycoloylneuraminic acid to the lumenal membrane of rat small intestinal enterocytes has also been demonstrated (Hansson 1983). Studies of glycolipid changes in cell maturation along the crypt-villus axis (Glickman & Bouhours 1976; Bouhours & Glickman 1976; 1977; Breimer et. al 1981) demonstrated an increase in ceramide hydroxylation., in glucosylceramide, and in *N*-glycoloylneuraminic acid containing gangliosides, but no differences in blood group active fucolipids. In contrast, qualitative changes in expression of blood group H (Bouhours & Bouhours 1985) and A active glycosphingolipids were found both in the proximal (Bouhours et al. 1990a) and distal (Bouhours et al. 1990b) parts of rat small intestine during the third week of life.

Table 2. Classification of histo-blood group carrying core glycoconjugates

Glycoconjugate type	Inner core	Peripheral core	References concerning the intestines
Glycoproteins			
N-linked	GlcNAcβ1-*N*-Asn	type 1 (Galβ1-3GlcNAcβ1-) type 2 (Galβ1-4GlcNAcβ1-)	(Finne et al. 1989)
O-linked	GalNAcα1-O-Ser/Thr (mucin type)	type 1 (Galβ1-3GlcNAcβ1-) type 2 (Galβ1-4GlcNAcβ1-) type 3 (Galβ1-3GalNAcα1-) type 4 (Galβ1-3GalNAcβ1-)	(Podolsky 1985; Capon et al. 1989; Hounsell et al. 1989)
Glycosphingolipids			
Lactosylceramide	Galβ1-4Glcβ1-1Cer (Lacβ1-1Cer)		(Björk et al. 1987)
Lacto-series	GlcNAcβ1-3Lacβ1-1Cer	type 1(Galβ1-3GlcNAcβ1-) type 2 (Galβ1-4GlcNAcβ1-) type 3 (Galβ1-3GalNAcα1-)	(Karlsson & Larson 1981; Björk et al. 1987)
Globo-series	Galα1-4Lacβ1-1Cer	type 4 (Galβ1-3GalNAcβ1-)	(Larson 1986; Larson et al. 1990)
*iso*Globo-series	Galα1-3Lacβ1-1Cer		(Hansson et al. 1987) (rat gastric mucosa)
Ganglio-series	GalNAcβ1-3Lacβ1-1Cer	type 4 (Galβ1-3GalNAcβ1-)	

(Modified from Clausen & Hakomori 1989)

Studies on human small and large intestinal specimens have established the presence of complex mixtures of acid (Keränen 1975; Holgersson et al. 1988) and non-acid (Siddiqui 1978; Björk et al. 1987; Holgersson et al. 1988) glycosphingolipids. Distinct differences between epithelial and stromal tissues as well as a composition of fucosylated compounds corresponding to the individuals blood group have also been demonstrated (Holgersson et al. 1988). Lactosylceramide was a minor component of the epithelial tissues in all cases but one. This unexpected dominance of lactosylceramide in small intestinal epithelial cells found in one individual was, based on the medical record, speculated to be caused by bacterial glycoside hydrolases (Björk et al. 1987).

Due to the problems involved in obtaining fresh human intestinal tissues for analysis of the intestinal glycosylation pattern it would be desirable to find an alternative source that mirror the glycoconjugate composition of the gastrointestinal cell membranes. Glycosphingolipids from fecal material from germ-free and conventional rats have been analysed and compared to the glycosphingolipid patterns from stomach, small and large intestine (Gustavsson et

al. 1986). Intestinal glycosphingolipids were chemically and immunologically very similar between the two groups of rats, apart from the presence of GM3 with *N*-glycoloylneuraminic acid in conventional rats. Feces of conventional rats retained a complete pattern of blood group A-, B- and H-active glycosphingolipids as found in sterile feces. This was unexpected with regard to previous reports (Hoskins & Zamcheck 1968). However, conventional feces contained less GM3 and more lactosylceramide of the same ceramide species, probably as a result of specific bacterial degradation. Feces from germ-free rats has been used to follow developmental changes in glycosphingolipid patterns seen in epithelial cells (Bouhours & Bouhours 1981; Larson & Midtvedt 1989) and essentially confirmed that the changes are independent of the presence of a bacterial flora. This is also in agreement with the experiences from mouse intestine (Sato et al. 1982).

The glycoconjugates of meconium, the first sterile stools of the newborn child, have been extensively studied. This material is composed mainly of sloughed cells from the fetal alimentary tract, intestinal secretions, but also swallowed material from the amniotic fluid. It is therefore a useful source in studies of the glycosylation pattern of fetal enterocytes (Karlsson & Larson 1979). Meconium contains complex mixtures of acid (Magnani et al. 1982; Prieto & Smith 1986; Fredman et al. 1989) and non-acid glycosphingolipids with 1 up to at least 11 sugars and with large amounts of blood group active fucosylated structures correlating to the blood group and secretor status of the individual (Karlsson & Larson 1978, 1981a, 1981b; Ångström et al 1982b, 1982c).

The protein linked (Capon et al. 1989; Hounsell et al. 1989) and free oligosaccharides (Herlant-Peers et al 1981) of human meconium have been found to be composed of complex carbohydrates containing both fucosylated, sialylated and sulfated structures.

1.5 Establishment of the Intestinal Microbiota

On no other occasions is the intestinal ecology, and thereby also the host, so vulnerable to invading pathogenic bacteria as during its developing stages. This occurs in two principally different situations, i.e. the physiological establishment of the microbial climax communities of the newborn childs exsterile intestines (Savage 1977) and the reestablishment of the flora after antibiotic therapy (Nord et al. 1986).

The microbial flora of the gastrointestinal tract comprise mainly of a complex mixture of procaryotic bacteria with minor amounts of eucaryotic cells, e.g. yeasts. The complexity of the intestinal microflora can only be estimated with available laboratory techniques (Olsen 1990). Intestinal bacteria belong to more than 400 different species (Moore & Holdeman 1974) and are thought to

outnumber the eucaryotic cells of the entire human body by one order of magnitude (Savage 1977).

The intestinal microflora is an open and dynamic ecological system living in metabolic balance with itself and its host. In this system a habitat at any given time will contain native (autochthonous, indigenous, resident) bacterial species and a variable set of transient (allochthonous) species that only temporarily fill an empty niche in the habitat. The allochthonous bacteria might be resident in niches higher up in the alimentary tract or be exogenous strains ingested with food or drink and should in either case not be considered as "normal" in the niche they presently occupy (Savage 1977). The term "normal microflora" might thus be misleading and "microbiota" has therefore been recommended as a more adequate way to denote the nonpathogenic microbial flora. The establishment of the intestinal microbiota is delicately regulated by both host derived and microbial factors (Alexander 1971) and is characterised by a successive colonisation of a series of bacterial strains during the first years of life (Savage 1977; Stark & Lee 1982; Lundequist et al. 1985). The control mechanisms of the initial phases of intestinal microbiotal development is still not clear but a role of the hosts humoral (Mellander et al. 1985) and cell mediated (van der Waaij 1988) immunosystem has been implicated.

The bacteria initially found associated to the sterile newborn child reflects the aerobic and anaerobic flora of the birth canal (Rotimi & Duerden 1981). The bacterial flora that first establish in high numbers in the intestines constitutes of *Escerichia coli, Clostridium welchii, Streptococci, Lactobacilli, Bacteroides* and *Bifidobacterium* strains (Smith & Crabb 1961; Rotimi & Duerden 1981). The composition of this microflora is almost identical among different mammals like cattle, pigs, sheep, cat, rabbit, mouse and human (Smith & Crabb 1961). *Bifidobacterium* strains then dominate the intestinal flora as long as the child is breast-fed (Bullen et al 1976) whereas *E. coli, Bacteroides, Clostridium* species and anaerobic streptococci have been reported to reach higher numbers in formula-fed infants (Stark & Lee 1982). Apart from diet, other exogenic factors like caesarean section (Bennet & Nord 1987), hospitalisation (Lefrock et al. 1979), antibiotic treatment (Bennet et al. 1986) and, in adults, inflammatory bowel disease (van de Merwe et al 1988) have profound effects on the intestinal bacterial ecology. At the time of weaning the microbiota grows more complex and metabolically active (Stark & Lee 1982; Midtvedt et al. 1988). The adult climax communities with a thousand-fold dominance of anaerobes, extreme complexity and a diverse set of biochemical activities may not establish for several years (Ellis-Pregler et al. 1975; Norin et al. 1985). Table 3 gives an overview of the dominating anaerobic bacterial genera in different parts of the adult gastrointestinal tract.

Table 3. Occurence of anaerobic bacteria (log_{10}/mL intestinal contents) in the human adult gastrointestinal tract.

Bacterial genera	Ventricle	Jejunum	Ileum	Colon
Peptococcus	0–4	0–6	2–6	2–8
Peptostreptococcus	0–4	0–6	2–6	2–8
Streptococcus	0–4	0–6	2–6	2–8
Acidaminococcus	0–4	0–6	2–6	2–8
Veillonella	0–4	0–6	2–6	2–8
Bifidobacterium	0–4	0–5	2–8	9–11
Eubacterium	-	0–3	2–4	4–6
Lactobacillus	0–4	0–4	3–7	4–8
Bacteroides	0–3	0–3	3–8	10–11
Fusobacterium	0–3	0–3	3–8	5–8
Total numbers	2–5	2–5	7–8	11–12

Adapted from Gustafsson 1982

1.6 Metabolic Aspects of the Intestinal Microbiota

One way of studying and describing the intestinal microbiota is by following the biochemical effects of the bacteria on their host. The intestinal microbiota is a highly metabolically active system which establishment can be monitored as a sequential introduction of new enzymatic activities into the gastrointestinal tract (Midtvedt 1986) paralleling the microbial succession. The term Microflora Associated Characteristics (MACs) has been presented to denote changes in the composition of the intestinal contents associated with colonisation by metabolically active microbial species (Midtvedt 1986). Germfree Associated Characteristics (GACs) are similarly used to describe the sterile situation. Among the typical MACs described are enzymatic modifications of steroids, degradation of mucins, inactivation of intestinal proteases and formation of short chain fatty acids (Midtvedt 1986; Midtvedt et al. 1988). There are also anatomical, histological and physiological changes like reduction in cecum size (Lindstedt et al 1965), morphological adaptation of the intestinal wall and increased cell turnover, as well as intensified muscular activity associated with the establishment of a physiological fecal flora (Midtvedt 1986).

These biochemical parameters are an important new and alternative tool to classical microbiological techniques for studying the status of the intestinal flora. Major effects on certain MACs have for instance been reported to be associated with peroral administration of antibiotics (Midtvedt et al. 1986).

1.7 Microflora Associated Aspects of Carbohydrates - Fermentation and Adhesion

Generation of energy (Fraenkel & Vinopal 1973; Doelle 1975; Salyers 1979) and attachment to surfaces (Savage 1987; Costerton 1983) are two crucial skills for bacteria in order to establish and survive in a specific habitat (Freter et al. 1983). The ability to utilise the glycoconjugates present in the environment is of importance for both of these processes.

A variety of complex carbohydrates enters the human colon. Some are of dietary origin whereas others are endogenous, i.e. goblet cell mucins and glycolipids, and glycoproteins from extruded epithelial cells. Since these compounds, in contrast to monosaccharides, are not absorbed in the small intestine, they are likely to be a primary source of carbon utilised for nutrients and energy by colonic bacteria. However, anaerobic bacteria utilising carbohydrates for their energy generating processes require monosaccharides. For this purpose some intestinal bacteria have developed a highly variable and ubiquitous set of monosaccharide releasing enzymes. These glycoside hydrolases will be described in the next section (1.8) whereas this section concentrates on the ways that the microbial flora can make use of carbohydrates.

Pasteur catalysed the birth of biochemical microbiology by his discovery of microbial fermentation in 1857. He described it as " la vie sans l'air" (life without air) since it can proceed in the abscence of oxygen. The breakdown of sugar into ethanol and carbon dioxide was for a long time the definition of fermentation. Today fermentation is defined as an ATP generating process in which organic compounds act as both donors of and acceptors for electrons.

The majority of the bacteria of the human colon are obligate anaerobes and most of them requires fermentable monosaccharides (Moore and Holdeman 1974; Holdeman et al. 1976). In fact, of the five dominating genera in the colon, i.e. *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus* and *Fusobacterium* (see Table 3), only the latter contains species that are predominantly non-saccharolytic (Salyers et al. 1977a). The repertoire of monosaccharides that can be fermented varies considerably among different bacterial species and determines to some extent the conditions under which bacteria will grow. This has been widely used as a mean of characterisation and identification of bacteria. Also, the ability to utilise unique monosaccharides in energy production probably provides an ecological advantage to a bacterial strain

(Freter et al. 1983). Anaerobic carbohydrate catabolism proceeds primarily via the glycolytic pathway and the hexose monophosphate shunt but also via unique strain specific metabolic steps (Fraenkel & Vinopal 1973; Doelle 1975). It also results in the production of short chain fatty acids thought to contribute to the regulation of the composition of the microbiota (Bullen et al. 1975; Perman 1989; Walker & Owen 1990).

Specific binding to carbohydrate epitopes was first demonstrated for the cholera toxin binding the sialic acid containing glycosphingolipid (ganglioside) GM1 (van Heyningen 1971; Holmgren et al. 1975). The number of known interactions between bacterial toxins and carbohydrates have been expanded through studies on *Escerichia coli* heat labile toxin binding GM1 (Moss et al. 1981; Griffiths et al. 1986), botulinum toxin (Simpson & Rapport 1971), and tetanus toxin (Van Heyningen 1974) binding polysialylated gangliosides. Recent reports also show binding of shiga toxin type 1 (Lindberg et al. 1987) to Gala1-4Gal containing glycolipids, *Escerichia coli* derived verotoxin (Waddell et al. 1990) binding to the globotriaosylceramide structure, and *Clostridium difficile* toxin A (Tucker & Wilkins 1991) binding to the histo-blood group determinants I, X, and Y.

Gangliosides have also been recognised as potential receptors for Sendai virus (Markwell et al. 1981; Holgersson et al. 1985) and Influenza virus (Bergelson et al. 1982; Huang 1983). Specific binding to non-acid glycosphingolipids has also been demonstrated for Sendai virus (Hansson et al. 1984).

Entamoeba histolytica, a protozoan being a major cause of infectious diseases in developing countries, also binds specifically to Gal- and GalNAc-terminated oligosaccharides of glycosphingolipids (Bailey et al. 1990) and glycoproteins (Ravdin et al. 1989).

Specific interaction between carbohydrates and bacteria are mediated by bacterial surface proteins called adhesins (Jones & Isaacson 1983). The first specific bacteria-carbohydrate interaction demonstrated was the D-mannose binding type 1 fimbriae which have been recognised since the 1950s by the ability of mannose to inhibit binding reactions of bacteria carrying this specific surface protein (reviewed by Duguid & Old 1980). In 1980 the first reports appeared establishing a specific binding of uro-pathogenic *Escherichia coli* to glycosphingolipids of the blood group P system (Leffler & Svanborg-Edén 1980; Källenius et al 1980). Using a novel thin-layer chromatography overlay technique (Magnani 1980; Hansson et al. 1985b) uro-pathogenic *E. coli* were shown to adhere to several slow moving structures in total glycolipid extracts from human kidney (Leffler et al. 1983) and the specific epitope was later defined as the globo-series characteristic Galα1-4Galβ1 sequence (Bock et al. 1985). The pap-adhesin of these bacteria, i.e. the P-fimbriae have been thoroughly characterised (Lund et al. 1987; Karr et al. 1989). Recent studies have demonstrated specific adhesion of

enterotoxigenic *E. coli* to the human colon carcinoma cell line Caco-2 (Darfeuille-Michaud et al. 1990). Mannose and Galα1-4Gal adhesion have been postulated to be of importance for adhesion of *E. coli* to human colonic epithelial cells (Wold et al. 1988). Resident *E. coli* strains of the colon appear to have the same serotypes and Galα1-4Gal binding specificities as uro-pathogenic strains (Agnes Wold, personal communication)

Lactosylceramide (Galβ1-4Glcβ1-1Cer) has been established as an *in vitro* receptor for many bacteria, among them several members of the intestinal microbiota (Hansson et al. 1983a), oral bacteria (Strömberg & Karlsson 1990) as well as several intestinal pathogens (Karlsson 1989). However, this interaction can not be easily inhibited by monovalent or by polyvalent albuminconjugated receptor oligosaccharides in solution (Strömberg & Karlsson 1990).

Bacteria associated with pneumonia, e.g. *Pseudomonas aeruginosa, Hemophilus influensae* and *Staphylococcus aureus* have been reported to bind glycosphingolipids containing the sequence GalNAcβ1-4Gal, e.g. asialo-GM1 isolated from lung tissue (Krivan et al. 1988). Recently *Pseudomonas aeruginosa* have been reported to bind to lactosylceramide (Baker et al. 1990) and oligosaccharide chains containing lacto-series type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) sequences (Ramphal et al. 1991). Lacto- and ganglio-series glycosphingolipids acts similarly as adhesion molecules for *Neisseria gonorrhoeae* and *Actinomyces* species *in vitro* (Strömberg et al. 1988; Deal & Krivan 1990; Strömberg & Karlsson 1990).

Binding to neuraminic acid (NeuAc) containing oligosaccharides of erythrocyte membrane glycoconjugates has been demonstrated for e.g. *Escherichia coli* (Parkkinen et al. 1986) and *Mycoplasma pneumonie* (Loomes et al. 1984).

1.8 Bacterial Glycoside Hydrolases in the Large Intestine

Intestinal bacteria and not their hosts are responsible for the degradation of mucin glycoproteins in the gastrointestinal tract. This was first shown by Lindstedt et al (1965) and Hoskins and Zamcheck (Hoskins & Zamcheck 1968; Hoskins 1968) comparing fecal excretion of oligosaccharides of germ-free and conventional rats. Salyers and co-workers later screened 188 *Bacteroides* strains (Salyers et al. 1977b), and 154 various strains of the *Bifidobacterium, Peptostreptococcus, Lactobacillus, Ruminococcus, Coprococcus, Eubacterium* and *Fusobacterium* genera (Salyers et al 1977a) for their ability to hydrolyse complex plant polysaccharides and mucins. Hydrolysis and fermentation of various plant polysaccharides including e.g. amylose, amylopectin, pectin, xylan, laminarin and guar gum was performed by various strains of the *Bacteroides, Bifidobacterium, Peptostreptococcus, Ruminococcus* and *Eubacterium* genera. Only *Bacteroides*

species degraded mucopolysaccharides like heparin, hyaluronic acid, and chondroitin sulfate. The only bacteria able to utilise intestinal mucins (porcine gastric mucin) were six out of nine tested strains of *Bifidobacterium bifidum*, a previously well known saccharolytic species (György et al. 1953) and two out of five *Ruminococcus torques* strains (Holdeman & Moore 1974). However, fermentation of free L-fucose and D-glucosamine, common constituents of many mucin oligosaccharides including colonic mucins, were performed by strains of several other species. This implies that the presence of mucin degrading bacteria are beneficial for a larger part of the microbiota by supplying other bacteria with appropriate monosaccharides. In further support of this, *in vitro* studies of bacterial growth (Harouny & Hoskins 1983) showed enhanced growth of *Bifidobacterium* and *Bacteroides* strains with hog gastric mucins only in the presence of a mucin degrading strain of *Ruminococcus AU* (later defined as *R. torques*). In contrast, the monosaccharide constituents of the mucin oligosaccharides stimulated growth even in the abscence of mucin degrading bacteria (Harouny & Hoskins 1983). Studies on carbohydrate contents in different parts of the intestines showed that a decrease in high molecular weight carbohydrates in the colon as compared to the small intestine of adult humans was not associated with an increase in free hexoses, hexosamines and sialic acids suggesting that these compounds were metabolised by the colonic flora (Vercelotti et al. 1977). Carbohydrate degrading and fermenting activities have also been found in colonic bacteria of hospitalised preterm infants with their characteristic flora of facultative anaerobes (*E. coli, Klebsiella, Serratia* and *Enterobacter* species) (Walker et al. 1989).

Hoskins and Boulding (1976a, 1976b) studied hydrolysis of hog gastric mucins and salivary glycoproteins by isolates of human fecal bacteria and found degradation of blood group ABH(O) and Le^a determinants. They also found evidence of an adaptation of the enzyme activities to the mucin oligosaccharide structures as determined by the blood group phenotype of the host, i.e. bacterial cultures from blood group A individuals degraded A antigens but not B antigens and vice versa. These blood group degrading bacteria were estimated to constitute approximately 1% of the fecal cultivatable flora (Miller & Hoskins 1981) and were identified as strains of the *Ruminococcus torques, Ruminococcus gnavus, Bifidobacterium bifidum and Bifidobacterium infantis* species (Hoskins et al. 1985). The bacteria appears to be responsible for a major part of the physiological hydrolyses of intestinal mucin oligosaccharides through their production and excretion of mixtures of α- and β-glycoside hydrolases. The enzymes studied by Hoskins and co-workers were found to be mostly constitutively produced in contrast to the glycoside hydrolases studied by Salyers which were inducible. Increased levels of α-galactosidases, β-glucosidases and α-L-fucosidases in human fecal supernatants has been reported to be associated with diets

containing the proper substrates for these enzymes (Salyers 1979). Using a continuous culture system and human intestinal bacteria MacFarlane et al. (1989) demonstrated elevated levels of α- and β-glycoside hydrolases and bacterial growth enhancement correlating to addition of porcine gastric mucin. These data imply a role for the sugar releasing enzymes in the autoregulation of the intestinal microbiota (Savage 1977; Tannock 1988).

In contrast to the abundance of glycoside hydrolases active on β-glycosidic linkages and repetitive α-glycosidic linkages found in polysaccharides like pectins, the production of specific blood group degrading α-glycoside hydrolases is limited to a few strains of fecal bacteria. Blood group decomposing enzyme activities were first described for *Lactobacillus bifidus* var. *Pennsylvanicus*, i.e. *Bifidobacterium bifidum* (György et al. 1954). Extracellular blood group ABH(O) and Lewis hydrolysing strains of the *Ruminococcus* and *Bifidobacterium* genera have been described by Hoskins and co-workers (see above). In addition, extracellular blood group H (Aminoff & Furukawa 1970) and A (Levy & Aminoff 1980) degrading enzymes have been isolated from *Clostridium perfringens*, and a cell-bound blood group B degrading enzyme has been isolated from a pathogenic strain of *Shigella flexneri* (Prizont 1982).

A rich and thoroughly studied microbial source of blood group decomposing α-glycoside hydrolases are spore forming bacteria present both in the gastrointestinal tract and in soil. A blood group H degrading α-fucosidase was first isolated from a strain of *Clostridium welchii* (Buchanan et al. 1956). Iseki and co-workers (see Iseki 1970) have isolated and characterised highly specific blood group A, B, H and Lewis decomposing glycoside hydrolases from bacteria of the *Clostridium* and *Bacillus* genera. They also showed that conversion of blood group A to blood group H by a strain of *C. tertium* A was achieved by the consecutive actions of a *N*-deacetylase and an α-D-galactosaminidase.

2. AIM AND RATIONALE OF THE STUDY

The aim of this study was to extend the knowledge of how glycoconjugates, represented by glycosphingolipids, might contribute to ecological stability in the gastrointestinal tract.

Based on the clinical experiences of severe infections as a consequence of antibiotic therapy and on the experimental experiences of germ-free research, the intestinal microflora have been assigned an important role in the human infectious defence. Ecological stability is crucial for the bacteria to be able to fulfill this task. Metabolically active bacteria have an important role in assuring this stable milleu by providing nutritional support and by defining a chemical environment optimal for the members of the microbiota. Bacteria of the human colonic flora, belonging to the *Ruminococcus* and *Bifidobacterium* genera, were

previously shown to have a unique niche in the colon being specialised in hydrolysis of complex fucosylated and sialylated oligosaccharides of gut mucin glycoproteins (see work by Hoskins et al. reviewed in section 1.8). Although a minor constituent of the fecal cultivatable bacteria (1%) they were shown to be responsible for a major part of the hydrolysis of the oligosaccharides of gut mucin glycoproteins. This implies a central role for these bacteria in the maintainance of a stable intestinal ecology.

Specifically this study investigates the involvement of bacterial glycoside hydrolases from the mucin degrading bacterial strains in the degradation of glycosphingolipids *in vivo* and *in vitro*. Glycosphingolipids are ubiquitous in the gastrointestinal epithelium and high amounts are continuously being shed into the gut lumen. They therefore constitute an ample substrate for these bacteria in the colon. Apart from being a possible source of nutrients these structures also contain carbohydrate sequences, among them lactose (Galβ1–4Glcβ1) hitherto not demonstrated in glycoproteins, which are of special interest since they can act as specific adhesion molecules for several bacteria *in vitro* (see section 1.7). Glycosphingolipids also have the advantage of being easier to isolate and structurally define than glycoproteins since each molecule only contains one oligosacchride chain. This makes it easier to monitor cleavage of single carbohydrate linkages which facilitates identification of single glycoside hydrolases in mixtures of enzymes.

The practical objectives of the study were to:

i) study the effects of weaning on the composition of fecally excreted glycosphingolipids

ii) through the hydrolyses of glycosidic linkages of struturally characterised glycosphingolipids and *para*-nitrophenyl glycosides elucidate the specificities of the α- and β-glycoside hydrolases produced in mixtures and isolated from culture supernatants from five strains of human fecal mucin-degrading bacteria, *Ruminococcus torques* strains IX-70 (ATCC No. 35915) and VIII-239, *Ruminococcus gnavus* strain VI-268 (ATCC No. 35913), *Bifidobacterium bifidum* strain VIII-210 (ATCC No. 35914) and *Bifidobacterium infantis* strain VIII-240

iii) optimise the conditions for glycosphingolipid *in vitro* degradations by the bacterial glycoside hydrolases.

iv) study the ability of the bacterial glycoside hydrolases to modify the glycosphingolipid structures of intact cell membranes.

3. METHODOLOGICAL ASPECTS

3.1 Preparation and Quantitation of Glycosphingolipids

In order to obtain pure glycosphingolipids there is a requirement for total glycosphingolipid fractions free of unpolar lipids (fatty acids, cholesterol and cholesterol esters), phospholipids, sphingomyelin and other glycoconjugates. Several preparative protocols have been described (e.g. Iwamori & Nagai 1978; Svennerholm & Fredman 1980; Hakomori 1983; Byrne et al. 1985; Karlsson 1987). The general procedure is based on the extraction of tissues using organic solvents, mild alkaline hydrolysis, (partitioning) and chromatography on silicic acid and anion exchange columns of native or acetylated lipid fractions (Handa 1963). Further separation of mixtures of non-acid and acid glycosphingolipids can be effectively achieved using Iatrobeads^{R} silica gel chromatography (Ando et al. 1976; Nagai & Iwamori 1980) and gradient elutions with mixtures of chloroform, methanol and water. Preparative HPLC techniques using different columns and solvent systems have also successfully been used to isolate non-acid glycolipids (Watanabe & Arao 1981) and gangliosides (Tjaden et al. 1977; Kannagi et al. 1987). Affinity chromatography utilising immobilised monoclonal antibodies (Bouhours et al. 1990a) and lectins (Torres & Smith 1988) have also been used for the purification of glycosphingolipids.

Quantitation of glycosphingolipids can be performed with densitometrical scanning of thin-layer chromatograms stained with copper acetate (Fewster et al. 1969; Sonnino et al. 1983; Papers II, III and IV this thesis), flourscence labelling of the sphingosine base with flourescamine (Naoi et al. 1974) and high-performance liquid chromatography of O-benzoylated glycolipids (McCluer & Jungalwala 1976; Gross & McCluer 1980).

3.2 Characterisation of Glycosphingolipids

The purity of a glycolipid fraction is primarily checked by thin-layer chromatography using chemical detection with anisaldehyde (Stahl 1961) and orcinol (Hakomori 1983) for non-acid glycosphingolipids and resorcinol (Svennerholm 1963) for gangliosides. A more specific and often more sensitive method is immunological detection with monoclonal (Brockhaus et al. 1981; Hansson et al. 1983b) or polyclonal antisera (Hansson et al. 1985a), bacteria (Hansson et al. 1985b), bacterial toxins (Magnani et al. 1980), viruses (Hansson et al 1984b) or lectins (Torres et al. 1988). Higher resolution can be achieved by eluting the thin-layer plate in two dimensions (Sonnino et al. 1983).

Thin-layer chromatographical techniques are relatively cheap and easily applicable on the laboratory bench. They also provide valuable information on the identity of a glycolipid, especially if glycolipids have been prepared and thoroughly characterised from the same tissue previously. However, thin-layer chromatography will not suffice to structurally determine completely unknown glycolipids. A complete structural characterisation of glycolipids includes determination of glycosyl residue number and composition, the positions and anomeric configurations (α or β) of glycosidic bonds, absolute configurations (D or L) and ring forms (pyranose or furanose) of the constituent sugars and finally the sequence of the glycosyl residues. In mammalian systems all sugar residues isolated hitherto except fructose are in the pyranose ring form and all except fucose are in the D form. The absolute configuration can be determined with gas chromatography of derivatised sugars (Leontien et al. 1978).

Identification of sugar residues and estimation of their molar ratios can be performed with gas chromatography after hydrolysis of the glycolipid molecule (Sweely & Walker 1964; Rickert & Sweely 1978; Torello et al 1980). By acid degradation of permethylated samples, reduction and finally acetylation the binding positions can be elucidated with gas chromatography-mass spectrometry (Stellner et al. 1973; Angel et al. 1987) of the resulting alditol acetates.

The rapid development and refinement of mass spectrometrical (MS) techniques have considerably potentiated the structural characterisation of glycolipids. Direct probe electron ionisation (Karlsson 1978) or chemical ionisation MS (Ariga et al. 1980) of permethylated (Hakomori 1964; Larson et al. 1987) or permethylated-reduced samples have been used for analysing glycolipids. Soft ionisation techniques like fast-atom bombardment (FAB) has been utilised for analyses of glycolipids (Dell 1987; Egge & Peter-Katalinic 1987; Ugorski et al 1989; Isobe et al 1989) and has proven superior to the electron ionisation techniques for analyses in the high molecular weight range. Recent developments further improving the MS methodology includes the introduction of tandem mass spectrometry (MS-MS) (Domon & Costello 1988), and direct coupling of HPLC equippment to the ion source (HPLC-MS) (Evans & McCluer 1987; Suzuki et al. 1989). Analysis of mixtures of glycolipids is performed after preseparation on thin-layer chromatograms (Kushi et al. 1988; Kubis et al. 1989; Påhlsson & Nilsson 1988), on HPLC (Evans & McCluer 1987; Suzuki et al. 1989), on supercritical fluid chromatography (Merritt et al. 1991), or by separation in the ion source by fractional evaporations (Breimer et al. 1979; Holgersson et al. 1989).

Oligosaccharides from glycolipids can be analysed after release from their lipid carriers using chemical (Hakomori 1983) or enzymatical (Ito & Yamagata 1989; Zhou et al. 1989) hydrolysis. Gas chromatography-mass spectrometry (GC-MS) have been successfully refined and utilised for structural analyses of high

molecular weight oligosaccharides and ceramides after endo-glucoceramidase treatment and permethylation (Bouhours et al. 1990a; Hansson et al. 1991).

Sugar sequences and anomerity of the glycosidic linkages may also be determined by utilising sequential degradations with exo- and endoglycoside hydrolases (Kobata 1979). Specific enzymatic hydrolysis can be combined with identification of the liberated sugars using e.g. HPLC and pulsed amperometric detection (Lee 1990) or radioactively labelled sugars (Radin 1972). Additional methods include analysis of the glycosylceramide products part remaining after enzyme incubation using thin-layer chromatography (Ito & Yamagata 1989; Papers II, III and IV this thesis).

The most powerful technique for structural characterisation of oligosaccharides is the nuclear magnetic resonance spectroscopy (NMR). This technique can provide information on both glycosidic composition, sequence, configuration (α or β) and type and position of glycosidic linkage. It has been successfully utilised for the structural analysis of a multitude of glycoconjugates (e.g. Dabrowski et al 1980; Herlant-Peers 1981; Breimer & Jovall 1985; Finne et al. 1989; Hounsell et al. 1989; Capon et al. 1989; Holgersson et al. 1990).

Most of the glycosphingolipids used as substrates for bacterial glycoside hydrolases in this study were isolated from human meconium (Karlsson & Larson 1978; 1979; 1981a; 1981b; Ångström et al. 1982b; 1982c; Larson 1986) and human erythrocytes (Ångström et al. 1986). They were structurally completely characterised using mass spectrometry of permethylated and permethylated/reduced derivatives, gas chromatography-mass spectrometry of partially methylated alditol acetates, and in most cases also proton nuclear magnetic resonance spectroscopy. Identifications of the lactoseries type 2 chain based structures from dog intestine, the blood group H active branched ten-sugar structure from rat small intestine and the sialylated structures from bovine brain (Paper III) were based on their Rf-values and chemical and immunological staining patterns on thin-layer chromatograms. However the non-acid glycosphingolipids from dog (McKibbin 1969; Sung & Sweely 1979; McCibbin et al. 1982; Hansson et al. 1983c) and rat (Breimer et al. 1982b) intestine as well as the ganglioside composition of bovine brain (Tettamanti et al. 1973) have previously been well characterised giving a litterature support for these structures.

3.3 Purification Procedures for Glycoside Hydrolases

Eventhough these methods are not a practical part of the thesis a short summary will be given here.

Whereas many bacterial glycoside hydrolases are produced constitutively (Hoskins et al. 1985) others have to be induced by growth in the presence of a proper substrate (Takegawa et al. 1989; see also Discussion of Paper III this

thesis). Bacterial glycoside hydrolases can be either extracellular or cellbound. Extracellular enzymes can be isolated from the culture supernatant (e.g. Wadström & Hisatune 1970; Fukuda & Matsumura 1976; Hoskins et al. 1985) whereas cellbound enzymes can be isolated only after the disruption of the cells (Berg 1981). The initial purification generally follow a standardised procedure with ammonium sulphate (or ethanol) precipitation followed by anion exchange chromatography and gel filtration in several steps. Similar protocols are also used for mammalian glycoside hydrolases (Conzelmann & Sandhoff 1987). Various affinity chromatography methods (Valisena et al.1982; Bishop & Desnick 1981; Sugai et al. 1989; Hopfer et al. 1989) and isoelectric precipitation (Wadström & Hirsatune 1970; Levy & Aminoff 1980; Berg 1981) are additional methods successfully used in the purification processes. Molecular cloning techniques offers a new approach to the production of pure enzymes (Ohmiya et al. 1988; 1989). However, these techniques require a reasonably pure enzyme fraction to start with in order to establish N-terminal sequences or monoclonal antibodies for construction of probes.

The mucin degrading strains used in this study, *Ruminococcus torques* strain IX-70 (ATCC No. 35915), *Ruminococcus torques* strain VIII-239, *Ruminococcus gnavus* (called *R. AB* in paper II) strain VI-268 (ATCC No. 35913), *Bifidobacterium bifidum* strain VIII-210 (ATCC No. 35914) and *Bifidobacterium infantis* strain VIII-240 were isolated by a maximum dilution technique from serial dilutions of fecal samples from healthy human adults. Bacteria were grown under anaerobic conditions with hog gastric mucins as carbohydrate source.

Purification of the glycoside hydrolase mixtures followed the standard protocol outlined above with ammonium sulphate precipitation and further fractionation on phenyl-Sepharose, hydroxyapatite, anion exchange chromatography and gel filtration. In the hydrophobic phenyl-Sepharose chromatography step Triton X-100 was added to break up hydrophobic interactions between different glycoside hydrolase components. High salt concentrations were used to remove remaining bacterial cell wall material (lipoteichoic acid) from the enzyme fractions (Ortiz et al. 1982). An α-*N*-acetylgalactosaminidase fraction from the enzyme mixture of *R. torques* IX-70 was enriched 90 times using this protocol and it appeared as a single band on denaturating SDS gel electrophoresis with a relative molecular weight of 209.000 and with a faint additional component.

3.4 Assays for Glycoside Hydrolase Activities and Specificities

Methods for monitoring hydrolysis of glycosidic linkages usually includes analysis of released colored (Paper III; Gatt & Rapport 1966) or flourogenic complexes (Desnick et al 1973). These methods are easy to perform, but

synthetical substrates often does not reflect the specificity and activity of an enzyme on the native substrate. Use of a biological substrate is therefore more reliable and in these cases enzyme activity can be monitored as release of radioactively labelled saccharides (Radin 1972) or ceramides (Scheel et al. 1982). Enzymatical (Gatt & Rapport 1966) or electrochemical (Lee 1990) detection of released saccharides and chemical detection of the glycosylceramide product(s) using thin layer chromatography (Ito & Yamagata 1989; Papers II, III and IV) are additional methods. The latter method is easy to perform, requires small amounts of substrate for detection and allows semiquantitation with scanning densitometry after spraying with a chemical reagent like copper acetate (Fewster et al 1969).

3.5 Effects of Experimental Conditions on Enzyme Activity

Every enzyme or group of enzymes have their own specific requirements, e.g. temperature, pH, ionic strength, divalent cations, other activators and cofactors, amount and nature of detergents, and presentation of the substrate (Tanaka & Suzuki 1975; Li & Li 1976; Gatt et al. 1978; Dixon & Webb 1979). If conclusions concerning the biological functions of an enzyme are to be made it is thus essential to mimic the *in vivo* conditions as closely as possible, and, in analogy, if enzymes are to be used as tools in *in vitro* experiments the conditions have to be properly optimised to give maximum yields.

Activators can work on the enzyme, e.g. by inducing conformational changes or stabilising the enzyme in an active form. They might also affect the substrate by presenting this compound in an optimal way for enzyme–substrate interactions. Detergents, a heterogenous group of amphiphilic compounds (Helenius & Simons 1975) have been shown to enhance glycoside hydrolase activities on both hydrophobic and hydrophilic substrates (Gatt & Rapport 1966; Wan Ho 1973; Ito & Yamagata 1989). This indicates that detergents execute effects both on the micellar organisation of the substrates as well as on the enzymes or their cofactors. It has been shown that non-denaturating detergents like bile acids and Triton X–100 bind to proteins, primarily through hydrophobic interactions (Helenius & Simons 1972; Makino et al. 1973).

4. PRESENT WORK AND DISCUSSION

4.1 Fecal Excretion of Intestinal Glycosphingolipids (Paper I)

The composition of glycosphingolipids was analysed in fecal samples from a child followed from 1 to 5 months of age. Initially the diet was exclusively breast milk but at three months of age solid food was introduced and at 5 months breast

milk was only a minor component of the total diet. As a comparison, analyses were made of fecal glycosphingolipids from a child, 23 months of age, fed only on solid food and from small and large intestinal epithelial cells and stroma from an other infant.

Non-acid fecal glycolipids of the nursed child, typed as a blood group BLe(a+b-) non-secretor, at 1 month of age was dominated by glucosylceramide and a pentaglycosylceramide structure carrying a blood group Le^a determinant (Figure 1). Elongated Le^a active structures were also detected. A similar pattern was also seen in the epithelial cell fractions from small and large intestines taken at autopsy. Di-, tri- and tetraglycosylceramides were all minor components. This pattern persisted until solid food was introduced in the diet at three months of age. At this time a dramatic shift in glycolipid pattern was seen as the more complex fucosylated glycolipids were diminished and simultaneously three lactosylceramide species appeared as major constituents. Lactosylceramide dominance was then conserved up to 5 months although a minor increase in the Le^a structure was detected. No apparent changes in glucosylceramide contents were seen. At 23 months of age lactosylceramide was a larger component than fucolipids in feces from a child fed entirely on solid food. However, the quantitative discrepancy was significantly smaller than the one seen during the first months after weaning.

The gangliosides were dominated by two structures, GM3, and a more slow moving band, identified later by mass spectrometry as GD3. From the time of weaning GM3 was gradually lost whereas GD3 persisted for a few months before it diminished. Very small amounts of GD3 were found after 23 months. Sulfatide had disappeared already after 1 month as compared to a meconium reference sample, whereas cholesterol sulfate remained unchanged during the entire study.

This study demonstrated for the first time the persistant fecal excretion of glycolipids from children up to 2 years of age. It also showed that glycolipids are undegraded in the intestines during the first period of life, at least as long as breast milk is the sole source of nutrients. In analogy with previous findings in meconium (Karlsson & Larson 1981b), the fecal glycosphingolipid composition corresponded to the individuals blood group and secretor status.

The findings were later confirmed in an extended study (Larson et al. 1990) following 6 children from birth through the weaning period. This showed that whenever formula or solid food was introduced, regardless of continued breast-feeding, the excretion of fucolipids and gangliosides diminished and lactosylceramide became the single dominating fecal glycolipid. These results also correlated to a change in excretion of short chain fatty acids, a major microflora associated characteristic (MAC) (Midtvedt et al 1988).

Marked changes in the glycolipid composition thus correlated to the introduction of solid food. Diet is the single most important factor for changes in

the composition of the colonic microbiota of infants (Stark & Lee 1982). The interindividual variations in fucolipid contents described in Paper I and the following study (Larson et al. 1990) might reflect variations in the composition of the intestinal microflora. A three year old boy with relapsing *Clostridium difficile* colitis has been shown to present a fecal glycolipid composition normally seen in meconium samples and in feces from breast-fed children (unpublished results). The association to changes in the intestinal flora known to occur early in the microbial succession (Savage 1989) and to metabolic processes connected to the establishment of new bacterial species (Midtvedt et al. 1988) strongly suggests that the changes in glycosphingolipid composition are a microflora associated characteristic that could be used to monitor bacterial succession and disruptions in the intestinal microbial ecology.

The influence of nutrient carbohydrates might be difficult to evaluate. It has been shown that milk oligosaccharides substancially contributes to the fecal oligosaccharides of breast-fed infants (Sabharwal et al. 1988). However it does not appear to influence the composition of glycosphingolipids (Larson et al. 1990).

4.2 Specificities of Glycoside Hydrolases of Mucin Degrading Bacteria of the Human Fecal Flora (Papers II and III)

Enzyme mixtures from five strain of the normal fecal flora (see section 3.2) were tested for the ability to degrade glycosphingolipids of different core chains (lactoseries type 1 and 2, globo, *iso*globo, galabio and ganglio), carrying different blood group determinants (A, B, H, Le^d, Le^a, Le^b, X, Y, Forssman and *para*-Forssman) or with different degrees of sialylation (mono- to tetrasialo). As a comparison, hydrolysis of synthetic pnp-glycosides were analysed. A summary of the glycosidase activities found in the different enzyme mixtures are given in Table 4.

Strong 1,3-α-*N*-acetylgalactosaminidase activities were found in the enzyme mixtures of the blood group A-degrading *R. torques* strains IX-70 and VIII-239. These were the only strains degrading both mono- and difucosyl A determinants on type 1 and type 2 core chains, the Forssman antigen and GalNAcα1-pnp. Weak and selective additional 1,3-α-*N*-acetylgalactosaminidases only degrading mono-fucosylated blood group A determinants were also found in the *R. gnavus* VI-268 and *B. infantis* VIII-240. *B. bifidum* VIII-210 did not degrade GalNAcα1- terminated structures at all. Blood group B-degrading 1,3-α-galactosidases hydrolysing monofucosyl B on type 1 chain and Galα1-pnp were found only in *R. gnavus* VI-268. Previous work has shown two isoforms of 1,3-α-galactosidases in the enzyme mixture from this strain responsible for hydrolysis of blood group B determinant in hog gastric mucin and Galα1-pnp respectively (Hoskins et al. 1985). All five strains were shown to produce 1,2-α-, 1,3-α- and

Table 4. Summary of extracellular glycoside hydrolase activities of five strains of human fecal bacteria of the *Ruminococcus* and *Bifidobacterium* genera

Enzyme activities	*R. torques* VIII-239	*R. torques* IX-70	*R. gnavus* VI-268	*B. bifidum* VIII-210	*B. infantis* VIII-240
1,3-α-GalNAc'ase*	+	+	+	–	+
1,3-α-Gal'ase	–	–	+	–	–
1,2-α-Fuc'ase	+	+	+	+	+
1,3-α-Fuc'ase	+	+	+	+	+
1,4-α-Fuc'ase	+	+	+	+	+
2,3-α-NeuAc'ase	+	+	+	+	+
2,8-α-NeuAc'ase	+	+	+	+	+
1,4-α-Gal'ase	–	–	–	–	–
1,3-β-Gal'ase	+	+	–	+	+
1,4-β-Gal'ase	+	+	+	+	+
1,1Cer-β-Glc'ase	–	–	–	–	–
1,3-β-GlcNAc'ase**	+	+	+	+	+
1,6-β-GlcNAc'ase	+	+	–	+	+
1,3-β-GalNAc'ase**	+	+	–	+	+
α-Ara'ase***	–	–	–	+	+
α-Glc'ase***	+	+	+	+	+
β-Glc'ase***	+	–	+	+	–

* Abbreviations: GalNAc'ase, *N*-acetylgalactosaminidase; Gal'ase, galactosidase; Fuc'ase, fucosidase; NeuAc'ase, *N*-acetylneuraminidase; 1,1Cer-β-Glc'ase, ceramidase; GlcNAc'ase, *N*-acetylglucosaminidase; Ara'ase, arabinosidase; Glc'ase, glucosidase.

** Data indicate the presence of both exo- and endo-glycosidase activities

*** Enzyme specificity identified by hydrolyses of *para*-nitrophenyl-glycosides. All other enzymes were identified through the release of saccarides from glycosphingolipids.

1,4-α-fucosidases degrading blood group H, Le^d X, Y, Le^a and Le^b active glycolipids. However, no Fucα1-pnp hydrolysing activities were detected in any of the strains.

All strains released α2-3 and α2-8 linked *N*-acetylneuraminic acids from mono-, di- and polysialylated gangliosides on a lactosylceramide, ganglio-tetraosylceramide or *neo*lactotetraosylceramide core. Only the two *R. torques* strains IX-70 and VIII-239 hydrolysed the internally positioned NeuAcα2- residue of GM1.

All five strains contained 1,3-β-*N*-acetylglucosaminidases as shown by the enrichment of the diglycosylceramide lactosylceramide. All strains but *R. gnavus* VI-268 also released *para*-nitrophenol from GlcNAcβ1-pnp. Only the two *R. torques* strains produced 1,4-β-galactosidases degrading lactosylceramide to

glucosylceramide and this to a limited extent (30% or less). Lactoseries type 1 and type 2 (Table 3) were the only core chains degraded by these strains. Four of the five strains were active on both chain types but *R. gnavus* VI-268 only degraded the lactoseries type 2 core chain. This indicates the presence of a 1,4-β-galactosidase and the lack of a 1,3-β-galactosidase. All strains but *R. gnavus* VI-268 also degraded Galβ1-pnp.

There was indirect evidence for the presence of an endo-1,3-β-*N*-acetylglucosaminidase and an endo-1,3-β-*N*-acetylgalactosaminidase in the enzyme mixtures from all strains but *R. gnavus* VI-268. The blood group A degrading strain *R. torques* IX-70 degraded a blood group B-active hexaglycosylceramide to lactosylceramide without any detectable intermediates. All strains but *R. gnavus* VI-268 degraded the β-Forssman structure (IV^3GalNAcβ-GbOse$_4$Cer) to GbOse$_3$Cer but not the Forssman (IV^3GalNAcα-GbOse$_4$Cer) nor the GbOse$_4$Cer. All strains but *R. gnavus* VI-268 hydrolysed the synthetic Galβ1-3GlcNAcβ1-pnp to varying extent. However, since the analysis of released sugars was not performed endo-glycosidase activities could not be definitely verified.

Additional α-arabinose releasing activities, as detected by release of *para*-nitrophenol from Araα1-pnp, were found in *B. bifidum* VIII-210 and *B. infantis* VIII-240. This is in analogy with previous reports on glycoside hydrolases produced by *Bifidobacterium* species (Salyers et al. 1977a). None of the strains degraded pnp-linked mannose.

There were two general findings in these studies. The first was the apparent preference for glycosphingolipids on type 1 and type 2 chains. These type chains, carrying a Galβ1-3/4GlcNAcβ1- sequence, are the dominating oligosaccharide cores of mucin glycoproteins (Podolsky 1985), membrane bound glycoproteins (Finne et al. 1989) and glycosphingolipids (Keränen 1975; Siddiqui et al. 1978; Björk et al. 1987; Holgersson et al. 1988) of the gastrointestinal tract. The second was the apparent accumulation of lactosylceramide, the normally minor component of the intestinal epithelium (Björk et al 1987; Holgersson et al. 1988), as a result of carbohydrate degradation.

The results implies an adaptation of the bacterial enzymes to the dominating endogenous oligosaccharide substrates of the gastrointestinal tract. Lactosylceramide has been shown to act as a low affinity receptor for several members of the intestinal microbiota, i.e. *Bacteroides*, *Clostridium*, *Fusobacterium*, *Lactobacillus* and *Propionibacterium* (Hansson et al. 1983a). An ecological advantage could be envisioned through the production of this structure that could possibly serve as an adhesion molecule and nidus for microcolony formation (Freter et al. 1983). To date nothing is known about the oligosaccharide adhesion characteristics, if any, of the mucin degrading strains. This is primarily due to difficulties in getting the bacteria metabolically labelled but such efforts are

being made. Released monosaccharides are used by bacteria as a carbon source and can enhance growth in an ecological system *in vitro* (Harouny & Hoskins 1983). The mucin degrading bacteria of this study are unique in their production of high amounts of α-glycoside hydrolases. The ability to utilise a specific nutritional substrate more efficiently than other strains have been suggested to give bacteria an ecological advantage in their habitat (Freter et al. 1983), and in the case of the mucin degraders released α-glycosides could serve such a purpose. Formation of disaccharides acting as growth promoting factors (i.e. Bifidus factor) (Springer et al. 1954; Lambert & Zilliken 1965) is an additional way that bacteria could benefit from oligosaccharide hydrolysis.

4.3 Optimisation of Glycosphingolipid *in vitro* Degradations by Bacterial Glycoside Hydrolases (Paper IV)

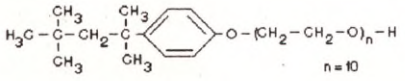
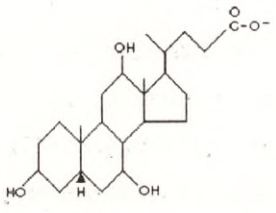
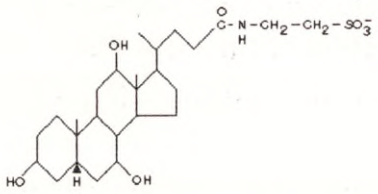
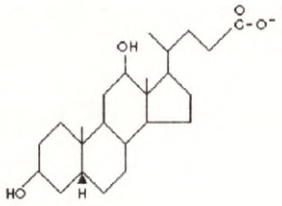
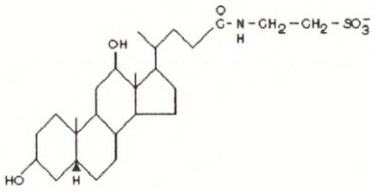
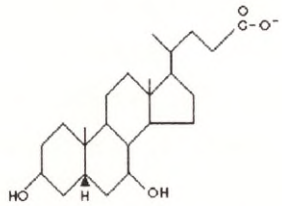
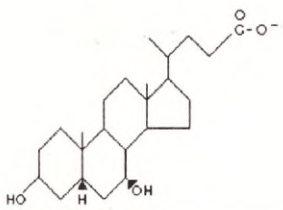
This study was aimed to optimise the experimental conditions for glycosphingolipid *in vitro* degradations by the mucin degrading strains. This was done in order to efficiently detect all glycoside hydrolases within the respective mixtures without prior separation of the enzymes. The investigation specifically addressed the question of the dependence of the glycoside hydrolases on type and concentration of detergents. A summary of the structures and critical micellar concentrations (cmc) (Helenius & Simons 1975) of the detergents used in this study is included in Table 5.

A critical dependence on type and concentrations of detergents was illustrated by the conversion of lactosylceramide to glucosylceramide. At 5 mM concentrations Titon X-100 and trihydroxylated bile salts (sodium cholate and sodium taurocholate) showed limited ability to promote release of the terminal Galβ1- residue (30% or less) from lactosylceramide and only with the enzymes of the *R. torques* strains VIII-239 and IX-70. In contrast, 5 mM concentrations of di-α-hydroxylated bile salts (sodium deoxy-, chenodeoxy-, and taurodeoxycholate) promoted a complete hydrolysis of lactosylceramide to glucosylceramide with enzymes from all strains but *R. gnavus* VI-268 within 1 hour. The 1,4-β-galactosidases of all five strains were completely inactive in the presence of the 7-β-epimer of chenodeoxycholic acid (sodium ursodeoxycholate). The detergent effects were concentration dependent with little or no hydrolytic activities detected below the reported cmc values for the respective detergents (Helenius & Simons 1975; Roda et al. 1983).

Glycolipid core chains shown to be resistant to hydrolysis in Papers II and III (i.e. galabio, globo, *iso*globo and ganglio) were also to a large extent resistant under the new optimised conditions. The only exceptions were the *iso*globotriaosylceramide structure being degraded by enzymes from *R. gnavus* VI-

268 and gangliotetraosylceramide being degraded by enzymes from *B. bifidum* VIII-210 and *B. infantis* VIII-240.

Table 5. Detergent structures and critical micellar concentrations

Detergent	Structure	Critical micellar conc. (cmc)
Triton X-100 (Polyethylene glycol *iso*octylphenyl ether)		0.3 mM (Helenius & Simons 1975)
Cholate (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid)		11 mM (Roda et al. 1983)
Taurocholate (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid N-(2-sulphoethyl) amide)		6 mM (Roda et al. 1983)
Deoxycholate (3α, 12α-dihydroxy-5β cholan-24-oic acid)		3 mM (Roda et al. 1983)
Taurodeoxycholate (3α, 12α-dihydroxy-5β cholan-24-oic acid N-(2-sulphoethyl) amide)		2.4 mM (Roda et al. 1983)
Chenodeoxycholate (3α, 7α-dihydroxy-5β-cholan-24-oic acid)		4 mM (Roda et al. 1983)
Ursodeoxycholate (3α, 7β-dihydroxy-5β-cholan-24-oic acid)		7 mM (Roda et al. 1983)

The major difference between the degradations performed in Triton X-100 (Papers II and III) and those done in the presence of bile salts was the hydrolysis of

lactosylceramide. It was not the purpose of this study to illustrate the chemical environmemt in the natural habitats of these mucin degrading bacteria. However, a comparison of the characteristic accumulation of lactosylceramide found in feces at the time of weaning (Paper I; Larson et al. 1990) and the extensive bacterial lactosylceramide degradation promoted by e.g. sodium deoxycholic acid, an apparently dominating detergent in human feces (Ali et al. 1966) *in vitro* could be considered to argue against a role of the mucin degraders in intestinal glycolipid degradation. The colonic pool of bile acids is however an extremely complex mixture (Carey 1973) of various derivatives at concentrations differing depending on e.g. diet, variations in intestinal absorptive function and intake of drugs (Hofmann & Poley 1972; Northfield & McColl 1973; McJunkin et al 1981) and being under the influence of microbial metabolism (Hylemon 1985).

The results suggest that the effects of detergents on the glycoside hydrolase activities and apparent specificities in glycolipid degradations are primarily exerted through the micellar presentation of the substrates and not through an interaction with the enzymes. However, a direct interaction with the enzymes was suggested by the stimulating effect of both Triton X-100 and sodium taurodeoxycholate on hydrolysis of pnp-glycosides at concentrtions below their respective cmc values. Distinct differences in solubilising and micellar properties have been described between different groups of detergents as well as among bile salts (Hofmann 1963; Helenius & Simons 1975; Montet et al 1979; Baillet-Guffroy et al. 1981) that could account for the observed effects.

The complete resistance to hydrolysis found for globotriaosylceramide and globoside is interesting with regard to the *in vitro* interaction of Galα1-4Galβ1- containing oligosaccharides with strains of uropathogenic *Escerichia coli* (Leffler & Svanborg-Edén 1980; Källenius et al. 1980) often originating from the gastrointestinal tract (Svanborg-Edén et al. 1989). Although very scarce in the intestinal epithelium (Björk et al 1987; Holgersson et al. 1988), globo-series glycolipids are found in human fecal extracts from birth up to adult age (Larson et al 1990). The complete resistance to enzymatic hydrolysis of a potential receptor structure may be the mechanism by which these bacteria can establish and survive in the colon even though they are potentially harmful to the host.

4.4 Degradation of Cell Surface Associated Glycoconjugates by Bacterial Glycoside Hydrolases (Paper V)

This paper addressed the question of whether the bacterial glycoside hydrolases are capable of modifying glycoconjugates of intact cell membranes. Human blood group A_1 erythrocytes were used as substrates for the blood group A-degrading enzyme mixture of *R. torques* IX-70, and for partly purified α-*N*-acetylgalactosaminidase (XI-117) and α-fucosidase (XI-50) fractions from this

mixture. Degradation of cell surface glycoconjugates was monitored by changes in agglutination patterns using standard blood typing reagents. Changes in immunological reactivities of glycosphingolipid fractions extracted from the erythroctes after enzyme treatment were detected by thin-layer chromatography using immunological staining with the same reagents.

The unfractionated enzyme mixture of *R. torques* strain IX-70 converted A_1 red blood cells to A_2-reactive cells within minutes at room temperature, as defined by the loss of hemagglutination with the *Dolichos biflorus* lectin (Bird 1952), and a retained agglutinability with anti-A antibodies. In contrast, purified enzyme fractions with similar α-*N*-acetylgalactosaminidase activities did not markedly diminish *D. biflorus* agglutination until after 3 hours of incubation at 37°C.

The *R. torques* IX-70 enzyme mixture degraded *D. biflorus* reactive glycosphingolipids but with a delay of 1 hour as compared to loss of hemagglutination. This change in binding to glycosphingolipids paralleled the appearence of *Ulex europaeus* lectin blood group H activity in the lipid extracts. Purified enzymes did not diminish *D. biflorus* binding glycosphingolipids within the 6 hour incubation time in spite of the complete loss of agglutinability with the lectin.

Papain pretreatment enhanced hydrolysis of glycosphingolipids. It also conserved strong blood group A and A_1 hemagglutinability regardless of glycoside hydrolase treatment, and uncovered *U. europaeus* reactive blood group H determinants. A_1 erythrocytes not hemagglutinated by *D. biflorus* due to glycoside hydrolase treatment with the enzyme mixture of *R. torques* IX-70 regained strong agglutinability with this lectin after protease treatment with papain.

Taken together this study showed that glycosidases of the *R. torques* strain IX-70 can degrade erythrocyte cell surface glycoconjugates without significantly affecting the viability of the cells. The loss of A_1 *D. biflorus* agglutinability did not correlate to a loss of medium chain glycosphingolipids binding the lectin. This suggests that the target molecules of the *D. biflorus* lectin and for the bacterial glycoside hydrolases is provided by glycoproteins (Finne et al. 1978; Järnefelt et al. 1978) and megaloglycolipids (Gardas & Koscielak 1973) rather than by medium chain glycosphingolipids Papain treatment disrupted the native (carbohydrate) topology of the erythrocyte cell membrane, and thereby exposed normally cryptic *D. biflorus* and *U. europaeus* reactive sites.

The glycosphingolipid degrading activities were found exclusively in the total IX-70 enzyme mixture. This might indicate that other enzymes than the specific 1,3-α-*N*-actylgalactosaminidases are required to increase the accessibility of the oligosaccharide chains. Alternatively the hydrolyses might not at all be performed by the purified α-glycoside hydrolases, but by other exo-α-glycoside hydrolases, or by endo-glycoside hydrolases (endo-β-galactosidases, endo-β-*N*-acetylglucosaminidases). The effects of the latter has been previously

demonstrated by the loss of gangliosides and blood group H antigens on slow moving glycosphingolipids of erythrocyte cell membranes using endo-β-galactosidase from *Escherichia freundii* (Fukuda et al. 1979).

Cell surface antigenicity is an essential problem of transfusion medicine and transplantation immunology. A reduction of cell surface antigens with a preserved cellular function would be most valuable in avoiding immunological complications in blood tranfusions and organ transplantations. Glycoconjugates have been implicated to play a role in the initial step of an infectious process, i.e. adhesion of bacteria, viruses or bacterial toxins to cell surfaces. The glycoside hydrolase producing bacteria could play a role in the prevention of the establishment of potential pathogens of the gut by reducing the number of adhesion sites for the invading strains. Whether that is the case or not remains to be elucidated, but the applicability of these sugar releasing enzymes in future studies of cell surface deantigenation is obvious.

5. CONCLUDING REMARKS AND FUTURE WORK

It is evident from these studies that bacteria of the intestinal microbiota are highly active in hydrolysis of intestinal glycosphingolipids. This was illustrated *in vivo* by the decomposition of blood group active fucolipids and gangliosides, and enrichment of lactosylceramide occuring at the time of weaning (Paper I).

Corresponding effects on glycolipids were exerted *in vitro* by glycoside hydrolase mixtures isolated from culture supernates from five *Ruminococcus* and *Bifidobacterium* strains of human fecal bacteria (Papers II-IV). These bacteria were previously shown to degrade fucosylated and sialylated mucin oligosaccharides of glycoproteins, and to be responsible for a major part of the hydrolysis of gut mucin glycoproteins. By utilising glycosphingolipids as substrates these bacteria have access to a rich additional source of oligosaccharides that could give them and associated bacterial strains an ecological advantage in their habitats (Freter et al. 1983). This could be envisioned through the release of nutrients in the form of monosaccharides to be fermented by bacteria of the colonic microbiota (Salyers et al. 1977a; 1977b; Salyers 1979; Hoskins & Harouny 1983), and the production of disaccharides acting as growth promoters (Springer et al.1954). Potential adhesion sites, like lactosylceramide, produced by bacteria from glycosphingolipids shed from the epithelium, and acting as niduses for microcolony formation could be of importance for the lumenal colonisation of the microbiota.

The methods employed in this study enabled analysis of the glycosylceramides but did not permit detection of the released sugars. This will be necessary in order to definitely establish the presence of endo-glycoside hydrolases through the release of di- or oligosaccharides. HPLC-PAD (pulsed

amperometric detection) methodology for detection of free saccharides (Lee 1990) is therefore presently being established in the laboratory.

The studies presented have given detailed information on the activities of the glycoside hydrolases in mixture which is of interest since that is the way they appear *in vivo*. However, further characterisation of the glycoside hydrolases will require purified enzymes. Affinity chromatography techniques using specific substrate analogs have proven very successful in obtaining highly purified glycoside hydrolase fractions (Bishop & Desnick 1981; Hopfer et al. 1990). Various cloning techniques have recently been employed for the purification of microbial glycoside hydrolases (e.g. Ohmiya et al. 1988; 1989). These methods also give information on the protein structure and sequence data of the genes encoding the enzymes. They also enables establishment of transformant bacterial strains producing only one or a few defined enzymes. Such strains would be very useful in ecological *in vitro* and *in vivo* studies as outlined below. Further, structural data of the protein would enable identification of the active site of the enzyme. This would make it possible to study the exact protein-carbohydrate interactions involved in the oligosaccharide degradation. Comparison of sequence data of the genes encoding these bacterial enzymes with the ever increasing reports of cDNA sequences for glycoside hydrolases from other species (e.g. Graham et al. 1988; Oshima et al. 1988; Fisher & Aronson 1989; Tsuji et al. 1989) would give interesting information on the evolutionary origin of these enzymes that are so abundant and vital in both procaryotic and eucaryotic life forms (e.g. Wan Ho et al. 1977; Cabezas et al. 1983; Conzelmann & Sandhoff 1987).

Even though the impact of the mucin degraders on the degradation of complex carbohydrates have been established (Hoskins et al. 1985; this thesis) their influence on the ecological development and stability is still unsettled. Further studies addressing this question should include evaluation of the establishment and maintainance of defined bacterial ecosystems in the presence and absence of the mucin degrading bacteria. The ability of potential pathogens to establish in such systems would also be valuable to study. Germ-free animals offer an opportunity to perform such studies *in vivo*. It also enables studies of the biochemical consequences of intestinal microbial establishment since both carbohydrates, proteins and steroids are known to be excreted underivatised from the sterile gastrointestinal tract (Midtvedt 1986). The access to transformant bacteria producing one or a few defined glycoside hydrolases would be very useful in such systems.

It would also be of great value to study the possible binding of glycoside hydrolase producing strains to glycoconjugates. If such interactions occur the next step would be to correlate the specificty of the binding with that of the enzymatic activity of the strain.

Many pathlogical conditions of the intestines involve changes in glycoconjugate composition. Thus, studies on mucin glycoproteins have shown distinct changes in oligosaccharide patterns associated with inflammatory bowel disease (Podolsky & Isselbacher 1984; Rhodes et al 1988), and with various degrees of gastrointestinal dysplasia (Boland et al 1982a, 1982b; Rhodes et al 1986), even in apparently normal areas adjacent to the diseased tissue. These changes, i.e. reduction in sulfated mucins and decreased O-acetylation of sialic acids, have been speculated to render the mucus coat less resistant, and thereby more permissive to injuries by exogenic factors like bacteria, toxins and carcinogens. In analogy to mucin glycoproteins, specific changes in glycolipid composition have been reported in association with inflammatory bowel disease (Stevens et al. 1986). Thus, lactosylceramide was found to be enriched in colonic biopsy material from patients with Crohn's disease. This could be explained by a weakend mucus barrier enabling intestinal bacteria to approach the epithelial cell lining and hydrolse the glycoconjugates of the apical cell membranes. Increased vulnerability of the epithlium due to bacterial mucus degradation has been hypothesised as part of the pathogenesis of this type of diseases (Rhodes et al 1988). In support of this, specific increases in activities of bacterial glycoside hydrolases have been associated to inflammatory bowel disease (Ruseler van Embden & van Lieshout 1987; Corfield et al. 1988; Ruseler van Embden et al. 1989).

Other diseases where intestinal bacterial metabolism of carbohydrates has been suggested to take part includes colon cancer (Gorbach & Goldin 1990), where it is thought to contribute to a longterm low concentration exposure of potential carcinogens in the colon increasing the risks of malignancy.

Because the intestinal microbiota is an integral part of the infectious defence mechanisms of the human body, changes in this ecosystem have great impact on the health of the host. Diarrheal diseases offers a major health problem throughout the world, and has been estimated that diarrhea are responsible for the deaths of 12.600 children per day in developing countries (Guerrant et al. 1990). A major cause of these diseases in the developing world is enterotoxigenic *E. coli*. These bacteria and their toxins have also been associated to hemolytic uremic syndrome (Karmali et al. 1983) and haemorrhagic colitis (Riley et al. 1983). In developed countries an ever increasing use of antibiotics have established multiresistant superinfections, among them *C. difficile* pseudomembranous colitis (Borrielo & Larson 1981; Lyerly et al. 1988) as a major therapeutic problem. Both the verotoxin of enterotoxigenic *E. coli* (Waddell et al. 1990), and the C. difficile toxin A (Tucker & Wilkins 1991) have been reported to bind glycolipids that can be found in the intestines (Holgersson et al. 1988; Finne et. al. 1989; Larson et al. 1990) Regardless of the type of agent causing the diarrheal diseases they are all associated with a rupture of the normally stable intestinal microbiota.

Metabolically active bacteria most likely play a crucial part in the maintainance of the intestinal microbiota and in the colonisation resistance of the gut. This could be exerted by production of growth promoting compounds for the resident microflora that thereby can out-grow invading bacteria and opportunistic strains of the normal flora. Bacteria of the intestinal microflora could also produce potential antimicrobial compounds down-regulating invading bacteria (van der Waaij 1989).

Increased knowledge of the biochemical basis for intestinal host-microbial interactions will extend our understanding of the pathogenesis of diarrheal diseases, and it will be of interest in the future studies of the causes of inflammatory bowel disease and colon cancer. Glycoside hydrolases or other endogenous bacterial enzymes might also be of use in the therapy of antibiotic associated diarrheal diseases and enterocolitis by facilitating the reestablishment of the resident intestinal microflora.

På grund av upphovsrättsliga skäl kan vissa ingående delarbeten ej publiceras här.
För en fullständig lista av ingående delarbeten, se avhandlingens början.

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